CONSERVATION GENETICS OF ENCLOSED BLACK RHINOCEROS POPULATIONS IN KENYA

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DECLARATION

I hereby declare that the work has been done by myself and no portion of the work contained in this Thesis has been submitted in support of any application for any other degree or qualification on this or any other university or institution of learning.

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This thesis is dedicated to my parents
who have given me everything

Abstract

Population size is a major determinant of extinction risk with small populations experiencing an inherently higher risk of extinction due to genetic threats and stochastic fluctuations in survival, fecundity and environmental conditions. For many species, natural populations are experiencing a dramatic decline in numbers and distribution as a direct result of human activities. The severity of the threats facing some species necessitates the movement of remaining individuals into protected areas or captive breeding programs where resources can be concentrated to promote recovery. Moreover, increasing levels of population fragmentation has led to metapopulation management being regarded as an integral part of many conservation strategies, particularly for large vertebrates. The translocation of individuals between populations across an inhospitable matrix is often needed to offset the detrimental effects of small population size and to maintain natural evolutionary processes. Whilst the benefits of active conservation management involving the mixing of individuals from hitherto isolated populations has been demonstrated in a number of cases, the impact on historic population structure and the potential for outbreeding depression is often poorly understood. Moreover an increasing body of theoretical and empirical work is demonstrating that mate selective choices are mediated not only by additive effects but by non-additive effects, most specifically the amount of genetic similarity between individuals. Recent studies on natural populations have demonstrated that there is a fitness cost associated with choosing maximally dissimilar mates and that even for intrapopulation breeding, individuals exercise a preference for mates of intermediate similarity. Populations subject to active conservation management are typically small admixed populations where individuals are presented with a limited number of potential mates, representing a greater spectrum of genetic divergence than would typically be present in non-managed populations. With many *in situ* and *ex situ* conservation programs reporting poor population growth rates linked to low or declining reproduction understanding the genetic influences on mate choice in these populations is potentially of great importance.

This thesis examines (1) the effects of active conservation management on levels of genetic diversity and (2) historic population structure in the eastern black rhinoceros (*Diceros bicornis michaeli*). It also examines the relative influences of additive and non-additive effects on female mate choice in this actively managed conservation priority species. The eastern black rhinoceros (*Diceros bicornis michaeli*) has been subject to one

of the severest human induced declines of any mammalian species. The subspecies formally ranged across East Africa from northern Tanzania to Somalia, with its largest populations in Kenya. A significant increase in poaching of rhinoceros for their horn during the 1970s and 1980s eliminated *D. b. michaeli* from Sudan, Ethiopia, Somalia, Uganda and Rwanda. Extensive populations in Tanzania were reduced to just two small populations and the subspecies was reduced from an estimated Kenyan population of 20,000 in 1970 to just 380 by 1987. In the face of the imminent extinction of the Kenyan population, the Kenya Wildlife Service (KWS) implemented a policy of moving all animals outside protected areas into fenced sanctuaries where resources could be concentrated to counter the poaching threat. The sanctuary system proved successful and as of 2006 Kenya had approximately 540 black rhinoceros protected within 14 separate populations.

With the sanctuary system largely successful in countering the continued threat of poaching, emphasis has shifted to metapopulation management to ensure the viability of the small isolated populations within the sanctuary system.

To assist in the effective metapopulation management of these isolated populations, data is presented here both on the current levels of genetic diversity and the range of historic genetic diversity captured within five enclosed sanctuary populations. A total of 166 individually identified black rhinoceros were genotyped for 9 microsatellite loci and a 507 bp segment of the mtDNA control region, with the majority of the genotyping conducted on DNA extracted from faeces. To assist in the identification of faecal samples from mixed sex pairings a simple, accurate, single-stage 5′-exonuclease assay for gender determination in the black rhinoceros from low-copy template DNA is presented. Genetic analysis and the examination of translocation records shows that the five sanctuaries are comprised of historic populations from three geographic regions within the country and that significant admixture has occurred between these historically divergent populations. These historically divergent populations are shown by molecular dating to have originated from the south of the country following an expansion which is putatively linked to the contraction of the Pleistocene forests approximately 300 KYA. Examination of mutation bias in the species reveals low levels of mutagenesis in concordance with other studies and evidence of ectopic gene conversion between eutherian sex chromosomes. The current metapopulation retains significant levels of genetic diversity for both nucleic ($A = 5.0$, $H_E = 0.689$) and organellar ($\pi = 0.007$) genomes, with levels of diversity in individual populations related to

the amount of admixture of former populations. Parentage analysis was undertaken for 107 individuals from three sanctuary populations representing approximately 16 years of successful reproductive activity in these populations. It is demonstrated that in the black rhinoceros male genetic diversity is a significant predictor of reproductive success and that females balance male genetic quality with intermediate levels of genetic similarity in admixed populations. This is the first time these effects have been investigated in a conservation priority species subject to active management and it is anticipated these results will have a profound impact on future management strategies for the species. In particular the overall results of this thesis provide a framework whereby the management of the Kenyan black rhinoceros metapopulation can be guided by the way rhinoceros are shown to manage their own reproductive success.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

1.1 Black rhinoceros

The black rhinoceros (*Diceros bicornis*) has been subject to one of the severest human induced declines of any mammalian species and has been listed as *Critically Endangered* in the *IUCN Red List* since 1996 (IUCN 2008). The species was once common outside of the rainforest belts throughout Sub-Saharan Africa, but is now restricted to just 8 countries, with possibly a few individuals existing outside known range states (Figure 1.1). At the time of European settlement the species numbered several hundred thousand but experienced a severe decline as a result of hunting and land clearance for farming (Emslie & Brooks 1999). However the species still existed in large, contiguous populations until a significant increase in commercial poaching during the 1970s and 1980s reduced numbers by approximately 96% (Western & Sindiyo 1972, Emslie & Brooks 1999). An estimated population of 65,000 in 1970 was reduced to fewer than 2,500 animals by 1992 (Gakahu 1993, Vigne & Martin 2006) (Table 1.1). Remaining populations, subdivided into four subspecies were left small and fragmented, with the western subspecies *D. b. longipes* possibly succumbing to poaching in the last few years and now listed as probably extinct with no animals in captivity (Emslie & Brooks 1999, Lagrot *et al.* 2007). As of 2010 the three remaining subspecies are now found only in eastern and southern African countries; with *D. b. bicornis* occurring in Namibia and South Africa (N=1,920), *D. b. minor* in South Africa, Zimbabwe and a small remnant population in southern Tanzania (N=2,220) and *D. b. michaeli* in Kenya with two populations in northern Tanzania (N=740) (Emslie 2011).

1.2 Poaching

The extensive poaching during the 1970s and 1980s was in response to an increase in demand for rhinoceros horn, particularly in the Middle East. Yemen has been the world's largest importer of rhinoceros horn since 1970s; Yemeni men highly value ornate daggers called *jambiyas* which have a handle made of rhinoceros horn (Vigne & Martin 2001). Before the 1970s these expensive status symbols were too expensive for most Yemenis to afford. The income generated during the 1970s oil crisis meant that the large number of migrant Yemenis working in Saudi Arabia could now afford *jambiyas* and this was the driving factor behind the increase in poaching (Vigne & Martin 2000). It is estimated that 67,050 kg of rhinoceros horn was imported into Yemen between

Table 1.1. Black rhino population estimates, by country, 1980-1997.

	1980	**1984**	**1987**	**1991**	**1992**	**1993/4**	**1995**	**1997**
Angola	300	90	–	50	50	10	–	0?
Botswana	30	10	<10	<10	5	4	0?	0?
Cameroon	110	110	30?	50	35	27	7	10[a]
CAR	3,000	170	10	5	0	–	–	–
Chad	25	5	3	0?	0	–	–	–
Ethiopia	20	10	–	0?	0?	5	1[a]	0?
Kenya	1,500	550	381	398	414	417	420[a]	424[a]
Malawi	40	20	25	5	0?	2	2	3
Mozambique	250	130	–	50	50	45	–	13[a]
Namibia	300	400	449	479	489	583	598	707
Rwanda	30	15	15	–	15	10	4	4
Somalia	300	90	–	0?	0	–	–	–
South Africa	630	640	577	771	819	897	1,024	1,043
Sudan	300	100	3	–	–	0	–	–
Swaziland	0	0	6	6	6	4	9	10
Tanzania	3,795	3,130	275	185?	127	132	32a	46
Uganda	5	0?	–	3	0	–	–	–
Zambia	2,750	1,650	>106	40?	40	33	0?	0?
Zimbabwe	1,400[b]	1,680[b]	>1,775	1,400	425	381[c]	315[c]	339
Total	**14,785**	**8,800**	**3,665**	**3,450**	**2,475**	**2,550**	**2,410**	**2,600a**

Totals have been rounded to the nearest 5 rhino. a Minimum estimates – speculative estimates for populations are not included. *b* The 1980 and 1984 official population estimates for Zimbabwe reflect minimum population estimates. In 1980 the true figure may have been more than 2,500. *c* The 1993/4 estimates for two Zimbabwe areas have subsequently been shown to be gross over-estimates, while the total number of black rhinos in known populations in intensive protection zones and sanctuaries remained stable from 1993/4 to 1995 and have since been increasing. ? estimate – reliable data unavailable. – data unavailable. (reproduced from Emslie & Brooks 1999)

1970 and 1997, representing over 22,000 rhinoceros, although it is accepted that this figure is an estimate and the true figure could be much higher (Martin *et al.* 1997).

1.3 *Diceros bicornis michaeli*

Due to East Africa's close socioeconomic links with the Middle East, the eastern subspecies *D. b. michaeli* was particularly badly hit by poaching (Western & Sindiyo 1972, Western 1982, Emslie & Brooks 1999). *D. b. michaeli* historically had its largest populations in

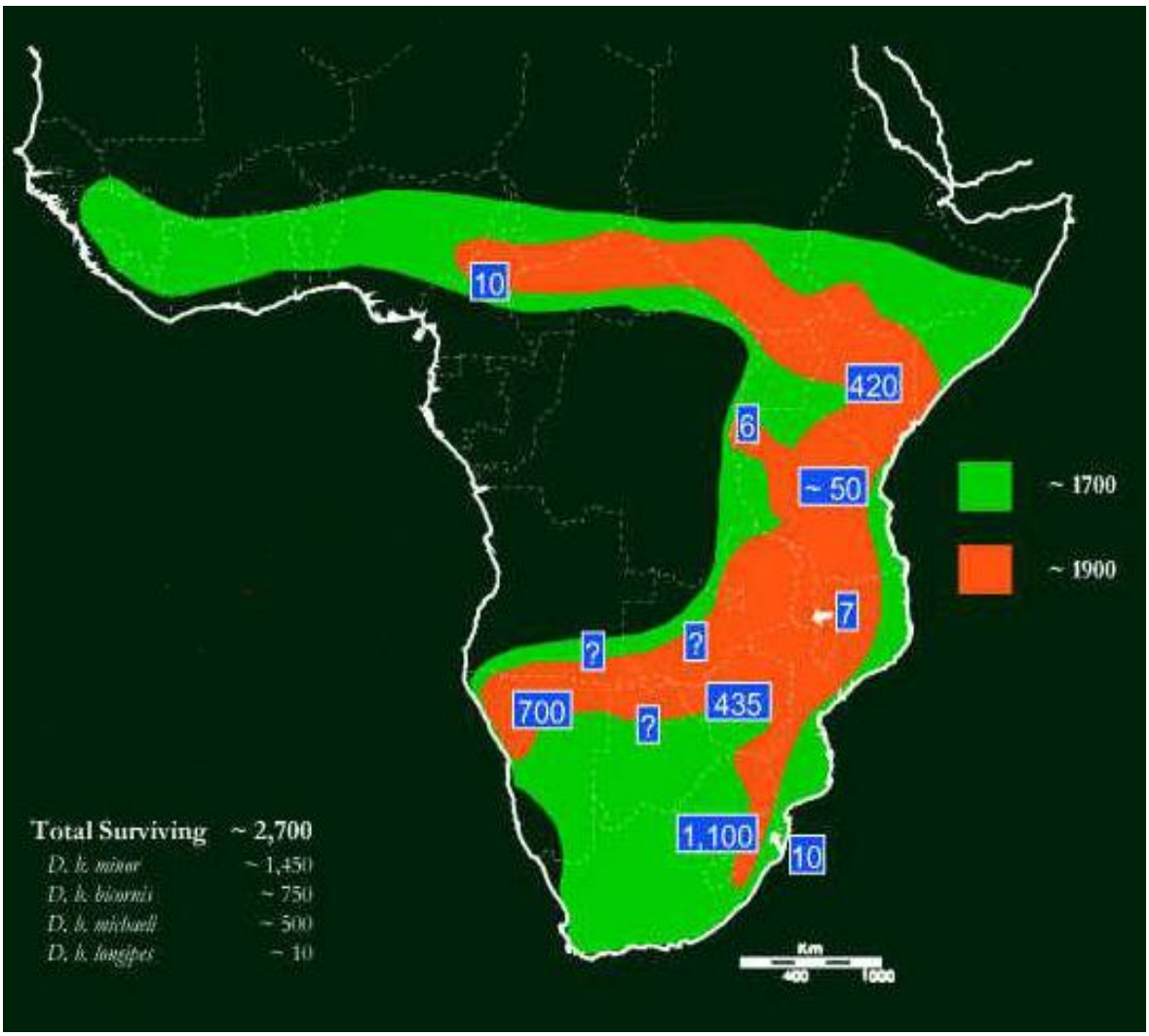

Figure 1.1. Black rhino historic distributions and country totals. Green area represents approximate black rhino distribution in ~1700 and red area represents approximate distribution in ~1900. Numbers in blue boxes represent approximate country totals in 2000 (reproduced from Foose 2000).

Kenya, but its range extended from northern Tanzania to Rwanda, Sudan, Ethiopia and Somalia. The subspecies is now extinct in Rwanda , Sudan, Ethiopia and Somalia, with only a couple of populations remaining in northern Tanzania (Emslie & Brooks 1999). Kenya had a population of over 20,000 black rhinoceros in 1970 but this was reduced to just 380 individuals by 1987, with the Tsavo National Park population reduced from approximately 9,000 to just 14 over this period (Brett 1993, Gakahu 1993, Emslie & Brooks 1999). Established trade routes with Somalia and Sudan provided overland access for the poached horn and the frequent dhow traffic along the coast provided a direct link with the Middle East (Martin & Vigne 2003). In 1984 at the height of the poaching the Wildlife Conservation and Management Department (WCMD) of the Kenyan government convened the Save the Rhino Committee (SRC). The committee implemented a strategy

(Kenya Rhino Project) of protecting all remaining black rhinoceros in the country within designated sanctuaries, where resources could be concentrated to counter the poaching threat (Leader-Williams 1989, Brett 1993). Initially four small fenced intensively protected areas were established within existing national parks and one private ranch and were stocked with animals from outside protected areas. In 1993 the Kenya Wildlife Service (KWS) (which superseded the WCMD) published the Conservation Strategy and Management Plan for Black Rhino with the aim of expanding the sanctuary system (Anon. 1993). Cooperation between KWS and NGOs led to the establishment of a number of new sanctuaries on private and government land with outlying animals and overstocked existing sanctuaries used to stock these new areas. Remnant populations in unfenced areas such as the Masai Mara National Reserve, Tsavo East National Park and the Aberdares National Park were provided with increased security and monitoring resources (Okita-Ouma *et al.* 2007). In 2000 KWS published the second black rhinoceros strategy (2000 to 2005) which placed an emphasis on establishing a standardised monitoring system for all rhinoceros within the sanctuary system, moving all remaining outlying animals into sanctuaries and a target set of at least 500 animals by the end of the strategy period (Anon. 2000). An individual-ID based monitoring system was implemented in all rhino conservation areas with information pertaining to births, deaths, calving periods and GIS data on individual sightings recorded into a central database (Amin *et al.* 2001, Okita-Ouma *et al.* 2007). Over the period of the second strategy, the Kenyan black rhinoceros population achieved an average growth rate of 9.43% and at the beginning of 2006, Kenya had a total of approximately 540 *D. b. michaeli* (~85% of the wild population) contained in 14 separate populations (Okita-Ouma *et al.* 2007).

With the sanctuary system largely successful in protecting remaining animals from the continuing threat of poaching, emphasis has shifted to biological management in order to promote recovery (Okita-Ouma *et al.* 2007). The latest conservation and management strategy for the Kenyan black rhinoceros population (2007-2011), aims for a minimum population growth rate of 6% per annum with a total population of 700 rhinos by 2011, and a long-term goal of 2000 animals as a viable metapopulation (Okita-Ouma *et al.* 2007). The core of the latest strategy is the active management of distinct populations as a metapopulation through the translocation of animals. This is to be undertaken in order to keep current sanctuaries below their ecological carrying capacity (ECC) and to maintain genetically and demographically viable populations through the controlled exchange of breeding individuals (Okita-Ouma *et al.* 2007). The latest strategy focuses on keeping

current populations below 75% of their ecological carrying capacity (ECC) in order to promote sustained population growth. With many sanctuaries currently exceeding this limit, emphasis is now on restocking former free-ranging areas in the form of Intensive Protection Zones (IPZ) where animals are placed into non-fenced areas but with high levels of security to ensure protection. With a target of only 150 animals within IPZs by the end of the current strategy, the sanctuary system still remains at the core of Kenyan black rhino conservation project. The separate black rhinoceros populations within the sanctuary system need to be managed as a single metapopulation to counter the elevated local extinction risks associated with small isolated populations (Gilpin & Soule 1986).

1.4 Metapopulation management

Population size is a major determinant of extinction risk with small populations experiencing an inherently higher risk of extinction due to genetic threats and stochastic fluctuations in survival, fecundity and environmental conditions (reviewed in Keller & Waller 2002). In light of the evidence for the impact of population size on population persistence the concept of minimum viable population size (MVP) is fundamental to many conservation management programs (Shaffer 1981, Shaffer *et al.* 2000). Minimum viable population size is defined as the minimum population size needed for a predefined probability of persistence for a given length of time, with MVPs generally derived through population viability analysis (PVA) (Shaffer 1981, Reed *et al.* 2003, Flather *et al.* 2011). However there is considerable debate about the utilitarian value of MVPs and the validity of estimates derived from PVAs due to paucity in long-term monitoring studies which are needed to determine accurate MVPs (reviewed in Flather *et al.* 2011, but also see Brook *et al.* 2011). Despite the debate about the validity of MVPs there is a consensus in the literature that minimum populations of several thousand are needed to ensure persistence in the long-term, i.e. >99% chance of persistence over 40 generations (Reed *et al.* 2003, Reed *et al.* 2004, Brook *et al.* 2006, Traill *et al.* 2007, Flather *et al.* 2011, Brook *et al.* 2011, Garnett & Zander 2011). Reed *et al* (2003) derived an MVP for the black rhinoceros of 6199 individuals for >99% probability of persistence for 40 generations, a value which is larger than the current combined population size of all three subspecies (N=4,880) (Emslie 2011). Current MVP estimates are of an order of magnitude larger than previous guidelines of an effective population size (N_e) of >50 to ensure population survival in the short-term and minimise the risk of inbreeding and 500 to retain evolutionary potential (Franklin 1980).

Whilst the impacts of random variances in demographic processes and environmental conditions on the persistence of small populations are well documented, the relative impacts of genetic factors are still the matter of some debate (Lande 1988, O'Brien 1994, Amos & Harwood 1998, Amos & Balmford 2001a, Frankham *et al.* 2002, Blomqvist *et al.* 2010). Genetic theory predicts that persistence at small population size after a bottleneck will lead to the erosion of genetic variability over time as a consequence of genetic drift and the random sampling of gametes (Fisher 1930, Wright 1931, Frankham 1996). A correlation between fitness and genetic variability has been demonstrated in many taxa, with drift subsequently expected to lead to a decline in fitness in small populations over time (Reed & Frankham 2003). However whether drift and an associated decline in fitness actually occur over time frames important for conservation is contentious (Lande 1988, Amos & Balmford 2001a, Reed & Frankham 2003, Spielman *et al.* 2004, Johansson *et al.* 2007, Rogell *et al.* 2010). Although the relative importance of genetic drift to conservation is the matter of some debate; the effects of inbreeding and resultant inbreeding depression in small populations on a time-scale relevant to conservation managers has been demonstrated in a range of laboratory, *ex situ* and wild populations (Charlesworth & Charlesworth 1987, Ralls *et al.* 1988, Saccheri *et al.* 1998, Keller & Waller 2002, Reid *et al.* 2007b, Blomqvist *et al.* 2010). Mating between relatives is an inevitable consequence of small population size and whilst inbreeding in the absence of selection does not alter allele frequencies from generation to generation, it leads to an increase in homozygosity through the redistribution of genotype frequencies (Charlesworth & Charlesworth 1999). Increasing homozygosity can result in inbreeding depression whereby fitness is reduced either by the increased expression of deleterious recessive alleles and/or as a consequence of a reduction in heterosis (Charlesworth & Charlesworth 1999).

Due to the large body of empirical evidence for inbreeding depression in a wide range of captive and wild populations, mitigation of the effects of inbreeding in small populations has been the primary focus for the genetic management of small endangered populations (Edmands 2007). Studies have shown that the fitness of small inbred populations can be increased significantly by the immigration of a relatively small number of unrelated immigrants (Westemeier *et al.* 1998, Madsen *et al.* 1999, Ingvarsson 2001, Vila *et al.* 2003, Johnson *et al.* 2010). In some cases the fitness benefits to the population are beyond that of the demographic contribution of the immigrant, a phenomena termed genetic rescue (reviewed in Tallmon *et al.* 2004).

The potential benefits of translocating individuals between genetically distinct populations are apparent in reducing inbreeding and promoting the recovery of small populations. Indeed many conservation breeding programs utilise breeding protocols that seek to maximise genomic divergence by mating genetically dissimilar individuals (Neff 2004, Boakes *et al.* 2007). This practice has to be weighed against the possible negative impact of outbreeding depression resulting from the mixing of genetically divergent populations. Until relatively recently a lack of empirical evidence for the manifestation of outbreeding depression in wild populations meant that its perceived implications for population persistence were secondary to those of inbreeding depression (reviewed in Edmands 2007). Outbreeding depression is the reduction in fitness from the mating of genetically divergent individuals either through extrinsic depression caused by the dilution of local adaptation and/or intrinsic depression caused by the disruption of epistasis in co-evolved gene complexes. (Lynch 1991, Fenster *et al.* 1997, Turelli & Orr 2000). Whilst the phenomenon of outbreeding depression has received more attention of late, data on its potential impact for population persistence is still significantly less than the body of evidence for inbreeding depression (Edmands 2007). Investigating outbreeding depression in wild populations is hampered by the apparent lack of depression in some taxa in the early stages of outcrossing (Allendorf & Luikart 2007). In F_1 generations from interpopulation crosses, epistasis is not disrupted as parental chromosomes are intact and it is not uncommon for heterosis to be exhibited in the F_1 generation (Coulson *et al.* 1998, Edmands *et al.* 2005). Outbreeding depression may not become apparent until subsequent generations when recombination between divergent parental chromosomes leads to a disruption of epistasis and a subsequent decline in fitness (Marshall & Spalton 2000, LeBas 2002, Edmands & Deimler 2004, Goldberg *et al.* 2005, Monson & Sadler 2010). Whilst long-term studies on outbreeding depression are rare, at least one study has shown that following interpopulation crosses in *Chamaecrista fasciculate* significant outbreeding depression was delayed until the F_3 after the F_1 had demonstrated heterosis (Fenster & Galloway 2000). If age at first male reproduction is used to approximate generation time, the generation time for the black rhinoceros is 7 years (Goetting-Minesky & Makova 2006). If outbreeding depression were not manifested in the black rhinoceros until the F_3 this would account for approximately 21 years, which in some cases is longer than many populations have been mixed under the Kenyan sanctuary system.

The two opposing but not necessarily mutually exclusive phenomena of inbreeding and outbreeding depression pose a serious dilemma for the management of the black rhinoceros metapopulation in Kenya (Marshall & Spalton 2000). Managers have to decide between active translocation to offset inbreeding and increase fitness or to avoid the mixing of genetically divergent populations in order to maintain local adaptation and the functioning of co-evolved gene complexes. The problem is further compounded by variance in susceptibility to inbreeding and outbreeding depression not just between different taxa but also within taxa depending on demographic history, environmental conditions and the fitness characters measured (Armbruster & Reed 2005, Allendorf & Luikart 2007). Taxa with expansive former ranges and historic low levels of inbreeding are expected to have a high genetic load and subsequently be more prone to the effects of inbreeding depression when reduced to small population sizes (Keller & Waller 2002, Boakes *et al.* 2007). Similarly those taxa which have historically had low interpopulation gene flow would be expected to have diverged more than those populations with high levels of gene flow and would subsequently be expected to suffer more from the breakdown of local adaptations through outbreeding (Edmands 2002, Mendleson *et al.* 2004). Environmental conditions are known to impact on the expression of inbreeding depression, with stressful conditions resulting in a more pronounced expression of the deleterious effects of inbreeding depression. Armbruster and Reed's (2005) review of the effects of environmental conditions on inbreeding depression based on 34 studies, found a 69% increase in inbreeding depression in stressful *vs* benign environments. Interestingly the opposite seems to be the case for outbreeding, with the detrimental effects of outcrossing in many cases apparently suppressed in stressful conditions (Edmands 2007). Several studies have shown that in stressful conditions heterosis is enhanced and one study has demonstrated a reduction in outbreeding depression (Barlow 1981, Armbruster *et al.* 1997, Edmands & Deimler 2004).

In light of the potentially detrimental effects of inbreeding and outbreeding depression, effective metapopulation strategies to promote persistence and recovery in endangered species are greatly facilitated by knowledge of the amount of divergence between populations and the genetic status of the populations within the system. The Kenyan black rhinoceros populations protected within the existing sanctuary system are a presumed mixture of historic populations. How representative the current sanctuary populations are of former free ranging populations or to what degree historic population structure has been maintained is unknown. In order for the Kenya Wildlife Service to implement an effective

management strategy, information is needed not just on the genetic status of the current populations, but also to what effect the translocation of animals has had on former population structure and to what degree this has led to the admixture of previously divergent populations.

1.5. Rhinoceros population growth

Many *in situ* and *ex situ* rhinoceros conservation programs are reporting poor population growth rates linked to either low or declining reproduction in small populations (Rabinowitz 1995, Adcock *et al.* 1998, Walpole *et al.* 2001, Mills *et al.* 2006, Swaisgood *et al.* 2006, Reid *et al.* 2007a, Okita-Ouma *et al.* 2008). The *ex situ* Sumatran rhinoceros (*Dicerorhinus sumatrensis*) program is deemed to have failed (Rabinowitz 1995); in 1984 with the *in situ* population experiencing a dramatic decline a captive breeding program was established for the species (Foose & Van Strien 1997). Forty wild Sumatran rhinoceros captured in Malaysia and Indonesia were used to establish captive breeding programs in the UK, US, Malaysia and Indonesia (Khan *et al.* 1999). However despite intensive research into breeding and reproduction in the species only one calf was born from the forty captive animals (Rabinowitz 1995, Foose & Van Strien 1997, Khan *et al.* 1999, Roth 2002, Steinetz *et al.* 2005). Sumatran rhinos are induced ovulators and were exceptionally difficult to manage in captivity. There was high mortality in the *ex situ* population and the four surviving captive rhinoceros were transported back to a purpose built breeding center in Sumatra (Rabinowitz 1995, Steinetz *et al.* 2005) . Similarly the *ex situ* program for the white rhinoceros (*Ceratotherium simum*) is classed as failing with the current population having a -3.5 % growth rate (Schwartzenberger *et al.* 1999, Swaisgood *et al.* 2006). Much of the reproductive problems with the captive population reside with poor reproduction in F_1 females born to previously wild F_0 (Swaisgood *et al.* 2006). Despite several studies on captive white rhino examining the underlying causes behind low reproduction no definitive cause has been ascertained although given that males show similar preference for F_1 females as $F_{0,}$ reproductive failure appears to occur post-copulation (Patton *et al.* 1999, Brown *et al.* 2001, Swaisgood *et al.* 2006). Whilst there is no literature available on current *ex situ* black rhinoceros reproduction rates, poor or negative population growth has been reported for several *in situ* populations including the Ngorongoro Crater (Tanzania) (Mills *et al.* 2006), Masai Mara (Kenya) (Walpole *et al.* 2001), Ngulia Rhino Sanctuary (Kenya) (Okita-Ouma *et al.* 2008), Hluhluwe-Umfolozi Park (South Africa) (Reid *et al.* 2007a) and Pilanesberg National Park (South Africa) (Adcock *et al.* 1998). As in the case of *ex situ*

populations the proximate causes underlying poor population growth in many *in situ* black rhinoceros populations are far from clear. The black rhinoceros population in Ngorongoro Crater declined from approximately 110 animals in 1960 to 20 by the mid 1970's as a result of poaching, however since then in the absence of poaching the population has never increased above 22 animals (Mills *et al.* 2006). Several factors have been postulated as possible causes for the lack of population growth including predation by hyenas, loss of calving areas through habitat change and competition from elephant and buffalo, however no definitive cause has been identified (Mills *et al.* 2006). Similarly the Masai Mara population was reduced from over a 100 in the 1960s to just 20 animals in 1988 as a result of poaching (Walpole *et al.* 2001). With increased security resources made available in the early 1990s the population partially recovered to 31 animals in 1994 but then decreased to 22 animals in 1999 (Okita-Ouma *et al.* 2007). Again the proximate cause for the lack of population recovery are not clear but the possible dispersal of the Mara animals into northern Serengeti has been suggested as a possible explanation although lack of sufficient monitoring led to uncertainty about the cause for the lack of recovery (Walpole *et al.* 2001).

Growth rates in black rhinoceros populations are thought to be heavily density-dependent and poor or declining population growth rates in several black rhinoceros populations have been postulated to be caused by the ecological carrying capacity (ECC) of the area being exceeded (Emslie & Brooks 1999, du Toit 2006, Okita-Ouma *et al.* 2007, Emslie *et al.* 2009). The Ngulia Rhino Sanctuary is a classic example of a rhinoceros population with limited population growth due to the ECC of the sanctuary being exceeded. In 2005 Ngulia contained 65 black rhinoceros and over 250 elephants in an area of 62 km^2 (Okita-Ouma *et al.* 2008). The availability of rhinoceros food plants had declined by 59%, with the rhinoceros population growth rate dropping to 3.1% between 2003 and 2005 compared to 7.7% between 1998 and 2001 (Okita-Ouma *et al.* 2008). With the ECC of sanctuary obviously exceeded, KWS removed 200 elephants and expanded the sanctuary to 88 km^2. The Ngulia rhinoceros sanctuary may however represent an extreme example of poor growth rate linked to exceeding ECC. A meta-analysis of three Kenyan black rhinoceros sanctuaries examining the interrelationship between growth rate and density failed to find that density dependence had an impact on the estimated growth rate in any of the three populations examined, despite a high variance in growth rate and density among the three populations (Okita-Ouma *et al.* 2009). The Hluhluwe-Umfolozi Park in South Africa is one of the most important black rhinoceros areas in Southern Africa and the source

population for many of the current 25 *D.b minor* populations in the region (Reid *et al.* 2007a). The park has been experiencing a negative growth rate of -1.29 since 1990, with a steady decline from 429 individuals in 1993 to 325 in 2000 (Reid *et al.* 2007a, Linklater & Hutcheson 2010). Despite reducing the population below the ECC, the population growth rate has not recovered due to the potential disruption of breeding pairings through the removal of animals (Linklater & Hutcheson 2010).

Given the number of rhinoceros conservation programs both *in situ* and *ex situ* which are reporting poor or negative growth rates linked to poor breeding performance, an examination of the genetic factors influencing mate choice and reproduction in *in situ* populations would be extremely valuable in helping to understand the driving factors behind the variance in rhinoceros reproduction.

1.6 Genetic influences on mate choice

In most species females are predicted to be the choosier sex when it comes to mate choice, due to a higher investment in gametes and the raising of offspring (Tregenza & Wedell 2000). Mate choice is critical in gaining direct or indirect benefits to maximise reproductive success and the fitness of offspring (Andersson 1994). Direct benefits can entail the provision of resources such as food, parental care and protection from predation. Recently the indirect benefits of mate choice have received considerable attention, most particularly the fitness benefits conferred to offspring from inherited alleles (reviewed in Tregenza & Wedell 2000). Previous work has focussed on the dichotomy between the additive and non-additive genetic effects involved in mate choice (reviewed in Mays & Hill 2004). Additive effects or the 'good genes' hypothesis is the choice by females for males with superior genetic quality, typically advertised through ornamental traits such as antler size in deer or coloured plumage in birds. The paradox of mate selection for additive traits (termed the 'lek paradox') is that variation for these traits will soon become exhausted as increased mating success by males bearing them will ultimately lead to fixation (Tomkins *et al.* 2004). A solution to the lek paradox is the non-additive 'compatible genes' hypothesis where the genetic quality of an individual is less important than the interaction between male and female genotypes (Mays & Hill 2004, Charpentier *et al.* 2008a). Disassortative mate choice whereby females select mates with dissimilar genotypes to their own is a potential mechanism whereby females can increase heterozygosity and thereby the fitness of their offspring as well as avoiding inbreeding and

the risk of deleterious recessive genes being expressed (Penn 2002, Mays & Hill 2004, Garcia-Navas *et al.* 2009). Studies on disassortative mate selection have focussed on the increased reproductive success and fitness benefits associated with increased heterozygosity in many taxa; heterozygote advantage or heterozygosity fitness correlates (HFC) (reviewed in Chapman *et al.* 2009). Several recent studies have found heterozygosity to be correlated with reproductive success (Seddon *et al.* 2004, Charpentier *et al.* 2005, Kempenaers 2007, Garcia-Navas *et al.* 2009, Thoss *et al.* 2011), survival (Coltman *et al.* 1998, Townsend *et al.* 2009, Huchard *et al.* 2010) and disease resistance (Reid *et al.* 2005, Charpentier *et al.* 2008b).

Much of the work on the 'compatible gene' hypothesis has focussed on disassortative mate choice for the major histocompatibility complex (MHC). The MHC is a multigene family which is a fundamental part of the immune system in vertebrates (Havlicek & Roberts 2009). Glycoproteins encoded by MHC genes are responsible for recognising and binding to peptides of foreign origin and then presenting these peptides to T-cells which initiates an immune response. The amino-acid sequence of the peptide binding region determines which antigens can be bound and subsequently which pathogens the host can initiate an immune response against (Janeway *et al.* 1999). The MHC loci are extremely polymorphic and express high intra-population polymorphism; diversity is selectively maintained and whilst there is some debate about the exact selective pressure maintaining polymorphism a general consensus is that polymorphism is maintained through some form of balancing selection (Havlicek & Roberts 2009). MHC genes are co-dominantly expressed and therefore individuals with high levels of heterozygosity for MHC loci are expected to be at an advantage against a greater range of pathogenic pressure (heterozygote advantage) (Havlicek & Roberts 2009). Initially work on disassortative MHC-based mate selection was undertaken under laboratory conditions and reported that female mice exercised mate preference for males with dissimilar MHC-genotypes (reviewed in Jordan & Bruford 1998). The main criticism of this early work is that it was undertaken on inbred laboratory mice which were ultimately derived from a very small gene pool (Hurst 2009, Roberts 2009). However subsequent studies on natural populations have reported MHC-disassortative mate preferences and lower homozygosity than expected under random mating in a range of taxa including birds (Freeman-Gallant *et al.* 2003), humans (reviewed in Havlicek & Roberts 2009), non-human primates (Schwensow *et al.* 2008, Huchard *et al.* 2010, Setchell *et al.* 2010), mice (Penn & Potts 1999), reptiles (Olsson *et al.* 2003, Miller *et al.* 2009) and fish (Landry *et al.* 2001, Consuegra & Garcia de Leaniz 2008).

Evidence for MHC-based dissassortive mate selection has led to a body of work on how individuals are able to assess the MHC-genotypes of potential mates. Research on laboratory mice which has been supported by a couple of studies on natural populations indicates that scent plays a critical role in mediating sexual behaviour (Hurst *et al.* 2001, Charpentier *et al.* 2008a, Hurst 2009, Setchell *et al.* 2011). MHC products have been linked to individual odour-types which are a result of the excretion of MHC-gene products in bodily secretions (Hurst 2009, Setchell *et al.* 2011). Evidence suggests that the mammalian vomeronasal organ (VNO) has the ability to assess these volatile MHC odour-types in addition to other volatile proteins such as major urinary proteins (MUPs) another multigene family which have been shown to convey information used to assess kin and genetic heterozygosity (Sherborne *et al.* 2007, Hurst 2009).

Whilst the number of studies examining mediation of mate selection by functional gene complexes is rather scant, there is a much larger body of work utilising neutral markers which have demonstrated similar trends to the work on functional genes (Mays & Hill 2004, Hansson *et al.* 2004). A large number of studies have reported heterozygosity fitness correlates linked to microsatellite heterozygosity (reviewed in Chapman *et al.* 2009). Moreover dissassortive mate selection has been reported using measures of relatedness based upon microsatellite genotypes for a range of taxa (Mainguy *et al.* 2009, Wilmer *et al.* 2000, Amos *et al.* 2001, Blomqvist *et al.* 2002, Foerster *et al.* 2006, Roberts *et al.* 2006, Bishop *et al.* 2007). Whilst examples of HFCs based on MHC genotypes is an example of the direct effect hypothesis, whereby the markers themselves are responsible for observed fitness correlates (Grueber *et al.* 2008), HFCs based on correlations with neutral markers are attributed to the indirect effect hypothesis (Hansson *et al.* 2004). The indirect hypothesis can be subdivided into the general effect hypothesis whereby an HFC for the neutral markers examined is representative of genomic heterozygosity; or the local effect hypothesis where the neutral marker under study is in linkage disequilibrium with a fitness locus (Hansson *et al.* 2004, Grueber *et al.* 2008). The general effect hypothesis is most evident in cases of inbreeding where homozygosity across the whole genome is increased (Grueber *et al.* 2008). Local effects however may still confer HFCs in the absence of inbreeding (Hansson & Westerberg 2002). Recent studies have shown that linkage disequilibrium can extend over large regions of the chromosome and can be maintained for hundreds of generations (reviewed in Chapman *et al.* 2009). Moreover increased linkage disequilibrium can be expected in populations which have recently undergone a severe

bottleneck or have been recently admixed; situations which are common in conservation priority species (Grueber *et al.* 2008).

Recent theoretical and empirical work shows that in many cases mate selection based purely on maximum dissimilarity or 'superior' genes is rather simplistic and that the genetic factors underlying mate selection are rather more complex (Roberts 2009). In particular several studies have shown that additive and non-additive benefits are actually compounded in natural populations with individuals (particularly females) selecting dissimilar partners which are modulated by additive benefits of high genetic diversity. Roberts *et al* (2006) study on MHC loci in humans and peafowl at microsatellite loci showed that average levels of allele sharing and relatedness were significantly correlated with heterozygosity. Hoffman *et al* (2007) similarly found that female fur seals actively balance mate choice according to genetic diversity (IR) and dissimilarity with conspecifics. Moreover recent work strongly suggests that the most genetically complementary mate is not the maximally genetic dissimilar mate. The manifestation of outbreeding depression when individuals with divergent genetic backgrounds mate as a consequence of dilution of locally-adapted genomes and/or the disruption of epistasis in co-evolved gene complexes is well documented (Marshall & Spalton 2000, LeBas 2002, Edmands & Deimler 2004, Goldberg *et al.* 2005, Monson & Sadler 2010). Even for intrapopulation mating there is recent compelling evidence that individuals will select mates with intermediate levels of dissimilarity (Neff 2004, Roberts 2009). Studies on sticklebacks have shown that individuals with very high numbers of MHC alleles have a higher parasitic load and are less desirable as mates (Milinski 2003), with tetraploid *Xenopus* frogs shown to silence half their MHC genes (reviewed in Penn & Potts 1999). Thymic selection on T-cell clones has been shown in a number of studies to reduce pathogen resistance in individuals with very high numbers of MHC alleles (Nowak *et al.* 1992, Penn & Potts 1999) (Figure 1.2). Furthermore two other distinct (but not necessarily exclusive) models of balancing selection for MHC polymorphism have been proposed other than heterozygote advantage, which favour locally adapted genotypes over maximally dissimilar genotypes. Under the frequency-dependent model (Red Queen hypothesis), rare alleles are favoured by selection as parasites which are able to evade the most common MHC genotypes are at a selective advantage (Apanius *et al.* 1997, Spurgin & Richardson 2010). Pathogens which are able to rapidly evolve peptides which don't bind with the host-genotype MHC products will rapidly spread through the host population. The spread of the pathogen is then brought under control by the spread of a previously rare allele which enables the host to counter the

variant form. Under the fluctuating-selection model, polymorphism is maintained in the absence of heterozygote advantage due to spatial and temporal variance in a particular pathogenic pressure, therefore under this model locally adapted genotypes will be favoured. Recent empirical studies on natural populations have produced results consistent with the intermediate strategy of mate selection (Roberts 2009). In a controlled experiment on near-natural conditions using sticklebacks, mating was random with regards to the genetic background (microsatellites) but significantly non random with regards to MHC-genotype.

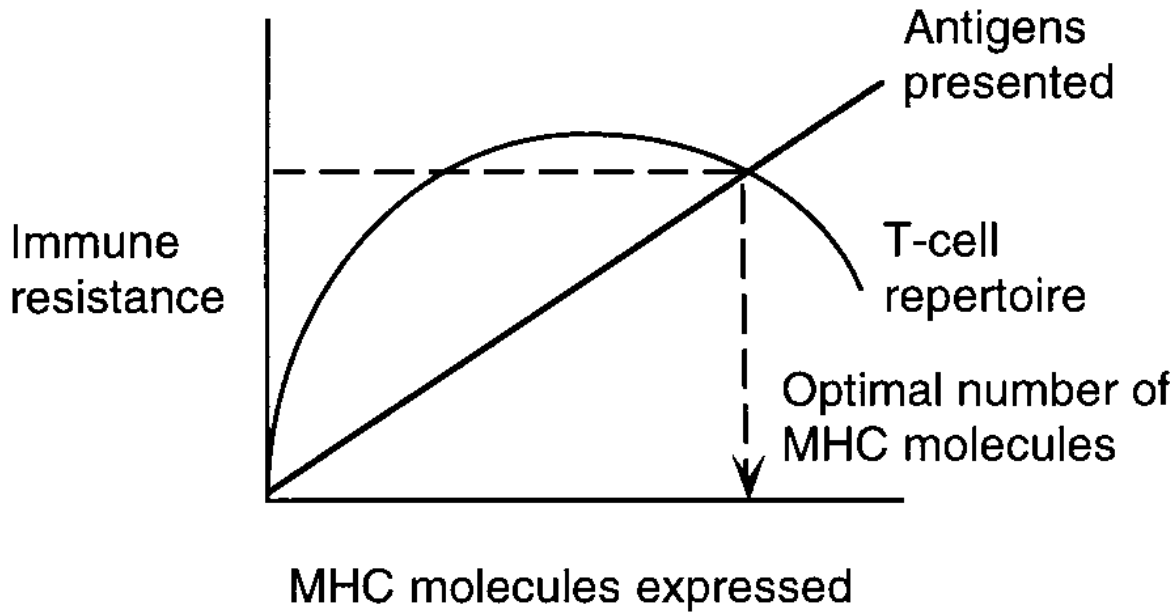

Figure 1.2 Increasing the number of MHC molecules expressed during ontogeny will initially increase immunological resistance by increasing both the diversity of antigens presented and increasing the number of T-cells preserved during thymic selection. However, at some point, increasing the number of MHC molecules expressed should cause a net loss of T-cells as negative thymic selection exceeds positive selection. This trade-off between increasing antigens presented and T-cell depletion is thought to maintain multiple MHC loci and prevent the further duplication of MHC loci. It also suggests that selection might favor individuals with an optimal number of loci and an optimal level of MHC heterozygosity (reproduced from Penn & Potts 1999).

Most significantly the mating distributions were at an optimal level of divergence with no selection for similar or dissimilar mate strategies (Eizaguirre *et al.* 2009). In wild tiger salamanders mating preference was for MHC-similar individuals but the population had no homozygote excess which again indicates mate selection for an intermediate level of similarity (Bos *et al.* 2009). Whilst these studies are probably examples of direct effects,

Neff (2004) demonstrated stabilising selection for genomic divergence in wild bluegill sunfish using microsatellite markers.

The factors influencing mate choice are very much context dependent, with individuals probably excising mate selection by comparative evaluation of the choices available (Bateson & Healy 2005). However a consensus is emerging whereby additive and non-additive indirect benefits of mate choice are compounded. Moreover there is compelling theoretical and empirical evidence that mate choice should ultimately be based upon intermediate levels of genetic dissimilarity.

1.7 Thesis aims

To date the genetic factors influencing mate choice have not been examined in a population subject to active conservation management. With the recovery of endangered species dependent on reproductive rate in the absence of deterministic factors driving decline, the genetic influences on mate choice are potentially of great importance. Many *in situ* and *ex situ* conservation programs are reporting low or declining population growth in endangered species as a result of poor reproductive rates. The genetic factors influencing mate choice in such populations are potentially profound as many populations are small admixed populations where individuals are presented with a limited number of potential mates, representing a great spectrum of genetic divergence than would typically be present in non-managed populations. Particularly the potential for preferential mate selection for mates with intermediate levels of genetic divergence has profound implications for conservation breeding protocols which typically seek to maximise genomic divergence by mixing hitherto isolated populations to mitigate the effects of small population size (Neff 2004).

The Kenyan black rhinoceros metapopulation is typical of such small admixed populations, where the amount of admixture in current populations or the historic population structure before human intervention is unknown.

The primary aims of this thesis are to;

1) Determine the level of genetic diversity and range of historic populations captured within the current Kenyan black rhinoceros metapopulation

2) Determine the level of admixture which has occurred between historic populations

3) Reconstruct pedigrees of potentially admixed sanctuary populations using genetic markers

4) Examine the underlying genetic influences behind mate selection in these populations to determine the relative effects of additive and non-additive benefits and to examine the preferential level of dissimilarity between mates.

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Chapter 2

Study Populations

2.1 Introduction

Of the fourteen populations of black rhinoceros in Kenya, animals from five populations were sampled during this study (Appendix 2.1). Three populations formed the core of the work; at least 92% of the animals in each of these populations were sampled and genotyped. In total, 166 black rhinoceros were sampled and genotyped during the study, which represents ca. 31% of the Kenya black rhinoceros population as of 2005 (*n*=539) (Table 2.1) (Okita-Ouma *et al.* 2007). Sampling and subsequent genotyping was conducted on positively identified animals in all cases. Genotyping was undertaken on DNA derived from a variety of biological sample types; genotyping of DNA extracted from faeces was the method of choice for two of the three core study populations. Where available sources of high copy number DNA were used in the work: mostly these took the form of tissue collected during veterinary procedures such as ear notching for identification or during translocations between reserves. In addition to tissue and faeces, 20 serum samples collected from the founder animals of the Mugie Rhino Sanctuary were also genotyped (Table 2.2).

Table 2.1. Black rhinoceros populations sampled and percentage of population included in study.

Population	n_p	***n***	**%**
1. Ol Pejeta Conservancy (OPC)			
Existing population (2005)	50	46	92%
New introduced population (2007)	24	24	100%
2. Lewa Wildlife Conservancy (LWC)	45	42	94%
3. Mugie Rhino Sanctuary (MRS)	28	28	100%
4. Ngulia Rhino Sanctuary (NRS)	54	19	35%
5. Nairobi National Park (NNP)	74	7	9.50%
Total	**275**	**166**	**60%**

n_p = census population size. n = number of animals sampled. % = percentage of census size sampled.

Table 2.2. Sample type by population.

Population	n_f	n_t	n_s	n_{ft}
1. Ol Pejeta Conservancy (OPC)	22	48	0	15
2. Lewa Wildlife Conservancy (LWC)	33	9	0	6
3. Mugie Rhino Sanctuary (MRS)	8	0	20	0
5. Ngulia Rhino Sanctuary (NRS)	0	19	0	0
6. Nairobi National Park (NNP)	0	7	0	0
Total	**68**	**79**	**20**	**21**

n_f = number of animals genotyped from DNA extracted from faeces. n_t = number of animals genotyped from DNA extracted from tissue. n_s = number of animals genotyped from DNA extracted from serum. n_{ft} = number of animals genotyped from DNA extracted from both faeces and tissue.

2.2. Ol Pejeta Conservancy

2.2.1 Overview

Ol Pejeta Conservancy (OPC) ($36^{O}55$'E $00^{O}02$'N) is a 365 km^2 wildlife conservancy located in the Laikipia District of Kenya on a plateau between Mount Kenya and the Aberdare mountains. The main entrance to the conservancy is approximately 20 km from the town of Nanyuki, which is located at the foot of Mount Kenya. The equator bisects the southern quarter of the conservancy.

At the beginning of the study in 2004 Ol Pejeta Conservancy was subdivided into Sweetwaters Game Reserve (SWG) and Ol Pejeta Ranch both of which were privately owned by Lonrho Africa PLC. The reserve was originally part of the ranch before it was designated as a game reserve in 1989: predominantly for the conservation and management of black rhinoceros. The 96 km^2 reserve received 19 founding animals between 1989 and 1993 with the majority coming from either neighbouring Solio Game Reserve (SGR) or Nairobi National Park (NNP) with one male originating from Lewa Wildlife Conservancy (LWC) (Appendix 2.2). In 2004, the Ol Pejeta Ranch and Sweetwaters Game Reserve were purchased by the UK-based conservation organisation 'Flora and Fauna International' and became the Ol Pejeta Conservancy. In 2007, the former dividing fence between the reserve and ranch was removed and the conservation area was expanded into the former ranch to form a 365 km^2 conservancy and the largest black rhinoceros sanctuary in East Africa. In February 2007, an additional 24 black rhino (NOPC) were translocated into the new area from neighbouring Solio Game Reserve.

2.2.2 Climate

The Laikipia region normally experiences two periods of heavy rainfall each year: the short rains occur March to April and the long rains October to December. The average yearly rainfall ranges from 600 mm to 800 mm. Ol Pejeta Conservancy is at an altitude of approximately 1,600 m above sea level and temperatures range from 7.6 ^{O}C at night to 29 ^{O}C during the day. Day time temperatures tend to rise as the wet season approaches and decrease with the onset of rain.

2.2.3 Habitat

Laikipia District is characterised by two main soil types: 'black-cotton' and 'red-sand loam'. Ol Pejeta Conservancy consists entirely of 'black-cotton' soil; this has high clay content with high levels of calcium carbonate, which impedes drainage (Young *et al*, 1995).

The Ewaso Ngiro River flows year-round from south to north close to the western boundary of the former reserve. During the dry season the water level is low enough to permit the movement of animals across the river at a number of crossing points. During the rainy season, the river, which is fed by heavy rains from the Aberdares Mountains, is impassable for long periods except via the conservancy's single bridge spanning the river in the south. A number of seasonal streams flow into the river; these have carved longitudinal depressions, running east to west, across the former reserve. Throughout the conservancy, dams and bore holes provide year-round water for wildlife and cattle.

The conservancy can be broadly divided into three main habitat types: *Acacia drepanolobium* bush, *Euclea divinorum* bush and grass plains. The areas with the highest soil water content, typically the stream depressions, are characterised by *Euclea divinorum* bush with associated shrubs such as *Rhus natalensis, Scutia myrtina* and *Rhamnus staddo*. With increasing elevation and drier soil content the vegetation transitions into *Acacia drepanolobium* bush and finally into grassland plains, which are dominated by *Thermeda triandra* and, in more heavily grazed areas, by C*yndon dactylon*. Riverine vegetation comprises dense *Euclea* bush and *Acacia xanthophloea* trees, with scattered *A. xanthophloea* trees found throughout the conservancy in wetter areas (Figure 2.1).

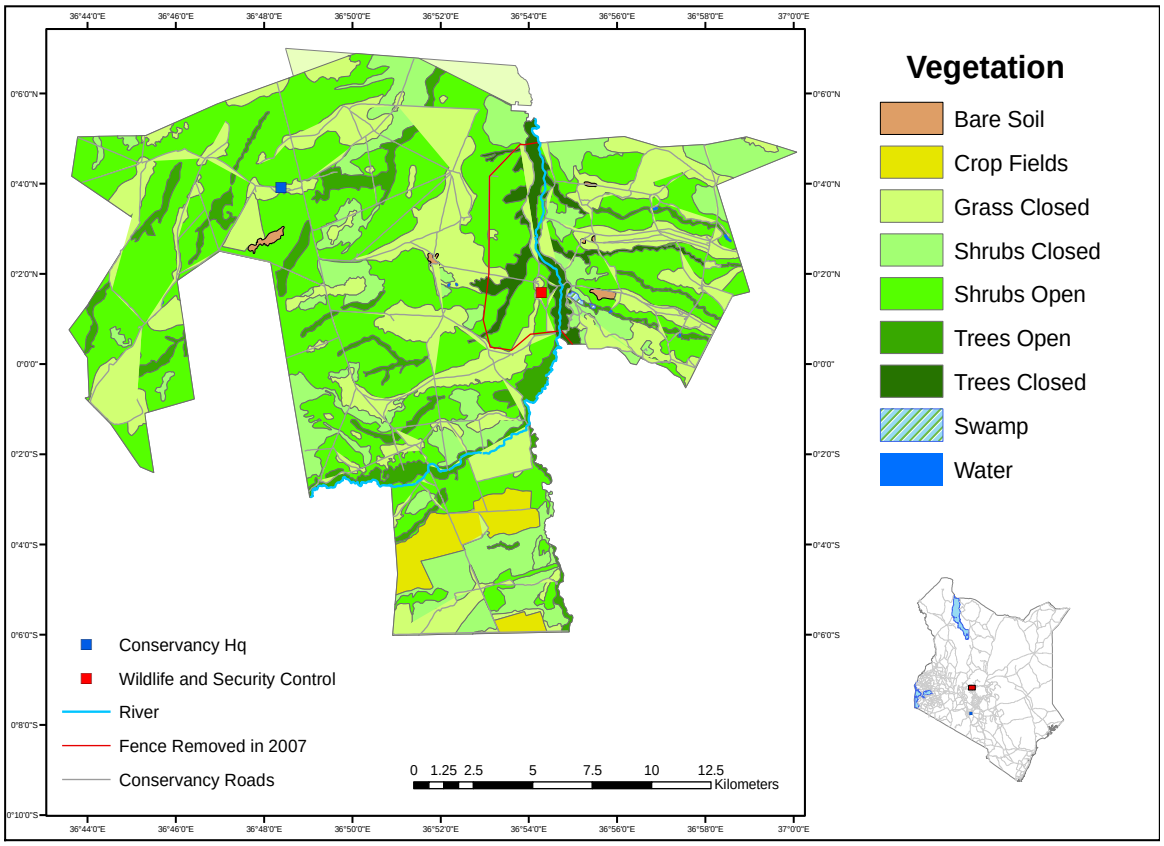

Figure 2.1. Vegetation map of Ol Pejeta Conservancy showing former dividing fence between the old ranch and reserve**.**

2.2.4 Sample collection

The main period of faecal sampling took place at Ol Pejeta between May and October 2004 and June and September 2005. Additional faecal samples were collected between June and August 2006. At the start of the study, Ol Pejeta Conservancy had 48 recorded black rhinoceros within the Sweetwaters Game Reserve. These animals were monitored and protected daily by a team of approximately 28 armed rangers whose responsibility was to locate and identify all the rhinoceros within their patrol area. A number of the founding animals within the reserve had been ear notched during their translocation to facilitate monitoring and identification. However, these were relatively few in number (N ~ 18) with the majority of the 48 animals (N ~ 30) un-notched, particularly the sub-adults and calves. Monitoring and identification of un-notched animals relied on a combination of association with notched animals (e.g. mothers and calves), distinctive body characteristics, particularly horn shape, size, sex and home range.

Monitoring of black rhinoceros in Sweetwaters Game Reserve was further complicated by the thick vegetation, particularly *Euclea divinorum* and associated shrubs: this is the black rhinoceros' preferred habitat particularly during the day. The difficulties of monitoring the

animals within Ol Pejeta were highlighted by the identification of an additional three animals in 2006 that had not been previously recorded; one adult female, and one sub-adult and calf which were presumed to be the offspring of the adult female.

At the start of the study there were no tissue samples available for any of the black rhinoceros within the Ol Pejeta Conservancy. The initial stages genotyping was to rely solely on DNA extracted from faeces; therefore the fieldwork at Ol Pejeta was undertaken with the aim of collecting fresh faecal samples from every black rhinoceros within the reserve.

All sample collection conducted at Sweet Waters Game Reserve was accomplished on foot with the aid of one of the black rhinoceros monitoring patrols. The area to be searched each day would be decided upon depending on a particular target animal and in accordance with the monitoring patrols remit of monitoring all the animals in a given area. Searches of the target area were broken down into several phases. Initially the roads and areas around water holes were checked for signs of fresh rhino spoor; if any were found then these would be followed in an attempt to locate the animal. If no fresh spoor was found, the patrols moved into an area where, based on their experience, they believed a particular rhino may be located and a sweep of the area was undertaken.

When a rhinoceros was located, it was followed until it did one of the following: defecated, went down on a bedding site (usually around mid-morning) or detected our presence and ran away. It would appear that rhinoceros usually defecate just before going down on a bedding site, around mid morning or just after getting up from it, around mid afternoon. If the animal went down onto a bedding site without defecating, then the location would be recorded on a GPS and an attempt would be made to collect a sample in the afternoon.
When an animal was observed defecating, a sample was collected once the animal had left the area. All the sample collection equipment was carried in pouches on army webbing. This method enabled unused pots, used pots, gloves, forceps, waste etc to be kept physically separated in order to minimise the chances of cross-contamination. Two samples of about 5 g each were collected from each animal, following the same procedure as that used to sample the captive animals' faeces. In those instances where contamination from another stool was a possibility, then a sample was only taken from the inside of the stool. The identity of the animal was recorded according to a scoring system based on the reliability of the identification (ID); that is, ear notches, size, sex, mother calf pairings etc.

The date, time, location, sex and other pertinent information were also recorded including possible alternative IDs where identification was ambiguous. Gloves were worn throughout the sample collection procedure and new, sterile forceps and spatulas were used for each sample. About 5 g of sample were added to approximately 20 g of silica gel and the pots were sealed with Parafilm™ (SPI Supplies). The pots were then labelled and taken back to the research centre for storage at room temperature. At every stage of sample collection, every effort was made to reduce the chances of cross contamination.

The description above is an example of an ideal sample collection episode, with regards to individual identification. However, there were many instances when this was not the case. In nearly 60% of cases, the identity of the animals from which samples originated was questionable: this was mainly due to an incomplete sighting of the target animal. It became apparent during the course of sample collection, that the typing of sex genes would prove invaluable in identification information of a given sample.

If a fresh sample was found before the location of an animal, it was collected and every effort was made to locate the animal in the vicinity. If an animal was found within the vicinity then its identification was recorded next to that of the sample, according to a predefined scoring system. The scoring system was based upon a number of points being allocated to a sample: four points was the highest score and was assigned in those cases where identity was certain, this went down the scale to one point, which was assigned to samples where the identity was completely unknown. Sex gene typing would quickly rule out any animals of the wrong sex in those instances where the animal could be sexed by the patrols. Similarly, sex gene typing, coupled with very good home range data, would enable the identity of some samples to be determined, such as in those cases where samples from a mother and calf or two sub adults were collected. In some cases it was difficult to distinguish between the middens of mothers and larger calves; again, sex gene typing would assist in assigning the correct genotype to a given animal.

Some of the animals in Ol Pejeta were relatively easy to locate and follow until they produced a sample, with the patrols finding them every three to four days. However, some of the animals were very shy and difficult to find and might not be found for up to a month at a time: this made the sample collection very difficult for these animals and unfortunately a couple of individuals were missed from the study. Over the period of the fieldwork, a total of 82 fresh faecal samples were collected corresponding to 46 animals (Figure 2.2).

Towards the end of the sample collection at Ol Pejeta the rhinoceros monitoring protocols were changed under the direction of a new wildlife manager: the number of monitoring patrols was increased, as was the training in identification and monitoring therefore the requirements for the maximum number of days between individual sightings was reduced.

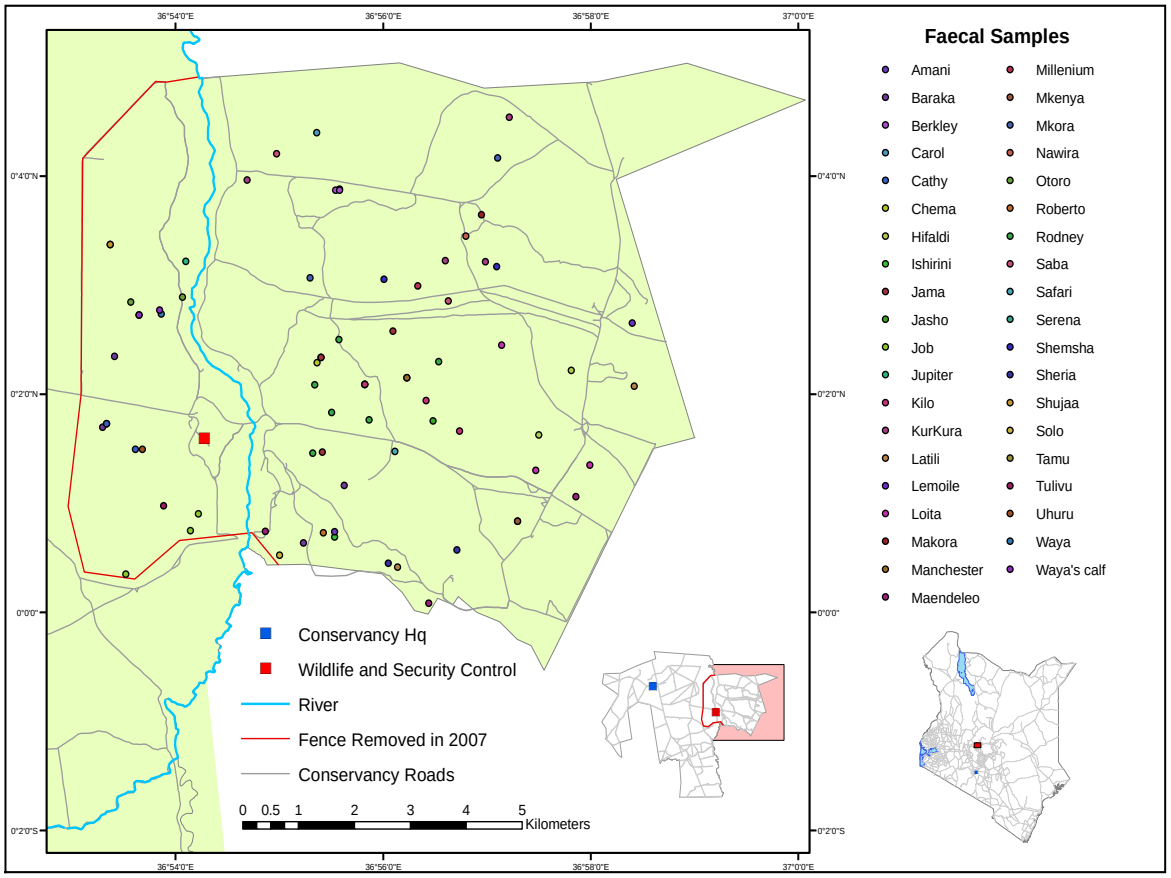

Figure 2.2. Map showing faecal samples collected for Ol Pejeta Conservancy

2.2.5 Tissue samples

Whilst the new management guidelines for the monitoring of black rhino within Ol Pejeta came into force after the main faecal sample collection had finished, a large number of 'clean' (un-notched) animals were ear notched in 2006. This ear notching exercise was undertaken to improve monitoring efficiency and was targeted predominantly at sub-adult animals as these were the most difficult to positively identify. In total, 19 animals were ear notched in the exercise, including the three newly discovered animals. Ear tissue samples from all these individuals preserved in 70% (v/v) ethanol were made available to the study. Many of the animals from which these samples derived also had faecal samples collected during the prior fieldwork; this enabled an examination of the reliability of the faecal sampling (Table 2.3). In addition, an old dominant male (Otoro) had died and been buried in the reserve during the sample collection period when the researcher was not present in

Kenya. This animal was exhumed by the researcher and a skin sample collected. A further round of ear notching was conducted in 2008 on five animals that were too young for notching in 2006; these ear tissue samples were also made available to the study.

Table 2.3. Samples collected from animals at Ol Pejeta showing sample type and the best sample score.

	Name	Sex	ID Code	Age class	Mother	Father	Birth Date	Origin	Sample	Sample Score
1	Morani	M	4001	A	Amboseli cow	Amboseli bull	5/19/76	Amboseli	faecal	****
2	Baraka	M	4017	A	Carol	OPC bull	11/20/94	OPC	faecal	****
3	Safari	M	4024	SA	Carol	OPC bull	11/26/98	OPC	faecal	****
4	Ishirini	F	4019	A	Chema	OPC bull	5/17/96	OPC	faecal	***
5	Mkora	F	4014	A	Kilo	OPC bull	3/1/94	OPC	faecal	***
6	Latili	F	4025	SA	Kilo	OPC bull	2/25/99	OPC	faecal	***
7	Waya	F	4018	A	Mia	OPC bull	2/4/95	OPC	faecal	***
8	Carol	F	46	A	NNP cow	NNP bull	6/15/80	NNP	faecal	**
9	Tamu	F	4005	A	Saba	SGR bull	9/1/91	OPC	faecal	****
10	Jupiter	M	2512	A	Shaba	LWC bull	5/16/86	LWC	faecal	***
11	Jama	M	4023	A	Shemsha	OPC bull	10/25/97	OPC	faecal	***
12	Rodney	M	4002	A	SGR cow	SGR bull	1/1/82	SGR	faecal	****
13	Kilo	F	4007	A	SGR cow	SGR bull	1/1/70	SGR	faecal	****
14	Kurkura	M	4008	A	SGR cow	SGR bull	1/1/87	SGR	faecal	***
15	Loita	M	4009	A	SGR cow	SGR bull	1/1/90	SGR	faecal	***
16	Shemsha	F	4011	A	SGR cow	SGR bull	1/1/83	SGR	faecal	***
17	Tulivu	F	4547	A	SGR cow	SGR bull	1/1/84	SGR	faecal	***
18	Hifadhi	M	4020	A	Tamu	OPC bull	9/4/96	OPC	faecal	***

	Name	Sex	ID Code	Age class	Mother	Father	Birth Date	Origin	Sample	Sample Score
19	Maendeleo	M	4027	SA	Tamu	OPC bull	9/10/99	OPC	faecal	***
20	Waya's calf	F	4043	C	Waya	OPC bull	5/15/05	OPC	faecal	***
21	Chema	F	4550	A	SGR cow	SGR bull	1/1/73	SGR	faecal	**
22	Job	M	4010	A	SGR cow	SGR bull	1/1/80	SGR	faecal	****
23	Solo	F	4030	SA	Carol	OPC bull	4/24/01	OPC	faecal/tissue	***
24	Berkeley	F	4038	C	Carol	OPC bull	6/15/03	OPC	faecal/tissue	**
25	Sheria	M	4026	SA	Chema	OPC bull	6/15/99	OPC	faecal/tissue	***
26	Jasho	F	4034	SA	Chema	OPC bull	3/15/02	OPC	faecal/tissue	**
27	Amani	F	4044	C	Ishirini	OPC bull	6/15/05	OPC	faecal/tissue	***
28	Manchester	F	4033	SA	Kilo	OPC bull	11/13/01	OPC	faecal/tissue	**
29	Serena	F	4041	C	Kilo	OPC bull	12/12/04	OPC	faecal/tissue	**
30	Nawira	M	4039	C	Mkora	OPC bull	3/23/04	OPC	faecal/tissue	***
31	Millenium	F	4029	SA	Saba	OPC bull	2/21/00	OPC	faecal/tissue	**
32	Mkenya	M	4042	C	Tamu	OPC bull	4/4/05	OPC	faecal/tissue	***
33	Uhuru	M	4028	SA	Tulivu	OPC bull	1/13/00	OPC	faecal/tissue	***
34	Cathy	F	4035	SA	Tulivu	OPC bull	5/25/02	OPC	faecal/tissue	***
35	Shujaa	M	4040	C	Tulivu	OPC bull	7/31/04	OPC	faecal/tissue	***
36	Otoro	M	4003	A	SGR cow	SGR bull	1/1/80	SGR	faeces/tissue	***
37	Saba	F	4511	A	SGR cow	SGR bull	1/1/76	SGR	faeces/tissue	**

	Name	Sex	ID Code	Age class	Mother	Father	Birth Date	Origin	Sample	Sample Score
38	Murembo	F	4047	A	UNK	UNK	1/1/91	UNK	tissue	
39	Benja	M	4037	SA	Ishirini	OPC bull	8/10/02	OPC	tissue	
40	Juba	M	4031	SA	Mkora	OPC bull	8/22/01	OPC	faeces/tissue	***
41	Tatizo	F	4045	A	Murembo	UNK	1/1/98	OPC	tissue	
42	Balozi	M	4046	SA	Murembo	OPC bull	1/1/01	OPC	tissue	
43	Roberto	F	4021	A	Saba	OPC bull	11/2/96	OPC	faeces/tissue	**
44	Batian	F	4032	SA	Shemsha	OPC bull	8/24/01	OPC	faeces/tissue	**
45	Lemoile	F	4036	SA	Tamu	OPC bull	7/28/02	OPC	faeces/tissue	***
46	Tulia	M	4022	A	Tulivu	OPC bull	5/1/97	OPC	faeces/tissue	****

Sample Score refers to the reliability of sample identification. * = identity of sample unknown. ** = typically sample found prior to sighting of animal, with animal located by tracking spoor from sample area. *** = sample collected from animal where identification is thought to be reliable but animal may have only been followed for a short time or where both sets of ear notches were not visible. **** = sample identification is virtually certain with animal followed for long period of time and both sets of ear notches were visible.

2.2.6 New population (NOPC)

Following the expansion of the rhino sanctuary into the former ranch area, 24 black rhinoceros were translocated from neighbouring Solio Game Reserve into the new area in February 2007 (NOPC) (Figure 2.3). These animals were free released (that is, they were not kept in bomas prior to release) at a number of predetermined release points. All of these animals were fitted with horn radio transmitters prior to release to facilitate their monitoring. This was particularly important for the new animals that were free released into such a large area as they would be expected to move around considerably prior to settling. Tissue samples and the GPS coordinates of all sightings for 18 months post release from all individuals were made available to the study.

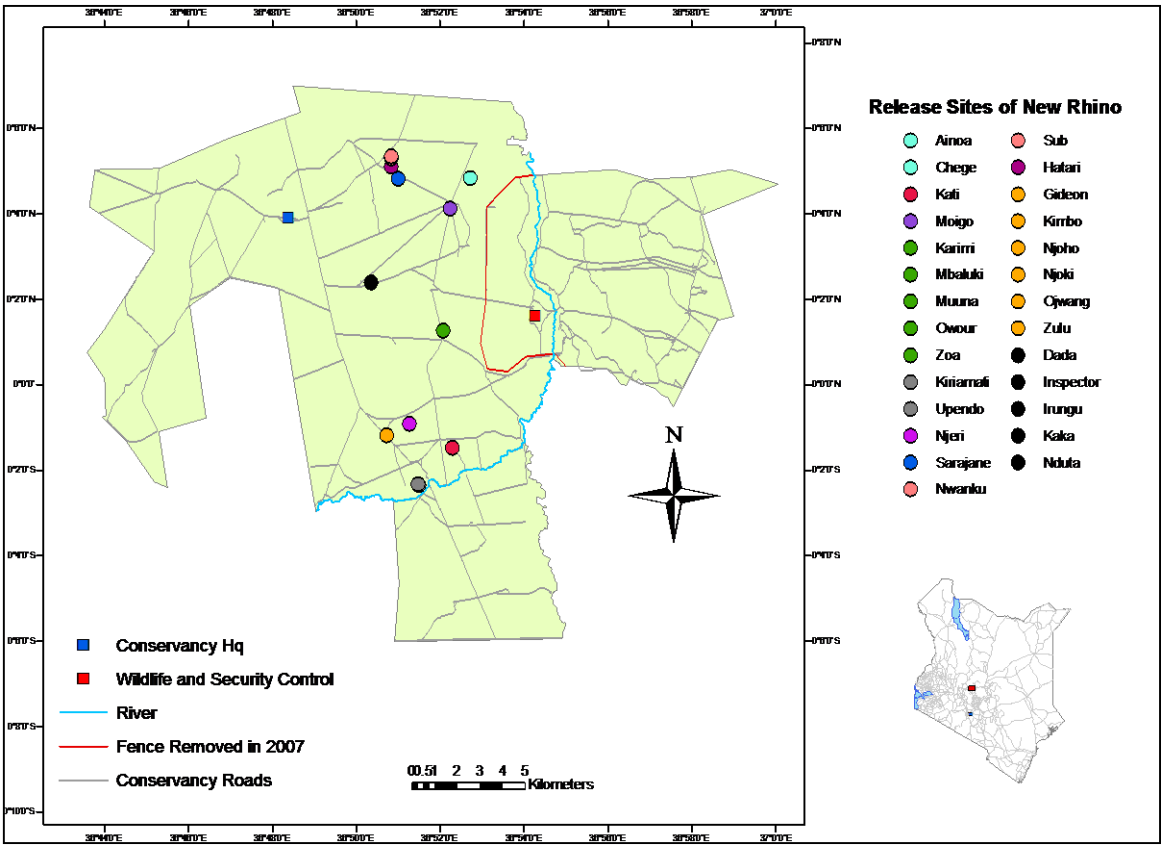

Figure 2.3. Release sites of newly introduced black rhinoceros into Ol Pejeta Conservancy (2007).

2.3 Lewa Wildlife Conservancy

2.3.1 Overview

Lewa Wildlife Conservancy (LWC) is a 267 km^2 private wildlife conservancy in Isiolo District, approximately 20 Km south of Isiolo town. Formerly a 162km^2 private family ranch, a 20km^2 fenced black rhinoceros sanctuary was established within the ranch in 1983, into which 15 founding black rhinoceros were translocated from the north of Kenya and Solio Game Reserve (Appendix 2.3). In 1994, the black rhino sanctuary was expanded to cover the entire ranch area and created the Lewa Wildlife Conservancy. The conservancy now includes 37 km^2 of additional land and the 57 km^2 Ngare Ndare Forest which forms a corridor for wildlife moving between the Mount Kenya forest and the Samburu lowlands to the north.

2.3.2 Climate

Lewa Wildlife Conservancy experiences similar yearly rainfall patterns as those experienced further south with normally two distinct wet seasons per year. However, the climate is more arid than the Laikipia district with the average yearly rainfall between 1985 and 2009 of 514 mm from 224 mm in 1987 and to 1,240 mm during the El Nino year of 1997. The conservancy is at an altitude of 1,700m above sea level with temperatures ranging between 10 ^{O}C to 35 ^{O}C during the day.

2.3.3 Habitat

The conservancy management classifies the conservancy into four main habitat types: plains, hills and slopes, riverine and the Ngare Ndare Forest (Figure 2.4). The Ngare Ndare forest is a dense *Juniperus procera-Olea Africana* forest that has recovered substantially from the effects of fire and logging and now has a number of secondary growth species such as *Dodonea* and *Rhus*. The riverine habitat is dominated by *Acacia xanthophloea* trees which are protected in certain areas by elephant exclusion zones. The hill and slopes habitat has approximately 40% tree cover comprised of a variety of Acacia species, including *A. brevispica, A. mellifera, A. nilotica, A.seyal, A. tortilis*. The plains vegetation has suffered a significant decline in tree cover down from approximately 35% in 1980 to about 2% in 2006. A combination of fire and browsing pressure is thought to be responsible for this decline. The trees on the plains are dominated by *Acacia drepanolobium* in the 'black-cotton' soil areas and *Acacia seyal* in the red loam areas. The grasses are dominated by the *Pennisetum* species, *P. stramineum* and *P. mezianum* and

their dominance, which is characteristic of more wooded areas, is believed to be a function of the recent decline in woody vegetation (Giesen *et al.* 2007).

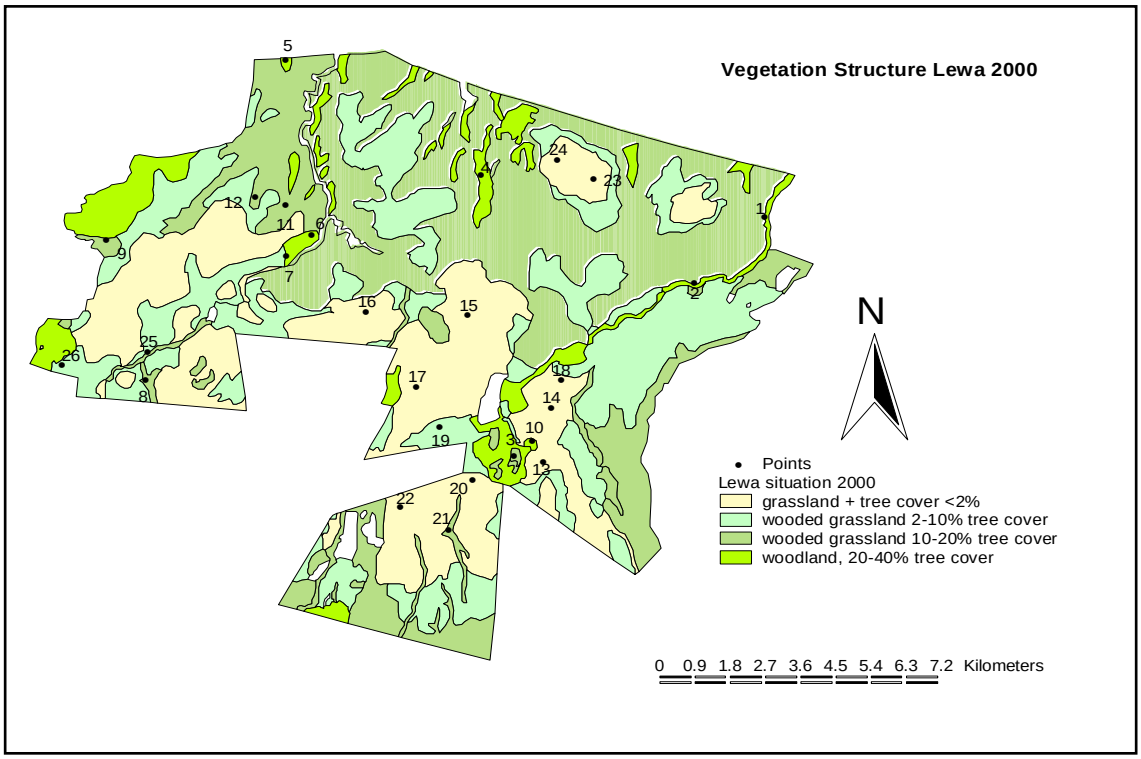

Figure 2.4. Vegetation map of Lewa Wildlife Conservancy (Giesen *et al.* 2007).

2.3.4 Sample collection

At the beginning of the study there was no tissue or other biological material available for genotyping for any of the black rhinoceros at Lewa; consequently, the genetic work at Lewa was initially undertaken solely on DNA extracted from faeces. The faecal sample collection at Lewa was broadly similar to that undertaken at Ol Pejeta Conservancy with the primary aim of obtaining fresh faecal samples from positively identified animals for every black rhinoceros within the conservancy.

The protocols for preserving the samples and scoring the reliability of the sighting associated with each sample were the same as those used at Ol Pejeta Conservancy. However, given the relatively open nature of the topography and vegetation at Lewa Downs compared to Ol Pejeta, a different approach was adopted for sample collection. Sample collection at Lewa Downs was predominantly vehicle-based and conducted with the assistance of a single monitoring patrolman, which enabled a wider area to be covered each day.

Before first light each morning, we positioned ourselves on top of a hill and used this position to look for rhinoceros over a relatively large area. This was particularly effective very early in the morning when the animals were out in the open before moving into

thicker bush as the temperature began to rise. If a rhinoceros was spotted we would then move closer and follow it on foot. The relevant patrol for the area was called in to provide a positive identification. The radio was monitored and if we were not involved in sample collection with a particular animal or we had not spotted one, we would move to any sightings by the other patrols.

During the sample collection a couple of trails were found that were obviously heavily utilised by rhinoceros for moving between thicker vegetation and the more open areas, which they are assumed to utilise at night. These trails had a large number of fresh dung middens and fresh spoor. For several days these trails were monitored from at least an hour before sun rise, using the vehicle as a hide, in an attempt to positively identify an animal defecating. However, despite great care being taken in positioning the vehicle so as not to be visible to any animal using the trails and positioning the vehicle downwind of the presumed direction of travel of the animals, no success was achieved using this method.

Faecal samples were also collected from a lone male rhinoceros originally from Lewa Conservancy, but now at Il Ngwesi Conservancy which borders Lewa.

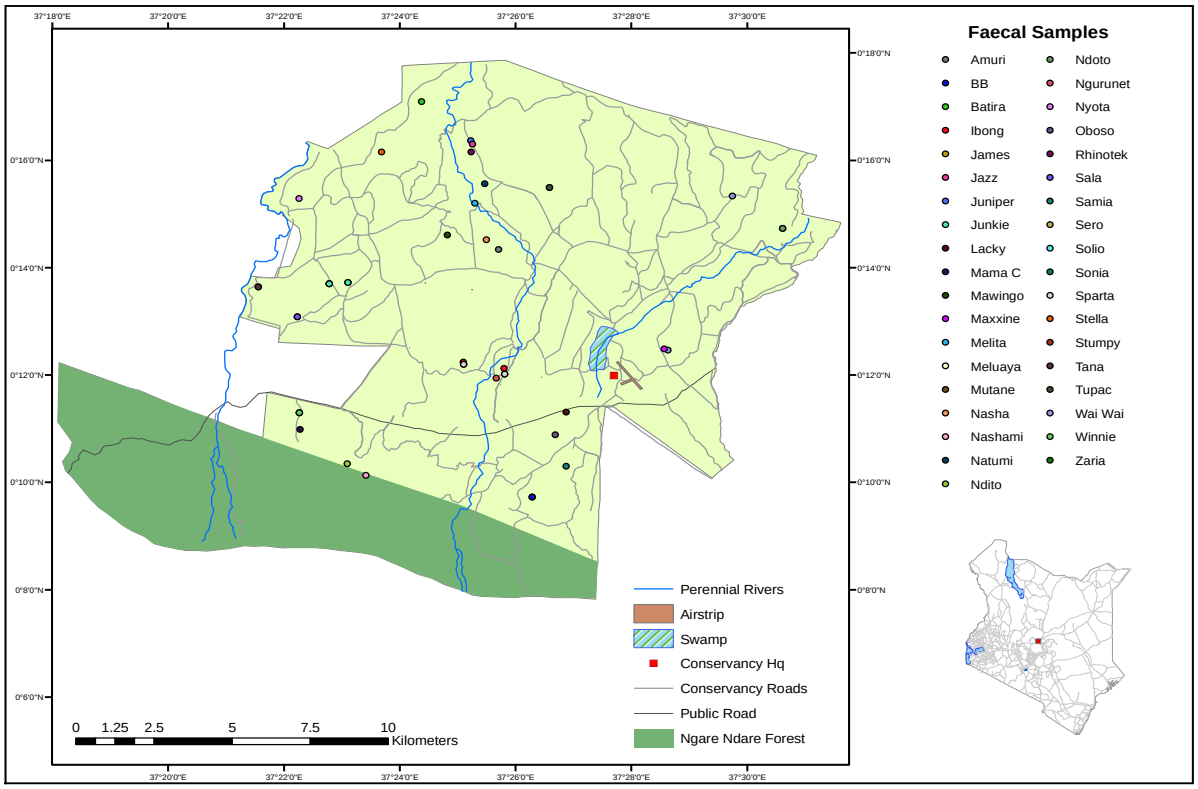

Figure 2.5. Map showing faecal samples collected for Lewa Wildlife Conservancy.

Samples were also collected from a young rhinoceros calf that the Craig family were hand rearing: it had been removed from its mother due to concerns over her ability to raise it.

In total 54 faecal samples were collected from Lewa representing a presumed 42 animals out of the 45 black rhinoceros present in the conservancy at the time of the sample collection (Figure 2.5). It was not possible to collect samples from two young calves despite samples being collected from their mothers. In addition one sub-adult was not sampled despite a number of attempts in which to collect a sample; the animal was followed on several occasions for a number of hours but did not defecate whilst being observed (Table 2.4).

2.3.5 Tissue samples

After the faecal sample collection was completed, the conservancy management in collaboration with Kenya Wildlife Service vets under took ear notching of five sub adult rhinoceros in 2006 and a further four in 2008. Tissue samples from these procedures were made available to the study for genotyping.

Table 2.4. Samples collected from animals at Lewa showing sample type and best sample score

	Name	Sex	ID Code	Age	Age class	Mother	Father	Birth Date	Origin	Sample	Sample Rating
1	Amuri	M	3508	19.7	A	Mama Kali	Ol Jogi bull	19/06/1987	Ol Jogi	Faecal	***
2	Batira	M	2537	7.8	A	Juniper	LWC bull	23/04/1999	LWC	Faecal	***
3	BB	F	2551	3.3	SA	Zaria	LWC bull	08/10/2003	LWC	Faecal	****
4	Ibong	M	3507	21.8	A	Mama Kali	Ol Jogi bull	05/05/1985	Ol Jogi	Faecal	***
5	James	M	3506	23.3	A	Mama Kali	Ol Jogi bull	11/10/1983	Ol Jogi	Faecal	***
6	Juniper	F	2509	18.6	A	Juno	LWC bull	28/06/1988	LWC	Faecal	***
7	Lacky	M	2531	10.7	A	Zaria	LWC bull	04/06/1996	LWC	Faecal	***
8	Mawingo	F	507	17.7	A	SGR cow	SGR bull	01/06/1989	Solio	Faecal	****
9	Maxxine	F	2545	4.7	SA	Waiwai	LWC bull	13/06/2002	LWC	Faecal	***
10	Melita	M	2524	23.1	A	SGR cow	SGR bull	01/01/1984	Solio	Faecal	**
11	Meluaya	F	2530	11.0	A	Juniper	LWC bull	25/01/1996	LWC	Faecal	****
12	Mutane	M	2525	18.1	A	SGR cow	SGR bull	01/01/1989	Solio	Faecal	***
13	Nasha	M	2543	6.3	SA	Solio	LWC bull	07/11/2000	LWC	Faecal	***
14	Nashami	F	2533	8.6	A	Stumpy	LWC bull	16/07/1998	LWC	Faecal	***
15	Natumi	F	2536	8.4	A	Solio	LWC bull	26/09/1998	LWC	Faecal	**
16	Ndito	F	2523	17.1	A	SGR cow	SGR bull	01/01/1990	Solio	Faecal	***
17	Nyota	F	2517	15.2	A	Stumpy	LWC bull	01/12/1991	LWC	Faecal	***
18	Oboso	F	2541	6.3	A	Zaria	LWC bull	09/10/2000	LWC	Faecal	****

	Name	Sex	ID Code	Age	Age class	Mother	Father	Birth Date	Origin	Sample	Sample Rating
19	Rhinotek	F	2544	5.5	SA	Juniper	LWC bull	16/08/2001	LWC	Faecal	***
20	Samia	F	2534	8.4	A	Sonia	LWC bull	10/09/1998	LWC	Faecal	****
21	Seiya	F	2538	7.8	A	Ndito	LWC bull	26/04/1999	LWC	Faecal	**
22	Sero	M	2539	7.4	A	Nyota	LWC bull	23/09/1999	LWC	Faecal	***
23	Solio	F	2505	31.1	A	SGR cow	SGR bull	01/01/1976	Solio	Faecal	**
24	Sonia	F	2516	15.5	A	Solio	LWC bull	23/08/1991	LWC	Faecal	***
25	Sparta	M	2549	3.8	SA	Sonia	LWC bull	09/05/2003	LWC	Faecal	***
26	Stumpy	F	2504	38.1	A	SGR cow	SGR bull	01/01/1969	Solio	Faecal	***
27	Tana	F	2542	6.3	SA	Stumpy	LWC bull	10/10/2000	LWC	Faecal	***
28	Tula	F	2554	2.8	C	Mawingo	LWC bull	01/05/2004	LWC	Faecal	****
29	Waiwai	F	2528	11.6	A	Solio	LWC bull	04/07/1995	LWC	Faecal	****
30	Winnie	F	2557	2.5	C	Ndito	LWC bull	27/08/2004	LWC	Faecal	**
31	Zaria	F	2514	18.9	A	Solio	LWC bull	09/03/1988	LWC	Faecal	****
32	Ngurunet	M	2535	died	2004	Zaria	LWC bull	15/09/1998	LWC	Faecal	**
33	Stella	M	2529			Stumpy	LWC bull	09/10/1995	LWC	Faecal	**
34	Jazz	M	2553	3.3	SA	Juniper	LWC bull	14/10/2003	LWC	Faecal/Tissue	**
35	Junkie	M	2552	3.4	SA	Stumpy	LWC bull	03/10/2003	LWC	Faecal/Tissue	***
36	Mama C	F	2548	4.6	SA	Ndito	LWC bull	22/07/2002	LWC	Faecal/Tissue	***
37	Ndoto	M	2555	2.7	C	Meluaya	LWC bull	05/06/2004	LWC	Faecal/Tissue	***

	Name	Sex	ID Code	Age	Age class	Mother	Father	Birth Date	Origin	Sample	Sample Rating
38	Sala	F	2550	3.3	SA	Solio	LWC bull	07/10/2003	LWC	Faecal/Tissue	**
39	Tupac	F	2559	1.6	C	Natumi	LWC bull	08/07/2005	LWC	Faecal/Tissue	*
40	Borana	M	2556	2.5	C	Waiwai	LWC bull	07/08/2004	LWC	Tissue	
41	Sonia's calf 3	M	2561	1.3	C	Sonia	LWC bull	19/10/2005	LWC	Tissue	
42	Stumpy's calf 7	M	2565	1.1	C	Stumpy	LWC bull	25/12/2005	LWC	Tissue	

Sample Score refers to the reliability of sample identification. * = identity of sample unknown. ** = typically sample found prior to sighting of animal, with animal located by tracking spoor from sample area. *** = sample collected from animal where identification is thought to be reliable but animal may have only been followed for a short time or where both sets of ear notches were not visible. **** = sample identification is virtually certain with animal followed for long period of time and both sets of ear notches were visible.

2.4 Mugie Rhino Sanctuary

2.4.1 Overview

Mugie Rhino Sanctuary is part of the privately owned Mugie Ranch located in northern Laikipia, approximately 300km north of Nairobi ($0^0$74'N, $36^0$65'E). The ranch is 200 km^2 and subdivided into a working cattle ranch and a 93 km^2 black rhino sanctuary, the two areas are bisected by the unpaved road which runs between Rumeruti and Maralal in Samburu District. The rhino sanctuary was previously managed as part of the working ranch, but in 2002 was subdivided when two white rhino were introduced. In 2004, the sanctuary, now surrounded by a 2 m high electric fence received a founding black rhino population of 20 animals from Nairobi National Park, Lake Nakuru National Park and Solio Game Reserve.

2.4.2 Climate

Mugie experiences the same two wet seasons and dry seasons per year as the other sanctuaries in the study. The sanctuary receives slightly more rain than other areas in Kenya on the same latitude and receives between 600 - 800 mm of rain per year, which is the same as Ol Pejeta despite being slightly further north than Lewa. Temperatures in Mugie are also very similar to Ol Pejeta. The sanctuary has an average elevation of 1825 m and there is little variation in the topography of this area.

2.4.3 Habitat

The sanctuary comprises areas of sand red loam and the characteristically poor-draining 'black-cotton' clay soil; it supports a range of heterogeneous habitats ranging from open grassland plains, to shrubland and closed woodland. The dominant woody species are *Euclea divinorum*, *Acacia nilotica*, *Olea africana* and *Croton dichogamusi*. Grasslands are dominated by *Themeda triandra*, and the *Pennisetum* species, *P. stramineum* and *P. mezianum*.

There are a number of streams and springs within the sanctuary and in 2009 a 63 ha dam was created, which provides water year round even in drought conditions. The conservancy supports a range of fauna in addition to the black rhinoceros, including elephant, buffalo, Grevy and Plains zebra, Beisa oryx, lion and cheetah. All wildlife, except rhinoceros, is able to move out of the sanctuary through corridors in the fence designed to prohibit the movement of rhino.

2.4.4 Founder population

In 2006, serum samples collected from the 20 founder animals that were translocated in 2004 from Nairobi National Park (NNP), Lake Nakuru National Park (LNP) and Solio Game Reserve (SGR) were made available for genotyping in this study (Table 2.5). The serum samples, approximately 5 ml, were stored at -20^{O}C in the laboratory at the International Livestock Research Institute in Nairobi prior to shipment to the UK for extraction and downstream analysis.

2.4.5 Animals born since foundation

In 2009 a faecal sample collection kit was sent out to Claus Mortensen, the manager of Mugie, in order for faecal samples to be collected from the 8 calves that have been born since the population's foundation (Table 2.6). The kit contained sterile forceps, gloves, sample pots, silica, samples recording sheets and a detailed protocol for sample collection. Claus oversaw the collection of fresh faecal samples from all 8 calves. Within two weeks of collection the samples preserved in silica were shipped back by courier (under DEFRA licence) to laboratories at Manchester Metropolitan University. The sample recording sheets included the names and IDs of both the subject animal and its the mother, the time and date of collection, notes on the reliability of sample identification and GPS coordinates of the collection site. (Figure 2.6).

Table 2.5. Mugie Rhino Sanctuary founder animals

	Capture date	ID Code	Name	Birth Date	Origin	Sex	Mother ID Number	Sample Type
1	20/07/2004	121	Mary	13/04/2001	NNP	F	21	Serum
2	20/07/2004	116	Chebii	15/03/2000	NNP	F	104	Serum
3	20/07/2004	118	Mwanzia	06/07/2000	NNP	M	25	Serum
4	22/07/2004	106	Russelas	15/06/1998	NNP	M	20	Serum
5	23/07/2004	120	Laupere	01/11/2000	NNP	M	139	Serum
6	23/07/2004	115	Parri	03/01/2000	NNP	F	20	Serum
7	27/07/2004	530	Kyela	15/06/1990	LNP	F	523	Serum
8	27/07/2004	567	Laban	15/12/2001	LNP	M	530	Serum
9	29/07/2004	548	Beth	01/10/1997	LNP	F	532	Serum
10	29/07/2004	571	Mugie	11/05/2002	LNP	M	548	Serum
11	31/07/2004	502	Kiserian	01/01/1974	LNP	M	UNK	Serum
12	31/07/2004	504	Ridgeback	01/01/1982	LNP	M	UNK	Serum
13	31/07/2004	503	Tatu	01/01/1979	LNP	M	46	Serum
14	02/08/2004	575	Mayiende	01/01/2001	LNP	F	535	Serum
15	02/08/2004	537	Leakey	01/03/1994	LNP	M	508	Serum
16	01/08/2004	555	Helga	01/12/1997	LNP	F	530	Serum
17	10/08/2004	2576	Baraka	01/06/1976	SGR	M	UNK	Serum
18	10/08/2004	2577	Solio	01/07/1998	SGR	F	UNK	Serum

	Capture date	ID Code	Name	Birth Date	Origin	Sex	Mother ID Number	Sample Type
19	10/08/2004	2578	Toyo	01/06/1989	SGR	M	UNK	Serum
20	10/08/2004	2579	Ndittolie	01/06/2001	SGR	F	UNK	Serum

NNP = Nairobi National Park, LNP = Lake Nakuru National Park, SGR = Solio Game Reserve

Table 2.6. Faecal samples collected from calves born at Mugie since 2004

	Name	ID No	Sex	Location	Maternal ID	Sample	Collection Notes
1	Simeon	9006	M	Mugie	Solio / 2577	Faecal	One hour old. Positive ID
2	Un-named 2	9005	M	Mugie	Helga / 555	Faecal	One hour old. Positive ID
3	Sammy	9003	M	Mugie	Beth / 548	Faecal	40 min old. Positive ID
4	Un-named 5	9008	F	Mugie	Kyela / 530	Faecal	40 min old. Positive ID
5	Un-named 1	9004	F	Mugie	Chebii / 116	Faecal	50 min old. Positive ID
6	Alfie	9010	M	Mugie	Mayiende / 575	Faecal	40 min old. Positive ID
7	Paula	9007	F	Mugie	Parri / 115	Faecal	One hour old. Positive ID
8	Un-named 6	9009	F	Mugie	Beth / 548	Faecal	2 hours old. Positive ID

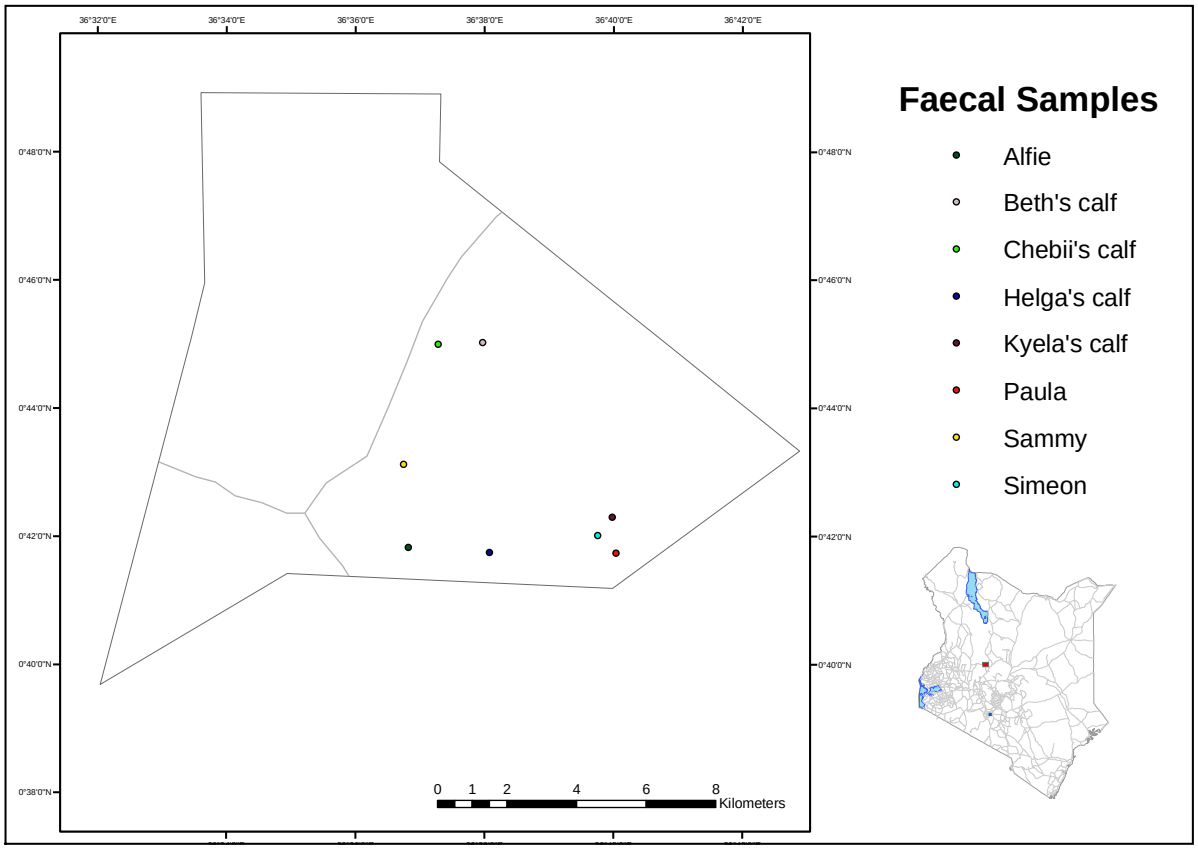

Figure 2.6. Map showing faecal samples collected for Mugie Rhino Sanctuary

2.5 Ngulia Rhino Sanctuary

2.5.1 Overview

Ngulia Rhino Sanctuary was established in 1985 as a small 3 km^2 fenced area within Tsavo West National Park. The sanctuary was initially established to rescue vulnerable black rhinoceros from nearby Kibwezi outside of the national park. Three females from Kibwezi were released into the enclosed area in 1986. With the capture of an additional three females from the east periphery of the park the sanctuary was expanded to 20 km^2 in 1987. The area was further expanded to 63 km^2 in 1990 with the release of a further ten animals; three permanent pipe-fed water holes were also established within the sanctuary.

The sanctuary had a temporary electric fence, which was lower in height compared to that of other established rhino sanctuaries in Kenya, and it was designed solely to contain the animals that were protected within the National Park. Consequently, in the early days of the sanctuaries establishment, there were a number of break-outs of rhino and predominantly as a result of elephants within the sanctuary breaking through the fence.

Due to the dense bush cover within Ngulia monitoring of the rhino population has always been problematic with initial monitoring attempted through footprint identification. In 2007, a new monitoring method was implemented in the newly expanded sanctuary (88 km^2) that consisted of photographing animals at the water holes during the dry season at night during a full moon (Mulama & Okita 2002). This monitoring system derived a population estimate of 61 animals (Appendix 2.4). Subsequently, this method has been adopted as the main monitoring method for Ngulia: full moon censuses of the rhino population are regularly undertaken.

2.5.2 Climate

The average annual rainfall for Ngulia is approximately 600 mm with a marked seasonal difference during the wet and dry seasons. The temperature ranges between 28 °C and 34 ^{O}C, with 46 to 73% humidity.

2.5.3 Habitat

The vegetation in the sanctuary is dominated by *Commiphora africana* and *Bauhinia taitensis*. With more than 50% of the small shrubs and trees forming dense stands around the three permanent water points are more open grasslands consisting of perennial species. The sanctuary has a very high density of elephants (N~250), which has led to a substantial reduction in browse availability. Consequent to this, the rhino population as a whole lost body condition and the breeding rate started to level off. In response to this problem the Kenya Wildlife Service, in collaboration with the Zoological Society of London, removed 200 elephants from the sanctuary in 2006.

2.5.4 Tissue samples

In 2008, an ear notching exercise was undertaken by the Kenya Wildlife Service to notch 19 adult and sub-adult rhino to facilitate monitoring and identification. Some of these animals were released into the newly-established Intensive Protection Zone (IPZ) outside of Ngulia in Tsavo West National Park. Tissue samples from these 19 animals were preserved in 70% (v/v) ethanol and shipped, under DEFRA licence, to the laboratory at Manchester Metropolitan University for genotyping (Table 2.7).

Table 2.7. Tissue samples for 19 black rhino from Ngulia Rhino Sanctuary

	Name	ID	Birth Date	Sex	Mother	Mother ID	Origin	Location	Sample Type
1	Adan	5016	01/07/1994	M	Chelangat	5004	Born-TW	IPZ	Tissue
2	Brett	5018	01/08/1995	M	Kaleah	5013	Born-TW	IPZ	Tissue
3	Miss Goss	5038	01/04/1999	F	Mrs. Maktau	5011	Born-TW	IPZ	Tissue
4	Sarah	5047	15/05/2002	F	Unknown	UNK	Born-TW	Ngulia	Tissue
5	Sachin	5048	04/02/2003	M	Wamboi	5009	Born-TW	Ngulia	Tissue
6	Maria	5049	04/04/2003	F	Wanjiku	56	Born-TW	IPZ	Tissue
7	Mboya	5051	01/05/2004	M	Unknown	UNK	Born-TW	Ngulia	Tissue
8	Bambo	5017	01/07/1995	M	Mrs. Maktau	5011	Born-TW	Ngulia	Tissue
9	Saumu	5032	01/07/1998	F	Shangigi	5022	Born-TW	Ngulia	Tissue
10	Keroken	5035	08/01/1999	M	Mangelete	5015	Born-TW	Ngulia	Tissue
11	Denny	5044	30/07/2001	M	Khadija	5019	Born-TW	Ngulia	Tissue
12	PPK	5045	15/04/2002	M	Leso	5001	Born-TW	Ngulia	Tissue
13	Hope	5060	01/03/2005	F	Achieng	5003	Born-TW	Ngulia	Tissue
14	Sinei	5031	05/04/1997	M	Sveda	32	Born-TW	IPZ	Tissue
15	Dr. Rajan	5033	01/04/1998	M	Ormanya	110	Born-TW	Ngulia	Tissue
16	Boit	5056	01/06/2004	M	Wairimu	34	Born-TW	Ngulia	Tissue
17	Kech	5014	01/01/1993	M	Mrs. Maktau	5011	Born-TW	Ngulia	Tissue
18	Amayo	5034	01/04/1998	F	Kadogoo	13	Born-TW	IPZ	Tissue

	Name	ID	Birth Date	Sex	Mother	Mother ID	Origin	Location	Sample Type
19	Muia	5050	03/04/2003	M	Unknown		Born-TW	Ngulia	Tissue

2.6 Nairobi National Park

Seven tissue samples were obtained from animals originally from Nairobi National Park; these animals were translocated to Laikipia Ranch in 2006. Unfortunately, these animals either died during the translocation or perished due to adverse weather conditions at the site of release. No further information is available for these samples.

References

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Okita-Ouma B, Amin R, Kock R (2007) Conservation and Management Strategy for the Black Rhino and Management Guideline for the White Rhino in Kenya (2007-2011). Unpublished report. Nairobi: Kenya Wildlife Service.

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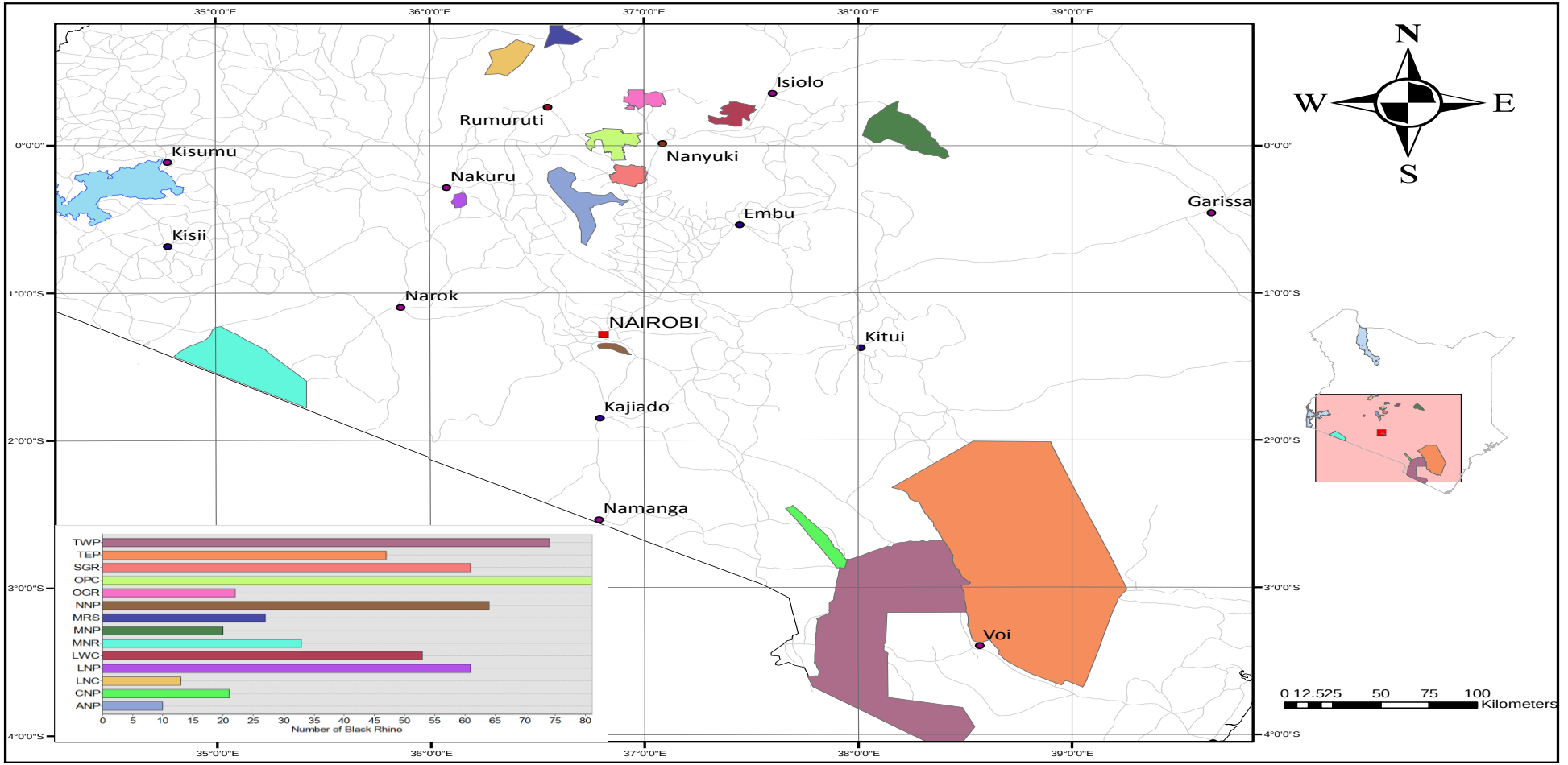
Appendix 2.1. Map showing black rhinoceros areas in Kenya.



TWP = Tsavo West National Park, TEP = Tsavo East National Park, SGR = Solio Game Reserve, OPC = Ol Pejeta Conservancy, OGR = Ol Jogi Game Reserve, NNP = Nairobi National Park, MRS = Mugie Rhino Sanctuary, MNP = Meru National Park, MNR = Mara National Reserve, LWC = Lewa Wildlife Conservancy, LNP = Lake Nakuru National Park, LNC = Laikipia Nature Conservancy, CNP = Chyulu Hills National Park, ANP = Aberdares National Park.

Appendix 2.2. Ol Pejeta Conservancy black rhinoceros population history (2005).

ID	Name	Sex	Mother	D.O.B	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02	'03	'04	'05
4505	TISA	F	UNK	01-01-85		SGR	D-F														
4511	SABA	F	UNK	01-01-76		SGR	c-9		18		20	c-11	22		24	c-2	26		28		30
4547	TULIVU	F	UNK	01-01-84		SGR			10		12		c-5		16.0	c-1	18	c-5	20	c-7	22
4006	MIA	F	UNK	01-01-85		SGR			9		c-2		13	D-P							
4047	UNKNOWN	F	UNK	01-01-91										c-1							
4005	TAMU	F	Saba	01-09-91					2		4	c-9	6		c-9		10	c-7	12		c-4
28	BEHEWA	F	UNK	01-01-89			NNP	D-F													
37	MUKOROFI	F	UNK	01-01-80				NNP	14	D-F											
46	CAROL	F	UNK	15-06-80				NNP	13	c-11	15		17	c11	19		c-4		c-6		25
4007	KILO	F	UNK	01-01-70					SGR	c-3	26		28		c-2		32		34	c-12	36
4011	SHEMSHA	F	UNK	01-01-83					SGR	c-7	13		c-10		17		c-8		21		23
4530	METRIC	F	UNK	01-01-83					SGR	D-F											
4550	CHEMA	F	UNK	01-01-73					SGR	c-5	23	c-5	25		c-6		29	c-3	31	D-P	
4014	MKORA	F	Kilo	01-03-94							2		4		6		c-8		10	c-3	12
4018	WAYA	F	Mia	04-02-95							1		3		5		7		9		11
4019	ISHIRINI	F	Chema	17-05-96									1		3		5	c-8	7		c-6
4021	ROBERTO	F	Saba	02-11-96									1		3		5		7		9
4045	UNKNOWN	F	UNK	01-01-98											2		4		6		8
4025	LATILI	F	Kilo	25-02-99											1		2		4		7
4029	MILLENIUM	F	Saba	21-02-00													2		4		6
4030	SOLO	F	Carol	24-04-01													1		3		5
4032	BATIAN	F	Shemsha	24-08-01													0.4		2		4
4033	MANCHESTER	F	Kilo	13-11-01													0.1		2		4
4034	JASHO	F	Chema	15-03-02															2		4
4035	CATHY	F	Tulivu	25-05-02															1.5		3
4036	LEMOILE	F	Tamu	28-07-02															1.4		3
4038	BERKLEY	F	Carol	15-06-03															0.5		2
041	KILO'S CF	F	Kilo	12-12-04																	1

ID no	Name	Sex	Mother	D.O.B	1989	90	91	92	93	94	95	96	97	98	99	00	01	02	03	04	05
4001	MORANI	M	UNK	19-05-76	NNP		15		17		19		21		23		25		27		29
4002	RODNEY	M	UNK	01-01-82	SGR		10		12		14		16		18		20		22		24
4003	OTORO	M	UNK	01-01-80	SGR		12		14		16		18		20		22		24		26
4004	MOYAK	M	UNK	01-01-84	SGR		8		10		12		14		16		18		20		22
2512	JUPITER	M	UNK	16-05-86			LWC		7		9		11		13		15		17		19
4013	NO NAME	M	Carol	16-09-92				D													
4008	KURKURA	M	UNK	01-01-87					SGR		9		11		13		15		17		19
4009	LOITA	M	UNK	01-01-90					SGR		6		8		10		12		14		16
4010	JOB	M	UNK	01-01-80					SGR		16		18		20		22		24		26
4012	SMITH	M	Shemsha	01-01-90					SGR		6	D									
4015	NO NAME	M	Chema	27-05-94						D											
4016	BAHATI	M	Shemsha	01-07-94							D										
4017	BARAKA	M	Carol	20-11-94							1		3.1		5		7		9		11
4020	HIFADHI	M	Tamu	04-09-96									1.3		3		5		7		9
4022	TULIA	M	Tulivu	01-05-97									0.7		3		4		6		8
4023	JAMA	M	Shemsha	25-10-97									0.2		2		4		6		8
4024	SAFARI	M	Carol	26-11-98											1		3		5		7
4026	SHERIA	M	Chema	15-06-99											0.5		2		4		6
4027	MAENDELE	M	Tamu	10-09-99											0.3		2		4		6
4028	UHURU	M	Tulivu	13-01-00													2		4		6
4031	JUBA	M	Mkora	22-08-01													04		2		4
4046	UNK	M	UNK	01-01-01													1		3		5
4037	BENJA	M	Ishirini	10-08-02															1		3
4039	MK'S CF	M	Mkora	23-03-04																	2
4040	TUL'S CF	M	Tulivu	31-07-04																	1
4042	TAMU'S CF	M	Tamu	01-04-05																	1

=Calving-month of | Ex-Source =Introduction-place of origin | **D-Cause** =Death-cause | **To-Dest'n** =Removed-Destination

Appendix 2.3. Lewa Wildlife Conservancy black rhinoceros population history (2005).

ID no	Name	Sex	D.O.B	'84	'85	'86	'87	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02	'03	'04	'05
2504	STUMPY	F	01-Jan-69	SGR	3				21.0		12		25.0		10	28.0		7	31.0	10	33.0	34.0	10		37.0
2505	SOLIO	F	01-Jan-76	SGR	2			3	14.0		8		18.0		7	21.0		9	24.0	11	26.0	27.0	10		30.0
2503	SHABA	F	01-Jan-70	Shaba		6			20.0		22.0	2	24.0			D									
2506	JUNO	F	01-Jan-61	SGR				6	29.0		31.0	2	D												
2502	RONGAI	F	01-Jan-66	Prt-jn				2	24.0		D														
2500	SABACHI	F	01-Jan-80		Wamba	D																			
507	MAWING	F	01-Jun-89				SGR		0.6		2.6		4.6			7.6	8		10		12.6	7		5	
2508	SAMIA	F	15-Feb-85						4.9		6.9		8.9	3	D										
2509	JUNIPER	F	28-Jun-88						1.5		3.5		5.5			1			4		8	14.5	10		17.5
2513	JILALE	F	18-Feb-88						1.9		3.9		5.9			8.9	7	D							
2514	ZARIA	F	09-Mar-88						1.8		3.8		5.8			6		9	11.8	10	13.8	14.8	10		17.8
2522	NARASHA	F	01-Jan-90								2.0		4.0	9	D										
2516	SONIA	F	23-Aug-91								0.4		2.4			5.4		9	8.4		10.4	11.4	5		
2517	NYOTA	F	01-Dec-91								0.1		2.1			5.1			9		10.1	6			14.1
2523	NDITO	F	01-Jan-90													7.0			4		12.0	7		9	16.0
2527	TASHA	F	21-Sep-94											D											
2528	WAIWAI	F	04-Jul-95													1.5			4.5		6.5	6		6	10.5
2530	MELUAYA	F	25-Jan-96													0.9			3.9		5.9	6.9		5	9.9
2533	NASHAMI	F	16-Jul-98																1.5		3.5	4.5			7.5
2534	SAMIA2	F	10-Sep-98																1.3		3.3	4.3			7.3
2536	NATUMI	F	26-Sep-98																1.3		3.3	4.3			7
2538	SEIYA	F	26-Apr-99																0.7		2.7	3.7		11	6.7
2541	OBOSO	F	09-Oct-00																		1.2	2.2			5.2
2542	TANA	F	10-Oct-00																		1.2	2.2			5.2
2544	RHINOTE	F	16-Aug-01																		0.4	1.4			4.4
2545	MAXXINE	F	13-Jun-02																			0.6			3.6
2548	MAMA C	F	22-Jul-02																			0.4			3.4

ID no	Name	Sex	D.O.B	'84	'85	'86	'87	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02	'03	'04	'05
2550	SALA	F	07-Oct-03																						2.2
2551	BB	F	08-Oct-03																						2.2
2557	NDITO CALF 3	F	27-Sep-04																						1.3
2554	TULA	F	01-May-04																						1.7

ID	Name	Sex	Date born	'84	'85	'86	'87	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02	'03	'04	'05
3505	JAMES	M	11-Oct-83										OJR								18		20		22
3507	IPONG	M	05-May85										OJR								16		18		20
3508	AMURI	M	19-Jun-87										OJR								14		16		18
3509	AKILI	M	12-Jul-87										OJR					TE							
4523	OL BAYU	M	01-Jan-75	SGR							D														
501	AMBONI	M	01-Jan-70	Prt-jn			LNP																		
4001	MORANI	M	19-May-76	NNP		OPC																			
2599	WAMBA	M	01-Jan-74	wam	D																				
2521	MUGAMB	M	01-Jan-67											SGR	D										
2501	GODOT	M	01-Jan-82	kit	4		6.0	MNP																	
2510	KELELE	M	06-Jan-84	Prt	2		4		6		8		10		12		14		16		16		MNP		
2524	MELITA	M	01-Jan-84											SGR	12		14		16		18		20		22
2511	BAHATI	M	22-Mar-85				2		4	D															
2512	JUPITER	M	16-May-86				1		3		5		7		9		11		13		15		17		19
2525	MUTANE	M	01-Jan-89											SGR	7		9		11		13		15		17
4532	SOPAT	M	01-Jan-76							SGR	D														
2515	KIKWAR	M	01-Jan-82							math	D														
2518	SHIMBA	M	07-Feb-92										2		4		D								
2519	JUNO C3	M	10-Feb-92									D													
2520	KENO	M	01-Jan-81										math		15	mth									
2526	SAMUEL	M	01-Mar-94												D										
2529	STELLA	M	09-Oct-95												0.2		2.2		4		6		8		10

ID	Name	Sex	Date born	'84	'85	'86	'87	'88	'89	'90	'91	'92	'93	'94	'95	'96	'97	'98	'99	'00	'01	'02	'03	'04	'05
2531	LACKY	M	04-Jun-96														1.6		3		5		7		10
2555	MEL'S CF	M	01-May-04																						2
2535	NGURUNET	M	15-Sep-98																1		3		5	D	
2537	BATIRA	M	23-Apr-99																		2		4		7
2539	SERO	M	23-Sep-99																		2		4		6
2540	OMNI	M	24-Oct-99																		2	ngi			
2543	NASHA	M	07-Nov-00																		1		3		5
2546	FOLLY	M	22-Jun-02																				1		3
2547	MAWS CF	M	16-Jul-02																			D			
2552	JUNKIE	M	03-Oct-03																						2
2553	JAZZ	M	14-Oct-03																						2
2549	SPARTA	M	09-May-03																						2
2558	SYRAH	M	01-Nov-04																						1
2561	SON CF 3	M	19-Oct-05																						
2560	MAWS CF	M	10-Oct-05																						

=Calving-month of | Ex-Source | =Introduction-place of origin | D-Cause | =Death-cause | To-Dest'n | =Removed-Destination

Appendix 2.4. Ngulia Rhino Sanctuary Population History (2009).

No	Name	ID	Birth Date	Sex	Mother	Origin	location	Status
1	Mark	5	03/01/1967	Male	UNK	Ex-Nairobi	Ngulia	Dead
2	Kadogoo	13	01/06/1989	Female	UNK	Ex-Nairobi	Ngulia	Live
3	Observation	14	09/05/1977	Male	UNK	Ex-Nairobi	Ngulia	Dead
4	Sveda	32	07/01/1989	Female	UNK	Ex-Nairobi	Ngulia	Live
5	Wairimu	34	02/01/1986	Female	UNK	Ex-Nairobi	Ngulia	Live
6	Hyrax	41	01/06/1978	Male	UNK	Ex-Nairobi	Ngulia	Live
7	Chris	42	01/06/1978	Male	UNK	Ex-Nairobi	IPZ	Live
8	Simon	51	02/01/1985	Male	UNK	Ex-Nairobi	IPZ	Live
9	Jeremy	54	01/06/1986	Male	UNK	Ex-Nairobi	Ngulia	Dead
10	Wanjiku	56	01/06/1989	Female	UNK	Ex-Nairobi	Ngulia	Live
11	Ormanya	110	15/06/1990	Female	UNK	Ex-Nairobi	Ngulia	Live
12	Leso	5001	01/06/1978	Female	UNK	Ex-Kibwezi	Ngulia	Live
13	Mbai	5002	01/06/1984	Female	UNK	Ex-Kibwezi	Ngulia	Live
14	Achieng'	5003	01/06/1983	Female	UNK	Ex-Kibwezi	Ngulia	Dead
15	Chelangat	5004	01/02/1979	Female	UNK	Ex-Taita	Ngulia	Live
16	John	5006	01/01/1977	Male	UNK	Ex-TW	Ngulia	Dead
17	Bill	5007	10/01/1980	Male	UNK	Ex-TW	Ngulia	Live
18	Oliver	5008	15/01/1987	Male	Leso	Ex-TW	Ngulia	Live

No	Name	ID	Birth Date	Sex	Mother	Origin	location	Status
19	Wamboi	5009	02/01/1990	Female	Mbai	Born-TW	Ngulia	Live
20	Cellen	5010	01/06/1990	Female	Achieng'	Born-TW	Ngulia	Live
21	Mrs. Maktau	5011	01/06/1980	Female	Unknown	Ex-Maktau	Ngulia	Live
22	Mtoro	5012	01/01/1993	Male	Leso	Born-TW	Ngulia	Live
23	Kaleah	5013	01/06/1982	Female	UNK	Born-TW	Ngulia	Live
24	Kech	5014	01/01/1993	Male	Mrs. Maktau	Born-TW	Ngulia	Live
25	Mangelete	5015	01/07/1983	Female	UNK	Ex-Mangelete	Ngulia	Live
26	Adan	5016	01/07/1994	Male	Chelangat	Born-TW	IPZ	Live
27	Bambo	5017	01/07/1995	Male	Mrs. Maktau	Born-TW	Ngulia	Live
28	Brett	5018	01/08/1995	Male	Kaleah	Born-TW	IPZ	Live
29	Khadija	5019	01/07/1995	Female	Achieng'	Born-TW	Ngulia	Live
30	Bahati	5020	01/01/1989	Female	UNK	Ex-Nairobi	Ngulia	Live
31	Gari	5021	14/04/2000	Male	Chelangat	Born-TW	Ngulia	Live
32	Shangigi	5022	01/06/1990	Female	UNK	Ex-Amboseli	Ngulia	Live
33	Rebecca	5023	01/01/1996	Female	Mangelete	Born-TW	Ngulia	Live
34	Rose	5024	01/01/1996	Female	Wairimu	Born-TW	Ngulia	Live
35	Dublin	5025	01/07/1997	Female	Wanjiku	Born-TW	Ngulia	Live
36	Negussie	5026	01/09/1997	Male	Kaleah	Born-TW	Ngulia	Live
37	Taye Teferi	5027	01/09/1997	Male	Leso	Born-TW	Ngulia	Live

No	Name	ID	Birth Date	Sex	Mother	Origin	location	Status
38	Isiche	5028	01/08/1997	Male	Mrs. Maktau	Born-TW	Ngulia	Live
39	Bakari	5029	01/10/1997	Male	Mbai	Born-TW	IPZ	Live
40	Kimondo	5030	01/01/1996	Female	Bahati	Born-TW	Ngulia	Live
41	Sinei	5031	05/04/1997	Male	Sveda	Born-TW	IPZ	Live
42	Saumu	5032	01/07/1998	Female	Shangigi	Born-TW	Ngulia	Live
43	Dr. Rajan	5033	01/04/1998	Male	Ormanya	Born-TW	Ngulia	Live
44	Amayo	5034	01/04/1998	Female	Kadogoo	Born-TW	IPZ	Live
45	Keroken	5035	08/01/1999	Male	Mangelete	Born-TW	Ngulia	Live
46	Susan	5036	04/02/1999	Female	Cellen	Born-TW	Ngulia	Live
47	Werikhe	5037	12/03/1999	Male	Kaleah	Born-TW	Ngulia	Live
48	Miss Goss	5038	01/04/1999	Female	Mrs. Maktau	Born-TW	IPZ	Live
49	Shamira	5039	15/02/2000	Female	Wairimu	Born-TW	Ngulia	Live
50	Bashir	5040	15/07/2000	Male	Wamboi	Born-TW	Ngulia	Dead
51	Terry	5041	15/08/2000	Male	Achieng	Born-TW	IPZ	Live
52	Najma	5042	15/01/2001	Female	Rebecca	Born-TW	Ngulia	Live
53	Lucy	5043	15/04/2001	Female	Bahati	Born-TW	Ngulia	Live
54	Denny	5044	30/07/2001	Male	Khadija	Born-TW	Ngulia	Live
55	PPK	5045	15/04/2002	Male	Leso	Born-TW	Ngulia	Live
56	Chepkwony	5046	15/04/2002	Male	Kadogoo	Born-TW	Ngulia	Live

No	Name	ID	Birth Date	Sex	Mother	Origin	location	Status
57	Sarah	5047	15/05/2002	Female	Unknown	Born-TW	Ngulia	Live
58	sachin	5048	04/02/2003	Male	Wamboi	Born-TW	Ngulia	Live
59	Maria	5049	04/04/2003	Female	Wanjiku	Born-TW	IPZ	Live
60	Muia	5050	03/04/2003	Male	UNK	Born-TW	Ngulia	Live
61	Mboya	5051	01/05/2004	Male	UNK	Born-TW	Ngulia	Live
62	UNK	5052	15/06/2004	UNK	UNK	Born-TW	Ngulia	Live
63	Kamu	5053	20/08/2004	Female	Mrs. Maktau	Born-TW	Ngulia	Live
64	Atoti	5054	20/08/2004	Male	Kadogoo	Born-TW	Ngulia	Live
65	Clare	5055	23/08/2004	Female	UNK	Born-TW	Ngulia	Live
66	Boit	5056	01/06/2004	Male	Wairimu	Born-TW	Ngulia	Live
67	Faith	5057	14/02/2005	Female	UNK	Born-TW	Ngulia	Live
68	Georgina	5058	01/09/2004	Female	Mangelete	Born-TW	IPZ	Dead
69	Ruth	5059	01/09/2004	Female	Sveda	Born-TW	Ngulia	Live
70	Hope	5060	01/03/2005	Female	Achieng	Born-TW	Ngulia	Live
71	Gabi	5061	01/05/2005	Female	Wanjiku	Born-TW	Ngulia	Dead
72	Catherine	5062	01/05/2005	Female	UNK	Born-TW	Ngulia	UKN
73	Wanjiku's calf	5064	01/02/2008	Female	Wanjiku	Born-TW	Ngulia	Live
74	Maktau Calf	5065	01/02/2008	Female	Mrs. Maktau	Born-TW	Ngulia	Live
75	Cedric	5066	01/02/2008	Male	UNK	Born-TW	Ngulia	Live

No	Name	ID	Birth Date	Sex	Mother	Origin	location	Status
76	Mbai cf	5067	01/04/2008	Unknown	Mbai	Born-TW	Ngulia	Live
77	Reiner	5068	01/04/2008	Male	UNK	Born-TW	Ngulia	Live
78	Blythe	5069	01/05/2008	Female	Wamboi	Born-TW	Ngulia	Live
79	Saum's calf	5070	01/05/2008	Female	Saumu	Born-TW	Ngulia	Live
80	UNK 1	5071	01/06/2008	Female	UNK	Born-TW	Ngulia	Live
81	Sveda Calf	5072	01/07/2007	UNK	Sveda	Born-TW	Ngulia	Live
82	Wairimu's cf	5075	01/02/2008	Female	Wairimu	Born-TW	Ngulia	Live
83	Shamira's cf	5076	01/02/2008	Female	Shamira	Born-TW	Ngulia	Live
84	Ormanya's cf	5077	01/02/2008	Female	Ormanya	Born-TW	Ngulia	Live
85	Mangelete's cf	5078	01/01/2009	UNK	Mangelete	Born-TW	Ngulia	Live
86	Kadogo's cf	5079	01/01/2009	Male	Kadogoo	Born-TW	Ngulia	Live
87	UNK 2	5080	01/01/2009	UNK	UNK	Born-TW	Ngulia	Live

Chapter 3

A reliable, single stage method for gender determination in black rhinoceros from low-copy template DNA

3.1 Abstract

I report the development of a simple, accurate, single-stage 5′-exonuclease assay for gender determination in black rhinoceros from low-copy template DNA. The assay targets a single nucleotide polymorphism in the last exon between the sex-linked zinc finger homologues in the black rhinoceros. I demonstrate that this method offers significant advantages over other methods of molecular sexing both in terms of accuracy in assigning gender from degraded templates, but also in the high throughput nature of the system, which greatly facilitates the multiple-tubes approach necessary for accurate gender determination from samples obtained using non-invasive methods.

3.2 Introduction

The black rhinoceros (*Diceros bicornis*) has been subject to one of the severest human induced declines of any mammalian species A significant increase in commercial poaching during the 1970s reduced numbers from an estimated population of 65,000 to approximately 2,500 animals in just two decades (Emslie & Brooks 1999). The remaining populations are vulnerable to the persistent threat of poaching and require intensive security and monitoring to ensure their persistence (Okita-Ouma *et al.* 2007). For many populations, monitoring is extremely difficult due to the secretive nature of the animals and the thick vegetation in the areas they inhabit (Mulama & Okita 2002). Consequently, for some populations baseline data on population sizes and sex ratios are absent (Foose 2001). Moreover with populations small and fragmented, metapopulation management is necessary in most rangeland states in order to maintain population viability (Emslie & Brooks 1999, Okita-Ouma *et al.* 2007). The application of molecular genetic techniques, particularly the genotyping of DNA from faeces, has the potential to greatly facilitate the conservation and management of this endangered species (Cunningham *et al.* 2001, Harley *et al.* 2005). An accurate, simple, inexpensive high through-put method for sexing black rhinoceros from low-copy DNA template would therefore be a valuable tool for the monitoring and management of populations.

In mammals, molecular sexing is accomplished by the detection of Y-linked markers, usually through PCR amplification and detection by agarose gel electrophoresis (Taberlet *et al.* 1993, Reed *et al.* 1997, Gibbon *et al.* 2009). However, such an approach has significant shortcomings when applied to low-copy template DNA such as that obtained from faeces, which is often of low quality and quantity (Taberlet *et al.* 1999). Allelic dropout or low amplification yield of Y-linked markers may lead to the incorrect gender

determination in low-copy DNA samples (Broquet & Petit 2004). Several strategies have been proposed to increase the accuracy of molecular sexing from low-copy DNA templates, such as the incorporation of a positive control in the form of X-linked markers as indicators of amplification success and the adoption of a multiple-tubes approach to counter allelic dropout (Taberlet *et al.* 1996, Schmidt *et al.* 2003). However, even with a positive control, differences in the size of amplification products and amplification efficiency can lead to erroneous results from degraded low-copy DNA (Rosel 2003, Morin *et al.* 2005). Recently, a number of techniques have been published that offer increased sensitivity and throughput to molecular sexing (Morin *et al.* 2005, Peppin *et al.* 2010). Peppin *et al* (2010) identified a 7-bp size polymorphism between the X and Y homologues of a zinc finger intron in the black and white rhinoceros which could be used in short tandem repeat (STR) profiling as a means of molecular sexing the two species. This method offers several advantages for low-copy template molecular sexing as the marker employed is short in length (102 bp) and therefore more suitable for degraded DNA than are other markers used in the more commonly used PCR-RFLP methods which are typically 250-800 bp in length (Peppin *et al.* 2010). The use of fluorescence tagged primers, and the separation and detection of the two homologues by capillary electrophoresis also offers the potential of greater sensitivity for the detection of low amplification yields and higher throughput than agarose gel based methods. Whilst the STR profiling method developed by Peppin *et al* (2010) does have advantages over many methods, the 5′-exonuclease assay technique developed by Morin for the molecular sexing of cetaceans offers greater benefits in terms of simplicity, cost and speed of analysis. Morin *et al* (2005) developed a 5′-exonuclease real-time PCR assay that utilises double-labelled fluorogenic or TaqMan® probes to detect a single nucleotide polymorphism (SNP) between the sex-linked zinc finger homologues in cetaceans. This method has the benefits of increased sensitivity of detection and small marker size, but is also a single stage system. The ability to process a large number of samples at moderate cost in a single system offers real benefits for increasing accuracy of low-copy DNA gender determination by facilitating the application of a multiple tests to counter allelic dropout in degraded samples.

Here the development of a 5′-exonuclease assay which targets a SNP in the last exon of the ZFX and ZFY homologues in the black rhinoceros is reported.

3.3 Method

Tissue and faecal samples were collected in Kenya from wild *D. b. michaeli* of known sex, as part of a larger conservation genetics project on the species. Tissue samples were taken from the ears of immobilised black rhinoceros by Kenya Wildlife Service vets as part of routine ear notching procedures to aid identification and monitoring. Faecal samples were collected from positively identified animals of known sex that were observed defecating, which ensured correct correlation of SNP findings and sex of the animal for initial validation purposes.

Tissue samples from six male and six female rhinoceros of confirmed sex were used for the ZFX and ZFY sequencing. Whole genomic DNA extractions from the tissue were performed using the Qiagen DNeasy Blood &Tissue Kit (Qiagen, Crawley, UK), using the supplied reagents and protocols.

ZFX and ZFY multiplex amplifications were carried out in 50 µl containing 1 x *Pfx* reaction buffer, 100 µM each dNTP, 0.5 µM each of forward primer ZFY0097 (CATCCTTTGACTGTCTATCCTTG, (Palsbøll *et al.* 1992)) and reverse primer P2-3EZ (GCACTTCTTTGGTATCTGAGAAAGT; (Aasen & Medrano 1990)), 1.5 units of *Pfx* DNA polymerase and 0.1 µg/µl of bovine serum albumin (BSA; Sigma-Aldrich) and 1.0 µl of template. A MJ Research PTC-200 thermal cycler was used with the following profile: denaturation at 94 ^{o}C for 45s, followed by 25 cycles of denaturation at 94 ^{o}C for 45s, annealing at 59 ^{o}C for 45s, extension at 72 ^{o}C for 1min 15s and a final extension at 72 ^{o}C for 10 min.

The multiplex amplification products were ligated into pCR®4-TOPO® plasmids (Invitrogen) to permit identification of the separate ZFX- and ZFY-specific sequences: amplicons from each individual animal were cloned twice. PCR amplicons were incubated with 1 unit of *Taq* polymerase (Invitrogen) at 72 ^{o}C for 10 min to generate the non-templated 3' adenine nucleotide addition. The amplicons were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The TOPO TA Cloning Kit for Sequencing (Invitrogen) was used for cloning: purified PCR amplicons were ligated into the plasmid vector and transformed into TOP10 One Shot Cells (Invitrogen) according to the manufacturer's instructions. In order to determine successful amplicon insertion into the plasmids, 5 µl of recombinant plasmid DNA,

isolated using Wizard Plus SV Minipreps Purification System (Promega), was digested with 10 units of *Eco* RI restriction endonuclease (Invitrogen). A 5 µl aliquot of the digestion reaction was electrophoresed through 2% agarose to identify the *Eco* RI-released amplicon insert. Using the recombinant plasmid DNAs, the inserted amplicons were sequenced in both directions using the PCR primers detailed above. The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used according to the manufacturer's instructions. Sequences were determined by electrophoresis on an ABI 3100 Capillary Sequencer (Applied Biosystems). Sequences were aligned and the vector DNA excised *in silico* using Geneious Pro ver.4.04.

The ZFX sequences (Genbank accession EU284593) were invariant for both males and females and the ZFY sequences (Genbank accession EU284594) were similarly identical for all cloned and sequenced males. The primers and probes for the 5′ -exonuclease assay were designed according to published guidelines (Livak *et al.* 1995, Morin *et al.* 1999, Smith *et al.* 2002). The assay targeted a 94-bp region of the aligned Zfx and Zfy sequences with a synonymous nucleotide difference at nucleotide 402. The primers and probes were designed and selected using File Builder (Applied Biosystems), primers were designed to anneal at 60 ^{o}C and to flank an oligonucleotide probe designed to anneal 7 ^{o}C above the annealing temperature (T_a) of the primers (Morin *et al.* 2005). The forward primer (ZFXY_Rhino_12F) sequence was GTCCACAGCAAGAACTTTCCTCATA and the reverse primer sequence was (ZFXY_Rhino_12R) CAGTATGGATTCGCATGTGCTTTT. Probes were synthesised with 5' reporter dye (FAM for ZFX_Rhino and VIC for ZFY_Rhino) and a 3' quencher (NFQ). The probe sequences were (ZFX_Rhino) FAM- AAACCTTTACCACACTCC-NFQ and (ZFY_Rhino) VIC- ACCTTTACCGCACTCC-NFQ (Figure 3.1). The 5'-exonuclease assays were performed in 25 µl reactions containing 1 x PCR buffer (HotStar Taq, Qiagen), 200 µM each dNTP, 1.25 units of HotStar Taq polymerase (Qiagen), 0.4 µg/µl of BSA and 20 x TaqMan Assay (Applied Biosystems). Amplifications were performed in a MX3000P Real-Time PCR System (Stratagene) using a 2-step thermal profile, with an initial incubation of 15 min at 95 ^{o}C followed by 50 cycles of 95 ^{o}C for 20s and 60 ^{o}C for 1 min 30s.

The assay was validated using tissue samples from 16 animals of known sex: 8 samples were from confirmed males and 8 from females. The assay was then tested for its effectiveness and accuracy in assigning gender from low-copy DNA templates on a subset

of 24 faecal samples from animals of known gender (12 male and 12 female). All tests with faecal material were performed according to a limited multiple-tubes approach with all reactions carried out in duplicate. All tests incorporated two negative controls.

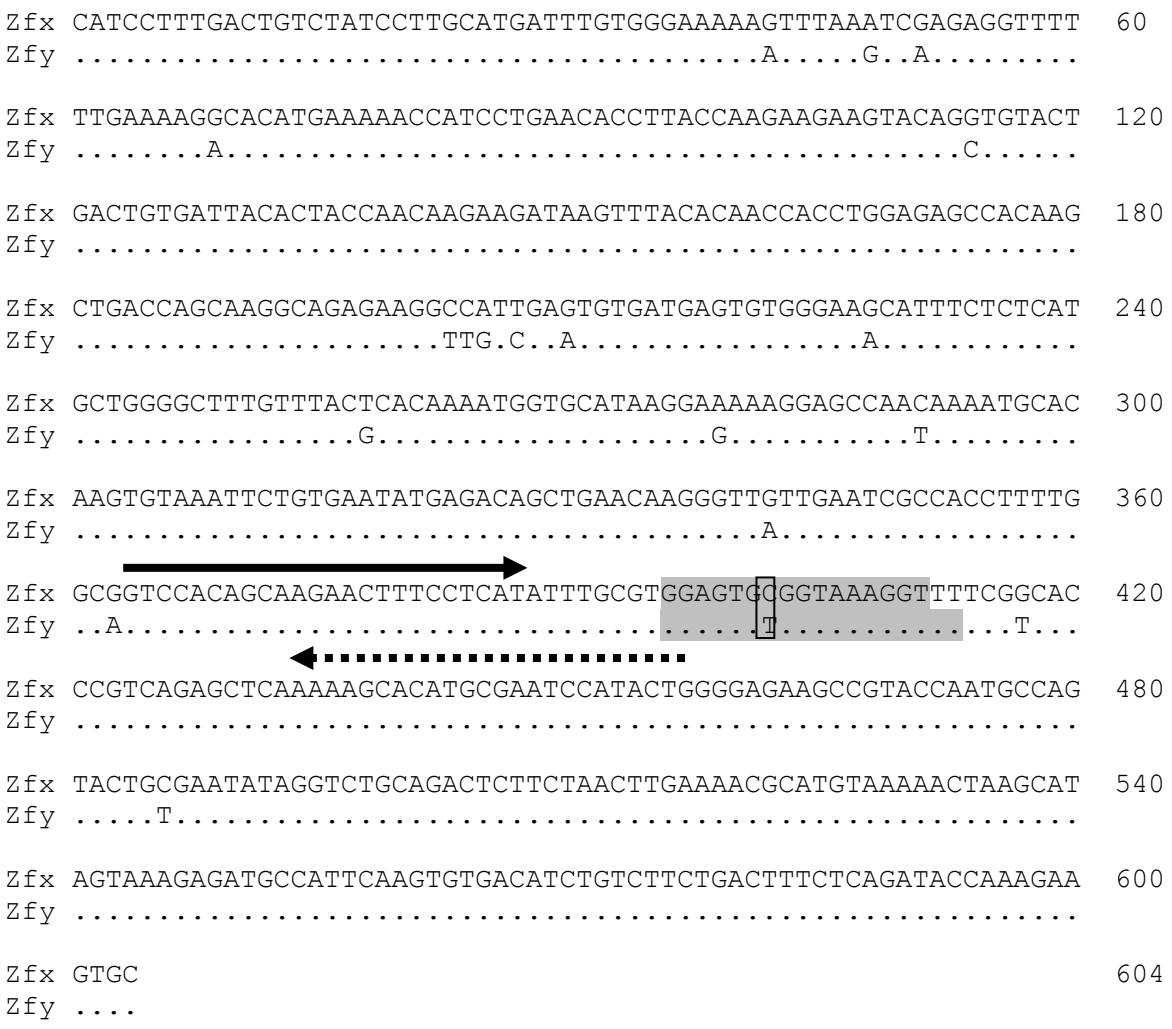


Figure 3.1. Alignment of exon 4 of Zfx and Zfy of black rhinoceros detailing the region amplified using primers ZFXY_Rhino_12F (solid arrow and ZFXY_Rhino_12R (dashed arrow) The grey shaded regions show the binding sites of the sex-specificTaqman® probes; and the relevant synonymous nucleotide difference is shown boxed.

3.4 Results and discussion

The 5'-exonuclease assay method proved to be an accurate and reliable method for gender determination using tissue samples obtained by invasive methods with a circa 94% success rate (15/16) in the trials with positive controls. The failed assay was for a female where no FAM signal was detected corresponding to the X-linked probe. Assignment of gender was correct and unambiguous for all reactions which produced positive results. For both male and female tissue samples the mean FAM (ZFX) fluorescent signal output was 6,000

dR (0.5 dRn) and the mean fluorescent output for HEX (ZFY) in positive male samples was 400 dR (0.03 dRN) and below 40 dR (0.005 dRN) for all female samples (Figure 3.2).

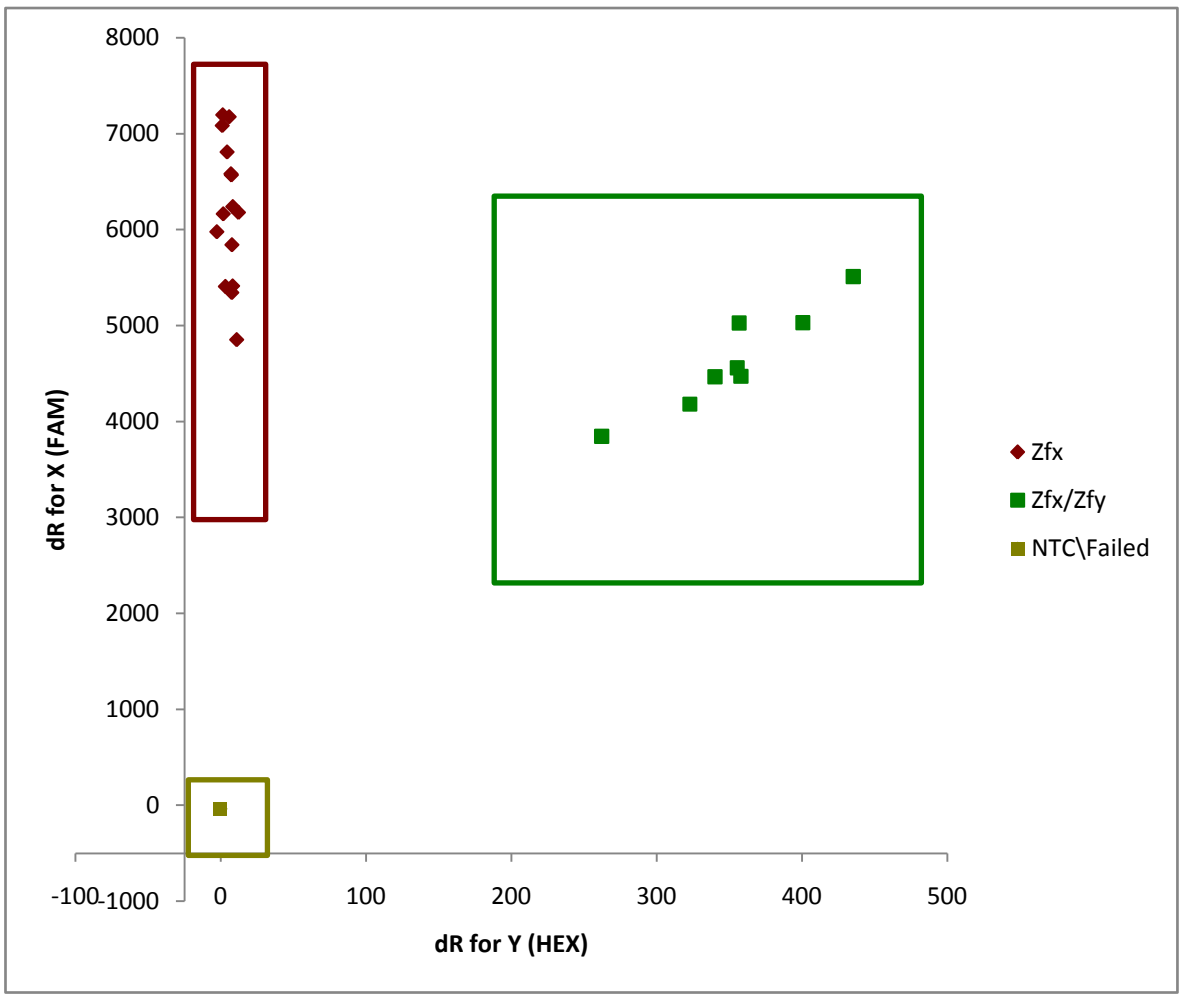


Figure 3.2. A plot of fluorescence values (dR) for the initial validation tests using16 tissue samples: 8 males and 8 females. All sexes are correctly identified with one sample failing to amplify**.**

The assay was successful in enabling accurate gender determination of individual rhino from faecal DNA (Figure 3.2). Whilst the assay was successful in enabling the sexing of individuals, the number of failed reactions for the ZFX probe (internal positive control) was higher than those for the high-copy DNA tests using tissue, with a success rate of ca. 87.5% (21/24). The fluorescence values for both probes tended to be more variable and slightly lower than for the high-copy tests, which in some cases resulted in potentially ambiguous scoring.The faecal DNA dR values ranged from 540 to 8600 for HEX and 160 to 940 for FAM compared to the high copy DNA test where the values for HEX were 4900 to 7200 and 250 to 450 for FAM. Similarly, in some cases the assay failed for one of the probes with the other homolog being scored, in the case of males this resulted in either the

Y probe showing a high fluorescent value with the X probe failing or vice versa. In cases where the Y probe alone failed, this could easily result in the mis-sexing of an individual. However in the tests with male faecal samples the instances where the ZFY probe failed to amplify whilst the ZFX probe gave a positive result occurred only in two reactions out of the 48 total reactions and for two separate animals.

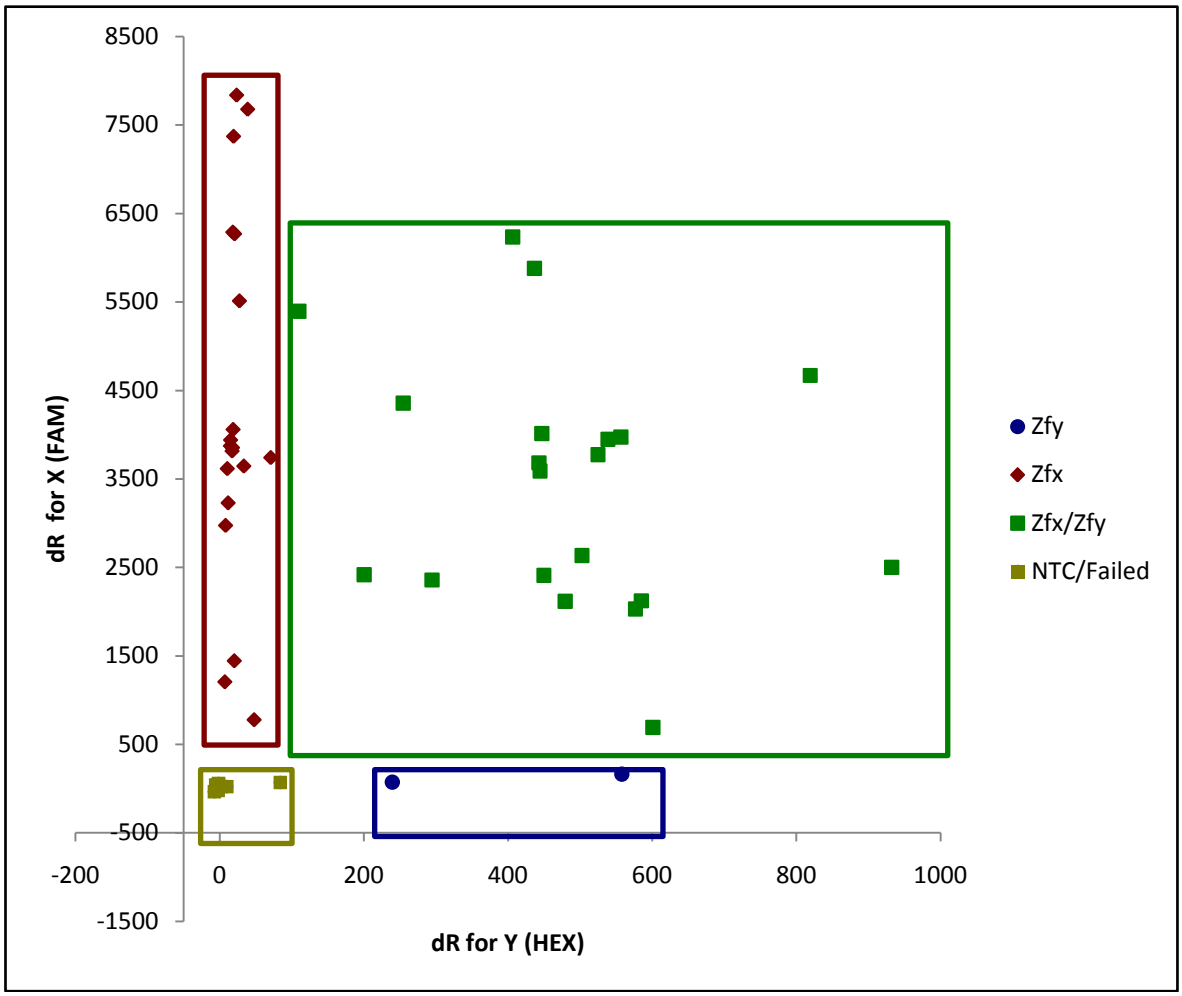


Figure 3.3. Plot of fluorescence values (dR) for initial low-copy DNA template validation test using 24 faecal DNA samples: 12 females and 12 males. All reactions performed in duplicate.

In these instances the limited multiple-tubes approach ensured correct gender assignment. The trial results of the 5'-exonuclease assay on low-copy DNA demonstrates that in males it is possible for only the X-linked allele to be successfully detected with the Y-linked probe failing (Figure 3.3); therefore, despite the inclusion of a positive control, sex may be incorrectly assigned in cases of low-copy DNA typing even with an extremely sensitive system. In light of this, a high through-put system such as the one described here, which is capable of detecting very low quantities of DNA, is desirable for molecular sexing where a large number of replicates is essential to ensure accuracy.

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Chapter 4

Demographic history of the Kenyan black rhinoceros (*D. b. michaeli*) inferred from mtDNA and microsatellite genotyping

4.1 Abstract

The black rhinoceros (*Diceros bicornis*) has been subject to one of the severest human induced declines of any mammalian species. A significant increase in poaching during the 1970s reduced the Kenyan population by over 99%, from a population of approximately 20,000 animals in 1970 to just 380 animals in 1987. At the beginning of 2006, the recovering population numbered approximately 540 animals (~85% of the wild population), with the majority protected within designated fenced sanctuaries. These isolated populations are comprised of a mixture of individuals from across the species former range in Kenya and managed within a sanctuary system to protect them from the continuing threat of poaching. To assist in the effective metapopulation management of these isolated populations, data is presented here both on the current levels of genetic diversity and the range of historic genetic diversity captured within five enclosed sanctuary populations. A total of 166 black rhinoceros were genotyped for 9 microsatellite loci and a 507 bp segment of the mtDNA control region. Genetic analysis and the examination of translocation records shows that the five sanctuaries are comprised of historic populations from three geographic regions within the country and that significant admixture has occurred between these historically divergent populations. The metapopulation retains significant levels of genetic diversity for both nucleic ($A = 5.0$, $H_E = 0.689$) and organelle ($\pi = 0.007$) genomes, with levels of diversity in individual populations related to the amount of admixture of former populations. The data presented should facilitate translocation strategies to maximise reproductive output whilst avoiding possible inbreeding and outbreeding depression within these populations.

4.2 Introduction.

Natural populations of many species are experiencing dramatic declines in numbers and distribution as a direct result of human activities (Pimm *et al.* 2001, Ceballos & Ehrlich 2002). The severity of the demographic declines for some species has necessitated the movement of remaining individuals into protected areas and captive breeding programs where resources can be concentrated to prevent extirpation and promote recovery (Balmford *et al.* 1995). Moreover, increasing fragmentation of populations, and the concomitant reduction in dispersal between previously connected areas, has led to the concept of metapopulation management being regarded as an integral part of many conservation strategies, particularly for large vertebrates; metapopulation management utilises active translocations to offset the detrimental effects of small population size and, more generally, to maintain natural evolutionary processes by allowing individuals to maintain "dispersal" across an otherwise inhospitable matrix (McCullough 1996, Margules & Pressey 2000, McCarthy & Possingham 2007, Newmark 2008). Endangered species management is therefore increasingly reliant on the mixing of individuals from different historic populations. For many conservation priority species information on historic population structure and the wider genetic consequences of mixing potentially genetic divergent populations is unknown (Crandall *et al.* 2000).

Given the importance of genetic diversity for population persistence (Saccheri *et al.* 1998, Frankham 2005), and for response to future environmental challenges, a particular concern for any population experiencing a severe demographic reduction is the concomitant increase in rate of genetic erosion and a greater risk of inbreeding (Frankham 1996, Saccheri *et al.* 1998, Reed & Frankham 2003, Allendorf & Luikart 2007). In vertebrates, inbreeding typically has negative consequences, such as unmasking deleterious recessive alleles (Charlesworth & Charlesworth 1999). Indeed, several high-profile studies have increased the fitness of inbred populations by the augmenting the inbred population with a relatively small number of unrelated individuals, a phenomenon termed genetic rescue (Westemeier *et al.* 1998, Madsen *et al.* 1999, Ingvarsson 2001, Vila *et al.* 2003, Johnson *et al.* 2010). Conversely, mixing individuals from divergent genetic backgrounds may have negative consequences. In such instances the apparent reduction in fitness (outbreeding depression) is thought to be a consequence of dilution of locally-adapted genomes and/or the disruption of epistasis in co-evolved gene complexes (Lynch 1991, Fenster *et al.* 1997, Turelli & Orr 2000). Few studies have quantified outbreeding depression in natural

populations; with the main problem arising from an apparent fitness benefit (possibly associated with heterosis) in early generations of outcrossing and outbreeding depression only becoming manifest after several generations as recombination disrupts epistasis (Allendorf & Luikart 2007). Nonetheless it is extremely important to consider for metapopulation management with a number of recent studies highlighting the negative consequences of mixing genetically divergent populations (reviewed in Edmands 2007). Incorporating information on the genetic background of species is therefore important but poses serious difficulties for the metapopulation management of conservation priority species. Managers have to balance decisions between active translocation to offset potential inbreeding effects, which could have catastrophic effects in small populations, while avoiding mixing individuals from populations that are "too genetically divergent". Of course, what constitutes "too divergent" often is not known, particularly as outbreeding effects may not be evident until several generations after breeding (Edmands 2007). Indeed, for many managed populations the historic baseline patterns of genetic divergence prior to anthropogenic habitat alteration have not been quantified. Effective metapopulation strategies require knowledge of the amount of divergence between populations and the genetic status of the populations within the system, only then can animal movements be implemented which mirror as closely as possible the natural process of dispersal (Crandall *et al.* 2000).

The black rhinoceros (*Diceros bicornis*) has been subject to one of the severest human induced declines of any mammalian species and is a prime example of a species whose presumed historically isolated populations have been extensively mixed as part of crisis management to prevent extinction (Leader-Williams 1989). The species is currently regarded, and thus managed, as four separate subspecies, although there was considerable overlap between their ranges (Emslie & Brooks 1999). At the beginning of the 20th century the black rhinoceros was common throughout sub-Saharan Africa in areas outside the rainforest belts. During the 1970s and 1980s an increase in demand for rhinoceros horn led to a huge rise in the level of commercial poaching and a concomitant sharp reduction in black rhinoceros numbers by approximately 96% - from an estimated total population of 65,000 in 1970 to fewer than 2,500 animals by 1992 (Gakahu 1993, Emslie & Brooks 1999).

The East African subspecies *D. b. michaeli* was particularly affected by poaching (Western & Sindiyo 1972, Western 1982, Emslie & Brooks 1999). Historically, *D. b. michaeli* had

its largest populations in Kenya, although its range extended from northern Tanzania to Sudan, Ethiopia and Somalia. Up until 1970, Kenya had approximately 20,000 *D. b. michaeli* but poaching reduced this number to just 380 by 1987 (Brett 1993, Gakahu 1993, Emslie & Brooks 1999). In 1984, at the height of the poaching crisis, the Kenyan government implemented a policy of protecting remaining black rhinoceros within specially-designated sanctuaries, so that resources could be concentrated to counter the poaching threat (Leader-Williams 1989, Brett 1993). During this period animals from populations across Kenya were mixed in 5 initial sanctuaries, on both private and government land. The sanctuary system proved successful and by 2006, Kenya had a recovering population of approximately 540 black rhinoceros (~85% of the wild population) contained within 14 geographically separate populations (Okita-Ouma *et al.* 2007). With the threat of extirpation apparently mitigated, there is a shift in emphasis from crisis management to biological management in order to still further promote recovery (Okita-Ouma *et al.* 2007).

The Kenyan black rhinoceros populations protected within the existing sanctuary system are a mixture of individuals from different regions of Kenya. However, neither the level of divergence among historic populations nor the degree to which the current sanctuary populations represent former free-ranging populations are known. Consequently information is needed not just on the genetic status of the current populations, but also to what effect the translocation of animals has had on former population structure and to what degree this has led to the admixture of previously divergent populations.

Previous studies examining genetic diversity in captive and wild black rhinoceros populations have produced conflicting results, some studies have found low levels of genetic diversity within populations based on allozymes (Merenlender *et al.* 1989) , mtDNA (Ashley *et al.* 1990) and microsatellites (Nielsen *et al.* 2008), whilst other studies have found high levels of diversity for the same markers (Swart *et al.* 1994, Brown & Houlden 2000) (Garnier *et al.* 2001, Harley *et al.* 2005). Whilst these studies were conducted on a mixture of populations with differing demographic histories, the relationship between the admixture of historic populations and levels of genetic diversity have not been examined in the black rhinoceros. Moreover there has been no comprehensive study examining genetic diversity measures for a single black rhinoceros metapopulation or an examination of the impact conservation strategies have had on

former population structure (Merenlender *et al.* 1989, Ashley *et al.* 1990, Swart *et al.* 1994, Garnier *et al.* 2001, Harley *et al.* 2005).

Results are presented here from the most comprehensive genetic study of any black rhinoceros subspecies to date, with genotypes from 166 identified black rhinoceros from 5 Kenyan sanctuaries approximately 31% of the Kenyan population. Using a combination of genetic markers (9 microsatellite loci and a 507 bp segment of mitochondrial DNA [mtDNA] control region) to (1) determine historical population structure in the Kenyan black rhinoceros and (2) assess population composition and the level of admixture that is a consequence of sanctuary management. These data are presented as baseline information for the effective metapopulation management of the black rhinoceros in Kenya with the aim of facilitating the recovery of this endangered species.

4.3. Materials and methods

4.3.1 Sample collection and DNA extraction

The Kenya Wildlife Service (KWS) maintains a comprehensive database of all black rhinoceros within the sanctuary system with extensive training given to sanctuary personnel on the identification and monitoring of individual animals. One hundred and sixty-six individually-identified black rhinoceros were sampled from five sanctuary populations in Kenya (Table 4.1). Faeces was the predominant source of DNA from Lewa Wildlife Conservancy (LWC) and Sweet Water Game Reserve (SWG) now called Ol Pejeta Conservancy (OPC). In these two areas, animals were tracked on foot and, once located, identified by either distinctive ear notches or horn shape. For every positively-identified animal, two ~5g samples of faeces were collected from the outside of the fresh dung pile. Samples were preserved with approximately 5:1 ratio of desiccating silica:faeces and kept for up to six weeks at room temperature prior to DNA extraction. Faecal samples were supplemented where possible by tissue samples collected from animals immobilised during routine veterinary procedures; for 21 individuals both faecal and tissue samples were collected which enabled assessment of the accuracy of the faecal DNA typing. In addition tissue samples were collected from all founder animals for a new population of black rhinoceros (NOPC) that was introduced into an expanded area of OPC (in 2007), from 19 animals at Ngulia Rhino Sanctuary (NRS) and from 7 animals from Nairobi National Park (NNP). Tissue samples were stored in 70% ethanol at room temperature for up to 12 weeks prior to DNA extraction. Serum samples were provided by

KWS from the 20 founding black rhinoceros for the newly established (in 2004) Mugie

Table 4.1. Sampled black rhinoceros populations, including census population size, number and percentage of animals sampled and sample type.

Population	Code	n_p	n	%	n_f	n_t	n_s	n_{ft}
1. Ol Pejeta Conservancy	OPC	78	74	95%				
Sweet Waters Game Reserve	SWG	50	46	92%	27	19	0	15
Introduced Population (2007)	NOPC	25	25	100%	0	25	0	0
2. Lewa Wildlife Conservancy	LWC	45	42	93%	33	9	0	6
3. Mugie Rhino Sanctuary	MRS	28	27	96%	8	0	20	0
4. Ngulia Rhino Sanctuary	NRS	54	19	35%	0	19	0	0
5. Nairobi National Park	NNP	74	7	9.5%	0	7	0	0
Total		**275**	**166**	**c. 60%**	**68**	**79**	**20**	**21**

n_p = census population size (2006). n = number of animals sampled. % = percentage of census size sampled. n_f = number of animals genotyped from DNA extracted from faeces. n_t = number of animals genotyped ^{froim} DNA extracted from tissue. n_s = number of animals genotyped from DNA extracted serum. n_{ft} = number of animals genotyped from both faeces and tissue.

Rhino Sanctuary (MRS); in addition faecal samples were collected in 2009 from seven calves born at MRS. DNA was extracted from faecal samples using a QIAamp® DNA Stool Mini Kit (Qiagen) with modifications to the manufacturers' protocol of (1) extending the initial lysis at 55°C to overnight and (2) making two 50-µl elutions in 1xTE buffer after 15 min incubation. Three separate extractions were performed on each faecal sample. DNA extractions from tissue and serum were performed using a Qiagen DNeasy Blood & Tissue Kit and the ZR Serum DNA Kit™ (Zymo Research) respectively, both according to the manufacturers' instructions.

4.3.2 Genotyping

A 507 bp segment of the mitochondrial DNA (mtDNA) control region was amplified using the primers mt15996L (Campbell *et al.* 1995) and mt16052H (Moro *et al.* 1998). PCRs were performed in 25-µL (2 µl of faecal DNA extract or 200 ng of tissue/serum DNA) reactions containing, 2.5 µg BSA, 200 µM each dNTP, 2 mM $MgCl_2$, 2.5 µl 10x Qiagen® PCR Buffer , 1 µM each primer and 0.625 units of Qiagen® HotStarTaq™. Thermal cycling conditions were: 95°C for 15 min followed by either 30 cycles for tissue DNA or 40 cycles for faecal and serum DNA of (94°C, 60 s; 65°C, 30 s; 72°C for 60 s), with a final extension of 10 min at 72°C. PCR products were treated with ExoSap (USB) and then sequenced using BigDye v.3.1 chemistry (Applied Biosystems) according to the manufacturers' instructions and using 1.6 pmol of mt15996L and mt16502H in separate

Table 4.2. Characteristics of microsatellite loci

Locus	Repeat Motif	Dye	T_a (ºC)	Mg^{2+} (mM)	Size (bp)	N_a	H_o	H_E
BR17*	$(AT)_5(GT)_{18}$	PET	59	2.5	127-137	6	0.730	0.732
DB5ǂ	$(CA)_{13}$	PET	59	2.0	187-209	10	0.822	0.838
DB1ǂ	$(CA)_{14}$	NED	59	2.0	118-130	6	0.691	0.705
DB66ǂ	$(CA)_7TA(CA)_{16}$	VIC	57	2.0	182-208	8	0.825	0.794
BR4*	$(CA)_{19}$	VIC	46	2.0	117-147	13	0.751	0.819
BR6*	$(CA)_{15}$	6-FAM	50	2.0	139-145	4	0.736	0.642
DB52ǂ	$(CA)_{21}$	VIC	63	2.0	209-225	9	0.736	0.744
DB23ǂ	$(CA)_{12}$	6-FAM	55	1.5	174-185	4	0.412	0.431
DB44ǂ	$(CA)_4G(CA)_{16}$	PET	64	2.0	172-192	6	0.355	0.724
DB14ǂ	$(CA)_{13}$	6-FAM	60	2.5	283-289	3	0.340	0.522

Dye = 5' fluorescent label. T_a = optimal PCR annealing temperature. Mg^{2+} = optimal PCR magnesium chloride concentration. Size = size range of alleles. N_a = number of alleles per locus. H_O = observed heterozygosity. H_E = expected heterozygosity. *Cunningham *et al*. 1999. ǂ Brown & Holden 1999.

reactions. Sequencing products were purified and then electrophoresed on an ABI3130xl capillary sequencer. All sequences were edited, assembled and aligned in Geneious v.3.7.

Every sample was genotyped at ten microsatellite loci (Brown & Houlden 1999, Cunningham *et al.* 1999) (Table 4.2). The genotyping protocol followed a modified multiple tubes approach for the low-copy DNA (i.e. derived from faeces or serum) (Taberlet *et al.* 1996). The two samples collected from each individual were genotyped separately with the two separate genotypes pooled to give the individual genotype, each sample was genotyped 6 times at all 10 loci, i.e. each individual was genotyped for a total of 12 replicate reactions. PCRs were performed in 25-µL final reaction volume containing (2 µl of faecal DNA extract or 200 ng of tissue/serum DNA) reactions containing, 0.1µg/µl BSA, 200 µM each dNTP, 2.0-2.5 mM $MgCl_2$, 2.5 µl 10x Qiagen® PCR Buffer , 1 µM each primer and 0.625 units of Qiagen® HotStarTaq™ 0.5-1.0µm each primer (with the forward primer 5'-labelled with either NED, PET, 6-FAM or VIC–Applied Biosystems). Thermal cycling conditions were: 96°C for 15 min, followed by either 30 cycles for tissue DNA or 40 cycles for faecal and serum DNA of (94°C, 1min; T_a°C, 30s; 72°C for 1min), where T_a is the published locus-specific annealing temperature. PCRs on tissue DNA extracts were carried out with the reaction conditions described above, but with 25 cycles. PCR products were pooled into one of two genotyping panels (depending on allelic size range and the 5' fluorescent dye) along with Genescan-500 LIZ size standard (Applied Biosystems) and separated using capillary electrophoresis on an ABI3130xl genetic

analyser (Applied Biosystems). Allele sizes were determined using the cubic model in Genemapper v.3.0 genetic analysis software (Applied Biosystems).

4.3.3 General data quality and basic diversity statistics

Basic measures of mtDNA genetic diversity within populations, haplotype diversity (*h*) and nucleotide diversity (π) (Nei 1987), were calculated using ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010).

Microsatellite data were examined for mistyped alleles, large allelic dropout and null alleles using MICROCHECKER v.2.2.3 (Van Oosterhout *et al.* 2004). Estimates of allelic dropout for faecal DNA samples were derived also by comparing genotypes obtained from faecal and tissue samples collected from the same animal (n=21 individuals). For each sanctuary population, exact tests for Hardy-Weinberg equilibrium and genotypic linkage disequilibrium between all pairs of microsatellite loci were calculated using GENEPOP v.4.0 (Raymond & Rousset 1995, Rousset 2008) with Markov chain parameters of 1,000 dememorisations, 100 batches and 1,000 iterations per batch. Sequential Bonferroni corrections were applied to maintain a population-specific error rate of α=0.05 (Rice 1989).

Genetic polymorphism at microsatellite loci for each population was calculated as the mean number of alleles per locus (*A*), observed heterozygosity (H_O) and expected heterozygosity (H_E), and allelic richness (*Ar*) (Leberg 2002); Wright's (1931) inbreeding coefficient F_{IS} within populations was also estimated. All estimates of genetic diversity were calculated using FSTAT v.2.9.3.2 (Goudet 1995), except for estimates of H_O and H_E, which were calculated using ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010).

4.3.4 Population structure

Geographic origins were assigned to specific mtDNA haplotypes using the subset of older individuals that could be assigned an accurate location of origin prior to being moved into a sanctuary, as determined from a detailed examination of sanctuary studbooks and translocation records. A small remnant population of black rhinoceros in the Chyulu Hills (CNP) were genotyped as part of a separate molecular tracking project, a comparison of the mtDNA haplotypes in the current sanctuary populations with the mtDNA haplotypes of the animals from Chyulu Hills (CNP) facilitated the assignment of areas of origin (Wandera 2010).

Mitochondrial DNA pairwise genetic differences (Φ_{ST}) were calculated as a distance matrix between haplotypes using a K2P model (Kimura 1980) (α=0.5) with associated significant levels and 10,000 permutations in Arlequin v.3.5.1.2 (Excoffier & Lischer 2010). Pairwise distances between haplotypes were calculated using MEGA v.4.1 (Tamura *et al.* 2007). Genetic differentiation among the sanctuary populations, based on microsatellite genotypes, was quantified using pairwise F_{ST} (Weir & Cockerham 1984), calculated using Arlequin v.3.5.1.2

The pattern of spatial genetic structure based on differences among all individuals was assessed using the Bayesian, model-based clustering approach implemented by STRUCTURE v.2.3.1 (Pritchard *et al.* 2000). Eight independent runs of STRUCTURE were made, to assess output consistency and to calculate ΔK (Evanno et al. 2005). Initially, the number of clusters (K) was varied from 1 up to 8 using the admixture model and correlated allele frequencies. All model runs were based on 700,000 iterations after an initial burn-in period of 50,000 iterations to ensure convergence of the MCMC. The most pronounced partition (level of population subdivision) of the data set was identified using the method of Evanno et al. (2005), with ΔK calculated using STRUCTURE HARVESTER v.0.56 (http://taylor0.biology.ucla.edu/struct_harvest).

4.3.5 Phylogeographic analysis

Pairwise comparisons of mutations between haplotypes were used to construct a minimum spanning network in which haplotypes are the nodes of the network. The haplotype network was estimated using NETWORK v.4.6 (http://www.fluxus-engineering.com), and by assigning equal weights to all variable sites and with default values for the epsilon parameter (epsilon=0).

Phylogenetic relationships and estimates of divergence times between mtDNA haplotypes were performed via a two-stage Bayesian analysis using BEAST v.1.5.4 (Drummond & Rambaut 2007). Divergence time estimates were split into interspecific and intraspecific analyses, with appropriate demographic models applied to each data set. The interspecific analysis across *Perissodactyla* was performed using fossil calibration points on a concatenated set of 3 mtDNA genes (control region, 12S and NADH3) in order to determine priors for the subsequent intraspecific analysis of the Kenyan mtDNA haplotypes (Appendix 4.1). *Acinonyx jubatus* was used as an outgroup for the interspecific

analysis due to the strong support for *Perissodactyla* and *Carnivora* as sister taxa (Xu *et al.* 1996, Arnason *et al.* 2008). The concatenated sequence alignment was analysed with separate nucleotide models for each gene region (control region - GTR+G; 12S - GTR+G; NADH3 - HKY+G) as determined by the hierarchical log-likelihood test in MODELTEST v.3. An uncorrelated lognormal relaxed clock was used (Drummond *et al.* 2006)**,** with a Yule speciation prior applied across the tree; the analysis was performed three times each for 50,000,000 generations with trees sampled every 1,000 generations following a discarded burn-in of 5,000,000 generations. The independent runs were combined using LOG COMBINER v. 1.6.1 (Drummond & Rambaut 2007) to estimate posterior values and the effective sample size (ESS) was checked with TRACER v.1.5 (Rambaut & Drummond 2007). Maximum clade credibility (MCC) trees were identified and annotated using TREE ANOTATOR v.1.4.8 (Drummond & Rambaut 2007), with the mean times to most recent common ancestor (tMRCA) estimated by mean values and 95% highest posterior density (HPD). Intraspecific analysis for the Kenyan mtDNA control region haplotypes was performed using the tMRCA estimate for *D. b. michaeli* and *D. b. minor* obtained from the inter-specific analysis as a normal prior (1.2 MYA, ± 0.2 MYA). A coalescent prior was used for the tree with the constant growth and exponential growth population models both tested; a coalescent constant growth tree prior was selected for the final analysis as this yielded the highest posterior probabilities, although results from both models were very similar. A strict clock model was applied and a uniform prior was set on the substitution rate of 0.15 to 0.30 substitutions per site per million years based on the observed variance of control region mutation rates in primates (Ho *et al.* 2005). All other aspects of the analysis were identical to the interspecific analysis. The topology of the resultant intraspecific BEAST tree was confirmed by comparison with the topology of trees generated by a maximum likelihood approach in PhylML (Guindon & Gascuel 2003) and a non-molecular clock Bayesian inference approach implemented in MrBayes v.3.1.2 (Huelsenbeck & Ronquist 2001).

4.4. Results

4.4.1 Data quality

Two lineages in OPC demonstrated mtDNA heteroplasmy (*n*=16 individuals); these haplotypes (DB04 and DB06) differed by a single transition (G→A) at nucleotide position 246. Given the limited nucleotide divergence between the haplotypes (0.2%) animals from both lineages were pooled as the single haplotype DB06, which has the highest frequency

of occurrence. One serum sample from MRS produced a complete microsatellite genotype but failed to produce a useable mtDNA sequence; due to the limited amount of serum it was not possible to extract further DNA and produce the mtDNA haplotype of this sample. Comparison between genotypes obtained from faecal samples and complementary tissue samples (N = 21) indicated that the error rate per multilocus genotype was <2% (Pompanon *et al.* 2005). Of the 136 faecal samples collected, only 17 (*ca*. 12%) failed to produce a microsatellite genotype.

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Table 4.3 Microsatellite and mtDNA diversity indices for each population

	Microsatellite DNA (9 loci)						mtDNA control region		
Population	N	A	R_S	H_O	H_E	F_{IS}	N_h	h (SE)	π
LWC	42	4.6	3.87	0.622	0.637	0.024	4	0.395 (0.080)	0.004
SWG	46	5.2	4.37	0.685	0.710	0.036	4	0.662 (0.048)	0.006
NOPC	25	5.7	4.43	0.724	0.674	0.076	2	0.513 (0.037)	0.006
OPC	71	5.9	4.50	0.700	0.703	0.005	4	0.618 (0.038)	0.006
MRS	27	5.4	4.57	0.716	0.757	0.055	7	0.822 (0.044)	0.008
NNP	7	3.6	3.67	0.571	0.673	0.161	3	0.714 (0.127)	0.004
NRS	19	5.6	4.44	0.682	0.683	0.001	4	0.731 (0.058)	0.007
Total	166	5.0	4.23	0.667	0.689	0.034	9	0.733 (0.026)	0.007

N = number of sampled animals from each population. A = allelic diversity. R_S = allelic richness. H_O = observed heterozygosity. H_E = expected heterozygosity. F_{IS} = Wright's inbreeding coefficient. N_h = number of haplotypes. h = haplotype diversity. π = nucleotide diversity.

Genotyping all samples for 10 microsatellite loci revealed 166 unique microsatellite genotypes that represents some 31% of the known Kenyan black rhino population at the beginning of 2006 census (Okita-Ouma *et al.* 2007). Significant allelic dropout was not detected. One locus (DB44) suffered from null alleles, large, positive value of average F_{IS}=0.456 and significant deviations from expected Hardy-Weinburg equilibrium conditions and was therefore excluded from genetic analyses. Significant (P<0.05 after sequential Bonferroni correction) linkage disequilibrium was identified between just one pair of loci in one population (Br17 & Br4, MSR) and there was no evidence of significant deviation from expected HWE (P>0.05) at any of the nine remaining microsatellite loci.

4.4.2 Diversity indices

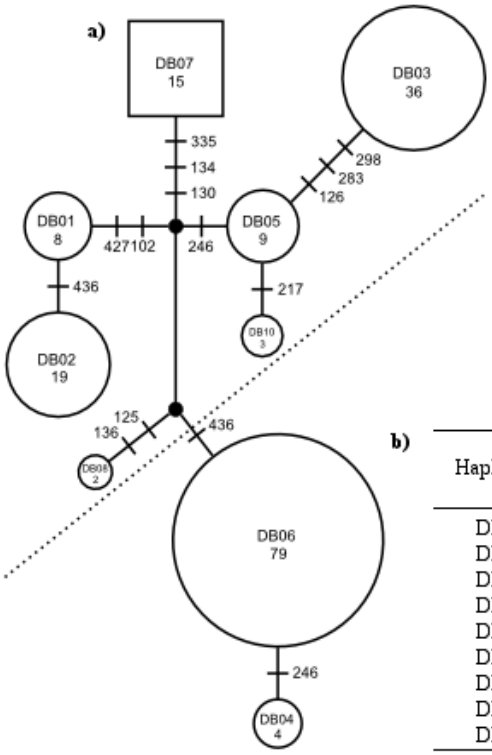
We identified 8 mtDNA haplotypes in these samples of Kenyan black rhinoceros. Thirteen sites in the 507 bp of the d-loop (2.8%) were polymorphic, of which 7 were parsimony informative (Figure 4.1); all 13 polymorphic sites were transitions and no indels were

detected. Sequence divergence between haplotypes ranged from 0.2% (one substitution) up to 1.7%, with a mean value of 1.1%. Haplotype diversity (*h*) within sanctuary populations ranged from 40% to 84% (mean *h*=73.3% ± 2.6%) and nucleotide diversity (π) within sanctuary populations varied between 0.4% and 0.9%, with an average π=0.7%.

All nine microsatellite loci were polymorphic, with 3 to 13 alleles per locus over the entire sample, allelic diversity (*A*) ranged from 3.6 alleles per loci to 5.9 per locus, with allelic richness (R_S) standardised to 7 individuals ranging from 3.7 to 4.6. Average expected heterozygosity (H_E) for all populations was greater than 0.6 (0.64 to 0.72) with an overall average H_E=0.69 (Table 4.3). Discounting the small sanctuary of NNP (*n*=7 individuals), the LWC population was least diverse at microsatellite (R_S=3.87, H_E=0.637) and mtDNA loci (N_h=4, *h*=0.4, π=0.004). The smaller and more recently founded MRS (est. 2006) population was the most diverse population for both microsatellites (R_S= 4.57, H_E = 0.756) and mtDNA (N_h = 7, *h* = 0.822, π = 0.008) from 7 haplotypes. The introduction of the new population (NOPC) (2007) (R_S = 4.43, *h* = 0.513) to the established Ol Pejeta population (SWG) (est. 1989) (R_S = 4.37, *h* = 0.662) had a positive effect on diversity measures for the population as a whole (R_S= 4.5, *h* = 0.618). The sample of 19 animals from the large NRS population (*n* = 54) also showed high diversity measures (R_S= 4.44, *h* = 0.731) for both genomes, with heterozygosity levels slightly below the average for all populations (H_E = 0.683).

4.4.3 Population structure

A detailed examination of translocation records enabled the putative area of origin for all 9 mtDNA haplotypes to be ascertained (Figure 4.1). Solio Game Reserve (SGR) in Laikipia District was one of the original fenced black rhinoceros sanctuaries (est. 1970) in Kenya and the main founder population for LWC and OPC; also, three animals from SGR were part of the MRS founder population and the entire NOPC population also came from SGR (Patton 2010). Translocation records for the founding animals of SGR indicated that animals were sourced from two geographically separate areas: (1) Laikipia District in the central highlands and (2) Chyulu Hills area (south east Kenya) (Patton 2010). Animals at OPC, LWC, MRS and NOPC (which originated from SGR) possessed three haplotypes DB03, DB04 and DB06. The Chyulu Hills molecular tracking project found haplotype DB03 in the remnant Chyulu Hills population and therefore it was assumed that haplotypes DB04 and the closely-related DB06 (*P* distance=0.2%) originated from the central highlands (Wandera 2010).



b)

Haplotype	1 0 2	1 2 5	1 2 6	1 3 0	1 3 4	1 3 6	2 4 3	2 4 6	2 4 8	2 4 4	3 3 5	4 2 7	4 2 8	Putative origin
DB01	A	C	C	T	A	G	A	T	G	T	C	C	A	Nairobi
DB02	·	·	·	·	·	·	·	·	·	·	·	·	C	Nairobi
DB03	G	·	T	·	·	·	G	·	A	G	·	T	·	Chyulu Hills
DB04	G	·	·	·	·	·	G	C	·	·	·	T	C	Central Highlands
DB05	G	·	·	·	·	·	G	·	·	·	·	T	·	Kibwezi / Tsavo East
DB06	G	·	·	·	·	·	·	C	·	·	·	T	C	Central Highlands
DB07	G	·	·	C	G	·	·	·	·	·	T	T	·	Amboseli
DB08	G	T	·	·	·	A	·	C	·	·	·	T	·	Tsavo West or Bura
DB10	G	·	·	·	·	·	·	·	·	·	·	T	·	Kibwezi / Tsavo

Figure 4.1. Kenyan black rhinoceros mtDNA control region haplotypes. A) Median joining network of mtDNA sequences; open nodes are haplotypes labelled with total number of individuals with node size proportional to frequency in current population, square node represents the root as derived by both Bayesian and maximum likelihood phylogenetic analysis. Black nodes depict median joining vectors of unsampled haplotypes with single base substitutions represented by slashes on the branches. Dotted line separates central highland haplotypes from southern haplotypes. B) Variable sites found in a fragment of 507 bp of the mtDNA control region in 165 black rhinoceros and putative geographic areas of origin within Kenya.

All founding animals for NNP were captured within the vicinity of the sanctuary and genotyping of animals at OPC that had been translocated from NNP confirmed the origin of haplotypes DB01 and DB02 in the Nairobi area. Haplotype DB07 was assigned to the Amboseli area on the basis of an orphaned animal at OPC that originally came from the Amboseli National Park, while the mother of three males at LWC originated from the Kibewzi/Tsavo East area of the country provided a link for haplotype DB10 to this area; DB05 which is similar to DB10 (*P* distance=0.2%) was also associated with Kibewzi/Tsavo East from the translocation records for NRS. The remaining haplotype

DB08 was linked putatively to the east of the country around Bura, based on the genotyping of a single individual at NRS.

Assigning geographic areas of origin to mtDNA haplotypes revealed that the current, sanctuary populations are now comprised of a mixture of animals from different historic populations (Figure 4.2). The founder animals of the MRS population were composed of the most geographically divergent haplotypes, with haplotypes originating from Nairobi (N = 3, 15.8%), Amboseli (N = 6, 31.6%), Tsavo East (N = 1, 5.2%), Chyulu Hills (N = 5, 26.3%) and the central highlands (N = 4**,** 21.1%). In contrast the LWC population was predominantly comprised of the central highlands haplotype (N = 40, 77%) with the remaining 23% consisting of three haplotypes from the south of the country. The SWG population consisted of a diverse mixture of haplotypes originating from three main areas, Nairobi (N = 11, ca. 24%), Chyulu Hills (N = 12, 26%) and the central highlands (N = 23, 50%). The NOPC population was split between Chyulu Hills haplotype (N = 11, 44%) and the central highlands (N =14, 56%), consequently the combined OPC population contained a similar composition to the established SWG population. The sampled NRS and NNP populations were composed of haplotypes from the Nairobi area, the south east of the country and the area around Bura in the east, with no haplotypes from the central highlands detected.

Values of F_{ST} varied between 0.013 and 0.127, and except for the distance between OPC and the components of NOPC and SWG, and between the two national park populations there was significant population subdivision ($P < 0.05$) at microsatellite loci for all sanctuary populations. Pairwise Φ_{ST} values for mtDNA haplotypes revealed similar significant differences ($P < 0.05$) between populations with values ranging from -0.008 to 0.501 (Table 4.4). Overall there was a mixture of low to moderate genetic differentiation among sanctuaries with strong similarities between distance measures for both genomes. Distance between populations therefore appears to be a function of historic population composition rather than a simple isolation by distance model. At present the largest distances for both microsatellite and mtDNA measures are between the private sanctuaries and the national park populations, with moderate differentiation between the three private sanctuaries. The largest differences were between NNP and LWC (F_{ST} = 0.1267, Φ_{ST} = 0.501), NRS and LWC (F_{ST} = 0.089, Φ_{ST} = 0.27) and OPC and NNP (F_{ST} = 0.093, Φ_{ST} = 0.326). Comparatively the two national parks (NNP, NRS) showed nonsignificant levels of genetic differentiation for both genomes (F_{ST} = 0.013, Φ_{ST} = 0.103).

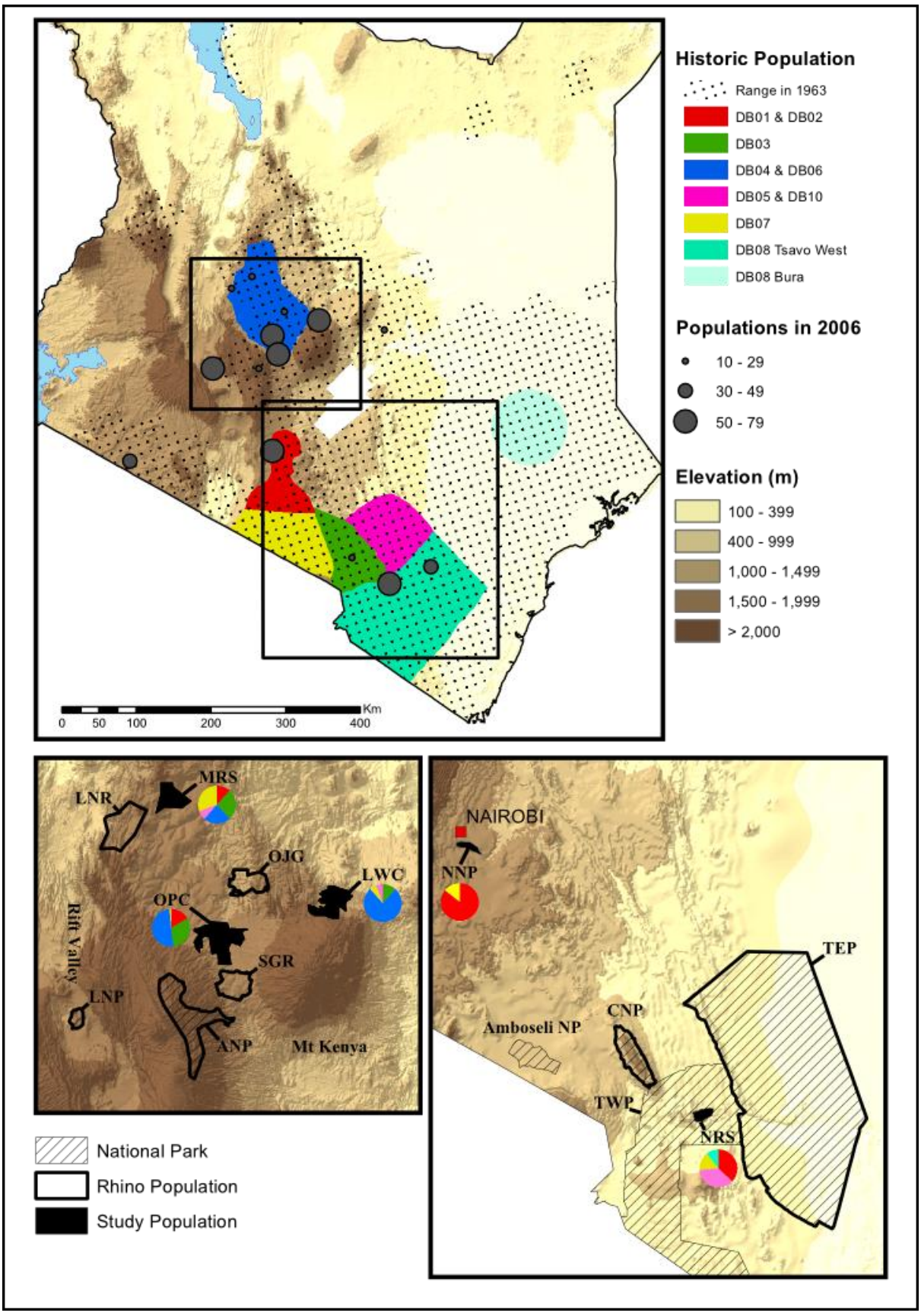


Figure 4.2. Map showing historic population structure of sampled Kenyan black rhinoceros. Insert maps show current sanctuary populations and relative composition of historic populations.

A two cluster model captured the majority of the genetic structure for the current sanctuary populations with a four cluster model also giving a high ΔK value (Appendix 4.2). Examination of the cluster assignment and mtDNA haplotype for presumed non-admixed individuals born before the translocation program shows that the two cluster model captures the genetic structure of the historic central highland populations and the historic southern Kenyan populations (Figure 4.3).

Table 4.4. F_{ST} values for microsatellite DNA (above the diagonal) and Φ_{ST} for mtDNA (below the diagonal) population pairwise comparisons

	LWC	**OPC**	**NOPC**	**SWG**	**MRS**	**NRS**	**NNP**
LWC		0.038**	0.035**	0.049**	0.078**	0.089**	0.127**
OPC	0.081**		0.000	-0.005	0.042**	0.061**	0.093**
NOPC	0.146**	0.007		0.019**	0.053**	0.070**	0.107**
SWG	0.077**	-0.008	0.048		0.042**	0.064**	0.093**
MRS	0.231**	0.112**	0.108*	0.120**		0.054**	0.052**
NRS	0.270**	0.160**	0.246**	0.123**	0.071*		0.013
NNP	0.501**	0.326**	0.455**	0.269**	0.265**	0.103	

Significant values. $^{**}P < 0.01$, $^{*}P < 0.05$

The two clusters show extensive admixture for those animals originating from SGR as indicated by the extensive mixing for NOPC population. Apart from the admixture which has occurred in SGR there appears to be a limited amount of mixture between the historic central highland population and the southern populations in general. The majority of individuals in LWC are assigned to the historic central highland population with the historic southern populations represented either by individuals with southern haplotypes or their presumed offspring. In accordance with the translocation records and mtDNA haplotypes and with the exception of one individual in NRS with haplotype DB08 from the east of the country; the national park populations and those individuals from MRS which originated from NNP, have no assignment to the historic central highland population. Those animals which were moved from Lake Nakuru National Park (LNP) into MRS demonstrated mixture for both clusters which is in accordance with translocation records for LNP showing that animals moved from SGR comprised the majority of the LNP founders.

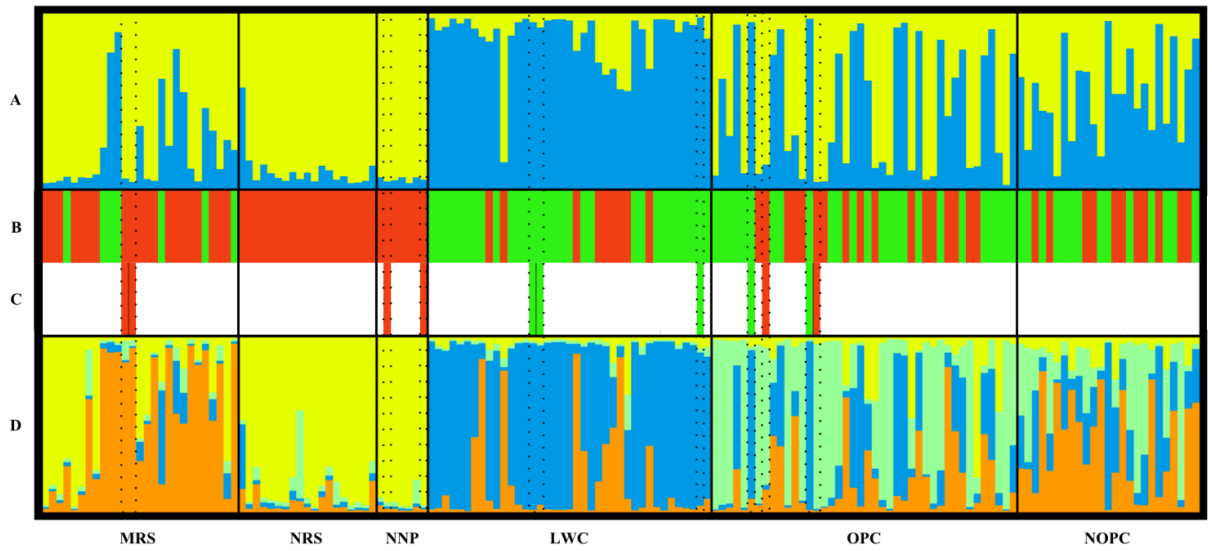


Figure 4.3. Output from model-based clustering analysis in STRUCTURE. A) K=2 . B) Individual's mtDNA haplotype grouped according to southern historic origin (red) and central highlands historic origin (green). C) Individuals born before translocation programme was implemented and therefore presumed not to be admixed, grouped according to south origin (red) and central highlands (green). D) K=4.

4.4.4 Phylogenetic Analysis and tMRCA Estimates

The interspecific phylogenetic analysis produced a maximum clade credibility tree with more than 95% posterior support for all nodes, with the exception of the node between the sister pairings of *R. unicornis*/*R. sondaicus* and *D. sumatrensis*/*C. antiquitatsis*. The posterior support for this node was 71%. The topology of the BMCMC tree was supported by the comparative maximum likelihood tree produced by PhylML, with a similar high support for all nodes other than the node for the split of the Asian rhinoceros species into sister clades (data not shown). Interspecific molecular clock analysis yielded a mean tMRCA (95% HPD) estimate for *Perisodactyla* and *Carnivora* of 85.69 MYA with a large range of 75.32 MYA between lower and upper 95% HPD limits (Table 4.5).

Estimates for tMRCA within *Rhinocerotidae* gave a mean tMRCA (95% HPD) estimate for *D. b. michaeli and D. b. minor* of 1.24 MYA (0.303 - 2.538). Subsequently the mean tMRCA estimate for the two black rhinoceros subspecies of 1.2 MYA was used as normal prior with a standard deviation of 0.2 MYA for the intraspecific analysis of the Kenyan *D. b. michaeli* haplotypes.

Table 4.5. tMRCA estimates from inter and intra specific mtDNA alignments (in million years ago).

Evolutionary Event	tMRCA MYA (95% HPD)
Interspecific Analysis	
Carnivora – Perissodactyla	**85.69 (53.519 - 128.838)**
Ceratomorpha – Hippomorpha	55.542 (51.626 - 59.421)
African Rhino - Asian Rhino	25 (17.846 - 33.296)
Ceratotherium – Diceros	9.952 (4.073 - 17.144)
E. caballus – E. asinus	3.424 (2.496 - 4.366)
C .s. simum – C. s. cottoni	2.39 (0.608 - 4.84)
D. b. michaeli – D. b. minor	1.24 (0.303 - 2.538)
Intraspecific Analysis	
D. b. michaeli – D. b. minor	1.183 (0.986 – 1.381)
DB02 / DB03	0.332 (0.114 – 0.597)
DB02 / DB06	0.301 (0.095 – 0.548)
DB02 / DB07	0.321 (0.085 – 0.591)
DB02 / DB10	0.304 (0.100 – 0.551)
DB03 / DB06	0.302 (0.085 – 0.563)
DB03 / DB07	0.343 (0.115 – 0.614)
DB03 / DB10	0.205 (0.037 – 0.489)
DB06 / DB07	0.315 (0.096 – 0.570)
DB06 / DB10	0.275 (0.073 – 0.504)
DB06 / DB08	0.182 (0.029 – 0.400)

Posterior support for nodes within the intraspecific analysis was generally lower than that for the interspecific analysis. Only the nodes for the split between *D. b. minor* and *D. b. michaeli*, and for those haplotypes which differed by 0.2% (one substitution) achieved support higher than 90%. Amongst the more divergent haplotypes posterior support was as low as 0.188 for the node between the [DB03, DB09, DB05, DB10] clade and the [DB04, DB06, DB08] clade (Figure 4.4). However the topology of the BMCMC tree was supported by the maximum likelihood tree created in PhylML, although with similar low support for the higher nodes within the *D. b .michaeli* haplotypes (data not shown). The Amboseli haplotype is indicated as the basal haplotype, with a split between the Nairobi haplotypes and the south eastern/central highlands haplotypes. The mean clock rate for the control region across all haplotypes and *D. b. minor* was 0.028 substitutions per site per million years, with the rate varying between 0.026 and 0.027 across the *D. b. michaeli* haplotypes. Mean tMRCA estimates (95% HPD) varied from 0.05 MYA for the Laikipia Haplotypes (DB04 and DB06) to 0.343 MYA between the Chyulu haplotype (DB03) and the Amboseli haplotype (DB07). All mean intraspecific tMRCA estimates had large ranges for lower and upper 95% HPD values.

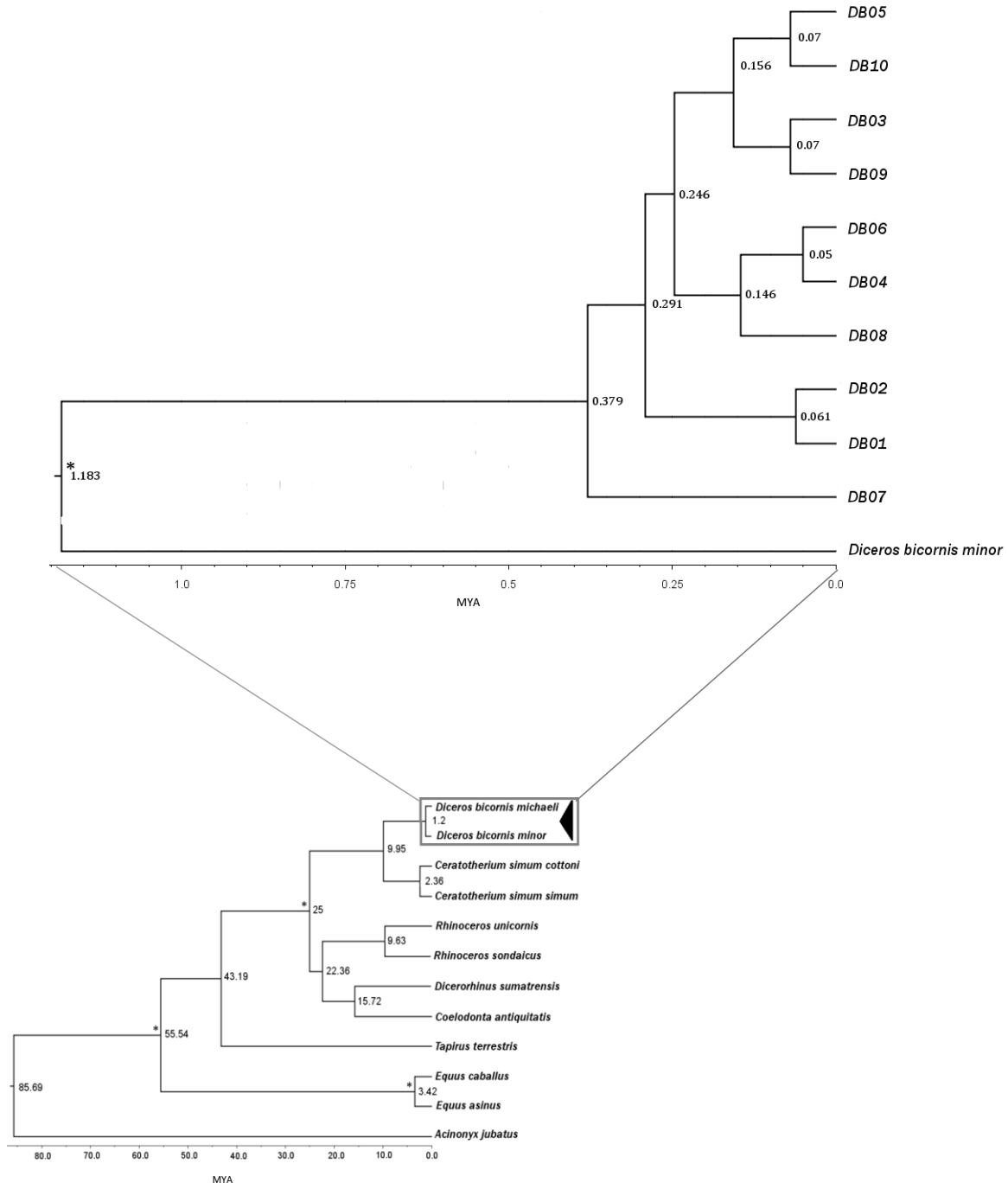


Figure 4.4. MCC trees for phylogenetic analysis of inter and intra specific divergence times. Interspecific MCC tree is presented at the bottom and the expanded intraspecific MCC tree is presented above. Node labels denote mean estimated divergence times (million years ago). * indicates estimated divergence priors. See text for information of posterior probabilities.

The minimum spanning network supported the general relationships between haplotypes shown by the ML and BMCMC trees. The network contained two median vectors; one separating DB07 from the other haplotypes and the other separating DB08 from the two central highlands haplotypes (Figure 4.1).

4.5 Discussion

Conservation biology is by definition a crisis discipline, in many instances constraints of time and resources require management decisions to be implemented without a full investigation of the potential consequences of a particular strategy (Soule 1985, Reed *et al.* 2003). The translocation of animals between hitherto isolated populations is a common management practice which is often necessary but frequently undertaken without the consideration of genetic management (Moritz 1999). The black rhinoceros in Kenya is such an example of a species where circumstances necessitated the movement of animals into more secure areas with the resultant mixture of previously isolated populations. Furthermore the current admixed population is typical of many conservation priority species where small population size and no scope for natural dispersal requires an active metapopulation management strategy to maintain long-term population viability (McCullough 1996, Margules and Pressey 2000, McCarthy and Possingham 2007). In order to understand the effects of management practices on historic population structure, I have undertaken the largest genetic study to date on a black rhinoceros metapopulation. I have found that the current Kenyan populations within the sanctuary system are a mixture of historic populations from three broad geographic regions of the country, with extensive interbreeding having occurred between these separate historic populations. The mixture of populations within the current sanctuaries has maintained high levels of genetic diversity and heterozygosity despite a severe population bottleneck. However given the extent of admixture between genetically divergent populations the potential for future outbreeding depression cannot be excluded.

4.5.1 Historic Population Structure

The phylogeny for both the median joining network and the Bayesian analysis supports the inferred geographic origins of the mtDNA haplotypes and the relationships between historic populations. The close relationship between the Amboseli haplotype (DB07) and the Nairobi haplotypes (DB01, DB02) reflects the recently diminished seasonal migration of animals between these two areas and northern Tanzania (Georgiadis 1995, Kristjanson *et al.* 2002). The Chyulu Hills (DB03, DB09) and the Tsavo East (DB05, DB10)

haplotypes demonstrate a close phylogenetic relationship, which is indicative of their close proximity to one another and the presumed movement of animals between these two areas. Interestingly the central highlands haplotypes (DB04, DB06) shows a close phylogenetic relationship with the Bura eastern haplotype (DB08) and suggests that the steep topography and forested areas of the southern central highlands may have acted as a landscape barrier to the dispersal of the black rhinoceros. Conversely the areas to the north and east of the central highlands are characterised by much more open savannah type habitat which adjoins the present day Tsavo National Parks.

My tMRCA estimates for the split between the two African rhinoceros species (9.9 MYA) are more recent than Willersley *et al* (2009) estimate of 15 MYA based on whole mtDNA genome analysis but closer to estimates from the fossil record of about 7.0 MYA (Bishop 1971, Cooke 1972). Moreover my estimate for the spilt between *D. b. minor* and *D. b. michaeli* (1.2 MYA) is in accordance with the estimate obtained by Brown & Houlden (2000) of between 0.93 and 1.3 MYA. Phylogenetic analysis and tMRCA estimates indicate a colonisation and population expansion event in the south of the country approximately 300 kyr. Whilst the interpretation of divergence estimates has to be undertaken with some caution (Templeton 1993, Pulquerio & Nichols 2006), evidence for the colonisation and expansion of the black rhinoceros around this period is in concordance with a large body of work on the influence of Pleistocene conditions on the evolutionary history of African mammals (Lorenzen *et al.* 2010, Arctander *et al.* 1999, Flagstad *et al.* 2001, Nersting & Arctander 2001, Muwanika *et al.* 2003, Okello *et al.* 2005, Nyakaana *et al.* 2002, Lorenzen *et al.* 2007). My results tentatively suggest that the black rhinoceros in Kenya was affected by a change in habitat conditions linked to a change in the Pleistocene climate and that the phylogenetic and tMRCA results possibly reflect a recolonisation from a southern refugia following an improvement in environmental conditions. The results indicate that the black rhinoceros recolonised Kenya from northern Tanzania in the proximity of the present day Amboseli National Park around 300 kyr and from here expanded to encompass the historic ranges covered in this study. This interpretation is in concordance with Arctander *et al* (1999) whose phylogeographic study of three African bovids indicated recolonisation and expansion by wildebeest from a southern refugia in the Amboseli-Nairobi area less than 600 kyr. With the Rift Valley as a potential landscape barrier to the west, the close relationship between the central highland haplotype and eastern haplotype indicates that the forested central highlands were colonised much later than the south of the country and probably from populations expanding from the eastern

part of the country (Pitra *et al.* 2002, Antunes *et al.* 2008). The historic central highlands population has been isolated from the southern populations for a considerable period of time. Both the tMRCA estimates and the STRUCTURE and distance measures based on historic population composition of the current sanctuaries indicate that there is a significant genetic difference between the rhinoceros originating from these two areas.

4.5.2 Current Sanctuary Populations

All of the examined sanctuary populations represent a mixture of former historic populations with varying levels of admixture between historic populations. The mixture during the 1970s of animals originally from the Chyulu Hills region and the central highlands within the Solio Game Reserve had a profound effect on the levels of admixture in the current sanctuary populations. Animals from SGR have been used as founders for many of the existing sanctuaries in Kenya including OPC, MRS, LWC and LNP. Examination of the founders from SGR for MRS and OPC indicates that extensive admixture occurred amongst the small number of founding SGR rhinoceros. The current national park populations however show little evidence of admixture even for the four cluster model of genetic structure.

Interestingly the former extensive populations from the west and the north of the country are not represented in the sampled sanctuary populations (Brett 1993, Walpole *et al.* 2001). A single male at OPC is confirmed as having originated from the north of country, however due to misidentification in the field, this animal was not sampled as part of the study. An absence of haplotypes from these areas in the current study is possibly a reflection of localised management practices and these historic populations may well be represented in other protected areas, such as the Maasai Mara and Lake Nakuru National Park. Although ten animals from Lake Nakuru National Park which were moved to MRS in 2006, were genotyped as part of this study and had haplotypes corresponding to the geographic regions already identified.

It is apparent that distances measures between populations support the broad differentiation of current populations based on relative compositions of central and southern historic populations. F_{ST} values for microsatellite data show there is no significant differentiation between the national park populations and only little differentiation between LWC and OPC, possibly due to the relatively high content of central highland genes in the OPC population. MRS shows moderate differentiation from LWC and NOPC probably due to

the relative lack of Nairobi area genes in both of these populations compared to MRS. However differentiation between the complete OPC population and MRS is low, presumably due to the inclusion of the Nairobi genes contained within the original SWG population. Population differentiation for mtDNA data supports the microsatellite F_{ST} values, although moderate population differentiation based on microsatellite F_{ST} values are shown to be strong or very strong for Φ_{ST}, which is presumably a function of the admixture of nucleic genes between historic populations.

Bottleneck events of the magnitude experienced by the Kenyan black rhinoceros population are usually accompanied by reductions in genetic diversity resulting in elevated risks of extinction (Gilpin & Soule 1986). The results of this study demonstrate that high levels of genetic diversity are preserved within the remnant eastern black rhinoceros population in Kenya, despite over a 99% reduction in numbers in 17 years. Previous genetic studies on the black rhinoceros have produced conflicting results, with some studies indicating low levels of genetic diversity within populations based on allozymes (Merenlender *et al.* 1989), mtDNA (Ashley *et al.* 1990) and microsatellites (Nielsen *et al.* 2008), whilst other studies have found high levels of diversity in allozymes (Swart *et al.* 1994), mtDNA (Brown & Houlden 2000) and microsatellites (Garnier *et al.* 2001, Harley *et al.* 2005). Discrepancies in the results from previous studies might be due to differing demographic histories of sample populations, unrepresentative sample sizes or due to differences in marker variability in the case of the mtDNA studies. No previous study has however examined the effect of the mixing of historic populations on diversity measure. Two previous studies with the largest sample sizes found levels of microsatellite diversity comparable to those shown in this study (Garnier *et al.* 2001, Harley *et al.* 2005). Harley *et al* (2005) using the same microsatellite markers as used in this study, found expected heterozygosity of 0.675 and allelic diversity of 5.56 in a population of 19 *D. b. michaeli* in South Africa founded from just 6 individuals from Kenya. Similarly Garnier's *et al* (2001) study of a single population of 35 *D. b. minor* founded with 12 individuals from a variety of locations in the Zambezi Valley found expected heterozygosity measures of 0.614 and an allelic diversity of 4.0 for ten microsatellite loci. Our results (H_E = 0.689, A = 5.0) although based on much larger sample sizes are comparable with both of these studies. Brown and Houlden (2000) analysed a 450 bp segment of the same mtDNA control region examined in this study and found higher levels of haplotype diversity (h = 0.86) amongst a captive population of *D. b. minor* (N= 9) also originally from the Zambezi Valley. Previous studies have suggested that the Zambezi Valley rhino population is particularly

important due to its high levels of genetic variation and historic large population size (Swart *et al.* 1994, Garnier *et al.* 2001). The apparent recolonisation of Kenya by black rhinoceros during the middle Pleistocene indicates a possible bottleneck of the eastern subspecies which could potentially account for the differences in mtDNA diversity measures between the *D. b. minor* populations in the Zambezi Valley and the *D. b. michaeli* examined in this study.

The relationship between mtDNA haplotype diversity and microsatellite diversity indicates that the levels of genetic diversity within populations are related to the amount of admixture of historic populations. Examination of the studbook and mtDNA analysis for LWC show that the founders of the population included rhinoceros from the north of the country, Chyulu Hills, Amboseli and the central highlands. Demographic stochasticity, variance in reproductive output and lineage sorting (Allendorf & Luikart 2007) has resulted in a low haplotype diversity, with 77% of the population having a haplotype from the central highlands. The levels of LWC nucleic diversity are consequently low with an allelic richness of 3.87 and an expected heterozygosity measure of 0.637. Whilst these diversity indices are comparable with the studies that have reported high levels of genetic diversity in other black rhinoceros populations, they are the lowest amongst the sampled Kenyan populations (Garnier *et al.* 2001, Harley *et al.* 2005). Although LWC is the oldest sanctuary in the study (est. 1984) the time between its foundation and 2006 when the study was undertaken represents only about 3 generations of black rhinoceros (Goetting-Minesky & Makova 2006). Therefore whilst demographic stochasity and lineage sorting appears to have had an impact on diversity indices, there is no evidence of inbreeding (F_{IS} = 0.024) within this population given the relatively small number of generations since its foundation. In contrast to LWC, the SWG population which was established in 1989 is comprised of mtDNA genotypes representing historic populations from the central highlands (50 %), the south east of the country (24%) and the areas around Nairobi (26%). The newly introduced population (NOPC) is comprised of 44% (N= 11) of animals with a south eastern haplotype (DB03) and 56% (N=14) with a central highlands haplotype (DB06). Consequently the total OPC population represents a considerable period of rhinoceros evolution within Kenya, which is reflected in the diversity indices for both nucleic and organelle genomes. The link between diversity of geographic origin and genetic diversity within the admixed Kenyan populations is further reinforced by the relatively new MRS population which is comprised of mtDNA haplotypes from all sampled geographic areas with the exception of Bura. The MRS population has the highest

levels of genetic diversity of any of the sampled black rhinoceros populations with a haplotype diversity of 0.822, an allelic richness of 4.57 and an expected heterozygosity of 0.757.

4.6 Conservation Implications

From the results of this study it is apparent that the rhino rescue strategy implemented in 1984 was successful not just in countering the poaching threat but also in preserving high levels of genetic diversity within the newly established sanctuary populations. Current populations on private land and within two national parks appear to have preserved a good representation of the genetic diversity that was present within the southern part of the country and the area of the central highlands prior to the dramatic decline in numbers during the 1970s and 1980s. The preservation of genetic diversity through the mixing of historic populations has been at the expense of maintaining historic population integrity with some current populations showing an extensive admixture of populations from a range of geographic areas.

The relatively long divergence times between historic populations must raise concerns about possible outbreeding depression and a resultant effect on fitness and reproductive output. The long generation time of the black rhinoceros means that the effects of outbreeding and indeed inbreeding might not become evident for several years. In light of this and in the absence of specific data on the effect of admixture on reproductive performance, it might be prudent to manage the two national park populations separately from the private sanctuaries at least in the short term. Metapopulation management between OPC, MRS, SGR and possibly LNP would have little impact on current population structure given the levels of admixture within these populations. Although LWC demonstrates distinctive population structure and genetic distance from other populations, this is not a representation of an historic preserved population. Certain individuals from OPC or MRS could be used to supplement the LWC population with little presumed effect on levels of admixture. The results presented here demonstrating extensive admixture between historic populations have to be evaluated in light of what would have happened if the current sanctuary system wasn't implemented. Given the decline that the subspecies was experiencing at the time of the establishment of the sanctuary system it is probably that the subspecies in Kenya would have suffered the same fate it did in the rest of its range. The sanctuary system has undoubtedly been a great success however it is

recommended that future strategies where animals are moved into protected areas should be undertaken with consideration of population structure.

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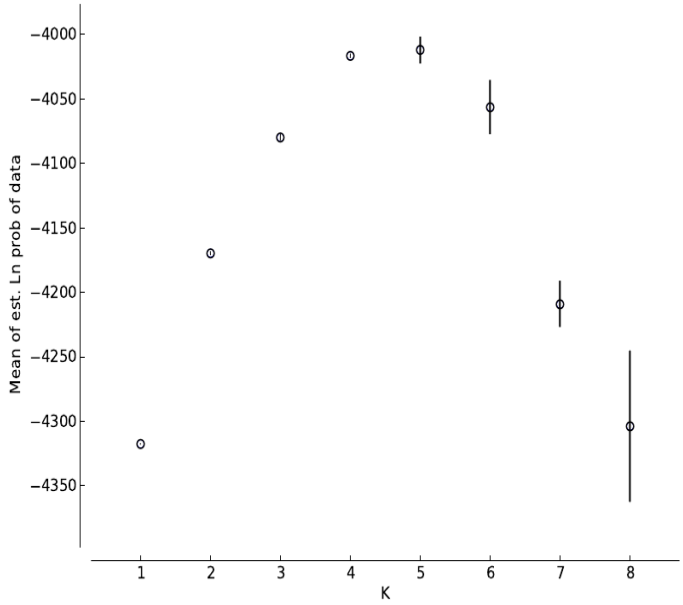
Appendix 4.1. mtDNA sequences used for interspecific analysis of tMRCA estimates for *Perissodactyla*.

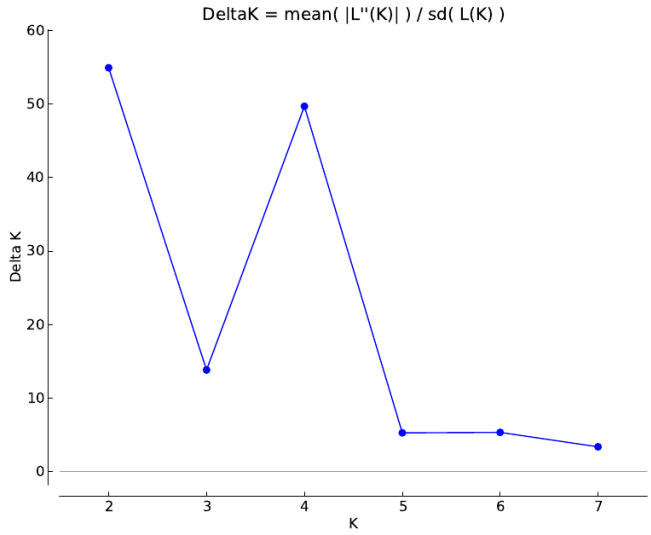
	Mitochondrial Gene								
	12S rRNA			Control Region			ND3		
Taxa	**GenBank Accession Nos.**	**Sequence Length**	**Reference**	**GenBank Accession Nos.**	**Sequence Length**	**Reference**	**GenBank Accession Nos.**	**Sequence Length**	**Reference**
Diceros bicornis michaeli	FJ608807	840	Groves *et al.* (2010)	DB06*	507		FJ608801	914	Groves *et al.* (2010)
Diceros bicornis minor	FJ608808	840	Groves et al. (2010)	AY742832	417	Fernando *et al*. (2006)	FJ608803	914	Groves *et al.* (2010)
Ceratotherium simum cottoni	FJ608806	840	Groves et al. (2010)	FJ004919	722		FJ608800	913	Groves *et al.* (2010)
Ceratotherium simum simum	Y07726	-	Xu & Arnason (1997)	Y07726	-	Xu & Arnason (1997)	Y07726	-	Xu & Arnason (1997)
Dicerorhinus sumatrensis	FJ905816	-	Willerslev *et al*. (2009)	FJ905816	-	Willerslev *et al*. (2009)	FJ905816	-	Willerslev *et al*. (2009)
Coelodonta antiquitatsis	FJ905813	-	Willerslev *et al*. (2009)	FJ905813	-	Willerslev *et al*. (2009)	FJ905813	-	Willerslev *et al*. (2009)
Rhinoceros sondaicus	FJ905815	-	Willerslev *et al*. (2009)	FJ905815	-	Willerslev *et al*. (2009)	FJ905815	-	Willerslev *et al*. (2009)
Rhinoceros unicornis	X97336	-	Xu *et al*. (1996)	X97336	-	Xu *et al*. (1996)	X97336	-	Xu *et al*. (1996)
Tapirus terrestris	AJ428947	-		AJ428947	-		AJ428947	-	
Equus asinus	X97337	-		X97337	-		X97337	-	
Equus caballus	X79547	-	Xu & Arnason (1994)	X79547	-	Xu & Arnason (1994)	X79547	-	Xu & Arnason (1994)
Acinonyx jubatus	AY463959	-	Burger *et al.* (2004)	AY463959	-	Burger *et al.* (2004)	AY463959	-	Burger *et al.* (2004)

Appendix 4.2. Graphical output from STRUCTURE HARVESTER for model-based clustering method implemented in STRUCTURE. (A) Mean L(K) (± SD) for STRUCTURE output for 8 runs for each K value . (B) ΔK value for STRUCTURE with 8 runs for each K value, modal value indicates true value of K, in this case is K=2.

A



B



Chapter 5

Female black rhinoceros exercise mate preference for males with high genetic diversity and intermediate levels of genetic distance in admixed populations

5.1 Abstract

Many conservation programs for large vertebrates are reporting poor population growth rates linked to low or declining reproduction in small populations. The relative influences of additive and non-additive genetic effects on mate choice have received considerable attention, with empirical studies demonstrating that these effects are often confounded in natural populations. The recent reporting of fitness costs associated with very high heterozygosity in some natural populations indicates that mate choice in some taxa may be mediated by intermediate levels of genetic dissimilarity between individuals. These recent findings have potentially profound implications for conservation programs which often involve the mixing of individuals from hitherto isolated populations. By definition populations subject to conservation management are often small and individuals are typically presented with a limited number of potential mates from divergent genetic backgrounds. The influences of additive and non-additive effects on mate choice have been examined in three admixed black rhinoceros populations subject to active conservation management. Parentage analysis was carried out on a 107 eastern black rhinoceros (*Diceros bicornis michaeli*), representing their reproductive performance over ca. 16 years. It is demonstrated that male genetic diversity is a significant predictor of reproductive success and that females balance male genetic quality with intermediate levels of genetic similarity in admixed populations. These results are significant for conservation programs which typically mix animals from divergent backgrounds to offset the detrimental effects of small population size.

5.2 Introduction

In the absence of deterministic factors driving decline, the recovery of endangered populations is mediated by reproductive output (Spielman *et al.* 2004). Three of the five extant rhinoceros species are currently classified as critically endangered and all are subject to intensive *in situ* and *ex situ* conservation efforts (IUCN 2008). Many programs however are suffering from poor population growth rates as a result of either low or declining reproduction in small populations. The *ex situ* program for the Sumatran rhinoceros is deemed to have failed and the current white rhinoceros captive program is classed as failing with a -3.5% growth rate (Rabinowitz 1995, Swaisgood *et al.* 2006). Similarly many *in situ* programs which typically involve the metapopulation management of small populations are experiencing poor growth rates, particularly for the black

rhinoceros (Mills *et al.* 2006, Walpole *et al.* 2001, Okita-Ouma *et al.* 2008, Reid *et al.* 2007a, Adcock *et al.* 1998). Whilst some studies have identified causes such as high mortality, skewed sex ratios or density dependence as limiting growth (Adcock *et al.* 1998, Okita-Ouma *et al.* 2008, Okita-Ouma *et al.* 2009) for many populations the factors inhibiting recovery are unclear (Walpole *et al.* 2001, Mills *et al.* 2006, Swaisgood *et al.* 2006, Linklater & Hutcheson 2010).

Genetic influences on mate choice and reproduction have received considerable attention in recent years, with much of the focus on the dichotomy between the additive and non-additive effects underlying mate choice (reviewed in Mays & Hill 2004). In most species females are predicted to be the choosier sex due to a typically higher investment in gametes and the raising of offspring (Tregenza & Wedell 2000). Additive effects or the 'good genes' hypothesis is the choice by females for males with superior genetic quality that will convey the greatest fitness benefits to offspring (Mays & Hill 2004). The paradox of mate selection for additive traits (termed the 'lek paradox') is that variation for these traits will soon become exhausted (Tomkins *et al.* 2004). A solution to the lek paradox is the non-additive 'compatible genes' hypothesis where the genetic quality of an individual is less important than the interaction between male and female genotypes (Mays & Hill 2004, Charpentier *et al.* 2008a). Disassortative mate choice whereby females select mates with dissimilar genotypes to their own is a strategy whereby females can avoid inbreeding and increase the heterozygosity of their offspring (Penn 2002, Mays & Hill 2004, Garcia-Navas *et al.* 2009). Studies on disassortative mate selection have reported increased fitness associated with heterozygosity in many taxa; heterozygote advantage or heterozygosity fitness correlates (HFC) (reviewed in Chapman *et al.* 2009). Several studies have found heterozygosity at fitness loci (MHC loci in particular) and neutral markers to be correlated with reproductive success (Seddon *et al.* 2004, Charpentier *et al.* 2005, Kempenaers 2007, Garcia-Navas *et al.* 2009, Thoss *et al.* 2011), survival (Coltman *et al.* 1998, Townsend *et al.* 2009, Huchard *et al.* 2010) and disease resistance (Reid *et al.* 2005, Charpentier *et al.* 2008b). It is generally thought that the interrelationship between neutral markers and HFCs is either due to linkage disequilibrium between neutral markers and fitness loci (local effect) or where neutral marker heterozygosity is reflective of genomic heterozygosity (general effect) (Hansson *et al.* 2004, Grueber *et al.* 2008). General effects are expected to be most evident in cases of inbreeding with linkage disequilibrium and therefore local effects increasing in recently admixed populations (Grueber *et al.* 2008). Several studies have shown that additive and non-additive benefits are actually confounded in natural

populations, with individuals selecting dissimilar partners which are modulated by the additive benefits of high genetic diversity. Roberts *et al* (2006) study on MHC loci in humans and peafowl at microsatellite loci showed that average levels of allele sharing and relatedness were significantly correlated with heterozygosity. Hoffman *et al* (2007) similarly found that female fur seals actively balance mate choice according to genetic diversity (IR) and dissimilarity with conspecifics. Recent work however suggests that the most genetically compatible mate is not necessarily the maximally dissimilar one (Roberts 2009). A decline in fitness (outbreeding depression) associated with the mating between individuals with divergent genetic backgrounds as a consequence of the dilution of locally-adapted genomes and/or the disruption of epistasis in co-evolved gene complexes is well documented (reviewed in Edmands 2007). However even for intrapopulation mating there is recent compelling evidence that individuals will select mates with intermediate levels of dissimilarity (Neff 2004, Roberts 2009). Studies on sticklebacks have shown that individuals with very high MHC heterozygosity have a higher parasitic load and are less desirable as mates (Milinski 2003, Havlicek & Roberts 2009). Selective pressure against high MHC heterozygosity is also apparent in tetraploid *Xenopus* frogs which have been shown to silence half their MHC genes (Du Pasquier *et al.* 1989, Penn & Potts 1999). Thymic selection on T-cell clones reduces pathogen resistance in individuals with very high numbers of MHC alleles and is a probable cause for selection against highly dissimilar mates (Nowak *et al.* 1992, Penn & Potts 1999). Moreover both the frequency-dependent and fluctuating selection models for MHC balancing selection also suggest the evolution of mate selective preference for locally adapted genotypes over maximally dissimilar ones (Apanius *et al.* 1997, Huchard *et al.* 2010, Spurgin & Richardson 2010). Recent empirical studies on natural populations examining mate selective choices according to both fitness loci and neutral markers have produced results consistent with the intermediate strategy of mate selection (Neff 2004, Bos *et al.* 2009, Eizaguirre *et al.* 2009, Roberts 2009).

To date the genetic factors influencing mate choice have not been examined in a population subject to active conservation management. Often such populations are small admixed populations where individuals are presented with a limited number of potential mates, representing a greater spectrum of genetic divergence than would typically be present in non-managed populations. In particular the potential for preferential choice for mates with intermediate levels of genetic divergence has profound implications for conservation breeding protocols which typically seek to maximise genomic divergence by

mixing hitherto isolated populations to mitigate the effects of small population size (Amos & Balmford 2001b, Neff 2004, Boakes *et al.* 2007).

I have examined the influence of male genetic quality estimated as internal relatedness (IR) and measures of genetic relatedness on female mate choice in three populations of eastern black rhinoceros (*Diceros bicornis michaeli*) in Kenya. *D. b. michaeli* has experienced one of the most dramatic declines of any mammalian taxa; the Kenyan population was reduced by poaching from over 20,000 animals in 1970 to just 380 by 1987 (Brett 1993, Gakahu 1993, Emslie & Brooks 1999). In response to the imminent extinction of the subspecies the Kenyan government implemented a policy of moving all remaining animals into fenced sanctuaries where resources could be concentrated to counter the poaching threat (Leader-Williams 1989, Brett 1993). The policy proved successful and in 2005 Kenya had 539 animals, protected within 14 separate populations representing ~84% of the world's total population (Okita-Ouma *et al.* 2007). The animals protected within the sanctuaries are a mixture of historically isolated populations from across the species former range in Kenya and are managed as a single metapopulation. The admixed Kenyan sanctuary populations provide an ideal situation to examine the factors influencing female mate choice within a typical conservation program.

One hundred and seven individual identified black rhinoceros from 3 Kenyan sanctuaries were genotyped for 9 microsatellite loci, with maternity and paternity assigned for 61 offspring. This is the first study to examine the effects of genetic diversity and relatedness on female mate choice in admixed conservation priority populations.

5.3 Methods

5.3.1 Sample collection

A total of one hundred and seven individually identified rhinoceros from three Kenyan black rhinoceros sanctuaries were sampled and genotyped. Black rhinoceros from Lewa Wildlife Conservancy (LWC), Mugie Rhino Sanctuary (MRS) and Ol Pejeta Conservancy (OPC) were genotyped from a combination of faeces (n = 65 individuals) tissue (n = 22 individuals) and serum (n = 20 individuals) collected between 2004 and 2009. All breeding adults within the three populations were sampled with a large proportion of offspring also sampled; sampling represented 92% of the OPC population (n = 41 individuals), 93%

LWC population (*n* = 39 individuals) and 96% of MRS population (*n* = 27 individuals) as of 2006.

5.3.2 Home range estimates and population data

The Kenya Wildlife Service (KWS) maintains a database for all black rhinoceros within the sanctuary system based upon an individual-ID based monitoring system (Amin *et al.* 2001). The database was interrogated to obtain information on mother-calf pairings and key performance indicators such as age at first calving and inter-calving intervals. Home range estimates were calculated using GPS positions collected by the monitoring patrols using minimum convex polygons (MCP) in order to capture maximum home range regardless of spatial behaviour (Frere *et al.* 2010).

5.3.3 Genotyping

DNA was extracted from faecal samples using a QIAamp® DNA Stool Mini Kit (Qiagen) with modifications to the manufacturers' protocol of (1) extending the initial lysis at 55°C to overnight and (2) making two 50-µl elutions in 1xTE buffer after 15 min incubation. Three separate extractions were performed on each faecal sample. DNA extractions from tissue and serum were performed using a Qiagen DNeasy Blood & Tissue Kit and the ZR Serum DNA Kit™ (Zymo Research) respectively, both according to the manufacturers' instructions.

Every sample was genotyped at ten microsatellite loci. For faecal DNA samples, the four replicate extracts with the highest DNA concentration were used to generate an individual's genotype. Moreover, the genotyping protocol followed a modified multiple tubes approach for the low-copy DNA (i.e. derived from faeces or serum) with every sample genotyped six times at all 10 loci (Taberlet *et al.* 1996). PCRs were performed in 25-µL final reaction volume containing (2 µl of faecal DNA extract or 200 ng of tissue/serum DNA) reactions containing, 0.1µg/µl BSA, 200 µM each dNTP, 2.0-2.5 mM $MgCl_2$, 2.5 µl 10x Qiagen® PCR Buffer, 1 µM each primer and 0.625 units of Qiagen® HotStarTaq™ 0.5-1.0µm each primer (with the forward primer 5'-labelled with either NED, PET, 6-FAM or VIC–Applied Biosystems. Thermal cycling conditions were: 96°C for 15 min, followed by either 30 cycles for tissue DNA or 40 cycles for faecal and serum DNA of (94°C, 1min; T_a°C, 30s; 72°C for 1min), where T_a is the published locus-specific annealing temperature. PCRs on tissue DNA extracts were carried out with the reaction conditions described above, but with 25 cycles.

Microsatellite data were examined for mistyped alleles, large allelic dropout and null alleles using MICROCHECKER v.2.2.3 (Van Oosterhout *et al.* 2004). Estimates of allelic dropout for faecal DNA samples were derived also by comparing genotypes obtained from faecal and tissue samples collected from the same animal (n=21 individuals). For each sanctuary population, exact tests for Hardy-Weinberg equilibrium and genotypic linkage disequilibrium between all pairs of microsatellite loci were calculated using GENEPOP v.4.0 (Raymond & Rousset 1995, Rousset 2008) with Markov chain parameters of 1,000 dememorisations, 100 batches and 1,000 iterations per batch. Sequential Bonferroni corrections were applied to maintain a population-specific error rate of α=0.05 (Rice 1989).

5.3.4 Parentage analysis, relatedness and estimates of inbreeding

The database for each population was initially interrogated to determine mother-offspring pairings and candidate males for paternity testing, with all males over the age of 5 years considered as possible candidate fathers (Garnier *et al.* 2001). Parentage assignments were performed using likelihood analysis in the computer software CERVUS ver. 3.0.3, with a genotyping error rate of 1% (Marshall *et al.* 1998). Mother-offspring relationships were tested using all females of 2 years and older at the time of offspring conception as possible candidates (Garnier *et al.* 2001). Critical LOD scores were determined by simulation for 100,000 offspring, all mother-offspring pairings with >95% probabilities were accepted. Paternity assignment was undertaken using known mother-calf pairings, simulation for 100,000 offspring to determine critical LOD scores and a genotyping error rate of 1%.

Seven estimates of relatedness were tested using COANCESTRY v.1.0.0.1 (Wang 2010a) for mating pairs, and only for non-mating pairs that co-inhabited the same reserve (*i.e.* only pairs that had an opportunity to breed). The triadic likelihood estimator (TrioML) (Wang 2007) was chosen as it best described known relatedness from the black rhinoceros pedigrees. In addition the estimator of Lynch & Ritland (1999) (LR) was also adopted as it has been shown to perform consistently well (Csillery *et al.* 2006, Mainguy *et al.* 2009), it provides estimates of relatedness over a wider spectrum of genetic similarity and conformed closely with the TrioML estimates (R = 0.84).

Estimates of inbreeding (f) were calculated using COANCESTRY v.1.0.0.1 (Wang 2010a) for four estimates of inbreeding; the triadic likelihood estimator (TrioML) (Wang 2007), the

dyadic likelihood estimator (DyadML) (Milligan 2003), the moment estimator by Lynch & Ritland (LR) (1999) and a moment estimator by Ritland (R) (1996a). Standard errors were obtained by 1,000 bootstrap replicates.

5.3.5 Best predictors of fitness

Generalised linear models (GLMs) were used to identify the predictor variables that explained the greatest proportion of the variance in the number of offspring produced by each individual of breeding age (OFF) (response variable fitted with a Poisson distribution). Six predictor variables were available for model selection: age (AGE, years), home range size (HOM, km^2), number of males with overlapping home ranges (MOH), number of females with overlapping home ranges (FOH), and heterozygosity (IR or MLH, see below); the contemporary reserve population (POP) (LWC, MRS and OPC) was included as a random factor. Model selection was completed for two common estimators of heterozygosity: (1) internal relatedness (IR) and (2) multilocus heterozygosity (MLH). IR is an adaptation of the Queller and Goodnight (1989) relatedness measure and is an estimate of parental relatedness according to allele sharing weighted by frequency relative to random expectations (Amos *et al.* 2001). Multilocus heterozygosity (MLH) is simply the proportion of heterozygous loci within an individual, although proved to a be a simple robust measure it does not correct for differences in numbers or frequencies of alleles (Di Fonzo *et al.* 2011). Both IR and MLC were highly correlated with heterozygosity (R=1.0, R=0.6 respectively). Variables were retained after verification that multicollinearity was not an issue by using calculating variance inflation factors (VIFs) using the CORVIF function in the AED package (Zuur *et al.* 2009), where VIF>3 (Zuur *et al.* 2009) or >5 (O'Brien 2007) indicates a potential problem with multicollinearity. Note that the measures of heterozygosity IR and MLH are significantly correlated (R=-0.912, df=43, P=<$2.2x10^{-16}$) but were never included in the same models.

Selection of terms in the models was based on minimising corrected Akaike's information criterion (AICc) using the DREDGE function within the package MUMIN in *R* v.2.12.1 (Barton 2011). Model selection was performed separately for males and females. Initially I selected the model with the fewest predictors that was within 2ΔAICc of the model with the lowest overall AICc and then, if necessary, explored the effect of single variables by dropping each variable in turn and then re-evaluating the model. Explained deviance (pR^2) of the final GLMs was calculated as 100*(null deviance-residual deviance)/null deviance (Zuur et al. 2009).

Identity disequilibrium (ID), the extent of correlation in heterozygosity across loci, was measured as g_2 using RMES software (David *et al.* 2007) and 10,000 randomisations. The relative importance of local and general effects was assessed using an *F*-ratio test that compared a single (*i.e*. MLH) with a multiple (*i.e*. single locus heterozygosities expressed as 0 or 1) regression of heterozygosity against offspring number (see Szulkin *et al.* 2009), using *R* v.2.12.1. The potential impact of inbreeding upon fitness was estimated using the equations provide by Szulkin *et al*. (2009) that use the basic descriptors of the HFC based on MLH (*i.e*. mean and variance of MLH, and the regression slope and the coefficient of determination, and the estimate of g_2): the squared correlations between (1) the number of offspring produced (*i.e*. fitness trait) and inbreeding ($r^2_{W,f}$), and (2) between heterozygosity and inbreeding ($r^2_{H,f}$), and also (3) the potential inbreeding load ($\beta_{W,f}$). Average inbreeding was assumed to be negligible (*i.e*. f=0), which results in a conservative estimate of $\beta_{W,f}$, but nonetheless one which is reasonable unless black rhino populations have particularly high levels of inbreeding (see Szulkin *et al*. 2009 for examples).

To determine whether relatedness influenced mate choice the relatedness among mating and non-mating pairs of rhinoceros was calculated by resampling the appropriate estimates of pairwise relatedness. To test whether mating pairs were more related than random mating pairs, permutation tests were conducted in R using 10,000 randomisations for TrioML and positive LR relatedness measures. To examine whether mating pairs were less related than random mating pairs, negative LR estimates were transformed to absolute values and positive values to 0, with permutation texts conducted in R using 10,000 randomisations. All randomisation tests were conducted between potential mating pairs from the same reserve.

5.4 Results

Breeding data for 27 females and 18 males, representing their reproductive activity over a ca. sixteen and a half year period was examined (May 1990 to November 2006). Females with a complete reproductive history within the study populations (n = 13) demonstrated a high degree of variance for key breeding performance indicators, with an average age of first calving of 83.2 months (SD 13.8). Similarly there was considerable variation in average inter-calving interval with the shortest period of 27 months between calves and the longest at 63 months with a mean of 38.2 months (SD 9.6) across all three populations.

Breeding indicators are based solely on successful breeding and do not account for the possibility of failed pregnancies.

5.4.1 Genotyping error rates, parentage and inbreeding

The mean genotyping error rate across all loci was 0.13% (range 0.0-0.24%). Most (>99%) instances of genotyping error were a result of allele dropouts in one of the two genotyping rounds and the few ambiguous genotypes were resolved by further PCRs. The presence of null alleles was detected for one locus (DB44) with a large positive value of average F_{IS} (F_{IS} =0.456) and significant deviations from HWE, this locus was therefore excluded from further analysis. Significant (P<0.05 after sequential Bonferroni correction) linkage disequilibrium was identified between just one pair of loci in one population (Br17 & Br4, MSR) and there was no evidence of significant deviation from expected HWE (P>0.05) for any of the nine remaining microsatellite loci.

Field observations of mother-offspring pairs were confirmed by the genetic data for all pairings for the MRS and LWC populations. The genetic data identified two mistakes in mother-offspring pairings for the OPC studbook, both for offspring born in 1996 (Appendix 5.1). In those instances maternity analysis was performed in CERVUS using the most likely father as known parent based on critical LOD scores and all females of reproductive age (> 2 years) at the time of conception as potential candidates (Garnier *et al.* 2001). A unique genotype was also identified born to female 4011 which could not be identified from the studbook. Paternity was assigned to 61 out of 62 offspring sampled, from a total of 73 offspring born in the three populations from August 1991 to November 2006. Critical delta scores for paternity without known mother gave confidence levels of > 95 % for 46 offspring, 10 >80% confidence and 6 with <80% confidence. Assignments with known parent included gave critical delta values with greater than 95% confidence for 61 offspring with the remaining unassigned offspring in LWC having a confidence level of <80%. It is postulated that this offspring was sired by a male in the early 90s who was moved out of the population and not sampled. For breeding males with a complete reproductive history there was considerable variance for the age of first reproduction with an average age of 129 months (SD 44.4) with the earliest at 75 months and the latest at 215 months. Reproductive success also varied widely between breeding males, in MRS only two males sired offspring from a total of seven males of reproductive age, with one male fathering 66.6% of all offspring (n = 4). In OPC two males sired 55% (n = 16) of the offspring from the 29 sampled with one male only siring a single calf. Similarly in LWC a

single male sired 37% of the offspring (n = 10) with one male not siring any of the 27 sampled offspring.

All four estimates of inbreeding (f) were highly correlated with the greatest difference between the Ritland (1996b) moment estimator and the dyadic likelihood estimator (DyadML) (Milligan 2003) (R=0.84). Inbreeding was very low for all three study populations with MRS having the highest average measure of inbreeding for the four estimates (f=0.061) and OPC have the lowest (f=0.037) (Table 5.1).

Table 5.1. Estimates of inbreeding (f) for study populations.

Population	***n***	**Ritland**	**LynchRd**	**TrioML**	**DyadML**
LWC	**39**	-0.01 (0.022)	-0.001 (0.033)	0.097 (0.01903)	0.108 (0.022)
MRS	**27**	0.035 (0.040)	0.019 (0.019)	0.091 (0.01085)	0.098 (0.012)
OPC	**41**	-0.007 (0.018)	-0.014 (0.019)	0.08 (0.00709)	0.089 (0.009)
All	**107**	**0.002**	**-0.004**	**0.089**	**0.099**

n=population size

5.4.2 Best predictors of fitness

VIFs varied between 1.54 and 3.04 for the male predictors, with low correlations (R=+/-0.4 or less) between all pairs of variables except between IR and HOM (R=-0.742, df=15, P=6.49x10^{-4}) and IR and MLH (R=0.714, df=15, P=0.0013); nonetheless, with the largest VIF equal to 3 all predictors were retained for the initial analysis. Model selection yielded slightly more complicated results for males than for females and returned a GLM that accounted for nearly 70% of the variation in offspring production by males and which included two significant predictors (IR and the factor POP) as well as one non-significant (FOH) predictor (Table 5.2). Omitting FOH from this best model resulted in a reduction of 1% of explanatory power (indeed, FOH was not-significant in a GLM as a single explanatory variable); by contrast, removal of IR and the factor POP resulted in pR^2 being reduced by 30% and 20% respectively. IR had the most explanatory power and as a single predictor in a GLM accounted for almost half of the variation in production of offspring by males (Table 5.2, Figure 5.1); similar results were obtained when MLH was used as the measure of heterozygosity, with MLH explaining about 40% of the variation in production of offspring by males.

Unlike females, age had no significant impact upon offspring production by mature male rhinos (OFF=1.245-0.0004AGE, P=0.987, pR^2=0; Figure 5.1). However, because of the significant, negative correlation between IR and male home range size, HOM acts as a significant predictor of the number of offspring produced by males (OFF=0.249+0.052HOM, P=7.7x10^{-4}; Figure 5.2), although the fit of the GLM is poorer (pR^2=17, AICc=95.36) than for GLMs that included either IR (ΔAICc=15.66) or MLH (ΔAICc=9.05) as the explanatory variables. Females tend to maintain larger home ranges than the males, but show no significant relationship between either IR and HOM (R=0.103, df=25, P=0.610; Figure 2) or OFF and HOM (OFF=3.033-0.008HOM, P=0.752, pR^2=0.41%).

Table 1. Final GLMs after model selection (see *Methods*) to identify predictors of either the total number of offspring (OFF) or the standardised number of offspring (OFFs) produced by black rhinoceros. Significant predictors included: AGE, age (in years) of parent; HET, heterozygosity (either IR or MLH); FOH, number of females with an overlapping home range; fPOP, population factor. pR^2 is the proportion of variation explained by the model.

		Response	intercept	AGE	HET	FOH	fPOP1	fPOP2	pR^2
Female	†	OFF	0.328	0.033**					0.342
		OFFs	-1.451*	0.072**					0.255
Male	IR	OFF	0.090		-3.294***	-0.073	1.540***	1.065*	0.717
		‡	-0.305		-2.867***		1.522**	1.220**	0.686
		OFFs	-0.856		-9.439***				0.609
	MLH	OFF	-3.121***		0.508***	-0.101	1.657***	1.440**	0.718
		‡	-3.075***		0.419***		1.604***	1.618***	0.678
		OFFs	-9.345***		1.367***				0.614
		ln(OFFs)	-1.885*		0.473***				0.499

*P<0.05, **P<0.01, ***P<0.001
†final model included neither IR nor MLH.
‡GLM repeated for the model above but with the non-significant term (FOH) omitted.

Significant ID was detected in males (g_2=0.0184, P=0.047) but not females (g_2=0.0090, P=0.104). There was no evidence for local effects to the apparent male HFC (F-ratio test=0.842, df=8,8, P>0.05). Using MLH as the sole predictor of OFF (*i.e.* a slope of 0.525), variation in inbreeding accounts for almost all ($r^2_{W,f}$=0.985) of the total variance in the numbers of offspring sired by male black rhinos; in addition, MLH correlated well with inbreeding ($r^2_{H,f}$=0.401). Since HFC is the product of these two correlations, it is not surprising that a strong HFC was detected, with MLH accounting for almost a third (~40%) of the variance in offspring production. The inbreeding load ($\beta_{W,f}$) for males was

calculated to be -8.951, which, for example, indicates a reduction of 1 or 2 offspring if males engage in half- or full sib matings (*i.e. f*=0.125 or 0.25) respectively.

I examined both age and IR in males and females and noted some residuals. Particularly for females, there appeared to be a qualitative effect of IR upon the number of offspring. However, removal of the two females with the two high IR values (and also 4 and 5 offspring) and rerunning the model selection procedure yielded a final model with just age as an explanatory variable.

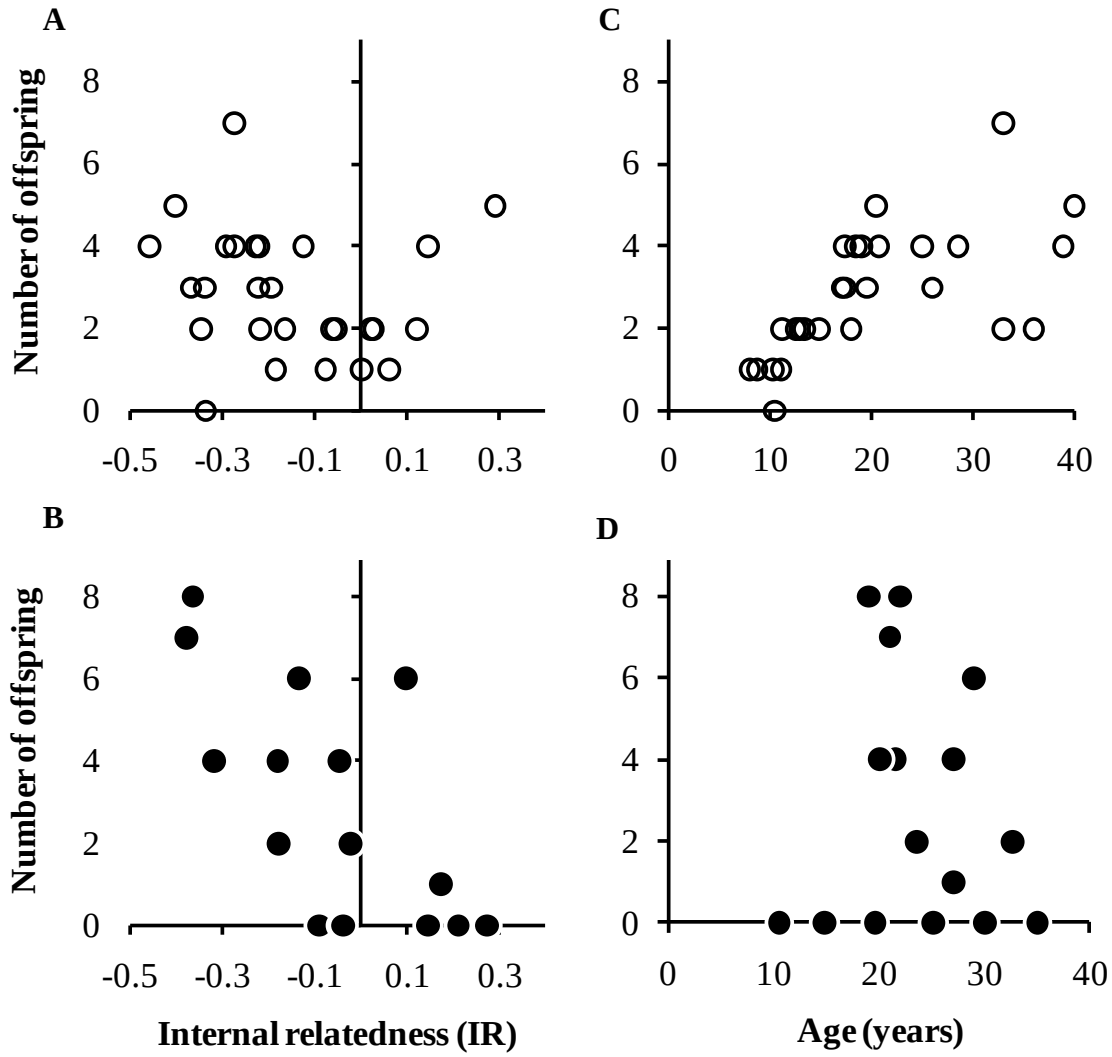


Figure 5.1. Plots showing relationship between A) number of offspring and female IR. B) number of offspring sired and male IR. C) number of offspring and female age. D) number of offspring sired and male age.

Average relatedness (*r*) among all pairs of potentially breeding rhinoceros was low for both relatedness measures (TML r = 0.022, SD = 0.041; LR r= 0.051, SD = 0.104). The difference between actual mating pairs compared to random pairs for TML was

significantly different (P=0.033) and non significant for the positive LR measures (P=0.183). Parentage analysis revealed no instances of offspring breeding with their parents and no instances of either full or half siblings breeding. Average relatednesss for negative LR estimates between mating pairs (r = -0.0233, SD = 0.055) was significantly less (P = 0.0085) than for random mating pairs (Figure 5.3).

5.5 Discussion

Genetic diversity is crucial for population survival. Here I show that genetic diversity drives reproductive performance in male black rhinoceros and that females balance male genetic quality with intermediate levels of genetic similarity in admixed populations. These findings are in concordance with recent studies examining genetic influences on mate choice which show that additive and non-additive effects are confounded in many natural populations (Roberts *et al.* 2006, Hoffman *et al.* 2007). Moreover the preferential selection for mates with intermediate levels of genetic similarity suggests a mechanism whereby mate choice is mediated by a fitness cost of selecting

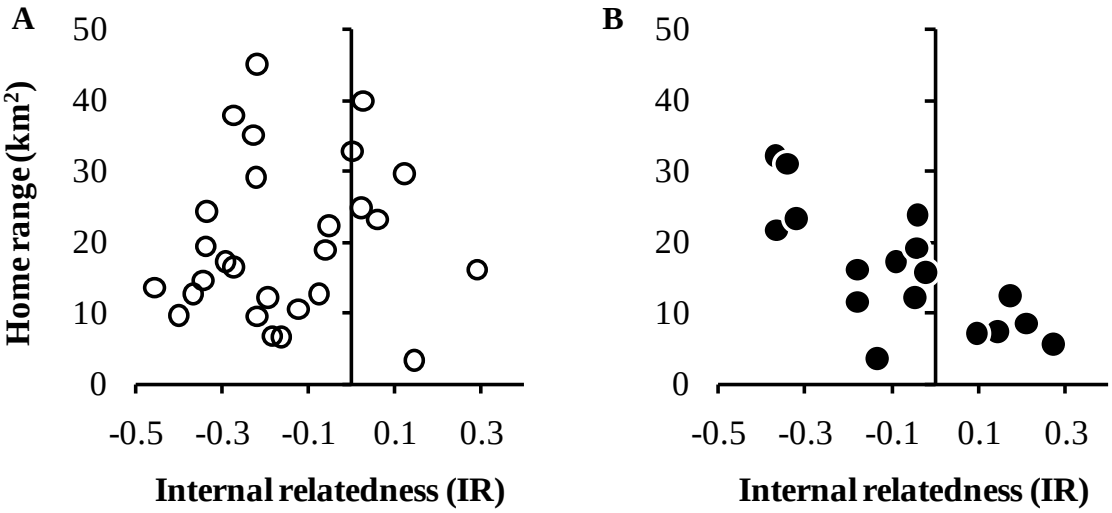


Figure 5.2. Plots showing A) relationship between female IR and home range size. B) relationship between male IR and home range size.

maximum dissimilar mates in these populations (Penn & Potts 1999, Milinski 2003, Roberts 2009).

All the individuals within the study populations are intensively monitored with a maximum of one week between sightings of an individual before additional resources are allocated to

locate an 'overdue' individual. It would therefore appear that the results presented here are a factor of successful conception rather increased survival of offspring. However in the absence of behavioural data on mating events it is impossible to speculate whether selection happens before or after mating. Work on *ex situ* populations of white rhinoceros indicates that reproductive failure occurs post-copulation (Swaisgood *et al.* 2006) and that Sumatran rhinoceros are induced ovulators (Rabinowitz 1995) indicating a possible post-copulation mechanism of selection.

Much debate surrounds the indirect mechanisms for observed correlations between HFCs and neutral markers, with HFCs for microsatellites postulated as either being indicative of genomic diversity (general effect) or in linkage disequilibrium (LD) with fitness-trait loci (local effect) (Hansson & Westerberg 2002). General effects should be most evident in cases of inbreeding, although MLH has been shown to be weakly correlated with inbreeding (Slate *et al.* 2004). The results presented here are from populations which have experienced a severe population decline, but mixing of individuals from different populations and apparent inbreeding avoidance by the black rhinoceros appears to have alleviated inbreeding effects. Moreover, lack of polygyny and a long generation time may have reduced genetic erosion. Slate *et al* (2004) suggested that other measures of

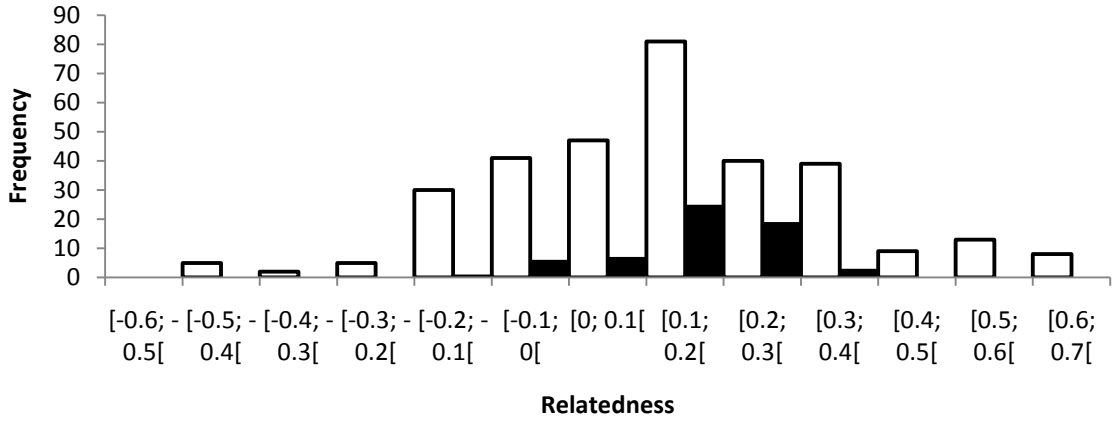


Figure 5.3. Frequency relatedness distribution based for all potential matings within a population (open bars) and actual matings within a population (solid bars), using the relatedness estimator of Lynch and Ritland (1999).

microsatellite variability might be more suitable indicators of *f* such as the internal relatedness (IR) measure used here. Both MLH and IR were strong predictors of male mating success in this study which suggests that the observed correlations are due to something other than inbreeding. Given the relatively small size of the populations

examined, the intensive monitoring they are subject to and the high probabilities obtained for parentage assignment it seems unlikely that the results reported are due to bias in paternity assignment (Slate 2009, Wang 2010b). In the absence of apparent general effects, LD between some of the microsatellite loci examined and fitness-trait loci is the most probable explanation for the observed correlations between heterozygosity and reproductive success (Grueber *et al.* 2008). The study populations are a mixture of former historic populations, most prominently populations from the south of the country and the central highlands (Cain 2012). Expansion into the Kenyan central highlands with the retreating of the Pleistocene forests around 180 KYA appears to have resulted in two distinct populations captured within the current sanctuary populations. The recent mixing of these two distinct populations would be expected to increase LD within the current admixed populations (Grueber *et al.* 2008). The local effects hypothesis is supported by the significant mating preference for individuals of intermediate genetic dissimilarity. Theoretical and recent empirical studies indicate that even in the absence of fitness costs associated with the breakdown of epistasis, individuals should preferentially choose locally adapted genotypes and/or select against maximally dissimilar mates (Penn & Potts 1999, Milinski 2003, Neff 2004, Bos *et al.* 2009, Eizaguirre *et al.* 2009, Roberts 2009).
Given the recent admixture of these two historic populations within the study populations it seems probable that individuals are selecting for mates with certain fitness-loci linked to these two historic populations which are in LD with the neutral markers under study.

Research on laboratory mice and a couple of studies on natural populations have demonstrated that scent plays a critical role in mediating sexual behaviour in mammals (Hurst *et al.* 2001, Penn 2002, Charpentier *et al.* 2008a, Hurst 2009, Setchell *et al.* 2011). MHC products have been linked to individual odour-types which are a result of the excretion of MHC-gene products in bodily secretions (Hurst 2009, Setchell *et al.* 2011). Evidence suggests that the mammalian vomeronasal organ (VNO) has the ability to assess these volatile MHC odour-types in addition to other volatile proteins such as major urinary proteins (MUPs), another multigene family which have been shown to convey information used to assess kin and genetic heterozygosity (Sherborne *et al.* 2007, Hurst 2009). Olfactory communication is fundamental to black rhinoceros social, spatial and mating behaviour and a potential mechanism whereby individuals can access genetic quality and similarity (Goddard 1967, Kiyasu & Kohshima 2002, Linklater *et al.* 2006). Black rhinoceros invest considerable effort in scent broadcasting by spraying pungent urine onto vegetation and defecating at dung piles or middens; moreover males in particular scrape

their back feet through their faeces upon defecation a behaviour which aids in scent broadcasting (Tatman *et al.* 2000)

Although females exhibited a significant preference for males with high genetic diversity measures and intermediate levels of genetic similarity, female rhinoceros were not monogamous and in some instances mated with up to three different males during the period covered by the study. In most instances females preferentially mated with males that they shared overlapping home ranges with; however a number of females had overlapping ranges with males with which they have never produced offspring. Previous work on black rhinoceros ranging behaviour indicates that the species is very philopatric, a potential factor in the strong genetic structure between historic populations and mate choice for intermediate dissimilarity (Linklater & Hutcheson 2010, Patton *et al.* 2010). Our study shows that while females have definite home ranges they will travel relatively short distances to mate with desirable males; several females at LWC and OPC mated with males in other areas of the reserve. In the absence of data on spatial variance in habitat quality within the reserves it is impossible to speculate whether this behaviour is a function of density dependence in the area of desirable males or whether these females obtained direct benefits from overlapping with males they didn't breed with. Whilst there was a strong relationship between male IR and home range size which indicates a mechanism whereby more heterozygous males can secure access to more females, home range overlap did not come out in the model as a significant predictor. Behavioural studies demonstrate that intersexual dominance is female biased in the black rhinoceros and therefore actual mating does not appear to be a function of encounter rate or male dominance, but rather an active choice by females (Berger & Cunningham 1998).

With many rhinoceros conservation programs reporting poor population growth rates in the absence of deterministic factors driving decline (i.e. poaching) understanding the influence of male diversity and genetic similarity on mate preference in wild populations of rhinoceros is significant in designing breeding protocols to maximise recovery (Grahn *et al.* 1998). The populations examined in this study are typical of many such populations which are subject to conservation management where hitherto isolated populations are mixed in order to promote recovery and offset the detrimental effects of small population size. Within such managed populations individuals are typically presented with a smaller number of potential mates representing a wider spectrum of genetic similarity than would be present in natural populations (Amos & Balmford 2001b, Boakes *et al.* 2007). However

the populations within this study were comprised of just two main historic populations and had a good number of breeding males with high diversity which represented a broad spectrum of genetic similarity from the two historic populations. Whilst no link was found between reproductive output and available mate choice in this study, this could be due to the quality of potential mates within these relatively large rhinoceros populations. Whether these factors do have an impact in smaller populations with more limited choice of potential mates is an important area of future research. There is also evidence of reproductive slow down in females as a function of senescence and the age of breeding females in populations with low growth rates should be considered.

The discovery of female mate choice based on male genetic quality and intermediate genetic relatedness is potentially extremely important for the metapopulation management of both *in situ* and *ex situ* populations of not just this taxa but other such managed species. Small populations which can no longer disperse across an inhospitable matrix rely on human intervention for maintaining genetic viability. A mechanism whereby reproductive output and ultimately the recovery of endangered populations is mediated not only by mate choice for high quality mates but by access to mates with intermediate levels of genetic relatedness has ramifications for all such conservation management practices. In light of the evidence for genetic effects mediating mate selection in these admixed populations, a metapopulation management strategy of selecting candidate females for translocation based on breeding performance indicators would appear to offer the best solution for maximising reproductive output.

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Appendix 5.1. Parentage assignment for 62 black rhinoceros from three Kenyan populations.

Population	*m*	*sm*	*off*	D.O.B	Paternity	1st LOD	2nd LOD	Nm	ΔLOD	Confidence	Trio LOD	Trio ΔLOD	Confidence Trio	*hr*
MRS	2577		9006	15/12/2006	504	2.24	-0.671	1	2.24	+	2	1.62	*	X
MRS	555		9005	22/11/2006	504	0.977	-2.69	2	0.977	+	2.7	2.7	*	Y
MRS	530		9008	28/01/2007	2576	3.82	-2.25	0	3.82	*	4.38	4.38	*	Y
MRS	116		9004	01/11/2006	504	4.54	3.03	1	4.54	*	4.94	4.52	*	Y
MRS	575		9010	01/10/2008	2576	4.13	-3.14	2	4.13	*	5.45	5.45	*	Y
MRS	548		9009	19/02/2008	504	4.6	-4.18	3	4.6	*	6.96	6.96	*	Y
LWC	2504		2552	03/10/2003	2525	4.36	-5.71	3	4.36	*	5.99	5.99	*	Y
LWC	2528		2556	07/08/2004	2524	2.49	-1.95	1	2.49	*	4.30	4.30	*	Y
LWC	2505		2550	07/10/2003	3508	1.57	-2.36	1	1.57	+	3.48	3.48	*	y
LWC	2530		2555	05/06/2004	2524	0.46	-3.97	2	0.46	+	3.47	3.47	*	Y
LWC	2536		2559	08/07/2005	2524	0.63	-2.03	1	0.63	+	3.10	3.10	*	Y
LWC	2504		2565	25/12/2005	2525	3.44	-2.05	2	3.44	*	4.66	4.66	*	Y
LWC	2509		2553	14/10/2003	3508	3.72	-4.09	2	3.72	*	6.81	6.81	*	y
LWC	2509		2567	19/01/2006	3507	6.62	0.37	1	6.25	*	10.06	10.06	*	x
LWC	2523		2548	22/07/2002	2524	2.29	-2.63	2	2.29	*	5.33	5.33	*	X
LWC	2514		2531	04/06/1996	2524	2.02	-4.01	2	2.02	*	4.15	4.15	*	y
LWC	2505		2528	04/07/1995	2502	2.74	-4.47	2	2.74	*	4.95	4.95	*	?
LWC	2504		2542	10/10/2000	2502	-1.14	-3.37	1	0	-	0.95	0.95	*	?
LWC	2517		2539	23/09/1999	2525	4.21	-3.20	2	4.21	*	7.03	7.03	*	Y
LWC	2505		2516	23/08/1991	2502	-0.90	-2.05	1	0	-	1.33	1.33	*	?
LWC	2516		2549	09/05/2003	2524	1.56	-3.19	2	1.56	+	4.25	4.25	*	x
LWC	2505		2543	07/11/2000	3508	4.91	-3.68	3	4.91	*	6.75	6.75	*	y
LWC	2505		2536	26/09/1998	2502	0.73	-3.68	2	0.73	+	2.77	2.77	*	?
LWC	507		2540	24/10/1999	2525	3.01	0.92	0	2.09	*	3.90	3.78	*	y
LWC	507		2554	01/05/2004	2524	3.13	-1.95	2	3.13	*	5.93	5.93	*	x
LWC	2505		2514	09/03/1988	2525	-0.82	-2.61	1	0	-	-2.79	0.00	-	?
LWC	2514		2551	08/10/2003	2524	1.34	-1.80	1	1.34	+	0.66	0.66	*	y
LWC	2523		2557	27/08/2004	3507	1.90	1.25	0	0.65	+	4.99	4.99	*	X
LWC	2514		2541	09/10/2000	2524	2.05	-3.66	2	2.05	*	3.10	3.10	*	y
LWC	2509		2537	23/04/1999	3508	0.25	-4.40	3	0.25	+	3.38	3.38	*	y
LWC	2528		2545	13/06/2002	2524	3.47	0.99	1	2.48	*	5.80	5.80	*	Y
LWC	2509		2530	25/01/1996	2502	2.40	-5.84	3	2.40	*	5.70	5.70	*	?
LWC	2504		2517	01/12/1991	2502	2.58	-3.72	2	2.58	*	4.85	4.85	*	?

Population	*m*	*sm*	*off*	D.O.B	Paternity	1st LOD	2nd LOD	Nm	ΔLOD	Confidence	Trio LOD	Trio ΔLOD	Confidence Trio	*hr*
OPC	4007		4014	01/03/1994	4003	4.82	-10.49	3	4.82	*	7.86	7.86	*	X
OPC	4005	4550	4019	17/05/1996	4008	2.17	-5.41	2	2.17	*	4.50	4.50	*	X
OPC	4007		4025	25/02/1999	4009	3.00	3.87	0	0	-	6.08	2.35	*	y
OPC	4011	4005	4020	04/09/1996	4008	2.09	-6.26	2	2.09	*	4.83	4.83	*	y
OPC	4511		4005	01/09/1991	4002	3.12	-7.43	2	3.12	*	6.20	6.20	*	y
OPC	46		4024	26/11/1998	4003	2.59	-3.37	1	2.59	*	5.66	5.66	*	
OPC	46		4017	20/11/1994	4003	5.18	-6.99	2	5.18	*	7.77	7.77	*	X
OPC	4511		4021	02/11/1996	4010	-0.11	-4.35	2	0	-	1.49	1.49	*	X
OPC	4011		4032	24/08/2001	4010	6.59	-7.98	3	6.59	*	8.96	8.96	*	X
OPC	4005		4036	28/07/2002	4009	2.94	0.44	1	2.49	*	4.50	4.50	*	y
OPC	4045		4045	~01/01/1998	4008	1.57	-9.55	3	1.57	*	3.98	3.98	*	y
OPC	4547		4035	25/05/2002	4003	3.30	-6.54	2	3.30	*	5.34	5.34	*	y
OPC	4014		4031	22/08/2001	4008	3.69	-7.76	2	3.69	*	5.22	5.22	*	y
OPC	4547		4028	13/01/2000	4010	5.52	-6.06	2	5.52	*	6.03	6.03	*	y
OPC	4547		4022	01/05/1997	4010	3.94	-4.84	2	3.94	*	5.35	5.35	*	y
OPC	4550		4026	15/06/1999	4003	3.15	-10.65	3	3.15	*	6.80	6.80	*	X
OPC	4005		4027	10/09/1999	4009	5.78	1.48	1	4.30	*	8.44	8.44	*	y
OPC	4011		unk	unk	4008	3.13	-6.36	2	3.13	*	4.30	4.30	*	y
OPC	4045		4046	~01/01/2001	4008	3.48	-6.50	2	3.48	*	5.55	5.55	*	y
OPC	4007		4033	13/11/2001	4010	3.63	-2.18	1	3.63	*	5.99	5.99	*	y
OPC	46		4030	24/04/2001	4009	4.94	-16.11	4	4.94	*	7.95	7.95	*	Y
OPC	4550		4034	15/03/2002	4010	3.63	-10.43	3	3.63	*	7.23	7.23	*	X
OPC	4019		4037	10/08/2002	4009	6.09	-7.17	2	6.09	*	9.36	9.36	*	y
OPC	46		4038	15/06/2003	4008	3.83	-10.14	3	3.83	*	6.30	6.30	*	Y
OPC	4005		4042	04/04/2005	4009	3.06	1.93	0	1.12	*	4.44	4.44	*	y
OPC	4019		4044	15/06/2005	4009	4.41	-3.73	1	4.41	*	7.38	7.38	*	y
OPC	4547		4040	31/07/2004	4003	4.51	-3.31	1	4.51	*	6.66	6.66	*	y
OPC	4014		4039	23/03/2004	4008	4.52	5.91	0	0	-	6.70	6.70	*	y
OPC	4007		4041	12/12/2004	4009	4.80	-14.10	4	4.80	*	6.52	6.52	*	y

m=genetic mother. *sm*=Mother given in studbook. *off*=offspring (unk=unknown) D.O.B=date of birth of OFF (unk=unknown). 1st LOD=LOD score for most likely father. 2nd LOD=LOD score for next most likely father. *Nm* = number of mismatches that discount next most likely father. ΔLOD= difference in LOD scores between most likely father and next most likely father. Confidence=paternity confidence; * = >95% probability, + = > 80% probability, - < 80% probability. TrioLOD=LOD score for mother and father combined. Trio ΔLOD=difference in LOD scores between most likely parentage assignment and next most likely parentage. Confidence Trio=parentage confidence; * = >95% probability, + = > 80% probability, - < 80% probability. *hr*=home range overlap between mother and father (? = home range data either unavailable or unreliable).

Chapter 6

Low male mutation bias in the black rhinoceros and evidence for ectopic gene conversion between eutherian sex chromosomes

6.1 Abstract

Much debate surrounds the causes for the observed variance in mutagenesis between different taxa. With differences in generation time and metabolic rate both postulated as being the cause for observed variances in mutation rate. However in many taxa both generation time and metabolic rate are a function of body size making it difficult to ascertain the relative effect of either on mutation rate. The extent of male mutation, whereby an increased meiotic rate in males is thought to be the main source of mutations in mammals, has been shown to be heavily dependent on generation time. An examination of variance in male mutation bias between different taxa provides a mechanism where the relative effects of generation time on mutagenesis can be examined. An understanding of factors influencing mutation rate differences between taxa is important in the assignment and evaluation of evolutionary significant units (ESUs) derived from genetic markers.

Rhinocerotidae is reported to have the lowest rates of mutatgenesis of any mammalian order. Here the male mutation bias in the black rhinoceros is examined for the sex-linked last exon for the zinc finger gene. Male mutation bias is compared to other orders and is found to be lower than would be expected from just generation time. Moreover evidence is presented which suggests that the X and Y linked zinc finger homologues have not been isolated within their sex chromosomes as long as postulated by other studies and that in fact ectopic gene exchange has occurred more recently between the eutherian sex chromosomes.

6.2 Introduction

The mammalian sex chromosomes are thought to have evolved from autosomal chromosomes prior to the divergence of eutherian mammals approximately 115-130 MYA (Page *et al.* 1987, Watson *et al.* 1993). As a result of inversions in the Y chromosome, recombination over 95% of the X and Y chromosomes has been suppressed (Schwartz *et al.* 1998, Lahn & Page 1999). A lack of recombination between the homologues has led to the divergence of these two ancestral chromosomes and the subsequent evolution of the heteromorphic sex chromosomes (Jablonka & Lamb 1990, Charlesworth 1996). Regions at the termini of the sex chromosomes still recombine during male meiosis and sequence similarity is thus maintained in these pseudoautosomal regions (PAR) (Lahn & Page 1999). The PARs in humans contain 13 active genes with significant differences in gene content between other eutheria, with an apparent total absence of a PAR in rodents (Poloumienko 2004, Raudsepp *et al.* 2004). As a result of degeneration due to a lack of

recombination outside of the PAR, the Y chromosome was thought to have lost most of its functional genes (Lahn & Page 1997, Engelstadter 2008). However, recent studies of the human Y chromosome indicate the presence of at least 27 protein coding genes outside of the PAR and only about 10 of these are thought to have a putative sex determination functions (Skaletsky *et al.* 2003, Raudsepp *et al.* 2004). Many of these functional Y genes have extensive sequence similarities with genes on the X-chromosome and are thought to be relict 'fossil' genes from the ancestral sex chromosomes. Studies have revealed there are 19 X-Y gene pairs on the human chromosomes that have extensive sequence similarities (Lahn & Page 1997, Lahn & Page 1999). Work on these homologous sex-linked genes in humans indicates that the suppression of recombination/gene transfer between the sex chromosomes did not occur as a singular event. Lahn and Page (1999) analysed K_s values for the 19 ancestral autosomal genes with X and Y homologues in comparison to their position on the X-chromosome. The clustering of K_s values into four groups according to position on the X-chromosome indicates that suppression of gene transfer due to Y-chromosomal inversion happened as four separate events. Similar work by Skaletsky *et al* (2003) also indicates that suppression of gene transfer happened as separate events, although they found the boundaries between the four strata less distinct. One of the most extensively studied of these pairs of sex-linked genes is the Zfy and Zfx zinc finger genes. The zinc finger genes are present on the sex chromosomes of all eutheria studied thus far (Pamilo & Bianchi 1993, Watson *et al.* 1993). In humans, ZFY is located at Yp11.32, approximately 200 kb from the pseudoautosomal region (Page *et al.* 1987), and ZFX located at Xp22.12, approximately 23 Mb from the PAR (Schlessinger *et al.* 1993). Chromosome mapping for other species shows that the position of the Zfy gene and its distance to PAR to be highly variable between different taxa (Raudsepp *et al.* 2004). The precise function of these zinc finger homologues is unknown but they are thought to act as sequence specific transcription activators (Mardon *et al.* 1990). The two genes code for similar proteins that contain two large domains: the amino-terminal domain and a carboxyl-terminal domain. The amino-terminal domain is rich in acidic residues and has been shown to activate transcription when bound to the DNA-binding region of yeast chromosomes (Mardon *et al.* 1990). The carboxyl-terminal domain contains 13 zinc fingers comprising 393 amino acids and is coded by the last exon at the 3' region half of the gene and probably binds to DNA in a sequence specific manner (Pamilo & Bianchi 1993, Luoh & Page 1994). The genes have been found to be ubiquitously expressed in all eutherian tissue studied thus far with ZFX escaping X-inactivation (Schneider-Gädicke *et al.* 1989). Lahn & Page (1999) estimated that in humans the X- and Y-chromosome zinc

finger homologues have been isolated from each other for approximately 100 million years (80 to 130 MYA), a result supported by the work of Slaketsky *et al* (2003). Whilst these studies suggest that the zinc finger homologues have remained isolated since the beginning of the radiation of eutheria, other studies have demonstrated that there has been more recent gene transfer between the two homologues, most probably as the result of ectopic gene conversion. Ectopic gene conversion is the non-reciprocal transfer of genetic information between homologous chromosomes which occurs most often as a repair mechanism in instances of double-strand DNA breaks (Hastings 2010). Phylogenetic analysis of the zinc finger homologues in humans, murines and the crab-eating fox demonstrated that the two zinc finger homologues had not evolved separately since the radiation of eutheria (Pamilo & Bianchi 1993). The clustering of both genes within the crab-eating fox lineage and the murine lineage suggests that there has been some gene transfer since their divergence. Also, in Pamilo and Bianchi (1993) study the clustering of the human ZFY gene outside of the mouse ZFY gene also suggests that there have been some homogenisation between the human zinc finger genes since the separation of Rodentia and Primates. More recently, Slattery *et al* (2000) demonstrated through phylogenetic analysis of the last exon of the zinc finger homologues that ectopic gene conversion had occurred relatively recently within the cat family, Felidae. In addition, similar phylogenetic analysis within the same study indicated gene transfer within primates and possibly rodents since their divergence (Slattery *et al.* 2000). Lawson & Zhang's (2009) study on gene conversion within the X-chromosome demonstrated a comparative phylogenetic approach was more reliable in detecting instances of ectopic gene conversion than the more commonly used predictive gene conversion programs. Given the contradictory results between the studies on the evolution of the human sex chromosomes and the studies of the zinc finger homologues in other species, broader analysis involving all available sequences for the terminal Zn-finger exon across orders is potentially valuable in understanding the evolution of the eutherian sex chromosomes. The characterisation of the last exon of the zinc finger homologues in the black rhinoceros (Cain 2011) combined with analysis of orthologous genes in other eutherian mammalian orders of varying divergence times is a potentially powerful tool for detecting gene conversion between the sex chromosomes. In this study, a phylogenetic approach across orders combined with divergence time estimates has enabled the rate and timing of gene conversions between the sex-linked zinc finger homologues to be quantified.

An examination of the differences in mutation rate between the X and Y-chromosome in the black rhinoceros will yield further insight into the molecular evolutionary rate of this

endangered species. Mitogenomic studies have indicated that *Perissodactyla* have the lowest mutation rate of all eutherian mammalian orders (Gissi *et al.* 2000). In addition, molecular clock studies, using relative rate tests and more sophisticated fossil calibrated Bayesian methods, have shown that there is substantial variation in mutation rate between taxa within the order and that these rates have varied temporally within taxa over the course of their evolution (Norman & Ashley 2000).

Several possible explanations have been put forward to explain the observed variances in mutagenesis between and within genomes; the most prominent of these is the generation time hypothesis. Most point mutations are thought to occur as a result of errors during DNA replication and are the source of subsequent genetic diversity that contributes to evolution (Bromham *et al.* 1996, Thomas *et al.* 2006). According to the generation time hypothesis, organisms with a short generation time and a high rate of germ-line cell division per unit of time will have faster molecular clocks than those with long generation times (Li *et al.* 1987, Wu & Li 1985). Given that spermatogenesis involves many more cell divisions than oogenesis, it is expected that mutations will accumulate faster in males than females and consequently evolution is thought to be 'male-driven' (Miyata *et al.* 1987). By the age of 40 years, humans males, for example, will have had approximately 610 germ cell divisions compared to 24 germ cell divisions in females (Lawson & Hewitt 2002). Quantification of the point mutation rate differences between sex-linked homologues or between sex-linked homologues and autosomal homologues has demonstrated this to be the case in many species (reviewed in see Li *et al.* 2002). However, some conflicting data means that the specific mechanisms and extent of male-driven evolution are controversial (Hurst & Ellegren 1998, Ellegren 2007). For example, the male-to-female ratio (alpha) of mutation rates in primates has been estimated to be 4-6 times higher in males than females (Huang *et al.* 1997, Nachman & Crowell 2000). Some more recent studies have found alpha to be only about 2 in humans, which is considerably less than the estimates for alpha in carnivores and birds (Bohossian *et al.* 2000). Given that the number of germ cell divisions within a species is a function of generation time, under male-driven evolution theory humans would be expected to have a much higher alpha number than carnivores and birds. Similarly, in rodents a mouse-rat comparison for synonymous mutation rates in X-linked genes compared to autosomal genes, produced the estimate for alpha as infinity (McVean & Hurst 1997, Wolfe & Sharp 1993).

The generation time hypothesis and associated mutagenesis has a significant impact on our understanding of the timing of evolutionary events. Molecular dating and the molecular clock hypothesis are potentially powerful tools for understanding evolutionary events and

have important applications in conservation biology. The application of genetic markers in the management of endangered species is now in routine use; classification of evolutionary significant units (ESUs) through the interpretation of molecular markers is a key component of conservations action plans for most species. The increasing evidence for wide variation in molecular evolution both within and among lineages has obvious implications for the application of molecular dating for species conservation (Bromham *et al.* 1996, Thomas *et al.* 2006). An understanding of the mutagenic processes in rhinoceros has important conservation implications for the management of conservation units. The low mutagensis found in mitogenomic studies of rhinoceros yields divergence times of current subspecies beyond that generally found for species in other mammalian orders. The drastic decline in rhinoceros numbers in recent history, predominantly as a result of poaching, now means that species recovery plans require the active metapopulation management of fragmented populations (Okita-Ouma *et al.* 2007). The characterisation of the extent of male mutation bias in the black rhinoceros provides valuable information about the processes of mutagensis in the nuclear genome and will therefore greatly facilitate the interpretation of mitogenomic studies in the context of defining management units (Ingvarsson 2001, Fredrickson *et al.* 2007).

6.3 Methods

The extraction, cloning and sequencing of the last exon of the zinc finger gene in the black rhinoceros has been described previously (Cain 2011).

The black rhinoceros Zfx/y sequences were BLAST searched (Altschul *et al.* 1990) and compared to available orthologous sequences for Felidae, Cetacea, Primates, Rodentia, Artiodactyla and *Equus caballus* (Appendix 6.1). The characteristics of this zinc finger exon were compared with a subset of sequences from the adjoining zinc finger intron: *D. bicornis* Zfx (DQ519374), Zfy (DQ520642); *E. przewalskii* Zfx (DQ519372), Zfy (DQ520642); *Pongo. pygmaeus* Zfx (DQ520720), Zfy (DQ520727) (Goetting-Minesky & Makova 2006) and *H. sapiens* ZFX (X58925), ZFY (X58926) (Shimmin *et al.* 1993). Homologous sequences were aligned by ClustalX as implemented in MEGA 4.0 (Tamura *et al.* 2007). Pairwise distance measures were calculated in Mega 4.0 (Tamura *et al.* 2007), measures of nucleotide diversity (π) (Nei & Li 1979), synonymous (K_S) and nonsynonymous (K_a) mutation rates were calculated in DnaSP ver. 5 (Librado & Rozas 2009).

Phylogenetic reconstruction was accomplished via Bayesian inference based upon posterior probabilities of phylogenetic trees using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Altekar *et al.* 2004). The best fitting model of evolution was determined by the hierarchical log-likelihood test in MODELTEST 3.5 (Posada & Crandall 1998). Out of the 28 models tested the Tamura-Nei model with a gamma distribution (TrN+G) for rate heterogeneity across sites was selected as the model of best-fit by MODELTEST (AIC = 3957.68). Because MrBayes cannot implement TRN+G, a general time reversible model with gamma distribution model (GTR+G) of nucleotide substitution was used for the analysis (AIC = 3959.44). The GTR+G model is similar to the TRN+G model and allows rates to vary across all six bidirectional DNA substitution types and incorporates heterogeneity in base composition (Rodriguez *et al.* 1990).

The Bayesian analysis of the zinc finger exons was undertaken with two independent runs each with one cold chain and three incrementally heated chains for 5,000,000 Metropolis-coupled Markov chain Monte Carlo steps with trees sampled every 100 generations (Huelsenbeck & Ronquist 2001). The heating parameter was adjusted to 0.05 to achieve better mixing. The results of the first 500,000 generations were discarded (burn-in), after checking that the stationarity of the lnL had been reached. Convergence between the two runs was assessed by examining the standard deviation of split frequencies (< 0.001). A consensus tree was constructed from the remaining 4,500,000 generations (45,000 reconstructed trees) with the calculation of the Bayesian posterior probabilities (BP) for all nodes. Male mutation bias (α_M) was calculated by inputting the Bayesian branch lengths into the equation $Y/X = 3\alpha/(2+\alpha)$ (Miyata *et al.* 1987).

In order to determine whether functional constraint was having an effect on the topology of the resultant Bayesian tree, a replicate analysis was conducted on the 4-fold and 2-fold degenerate sites. Available sequences were downloaded for the entire zinc finger homologues for *H. sapiens*, *Pan troglodytes* and *Bos taurus* using NCBI's HomoloGene. The X-linked homologues for the last exon were translated and aligned against the translated complete X sequences from HomoloGene using ClustalX in Mega 4.0 (Tamura et al. 2007), with an identical procedure carried out for the Y homologues. The exon sequences were then translated back and homologues from both chromosomes were combined and realigned using ClustalX. The 4-fold and 2-fold degenerate sites were extracted from the aligned sequences using Mega 4.0 (Tamura et al. 2007) and used for phylogenetic analysis.

The topology of the resultant Bayesian trees were checked for conformity with a neighbour-joining tree (Saitou & Nei 1987) constructed in Mega 4.0 (Tamura *et al.* 2007) with 1000 bootstrap replicas and a Tamura-Nei substitution model (Tamura & Nei 1993).

6.4 Results

The characteristics of the last zinc finger exon in the black rhinoceros have been described previously (Cain 2011). Briefly, the 604 bp open reading frame revealed 19 variable sites between Zfx and Zfy resulting in a 96.9% sequence similarity and number of substitutions per site (π) of 0.0315. The ORF contained 121 synonymous sites (K_S = 0.1562) and 482 nonsynonymous sites (K_a = 0.0042) with a K_S/K_a ratio of 37.2 between the two homologues. The GC content for the two sequences was comparable with Zfy having a GC content of 42.4% and Zfx having a slightly higher content at 43.7%.

Comparison of the black rhinoceros ORF with the orthologous human sequences reveals 30 variable sites with no InDels (Page *et al.* 1987, Schneider-Gädicke *et al.* 1989). Nucleotide diversity between the two human homologues is higher with π = 0.04967; K_S = 0.268, K_a = 0.0062 and a K_S/K_a ratio of 43.22. The GC content for the two human homologues is similar to that found in the black rhinoceros with a 42.7% GC content for ZFY and 44% for ZFX. Comparison with the domestic horse (*Equus caballus*) which is the only Perissodactyla other than the black rhinoceros for which sequence data for the last exon of the zinc finger is available, reveals significant differences (Shiue *et al.* 2000). The available *E. caballus* Zfx and Zfy 447 bp open reading frame is orthologous to positions 158 to 604 of the black rhinoceros exon and demonstrates a higher substitution rate. The *Equus* homologues have 20 variable sites compared with 14 for *Diceros* for the same portion of the ORF, with no InDels. *E. caballus* has a nucleotide diversity of π= 0.0447; with K_S value of 0.2239 and a K_a value of 0.0085 (K_S/K_a ratio of 26.34), compared to a K_S of 0.1489 and K_a of 0.0057 (K_S/K_a ratio of 26.12) and a nucleotide diversity of π=0.03132 for the same region in *Diceros*. The GC content for *Equus* is slightly higher than for *Diceros* with a content of 43.8% for Zfy and 44.7% for Zfx.

Comparison between the exon and a subset of available sequences for the adjoining zinc finger intron shows that there is a pronounced difference between the exon and intron in divergence rates between homologues (Table 6.1.) The percentage similarity between *Diceros bicornis* and *E. caballus* for the exon is 97.8% for Zfx and 95.1% for Zfy compared to 94.3% for the Zfx intron and 87.7% for Zfy between *Diceros bicornis* and *E.*

przewalskii. The number of nucleotide substitutions per site (π) between the Zfx and Zfy intron for *Diceros* was 0.1965 compared to 0.2665 for *E. przewalskii*. The differences between humans and black rhinoceros are even more pronounced with 96.4% and 93.7% similarity for Zfx and Zfy exons respectively, for introns the percentage similarity is 42% for Zfx and 43.3% for Zfy, with the number of nucleotide substitutions per site (π) between ZFX and ZFY for the human intron being 0.6954.

The male to female mutation rate ratio (α) was calculated for *D. bicornis* and *E. caballus* by substituting the ratio of Y-specific to X-specific branch lengths from the Bayesian analysis into the equation $Y/X = 3\alpha/(2 + \alpha)$ (Miyata et al. 1987). The Y/X ratio for the 447 ORF of the last exon in *D. bicornis* was 1.95, which gave an estimated male-to-female mutation rate ratio (α) of 3.71. For the orthologous ORF in *E. caballus* the Y/X ratio was 2.422 which gave an estimated male-to-female mutation rate ratio (α) of 8.38.

Table 6.1. Pairwise similarity between taxa for Zfx (in red) and Zfy for the last exon and adjoining intron

Exon	**1**	**2**	**3**	**4**	**Intron**	**1**	**2**	**3**	**4**
1. *D. bicornis*		95.1	93.1	93.7	***1.*** *D. bicornis*		87.7	57.6	43.3
2. *E. caballus*	97.8		91.7	92.4	***2.*** *E. przewalski*	94.3		56.7	43.9
3. *P. pygmaeus*	96.6	94.9		98.2	***3.*** *P. pygmaeus*	90.7	89.9		39.0
4. *H. sapiens*	96.4	95.1	99.8		***4.*** *H.sapiens*	42.0	43.2	42.0	

Analysis of uncorrected pairwise distances for all 42 species examined for Zfx showed that nucleotide diversity across all orders ranged from 0.2% to 9.4% with a mean average of 2.6% compared to Zfy which ranged from 0.2% to 10.6% with a mean of 3.8%. Between the main taxonomic groups examined; ungulates, cetaceans, primates and felids, Zfx showed the greatest conservance between groups compared to Zfy with the average percentage similarity between all groups being 95.5% for Zfx compared to 93.4% for Zfy (Table 6.2)**.** Similarly, the within groups differences were larger for Zfy than Zfx, with the average number of base pair differences per site for Zfx being 0.021 compared to 0.029 for Zfy. The percentage GC content for Zfx within groups is 43.4% for cetaceans, 45.1% for Felids, 44.2% for primates and 42.8% for ungulates. For Zfy, the GC content is 42.8% for cetaceans, 43.1% for Felids, 42.7% for primates and 42.9% for ungulates.

Table 6.2. Uncorrected pairwise distances between mammalian groups (Zfx in red).

	1	**2**	**3**	**4**
1. *Ungulates*		92.1	93.6	94.0
2. *Primates*	94.5		94.1	93.3
3. *Cetaceans*	95.3	96.0		95.3
4. *Felidae*	94.7	96.0	96.6	

The number of synonymous substitutions per site (K_S) for all species, excluding the *Rodentia*, was estimated using the method of Kumar in Mega 4.0 (Nei & Kumar 2000, Tumura *et al* 2007). In accordance with the results of the total pairwise distances of the zinc finger exon and intron, the Y linked homologue showed the highest rate of synonymous nucleotide substitution. For Zfx, the average number of synonymous substitutions per synonymous site across all taxa was 0.115, for Zfy it was 0.157. Within the examined groups of ungulates, primates, cetaceans and felids, the highest difference between the K_S values for the X homologue and Y homologue was in primates with a rate of 0.037 for X and 0.069 for Y (Table 6.3).

Table 6.3. Synonymous substitution rate (K_S) within mammalian groups.

	K_S	
	Zfx	**Zfy**
Ungulates	0.026	0.027
Primates	0.037	0.069
Cetaceans	0.016	0.038
Felids	0.015	0.034

The zinc finger sequences for *Rattus norvegicus* were omitted from the larger phylogenetic analysis due to apparent long-branch attraction of the *R. norvegicus* Zfy sequence to the Zfy sequence of *E. caballus*. Long-branch attraction is the grouping of two or more long branches due to errors in the phylogenetic methodology when dealing with rapidly evolving sequences (Bergsten 2005). Long-branch attraction between *R. norvegicus* Zfy and *E. caballus* Zfy was apparent in all phylogenetic tests. With *R. norvegicus* omitted, the phylogenetic analysis for the zinc finger exon across taxa revealed that the X and Y sequences bifurcated between orders for all taxa (Figure 6.1). Posterior support across the tree was generally high, particularly for the higher nodes with lower support for the some

interspecific relationships particularly within Felidae. Within Felidae, the Zfy and Zfx sequences for *Otocolobus manul* clustered together indicating the recent gene conversion between the two homologues within this species (Slattery *et al.* 2000). Perissodactyla and Carnivora were confirmed as sister taxa although with lower levels of support than for other inter-order nodes. The Bayesian tree topology was supported by the NJ tree: only the Bayesian tree is presented here (Figure 6.1). Phylogenetic analysis of the 2-fold and 4-fold degenerate sites proved to be phylogenetically uninformative. Across all 42 taxa there were 40 4-fold degenerate sites for the 604 bp ORF, with only two variable sites. There were 90 2-fold degenerate sites across the same ORF with 11 variable sites; phylogenetic analysis utilising these 11 sites resulted in a tree which demonstrated a significantly different inter-order topology from the Bayesian and NJ trees; consequently, the 4-fold and 2-fold sites were omitted from further analysis.

In order to investigate the relationship of the zinc finger homologues in the Rodentia to the other mammalian orders, a separate set of phylogenetic analysis was performed on a smaller number of taxa. This was done in an attempt to circumvent the long-branch attraction phenomenon demonstrated by the *R. norvegicus* Zfy sequence in the larger analysis. An identical analysis to that performed across all 42 taxa was performed on 11 taxa representing each order including Rodentia, with *E. caballus* omitted. The resultant Bayesian tree confirmed Rodentia and Primates as sister taxa with the zinc finger genes bifurcating within order for Rodentia (Figure 6.2). The tree topology was identical to that of the larger analysis with similar posterior support for nodes, but with the inclusion of Rodentia (Figure 6.2).

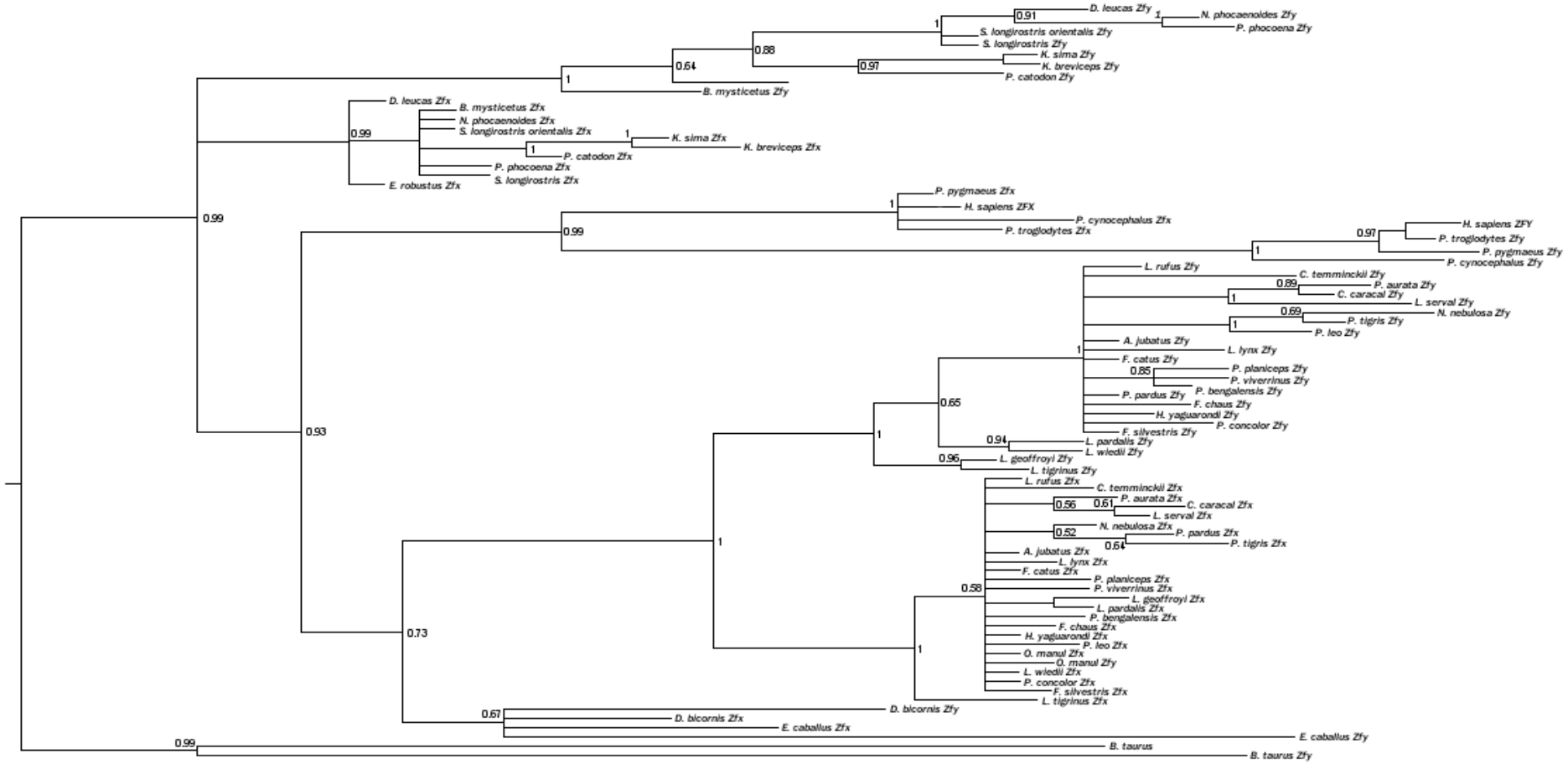

Figure 6.1. Bayesian phylogenetic tree of the last exon for Zfx and Zfy for 41 taxa. Posterior support is shown above each node. Tree is rooted with Zfx/y sequences for *Bos taurus*.

6.5 Discussion

A comparison of the black rhinoceros Zfy and Zfx exon with the domestic horse indicates that the structure of the zinc finger domain is highly conserved within Perissodactyla. There is a 97.8% similarity between the horse and rhinoceros Zfx compared to a 96.9% similarity for the Zfy. The Equidae and Rhinocerotidae diverged approximately 55 MYA (Norman & Ashley 2000) and show a 72.7% similarity for a 504bp segment of the mtDNA loop and a 89.2% similarity for a 840 bp segment of the transcribed mtDNA 12s gene (Cain 2011). Similarly, there is an 87.7% similarity between Equidae and Rhinocerotidae for the non-transcribed zinc finger intron examined by Goetting-Minesky *et al* (2005). The relatively high divergence for these transcribed and non-transcribed genes compared to that found between the zinc finger exon homologues indicates high selective pressure and functional constraint for the sequence specific binding region of the zinc finger carboxy-terminal domain in Equidae and Rhinocerotidae.

Despite a small divergence of the Zfx/y exon between *E. caballus* and *D. bicornis*, an examination of mutation rates shows unequal rates between the two species. The synonymous mutation rate (K_S) in Equids is 1.5 times higher than that found for the same ORF in *Diceros*, with a comparable difference shown for the nonsynonymous rate (K_a). This faster rate in Equids compared to Rhinocerotidae is in accordance with the differences found in other studies comparing the mutation rates of mitochondrial genes between the two taxa. Norman & Ashley (2000) found a higher mutation rate in Equids compared to Rhinocerotidae for the mitochondrial genes, cytochrome c oxidase (COII) and 12S. Due to differences obtained from molecular clock tests using fossil calibration points and relative rate tests within Perissodactyla, Norman & Ashley (2000) concluded that the mutation rate in Equids has sped up during the course of their evolution. An examination of the mutation rates for the last intron of the zinc finger homologues also reveals unequal rates of mutation between the taxa. The non transcribed intron shows higher rates of nucleotide substitutions per site between Zfx and Zfy for *E. caballus* than for *Diceros*. The amount that the *E. caballus* Zfx/Zfy exon K_S rate is higher than the *Diceros* K_S rate (1.5) is similar to the difference found for the non-transcribed intron (1.43). The similarity in rate differences for both the transcribed exon and the non-transcribed intron,

Figure 6.2. Bayesian phyologenetic tree for a subset of 11 taxa from the main 41 taxa analysis but including *R. norvegicus*. Posterior support for each node is given.

indicates that the evolutionary rate in Equids is uniformly higher for the entire zinc finger gene and the differences observed for the exon are not due to functional constraint. This is consequently reflected in differences for the male mutation rate; α_m for *Diceros* is 3.71 compared to 8.38 for *Equus*. It is apparent from the figures obtained for α_m in both species that they experience a male mutation bias although of apparently different magnitudes. The male mutation bias in *Diceros* is comparable to that found in Felidae ($\alpha = 3.76$) and Caprini ($\alpha = 2.93$) (Slattery *et al.* 2000, Lawson & Hewitt 2002). It is also similar to that found by Goetting-Minesky *et al* (2006) in their study of male mutation bias for three sex linked homologues in Perissodactyla where they found α_m in *Diceros* to equal 3.88. Similarly, they found a large difference in the estimates for male mutation bias between horses and rhinoceros with the values for α ranging from 2.90 to 6.07 across the order.

There is considerable debate behind the proximate cause for the observed rate heterogeneity both between and within genomes including the generation time hypothesis (Laird *et al.* 1969), metabolic rate hypothesis (Martin *et al.* 1992) and DNA repair efficiency (Britten 1986). Under neutral theory, rate heterogeneity between lineages has been linked to the generation time hypothesis (Li et al., 1987, Wu and Li, 1985). Given that most mutations occur as errors during DNA replication in germ cell division, it is postulated that those organisms with a shorter generation time will have a higher substitution rate for a given unit of time. Consequently, male mutation rate is a function of generation time (reviewed in Ellegren 2007). The rate of germ-line cell division (C) has not been quantified for Equids or Rhinocerotidae, however generation time as a function of the approximate age of first male reproduction has been shown to be a valid indicator of C in other species (Goetting-Minesky & Makova 2006).

A comparison of generation time and α_m in *Diceros* (3.71) with Felidae (3.76) and Caprini (2.93) shows similar α_m values despite a difference in generation time (Slattery *et al.* 2000, Lawson & Hewitt 2002). The average generation time for Felidae in the Slattery *et al* (2000) study is 1.93 (0.6-5) and in the Caprini the average generation time is 5.07 (1.5-7) (Goetting-Minesky & Makova 2006), compared to a generation time in *Diceros* of 8 years. The high value of α_m obtained for *E. caballus* (8.38) is similarly inconsistent with the generation time hypothesis, *E. przewalski* has a generation time of 5 years (Goetting-Minesky & Makova 2006). The male mutation bias in Equus is higher than that found in primates which have a generation time of approximately 8 years (Goetting-Minesky & Makova 2006). If the differences in generation time were the cause for the observed differences in α_m in Perissodactyla it would be expected that *Diceros bicornis* and primates

would have similar values for α_m with *E. caballus* having a similar male mutation bias to that observed in Caprini. Consequently it would appear that a difference in generation time between Equids and *Diceros* is not the primary cause for the difference in male mutation bias between the two taxa.

Whilst generation time has a direct relationship with germ-line replication (C) and subsequent male-to-female mutation bias, C is also affected by sexual selection which may have an impact on α_m (Bartosch-Harlid *et al.* 2003). Sperm competition is a fundamental part of sexual selection and in the presence of sexual selection, selection should favour increased sperm production (Moller & Briskie 1995). An increase in the germ-line meiotic rate in males as a result of sexual selection would be expected to have an impact on α_m. Alternatively, differences between the two taxa in DNA repair mechanisms may be acting to cause the observed differences (Britten 1986).

Phylogenetic analysis of the last exon for Zfx and Zfy between different mammalian orders reveals that the two homologues appear not to have evolved independently since their initial separation. If the Zfx and Zfy homologues had remained isolated since their divergence before the radiation of eutherians, the sex-linked genes would be expected to bifurcate according to X & Y across orders (Pamilo & Bianchi 1993, Charlesworth 1996, Slattery *et al.* 2000). The observed bifurcation within orders indicates that there have been numerous episodes of gene conversion between the X & Y linked zinc genes over the course of eutherian evolution. Genetic exchange between the non-recombining regions of the two sex chromosomes in eutherians has been implicated in other studies of the zinc finger homologues where high similarities within order have been observed (Pamilo & Bianchi 1993, Slattery *et al.* 2000). Goetting-Minesky *et al* (2006) failed to detect gene conversion in an examination of male mutation bias within Perssiodactyla with primates as a comparative order. The phylogenetic analysis in this study was undertaken separately for each order with no analysis presented across orders, consequently no gene conversion events were detected.

Using the phylogenetic analysis of the zinc finger homologues across a large number of mammalian taxa and approximate divergence dates, it is possible to estimate the dates of gene conversion events between the X and Y chromosome for Lahn & Page's (1999) stratum 3 genes. Due to the small number of well-defined fossil calibration points and the variance in results obtained from molecular studies, there is considerable debate concerning the timing of placental mammal radiation, (Kumar & Hedges 1998, Eizirik *et*

al. 2001, Springer *et al.* 2003, Arnason *et al.* 2008). In recent years there have been a number of molecular studies that have utilised a wide range of molecular markers, which have been calibrated with reliable fossil calibration points. Consequently, a general consensus has emerged in some areas of the literature regarding certain inter-ordinal divergence dates and relationships between most eutherian orders (Springer *et al.* 2003, Kitazoe *et al.* 2007, Arnason *et al.* 2008). The orders Cetacea and Artiodactyla are currently placed in the same clade, called Certartiodactyla, reflecting their close relationship (O'Leary & Geisler 1999). Although there is some debate as to whether Cetacea evolved from Artiodactyla or whether they are sister taxa, the divergence of Cetacea and Artiodactyla is generally accepted to have occurred approximately 60 MYA (A/C-60) with Cetaceans undergoing expansive radiation approximately 36 MYA (Arnason & Gullberg 1996, McGowen *et al.* 2009). Carnivora and Perisodactyla are believed to have diverged from a common ancestor with their basal divergence estimated at approximately 80MYA (Kumar & Hedges 1998, Eizirik *et al.* 2001, Flynn *et al.* 2005). Approximately 52 MYA there was a basal split in Carnivora into two monophyletic clades - caniforms and feliforms - and similarly the Perissodactyla diverged into Equidae and Rhincerotidae approximately 55 MYA (Flynn *et al.* 2005, Arnason *et al.* 2008). The orders Perisodactyla, Carnivora, Artiodactyla and Cetacea are grouped into the same clade or superorder Laurasiatheria, which diverged approximately 75 MYA (Kitazoe *et al.* 2007). Primates are currently assigned to the superorder Euarchontoglires (supraprimates) along with Rodentia with an estimated divergence time between Primates and Rodentia approximately 68 MYA (Eizirik *et al.* 2001). The basal divergence between Laurasiatheria and Euarcgontoglires is estimated to have occurred approximately 90 MYA (Arnason *et al.* 2008). Examination of the zinc finger phylogeny for Artiodactyla and Cetacea indicates that there was gene transfer between the two homologues between 60 MYA and 36 MYA as indicated by the bifurcation of the homologues between *Bos taurus* and Cetacea. In the case of Perisodactyla and Carnivora, there has been gene transfer between the two homologues since their basal divergence 80 MYA; although there has been no gene transfer in Perissodactyla since Equidae and Rhincerotidae separated 55 MYA. The lack of available zinc finger sequences for caniforms means that it is not possible to examine whether gene transfer between the homolgoues has occurred since the divergence of caniforms and feliforms. However, Slattery *et al* (2000) demonstrated that there has been relatively recent gene transfer between the two homologues within the felidform clade (Slattery *et al.* 2000). The grouping of both homologues within the primate order similarly indicates that gene conversion has occurred in Euarchontoglires since its divergence from

Laurasiatheria and earlier than 68 MYA based on the divergence dates for Primates and Rodentia. Studies by Slattery *et al* (2000) and Pamilo *et al* (1993) similarly found the grouping of zinc finger homologues within orders for Rodentia and Primates indicating that gene transfer has occurred much more recently in Euarchontoglires than implied by Lahn & Page (1999) and Skaletsky *et al* (2003).

Due to Y-chromosome inversion resulting in the cessation of recombination between the sex chromosomes, it is fair to postulate that the gene transfer demonstrated in this study is a result of ectopic gene conversion (Lahn & Page 1999, Slattery *et al.* 2000). Ectopic gene conversion is potentially important in maintaining the function of genes present in the non-recombining region of the Y-chromosome. It has been proposed that selection for alleles that are advantageous for males but disadvantageous for females is one mechanism behind the genetic differentiation of sex genes and the subsequent lack of recombination (Charlesworth 1996). However, the zinc finger genes are ubiquitously expressed and as a consequence of this it would appear that Zfx is not subject to X inactivation with both genes required for normal function (Jegalian & Page 1998, Skaletsky *et al.* 2003). It is predicted that in the absence of recombination, genes on the Y-chromosome would decay due to the effects of either 'Muller's ratchet' or by the fixation of deleterious mutations 'hitchhiking' with favourable mutations (Charlesworth 1978, Rice 1987). An increased male mutation rate, as demonstrated in this study, means that the erosion of the Y-chromosome probably occurs at a faster rate. Ectopic gene conversion is a potential mechanism whereby the function of ubiquitously expressed sex-linked genes can be maintained in the absence of recombination (Slattery *et al.* 2000). It is thought that ectopic gene conversion does not require the formation of a synaptonemal complex with reciprocal exchange (Jinks-Robertson & Petes 1986, McKim *et al.* 1998) and is therefore a potential mechanism for gene exchange in a non-recombining region of the genome such as the NRY.

6.6 Conclusion

The cloning and sequencing of the last exon for the zinc finger homologues in the black rhinoceros has yielded insights into the processes of molecular evolution governing these genes. A comparison of the synonymous and nonsynonymous substitution rates within Perisodactyla indicates strong conservation for the function of these genes within the order. Examination of mutation rates for zinc finger intron homologues and for transcribed and

non-transcribed mtDNA genes provides further evidence that the transcribed zinc finger exon in Perissodactyla is subject to strong selective pressure.

An elevated mutation rate for Equids compared to *Diceros* is also apparent from an examination of the divergence between and within species for the zinc finger homologues. Examination of the male-to-female mutation bias (α) has shown that male driven evolution for the black rhinoceros is lower than would be expected according to generation time. Similarly, Equids exhibit a much higher mutation bias than even primates, which also appears to be due to factors other than generation time. Whilst no direct link between the 'generation time hypothesis' is apparent, an accelerated mutation rate in Equids is supported by data from mtDNA genes and fossil calibration points. This acceleration of mutation rate during Equid evolution may possibly be linked to a trend of increased body size during the course of horse evolution (Norman & Ashley 2000). The male-to-female bias in rhinoceros, which is similar to that found in Felidae, may be linked to something other than generation time, such as metabolic rate, sexual selection or variance in the efficiency of DNA repair mechanisms. The confirmation of a reduced substitution rate within Rhinocertidae has important implications for the management of the endangered species within this family. The definition of Rhinocertidae management units and taxonomic classification, particularly those based on divergence time estimates, should be evaluated in light of a reduced rate of molecular evolution within the family.

The results presented here, which examined substitution patterns of the zinc finger homologues across mammalian orders, expands on other work which has called into question the hypothesis that the two zinc finger homologues have been separated for at least 100 MYA(Lahn & Page 1999, Skaletsky *et al.* 2003). The phylogenetic analysis of zinc finger homologues for Primates, Rodentia, Artiodactyla, Cetacea, Carnivora and Perissodactyla shows that the two homologues cluster together within orders. This work builds upon other work and demonstrates that, in the absence of recombination ectopic gene conversion between the sex chromosomes may be a mechanism by which transcribed genes on the Y-chromosomes escape Y-chromosome degeneration (Slattery *et al.* 2000). The results of this study demonstrate that there have been several independent ectopic gene conversion events in the zinc finger gene since the divergence of eutherian mammals. With the exception of the recent gene conversion within Felidae, the most recent of these events has occurred since the divergence of Artiodactyla and Cetacea approximately 60 MYA. Within Euarchontoglires ectopic gene conversion has occurred at least as recently as 68 MYA since the divergence of Primates and Rodentia.

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Appendix 6.1. Sex-linked zinc finger homologues by mamalian order from BLAST search of *D. b. michael Zf x/y exon 9*

Order	Species	Common name	Gene	Aligned sequence Length	GenBank accession number	Reference
Artiodactyla	*Bos taurus*	Cow	Zfx	604	AF032866	Poloumienko *et al.* 2004
	Bos taurus	Cow	Zfy	604	AF032366	Poloumienko *et al.* 2004
Carnivora	*Acinonyx jubatus*	Cheetah	Zfx	604	AF252986	Slattery *et al*. 2000
	Acinonyx jubatus	Cheetah	Zfy	601	AF253016	Slattery *et al*. 2000
	Caracal caracal	Caracal	Zfx	604	AF252992	Slattery *et al*. 2000
	Caracal caracal	Caracal	Zfy	601	AF253019	Slattery *et al*. 2000
	Catopuma temminckii	Asiatic golden cat	Zfx	604	AF252984	Slattery *et al*. 2000
	Catopuma temminckii	Asiatic golden cat	Zfy	601	AF253012	Slattery *et al*. 2000
	Felis catus	Domestic cat	Zfx	604	AF252989	Slattery *et al*. 2000
	Felis catus	Domestic cat	Zfy	601	AF253014	Slattery *et al*. 2000
	Felis chaus	Jungle cat	Zfx	604	AF252990	Slattery *et al*. 2000
	Felis chaus	Jungle cat	Zfy	601	AF253013	Slattery *et al*. 2000
	Felis silvestris	Wild cat	Zfx	604	AF252991	Slattery *et al*. 2000
	Felis silvestris	Wild cat	Zfy	601	AF253015	Slattery *et al*. 2000
	Herpailurus yaguarondi	Jaguar	Zfx	604	AF252987	Slattery *et al*. 2000
	Herpailurus yaguarondi	Jaguar	Zfy	601	AF253008	Slattery *et al*. 2000
	Leopardus pardalis	Ocelot	Zfx	604	AF252970	Slattery *et al*. 2000
	Leopardus pardalis	Ocelot	Zfy	601	AF252997	Slattery *et al*. 2000
	Leopardus tigrinus	Little spotted cat	Zfx	604	AF252971	Slattery *et al*. 2000
	Leopardus tigrinus	Little spotted cat	Zfy	601	AF252998	Slattery *et al*. 2000
	Leopardus wiedii	Margay	Zfx	604	AF252972	Slattery *et al*. 2000
	Leopardus wiedii	Margay	Zfy	601	AF252999	Slattery *et al*. 2000
	Leptailurus serval	Serval	Zfx	604	AF252993	Slattery *et al*. 2000
	Leptailurus serval	Serval	Zfy	601	AF253020	Slattery *et al*. 2000
	Lynx lynx	Eurasian lynx	Zfx	604	AF252974	Slattery *et al*. 2000
	Lynx lynx	Eurasian lynx	Zfy	601	AF253001	Slattery *et al*. 2000
	Lynx rufus	Bobcat	Zfx	604	AF252975	Slattery *et al*. 2000
	Lynx rufus	Bobcat	Zfy	601	AF253002	Slattery *et al*. 2000
	Neofelis nebulosa	Clouded leopard	Zfx	604	AF252979	Slattery *et al*. 2000

Order	Species	Common name	Gene	Aligned sequence Length	GenBank accession number	Reference
	Neofelis nebulosa	Clouded leopard	Zfy	601	AF253006	Slattery *et al*. 2000
	Oncifelis geoffroyi	Geoffroy's cat	Zfx	604	AF252973	Slattery *et al*. 2000
	Oncifelis geoffroyi	Geoffroy's cat	Zfy	601	AF253000	Slattery *et al*. 2000
	Otocolobus manul	Pallas's cat	Zfx	604	AF252994	Slattery *et al*. 2000
	Otocolobus manul	Pallas's cat	Zfy	604	AF252995	Slattery *et al*. 2000
	Panthera leo	Lion	Zfx	604	AF252980	Slattery *et al*. 2000
	Panthera leo	Lion	Zfy	601	AF253007	Slattery *et al*. 2000
	Panthera pardus	Leopard	Zfx	604	AF252982	Slattery *et al*. 2000
	Panthera pardus	Leopard	Zfy	601	AF253009	Slattery *et al*. 2000
	Panthera tigris	Tiger	Zfx	604	AF252983	Slattery *et al*. 2000
	Panthera tigris	Tiger	Zfy	601	AF253010	Slattery *et al*. 2000
	Prionailurus bengalensis	Leopard cat	Zfy	601	AF253004	Slattery *et al*. 2000
	Prionailurus bengalensis	Leopard cat	Zfx	604	AF252977	Slattery *et al*. 2000
	Prionailurus planiceps	Flat-headed cat	Zfx	604	AF252976	Slattery *et al*. 2000
	Prionailurus planiceps	Flat-headed cat	Zfy	601	AF253003	Slattery *et al*. 2000
	Prionailurus viverrinus	Fishing cat	Zfx	604	AF252978	Slattery *et al*. 2000
	Prionailurus viverrinus	Fishing cat	Zfy	601	AF253005	Slattery *et al*. 2000
	Profelis aurata	African golden cat	Zfx	604	AF252985	Slattery *et al*. 2000
	Profelis aurata	African golden cat	Zfy	601	AF253011	Slattery *et al*. 2000
	Puma concolor	Puma	Zfx	604	AF252988	Slattery *et al*. 2000
	Puma concolor	Puma	Zfy	601	AF253018	Slattery *et al*. 2000
Cetacea	*Balaena mysticetus*	Bowhead whale	Zfx	604	AF260784	Morin *et al*. 2005
	Balaena mysticetus	Bowhead whale	Zfy	604	AF260785	Morin *et al*. 2005
	Delphinapterus leucas	Beluga whale	Zfx	604	AF260788	Morin *et al*. 2005
	Delphinapterus leucas	Beluga whale	Zfy	604	AF260787	Morin *et al*. 2005
	Eschrichtius robustus	Grey whale	Zfx	604	AF260790	Morin *et al*. 2005
	Eschrichtius robustus	Grey whale	Zfy	604	AF260791	Morin *et al*. 2005
	Kogia breviceps	Pygmy sperm whale	Zfx	604	AF260793	Morin *et al*. 2005
	Kogia breviceps	Pygmy sperm whale	Zfy	604	AF260794	Morin *et al*. 2005
	Kogia simus	Dwarf sperm whale	Zfx	604	AF260797	Morin *et al*. 2005
	Kogia simus	Dwarf sperm whale	Zfy	604	AF260796	Morin *et al*. 2005
	Neophocaena phocaenoides	Finless porpoise	Zfx	604	AF260800	Morin *et al*. 2005
	Neophocaena phocaenoides	Finless porpoise	Zfy	604	AF260799	Morin *et al*. 2005

Order	Species	Common name	Gene	Aligned sequence Length	GenBank accession number	Reference
	Phocoena phocoena	Harbour porpoise	Zfx	604	AF260805	Morin *et al*. 2005
	Phocoena phocoena	Harbour porpoise	Zfy	604	AF260806	Morin *et al*. 2005
	Physeter catodon	Sperm whale	Zfx	604	AF260802	Morin *et al*. 2005
	Physeter catodon	Sperm whale	Zfy	604	AF260803	Morin *et al*. 2005
	Stenella longirostris	Spinner dolphin	Zfx	604	AF260808	Morin *et al*. 2005
	Stenella longirostris	Spinner dolphin	Zfy	604	AF260809	Morin *et al*. 2005
	Stenella longirostris orientalis	Eastern spinner dolphin	Zfx	604	AF260812	Morin *et al*. 2005
	Stenella longirostris orientalis	Eastern spinner dolphin	Zfy	604	AF260811	Morin *et al*. 2005
Perissodactyla	*Diceros bicornis michaeli*	Black rhinoceros	Zfx	604	EU284593	Cain *et al*. 2007
	Diceros bicornis michaeli	Black rhinoceros	Zfy	604	EU284594	Cain *et al*. 2007
	Equus caballus	Horse	Zfx	448	AF132292	Shiue *et al*. 2004
	Equus caballus	Horse	Zfy	448	AF133198	Shiue *et al*. 2004
Primates	*Homo sapiens*	Human	ZFX	604	M26946	Schneider *et al.* 1989
	Homo sapiens	Human	ZFY	604	J03134	Page *et al*. 1987
	Pan troglodytes	Chimpanzee	Zfx	604	XM520979	NW001251726*
	Pan troglodytes	Chimpanzee	Zfy	604	AY913763	Ebersberger & Meyer. 2005
	Papio cynocephalus	Yellow baboon	Zfx	604	X75174	Shimmin *et al.* 1994
	Papio cynocephalus	Yellow baboon	Zfy	604	X75173	Shimmin *et al.* 1994
	Pongo pygmaeus	Bornean orangutan	Zfx	604	X75169	Shimmin *et al.* 1994
	Pongo pygmaeus	Bornean orangutan	Zfy	604	X75176	Shimmin *et al.* 1994
Rodentia	R.norvegicus (Lewis)	Brown rat	Zfx	1181	X75171	Shimmin *et al.* 1994
	R.norvegicus (Lewis)	Brown rat		1175	X75172	Shimmin *et al.* 1994

*Predicted by automated computational analysis. Record is derived from a genomic sequence

Chapter 7

Conclusions

This dissertation reports the results of a conservation genetics project conducted on enclosed populations of the eastern black rhinoceros (*D. b. michaeli*) in Kenya. The work was undertaken with the aim of understanding what impact conservation practices have had on the historic population structure and to what extent genetic diversity has been maintained in current admixed populations following a severe population bottleneck. The effects of these management practices on the genetic mechanisms of mate choice have also been investigated in an attempt to guide future *in situ* and *ex situ* management for the species.

The majority of the genotyping on identified rhinoceros was undertaken on DNA extracted from faeces. Following a black rhinoceros on foot for extended periods of time through sometimes thick vegetation and collecting a fresh faecal sample without detection is not without its difficulties. This is particularly the case in those instances when pairings of animals made it extremely difficult to identify a particular sample with a particular individual. Pairings of animals are usually sub-adult males and females or mother and calves; in both instances animals often defecate at the same time a close distance apart. A reliable and high-throughput system of sex determination was invaluable in assigning the correct microsatellite genotypes and mtDNA haplotypes to the correct individual in many of these instances. Despite the investment in time and resources to develop the assay, the high-throughput nature of the system made it extremely user-friendly and simple to replicate, which ensured correct gender determination in light of allelic dropout. As the assay was only designed for use on *D. b. michaeli* it hasn't been tested on the other subspecies of black rhinoceros. Future work would be to obtain from the other two extant subspecies either low or high-copy DNA samples from individuals of known gender to test the assay. Unfortunately the United Kingdom only has *D. b. michaeli* in captivity so samples would have to be obtained from *in situ* populations or captive populations outside of the United Kingdom.

The characterisation of the last exon of the zinc finger homologues in the black rhinoceros for the design of the gender determination assay presented the opportunity to examine male mutation bias in the species. Previous authors have highlighted the low rate of mutagenesis in Rhinocerotidae and the low male mutation bias for the adjacent zinc finger intron. The definition of management units is often based on divergence time estimates obtained from molecular genetic analysis. Understanding the variance in mutatgenesis between taxa and the proximate causes for these differences are valuable in evaluating the results of such

analysis. Investigation of male mutation bias in relation to generation time demonstrated that indeed the black rhinoceros has a lower comparative male mutation bias that would be predicted by the species' generation time. Other postulated causes for variance in mutatgenesis between taxa are differences in metabolic rate and DNA repair mechanisms. Unfortunately information is unavailable for either of these two mechanisms in the black rhinoceros so it was not possible to ascertain whether they have an effect on mutation rate. An investigation of male mutation bias for other sex-linked homologues across all extant species of rhinoceros would possible yield important information on variation in mutatgenesis within Perissodactyla. This work would assist greatly in interpreting current management unit designations for the order.

The meta-analysis presented on ectopic gene conversion between the two sex-linked zinc finger homologues provides preliminary evidence for gene conversion between eutherian sex chromosomes. This work is in concordance with work presented by other authors examining the phenomena in a smaller range of taxa. Whilst the evidence for gene conversion seems compelling, especially in light of other authors reporting similar results for the non-transcribed zinc finger intron, the results are only presented as preliminary analysis. The results presented here for the transcribed exon might well be due to selective constraint for the functionality of the region investigated. Further work needs to be undertaken on a wider range of sex-linked homologues including both transcribed and non-transcribed regions to identify the pattern of bifurcation for all different regions of the eutherian sex chromosomes. A preliminary search of the NCBI database has identified available sequences for both introns and exons of DB X/Y and UTX/Y genes, although on a smaller range of taxa than the meta-analysis presented in this thesis.

The results presented on the demographic history of the Kenyan black rhinoceros reveal that the subspecies underwent a population expansion in the south of the country approximately 300 KYA putatively linked to a contraction in the Pleistocene forests. The subspecies moved into the central highlands much later, presumably as a result of the delayed contraction of the forests in this upland area; indeed remnants of the Pleistocene forests and associated fauna are still found on the Aberdares mountains and Mount Kenya in the central highlands. Phylogenetic analysis indicates that the historic central highlands population are more closely linked with former populations in the east of the country rather than the south. The current sanctuary populations which were genotyped during the study are now a mixture of former populations from the central highlands and the south of the

country. The movement of animals from the central highlands and the southern Chyulu Hills region into Solio Game Reserve (SGR) during the early 1970s has had a profound effect on the genetic structure of current sanctuary populations. The analysis of genetic structure in the current populations revealed very little discernable structure amongst individuals with a 'southern' haplotype. The most discernable genetic structure was according to relative composition of historic southern and central highland populations. These results may be indicative of the central highlands population being relatively isolated from the other Kenyan populations; the Rift Valley forms a considerable barrier to the west, the Aberdares Mountains and Mount Kenya form barriers to the south and east, with the arid rangelands to the north providing the most obvious means of dispersal. The mixture of individuals in SGR during the 1970s and the subsequent stocking of new sanctuaries from the SGR population over proceeding years has resulted in a marked difference between those populations which received animals from SGR and those which have not, i.e. the two sampled national park populations. The animals from MRS which originated Lake Nakuru National Park consisted of individuals with central highlands mtDNA haplotype and demonstrated genetic structure in accordance with central highland alleles. Initial active management in mixing individuals from different historic populations and subsequent translocations between populations has undoubtedly done much to preserve genetic diversity and offset inbreeding in these relatively small populations. This however has been at the expense of historic population structure, with the potential for future outbreeding depression impossible to quantify. This has to be put into context of the poaching threat facing the black rhinoceros at the time of the implementation of the sanctuary system and the consequences if it hadn't been implemented. Given the pervasive mixture of historic populations within the current sanctuary populations it would almost be impossible to manage the current sanctuary populations to maintain any former historic genetic structure. However the differences between the private, LWC, OPC and MRS populations with the sampled national park populations might lend itself to a metapopulation management strategy which considers whether populations had received animals from SGR. The results of this study demonstrate that conservation strategies have to take into account former population structure when moving animals into or between protected areas.

Examination of the translocation records for the sampled populations makes it apparent that the absence of historic populations from the north and west of the country is not surprising. A more extensive study on all of the current Kenyan black rhinoceros

populations using the approach implemented in this study would greatly facilitate the metapopulation management of the subspecies. Such a study would hopefully identify whether historic populations from the north and west of the country have been captured within the sanctuary system. A more expansive study would identify local differences in not just historic population structure but also in levels of genetic diversity and inbreeding within different populations. Such information would enable KWS to implement a more effective and targeted metapopulation management approach to ensure long-term viability.

This dissertation does not attempt to quantify genetic signatures of recent population contraction. Using the microsatellite data presented here, analysis using a Bayesian coalescent-based approach to detect and quantify signatures of contraction is being undertaken using MSVAR 0.4.1b. The preliminary analysis has yielded evidence of a severe population crash which appears to be more severe than the figures given in this dissertation, i.e. 20,000 in 1970 to < 400 by 1987 which may be in concordance with the reported severe decline in black rhinoceros as a result of European settlement. I am currently trying to source the latest version of the software (MSVAR 1.3) which will enable the quantification of specific population sizes and specific dates before the results are presented.

The results presented on the genetic influences on mate choice in the black rhinoceros demonstrate that male genetic diversity is a significant predictor of reproductive success and that females balance male genetic quality with intermediate levels of genetic similarity. In the absence of inbreeding these results appear to be consistent with some of the microsatellite loci examined being in linkage disequilibrium with fitness-trait loci. Whether these mate preferences are due to females actively seeking males with high diversity but locally adapted fitness-trait loci or whether it is a mediating preference against very high diversity is unclear. Further analysis needs to be conducted to examine local effects and if the loci in LD can be identified, specifically in relation to historic central highland or southern populations The results of this work are a fertile ground for future study. The most obvious and potentially the most important continuation is to examine these factor in black rhinoceros populations (*in situ* or *ex situ*) which are suffering from poor reproduction. Comparative analysis between the results presented here for populations with a relatively 'large' mate choice compared to smaller *ex situ* and *in situ* populations (e.g. the Ngorongoro population) would not only potentially ascertain whether available mate choice influences reproductive output but also guide management in

promoting recovery of these endangered populations. The examination of fitness-trait loci in these mixed populations, most specifically MHC genes would yield valuable insight into whether mate selection was mediated by locally adapted genotypes or a fitness cost associated with very high heterozygosity. An examination of whether high diversity (IR) correlates with phenotype, e.g. body mass and horn size would indicate whether high diversity conveys a fitness advantage in this species. A specific management recommendation to come from this preliminary work is for metapopulation management in Kenya to undertaken with reference to performance indicators in the selection of individuals for translocation. Currently the criteria of moving animals are based on the extent to which estimated ECC has been reached and the age and sex structure of the donor population. Even in the absence of specific genetic data such as that presented here, females with poor breeding performance indicators should be considered as prime candidates for movement to another sanctuary. The wider implications for this study are whether those populations which have poor overall performance rates are indeed due to a density dependent effect or indeed due to small population size and a lack of 'genetically' suitable mates. Whilst it is impossible to ascertain this from the results presented in this thesis, this is an area which requires further investigation based on the results of this work.