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**Investigating genetic diversity at neutral and adaptive DNA markers
in the severely bottlenecked Southern white rhinoceros
(*Ceratotherium simum simum*)**

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Doctor of Philosophy



Department of Molecular and Cellular Biology

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Southern white rhinoceros and calf, *Ceratotherium simum simum* (Burchell 1817)

Declaration

This thesis reports the results of original research that was conducted under the auspices of the Department of Molecular and Cell Biology, University of Cape Town, between June 2005 and January 2009. It has not been submitted for a degree at any other university. All the assistance received has been duly acknowledged.

Natalie Coutts

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Abstract

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Investigating genetic diversity at adaptive and neutral DNA markers in the severely bottlenecked Southern white rhinoceros (*Ceratotherium simum simum*)

Ph.D. thesis, University of Cape Town

Anthropogenic activities have severely affected many ecosystems. The Southern white rhinoceros (*Ceratotherium simum simum*) was virtually extirpated during the 19th century, with an historically widespread distribution severely reduced in southern Africa. By 1895, a single remnant population of ~100 individuals persisted in KwaZulu-Natal, South Africa. Successful conservation strategies and high fecundity facilitated rapid population recovery and the relocation of surplus individuals to other protected areas began in 1961. There are currently ~17 500 Southern white rhinoceroses worldwide and their recovery from the brink of extinction is “*internationally recognised as one of the world’s greatest conservation successes*”.

Southern white rhinoceroses from the original source population (Hluhluwe Imfolozi Game Reserve, South Africa) and three seeded populations across the southern African subregion were analysed to determine the population genetic effects of severe demographic decline. Data from neutral genetic markers together with adaptive major histocompatibility complex gene (MHC) sequences were used to investigate the following hypotheses; (1) founder events have resulted in reduced genetic diversity in seeded populations, (2) founder events and subsequent genetic drift in small populations have resulted in genetic differentiation between populations and (3) extant populations exhibit genetic evidence of their demographic bottleneck and/or founder events. A combination of classical population genetic parameters, together with population simulations and tests for predicted expectations of population decline were used to address these hypotheses.

Contemporary levels of genetic diversity in Southern white rhinoceroses are amongst the lowest reported in large mammals. Average values across all four populations included microsatellite allelic richness of 2.6 alleles per locus, 0.44 observed heterozygosity and a polymorphic informative content of 0.37. Only four mtDNA control region haplotypes were identified and displayed low nucleotide and haplotype diversity ($\pi = 0.003$; $h = 0.52$). Of particular interest was the functional monomorphism at the *DQA* and *DQB* MHC loci. Identical alleles were observed in Northern white

rhinoceroses, despite an estimated lineage divergence of 0.7 to 2 MYA. No significant reduction in microsatellite allele numbers or heterozygosity was observed in seeded populations relative to the source population. Genetic differentiation was greatest amongst seeded populations, relative to the period of time since each founding event, and is most likely the combined result of founder events and genetic drift in small and isolated populations. No statistical evidence of recent or historic genetic bottlenecks were detected in either the source or seeded populations, despite the documented demographic decline and subsequent founder events.

The principal management approach used in modern rhinoceros conservation is the protection of small populations, via a network of fenced reserves, with limited potential for dispersal and gene flow. Most Southern white rhinoceroses are owned by the private sector and maintained in discrete free-ranging populations with an average “population” size of ~11 individuals. A metapopulation management strategy, via the periodic translocation of breeding males, is typically implemented to buffer the effects of genetic drift and limiting subpopulation isolation. Substantial financial and logistical resources are required to translocate white rhinoceroses and the accurate identification of successfully breeding males is vital. Data from microsatellite markers were analysed to assign paternity in two seeded populations, Welgevonden Private Game Reserve, South Africa, and Matobo National Park, Zimbabwe, to (1) accurately identify breeding males for periodic translocations, (2) describe the mating system, reproductive skew and a genetic component of female mate choice and (3) confirm maternal relationships identified by observational data to corroborate a suspected calf-swapping event. Unfortunately, genetic diversity was not sufficient to resolve parental relationships based on this data, which in turn limited inferences regarding mating systems, reproductive skew or mate choice. Analysis of mother-calf combinations did, however, reveal unequivocal evidence of calf-swapping that raises a number of evolutionarily and ecologically interesting issues.

Southern white rhinoceroses are so genetically similar at the DNA markers used in this study that translocating breeding males may not be sufficient to limit the effects of genetic drift. In addition, simulations of the role of drift on maintaining genetic diversity in small populations suggest that existing diversity is unlikely to be maintained under the current metapopulation management strategy. A more effective conservation approach would be the investment of resources in larger populations, such as those in national parks with an average population size of ~123 individuals, where it is predicted that 77.5% of the existing allelic diversity and 94.8% of the observed heterozygosity would be retained after 200 years.

The Southern white rhinoceros is an African conservation flagship and was one of the first large mammals taken to the brink of extinction by anthropogenic activities. This study investigated the genetic outcomes associated with demographic recovery after a population bottleneck in large long-lived mammals. The data and comparative analyses with the Northern white rhinoceros, however, suggest that historic populations prior to their decline in the late 1900's were already characterised by low levels of genetic diversity. Reduced genetic diversity in contemporary Southern white rhinoceroses does not appear to be the result of recent anthropogenic decline and near extinction.

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Chapter 1

Introduction

The Rhinocerotidae Family (Order Perissodactyla)

The Rhinocerotoids represent one of the most diverse and successful groups of large herbivores over the past fifty million years, with approximately 60 genera and hundreds of species flourishing in virtually all available ecological niches worldwide; from tropical vegetation in the middle Eocene and savannas of the Miocene to the glacial conditions of the Ice Ages (Prothero 1993). The rhinocerotid species during the Eocene and Oligocene were small hornless browsers and it was only during the late Oligocene that paired horns evolved independently in two species, *Diceratherium* and *Menoceras* (Prothero 1993). The Rhinocerotidae, together with Tapiridae (tapirs) and Equidae (horses and asses), comprise the Order Perissodactyla. The extant Rhinocerotidae consists of five species within four genera distributed in Africa and Asia (Figure 1.1.): the white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceroses in Africa (*dicerotines*) and the Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran (*Dicerorhinus sumatrensis*) rhinoceroses in Asia (*rhinocerotinines*; Morales & Melnick 1994).

The divergence between the African and Asian lineages, based on mitochondrial DNA (mtDNA) analysis and allozyme studies, occurred approximately 26 million years ago (MYA; Tougaard *et al.* 2001; Merenlender *et al.* 1989) and the divergence between black and white rhinoceroses is approximately 3.4 MYA (O'Ryan & Harley 1993). Fossil records, however, suggest that the African rhinoceros lineage dates to the middle Miocene (about 18 MYA) with *Paradiceros mukirii* distributed across present-day Kenya and Morocco and the subsequent emergence of *Diceros*, known from deposits in Africa and Europe, approximately 10 MYA (Prothero 1993; Cumming *et al.* 1990). The white rhinoceros lineage is considered to be more recent with the earliest fossil records of *Ceratotherium simum* dating to the late Miocene (about 7 MYA) from deposits found only in Africa (Prothero 1993).

The two recognised white rhinoceros subspecies exhibit discontinuous distribution ranges (Figure 1.2.); the Southern white rhinoceros (SWR; *Ceratotherium simum simum*) occurs in the Savannah grasslands of southern Africa and the Northern white rhinoceros (NWR; *Ceratotherium simum*

cottoni) occurs north of the tropical forests of central Africa. The fossil record and cave paintings suggest that white rhinoceroses may have occupied a more continuous range across the African continent and it was climatic and habitat changes during the Plio-Pleistocene glacial cycles that separated the Northern and Southern lineages (Emslie & Brooks 1999). The status of the two white rhinoceros subspecies was designated based on morphological differences, with the Northern type having slightly longer legs, shorter body and less concave skull (Skinner & Chimimba 2005). An initial study on mtDNA restriction enzyme site patterns suggested that the two subspecies are genetically distinct, with 4% sequence divergence indicating that the two lineages diverged approximately 2 MYA (George *et al.* 1983). A more recent study of serum proteins and mtDNA restriction fragment length polymorphism suggested that the sequence divergence is closer to 1.4% (George *et al.* 1993), greatly reducing the estimated time since the lineages diverged to approximately 700 000 years.

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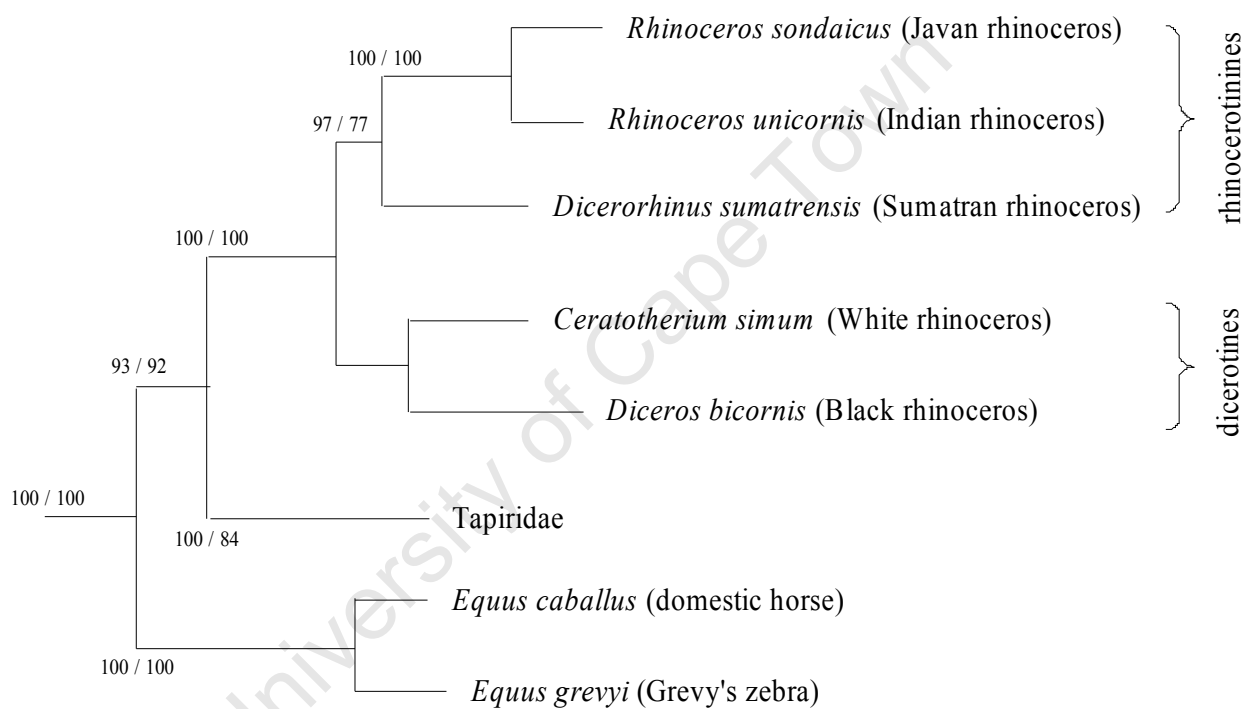


Figure 1.1.

Maximum-likelihood tree utilising a combined data set of 12S rRNA and *cytochrome b* mtDNA gene sequences (adapted from Tougaard *et al.* 2001). The numbers at the nodes indicate percentage support in maximum-likelihood and parsimony analyses, respectively.

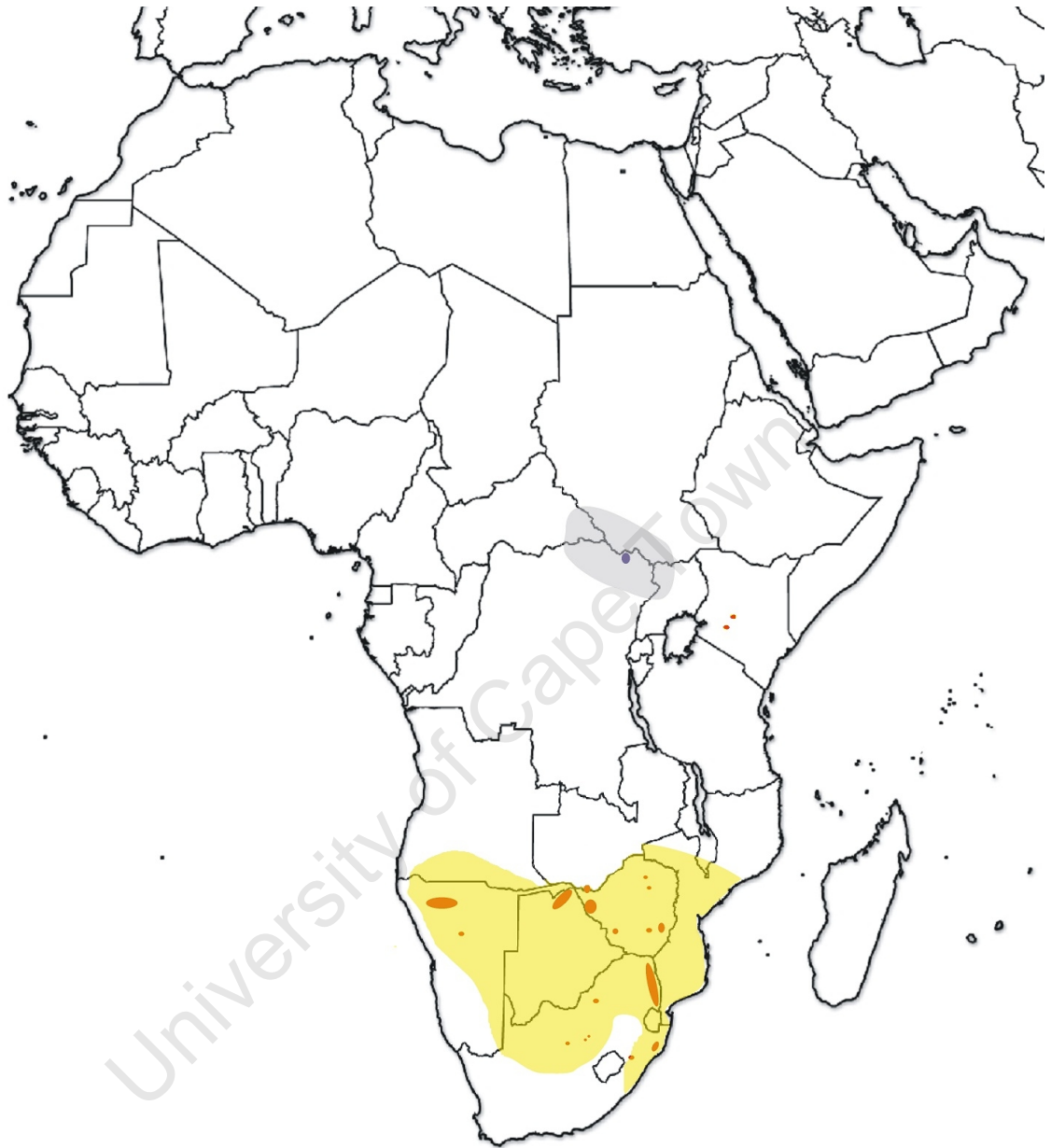


Figure 1.2.

A map of Africa indicating both the inferred historic and extant distribution ranges of Southern (*Ceratotherium simum simum*) and Northern (*Ceratotherium simum cottoni*) white rhinoceroses (adapted from International Rhino Foundation, accessed 14th July 2007 [<http://www.rhinos-irf.org/whiterhinomap/>]).

The Southern White Rhinoceros, *Ceratotherium simum simum* (Burchell, 1817)

William John Burchell first described the “white” or “square-lipped” rhinoceros after discovering it in 1812 near Chué Springs, approximately 50 km north of present-day Kuruman in the Northern Cape, South Africa (Rookmaaker 2000). The word “rhinoceros” is derived from the Greek “*rhis*” and “*keras*”, meaning “nose” and “horn”; thus the rhinoceros species were named for this characteristic feature (Bannister & Skinner 1985). White rhinoceroses are slate grey in colour and the name is purported to originate from the Afrikaans word “weit”, meaning “wide”, in reference to the species’ broad, squared-off muzzle for grazing, but the term was misinterpreted as “white” by English-speaking settlers during the 19th century (Skinner & Chimimba 2005). Another explanation may be that the name originated in reference to the darker-coloured black rhinoceros or as a derivation of an indigenous African name (Rookmaaker 2003).

The white rhinoceros is the second largest land mammal in Africa, with adult males having shoulder heights of about 1.8 m and weighing between 2 000 kg and 2 400 kg and adult females about 30% smaller than males (Skinner & Chimimba 2005; Owen-Smith 1988). Body length and chest girth of males increase with age until an asymptote is reached at about 14 and 20 years of age, respectively, and territorial males have significantly larger neck and chest girths than subordinate males (Rachlow *et al.* 1998). The paired horns grow continuously and consist of compacted keratin filaments with no bony core; the anterior horn is usually longer with regular wear keeping the average length at about 60 cm and the posterior horn at about 24 cm (Skinner & Chimimba 2005). The record length of white rhinoceros horns is 158.8 cm and 133.4 cm, respectively, for the front and back horns (Player 1972).

Unlike other extant rhinoceros species, white rhinoceroses exhibit a degree of sociality in the permanent associations formed between sub-adults, between sub-adults and adult females as well as between adult females and their calves; these associations can persist for only a few days or for several years (Owen-Smith 1988; Owen-Smith 1975). Sub-adults appear to preferentially associate with adult females and seem to benefit from the more extensive experience of the older individual, however, adult females accompanied by young calves do not tolerate the company of others (Shrader & Owen-Smith 2002). Social groups can vary in size, usually consisting of two to three individuals although larger groups of up to 15 have been observed (Skinner & Chimimba 2005; Shrader & Owen Smith 2002).

An ecological study by Owen-Smith (1971) in the Hluhluwe Imfolozi (formerly Umfolozi) Game Reserve in South Africa found that the social system of white rhinoceroses is based on a clearly delineated mosaic of adult male territories. White rhinoceroses maintain a territorial system that is based on a space-correlated dominance relationship among breeding males, where territories are defended against other dominant males but may be shared with subordinate males. This study revealed that about two-thirds of the adult males in the park occupied clearly defined territories that varied in size and were typically about 2 km² in area. An independent study in the Matobo National Park by Rachlow *et al.* (1999) showed that only about one-third of the adult males were territorial and that the territory sizes were much larger than those previously reported for territorial males. Rachlow *et al.* (1999) suggested that this may be the result of only 50% of the total area of the reserve being available as suitable habitat, and/or the fenced boundary that prevented the dispersal of young adult males to lower density areas on the periphery where they could have established territories of their own. Owen-Smith (1971) reported that territorial males usually only leave their territories if there is no access to water, taking a direct route along the same pathways every three to four days to reach water. Intrusion into other territories was tolerated provided that subordinate behaviour was displayed and thus territorial conflict was infrequent. Adult females nearing oestrus are kept within the confines of a specific territory and prevented from leaving, in this way courtship and mating could occur without interference from neighbouring territorial males.

All available habitat in Hluhluwe Imfolozi was occupied by territorial males, therefore subsidiary males in the population are forced to co-exist with a dominant male within its territory, possibly due to artificially high population densities (about five rhinoceroses/km²). Subsidiary males did not get the opportunity to reproduce and therefore the dominance relationship appeared to regulate reproductive competition between males and lessen the chance of injury arising from fights. Rachlow *et al.* (1999), however, showed that while there was extensive overlap between territorial and subsidiary males, there were no dominant “alpha” and subordinate “beta” males occupying the same territory in Matobo National Park. This study also revealed that the size of the home ranges occupied by adult females varied according to population density, with the largest home ranges found in the areas with the lowest density. These home ranges overlapped those of other adult females as well as the territories of a number of territorial males, indicating that either social factors or shared resources might affect the spatial distribution of adult females (Rachlow *et al.* 1999; Owen-Smith 1971).

White rhinoceros females reach sexual maturity at four to five years (Bannister & Skinner 1985). The youngest females observed to be in oestrus in the Hluhluwe Imfolozi population were 3.8, 4, and 4.5 years old, respectively, and 6.5 ± 0.5 years was the youngest age at first parturition (Owen-Smith 1988). In Matobo National Park, the age at first parturition varied between 6.5 and 11.5 years with the age increasing significantly at higher population densities (Rachlow & Berger 1998). Young females stay with their companions through their first oestrus and generally only leave these groups at first parturition (Owen-Smith 1988). The gestation period is about 480 days and results in the birth of a single calf weighing approximately 40 kg (Skinner & Meakin 1988). Birthing can occur at any time of the year, but in Hluhluwe Imfolozi there is a peak period between March and July (Stuart & Stuart 2000). Calves are produced every three to four years (Stuart & Stuart 2000), with the reported birth intervals in the Hluhluwe Imfolozi population varying between 22 months and about 3.5 years (Owen-Smith 1988). Adult females continue producing calves throughout their lifespan although birth intervals and infant mortality increases with age (Owen-Smith 1988). Calves leave their mothers at about 2 to 3.5 years of age and thereafter may form associations with other adult females or sub-adults and some pairs of adolescents stay together for several years (Owen-Smith 1988).

Sub-adult white rhinoceros males usually remain in groups until approximately eight years old when solitary tendencies start to develop and they begin to establish territories as either a dominant or subordinate male between 10 and 12 years, although some males stay with their sub-adult companions until as old as 11 or 12 years of age (Owen-Smith 1988). It is advantageous for male sub-adults to form cohesive pairs or even larger groups to diminish or avert aggressive challenges from territorial males, because solitary male sub-adults are more likely to be attacked or killed in these skirmishes (Shrader & Owen-Smith 2002). Young adult males are physically capable of breeding at seven to eight years of age, although this is usually delayed by social factors because dominant males tend to breed most of the available females in the population (Bannister & Skinner 1985). Sub-adult males do not normally display sexual interest in females, although exceptions have been observed in populations without other adult males (Owen-Smith 1971). The youngest territorial male observed in Hluhluwe Imfolozi was about 12 years of age (Owen-Smith 1988).

White rhinoceros longevity is estimated to be about 45 years (Owen-Smith 1988). Annual mortality rates of adult males, adult females, and calves were calculated to be 3.6%, 1% and 3.5%, respectively, in the Hluhluwe Imfolozi population (Owen-Smith 1988). Mortality rates of adult males, sub-adult males, adult females and sub-adult females, after being re-introduced to Moremi

Game Reserve in Botswana, were much higher at 33%, 25%, 10% and 9%, respectively (Pitlagano 2007). However, no adult mortalities were reported after re-introduction to Ongava Game Reserve in Namibia with a 8.6% mortality rate for calves born on the reserve since founding (*pers. comm.* A. Guerier). The density of established populations can increase exponentially as a result of high fecundity and low mortality rates (Kretzschmar 2001; Emslie & Brooks 1999; Rachlow & Berger 1998). The population density is regulated by the natural dispersal of male and female sub-adults and some adult males from high density to lower density areas, usually moving from the centre towards the periphery of the population (Skinner & Chimimba 2005; Owen-Smith 1988). This rate of dispersal was estimated at 7 to 10% per sub-adult per year for a population at high density and experiencing below average rainfall (Owen-Smith 1988; 1983; 1981). It was suggested that this dispersal is the result of limited resources rather than social pressures as both male and female sub-adults are affected. Shrader & Owen-Smith (2002) proposed that white rhinoceroses employed a “buddy system” to ameliorate the potential risks of dispersal. It was shown that sub-adults, when venturing out of known areas into unfamiliar habitat, were almost always accompanied by another individual and thereby decreasing the possibility of aggression from territorial males and increasing the likelihood of becoming acquainted with a new environment.

Population Bottlenecks and Demographic Recovery

Throughout recent history, all five extant rhinoceros species have been heavily persecuted for a number of reasons; these include the clearance of grasslands for agriculture, their demand as hunting trophies and for their meat, skin and horns (Leader-Williams 2003; Emslie & Brooks 1999). It has mostly been the huge demand for rhinoceros horn, used for handles of ceremonial daggers and in traditional Chinese medicines, that has resulted in historically widespread distributions being reduced to small fragmented populations (Emslie & Brooks 1999). Southern white rhinoceroses (SWR) were once continuously distributed north of the Orange River to the Zambezi River and as far south-east as Zululand in South Africa (Owen-Smith 1988), extending across present-day south-eastern Angola, south-western Zambia, Mozambique, Zimbabwe, Botswana, eastern Namibia, and South Africa. When SWR were first described by Burchell in 1817, individuals were reportedly found in great abundance (Lang 1924), however, this was one of the most severely affected species, with reports of 90 individuals killed in a single hunting trip and 60 being killed by a hunter in one season (Lang 1924). The SWR was generally considered to be on the verge of extinction by the end of the 19th century (Sclater 1890). In the words of Selous (1893), “some few white rhinoceroses no doubt still survive, but it is not too much to say that long before the close of the century the white rhinoceros will have vanished from the face of the earth”.

A single remnant population of between 20 and 100 individuals persisted in the region that forms present-day Hluhluwe Imfolozi Game Reserve in KwaZulu-Natal, South Africa (Skinner & Chimimba 2005; Emslie & Brooks 1999; Bannister & Skinner 1985). There is, however, little consensus in the literature regarding the actual number of surviving SWR, with various references stipulating either just a few, 20 or 50 individuals, or even as many as a hundred, and the years 1895, 1900, 1910 and 1920 have all been proposed as the date when the population reached its lowest numbers (Rookmaaker 2000). Rookmaaker (2000) suggested that an exaggeratedly low population size estimate was publicised to motivate public support for the conservation effort and that there were probably at least 200 individuals at that time. Regardless of the year or actual number, a single remnant population was granted protection with the proclamation of the Umfolozi Junction Reserve in 1895 (Rookmaaker 2000). High fecundity and negligible mortality rates facilitated rapid population growth rates allowing the population size to increase to over 400 by the early 1950s (Player 1972). In 1961, the successful development of translocation techniques during the world famous “Operation Rhino” made it possible to begin relocating surplus individuals to form seeded populations in other protected areas (Emslie & Brooks 1999; Player 1972).

By 2007, there were an estimated ~17 500 SWR surviving in the wild worldwide (Table 1.3. and Figure 1.4.; The International Rhino Foundation, accessed 9th May 2008 [<http://www.rhinos-irf.org/white/>]). Of these, more than 90% were located in South Africa, with small re-introduced populations in Botswana, Mozambique, Namibia, Swaziland and Zimbabwe, and populations founded outside the historical range in Kenya, Zambia and, very recently, Uganda (Table 1.3.; Amin *et al.* 2006; Emslie & Brooks 1999). Many seeded populations have, however, been extensively poached and are again under severe pressure. In South Africa, more than 20 SWR have been killed by poachers in the Kruger National Park between 2007 and 2008 and a further 12 SWR have been killed in KwaZulu-Natal during 2008 with four of these in the Hluhluwe Imfolozi Game Reserve (Gosling 2008). Poaching has increased alarmingly in Zimbabwe with about 70 rhinoceroses having been killed since the beginning of 2000, in contrast with the absence of poaching in that country for the seven years prior to 2000 (Lekotjolo 2008).

The rapid demographic recovery of SWR has been facilitated by a combination of high fecundity and low mortality rates; annual net population growth rates have been estimated at 6.5 to 15% in protected free-ranging populations (Kretzschmar 2001; Emslie & Brooks 1999; Rachlow & Berger 1998) and a current rate of about 7.5% reported in the Hluhluwe Imfolozi Game Reserve (*pers. comm.* S. Janse van Rensburg). It has been suggested that rapid growth rates are a function of initial low population densities in newly founded populations, which together with skewed sex ratios result in disproportionately large numbers of females successfully producing offspring (Kretzschmar 2001). Few, if any, SWR persist outside protected sanctuaries and game reserves and successful anti-poaching programs together with essentially non-existent dispersal potential, results in the rapid increase of population densities. Rachlow & Berger (1998) compared populations of high and low density in Matobo National Park and suggested that population density and population growth were inversely proportional. Female age at first parturition and calving intervals both increased significantly in the higher density population. It is therefore probable that annual growth rates in established populations stabilise once resource availability becomes limited and sex ratios approach parity; under this scenario proportionally fewer reproductively active females are likely to contribute to the population growth rate (Kretzschmar 2001).

SWR are more numerous than all the other extant rhinoceros taxa combined (Amin *et al.* 2006) and the species; recovery has been internationally recognised as “*one of the world’s greatest conservation successes*” (Emslie & Brooks 1999).

Southern White Rhinoceros (<i>Ceratotherium simum simum</i>) Total Population Size Estimates												
Country	1895	1929	1948	1968	1984	1987	1991	1992	1993/4	1995	1997	2003
Botswana	0	0	0	0	190	125	56	27	18	20	23	67
Ivory Coast	0	0	0	0	0	0	5	5	5	4	4	-
Kenya	0	0	0	0	33	47	57	74	87	122	137	218
Mozambique	0	0	0	0	1	-	-	-	-	-	-	2
Namibia	0	0	0	0	70	63	80	91	98	107	141	186
South Africa	20	150	550	1 800	3 234	4 137	5 057	5 297	6 376	7 095	7 913	10 536
Swaziland	0	0	0	0	60	80	60	46	33	41	50	61
Zambia	0	0	0	0	10	6	0	-	6	5	6	3
Zimbabwe	-	-	0	30	200	208	250	249	134	138	167	250
Total	20	150	550	1 800	3 798	4 666	5 565	5 789	6 757	7 532	8 441	11 350

Table 1.3.

Population size census counts for SWR in each range state from 1895 to 2003 (compiled by IUCN SSC African Rhino Specialist Group; Emslie 2004; Emslie & Brooks 1999; Roth 1967). A dash (-) indicates that the relevant data are unknown.

Southern white rhino numbers (1895 to present)

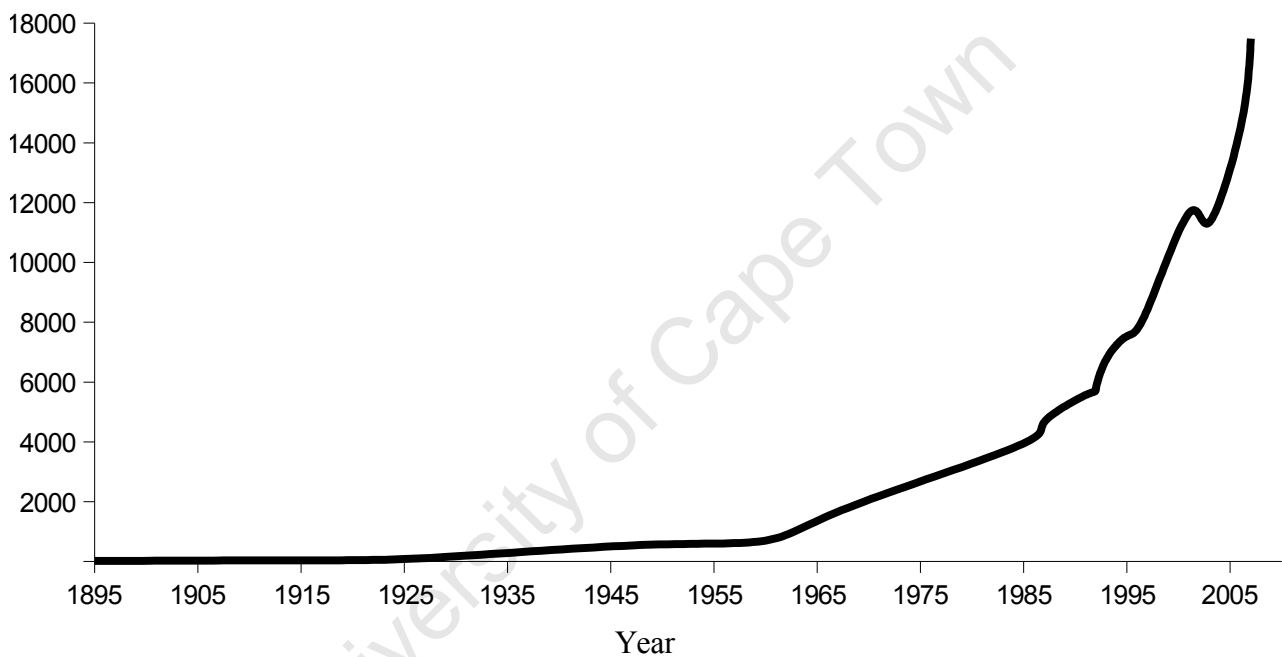


Figure 1.4.

The increase in total numbers in free-ranging populations of SWR from 1895 to present (adapted from IUCN 2008; Emslie 2004; Emslie & Brooks 1999; Bannister & Skinner 1985). The decrease in 2003 is due to sampling variance in the method used to estimate population size in the Kruger National Park, South Africa, and does not reflect an actual decrease in numbers (Emslie 2004).

Conservation Status

In 1975, the three Asian rhinoceros species and NWR were among the first species listed on the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I, prohibiting international trade in rhinoceroses and rhinoceros products (CITES Secretariat, accessed 12th May 2008 [<http://www.cites.org>]). The black rhinoceros and SWR were also included on CITES Appendix I in 1977, however after two decades of continued increase in numbers, the South African SWR populations were downlisted to Appendix II in 1995 (CITES Secretariat, accessed 12th May 2008 [<http://www.cites.org>]). This allowed for the trade in live animals to “approved and acceptable destinations” and for the limited export of hunting trophies (Emslie & Brooks 1999). In addition, by 1996 the International Union for Conservation of Nature (IUCN) had reclassified SWR from Vulnerable to Lower Risk/Conservation Dependent (IUCN 2008). Since 2002, SWR have been classified as Near Threatened (v3.1) in response to poaching in several range states and the continued high demand for rhinoceros horn (IUCN 2008).

For the past few decades, the rapid decline in rhinoceros populations has primarily been the result of poaching to meet the demand for illegal rhinoceros horn trade, but this decimation has been exacerbated by the loss or degradation of much of the species' natural habitat (Amin *et al.* 2006). According to Emslie & Brooks (1999), “the international community should monitor the sentences handed down in range states, rather than focus on legislative penalties”. The effectiveness of national legislation is entirely dependent on the manner in which it is enforced, and corruption in range states often allows poachers and dealers to evade punitive action (Emslie & Brooks 1999). For example, four poachers, after admitting to killing 18 rhinoceroses in different parts of central Zimbabwe, were granted bail and immediately absconded (Janicke 2008). Protection from poaching has been crucial and most surviving populations are located in fenced sanctuaries and game reserves where it is possible to form protection zones with effective law enforcement (Emslie & Brooks 1999). However, military-style anti-poaching measures are prohibitively expensive and adequate resources and personnel are seldom available in African countries, thus funds provided by donor agencies are vital to ensure continued effective protection and management of existing populations (Amin *et al.* 2006). Political instability and civil unrest in many African nations and the availability of weapons, together with increasing poverty, have resulted in the decimation of many rhinoceros populations by poachers (Amin *et al.* 2006). The untransformed land available for wildlife conservation in Africa is under huge demand to be converted to subsistence farming, grazing for livestock or for a number of other commercial uses (Amin *et al.* 2006).

Despite international legislation and domestic bans on commercial trade in rhinoceros products, rhinoceros horn is still used in traditional Chinese medicine (TCM) and for elaborately carved ceremonial daggers (jambiyas) much prized in Middle Eastern countries and all rhinoceros species are under constant threat (Emslie & Brooks 1999). To such an extent that even museum displays have been targeted with rhinoceros horns being stolen from century-old exhibits to supply the demand for the black market trade (Reuters 2008).

The international media has perpetuated the mistaken notion that rhinoceros horn is valued in Asian countries as an aphrodisiac (Martin & Martin 1982). It has actually been used for this purpose in only a few locations in India and, with domestic bans in place and soaring prices, this practise has virtually come to an end (Leader-Williams 2003). The primary use of rhinoceros horn is medicinal and has been used as an ingredient in TCM for centuries (Nowell *et al.* 1992). It has been difficult to reduce this demand as interference is often viewed as contempt for Eastern culture and, as many practitioners do not believe that effective substitutes for rhinoceros horn exist, it is considered to be a life-saving substance and not a luxury item (Emslie & Brooks 1999). Clinical trials, however, attempting to prove the efficacy of the pharmacological uses of rhinoceros horn have been inconclusive (Still 2003; Emslie & Brooks 1999). The emergence of many oil-based economies in the Middle East in the 1970s resulted in an increase in the demand for rhinoceros horn jambiyas, particularly in North Yemen (Emslie & Brooks 1999). Fortunately, a ministerial decree banning the importation of rhinoceros horn and an Islamic edict condemning the killing of rhinoceroses were passed and this legislation, together with an economic decline, resulted in a significant decrease in the demand for rhinoceros horn in the mid-1980s and 1990s (Martin *et al.* 1997).

By supplying individuals for re-introduction into the wild, captive breeding populations of endangered species should potentially serve as safety nets and/or genetic reservoirs in the event of the collapse of free-ranging populations. Growth rates and recruitment in captive populations of white rhinoceroses have, however, been exceptionally poor (Swaisgood *et al.* 2006; Emslie & Brooks 1999). Negative population growth rates of -3.5% have been reported in captivity; 658 SWR were translocated to sanctuaries outside of Africa between 1962 and 1994, but there were only 646 in captivity by 1997 (Emslie & Brooks 1999). Furthermore, it is more costly to conserve rhinoceroses in zoos than in the wild (Leader-Williams 1993). The failure of captive breeding programmes is particularly dire when compared to the successful demographic recovery of free-ranging populations. Extensive scientific research of both captive and free-ranging populations have attempted to improve husbandry and understanding of social and reproductive behaviour such as

mate choice, male-male competition and female cycle length and pregnancy in a concerted effort to increase productivity of white rhinoceroses in captivity (Hildebrandt *et al.* 2007; Swaisgood *et al.* 2006; Hermes *et al.* 2004; Kretzschmar *et al.* 2004; Seror *et al.* 2002; Kretzschmar *et al.* 2001; Patton *et al.* 1999; Rachlow *et al.* 1998).

As a result of the insufficient protection afforded to SWR in many range states, a suggested alternative anti-poaching measure for free-ranging populations has been the removal of rhinoceros horns. Namibia, Zimbabwe and Swaziland have started such programmes in an attempt to provide greater protection for their remaining rhinoceros populations (Berger & Cunningham 1994a). Horns regenerate at a rate of about 8.7 cm per rhinoceros per year, such that one year of regrowth equates to more than 0.5 kg, and poachers do not discriminate according to horn size and the costly process of dehorning would have to occur frequently for this type of management strategy to be effective (Berger & Cunningham 1994b). A population of 100 white rhino, with about 80 dehorned individuals, was decimated by poachers until just five rhinoceros were still surviving after 18 months (Rachlow 1993). The evolutionary functions of rhinoceros horns are poorly understood. The main hypotheses for these characteristic horned structures are that they play a role in (1) intrasexual aggression and territory defence, (2) mate choice or (3) protection against predators (Berger 1994). A study by Berger & Cunningham (1996; 1994a; 1994b) suggested that while dehorning black rhinoceros in Namibia might alleviate pressure from poaching, it resulted in a significant increase in calf mortality in populations sympatric with spotted hyaena (*Crocutta crocutta*). These findings, however, remain controversial due to the small sample sizes and criticisms of the statistical analyses utilised (Lindeque & Erb 1995; Loutit & Montgomery 1994a; 1994b).

It has also been a strategic manoeuvre to enhance the long-term security of SWR by including the private sector in the management of populations in several countries, as private ownership allows landowners to recover their costs via tourism, live sales and limited trophy hunting of surplus males (Emslie & Brooks 1999). Most of the surviving black and white rhinoceroses in Africa (70%) are conserved in State protected reserves while the remainder are either privately owned or managed on a custodianship basis for the State (Emslie & Brooks 1999). Approximately 20% of the white rhinoceroses in South Africa are owned by the private sector, and in Botswana, Kenya, Namibia and Swaziland there are more white rhinoceroses on privately owned land than in State reserves (Emslie & Brooks 1999). The translocation of white rhinoceroses to many private game reserves has greatly increased the amount of land available for conservation purposes (Amin *et al.* 2006). These small and isolated populations, however, need to be adequately managed and conserved, and their genetic

status and overall fitness is uncertain. Many extant “populations” are too small to be considered biologically or evolutionarily viable. By 1997, 74% of discrete free-ranging populations in Africa were owned by the private sector with an average “population” size of ~11 individuals as compared with ~123 individuals in State owned parks (Emslie & Brooks 1999).

While conservation efforts have increased the number of populations and available range area, these populations remain isolated as dispersal is restricted by fenced boundaries and/or human settlements. This is in contrast with prehistoric times when rhinoceroses were able to disperse freely and thereby facilitate gene flow (Owen-Smith 1988). For large mammals that range across vast areas at low densities in unrestricted conditions, game reserves simulate island ecosystems and some of these species would either reach unnaturally high densities or become susceptible to extinction (Owen-Smith 1988). Management strategies should be implemented to alleviate the “island effect” by creating “dispersal opportunities” (Owen-Smith 1988). One such method is the creation of dispersal sinks where specific areas are designated as vacuum zones and periodic culling or capture-relocation events remove all individuals and allow for the natural dispersal of other individuals from the remaining areas into the sink area (Owen-Smith 1988). The following advantages are then promoted: (1) individuals removed from the population are determined by natural selection, (2) population densities can adapt naturally to changes in the available resources, and (3) artificial population structure in the main population is averted (Owen-Smith 1988).

To successfully protect rhino populations from the threat of poachers, populations need to be kept within relatively small areas because guarding large and remote tracts of land is prohibitively expensive. It has been suggested that population sizes in games reserves should be kept below the ecological carrying capacity of the environment to prevent density-dependent factors affecting population growth rates (Rachlow & Berger 1998; Owen-Smith 1983). Megaherbivores, such as white rhinoceroses, require large areas and as a result most populations remain relatively small, less than that recommended for long-term population viability (Foose 1987). Another conservation strategy is that of a metapopulation management approach, where the periodic exchange of individuals between reserves helps to alleviate adverse genetic and demographic effects inherent in small and isolated populations by simulating dispersal and gene flow (Emslie 1994; Foose 1987; for examples see Madsen *et al.* 1999; Westemeier *et al.* 1998). However, the translocation of rhinoceroses is both financially costly and logistically difficult and the exchange of only successful breeding males would maximise the beneficial effects thereof whilst minimising the number of individuals needing to be translocated.

Conservation Genetics: Small Population Size and the Threat of Extinction

Darwin (1868) first contemplated the role of genetics in the conservation of natural populations when he suggested that fallow deer (*Cervus dama*) in British parks may lose vigour as a result of their isolation and small population sizes. It was not until the early 1980's, however, that the field of conservation genetics emerged in response to three publications that proposed the application of genetic principles to the conservation of biodiversity in natural populations (Schonewald-Cox *et al.* 1983; Frankel & Soulé 1981; Soulé & Wilcox 1980). The IUCN now recognises genetic diversity, together with species and ecosystem diversity, as crucial to the conservation of biodiversity.

Translocations for the Conservation of Endangered Species

Anthropogenic activities have severely affected many ecosystems; the over-exploitation of species and/or the destruction, modification or fragmentation of their habitat has resulted in precipitous declines in many populations and greatly increased the rate of local extinctions. Current extinction rates are estimated at 50 to 500 times greater than background rates and 3 000 to 30 000 species are expected to go extinct every year (Woodruff 2001). The primary function of wildlife conservation programmes is to conserve and sustain biodiversity at the ecosystem level, and genetic diversity at the species level (Frankham 1995a). For this reason, an important aspect of many conservation strategies is the translocation of endangered species to either augment existing populations that have been drastically reduced in numbers or to re-introduce individuals to former range areas in order to re-establish populations and prevent local extinctions (Griffith *et al.* 1989). A translocation is considered successful if it results in a new self-sustaining population, however small numbers of founder individuals is commonplace, and may result in reduced genetic diversity, i.e. a population bottleneck (Frankham 1996; Nei *et al.* 1975). The continued persistence of seeded populations is more likely when founded with a large number of genetically diverse individuals, there is a rapid rate of population increase, there are no potential sympatric competitors and the new environment is a suitable, high quality habitat that is well protected with minimal environmental fluctuations (Griffith *et al.* 1989). Furthermore, herbivorous species have a greater likelihood of being successfully translocated than carnivores or omnivores (Griffith *et al.* 1989). Seeded populations founded with exclusively wild-caught individuals have been found to be more successful ($P \leq 0.100$) than those founded with captive-reared individuals and the success of the former depended on the density of the source population, with 77% success rate from high density populations ($n = 109$), 36% from stable populations ($n = 49$) and 44% from declining populations ($n = 9$; Griffith *et al.* 1989).

Translocations and Founder Events Potentially Reduce Genetic Diversity

The conservation of genetic diversity is considered vital for two reasons, (1) to enable populations and species to evolve and adapt to changes in their environment and (2) because of the relationship between reduced heterozygosity and decreased population fitness that results via inbreeding in small populations (Reed & Frankham 2002). Frankel & Soulé (1981) proposed that as little as 10% decrease in genetic diversity from inbreeding in small populations could result in 10 to 25% loss of reproductive output in the population. This directly impacts the population size and reduces the likelihood of the continued persistence of that population. Seeded populations usually have reduced genetic diversity, predominantly due to the loss of rare alleles present in the source population but not sampled in the founder individuals or that were lost within a few generations due to genetic drift (Allendorf 1986). Fuerst & Maruyama (1986) advocate the preservation of allelic diversity, as opposed to heterozygosity, in founded populations but this would generally require greater numbers of founders and sufficient individuals of endangered species are seldom available. In reality, populations are usually founded with few individuals and population recovery can be very gradual.

Studies have reported that seeded populations generally exhibit lower levels of genetic diversity relative to the source population and this could potentially affect both the short- and long-term viability of the newly founded populations. Stockwell *et al.* (1996) reviewed 19 studies that examined the effects of translocations, and specifically founder events, on genetic diversity and reported a decrease in heterozygosity in 50% of the studies and a decrease in allelic diversity in 75% of the studies. Invasive species are expected to experience founder events and Dlugosch & Parker (2008) reviewed quantitative studies that compared levels of allelic richness and heterozygosity in 80 animal, plant and fungus species to show that populations of invaders have significantly reduced genetic diversity compared to conspecifics in native range areas and related non-invasive species. Numerous empirical studies have showed the loss of diversity in founded populations relative to their source population/s. For example, in a study of seeded populations of western mosquitofish (*Gambusia affinis*), there was significantly less diversity at 16 allozyme loci in comparison to the source populations (Stockwell *et al.* 1996). The re-introduction of moose (*Alces alces*) to Newfoundland and Cape Breton in Canada during the late 1800s and early 1900s resulted in 14 to 23% loss of heterozygosity after each single founder event and a 46% loss of heterozygosity following two successive founder events during the natural re-colonisation of the Avalon Peninsula, as compared with the source populations (Broders *et al.* 1999). Twenty desert bighorn sheep (*Ovis canadensis mexicana*) were introduced to Tiburon Island in the Sea of Cortez in 1975, this population has been used to source nine other populations since 1995, and has itself

lost 26% of the heterozygosity compared to the source populations in Arizona, U.S.A (Hedrick *et al.* 2001). Two populations of dice snakes (*Natrix tessellata*), illegally introduced to lakes in Switzerland, had significantly decreased allelic diversity and heterozygosity compared with two natural populations, and a greater reduction in genetic diversity in serially bottlenecked population than the population that had experienced a single bottleneck (Gautschi *et al.* 2002). In addition, this study revealed a greater occurrence of scale anomalies in the seeded populations than in natural populations and a significant association between scale anomalies and the loss of genetic diversity in the introduced populations (Gautschi *et al.* 2002). Herds of elk (*Cervus elaphus*) have been restored to many parts of the historical range in North America and a re-introduced population in Pennsylvania, founded with 34 individuals between 1915 and 1926, had few rare alleles and no unique alleles and had lost 61.5% of its heterozygosity compared to the two source populations (Williams *et al.* 2002). The genetic assessment of a small re-introduced population of the Mauritius Kestrel (*Falco punctatus*) revealed that levels of inbreeding increased by 2.6% and genetic diversity decreased by 1.6% every generation (Ewing *et al.* 2008). This study suggested that variation in re-introduced populations of endangered species should be monitored during recovery programmes.

In contrast to these studies, no further loss of diversity was observed in a genetically depauperate New Zealand passerine, the threatened South Island saddleback (*Philesturnus carunculatus carunculatus*), despite having experienced several sequential translocation events (Taylor & Jamieson 2008). The introduction of mammalian predators to the islands in the 1800s resulted in the extirpation of the species from all but one island. This remnant population was again under threat as a result of the invasion of rats in 1962 and 36 individuals were relocated to two other islands in 1964. This study proposed that while there may have been a reduction in allele numbers and/or heterozygosity during the first-order founding event, subsequent translocations have had no further deleterious effect on genetic diversity. The seven extant island populations differed only in allele frequencies with minimal loss of rare alleles among the populations. It is possible that genetically depauperate populations are less sensitive to founder effects as the few remaining alleles are well represented, i.e. at high frequency in the remaining individuals across the population, and no rare alleles are available to be lost during translocations (Taylor & Jamieson 2008). Historic population bottlenecks can, therefore, have a long-term effect impact on genetic diversity such that evidence of contemporary bottlenecks is not detectable. Taylor & Jamieson (2008) suggested that it should be possible to re-establish populations of endangered species that have had historically low levels of genetic diversity by sequential translocations, without further loss of alleles or heterozygosity.

Population Bottlenecks and the Associated Loss of Fitness

Populations that have experienced substantial reductions in effective population size are predicted to lose genetic diversity and the associated decrease in adaptive potential renders the population vulnerable to extinction (Nei *et al.* 1975; Maruyama & Fuerst 1985; Lacy 1997; Frankham *et al.* 2002; for a review see Hedrick & Kalinowski 2000). Many empirical examples have been reported in the literature. For example, Saccheri *et al.* (1998) showed that the risk of extinction in a large metapopulation of Glanville fritillary butterflies (*Melitaea cinxia*) in Finland increased significantly with a reduction in heterozygosity and inbreeding negatively impacted the rate of egg-hatching, larval survival and adult longevity in the many small, relatively isolated populations. The Greater Prairie Chicken (*Tympanuchus cupido pinnatus*) in Illinois, U.S.A., experienced a severe population bottleneck and the loss of genetic diversity was accompanied by a reduction in overall population fitness, as measured by fertility and egg-hatching rates, and the translocation of birds from a large genetically diverse population resulted in improved egg viability (Westemeier *et al.* 1998). A highly inbred and isolated population of adders (*Vipera berus*) in Sweden was on the verge of extinction due to inbreeding depression, manifested as deformed or stillborn offspring, when a number of adult males from large and genetically diverse populations were released for a period of three years, the increased levels of genetic diversity resulted in a dramatic increase in offspring viability as well as the resultant adult recruitment (Madsen *et al.* 1999).

The detrimental consequences of bottleneck events, although variable between different populations and species, are particularly significant for populations with few founder individuals and/or remain small for several successive generations (Maruyama & Fuerst 1985). These effects are surprisingly minimal if the population experiences a bottleneck of short duration, e.g. Indian rhino (*Rhinoceros unicornis*; Dinerstein & McCracken 1990) and African buffalo (*Syncerus caffer*; Wenink *et al.* 1998). The amplified effects of genetic drift is of particular concern in bottlenecked populations and results in the random loss of diversity in the population and, with inbreeding eventually becoming unavoidable, the further loss of diversity in individuals (Lacy 1997). The risk of extinction can also be greatly increased by genetic drift acting in small populations, were it can supersede natural selection as the prevailing force shaping evolutionary adaptation (Lacy 1997). “Mutational meltdown” is another consequence of genetic drift in small populations where mildly deleterious mutations become fixed by chance in the population and exacerbate the rapid loss of fitness and risk of extinction (Lynch & Gabriel 1990). A greater understanding of population bottlenecks, and the consequences thereof, has become increasingly essential as countless species continue to experience increased habitat fragmentation and population decline. Reed & Frankham (2002) reviewed 34 data

sets, each for three or more populations, that analysed fitness or a component of fitness relative to levels of heterozygosity, population size and/or heritability. They concluded that these surrogate fitness measurements were positively and significantly correlated with actual population fitness and explained 15 to 20% of the variation in fitness, and that fitness and potential for adaptation are reduced in small populations (Reed & Frankham 2002).

Loss of Fitness as a Result of Inbreeding Depression

Inbreeding depression is one of the most detrimental consequences of the loss of genetic variation arising from demographic bottlenecks. In the resultant small and isolated population, inbreeding becomes inevitable as individuals eventually become more closely related by descent, this can lead to increased levels of homozygosity and the expression of recessive deleterious alleles or the loss of heterozygote advantage (Charlesworth & Charlesworth 1987). Reduced viability and fecundity in a population or species ultimately increases the risk of extinction (Brook *et al.* 2002; Frankham *et al.* 2002; Frankham *et al.* 1999; Frankham 1995b). Inbreeding depression is considered a critical threat to the future of many endangered species, particularly in populations bred in captivity where an increased incidence of juvenile mortality and decreased adult fecundity has been observed (Hedrick & Kalinowski 2000; Frankham 1995a). There is extensive evidence of inbreeding depression in captive populations (Frankham *et al.* 2002; Charlesworth & Charlesworth 1987), with Ralls *et al.* (1979) reporting significantly increased juvenile mortality in inbred individuals compared with non-inbred individuals in 16 species of captive ungulates and Ralls *et al.* (1988) reporting that 36 of the 40 populations comprising 38 different species studied in zoos showed signs of decreased juvenile viability. For example, the South China tiger (*Panther tigris amoyensis*; Xu *et al.* 2007), considered extinct in the wild with just 73 individuals remaining in captivity, has demonstrated a significant decrease in fecundity with only 13 breeding individuals and 46% of juveniles surviving the first three months after birth. There is also evidence of inbreeding depression in natural populations (for a review see Keller & Waller 2002; for examples see Saccheri *et al.* 1998; Madsen *et al.* 1996; Keller *et al.* 1994) and in experimental studies (Frankham 1995a; Newman & Pilson 1997).

Purging of Deleterious Alleles during Population Bottlenecks

Two contested hypotheses that attempt to explain the loss of fitness associated with inbreeding are the partial dominance hypothesis and the overdominance hypothesis of classical population genetic theory. The partial dominance hypothesis postulates that inbreeding depression results from the increased expression of homozygous deleterious alleles that are no longer masked by heterozygous dominance and therefore affect components of fitness (Jones 1917). The over-dominance

hypothesis postulates that heterozygous individuals are more fit than homozygotes and that inbreeding increases the number of homozygous individuals and decreases the overall fitness of the population (East 1908). There is evidence in the literature to support both hypotheses, however it is generally agreed that the partial dominance hypothesis is better supported (Charlesworth & Charlesworth 1999). If so, then inbreeding depression in bottlenecked populations can theoretically be avoided if deleterious recessive alleles, i.e. the genetic load (Lynch & Gabriel 1990), are removed from the gene pool by successive generations of inbreeding due to a process of selection called purging.

Crnokrak & Barrett (2002) reviewed 28 studies of mammals, insects and plants and found that purging could render a population less susceptible to future inbreeding depression, in contrast to populations that maintained relatively higher frequencies of deleterious alleles. There was evidence of frequent and substantial purging depending on the way in which individual studies were conducted and analysed. Another hypothesis was therefore proposed, rebounds in fitness levels in highly inbred experimental populations may be due to adaptation to laboratory conditions and not purging of deleterious alleles (Crnokrak & Barrett 2002). The results of laboratory experiments of purging are extremely varied and investigations of natural populations show only that the severity of inbreeding depression varies greatly with the species involved and its recent history (Hedrick & Kalinowski 2000); it is therefore difficult to predict the effectiveness and consequences of purging in small captive and free-ranging populations (Leberg & Firmin 2007).

Experimental studies of the housefly (*Musca domestica*) showed that while the loss of fitness arising from a bottleneck was never recovered, subsequent bottlenecks had minimal additional effect (Reed & Bryant 2000). Consequently, it was suggested that captive breeding or restoration programmes should be founded with isolated and/or bottlenecked natural populations. Another experimental study of mosquitofish (*Gambusia affinis*), however, indicated that founder individuals from populations that had experienced one or more bottleneck events were less likely to successfully establish a new self-sustaining population ($P = 0.027$; Leberg & Firmin 2007). The fixation of deleterious alleles in the bottlenecked populations negatively impacted the survival of newly founded populations, but the relatedness of the founder individuals had no observable effect ($P = 0.723$; Leberg & Firmin 2007). In addition, inbreeding depression did not manifest less often in populations that experienced multiple sequential bottleneck events and therefore purging of deleterious alleles either did not occur or did not prevent inbreeding depression from occurring (Leberg & Firmin 2007).

Conclusion

The understanding of observed population genetic structure versus theoretical expectations is vital for the evaluation of anthropogenic and environmental disturbances in natural populations. The isolation of populations in fragmented or reduced habitats limits migration and gene flow between populations. This, in turn, increases the degree of genetic differentiation amongst populations and prevents the introduction of new genetic diversity. In general, species with minimal dispersal potential are naturally characterised by pronounced spatial genetic structuring whereas those with greater dispersal potential have less spatial structuring (Waples 1998). Bohonak (1999) reviewed 20 groups of different animal species and reported a significant association between increased dispersal and decreased differentiation and a moderate association with improved levels of genetic diversity. Translocations that are carried out to augment existing populations and/or introduce new genetic diversity can be used to promote “gene flow” among populations, which can then reduce the detrimental effects of inbreeding depression (Avisé 1994).

Organisation of this Thesis

In addition to the *Introduction* (Chapter 1) and *General Synthesis and Conclusions* (Chapter 5), this thesis consists of three chapters. Chapter 2, *Materials and Methods*, details the sampling of Southern white rhinoceros individuals from a number of populations in southern Africa, together with the molecular methodologies used in this study; molecular methods include microsatellite genotyping together with the production of mtDNA control region and MHC *DQA* and *DQB* exon 2 gene sequence data sets. With a focus on the population level Chapter 3, *Population Bottlenecks, Founder Events and Genetic Diversity*, details the use of molecular data to analyse adaptive and neutral genetic diversity and genetic differentiation within and among contemporary SWR populations; these represent both the source and seeded populations of SWR in southern Africa. This chapter also draws on a number of statistical approaches that identify signatures of genetic bottlenecks in natural populations and, using a simulation approach, investigates whether genetic variation is likely to be maintained given current management strategies. At the level of the individual, Chapter 4, *Southern White Rhinoceros Metapopulation Management Strategy*, describes the mating system and investigates possible reproductive skew in two seeded populations of SWR; microsatellite data are used to assign parentage and explore aspects of female mate choice. This data is used to inform metapopulation management programs that require accurate identification of breeding males for translocation to other reserves.

Chapter 2

Materials and Methods

Sample Populations

The SWR DNA samples analysed in this study were collected from five populations across the southern African subregion during the period 1988 to 2007 (Table 2.1. and Figure 2.1.); these included 28 from Hluhluwe Imfolozi Game Reserve (HIR) and 49 from Welgevonden Private Game Reserve (WGR) in South Africa, 18 from Waterberg Plateau Park (WPP) in Namibia and 49 from Matobo National Park (MNP) in Zimbabwe. HIR and WPP samples were obtained from skin biopsies taken from the pinna of the ear for identification purposes or blood samples collected during routine veterinary inspections and/or translocations. WGR and MNP samples were obtained from tissue collected by biopsy darting (Karesh *et al.* 1987) during a behavioural and ecological study carried out by Dr Janet Rachlow (Department of Fish and Wildlife Resources, University of Idaho, U.S.A) between 1994 and 1998. In addition, NWR samples were collected by Dr Kes Hillman-Smith, between 1999 and 2004, from 19 desiccated rhinoceros carcasses killed by poachers in Garamba National Park (GNP) in the Democratic Republic of the Congo (DRC). These NWR samples were included for comparative analyses.

Ear nicks and biopsy dart tissue were collected into 100% ethanol and stored at 5°C before undergoing DNA extraction. Whole blood samples were collected into Vacutainer® K3 EDTA Tubes (Becton Dickinson Vacutainer Systems) and stored at -20°C before undergoing DNA extraction.

The Hluhluwe Imfolozi Game Reserve is situated in central Zululand in KwaZulu-Natal Province, South Africa, in the junction between the Black and White Umfolozi rivers. This area formed much of the exclusive hunting preserve of Zulu kings Dingiswayo and Shaka and was protected by royal decree long before the official proclamation of the reserve in 1895 (Player 1972). The reserve initially consisted of three separate reserves; Hluhluwe Valley Reserve to the north, Umfolozi Junction Reserve to the south and between the two, however the Hlabisa Game Reserve was deproclaimed in 1907 due to pressure from surrounding farmers (Ezemvelo KwaZulu-Natal Wildlife, accessed 10th October 2008 [<http://www.kznwildlife.com/site/ecotourism/destinations/WildlifeParks/Hluhluwe/History.html>]). Hluhluwe and Umfolozi operated independently, with just an 8 km wide corridor connecting the two game reserves, until 1989 when the Corridor Game Reserve was proclaimed and the reserves amalgamated and renamed Hluhluwe Imfolozi Game Reserve, which now encompasses 960 km² of natural habitat (Ezemvelo KwaZulu-Natal Wildlife, accessed 10th June 2008 [<http://www.kznwildlife.com/site/ecotourism/destinations/WildlifeParks/Hluhluwe/History.html>]). The HIR SWR population recovered from the well-documented large-scale demographic bottleneck during the early 1900s and is currently maintained at about 1650 rhinoceroses by means of a source-sink management strategy (*pers. comm.* S. Janse van Rensburg), whereby rhinoceroses are only translocated to other protected areas from designated sink areas along the outer boundary of the reserve (Owen-Smith 1988; 1983).

The Welgevonden Private Game Reserve, situated in the Waterberg plateau in Limpopo Province, South Africa, forms part of the Waterberg Nature Conservancy established in 1990 and awarded International Biosphere status by the United Nations Educational, Scientific and Cultural Organisation (UNESCO) in 2001 (UNESCO, accessed 18th October 2008 [<http://www.unesco.org/mab/BRs/AFRBRs.shtml>]). The reserve is 340 km² in size but has recently removed boundary fences with neighbouring Marakele National Park and other private game reserves (*pers. comm.* H. Kilian) to allow wildlife free access across approximately 1500 km² of indigenous bushveld. The WGR SWR population was founded between 1994 and 1996 with 40 individuals and had a population size of 50 at the time of sampling. The current population size is estimated at about 43 rhinoceroses as 28 individuals have been sold to alleviate increased levels of aggression and fighting and five deaths were reported between 1999 and 2006 (*pers. comm.* H. Kilian).

Waterberg Plateau Game Reserve is located near Otjiwarongo in Namibia and was established in 1972 as a nature conservancy for the protection and breeding of rare and endangered species (Kandjii 2006). The reserve covers 405 km², with the virtually inaccessible Waterberg Plateau rising 200m above the surrounding area (Kandjii 2006). The WPP SWR population was founded in 1975 (the founder number is unknown) and the population consisted of 62 individuals at the time of sampling.

Matobo National Park is situated in Matabeleland in south-western Zimbabwe and encompasses 425 km² of the Matobo Hills. The park was established in 1953 and received UNESCO World Heritage Status in 2003 (UNESCO, accessed 18th October 2008 [<http://www.unesco.org/mab/BRs/AFRBRs.shtml>]). The MNP SWR population was founded between 1962 and 1967 with 12 individuals (seven females and five males) periodically released into a 105 km² fenced sanctuary called Whovi Game Park and in 1987, a further three females and one male were introduced to the population from Swaziland (*pers. comm.* J. Rachlow). A reported annual growth rate of 10.4% (Rachlow & Berger 1998) enabled the population size to increase rapidly and 20 individuals were removed from the population between 1975 and 1986 due to increased levels of aggression. In 1978 to 1979, a separate population of six rhinoceroses was established in MNP outside the fenced game park. The population size of both populations consisted of 52 individuals at the time of sampling.

Garamba National Park is located in the north-east of the DRC on the Sudanese border and is home to the last remaining free-ranging population of NWR (Amin *et al.* 2006). The park, encompassing 4 900 km², was established by Belgian royal decree in 1938 and received UNESCO World Heritage Status in 1980 (African Parks Foundation Annual Report Garamba National Park, accessed 23rd August 2008 [<http://www.africanparks-conservation.com>]). NWR appeared to be locally abundant when first reported in 1903 but formed a large part of the diet of the indigenous peoples (Emslie & Brooks 1999). The park afforded protection from the hunting pressure and by 1960 there were an estimated 1000 to 1300 rhinoceroses in GNP (Smith & Smith 1993). The Simba rebellion in 1964 resulted in the decimation of the population, but thereafter it gradually recovered again to about 490 individuals by 1976 (Smith & Smith 1993). Between 1978 and 1984, commercial poaching again reduced the population to 15, but an intense conservation effort by Garamba National Park Project and the *Institut Congolais pour la Conservation de la Nature* (ICCN) allowed these numbers to increase to 30 individuals in eight years (*pers. comm.* K Hillman-Smith). This successful effort maintained the population at ~30 rhinoceroses despite armed conflict in the area between 1992 and

2003 (*pers. comm.* K Hillman-Smith). The increasing pressure of poachers from the Sudan and many years of civil unrest in the DRC ultimately resulted in the extermination of most of the wildlife in the northern regions of the park with rhinoceros and elephant populations starting to be affected in the south by 2004 (*pers. comm.* K Hillman-Smith). It was during this period that the NWR samples were collected from poached carcasses. The African Parks Foundation, in partnership with ICCN, officially assumed management of the park in November 2005 and a survey found just four rhinoceroses, although one year later this number had remained unchanged at one adult female, two adult males and one adolescent male (African Parks Foundation Annual Report Garamba National Park, accessed 23rd August 2008 [<http://www.africanparks-conservation.com>]). The future of the NWR remains bleak.

Sample Site	Country	Sample size	Population size	Number of founders	Year of founding
Hluhluwe Imfolozi Reserve	South Africa	28	1 650	20 - 100	N/A
Welgevonden Game Reserve	South Africa	49	50	40	1994 - 1996
Waterberg Plateau Park	Namibia	18	62	unknown	1975
Matobo National Park	Zimbabwe	49	52	12	1962 – 1967
Garamba National Park	DRC	19	30	abundant	N/A

Table 2.1.

The number of samples analysed per population, estimated total population size at the time of sampling, date of founding for each seeded population and the number of founding individuals where applicable

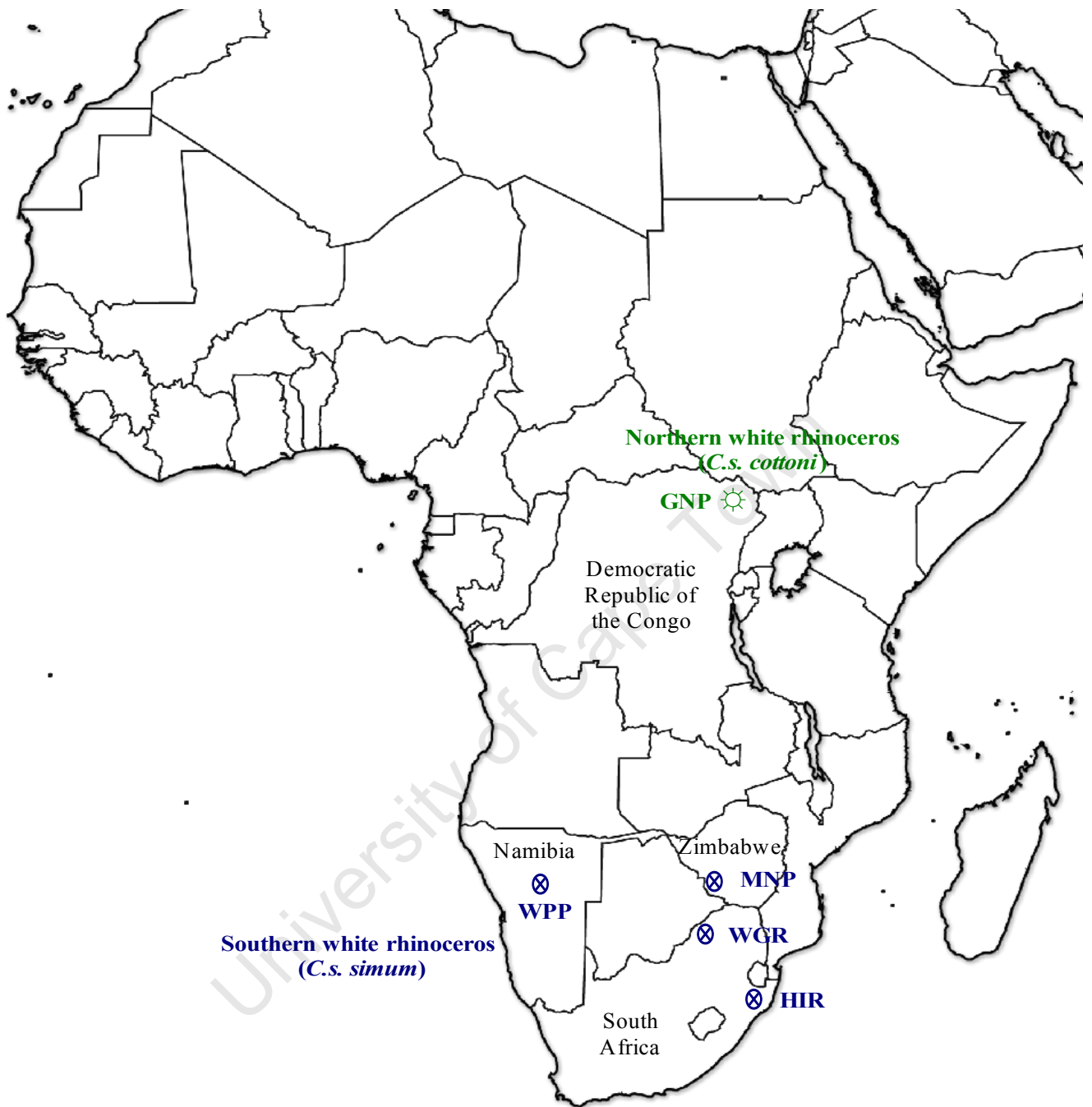


Figure 2.2.

A map of southern Africa indicating the SWR study populations (☉); Hluhluwe Imfolozi Game Reserve (HIR) and Welgevonden Private Game Reserve (WGR) in South Africa, Waterberg Plateau Park (WPP) in Namibia, Matobo National Park (MNP) in Zimbabwe and NWR (☉) in Garamba National Park (GNP) in the Democratic Republic of the Congo.

DNA Extraction

DNA was extracted from ear nick and biopsy dart tissue samples by means of Proteinase K digestion at 56°C followed by a standard phenol/chloroform protocol with ethanol precipitation (Sambrook *et al.* 1989). DNA was extracted from whole blood samples after the addition of red blood cell lysis buffer (150 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100, 50 mM Tris-HCl pH 7.5) to initiate erythrocyte lysis and thereafter a DNA salt precipitation process was employed (Miller *et al.* 1988). Desiccated tissue samples collected from poached NWR carcasses were first rehydrated in distilled water at 45°C overnight after which DNA was extracted using the above standard salt precipitation process.

All extracted DNA samples were resuspended in 1× TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) at 50°C for 15 minutes and stored at -20°C. DNA concentrations were estimated for all samples using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). Samples were visually checked for DNA quality using agarose gel electrophoresis and stained with Ethidium bromide. Reagent blank samples were included and processed through each DNA extraction and quantification procedure to control for contamination.

Microsatellite Marker Genotyping

Thirty-four polymorphic microsatellite loci were screened to identify markers that were polymorphic in white rhinoceroses. These markers included loci 7B, 7C, 12F, 32A, 32F (Florescu *et al.* 2003) and AY138542, AY138545 (Nielsen *et al.* 2007) isolated in the white rhinoceros; BR3, BR4, BR6, BR17, BR20 (Cunningham *et al.* 1999) and DB1, DB4, DB5, DB14, DB23, DB30, DB42, DB44, DB49, DB52, DB66 (Brown & Houlden 1999) isolated in the black rhinoceros; and Rh1, Rh2, Rh3, Rh4, Rh5, Rh6, Rh7, Rh8, Rh9, Rh10, Rh11 (Zschokke *et al.* 2003) isolated in the Indian rhinoceros. Thirteen of these microsatellite loci (Table 2.2.) proved to be polymorphic in the white rhinoceros and were included in this study; the remainder were either monomorphic, heterozygous for the same two alleles or failed to amplify white rhinoceros DNA.

The 13 microsatellite markers were individually amplified by polymerase chain reaction (PCR) using a GeneAmp® 2700 thermocycler (Applied Biosystems) to analyse individual and population level polymorphisms. PCR amplification was performed in thin-walled 0.2ml PCR tubes and contained the following reagents in a 20µl reaction volume: 10 to 50 ng genomic DNA, 0.25 units of GoTaq™ Flexi DNA polymerase (Promega), 1× reaction buffer (final concentration: 16 mM [NH₄]₂SO₄, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20), 0.3 µM fluorescently labelled (FAM™ or HEX™ fluorescent dye) forward and unlabelled reverse primers, 2 mM MgCl₂ and 0.2 mM dNTPs. The PCR protocol for ten of these loci consisted of one denaturing step at 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, the specific annealing temperature (Table 2.2.) for 30 seconds, 72°C for 45 seconds, and followed by a final extension step of 72°C for 10 minutes.

The PCR reaction for the three loci isolated in the Indian rhinoceros (Rh7, Rh8, Rh9) contained 0.05 µM forward primer with a M13 sequence appended to the 5' end (5' – TGTAACGACGGC CAGT - forward primer sequence - 3'), 0.2 µM reverse primer and 0.2 µM M13 primer labelled with either FAM™ or Hex™ fluorescent dyes (as per Schuelke 2000). The PCR protocol consisted of one denaturing step at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, the specific annealing temperature (Table 2.2.) for 30 seconds, 72°C for 45 seconds, and then eight cycles of 94°C for 30 seconds, 53°C for 30 seconds for M13 attachment, 72°C for 45 seconds and then followed by a final extension step of 72°C for 10 minutes. This protocol was proposed by Schuelke (2000) and reduces the costs involved in purchasing large numbers of fluorescently-labelled primers for screening microsatellite markers, as only the M13 primer is labelled. This primer is incorporated into the PCR product during the additional eight cycles of the PCR.

Amplified PCR products were visualised by automated fragment size analysis on an ABI Prism™ 373 Genetic Sequencer (Applied Biosystems) using polyacrylamide gel (6%) electrophoresis on a 6% polyacrylamide gel according to the specifications of the manufacturer. The resultant fragment size data were collected and analysed using GENESCAN™ 672 Data Collection Software v1.2.1 (Applied Biosystems). Microsatellite alleles were sized manually in relation to a fluorescent-labelled internal size standard (GeneScan™ Rox-350; Applied Biosystems). To ensure consistency of allele fragment size scoring between gels and that PCR amplifications were free of DNA contaminants, a positive control of known size together with a negative control were included in each run.

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Locus	Repeat Motif	T _A (°C)	N _A	Size Range (bp)	Fluorescent Dye	Primer Sequences (5'→3')	Reference
7B	(TG) _n	58	4	262 - 268	FAM TM	F: CCTCTGTGATTAAGCAAGGC R: ATGAACAGGAAGGAAGACGC	Florescu <i>et al.</i> (2003)
7C	(CT) _n (AT) _n	58	3	249 - 255	HEX TM	F: TGAACTCTGATGGAAATGAG R: AACAGGTCTTGATTAGTGC	Florescu <i>et al.</i> (2003)
32A	(CA) _n	58	3	244 - 258	HEX TM	F: CAGTCCTGCTGCATAAAATCTC R: GCAGTACAGCTAGAATCACC	Florescu <i>et al.</i> (2003)
AY138542	(AC) _n	50	6	174 - 186	FAM TM	F: GGCAAACTAAGAGAAGCTTG R: GATACCAAAGTGGAAATGG	Nielsen <i>et al.</i> (2007)
AY138545	(TA) _n (CA) _n	50	3	225 - 239	HEX TM	F: ACAGCTAGAATCACCAAAAC R: TCCTGCTGCATAAAATCTC	Nielsen <i>et al.</i> (2007)
BR6	(CA) _n	50	6	129 - 155	HEX TM	F: TCATTTCTTTGTTCCCCATAGCAC R: AGCAATATCCACGATATGTGAAGG	Cunningham <i>et al.</i> (1999)
DB1	(CA) _n	50	4	124 - 132	FAM TM	F: AGATAATAATAGGACCCTGCTCCC R: GAGGGTTTATTGTGAATGAGGC	Brown & Houlden (1999)
DB44	(CA) _n G(CA) _n	58	4	175 - 189	FAM TM	F: GGTGGAATGTCAAGTAGCGG R: CTTGTTGCCCATCCCTG	Brown & Houlden (1999)
DB49	(CA) _n	64	4	170 - 176	FAM TM	F: GTCAGGCATTGGCAGGAAG R: CAGGGTAAGTGGGGGTGC	Brown & Houlden (1999)
DB66	(CA) _n TA(CA) _n	55	4	198 - 208	HEX TM	F: CCAGGTGAAGGGTCTTATTATTAGC R: GGATTGGCATGGATGTTACC	Brown & Houlden (1999)
Rh7	(TG) _n	57	2	199 - 201	M13 tail	F: CCGTCACATATGACAGTGTGC R: GGGCAGCTTATGCTCAAGTC	Zschokke <i>et al.</i> 2003
Rh8	(TG) _n (AG) _n ANACA(GA) _n CA (GA) _n CA(GA) _n CG(TA) _n	55	5	156 - 164	M13 tail	F: ACACACCTTTATAAACAATATGGTCAC R: AGTCTCTAGTCAAAAGGGATTGC	Zschokke <i>et al.</i> 2003
Rh9	(TG) _n TT(TG) _n TA (TG) _n	55	5	141 - 153	M13 tail	F: TCTGGTACCACCAAATGTAGC R: ACGATTACGTCTTTCAGTTGC	Zschokke <i>et al.</i> 2003

Table 2.2.

Thirteen microsatellite markers amplified in SWR; the repeat motif, annealing temperature (T_A), number of alleles (N_A), allele size range in base pairs, specific fluorescent dye, forward (F) and reverse (R) primer sequences and reference are indicated.

Microsatellite Marker Genotyping Error Rate

Microsatellite marker genotype data sets typically incorporate a level of error and many studies of natural populations are likely to incorporate erroneous results (Marshall *et al.* 1998). Recent studies suggest that even minimal error rates can adversely affect estimates of genetic diversity, population structure, migration rates, kinship studies and parentage analyses (Creel *et al.* 2003; Piggott & Taylor 2003; Waits & Leberg 2000; Marshall *et al.* 1998; Taberlet *et al.* 1999). No consensus, however, currently exists on how genotyping errors should be identified or what measures should be employed to reduce the frequency of these errors.

There are a number of factors that contribute to genotyping errors. These include low quantity and/or quality template DNA, e.g. samples collected from non-invasive sources such as faecal material and shed hairs, where “allelic dropout” can be caused by one allele of an heterozygote randomly failing to amplify in the PCR reaction and the individual is mistakenly scored as a homozygote at the locus of interest (Gagneux *et al.* 1997; Walsh *et al.* 1992). Another cause of allele non-amplification is “null alleles”, which are caused by mutations in the primer binding region, such that heterozygous individuals may also appear to be homozygous at a particular locus (Dakin & Avise 2004). In addition, extraneous PCR artefacts, called “misprinting”, can be misinterpreted as actual alleles as well as the presence of “stutter bands”, resulting from *Taq* polymerase slippage during PCR, can interfere with the accurate scoring of microsatellite alleles (Bradley & Vigilant 2002; Goossens *et al.* 1998; Taberlet *et al.* 1996).

As a result, a number of quality control protocols to minimise levels of genotyping errors have been suggested. These include the multiple tube approach where DNA samples undergo several independent PCR amplifications (Frantz *et al.* 2003; Taberlet *et al.* 1996) and comparisons between genotypes derived from both blood and tissue from each individual sampled to check for consistency in the results (Fernando *et al.* 2003). Other approaches include using maximum likelihood analysis to determine which loci are most likely to incorporate errors and therefore only requiring the re-amplification of those loci rather than repeating all of the individual genotypes (Miller *et al.* 2002). The pre-screening of samples to determine DNA quantity and quality (Morin *et al.* 2001; Segelbacher 2002). The use of pilot studies, before undertaking a research study, to identify technical issues associated with non-invasive sampling and to calculate potential error rates (Taberlet & Luikart 1999). Computational simulations to estimate the effect of genotyping errors on the reliability and accuracy of the genotype data (Van Oosterhout *et al.* 2004; Valiere *et al.* 2002; Taberlet *et al.* 1996).

There are several ways to estimate error rates to account for genotyping errors in downstream analyses. These include re-genotyping a subset of individuals to compare with the original data set (Ghosh *et al.* 1997) and comparing known mother-offspring pairs to detect the frequency of genotype mismatches (Marshall *et al.* 1998), although this approach could be compromised by offspring fostering behaviour or egg-dumping by brood parasites. There are also more economical statistical tests, such as testing for deviations from expected Hardy-Weinberg equilibrium (HWE) to identify homozygous excess associated with allelic dropout or null alleles (Gomes *et al.* 1999).

In this study, the following steps were taken to minimise genotyping error. DNA was extracted from blood or tissue and the quality of the extracted DNA was visualised by agarose gel electrophoresis to determine the proportion of high molecular weight DNA versus sheared DNA. The quantity of genomic DNA used in PCR reactions was calculated using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). The probability of allelic dropout affecting allele scoring was therefore minimal. Null alleles were possible because several of the loci used were originally isolated in other rhinoceros taxa. The preferential amplification of small alleles (short allele dominance; Wattier *et al.* 1998) can also result in the over-representation of homozygotes in a data set. A single locus (DB66) revealed significant homozygous excess associated with allele non-amplification and had positive estimates of null allele frequencies ($P > 0.448$; CERVUS v3.0; Marshall *et al.* 1998). This locus was therefore excluded from parentage analyses, but not population genetic analyses as deviations from HWE in a single locus may indicate natural selection acting on a nearby gene and positive estimates of null allele frequency could denote homozygous excess without necessarily indicating the presence of a null allele. The likelihood of PCR artefacts arising from extraneous amplification was reduced as loci were independently amplified rather than in a multiplex PCR so that each reaction occurred at the optimal annealing temperature. The frequency of amplification errors has been found to increase significantly with PCR product size (Sefc *et al.* 2003), but the largest PCR product amplified in this data set was 268 bp in length.

Fluorescent gel allele scoring was relatively simple as there were no overlapping banding patterns between different loci to result in misinterpreted data. Only loci with different fluorescent dyes and differing in size by more than 50 bp were electrophoresed together on a single gel. Incorrect allele scoring can also be caused by stutter bands overlapping true alleles, so if two alleles of an apparently heterozygote individual only differed in size by a single repeat unit, then these “adjacent allele heterozygotes” were distinguished from homozygotes by comparison with known homozygous banding patterns characteristic of the particular locus. PCR amplifications that initially

failed were repeated up to three times and all reactions that produced ambiguous genotypes, for example faint or unclear bands, were also repeated. No SWR DNA samples failed to amplify at two or more loci and therefore no samples were excluded from the final data set. The frequency of missing single locus genotypes was 2.24% (42 out of a possible 1 872). The loci not typed were either due to DNA samples being used up or alleles that could not be accurately sized. Genotypes were recorded in a Microsoft® Excel spreadsheet and systematically confirmed as correct with the original fluorescent gel to avoid clerical error.

Genotype mismatches between known mother-offspring combinations were re-genotyped at least once. A number of these mismatches were corroborated in the WGR population with mothers and calves presenting different maternally inherited mtDNA control region sequences. It is therefore apparent that at least a proportion of these mismatches are not due to genotyping error, but rather indicate calf swapping between adult females. As a result, the genotypes of the entire WGR and MNP populations were randomly repeated for 12 loci (excluding DB66). The low level of allelic diversity observed in SWR makes this study particularly sensitive to error as candidate fathers could be excluded on the basis of a single mismatch (Taberlet *et al.* 1999; Marshall *et al.* 1998). Hoffman & Amos (2005) showed that even a genotyping error rate of 1% could prevent more than 20% of paternity assignments.

To identify possible genotyping errors, the program MICRO-CHECKER (Van Oosterhout *et al.* 2004) was used to assess the microsatellite genotypes generated in this study. An apparent deficiency of homozygotes arising from genotyping errors can often appear similar to those caused by inbreeding, assortative mating or a Wahlund effect. This software can distinguish between deviations arising from non-panmixia and those that are due to genotyping errors. The latter is caused by short allele dominance, stutter bands and null alleles and have specific “signatures” although allelic dropout is usually not dependent on allele size (Miller *et al.* 2002). There was no evidence of genotyping errors caused by stutter bands, short allele dominance (large allele dropout) or null alleles at any of the 13 microsatellite loci genotyped for this study ($p > 0.05$).

The terminology used in the literature to describe genotyping error rates is varied, being expressed as number of errors per allele to number of errors per reaction, and from the error rate per locus to that across multiple loci (Hoffman & Amos 2005). The error rate of the genotype data set used in this study, estimated by genotyping a random subset of 98 individuals, is expressed as the number of errors per allele at 0.667% (17 incorrectly genotyped alleles per 2 548 total alleles).

Mitochondrial DNA Control Region Sequencing

A 763 bp fragment of the hypervariable region 1 (HVR1) of the mtDNA control region was sequenced using primers (Table 2.3.) designed by aligning the complete white rhinoceros mtDNA sequence (Accession No. Y07726; Xu & Arnason 1997) with the program DNAMAN (Lynnon BioSoft, Quebec, Canada). The length of the control region in white rhinoceroses is approximately 1381 bp, depending on the number of tandem repeat motifs located at the 3' end (Xu & Arnason 1997). A total of 111 samples were sequenced, these included 22 from HIR, 35 from WGR, 14 from WPP and 31 from MNP.

The control region sequences were PCR amplified on a GeneAmp® 2700 thermocycler (Applied Biosystems) using thin-walled 0.2 ml PCR tubes. The control region PCR amplification contained the following reagents in a 50µl reaction volume: 0.1 to 0.5 ng genomic DNA, 0.5 units of GoTaq™ Flexi DNA polymerase (Promega), 1× reaction buffer (final concentration: 16 mM [NH₄]₂SO₄, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20), 0.5 µM forward and reverse primers, 2 mM MgCl₂ and 0.2 mM dNTPs. The PCR protocol consisted of one denaturing step at 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, the specific annealing temperature (Table 2.3.) for 45 seconds, 72°C for 45 seconds, and followed by a final extension step of 72°C for 10 minutes.

All PCR products were electrophoresed on a 2% agarose gel and the product band excised and purified using a Wizard SV Gel and PCR Clean-up System (Promega). The purified PCR products were then cycle sequenced with either the forward or reverse primer using the BigDye® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). The quantity of purified DNA used in each cycle sequencing reaction was estimated according to the relative intensity of the product band on a 2% agarose checking gel. PCR conditions for the cycle sequencing reactions were 25 cycles of 94°C for 30 seconds, 50°C for 5 seconds and 60°C for 4 minutes. After purification with Sentriseq Columns (Princeton), the cycle-sequenced products were run on an ABI 3130 Automated DNA Sequencer (Applied Biosystems) according to the specifications of the manufacturer.

Major Histocompatibility Complex Gene Sequencing

Direct sequencing of DNA is the most accurate way to detect, identify and characterise mutations; however, this is costly and labour-intensive and additional methods have been developed to rapidly screen large numbers of samples. One such method is single-strand conformation polymorphism (SSCP), which is a simple and reliable technique that provides a relatively inexpensive alternative to direct sequencing (Orita 1989a; 1989b).

SSCP analysis is a technique that is based on differences in conformation that occur once DNA has been denatured; these differences are due to self complementarity and intra-molecular interactions and even single nucleotide polymorphisms (SNP) will cause subtle changes in the tertiary structure. These changes in conformation can then be identified by a shift in the electrophoretic mobility of the DNA (Hayashi 1991).

Gene duplication is fairly common in the major histocompatibility complex (MHC; Kasahara 1999) and therefore SSCP gel electrophoresis was used to screen for polymorphisms in the second exon of the *DQA* and *DQB* homologues in the white rhinoceros and to determine whether one or more loci of each gene was PCR amplified. SWR from the HIR, WGR and MNP populations and five NWR samples from GNP were screened to identify the DNA motifs. Thereafter, samples exhibiting each individual motif were cloned and directly sequenced to determine the nucleotide sequences of each allele and the minimum number of MHC loci being amplified. The second exon of each gene was targeted for analysis as the high levels of polymorphism typically found in vertebrate MHC class II genes is mostly confined to this nucleotide sequence region that encodes the functionally important peptide binding region (Potts & Wakeland 1990; Van Eden *et al.* 1983).

SSCP Capillary Electrophoresis

The second exon of the *DQA* and *DQB* MHC gene homologues were PCR amplified on a GeneAmp® 2700 thermocycler (Applied Biosystems). PCR reactions were performed in thin-walled 0.2ml PCR tubes and contained the following reagents in a 25µl reaction volume: 10 to 50 ng genomic DNA, 0.5 units of GoTaq™ Flexi DNA polymerase (Promega), 1× reaction buffer (final concentration: 16 mM [NH₄]₂SO₄, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20), 0.3 µM forward (labelled with FAM™) and reverse (labelled with HEX™) primers, 2 mM MgCl₂ and 0.2 mM dNTPs. The PCR protocol consisted of one denaturing step at 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, the annealing temperature (Table 2.3.) for 30 seconds, 72°C for 45 seconds, and followed by a final extension step of 72°C for 10 minutes. The fluorescently-labelled PCR products were electrophoresed on a 2% agarose gel and the DNA dilution for subsequent automated capillary electrophoresis SSCP (CE-SSCP) was determined by the relative intensity of each product band. Capillary electrophoresis is more rapid, has a greater resolution and sensitivity compared with automated slab gel electrophoresis.

Initially, 1µl of dilute PCR product was combined with 15µl HiDi (deionised formamide; Applied Biosystems) and 0.5µl GeneScan-500 LIZ internal size standard (Applied Biosystems) and heat denatured at 95°C for 5 minutes and then rapidly cooled to 4°C. CE-SSCP analysis was then performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) using a 10× buffer with EDTA (Applied Biosystems), 9% Conformational Analysis Polymer (Applied Biosystems) and 100% Glycerol mixture. Three separate analyses, at different temperatures, were carried out for each sample and the heating mantle was set at 18°C, 30°C or 45°C per run. SSCP peak profiles were collected using ABI Data Collection software v3.0 (Applied Biosystems) and analysed with Genemapper v3.7 (Applied Biosystems). The relative position of each SSCP peak does not represent an absolute size of the fragment. DNA fragments that are labelled with different fluorophors can be detected which allows for the simultaneous identification of the SSCP peak profiles for both the forward and reverse DNA strands.

As originally reported by Orita *et al.* (1989a), environmental factors can affect the conformation of single-stranded DNA during gel electrophoresis; these include the temperature of the acrylamide gel or heating mantle for CE-SSCP, buffer concentration and the presence of denaturing agents in the gel. Wenz *et al.* (1998) showed that a particular mutation would respond differently, with respect to both magnitude and direction of mobility shift, to various temperatures and that repeating the SSCP analysis at different temperatures would greatly increase the sensitivity of mutation detection.

According to Inazuka *et al.* (1997), running a sample at 20°C and 30°C will result in 97% sensitivity with a 0.3% possibility of false positives. Therefore CE-SSCP was performed at three temperatures, namely 18°C, 30°C and 45°C, to identify and resolve each SSCP profile to determine the sequence variation. The SSCP data generated for each temperature were analysed separately and the motifs labelled A, B or C such that each sample was assigned a three-letter overall motif. These data were recorded in a Microsoft® Excel spreadsheet and systematically confirmed as correct to avoid clerical error. A total of 126 SWR from HIR, WGR and MNP, and five NWR from GNP were analysed by PCR-SSCP for the second exon of the *DQA* and *DQB* genes. A sub-set of samples exhibiting each of the unique motifs was cloned and sequenced to determine the actual nucleotide sequence of these motifs.

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Cloning and Sequencing

Samples selected for cloning and sequencing were PCR amplified using unlabelled primers (as previously described; Table 2.3.). PCRs were performed on a GeneAmp® 2700 thermocycler (Applied Biosystems) in thin-walled 0.2 ml PCR tubes and contained the following reagents in a 50µl reaction volume: 10 to 50 ng genomic DNA, 1.5 units of Expand High Fidelity PCR System (Roche), 1× reaction buffer (final concentration: 20 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Nonidet P40, 0.5% Tween 20, 50% glycerol, pH 8.0), 0.4 µM forward and reverse primers, 2 mM MgCl₂ and 0.2 mM dNTPs. The PCR protocol consisted of one denaturing step at 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, the annealing temperature (Table 2.3.) for 45 seconds, 72°C for 45 seconds, and followed by a final extension step of 72°C for 10 minutes. The Expand High Fidelity PCR System (Roche) consists of a blend of enzymes to improve the fidelity of the PCR amplification with a threefold greater accuracy (error rate approximately 4.8×10^{-6} compared with 1.6×10^{-6} for other *Taq* DNA Polymerases).

The PCR products were electrophoresed on a 2% agarose gel and the product band excised and purified using a Wizard SV Gel and PCR Clean-up System (Promega) and cloned using the pGEM-T Easy Vector System (Promega). The purified DNA was ligated overnight at 4°C and transformed into DH5α competent *Escherichia coli* cells according to the specifications of the manufacturer. Transformed cells were plated onto Luria agar plates containing 100 mg/ml ampicillin (Merck), IPTG (Fermentas) and X-Gal (Fermentas) for blue/white screening. White colonies were selected for colony PCR screening using M13 forward and reverse primers, which bind to sites within the vector, to confirm that a fragment of the correct size was inserted into the vector. The PCR was performed on a GeneAmp® 2700 thermocycler (Applied Biosystems) in thin-walled 0.2 ml PCR tubes and contained the following reagents in a 20µl reaction volume: 1µl of colony DNA suspended in Luria Broth, 0.5 units of Super-Therm DNA polymerase (Hoffman-La-Roche), 1× reaction buffer (final concentration: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol and stabilisers), 0.5 µM forward and reverse M13 primers, 1.5 mM MgCl₂ and 0.2 mM dNTPs. The PCR protocol consisted of one denaturing step at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, the specific annealing temperature (Table 2.3.) for 30 seconds, 72°C for 55 seconds, and followed by a final extension step of 72°C for 10 minutes.

For each sample, 4 to 8 clones were cycle sequenced with the forward M13 primer using the BigDye® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) as previously described.

Locus	Genome	Selection	T _A (°C)	N _A	Size Range (bp)	Primer Sequences (5' → 3')	Reference
Control region	mtDNA	Neutral	60	5	763	F: CCTAGCCTCACCATCAAC R: GTGTATATGCGTATGTCCTG	Designed for this study
MHC <i>DQA</i>	Nuclear	Balancing	48	4	246	F: CTGAICACITTGCCTCCTATG R: TGGTAGCAGCAGIAGIGTTG	Fraser & Bailey 1998
MHC <i>DQB</i>	Nuclear	Balancing	50	3	210	F: AAGGGCCAGTGCTACTTCACCAACG R: GTATCTGCACACTCTGTCCAACCTCG	Hořin & Matiašovic 2002

Table 2.3.

DNA markers used in this study to amplify SWR and NWR mitochondrial and nuclear gene regions; the annealing temperature (T_A), number of alleles (N_A), sequence size range in base pairs, forward (F) and reverse (R) primer sequences and reference are indicated.

Chapter 3

Demographic Bottlenecks, Founder Events and Genetic Diversity

Introduction

Natural selection is dependent on heritable phenotypic variation and associated differences in fitness within individuals of a species. Early studies detected surprisingly high levels of genetic diversity within and among both individuals and populations, for example protein loci were demonstrated to be polymorphic in humans (Harris 1966) and fruit flies (*Drosophila pseudoobscura*; Hubby & Lewontin 1966). This variation was unexpected because it was believed that natural selection retained only the fittest alleles in the population. Kimura (1968) proposed that most genetic diversity within the genome must be selectively neutral or otherwise the resultant genetic load would be immense and cause extinctions. Levels of genetic diversity are maintained in populations by new alleles arising from mutations and alleles being lost due to random genetic drift. Mutations that result in deleterious or advantageous alleles are either lost or become fixed, respectively, from the gene pool and do not significantly affect levels of genetic diversity.

Many natural populations have experienced population bottlenecks or are at risk of future demographic decline. Bottlenecks can be caused by natural founder events in island populations (Frankham 1997) and isolated mainland populations such as crater and cave populations (Packer *et al.* 1991; Avise & Selander 1972) or anthropogenically-induced founder events such as re-introduced populations (Ellegren *et al.* 1993). A reduction in effective population size limits evolutionary adaptive potential and the resultant inbred population is less likely to survive adverse environmental changes. This has been shown unequivocally in experimental studies of *D. melanogaster* (Frankham *et al.* 1999) and an empirical study of song sparrows (*Melospiza melodia*; Keller *et al.* 1994).

Soulé (1976) suggested that genetic diversity in natural populations may be related to population size, with larger populations generally retaining more diversity than smaller populations. The effective population size (N_e) of a population is therefore the best predictor of its ability to maintain genetic diversity. The N_e required to sustain evolutionary potential and prevent inbreeding depression, however, remains a controversial subject in conservation genetics. Soulé (1980) proposed that an N_e of 50 would be sufficient, however, signs of inbreeding depression have been

observed in long-term experimental *D. melanogaster* populations with N_e of ~ 50 (Latter & Mulley 1995). Franklin (1980) proposed the 50/500 rule; short-term N_e should not be less than 50, but the maintenance of mutation-drift equilibrium and long-term evolutionary potential would require an N_e of 500. Lande (1995) argued that 90% of new mutations were deleterious and that an N_e of 5000 would actually be required. Franklin & Frankham (1998) refuted this estimate as deleterious mutations had, for the most part, already been taken into account and the heritability values of 50% used by Lande (1995) were too high, and they supported the original estimate of 500. The conservation implications of reduced population size are unequivocal; population bottlenecks adversely affect the long-term survival of populations or species and should be prevented whenever possible. Re-introduced or translocated populations of endangered species should be founded with as many individuals as possible and kept at a sufficiently large N_e to maximise the chances of survival in the future. Small populations may therefore need to be managed as single larger metapopulation, which involves translocating animals between subpopulations. Levins (1970) introduced the term metapopulation to describe a “population of populations”, a collection of fragmented local populations that are under threat of extinction. The term is used in conservation to indicate a network of populations among which individuals could be translocated to conserve subpopulations through genetic and demographic augmentation (for examples see Madsen *et al.* 1999; Westemeier *et al.* 1998).

Numerous genetic studies of natural populations (described in Chapter 1) have reported empirical evidence supporting the theoretical predictions of demographic decline and reduced genetic diversity. These studies typically make use of selectively neutral DNA markers to measure comparative levels of genetic diversity within and among individuals and populations. Molecular markers include microsatellite markers, mtDNA sequences, and single-nucleotide polymorphisms (SNPs). Of these, microsatellite markers are the most powerful genetic marker currently available for the analysis of neutral variation in natural populations (Goldstein & Pollock 1997; Bruford *et al.* 1996; Jarne & Lagoda 1996). In addition to neutral markers, the analysis of loci that directly influence fitness, for example genes of the major histocompatibility complex, can elucidate local selective constraints that affect populations (Hedrick *et al.* 2001). The analysis of these adaptive loci greatly improve the understanding of the processes involved when selection acts on individuals and the potential for future adaptive change that these individuals would have. Both microsatellite markers and mtDNA control region sequences together with MHC exon 2 gene sequences are used in this study to examine genetic diversity and differentiation in contemporary SWR populations.

Microsatellite DNA Markers

Microsatellite markers were first described in the 1980s (Schlötterer 1998; for a review see Goldstein & Pollock 1997). These markers are tandem repeats of short motifs of between two and six nucleotide bases randomly distributed throughout the genomes of all organisms (Hancock 1999). Microsatellite loci show classic Mendelian inheritance with co-dominant allelic variants and are selectively neutral (Schlötterer & Wiehe 1999; Bruford & Wayne 1993).

High levels of variability at microsatellite loci are the result of rapid mutation rates, with mutation rates of 10^{-2} to 10^{-5} events per locus per replication reported in *E. coli* and yeast and 10^{-3} events per locus per generation in humans (Hancock 1999). Intra-allelic polymerase slippage during DNA replication (Levinson & Gutman 1987; Streisinger *et al.* 1966) and/or recombination between DNA molecules (Jeffreys *et al.* 1994; Smith 1976) are thought to be responsible for the high mutation rate. The potential variability at microsatellite loci has been reported by Schlötterer *et al.* (1991) with 58 alleles being identified at a single locus in a study on whale species.

A number of theoretical models have been proposed to explain the evolution of microsatellite loci. The infinite allele model (IAM; Kimura & Crow 1964) always introduces an allele state not previously found in the population by the addition of one or more tandem repeats to an existing allele whereas the stepwise mutation model (SMM; Ohta & Kimura 1973) produces allele states that may already occur in the population by the loss or gain of only a single tandem repeat. An intermediate model, the two phase model (TPM), proposes that mutations generate new allele states by the gain or loss of both one-step and multi-step tandem repeats (Di Rienzo *et al.* 1994). The application of these statistical models to microsatellite data has produced both contradictory and inconclusive results. In one of the first population studies on dinucleotide microsatellite diversity in humans, Edwards *et al.* (1992) showed that the SMM fit the observed allelic distributions better than the IAM. Shriver *et al.* (1993), however, suggested that while SMM was the best mutation model when considering human microsatellites of 3 to 5 bp repeat units, the IAM was a better fit for those with 1 to 2 bp repeat units. Di Rienzo *et al.* (1994) reported that a TPM comprised of mostly one-step mutations and a small proportion (0.05-0.20) of multi-step mutations was the best model of mutation for analysing most human dinucleotide microsatellites.

Laboratory analysis of microsatellite markers is PCR-based and while oligonucleotide primers are species-specific they can often be used to amplify loci in closely related taxa (Allendorf & Luikart

2007). PCR amplification is extremely sensitive and microsatellite loci can be amplified from minute quantities of target DNA or from significantly degraded DNA, such as DNA isolated from forensic material or ancient samples (Bruford & Wayne 1993). Microsatellite markers have been used extensively in the field of conservation genetics to describe levels of genetic diversity, hybridisation between species, divergence amongst populations, historic demography of populations, phylogeography, population bottleneck events, inbreeding and outbreeding and relatedness as well as inferring the effects of reproductive behaviour, social structure and dispersal on endangered species (Beaumont & Bruford 1999).

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The Mitochondrial DNA Control Region

The mitochondrial genome is a small, covalently closed circular molecule that encodes 37 genes, namely 13 messenger RNAs, two ribosomal RNAs and 22 transfer RNAs (Wilson *et al.* 1985). There is also a non-transcribed control region that regulates mtDNA replication and RNA transcription. Gene arrangement and genome size are highly conserved within taxonomic phyla (Wilson *et al.* 1985). The length of the white rhinoceroses mitochondrial genome is 16832 bp (Xu & Arnason 1997). Size differences are generally due to additions or deletions in the control region, which evolves more rapidly than the rest of the mitochondrial genome. This genome lacks the complicating features of repetitive DNA, introns, pseudogenes or spacer sequences typically found in the nuclear genome. MtDNA is maternally inherited without any recombination and therefore represents an unambiguous marker of maternal phylogeny (Allendorf & Luikart 2007).

The mtDNA matrilineal and haploid mode of transmission results in a considerably reduced effective population size (N_e) in comparison to nuclear markers and mtDNA markers are therefore more sensitive to the effects of genetic drift (Moritz 1994). MtDNA evolves approximately 10 times more rapidly than single-copy nuclear DNA in mammals (Allendorf & Luikart 2007). The genes encoded by the mtDNA genome are vital for the production of energy needed to sustain life and therefore any mutations causing alterations in the amino acid compositions are highly visible to purifying selection. Most mutations are therefore base substitutions in silent positions in protein coding genes and substitutions or addition/deletions in the non-transcribed control region.

Somatic cells contain hundreds to thousands of mitochondria and mutations can result in heteroplasmic states, however, empirical examples of heteroplasmy are rare (for example Bermingham *et al.* 1986). Heteroplasmy is thought to be the result of mutations, rather than paternal leakage of mtDNA, and is transitory because mtDNA is rapidly sorted in germ cell lineages (Chapman *et al.* 1982).

The Major Histocompatibility Gene Complex

The major histocompatibility complex (MHC) is a multigene family expressed in all vertebrates. Genes within this complex encode cell surface receptors that bind and transport foreign protein fragments to the cell membrane surface where the complex is recognised by T cells to activate an immune response (Ploegh & Watts 1998). The gene family consists of class I, class II and class III genes that form a single gene complex in mammals (Hess & Edwards 2002; Hughes & Yeager 1998). Class I genes are expressed on all nucleated somatic cells and activate immune response to intracellular pathogens by binding to endogenously derived peptides from viral proteins and cancerous cells in the cell cytoplasm (Sommer 2005). The class III gene region is comprised of a dense collection of immune and non-immune genes (Xie *et al.* 2003). The MHC class II genes are expressed on antigen-presenting cells of the immune system, B cells and macrophages, and present exogenously derived peptide antigens to helper T cells to activate immune response to extracellular parasites such as bacteria, nematodes and cestodes (Sommer 2005). MHC proteins recognise and activate immune responses against a multitude of different pathogens as a result of the sequence diversity in the functionally important peptide binding region (PBR; Potts & Wakeland 1990; Van Eden *et al.* 1983). The evolutionary, ecological and ethological processes that generate and maintain MHC diversity in natural populations, however, are still not completely understood (Edwards & Hedrick 1998; Potts & Slev 1995).

Balancing selection (or over-dominance) has been proposed as the driving force maintaining MHC diversity; balancing selection limits the fixation of alleles and promotes heterozygosity (Garrigan & Hedrick 2003; Muirhead 2001). Under a model of balancing selection the following genomic outcomes are predicted: (1) a greater number of nonsynonymous nucleotide substitutions, (2) even allele frequency distributions (Hedrick & Thomson 1983) and (3) allelic lineages typically outlasting the lifespan of the species (Garrigan & Hedrick 2003; Takahata & Nei 1990).

(1) Polymorphisms occur most frequently in the PBR with the rate of non-synonymous substitutions (d_N ; nucleotide sequence mutations that change the amino acid sequence) exceeding that of synonymous or silent substitutions (d_S ; Hughes & Nei 1989; Hedrick & Thomson 1983). The ratio of nonsynonymous to synonymous nucleotide substitutions per nonsynonymous and synonymous sites ($d_N : d_S$), a test proposed by Hill & Hastie (1987) and then Hughes & Nei (1989), is the most commonly used approach to detect long-term effects of balancing selection. Synonymous mutations are essentially neutral and the rate of evolution is expected to equal the mutation rate. Nonsynonymous mutations are susceptible to selection resulting in the removal of deleterious

changes by purifying selection; the rate is therefore less than the expected neutral rate ($d_N : d_S < 1$), whereas advantageous mutations are retained and the rate would be greater than the neutral rate ($d_N : d_S > 1$). This test has been applied in many studies of natural vertebrate populations to corroborate the effects of balancing selection acting on MHC genes; Bernatchez & Landry (2003) reviewed 48 of these studies and showed that only one example did not exhibit an excess of nonsynonymous to synonymous substitutions.

(2) Theoretical models of balancing selection predict that heterozygous individuals are more fit than homozygous individuals as the expression of different alleles facilitates the recognition and activation of the immune response against a greater number of pathogens (Doherty & Zinkernagel 1975). Natural selection acting on populations would therefore be reflected in an increased frequency of heterozygotes compared to that expected under HWE (Piertney & Oliver 2006; Hedrick *et al.* 2000b).

(3) The levels of polymorphism reported in many vertebrate species could only have been generated by a high substitution or mutation rate, especially in evolutionarily young species such as humans and the house mouse (*Mus domesticus*), but MHC genes do not evolve more rapidly than other genes (Klein *et al.* 2007). Two mouse species that diverged 1 to 2 MYA expressed similar or identical MHC alleles suggesting that the allelic lineages descended from a common ancestor and are older than the species in which they are expressed (Klein *et al.* 2007). Trans-species polymorphism (TSP) is the non-neutral retention of alleles across species and is used to analyse the effects of long-term selection on MHC genes (Bernatchez & Landry 2003; Edwards & Hedrick 1998). For example, Garrigan & Hedrick (2001) reported phylogenetic analyses that showed 12 MHC class I alleles in a population of Chinook salmon were derived from two allelic lineages that were also observed in four other Pacific salmon species. In addition, the most recent common ancestor of all 12 alleles existed approximately 15 MYA. TSP has been reported in a range of other vertebrates, including ungulates (Van Den Bussche *et al.* 2002) and pinnipeds (Hoelzel *et al.* 1999).

Balancing selection plays an important role in the generation of new diversity at MHC genes. The two models of selection proposed to explain this role, (1) parasite-mediated selection (Dean *et al.* 2002; Hess & Edwards 2002; Penn *et al.* 2002; Edwards & Hedrick 1998; Hughes & Yeager 1998; Apanius *et al.* 1997) and (2) sexual selection (Potts & Wakeland 1993; Hedrick 1992) are strongly contested, although not necessarily mutually exclusive (for a review see Piertney & Oliver 2006; Sommer 2005).

(1) MHC diversity and heterozygosity plays an integral role in the resistance or susceptibility to autoimmune disease and infectious pathogens (Apanius *et al.* 1997). The “heterozygote advantage or over-dominance” hypothesis suggests that heterozygous individuals have increased potential for antigen recognition and presentation (Penn *et al.* 2002; Parham & Ohta 1996; Potts & Slev 1995; Hughes 1992; Takahata *et al.* 1992). The “negative frequency dependant selection or rare allele advantage” hypothesis proposes a cyclical frequency dependent co-evolutionary “arms race” between host organisms and parasites (Penn & Potts 1999; Slade & McCallum 1992; Hill *et al.* 1991; Takahata & Nei 1990; Bodmer 1972; Clarke & Kirby 1966). MHC alleles that confer resistance to parasites provide a selective advantage and therefore spread throughout the population, applying selective pressure on parasites to evolve to avoid detection. At this point, the selective advantage shifts to new, rare alleles that are resistant to the parasites. For example specific MHC alleles appear to provide immune resistance to helminthic infections in hairy-footed gerbils (*Gerbillurus paeba*; Harf & Somer 2005).

(2) Sexual selection is a mechanism associated with the generation of MHC diversity and these genes are proposed as candidate genes for the genetic component of mate choice (Brown & Eklund 1994; see Chapter 4). For example, female White-tailed deer (*Odocoileus virginianus*) preferred the scent of MHC heterozygous males (Ditchkoff *et al.* 2001). MHC-mediated mate choice reportedly maintains diversity in many natural outbred populations (for a review see Piertney & Oliver 2006; Garrigan & Hedrick 2003; Bernatchez & Landry 2003; Apanius *et al.* 1997). The “heterozygote advantage” must, however, be balanced against the cost of expressing multiple alleles that could potentially cause autoimmune disease and an optimal level of MHC heterozygosity would provide the greatest level of fitness (Penn & Potts 1999). Reusch *et al.* (2001) proposed the “allele counting” hypothesis based on mate choice studies in three-spined sticklebacks (*Gasterosteus aculeatus*) where females chose genetically compatible mates by counting alleles to ensure an optimal number of alleles in their offspring.

A principle aim of evolutionary biology is to understand how natural selection shapes local adaptation and how selection interacts with mutation, migration and genetic drift. The adaptive potential within and among populations is critical for the long-term survival of a species. Previous studies show that levels of MHC diversity affect individual fitness, either via reproductive success or survival probability of progeny to infectious pathogens (Bernatchez & Landry 2003; Paterson *et al.* 1998). For example Aguilar *et al.* (2004) reported that San Nicolas Island foxes (*Urocyon littoralis dickeyi*) are the most genetically depauperate sexually reproducing mammal yet studied

and lack diversity at all hypervariable markers. The species does, however, show surprisingly high levels of heterozygosity at four of the five MHC loci examined. A $d_N : d_S$ substitution ratio in the PBR > 1 relative to non-PBR sites suggested that the effects of balancing selection could counterbalance genetic drift in small populations (Aguilar *et al.* 2004). Seddon & Baverstock (1999) showed that balancing selection maintained MHC diversity in small, isolated island populations of Australian bush rats (*Rattus fuscipes greyii*) with three of the populations having greater diversity than expected under neutrality.

Demographic bottlenecks in natural populations, depending on the extent and duration of the event, could decrease diversity in MHC genes and render individuals susceptible to infectious pathogens. This was reported in the classical example of the cheetah (*Acynonyx jubatus*; O'Brien *et al.* 1985). Limited MHC diversity has been described in other endangered species that have experienced severe demographic bottlenecks, for example the mountain goat (*Oreamnos americanus*; Mainguy *et al.* 2007), Przewalski's horses (*Equus przewalskii*; Hedrick *et al.* 1999), Mexican wolf (*Canis lupus baileyi*; Hedrick *et al.* 2000a) and Arabian oryx (*Oryx leucoryx*; Hedrick *et al.* 2000b).

Chapter Research Aims

The following hypotheses are tested in this chapter:

An unprecedented demographic bottleneck in the Southern white rhinoceros, together with subsequent founder events that aimed to restore populations to their historic distribution range, have resulted in the significant loss of neutral genetic diversity with seeded populations exhibiting less variation than the source population. Diversity at adaptive loci, shaped predominantly by balancing selection, is retained despite population decline.

Given the unprecedented historic demographic bottleneck and multiple subsequent founder events, the extensive loss of genetic diversity and increased potential for inbreeding depression within extant SWR populations, and particularly seeded populations, is predicted. As indicated by the high levels of fecundity and rapid recruitment rates in free-ranging populations SWR do not, however, currently demonstrate the expected effects of inbreeding depression (described in Chapter 1). The following research objectives, using data from both selectively neutral and adaptive DNA markers, are investigated in this chapter:

- 1.) To calculate levels of contemporary genetic diversity retained in populations of SWR in southern Africa according to expectations under a neutral model. Data from the source Hluhluwe Imfolozi Game Reserve population are compared with that from three seeded populations to determine whether population founding events have resulted in reduced diversity in seeded populations. Genetic diversity is estimated from 13 nuclear microsatellite loci, the mtDNA control region and exon 2 sequence data of the *DQA* and *DQB* MHC genes.
- 2.) To determine the extent of genetic differentiation among source and seeded SWR populations, relative to the time since each founding event as well as founder number. Genetic differentiation was quantified using a number of approaches including the co-efficients F_{ST} and R_{ST} together with a principal co-ordinates analysis (PCA) using microsatellite data and pairwise Φ_{ST} estimates, together with an AMOVA (analysis of molecular variance), based on mtDNA control region haplotypes.

- 3.) To determine whether SWR populations exhibit genetic evidence of demographic decline and test whether seeded populations have experienced a loss of adaptive and neutral genetic diversity as a result of founder events. Two statistical approaches, based on (1) deviations in levels of heterozygosity from that expected in an equivalent population at mutation-drift equilibrium and (2) the observed number of alleles relative to the total range in allele sizes, are used to analyse the microsatellite data. To inform our understanding of the future sustainability of this taxon under current management approaches, these data were also used to simulate the expected loss of diversity, via genetic drift, in hypothetical populations of different sizes seeded from the Hluhluwe Imfolozi Game Reserve population.

Molecular methods for all data analysed in this chapter are described in Chapter 2.

Analytical Methods

Analyses of Microsatellite Data

Contemporary levels of genetic diversity in SWR were estimated from neutral microsatellite loci using the program FSTAT v2.9.1 (Goudet 1995). Allele frequencies (Appendix I) and allelic richness (number of alleles per locus corrected for population size) were calculated for all populations. Levels of heterozygosity (H_{Obs} and H_{Exp}), gametic disequilibrium and deviations from Hardy-Weinberg proportions (HWE) for each locus, and in each population, were calculated in Arlequin v3.11 (Excoffier *et al.* 2005). Weir & Cockerham's (1984) inbreeding estimator (F_{IS}), measuring heterozygosity excess or deficit within subpopulations relative to HWE, was calculated in FSTAT v2.9.1. The polymorphic informative content (PIC) of the data, a measure of genetic diversity that incorporates both the number and frequency of alleles at a particular locus (Botstein *et al.* 1980), together with tests for the presence of null alleles and allelic dropout were determined using CERVUS v3.0 (Kalinowski *et al.* 2006; Marshall *et al.* 1998). Additional standard statistical tests were calculated in Statistica v7 (Statsoft) to determine whether measures of genetic diversity, described by allele number and heterozygosity, were significantly different between populations.

The degree to which allele frequencies in the three seeded population (WGR, WPP and MNP) had diverged from the source HIR population was used to investigate the effect of founding population size and time since founding on contemporary variation in SWR. Population structure was investigated using AMOVA (Weir 1996) in Arlequin v3.11 to determine how genetic variance was partitioned among the four populations; significance ($\alpha = 0.05$) was assessed using 10000 permutations. Levels of divergence between populations was quantified using F_{ST} (Weir 1996; Weir & Cockerham 1984), calculated in FSTAT 2.9.1 (Goudet 1995), and R_{ST} (Slatkin 1995), based on a step-wise mutation model that approximates the model of evolution observed at microsatellite loci, calculated in RstCalc (Goodman 1997). A PCA based on Nei's genetic distance (Nei 1972) was performed using GenAlEx v6.1 (Peakall & Smouse 2005).

Two different approaches were used to examine the data for genetic signatures of severe reductions in population size. Deviations in the levels of heterozygosity (heterozygosity excess; H_e), from that expected in an equivalent population at mutation-drift equilibrium (H_{eq}), were calculated in BOTTLENECK v1.2.02 (Piry *et al.* 1999). Statistical significance was determined using both a sign test and a Wilcoxon sign-rank test implemented in the program. The standardised differences test was not used as this requires at least 20 polymorphic loci, for fewer than 20 loci the Wilcoxon's test

has been shown to be the most powerful and appropriate test (Piry *et al.* 1999). During a population bottleneck, allelic diversity is lost at a faster rate than heterozygosity (Cornuet & Luikart 1996). This is predominantly the result of the initial loss of rare alleles, which contribute relatively little to heterozygosity; the predicted outcome is that H_e will therefore exceed H_{eq} at most loci. Heterozygosity excess lasts until a new equilibrium is reached in the population and only recent reductions in N_e can therefore be detected. “Recent” can be defined as the past 0.2 to 4.0 N_e generations (Cornuet & Luikart 1996). The TPM (Di Rienzo *et al.* 1994) of microsatellite evolution was applied as this is more appropriate than the strict SMM when analysing microsatellite data (Piry *et al.* 1999). The TPM parameters were set at 95% single-step mutations and 5% multiple-step mutations and with the variance among multiple steps set at 12 as suggested by Piry *et al.* (1999). To assess the robustness of this test, additional tests with the single-step mutation percentage set at 90% and 99% were performed. As a second approach, the mean ratio of the observed number of alleles (k) to their total range in allele size was calculated using Garza & Williamson’s (2001) M ratio test, where $M = k/r$. Bottlenecked populations are predicted to lose alleles at a faster rate relative to the total range in allele size, and thereby reduce the ratio M under the SMM. This bottleneck signature can persist for hundreds of generations if the N_e or mutation rate remains sufficiently low (Garza & Williamson 2001), enabling the detection of ancient bottlenecks that may be difficult to observe with the transient heterozygosity excess approach detailed by Cornuet & Luikart (1996). M_P_VAL (Garza & Williamson 2001) was used to calculate M with the following parameters: $\theta = 1$ ($4N_e\mu$), the proportion of one-step mutations (p_s) = 0.9 and average size of non-one-step mutations (Δg) = 3.5. To assess the robustness of this test, additional analyses were performed using θ values ranging from 0.1 to 10, p_s ranging from 0.8 to 0.99, and Δg ranging from 2.5 to 6 for 10 000 replicates. Significance was determined by estimating the critical ratio (M_c) using simulations in $CRITICAL_M$ (Garza & Williamson 2001) for $\theta = 1$ and $\theta = 10$, whereby 95% of 10000 simulations of an equivalent population at equilibrium had $M > M_c$.

A simulation-based approach was used to explore the potential future loss of allelic diversity and heterozygosity in SWR populations that were founded and subsequently maintained at 10, 50 or 100 individuals. Simulations were carried out over 200 years for 1000 iterations using the simulation software of BOTTLESIM v2.6 (Kuo & Janzen 2003). Simulations were performed for constant population size using the following life-history parameters: (1) The potential longevity is estimated at 45 years (Owen-Smith 1988), (2) age of sexual reproduction is 8 years (the median between females that reproduce at 6.5 ± 0.5 years and males that, while physically capable of breeding at 7

to 8 years, usually only reproduce later because older territorial males tend to breed with most females in the population (Owen-Smith 1988)), (3) generations were maximally overlapped with all individuals starting at a random age estimate, (4) random mating, (5) 1:1 male to female ratio (median estimate to reflect the 173:100 male to female ratio in HIR and 147:100 ratio in zoo populations (Owen-Smith 1988), and newly founded populations that usually include more females than males (Kretzschmar 2001; Rachlow & Berger 1998)).

Sequential Bonferroni corrections were not applied to multiple pairwise comparisons because this increases the probability of type II errors (β : the incorrect assumption that no differentiation exists), an effect that becomes progressively worse as p -values are discarded (Moran 2003).

Analyses of mtDNA Control Region Sequences

Nucleotide sequences were edited using the program Chromas Lite v2.01 (Technelysium (PTY) Ltd. [<http://www.technelysium.com.au>]) and aligned using a ClustalW multiple alignment method implemented in BioEdit Sequence Alignment Editor (Hall 1999). The four resulting control region haplotypes, determined from 102 SWR, have been deposited in GenBank (Accession No. FJ004915 – FJ004918).

Contemporary levels of genetic diversity in SWR at mtDNA control region sequences were estimated from the number of unique haplotypes identified, levels of nucleotide diversity (π , number of nucleotide differences between pairs of sequences; Tajima 1983) and haplotype diversity (h , equivalent to H_{Exp} for microsatellite markers; Nei 1987) within each population using DnaSP v4.10.3 (Rozas *et al.* 2003). Sequences gradually diverge from ancestral sequences as a result of nucleotide substitutions and/or small insertions or deletions and therefore the proportion of variable nucleotide sites is a simple measure of the divergence between sequences. The transition/transversion ratio was determined empirically. Population structure was inferred using AMOVA (Weir 1996) in Arlequin v3.11 (Excoffier *et al.* 2005) to determine how mtDNA control region haplotype variation is partitioned among the source population and three seeded populations. An exact test of pairwise population differentiation (Φ_{ST}) was also calculated using Arlequin v3.11.

Analyses of Major Histocompatibility Complex Sequences

SSCP data from the *DQA* and *DQB* MHC loci were visually assessed to determine the number of unique motifs resulting from the capillary runs at 18°C, 30°C and 45°C. Motifs were recorded and systematically confirmed as correct by rechecking the results with the original fluorescent SSCP peak profiles. Exon 2 *DQA* and *DQB* nucleotide sequences were edited in Chromas Lite v2.01 and aligned using the ClustalW multiple alignment method implemented in BioEdit.

Levels of genetic diversity in SWR MHC genes were initially estimated by calculating the number of unique alleles in each data set. The transition/transversion ratio was determined empirically. Putative PBR sites in SWR *DQA* and *DQB* exon 2 homologues were identified by amino acid alignments to reported PBR sites in human sequences (Brown *et al.* 1993). To test whether these loci are evolving under balancing selection, the $d_N : d_S$ ratio (Nei & Gojobori 1986) was calculated for PBR and non-PBR sites of the *DQA* and *DQB* exon 2 sequences in MEGA v3.1. (Kumar *et al.* 1993).

Individual nucleotide sequences were translated into amino acid sequences using BioEdit and aligned to the corresponding human leukocyte antigen system (HLA; Genbank Accession No. NC000006), canid (DLA; NC006594, *Canis familiaris*), bovid (BoLA; NC007324, *Bos taurus*), suid (SLA; NC010449, *Sus scrofa*) and equid (ELA; NC009163, *Equus caballus*) *DQA* and *DQB* exon 2 amino acid sequences obtained from GenBank using a ClustalW multiple alignment method. The naming of white rhinoceros alleles, discovered in this study, follows the nomenclatural rules of Klein *et al.* (1990) and Ellis *et al.* (2006).

Results

Genetic Diversity

Screening 32 microsatellite loci yielded thirteen loci that were polymorphic in the SWR, except locus Rh8 that was monomorphic in the WPP. Low levels of diversity characterised all four populations (Table 3.1.; Appendix I). The total number of alleles at each locus ranged from one to four with an average of 2.6 across all the populations. Allelic richness did not differ significantly among the populations (HIR 2.8; WGR 2.7; WPP 2.3; MNP 2.5; Mann-Whitney U test, pairwise t-test; $p > 0.05$) and no private alleles were observed. Heterozygosity per locus varied from 0.00 to 0.73 with mean H_{Exp} of 0.45 and H_{Obs} of 0.44 across all loci and populations, but no significant difference in H_{Obs} was observed between the populations (HIR 0.46; WGR 0.39; WPP 0.46; MNP 0.43; Mann-Whitney U test, pairwise t-test; $p > 0.05$). There is also no difference in allelic richness or H_{Obs} between the adult (>6 years), sub-adult (2.5 to 6 years) and calf (<2.5 years) age classes (age data only available for WGR and MNP) suggesting that genetic drift acting on recent generations has not resulted in an immediate depletion in genetic diversity between generations since founding (Mann-Whitney U test, pairwise t-test; $p > 0.05$). PIC values averaged across all the loci were 0.4 in HIR and ranged from 0.34 to 0.38 in the seeded populations. The PIC incorporates both heterozygosity and allele number and therefore these values were slightly lower than the heterozygosity as a result of the low levels of allelic richness. All of these genetic diversity parameters indicate that the three seeded populations show minimal loss of diversity relative to the source population, irrespective of founder number or time since founding.

Twelve of the microsatellite loci displayed expected Hardy-Weinberg genotypic proportions for all the populations (Table 3.1.). The exception was locus DB66 that deviated from HWE in three of the four populations and displayed significant estimates of null allele frequencies ($p = 0.448$) across all the populations. Weir & Cockerham's (1984) F_{IS} inbreeding estimator, measuring the excess of homozygotes within populations relative to HWE, showed balanced homozygote-heterozygote proportions for the HIR, WPP and MNP populations (0.068, -0.07 and 0.009, respectively). Only WGR exhibited an excess of homozygotes with an F_{IS} of 0.153. The F_{IS} values, however, showed balanced proportions for all populations (HIR, WGR, WPP and MNP values of -0.025, 0.065, -0.094 and -0.080, respectively) when recalculated excluding locus DB66.

There was significant ($p < 0.05$) gametic disequilibrium between pairs of loci within populations, however, none were consistent across all the populations. Of the 78 possible combinations of pairs of loci there was significant gametic disequilibrium at four pairs of loci in the HIR population, six

in WGR, four in WPP and 17 in MNP (Table 3.2.). This disequilibrium is not indicative of linkage but rather the result of nonrandom associations generated by genetic drift in small populations (Allendorf & Luikart 2007; Ledig *et al.* 1999).

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	Hluhluwe Imfolozi Reserve						Welgevonden Game Reserve						Waterberg Plateau Park						Matobo National Park					
	A	AR	H _{Obs}	H _{Exp}	PIC	HWE	A	AR	H _{Obs}	H _{Exp}	PIC	HWE	A	AR	H _{Obs}	H _{Exp}	PIC	HWE	A	AR	H _{Obs}	H _{Exp}	PIC	HWE
a _{7B}	3	3.0	0.64	0.53	0.47	0.75	3	3.0	0.59	0.59	0.52	0.84	3	3.0	0.67	0.50	0.42	0.27	3	3.0	0.61	0.55	0.48	0.57
a _{7C}	3	3.0	0.43	0.48	0.41	0.22	3	3.0	0.47	0.51	0.43	0.21	2	2.0	0.28	0.32	0.26	0.51	3	3.0	0.60	0.56	0.46	0.39
a _{32A}	2	2.0	0.48	0.51	0.38	1.00	2	2.0	0.50	0.45	0.35	0.52	2	2.0	0.39	0.51	0.37	0.37	2	2.0	0.43	0.41	0.33	1.00
b _{AY138542}	3	3.0	0.68	0.66	0.57	0.50	3	3.0	0.57	0.61	0.52	0.60	3	3.0	0.67	0.51	0.49	0.34	3	3.0	0.73	0.65	0.57	0.65
b _{AY138545}	2	2.0	0.55	0.48	0.36	0.65	2	2.0	0.47	0.50	0.37	0.77	2	2.0	0.44	0.51	0.37	0.66	2	2.0	0.46	0.42	0.33	0.73
c _{Rh6}	4	4.0	0.71	0.61	0.55	0.23	4	3.9	0.37	0.47	0.43	0.09	4	4.0	0.56	0.57	0.47	0.80	3	2.8	0.18	0.17	0.16	1.00
d _{DB1}	2	2.0	0.14	0.19	0.17	0.26	2	2.0	0.20	0.25	0.22	0.23	2	2.0	0.44	0.36	0.29	0.53	2	1.9	0.10	0.10	0.09	1.00
d _{DB44}	3	2.9	0.32	0.28	0.26	1.00	3	2.4	0.22	0.26	0.23	0.35	2	2.0	0.56	0.46	0.35	0.60	3	3.0	0.55	0.49	0.42	0.26
d _{DB49}	3	3.0	0.67	0.67	0.58	0.80	3	3.0	0.63	0.66	0.58	0.96	3	3.0	0.72	0.68	0.58	0.77	3	3.0	0.65	0.64	0.56	0.91
d _{DB66}	3	2.7	0.14	0.50	0.39	0.00*	3	2.8	0.14	0.52	0.41	0.00*	2	2.0	0.06	0.16	0.14	0.09	2	2.0	0.19	0.50	0.37	0.00*
e _{Rh7}	2	2.0	0.43	0.38	0.31	0.65	2	2.0	0.20	0.28	0.24	0.10	2	2.0	0.50	0.51	0.37	1.00	2	2.0	0.57	0.45	0.34	0.06
e _{Rh8}	3	3.0	0.21	0.40	0.35	0.01*	3	2.7	0.16	0.15	0.15	1.00	1	1.0	0.00	0.00	0.00	-	3	2.9	0.27	0.28	0.26	0.52
e _{Rh9}	4	3.6	0.64	0.57	0.46	0.68	4	3.1	0.55	0.54	0.43	0.26	2	2.0	0.72	0.51	0.37	0.15	2	2.0	0.26	0.29	0.25	0.6
Mean	2.8	2.8	0.46	0.48	0.40		2.8	2.7	0.39	0.45	0.38		2.3	2.3	0.46	0.43	0.34		2.5	2.5	0.43	0.42	0.36	

Table 3.1.

A summary of parameters of genetic diversity for each microsatellite locus indicating the number of individuals sampled (n), observed number of alleles (A), allelic richness corrected for population size (AR), observed (H_{Obs}) and expected (H_{Exp}) heterozygosity, polymorphic informative content (PIC) and Hardy-Weinberg equilibrium (*significant departure from HWE, $\alpha = 0.05$). Microsatellite loci were isolated in the white rhinoceros (^aFlorescu *et al.* 2003; ^bNielsen *et al.* 2008), black rhinoceros (^cCunningham *et al.* 1999; ^dBrown & Houlden 1999) and Indian rhinoceros (^eZschokke *et al.* 2003).

Population	7B & 32A	7B & BR6	32A & AY138545	7B & AY138542	7B & AY138542	AY138542 & AY138545	AY138542 & DB66	7B & DB44	7B & DB49	7B & DB66	7C & DB49	7C & Rh7	7C & Rh8	7C & Rh9	32A & Rh8	32A & Rh9	BR6 & Rh7	BR6 & Rh8	BR6 & Rh9	DB44 & DB49	7B & 32A	7B & 32A	7B & 32A	7B & 32A	7B & 32A	7B & 32A
HIR	-	0.03	0.00	-	-	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04	-	-	-	-	-
WGR	-	-	-	-	-	0.04	0.03	-	-	-	-	-	-	-	-	-	0.04	0.02	-	-	0.04	-	-	-	0.00	-
WPP	-	-	0.00	-	0.02	-	-	0.01	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MNP	0.00	-	-	0.01	-	0.00	-	-	0.04	0.02	0.02	0.02	0.00	0.02	0.01	0.00	-	-	0.01	0.00	-	0.04	0.01	0.00	-	0.01

Table 3.2.

Gametic disequilibrium ($p < 0.05$) observed between specific pairs of microsatellite loci for each SWR population.

Four unique mtDNA control region haplotypes (SWR1, SWR2, SWR3 and SWR4) were identified from 102 SWR (Table 3.3.; Appendix II), including 22 individuals from HIR, 35 from WGR, 14 from WPP and 31 from MNP. One of these haplotypes was present in a single individual from the source HIR population. These haplotypes are characterised by a total of seven polymorphic sites consisting of six transition and one transversion nucleotide changes across the 750 bp fragment. The haplotype diversity (h) was 0.62 and the nucleotide diversity (π) was 0.004 across all the sequences in the HIR population. In the seeded populations, $h = 0.53$ (3 haplotypes) and $\pi = 0.003$ in WGR, $h = 0.44$ (2 haplotypes) and $\pi = 0.003$ in WPP and $h = 0.49$ (2 haplotypes) and $\pi = 0.001$ in MNP (2 haplotypes differing by a single polymorphic site).

Haplotype	Nucleotide position							HIR	WGR	WPP	MNP	<i>N</i>
	1	1	3	3	5	6	6					
	3	5	3	9	9	7	9					
	1	9	6	2	6	5	4					
SWR 1	C	T	C	T	T	G	T	2	2	-	12	16
SWR 2	A	.	12	12	10	19	53
SWR 3	T	C	T	C	.	A	C	7	21	4	-	32
SWR 4	T	C	T	C	A	A	C	1	-	-	-	1

Table 3.3.

MtDNA control region haplotypes identified in SWR, indicating the nucleotide position of each segregating site, the nucleotide substitution, the number of each haplotype observed in the populations (HIR, WGR, WPP and MNP) and the total number of each haplotype (N).

To identify polymorphic trans-species lineages, alleles from the MHC *DQA* and *DQB* exon 2 were compared in samples from both SWR and NWR. Trans-species polymorphism can be used to determine the effects of long-term selection on MHC genes (Bernatchez & Landry 2003; Edwards & Hedrick 1998).

The *DQA* SSCP data from 126 SWR from the source HIR and seeded WGR and MNP populations were assigned six motifs: AAA (58.6%), BAA (27.6%), CCC (4.9%), BBB (4.1%), BAA (2.4%) and BCB (2.4%). A single additional motif was assigned to all NWR from the Garamba National Park (GNP). Exon 2 of the *DQA* gene was either directly sequenced or cloned and sequenced for six SWR and two NWR samples to identify the nucleotide sequences represented by each SSCP motif. Four unique sequences were identified. All of these sequences were observed in one NWR and it can therefore be assumed that there are at least two *DQA* loci in white rhinoceroses. Alignments of nucleotide (Figure 3.4.) and amino acid sequences (Figure 3.5.) showed that two alleles, Cesi-DQA*01 and Cesi-DQA*02, differed by a single synonymous T to C transition (193T>C) in a non-PBR site and the remaining two sequences, Cesi-DQA*03 and Cesi-DQA*04, differed by a synonymous G to A transition (155G>A) at a PBR site. When these two pairs of sequences were compared with each other and the human HLA-DQA1 sequence, there was a 5 bp deletion in the Cesi-DQA*03 and Cesi-DQA*04 sequences. There were also 43 nucleotide substitutions (25 transitions and 18 transversions) between the two pairs of sequences. The $d_N : d_S$ ratio could not be calculated for either pair of sequences as they each differed by a single synonymous mutation.

The amino acid translations of Cesi-DQA*01 and Cesi-DQA*02 alleles were consistent with functional *DQA* alleles in human and additional mammalian species (Figure 3.5.). The 5 bp deletion in the Cesi-DQA*03 and Cesi-DQA*04 sequences, however, altered the reading frame and introduced three stop codons to the translated amino acid sequence. This change in the reading frame affected 15 PBR sites and most likely results in a non-functional protein; in all likelihood these alleles constitute a *DQA* pseudogene in white rhinoceroses. The four nucleotide sequences, that represent a single amino acid sequence, were observed in 25 white rhinoceros samples. As a result, and regardless of the assigned SSCP motif, SWR and NWR appear to be functionally monomorphic at the MHC *DQA* locus.

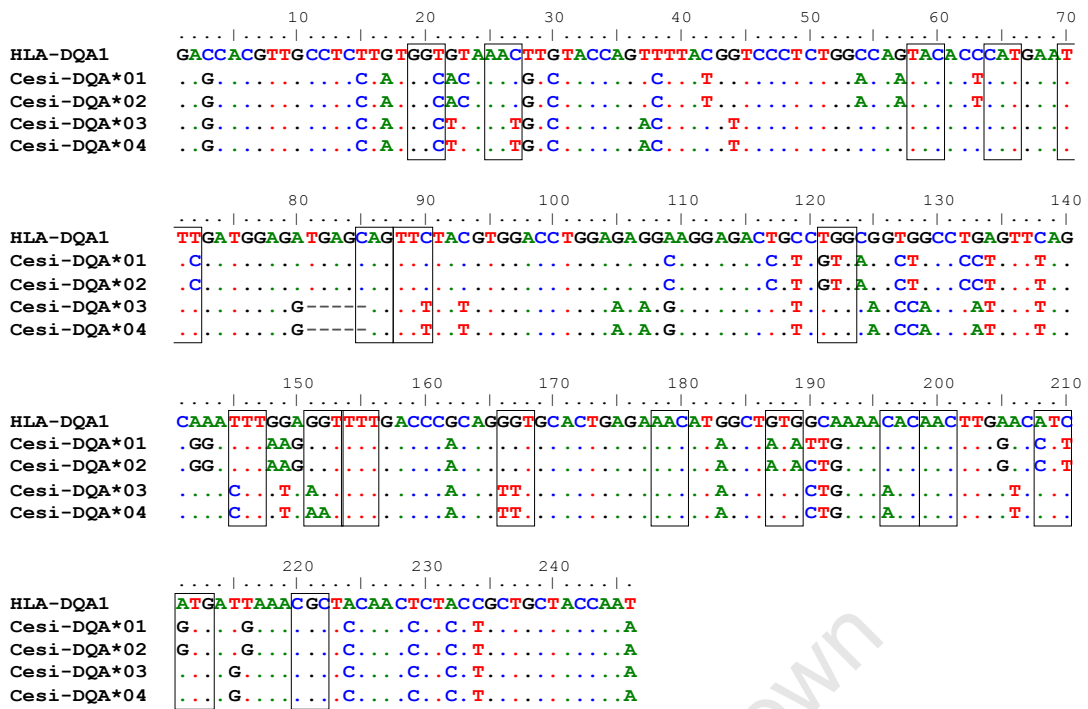


Figure 3.4.

Alignment of *DQA* allele nucleotide sequences to a human HLA-DQA1 sequence. Dots (.) show identity with the human sequence, hyphens (-) indicate the 5 bp deletion and the nucleotide positions of each putative PBR site are enclosed in a box.

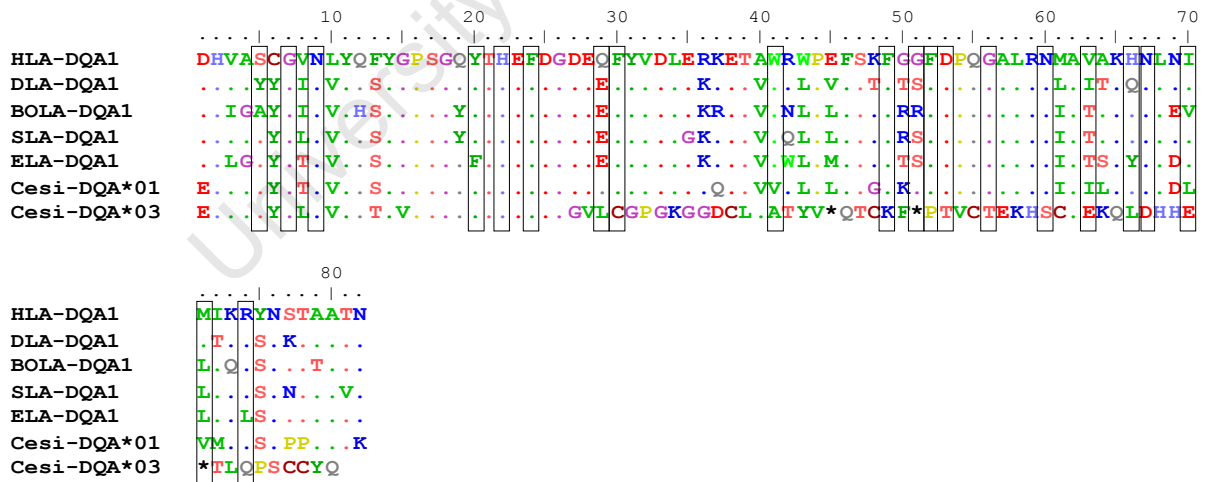


Figure 3.5.

Alignment of translated *DQA* amino acid sequences to human, canine, bovine, swine and equine DQA1 amino acid proteins. Dots (.) show identity with the human sequence, asterisks (*) indicate the inclusion of stop codons and the amino acid position of each putative PBR site is enclosed in a box.

The *DQB* SSCP data from 126 SWR from the source HIR and seeded WGR and MNP populations were assigned two motifs: AAA (95%) and BAA (5%). A single additional motif was assigned to all NWR from the Garamba National Park (GNP). The B motif is only occasionally observed at the lowest temperature and is probably the result of heteroduplex formations. Direct sequencing for 11 SWR individuals, 6 from HIR, 3 from WGR and 2 from MNP, that exhibited either AAA or BAA motifs, resulted in identical sequences with no ambiguous sites. NWR samples that were directly sequenced returned unresolved chromatograms and therefore 4 and 6 clones were sequenced for two samples, respectively. A single additional sequence was observed in NWR.

Alignments of nucleotide (Figure 3.6.) and amino acid sequences (Figure 3.7.) showed that the two sequences, Cesi-DQB*01 and Cesi-DQB*02, differed by three nucleotide changes: a synonymous A to T transversion (50A>T) at a PBR site, a nonsynonymous T to C transition (57T>C) that changed Tyrosine to Phenylalanine (Y17F) at a PBR site and a nonsynonymous A to G transition (112A>G) that changed Threonine to Alanine (T38A) at a non-PBR site. The sequence identified in all SWR, Cesi-DQB*01, had a 1 bp insertion at a non-PBR site that altered the reading frame but no stop codons were introduced and no PBR sites were affected. There were also a C to G transversion (C163G) and a T to C transition (T>C) downstream of the insertion. The Cesi-DQB*02 sequence, found only in NWR, aligned to the human HLA-DQB1 and additional mammalian sequences and was consistent with functional *DQB* loci sequences (Figure 3.7.). The $d_N : d_S$ ratio could not be calculated as only a single sequence was observed in SWR.

These results suggest two possible scenarios; firstly the sequence incorporating a 1 bp insertion may still encode a functional protein with all the required PBR sites and, as such, SWR are monomorphic at this locus or, alternatively, the data represent a single functional locus, identified in NWR, together with a *DQB* pseudogene in SWR. A mutation in the primer-binding region could have prevented the PCR amplification of the functional locus in SWR. A functional mRNA analysis is required to determine whether these loci are expressed in white rhinoceroses. Nonetheless, these results indicate unequivocally that SWR are genetically depauperate at the MHC *DQA* and *DQB* loci.

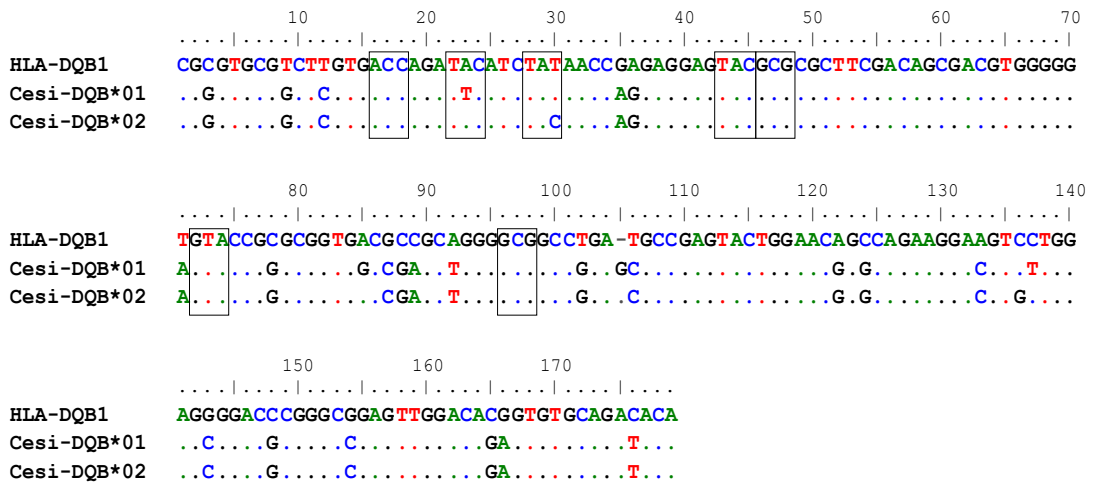


Figure 3.6.

Alignment of *DQB* nucleotide sequences to a human HLA-DQB1 sequence. Dots (.) show identity with the human sequence, a hyphen (-) indicates a 1 bp insertion and the nucleotide positions of each putative PBR site are enclosed in a box.

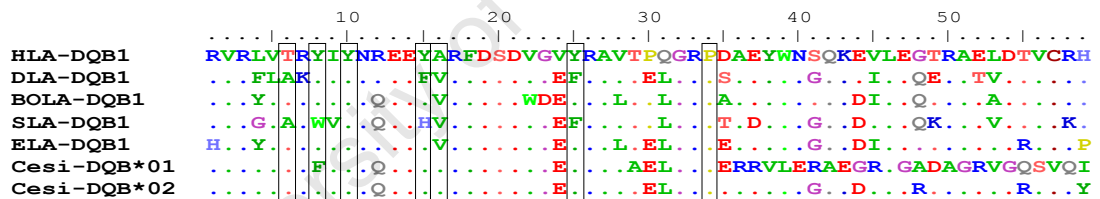


Figure 3.7.

Alignment of translated *DQB* amino acid sequences to human, canine, bovine, swine and equine *DQB*1 amino acid proteins. Dots (.) show identity with the human sequence and the amino acid position of each putative PBR site is enclosed in a box.

Founder Effects on Allele / Haplotype Frequencies

An AMOVA test of the microsatellite data indicated that variation within individuals accounted for 93% of the total variation detected. The overall F_{ST} of 0.049 indicated minimal differentiation among populations. The low number of alleles per locus and complete lack of private alleles suggests that differentiation between populations is the result of different allele distribution frequencies (Appendix I). Pairwise F_{ST} and R_{ST} estimates (Table 3.8.) varied from non-significant between HIR and WGR to highly significant between WPP and MNP and between the source population and each of the WPP and MNP populations ($p < 0.05$).

Population	HIR	WGR	WPP	MNP
HIR	-	0.003	0.044*	0.045*
WGR	0.002	-	0.068*	0.050*
WPP	0.053*	0.074*	-	0.106*
MNP	0.058*	0.044*	0.094*	-

Table 3.8.

Parameters of genetic differentiation (F_{ST} above diagonal and R_{ST} below diagonal) among SWR populations (* significant genetic differentiation, $\alpha = 0.05$).

These results were corroborated by a PCA of the microsatellite data based Nei's genetic distance amongst the SWR populations (Figure 3.9.) where 100% of the variance was explained by the first two components (81.09% and 18.91%, respectively). The HIR and WGR populations cluster together and are separate from both the WPP and MNP populations, verifying their genetic similarity and distinctiveness from the latter populations. The WGR population was recently founded, between 1994 and 1996, whereas WPP was founded in 1975 and MNP was founded between 1962 and 1967. The degree of differentiation between WPP and MNP reflects the effect of genetic drift in these isolated populations.

Principal Co-ordinates Analysis

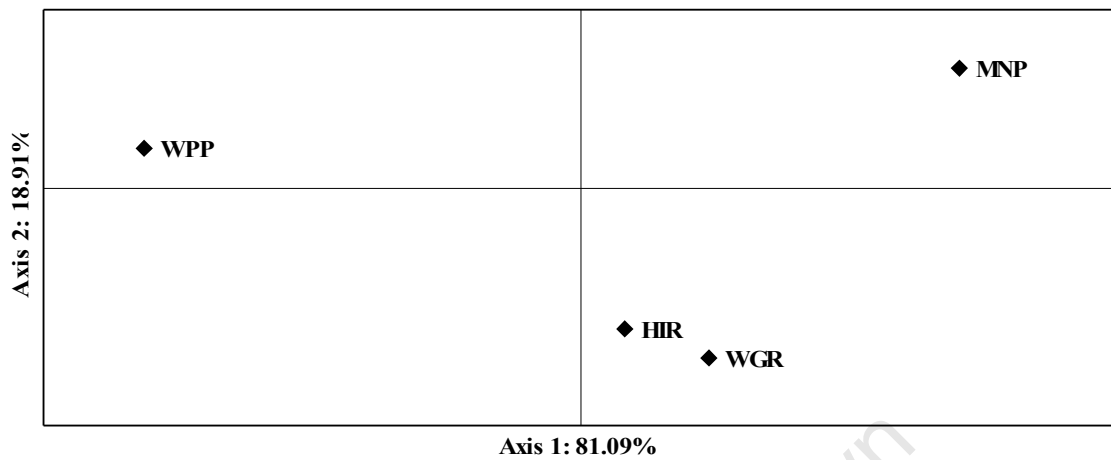


Figure 3.9.

A principal co-ordinates analysis (PCA) based on Nei's genetic distance amongst the SWR populations, PCA via Distance matrix with data standardisation, 100% of the variation is explained by the first two axes.

All four mtDNA control region haplotypes (Appendix II) were observed in the source HIR population and three of these were observed at different frequencies in the recently seeded WGR population. The haplotype SWR4 was found in a single individual in the source HIR population. Both WPP and MNP supported only two haplotypes; SWR1 was absent from WPP and SWR3 was absent from MNP. The differences in haplotype frequencies are indicative of founder events as a result of the random sampling of haplotypes in founder individuals and/or subsequent genetic drift in small populations where rare haplotypes are more likely to be lost (Figure 3.10.).

In contrast to the microsatellite data, an AMOVA test based on mtDNA control region sequences indicated that variation within populations accounted for 70.6% of the total variation detected and 29.4% among populations. And the overall Φ_{ST} of 0.294 indicated substantial differentiation in haplotype frequencies, with pairwise population Φ_{ST} estimates of 0.283 in HIR, 0.284 in WGR, 0.291 in WPP and 0.315 in MNP. The matrilineal and haploid mode of transmission of mtDNA markers results in a considerably reduced N_e in comparison to nuclear markers and are therefore more sensitive to the effects of genetic drift. Pairwise Φ_{ST} estimates (Table 3.11.) ranged from non-significant between the source HIR population and seeded WGR and WPP populations, to highly significant between HIR and MNP and between each of the seeded populations ($p < 0.05$).

Genetic differentiation is greatest between the two earliest seeded populations, WPP and MNP. WPP was founded in 1975 and MNP between 1962 and 1967, with no migration or introduction of new individuals. Thus, genetic drift has significantly altered the haplotype frequencies in each population since their founding, and different haplotypes have been lost from each population. There was non-significant differentiation between the source population and the recently founded WGR population and the WPP population. The extent of the differentiation is due to the relative frequencies of each haplotype with MNP, having SWR1 as the most common haplotype, being most different from the other populations as this haplotype is rare or absent from other populations.

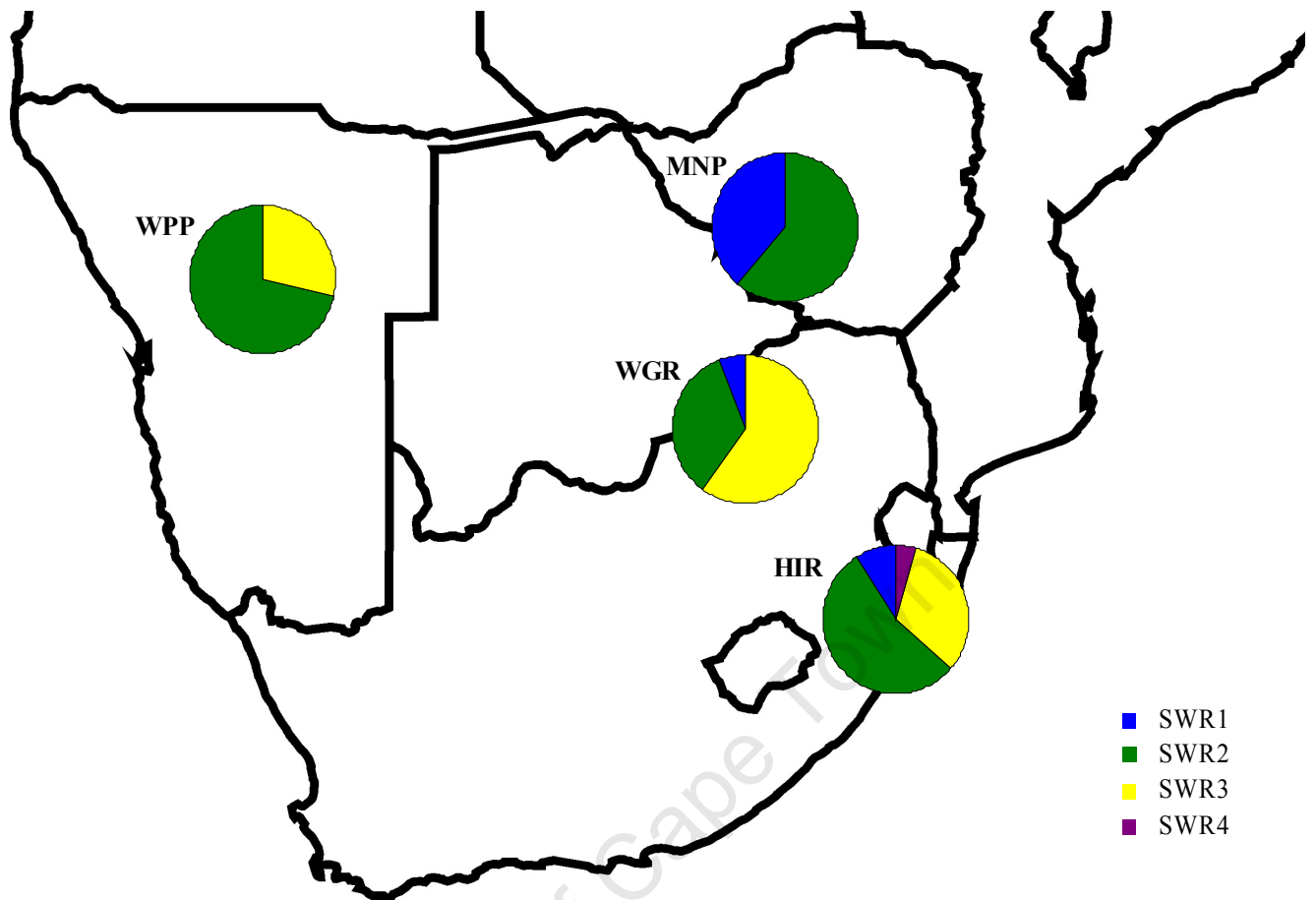


Figure 3.10.

Distribution frequencies of the mtDNA control region nucleotide sequence haplotypes as represented by pie charts for the source HIR population and WGR, WPP and MNP seeded populations.

Population	HIR	WGR	WPP	MNP
HIR	-			
WGR	0.066	-		
WPP	-0.044	0.131*	-	
MNP	0.333*	0.537*	0.334*	-

Table 3.11.

Pairwise Φ_{ST} estimates of genetic differentiation among SWR populations (*significant genetic differentiation, $\alpha = 0.05$).

Genetic Bottleneck Events

Despite the documented large-scale demographic decline in SWR population numbers, statistical analyses of allele frequency data revealed little support for the genetic effects of contemporary bottleneck events in the source HIR population (Table 3.12. and Table 3.13). This finding persisted regardless of the percentage of single-step mutations included in additional tests for deviations from H_{eq} or the parameters used to calculate the M ratio. No signature of a genetic bottleneck was found in the WGR and MNP populations despite being founded with 40 and 12 individuals, respectively. Only the seeded WPP population exhibited significant heterozygous excess for all tests and mutation models ($p < 0.05$), although the M ratio values for this population did not indicate a significant reduction in population numbers. The test for heterozygous excess compares the heterozygosity of the focus population to that of a theoretical population with similar allele frequencies at mutation-drift equilibrium. Previous analyses in this study have shown that these populations do not have significantly different allele numbers or heterozygosity and Cornuet and Luikart (1996) advise the use of at least 40 haploid genomes (20 diploid individuals) to achieve sufficient statistical power for analysis. These results may therefore be an artefact of the small sample size for WPP (18 individuals).

The M_c value, being the critical ratio generated by simulations where 95% of an equivalent population at equilibrium had $M > M_c$, calculated for these populations ranged between 0.43 ($\theta = 1$) and 0.47 ($\theta = 10$), which is less than the critical value of 0.68 reported by Garza & Williamson (2001). M ratios of between 0.70 and 0.72 (Table 3.13.) were calculated for SWR populations where, for 10000 replicates, a smaller ratio would be expected at equilibrium 100% of the time. These values exceed both the M_c calculated for these populations and the reported M_c , indicating that SWR populations do not exhibit reduced numbers of observed alleles relative to the total range in allele size, regardless of the parameters used in each analysis.

Despite large-scale demographic reductions in N_e in the HIR population approximately 13 generations ago and founder events between three and six generations ago in the seeded populations, neither statistical approach detected signatures of genetic bottlenecks in these populations.

	%	Hluhluwe Imfolozi Reserve		Welgevonden Game Reserve		Waterberg Plateau Park		Matobo National Park	
		SMM	TPM	SMM	TPM	SMM	TPM	SMM	TPM
Sign test	90	0.276	0.51	0.260	0.28	0.030*	0.05	0.302	0.47
	95	0.282	-	0.274	-	0.031*	-	0.296	-
	99	0.290	-	0.286	-	0.033*	-	0.486	-
Wilcoxon test	90	0.110	0.34	0.455	0.79	0.008*	0.013*	0.542	0.69
	95	0.191	-	0.588	-	0.011*	-	0.684	-
	99	0.305	-	0.735	-	0.011*	-	1.000	-

Table 3.12.

Heterozygosity excess in the source and seeded SWR populations assessed using the sign test and Wilcoxon test. *P* values are indicated for each analysis, the %SMM refers to the contribution of single-step mutations in TPM and hypens (-) indicate SMM analyses that are not applicable (*significant deviation from mutation drift equilibrium, $\alpha = 0.05$).

Hluhluwe Imfolozi Reserve		Welgevonden Game Reserve		Waterberg Plateau Park		Matobo National Park	
<i>M</i>	<i>M_c</i>	<i>M</i>	<i>M_c</i>	<i>M</i>	<i>M_c</i>	<i>M</i>	<i>M_c</i>
0.70	0.43	0.70	0.41	0.70	0.43	0.72	0.42

Table 3.13.

The *M* ratio test value for the source HIR and seeded populations, indicating the critical *M* value (*M_c*) calculated for each population.

As discussed in Chapter 1, by 1997 74% of discrete free-ranging populations in Africa were owned by the private sector and had an average “population” size of ~11 individuals compared with ~123 individuals in State owned parks. These “populations” are unlikely to be large enough to be considered biologically or evolutionarily viable; additionally, they are confined to fenced reserves with limited potential for dispersal and resultant gene flow. Simulations were therefore used to investigate the fate of genetic diversity in SWR populations founded and maintained at 10, 50 or 100 individuals for 200 years. Only data from HIR were used as HIR remains the main source of rhinoceroses for seeding programs.

The simulation results suggested that seeded populations of SWR are relatively resistant to the effects of genetic drift. Contemporary levels of genetic diversity in the HIR population are 2.8 alleles per locus and H_{Obs} of 0.46. The predicted levels of genetic diversity retained in seeded populations after 100 years, which equates to 12 to 13 generations, and 200 years, which equates to approximately 25 generations, are summarised in Table 3.14. and Figure 3.15. After 200 years, a population of 100 individuals would retain 77.5% of the existing number of observed alleles and 94.8% of the H_{Obs} , with 2.2 alleles per locus and H_{Obs} of 0.44 being retained. A population of 50 individuals would retain 62.7% of the observed alleles and 90.1% of the H_{Obs} , with 1.8 alleles per locus and H_{Obs} of 0.41 being retained. The population of 10 individuals, which represents the majority of contemporary SWR populations, would retain only 36.3% of the observed alleles and 62.7% of the H_{Obs} and have just 1.0 allele per locus and H_{Obs} of 0.29.

Population Size	Genetic diversity after 100 years				Genetic diversity after 200 years			
	Alleles retained	% Alleles lost	H_{Obs} retained	% H_{Obs} lost	Alleles retained	% Alleles lost	H_{Obs} retained	% H_{Obs} lost
100	2.5	10.7%	0.45	2.2%	2.2	12.4%	0.44	4.3%
50	2.2	21.4%	0.44	4.3%	1.8	35.7%	0.41	10.9%
10	1.5	46.4%	0.38	17.4%	1.0	64.3%	0.29	37.0%

Table 3.14.

The predicted loss of genetic diversity as measured by the number of alleles/locus and H_{obs} retained in hypothetical SWR populations founded and maintained at 10, 50 or 100 individuals over 100 and 200 years, respectively. The percentage of diversity lost for each scenario is also indicated.

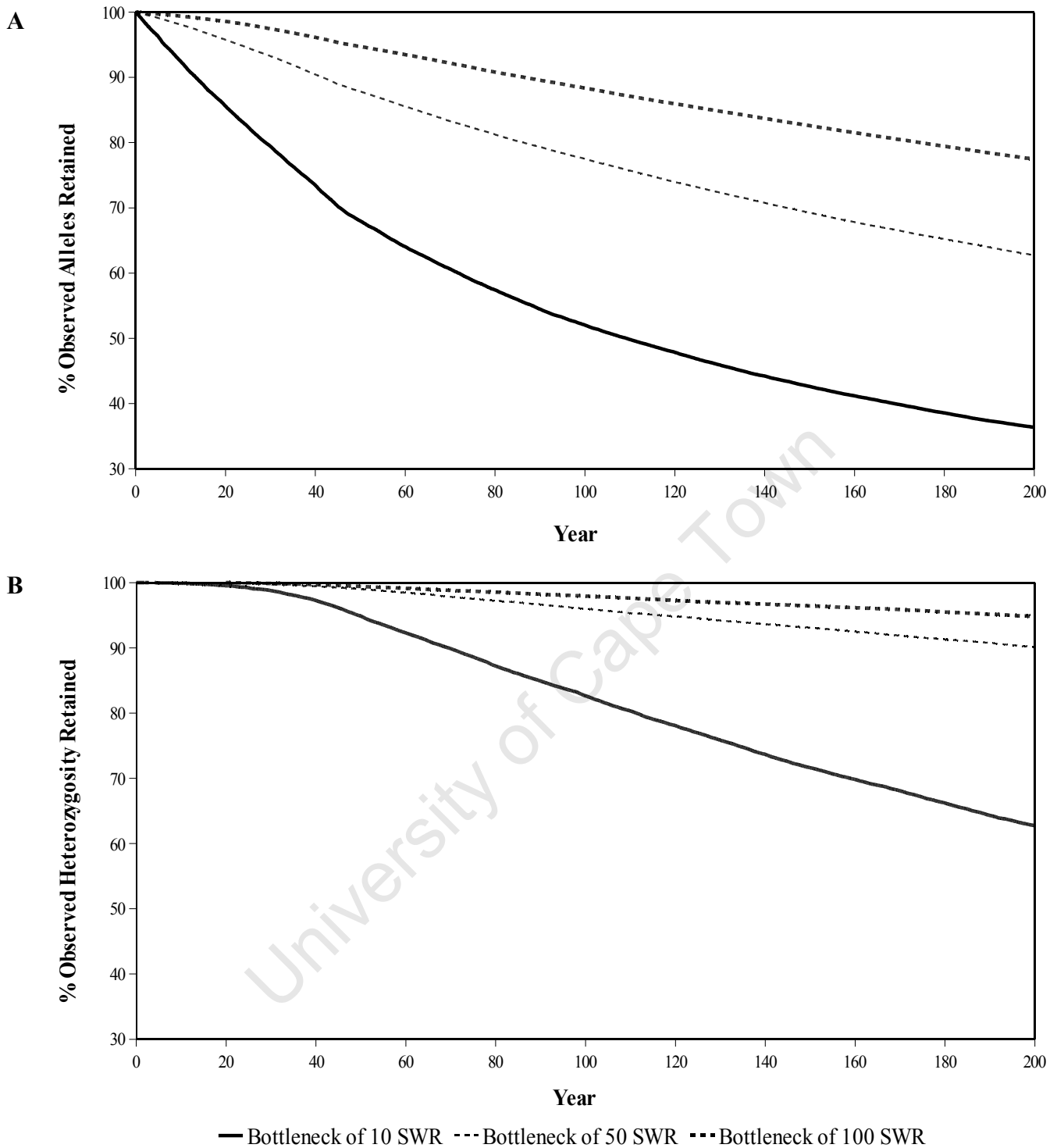


Figure 3.15.

A graphical representation of the predicted loss of genetic diversity measured by the percentage of alleles/locus (graph A) and H_{Obs} (graph B) retained in hypothetical SWR populations founded and maintained at 10, 50 or 100 individuals over 200 years.

Discussion

Genetic Diversity

The re-introduction of populations of threatened species is a vital component of modern conservation management (Griffith *et al.* 1989); however, this process may negatively affect genetic diversity in newly founded populations. Empirical studies show that seeded populations generally have reduced genetic diversity relative to the source population/s (for reviews see Parker 2008; Stockwell *et al.* 1996). Examples include studies of the dice snake (*Natrix tessellata*; Gautschi *et al.* 2002), elk (*Cervus elaphus*; Williams *et al.* 2002), bighorn sheep (*Ovis canadensis mexicana*; Hedrick *et al.* 2001) and moose (*Alces alces*; Broders *et al.* 1999). Bottlenecks deplete genetic diversity, initially as a result of the random sampling of individuals from the source population, and over time, from the effects of genetic drift acting in small populations. In an interesting contrast to these general observations, Taylor & Jamieson (2008) argue that an historical bottleneck (approximately 200 years ago) in a passerine bird species has depleted genetic variation to such a degree that contemporary bottlenecks have had no further noticeable effect. They propose that founding new populations of threatened species that exhibit minimal genetic diversity, and are therefore less sensitive to founder effects than species with greater levels of diversity, would reduce the increased risk of extinction resulting from stochastic demographic events in small populations.

The lack of diversity at genetic markers in SWR is noteworthy; analyses of microsatellite loci and mtDNA control regions have showed unequivocally that SWR populations are characterised by low variation, with few alleles or haplotypes and minimal heterozygosity. Of particular interest, is the lack of diversity at functionally important MHC *DQA* and *DQB* exon 2 sequences. These findings suggest that the SWR genome is, on the whole, likely to be characterised by similarly low levels of variation. Contemporary genetic diversity in the SWR is amongst the lowest reported in large mammals (Table 3.16.). It is particularly notable that while current SWR numbers are greater than all other extant rhinoceros taxa combined, the species has the lowest levels of genetic diversity. Comparative levels of diversity displayed by the South China tiger (*Panthera tigris amoyensis*), extinct in the wild, have resulted in severe inbreeding depression and low juvenile survivorship in captive populations (Table 3.16.; Xu *et al.* 2007). Despite low diversity at both neutral and selected markers, there is no evidence of inbreeding depression or decreased recruitment in contemporary SWR populations. Reported annual growth rates of free-ranging SWR populations are 6.5% to 15% and mortalities are mostly due to translocations, increased aggression in high density populations or the predation of very young calves.

Common Name	Scientific Name	IUCN Red List	Microsatellite Markers		MtDNA Control Region			Reference
			Alleles/locus	Overall H_{Obs}	N	h	π	
Southern white rhinoceros	<i>Ceratotherium simum simum</i>	NT	2.6 ^{13 loci}	0.44	4	0.52	0.003	This study
Black rhinoceros	<i>Diceros bicornis bicornis</i>	CR	4.0 ^{9 loci}	0.52	-	-	-	Harley <i>et al.</i> 2005
	<i>Diceros bicornis michaeli</i>	CR	5.6 ^{9 loci}	0.73	16	-	0.012	Harley <i>et al.</i> 2005 S.M. Muya (unpublished data)
	<i>Diceros bicornis minor</i>	CR	5.4 ^{9 loci}	0.44	5	0.86	0.004	Harley <i>et al.</i> 2005 Brown & Houlden (2000)
Indian rhinoceros	<i>Rhinoceros unicornis</i>	EN	4.2 ^{11 loci}	0.46	-	-	-	Zschokke <i>et al.</i> 2003
Sumatran rhinoceros	<i>Dicerorhinus sumatrensis</i>	CR	3.7 ^{10 loci}	0.52	-	-	-	Scott <i>et al.</i> 2004
Javan rhinoceros	<i>Rhinoceros sondaicus</i>	CR	-	-	3	-	-	Fernando <i>et al.</i> 2006
North Atlantic right whale	<i>Eubalaena glacialis</i>	EN	3.2 ^{16 loci}	0.31	5	0.87	0.006	Waldick <i>et al.</i> 2002
								Malik <i>et al.</i> 2000
South China tiger	<i>Panthera tigris amoyensis</i>	CR	2.5 ^{17 loci}	0.47	1	0	0	Xu <i>et al.</i> 2007
								Luo <i>et al.</i> 2004
African elephant	<i>Loxodonta africana</i>	VU	3.9 ^{9 loci}	0.44	43	0.85	0.020	Whitehouse & Harley 2001 Nyakaana <i>et al.</i> 2002
African buffalo	<i>Syncerus caffer</i>	LC	7.1 ^{14 loci}	0.73	124	0.92	0.049	Van Hooft <i>et al.</i> 2000
								Van Hooft <i>et al.</i> 2002

Table 3.16.

Levels of genetic diversity across a range of large endangered mammals. Genetic diversity is measured at microsatellite loci and the mtDNA control region and reported as the mean number of alleles per locus and observed heterozygosity (H_{obs}) for microsatellite loci and the number haplotypes (N) and haplotype (h) and nucleotide (π) diversity for mtDNA sequences. The IUCN conservation status of each species is reported (CR, critically endangered; EN, endangered; NT, near threatened; VU, vulnerable; LC, least concern), hyphens (-) indicate unknown data. References, where applicable, are listed for microsatellite marker studies and those based on mtDNA, respectively.

Of the 13 microsatellite loci analysed in this study only a single locus deviated from expected HW genotypic proportions; this locus also displayed evidence of null alleles. Deviations from HWE at a single locus may indicate selection on a nearby gene while significant estimates of null allele frequencies can denote homozygous excess without necessarily indicating the presence of a null allele. The remaining 12 microsatellite loci were all in HWE and displayed balanced heterozygote-homozygote proportions, as measured by F_{IS} . This is noteworthy considering the low allele number and, in some cases, the small founder number and current size of the study populations. Significant deviation from linkage equilibrium was indicated for a number of pairs of loci in each population; these associations were not, however, consistent across all the populations. Given the demographic history of these populations, this is unlikely to be the result of true physical linkage but rather the non-random association between alleles of different loci in bottlenecked populations as a function of low allelic diversity (Allendorf & Luikart 2007; Ledig *et al.* 1999).

Genetic diversity at the functionally important MHC class II genes confers resistance to pathogens and infectious diseases (Croisetiere *et al.* 2008; Cohen 2002; Penn *et al.* 2002; Hill *et al.* 1991). For example, the cheetah (*Acinonyx jubatus*) has low levels of neutral genetic diversity (O'Brien *et al.* 1983) considered to be the result of an ancient bottleneck during the late Pleistocene (Menotti-Raymond & O'Brien 1993). This genetic paucity is associated with inbreeding depression, reflected in extremely low sperm counts and abnormal sperm morphology (Wildt *et al.* 1983) and low juvenile survivorship due, in part, to increased disease susceptibility (O'Brien *et al.* 1985). Successful reciprocal skin grafts between unrelated individuals have indicated that cheetah MHC genes are similarly lacking in diversity, thereby reducing resistance to infectious diseases such as coronavirus-associated feline infectious peritonitis (O'Brien *et al.* 1985). Like the cheetah, SWR have limited diversity at neutral and adaptive DNA markers, and yet despite this, founded populations have greatly increased in numbers across most of the species' historical range states and do not display evidence of inbreeding depression or susceptibility to pathogens and infectious diseases. The impact of selection in small inbred populations depends largely on the number of deleterious recessive alleles present in a population, such alleles may have been purged from naturally outbreeding historical populations. As a result, contemporary SWR populations would not be affected by local inbreeding. Alternatively, the functional monomorphism at two adaptive MHC loci may reflect the retention of a high frequency allele through ancient and more recent bottlenecks and loss of rare alleles via drift in small populations.

San Nicolas Island foxes colonised three Channel Islands off the coast of southern California approximately 16 000 years ago and were introduced to another three islands by Native Americans 800 to 4300 years ago (Aguilar *et al.* 2004). Island foxes lack diversity at all hypervariable markers studied to date (discussed in Chapter 1). Yet the species still exhibits surprisingly high levels of heterozygosity at four of the five MHC loci examined; moreover $d_N : d_S$ ratios in the PBR relative to non-PBR sites were > 1 , providing evidence for ongoing balancing selection on these genes (Aguilar *et al.* 2004). These findings suggest that genetic drift in small populations can be counterbalanced via the effects of balancing selection elsewhere in the genome. In contrast, SWR exhibit reduced genetic diversity at hypervariable markers as well as a complete lack of functional diversity at two well characterised MHC loci.

Based on an extensive search of recent literature, this study is the first example of a mammalian species in which seeded populations exhibit no further loss of genetic diversity in comparison to the source population. Microsatellite allele and mtDNA control region haplotype frequencies varied between populations, as commonly observed after founder events, but with no evidence of a significant loss of diversity in seeded populations. These results suggest that a gene pool comprised of very few high frequency alleles or haplotypes, with no rare or private alleles or haplotypes, is unlikely to experience a further reduction in genetic diversity when subsets of individuals found new populations. Allele or haplotype frequencies fluctuate from generation to generation and are therefore only observable in SWR over a very long period of time because of the species' long generation time (~ 8 years). This is further supported by the lack of significant difference in levels of diversity in adult, sub-adult and calf age classes in two of the study populations, suggesting that genetic drift has not noticeably reduced diversity in recent generations. There are, however, mtDNA haplotypes absent from the WPP and MNP populations, which may be due to the random sampling of founder individuals. Alternatively, mtDNA markers, being haploid and maternally inherited, have a considerably reduced N_e relative to nuclear markers (1:4 if the ratio of breeding males to females is 1:1) and are more sensitive to the effects of genetic drift. Rare haplotypes in founder individuals may therefore have been lost from the gene pool since these populations were founded.

Bottlenecks and Founder Events: Implications for Conservation

Genetic diversity is predominantly lost in small populations as a result of genetic drift (Allendorf 1986). Selection is usually not sufficient to counter the effects of drift in populations of 100 or fewer individuals. Lacy (1987) therefore suggested that conservation programs should primarily aim to offset the effects of genetic drift almost to the exclusion of being concerned about selection

and mutation. Understanding gene flow and the movement of genetic diversity among populations is pivotal to the management of endangered species. Today, SWR persist as numerous small populations across southern Africa with virtually no gene flow, predisposing them to the effects of stochastic disturbances, genetic drift and the potential for inbreeding depression. The continued loss of allelic diversity can be limited by innovative management regimes that simulate gene flow via the periodic immigration of new individuals (i.e. translocations), thereby preventing isolation and reducing population differentiation. The relatively high level of genetic differentiation reported in this study draws attention to the role of genetic drift in contemporary SWR populations. The extent of genetic differentiation between populations corresponds to the time since founding; WGR was founded most recently with a large number of founder individuals and remains undifferentiated from the HIR source population, whereas WPP and MNP were each founded approximately forty years ago and reveal significant differentiation from both HIR and from each other.

One of the central findings of this study is that SWR do not retain the genetic signatures of recent population decline according to the two unrelated statistical approaches used for these analyses. The results are significant given the virtual eradication of SWR at the turn of the 19th century and the subsequent translocation program used to found new populations. Cornuet & Luikart's (1996) approach tests for heterozygous excess compared to the expected heterozygosity of an equivalent population at mutation-drift equilibrium. The main assumption of their model is that rare alleles are lost rapidly when N_e is reduced and therefore a transient heterozygous excess exists in populations before a new equilibrium is reached. Both source and seeded SWR populations did not show statistical evidence of a genetic bottleneck, which could only be explained if the numbers of alleles present in these populations were not affected by both initial population decline and subsequent founding events. In contrast to this approach, Garza & Williamson's (2001) M test calculates the ratio of the number of alleles relative to the total allele size range in a population. A reduction in N_e is predicted to reduce the number of alleles, and accordingly the ratio, as only the loss of the largest or smallest allele would affect the allele size range. Under a scenario where the pre-bottleneck gene pool was dominated by a few well-represented alleles, alleles would not easily be lost and the ratio would thus not be significantly affected.

The M ratio is expected to have a long recovery time while heterozygosity excess and allele frequency distributions would recover relatively quickly once heterozygosity reaches a new equilibrium. Demographic recovery is characterised by the appearance of new rare alleles and the addition of any such alleles would reduce the observable heterozygosity excess whereas these new

alleles are unlikely to increase the M ratio, unless they increased the overall allele size range of the locus. Populations that have primarily been influenced by historical decline(s) are more likely to exhibit low M ratios but non-significant heterozygosity excess, while recently reduced populations or those currently in decline will not have had time to recover from the genetic signatures associated with demographic bottlenecks. These results suggest that the documented historic bottleneck and subsequent founder events have had relatively limited effects on contemporary levels of genetic diversity in seeded SWR populations. Fluctuations in allele and haplotype frequencies are most likely the result of genetic drift in small and isolated populations. Historically, SWR were widely distributed across the grasslands of central and southern Africa and individuals could have migrated across vast areas. Migration and resultant gene flow would have offset the detrimental effects of localised inbreeding and genetic drift. Modern conservation practices, however, maintain SWR in small and isolated populations in fenced reserves and the potential for migration is limited. A metapopulation management strategy has been promoted for many endangered species. This strategy would maximise existing level of diversity and combat the effects of genetic drift via the relocation of breeding males between subpopulations to simulate gene flow and prevent subpopulation isolation (Foose 1987). This study, however, clearly demonstrates that individual SWR are so genetically similar that relocating breeding males may not be sufficient to prevent the continued loss of diversity; instead, translocations would serve to reshuffle limited allelic diversity between populations and may eventually prove ineffective in reducing genetic drift in the long-term. An exception to this would be the translocation of breeding males between small SWR “populations” that essentially consist of an extended family group; under these conditions unrelated breeding males would be crucial to prevent consanguineous matings.

In comparison with SWR, other extant rhinoceros taxa have retained high levels of genetic diversity despite dramatic population declines (Table 3.16.). Black rhinoceroses (*Diceros bicornis*) are one of the most endangered mammal species in Africa; an estimated 100 000 individuals in the 1960's have been reduced to ~2600 by 1997 (Emslie & Brooks 1999). Harley *et al.* (2005) reported high levels of genetic diversity in the four remaining subspecies and suggested that the recent and rapid bottleneck has had a limited effect on genetic diversity in contemporary populations. A metapopulation management strategy would therefore maintain diversity in small and isolated black rhinoceros populations by simulating gene flow and limiting subpopulation isolation. The Indian rhinoceros (*Rhinoceros unicornis*) is highly endangered with ~2400 individuals persisting in two main wild populations in Nepal and Assam, India (Zschokke *et al.* 2003). Zschokke *et al.* (2003) and Dinerstein & McCracken (1990) reported that the species has retained high levels of genetic

diversity despite this reduction in population size. The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is critically endangered, with the species number decreased by half during the 1990's (Morales *et al.* 1997) and only ~300 individuals still persist in Indonesia and Malaysia (Scott *et al.* 2004). The Javan rhinoceros (*Rhinoceros sondaicus*) is arguably the most endangered large mammal with <60 individuals persisting in two populations in Ujung Kulon, Indonesia, and Cat Tien, Vietnam (Fernando *et al.* 2006). Fernando *et al.* (2006) analysed mtDNA control region sequences and identified three haplotypes across the two populations, which is comparable to the number of haplotypes identified in this study in SWR, despite the great difference in numbers of extant individuals in these two species.

Management and Population Size in Southern White Rhinoceroses

Most contemporary SWR populations are owned by the private sector and have an average “population” size of ~11 individuals, the majority of which may therefore be related, whereas state-owned parks support populations of on average ~123 individuals (Emslie & Brooks 1999). Simulations were used to investigate the effects of genetic drift in hypothetical populations founded from HIR to evaluate the long-term effectiveness of this strategy. Simulations were performed using populations founded with 10, 50 or 100 individuals and maintained at a constant population size for 200 years (approximately 25 generations). The results demonstrated unequivocally that small populations rapidly lose existing diversity; in a population of 10 individuals, only 36.3% of the current observed alleles (and representing 1.0 allele/locus) and 62.7% of the H_{Obs} (0.29) would be retained. It is likely that a threshold would eventually be reached in small SWR populations where inbreeding depression could manifest. In contrast, populations maintained at 100 individuals are predicted to retain 77.5% of existing allelic diversity and 94.8% of the H_{Obs} , approaching the suggested values of 90 to 95% of the common alleles being retained in a population (Frankham 1995; Marshall & Brown 1975). Demographic recovery is a vital component of conservation management, however, the conservation of endangered species in small and/or fragmented populations needs to have integrated approach that is cognisant of all demographic, environmental and genetic factors that may increase the probability of extinction (Lande 1998).

Concluding Comments

The four key findings of this chapter are that (1) contemporary SWR populations, both source and seeded, do not retain signatures of genetic bottlenecks at neutral microsatellite loci, (2) there is no reduction in genetic diversity in seeded free-ranging populations of SWR in southern Africa, (3) SWR lack functional diversity at two loci within the MHC gene complex and share most alleles with the NWR and as a result (4) current management strategies may not be appropriate for the long-term conservation of SWR.

Seeded SWR populations do not exhibit reduced genetic diversity when compared to levels in the source HIR population. The current management strategy of founding new populations is therefore not expected to cause significant decreases in levels of genetic diversity. But, given the results from this study, the conservation of SWR in numerous small and isolated populations is unlikely to ensure the long-term persistence of SWR; increased genetic drift in small populations may eventually decrease levels of genetic diversity beyond a threshold with potentially catastrophic repercussions for the future of the species. A more effective strategy would be to maximise financial and logistical investment in maintaining larger populations - simulations demonstrate that populations of 50 to 100 or more individuals would limit the effects of genetic drift and improve the chances of existing genetic diversity in SWR persisting into the future.

The most likely explanation consistent with the observed data is that historic SWR populations, prior to their decline in the late 1900's, were already characterised by low levels of genetic diversity. Low levels of neutral and adaptive diversity are characteristic of both the Southern and Northern white rhinoceros lineages, suggesting two possible scenarios that may account for these findings. The first scenario is that low levels of genetic diversity were the result of an ancient bottleneck that predated the 19th century decline. Subsequent purging could have led to the rapid removal of lethal recessive alleles from populations, resulting in a genetically depauperate but fit lineage of white rhinoceroses. A second scenario is that while SWR were extirpated across their historical range, the overall decline did not critically affect the N_e of the remnant population that persisted in Umfolozi Reserve. Post-exploitation, this population could have increased in number without significantly affecting remaining levels of genetic diversity or fitness. Furthermore, it has been suggested that the reported census number was initially exaggerated to provoke public support for the proclamation of the Umfolozi Reserve and the conservation of the SWR. Low diversity in SWR does not therefore appear to be the result of recent anthropogenic decline and near extinction.

Chapter 4

Southern White Rhinoceros Management Strategy

Introduction

The analysis of genetic diversity is an important aspect of modern conservation approaches. In part, this is because maintaining diversity is considered crucial to the long-term survival of a species. Small and increasingly isolated populations of threatened species are vulnerable to genetic, demographic or environmental stochasticity that increase the probability of extinction (Gilpin & Soulé 1986). Genetic factors include the loss of genetic diversity, via genetic drift and/or localised inbreeding, necessary for both individual and species fitness, and to sustain adaptive potential. Demographic threats include biased sex ratios and fluctuations in individual reproductive success while environmental factors include disease epidemics and natural catastrophes such as floods or earthquakes. Detrimental genetic and demographic effects in small populations can be ameliorated by the translocation of individuals (Griffith *et al.* 1989). A management strategy where small populations are artificially managed as a metapopulation is typically implemented for small populations of endangered species (Emslie 1994; Foose 1987). The main aim of this strategy is to limit localised inbreeding and reductions in adaptive variation, primarily due to genetic drift, by simulating dispersal and gene flow. Examples of successful implementation of this strategy include the conservation of the Greater Prairie Chicken (*Tympanuchus cupido pinnatus*) in Illinois, U.S.A. (Westemeier *et al.* 1998) and the recovery of an isolated population of adders (*Vipera berus*) in Sweden (Madsen *et al.* 1999).

Most contemporary SWR are conserved in small seeded populations on private game reserves with only National Parks generally supporting larger populations; the result is isolated populations with limited potential for dispersal and gene flow (Emslie & Brooks 1999). The translocation of large mammals, such as SWR, is both financially and logistically challenging. The financial costs, including the use of helicopters, immobilising drugs, veterinary and support staff, vehicles and other equipment, have fluctuated in recent years (e.g. \$1250 to \$500 per SWR between 2000 and 2005 in the Hluhluwe Imfolozi Reserve; Spenceley & Barnes 2005). The accurate identification of breeding males using molecular techniques to assign paternity could therefore help to optimise available resources by only translocating males that have contributed to the population gene pool.

Field-based studies report that sexually receptive adult SWR females consort predominantly with territorial males (Rachlow *et al.* 1999), suggesting that only dominant males reproduce. Observations of SWR copulations are rare (Owen-Smith 1973) and molecular paternity assignments would either provide support for, or refute, the hypothesis that only dominant males reproduce, as well as inform the relative success of territorial and non-territorial male mating strategies and levels of reproductive skew in free-ranging SWR populations (Keller & Reeve 1994). Paternity assignments would also inform our understanding of the mating system employed by free-ranging SWR populations (Laloi *et al.* 2004); behavioural data alone are insufficient for this purpose because female home ranges overlap the territories of a number of males (Rachlow *et al.* 1999; Owen-Smith 1971) and females may consort with a number of males during oestrus (Patton *et al.* 1999).

In addition to informing the development of effective metapopulation management strategies in SWR, improved understanding of the mating system employed and relative success of male territorial versus non-territorial reproductive strategies in free-ranging populations would also serve to improve husbandry in zoos and captive-breeding facilities where reproductive success is poor.

Mammalian Mating Systems and Reproductive Skew

The mating system employed by a species or population together with the extent of reproductive skew greatly affect levels of relatedness and effective population size. Reproductive skew theory is a mathematical approach to understanding how reproduction is partitioned among members of social groups and takes into account the ecological, social, and genetic factors within animal societies. Highly skewed populations consist of a single or small group of reproductively successful individuals whereas reproductive success is more evenly distributed among individuals in low-skew populations (Keller & Reeve 1994). The observed patterns of reproduction may not always be consistent with the genetic identification of male and female breeders, which has been demonstrated in a broad range of species. In a study based on DNA fingerprinting, Pemberton *et al.* (1992) revealed field observations of red deer (*Cervus elaphus*) harems to be poor indicators of male reproductive success. Behavioural studies consistently overestimated the reproductive success of many subordinate males and underestimated the success of dominant males. The variance in male reproductive success was therefore much greater than originally suggested. Birkhead *et al.* (1990) investigated the frequency of extra-pair mating and brood parasitism in zebra finches (*Taeniopygia guttata*) using DNA fingerprinting data. And 11% of offspring and one third of all nests were the result of extra-pair maternity, resulting from either intraspecific brood parasitism or two or more females assuming ownership of a single nest. This frequency was greater than expected because observational data had revealed no evidence of the phenomenon. Westneat (1990; 1987a; 1987b) used DNA fingerprinting to reveal that extra-pair paternity accounted for up to 42% of all offspring in indigo buntings (*Passerina cyanea*), a species observed to be predominantly monogamous. Extra-pair mating and intraspecific brood parasitism generate opposing selective pressures in order to maximise fitness, i.e. selection for males that can successfully achieve extra-pair paternity and for male and female pairs that successfully parasitise other brooding pairs in opposition to selection for males to avoid being cuckolded and for male and female pairs to avoid being parasitised. These studies demonstrate that the use of molecular techniques in parentage analyses, together with observational data, is crucial for understanding the selective pressures shaping mating strategies in natural populations.

Mammalian species display a diverse range of reproductive and social behaviours. Males can exhibit obligate monogamy, unimale and group polygyny or promiscuity that are associated with behaviours such as mate guarding and the defence of territories, female groups or individual receptive females (Clutton-Brock 1989). Females can exhibit long-term monogamy, serial monogamy, polyandry or promiscuity (Clutton-Brock 1989). Reproductive strategies can vary

between individuals within a population and are therefore not characteristic of a species but rather the result of social and ecological adaptation of males and females to the local environment. Clutton-Brock (1989) suggested that males use different mating strategies in response to the following factors: (1) the importance of paternal assistance in the successful rearing of offspring, (2) the size of female ranges and core areas, (3) the size and stability of female social groups and (4) female density and distribution. The success of various male reproductive strategies depends on a combination of these factors and suggests that, with respect to SWR, either “facultative monogamy” or “polygyny” would be the most successful male reproductive strategy. For example, adult SWR females do not require paternal assistance to rear calves and therefore female range size and female group size influence male reproductive behaviour. Females inhabit home ranges that are sufficiently small to enable a single male to defend a territory that overlaps those of several females. Dominant SWR males establish and defend territories (discussed in Chapter 1); a common reproductive strategy used by polygynous ungulates to increase the probability of securing receptive females (Owen-Smith 1977). When all available habitat is occupied by territorial males, however, the remaining males are relegated to a non-territorial reproductive strategy. Rachlow *et al.* (1998) reported that territorial SWR males spend significantly more time associating with females of higher reproductive value than non-territorial males, suggesting that these two reproductive strategies may not be equally successful.

Maternal Investment and Maternal-Offspring Recognition Mechanisms

Sophisticated parent-offspring recognition mechanisms are crucial for group-living species where misidentification is possible and/or there is extensive parental investment. The quality and extent of maternal care affect individual and group female reproductive fitness. Lactation is the most fundamental aspect of mammalian maternal care (Gittleman & Thompson 1988) and mothers usually display selective investment by only providing nutrition for their own offspring. Mother-offspring combinations must therefore recognise one another to prevent costly misdirected maternal care that can result in reduced survival of true offspring. These recognition mechanisms have been investigated in natural populations of a number of social mammalian species, including Northern fur seals (*Callorhinus ursinus*; Insley 2001), grey seals (*Halichoerus grypus*; McCulloch & Boness 2000), Barbary macaques (*Macaca sylvanus*; Hammerschmidt & Fischer 1998), bottlenose dolphins (*Tursiops truncatus*; Sayigh *et al.* 1998) and Mexican free-tailed bats (*Tadarida brasiliensis*; Balcombe & McCracken 1992; Balcombe 1990). Some species, however, do not exhibit true recognition and parental care is based on spatial proximity within a specific location such as a nest or den. This mode of spatial proximity is observed in Belding's ground squirrel (*Spermophilus beldingi*; Holmes 1990).

Evidence suggests that parent-offspring recognition mechanisms are primarily based on olfactory or auditory cues; olfaction is mainly used for short-range identification and vocalisations for longer-range identification (Torriani *et al.* 2006; Searby & Jouventin 2003). For example, a study on Barbary macaques (*Macaca sylvanus*; Hammerschmidt & Fischer 1998) showed that mothers in a free-ranging population could discriminate the vocalisations of their offspring from those of unknown young. This adaptation has enhanced maternal care in many species by enabling mothers to keep track of their offspring via auditory cues while simultaneously engaging in group social interactions, foraging behaviour and predators avoidance.

Unidirectional parent-offspring recognition mechanisms have predominantly been identified in vertebrate species (Colgan 1983; Falls 1982), whereas evidence for bidirectional or mutual recognition are less common (Falls 1982; Shillito & Alexander 1975). One such example, however, is the Northern fur seal (*Callorhinus ursinus*; Insley 2001) where mothers and offspring routinely locate and recognise each other amongst hundreds or thousands of conspecifics in breeding colonies. Vocalisation playback experiments confirmed that recognition is mutual and has evolved under strong selective pressures within a system where 16% of adult females bite, often fatally, unknown offspring that approach them. There is also evidence that parent-offspring recognition

mechanisms can vary according to different anti-predatory strategies. For example in “follower species” such as domestic sheep (*Ovis aries*), mothers and offspring are capable of recognising one another using contact calls (Searby & Jounventin 2003), whereas in “hider species”, such as fallow deer (*Dama dama*), recognition is unidirectional and the ‘hiding’ fawn must distinguish the calls of its mother (Torriani *et al.* 2006).

Illmann *et al.* (2002) have demonstrated that both wild and domestic sows (*Sus scrofa*) can discriminate the vocalisations of their own offspring from those of unknown offspring in order to prevent allosuckling and to locate offspring that have become separated from the mother and are at increased risk from predators or starvation. Wild and domestic sows, in semi-natural conditions, remain isolated for a period of time after parturition and this behavioural adaptation is considered to be crucial for a period of olfactory and acoustic learning to differentiate offspring signals before returning to the family social group (Illmann *et al.* 2002).

Mammalian maternal investment includes not only fertilization, gestation and parturition, but also lactation and increased risk of predation as a result of caring for neonates. Maternal care is a vital aspect of evolutionary adaptation and parent-offspring recognition mechanisms serve to maximise individual fitness of adult females and survival of their offspring (Nowak *et al.* 2000).

Chapter Research Aims

The approach most likely to reveal accurate assessments of individual reproductive success in natural populations is to combine genetic analyses and long-term field observational data in parentage studies. This multi-faceted approach has elucidated the mating systems and strategies employed by a multitude of species and has provided unforeseen insight into the relative roles of natural and sexual selection and the evolution of mate choice. Understanding the factors involved in individual reproductive success is of particular importance to optimise demographic recovery in newly founded free-ranging or captive-breeding populations. Within this framework the following research objectives are investigated in this chapter:

- 1.) To assign parentage in the WGR and MNP seeded populations using a likelihood statistical approach; data from microsatellite loci are combined with observational data collated from a previous demographic study to identify reproductively successful SWR males. The translocation of breeding males between populations would facilitate the effective implementation of a metapopulation management strategy because few individuals would need to be translocated to maximise the beneficial effects of increased N_e that come about when management incorporates methods to simulate dispersal and gene flow.
- 2.) To describe the mating system and investigate levels of reproductive skew in free-ranging SWR populations. Parentage assignment is used to elucidate male and female reproductive success and thereby provide insight into the mating system of SWR, the proportion of individuals reproducing in the population, as well as confirming or refuting behavioural observations that only territorial males reproduce. The functional monomorphism identified at two major histocompatibility complex genes (described in Chapter 3) limited the investigation of a genetic component of female mate choice in SWR.
- 3.) To confirm maternal-calf relationships for individuals from WGR and MNP and investigate behavioural observations of a calf-swapping event between adult female SWR in the MNP population. These findings inform the understanding of the development and evolutionary implications of mother-offspring recognition mechanisms in SWR.

Molecular methods for all data analysed in this chapter are described in Chapter 2.

Analytical Methods

The use of data from microsatellite markers has become widespread in studies on paternity in natural populations (Marshall *et al.* 1998). Exclusion is the simplest technique of parentage analysis and is based on Mendelian inheritance with genotypic incompatibilities between putative parents and offspring excluding parent-offspring combinations until the last remaining non-excluded candidate parent is assigned (Luikart & England 1999). This approach is used extensively in human forensic analyses and paternity testing and has also been used successfully in paternity assignments in natural populations, for example in wild chimpanzees (*Pan troglodytes verus*; Vigilant *et al.* 2001). The greatest weakness of strict exclusion analysis is the increased probability of excluding the true parent as a result of genotyping errors, null alleles and/or mutations, that contribute to false exclusions. Exclusion may not resolve paternity unambiguously and a statistical approach is required for multiple non-excluded parental candidates. A statistical approach would also be required for populations that exhibit low levels of genetic diversity and when parental candidates have not all been sampled (Marshall *et al.* 1998). The exclusion approach alone is not appropriate for the analysis of parentage in this case as SWR are characterised by low allele numbers and individuals share common alleles at most of the microsatellite loci (detailed in Chapter 3).

Paternity can be assigned as a statistical probability using either a categorical or fractional likelihood approach. The categorical approach attempts to assign a single parent-pair to a particular offspring whereas the fractional approach divides an offspring amongst all the possible parents (Jones & Arden 2003). The categorical likelihood approach, implemented in the program CERVUS v3.0. (Kalinowski *et al.* 2007) is used in this study to assign parentage. CERVUS generates likelihood ratios for each candidate parent from microsatellite allele frequency data. These ratios are expressed as the natural logarithm of the ratio or LOD scores. The likelihood ratios calculated by version 3.0 utilise the revised likelihood equations of Kalinowski *et al.* (2007). A Monte-Carlo simulation, based on the observed allele frequencies and taking into account the number of candidate parents, the effects of a genotyping error rate, unsampled candidate parents and/or missing genotypes, is used to calculate the required critical difference in LOD scores (Marshall *et al.* 1998). This value is termed *Delta* (Δ LOD), being the difference in the LOD scores of the first and second most-likely parents, and is used to assign parentage at a given level of confidence relative to the critical *Delta* score calculated by simulation. Parentage can remain unassigned when the LOD scores of two or more most-likely parents are similar.

Critical Δ LOD-values were calculated for two scenarios, (1) unknown paternity and (2) both parents unknown. Analyses were performed for both strict (95%) and relaxed (80%) levels of statistical confidence with 10000 simulation cycles for 12 paternal candidate fathers in both populations. Analyses incorporated a genotyping error rate of 0.667%, allowed for each individual to have missing data for two of the 12 loci, locus DB66 was excluded from analysis due to an excess of homozygotes identified at this locus, and 98% of WGR and 97% of MNP loci were successfully genotyped. The proportion of paternal candidates was 0.90, allowing for the possibility that a single male is absent from each data set, as a territorial male died from injuries sustained in a territorial fight with a younger male was not sampled in the MNP population.

CERVUS calculates the polymorphic informative content (PIC), HWE, frequency of null alleles, average exclusion probabilities and probability of identity (P_{ID}). PIC estimates, calculated according to Botstein *et al.* (1980), were initially devised for use in genome scanning to measure the informativeness of markers, but are now commonly used in population studies to determine the value of each marker for identifying polymorphisms in a population. HW proportions and the frequency of null alleles were calculated to identify loci that could adversely affect accurate parentage assignments. The effectiveness of the microsatellite data set to resolve paternity was tested by calculating the average exclusion probabilities (P_{excl}), given only the genotypes of the offspring (total exclusionary power of the first parent) and given the genotypes of the offspring and one known parent (total exclusionary power of the second parent). All calculations assumed HWE. The P_{ID} measures the probability that identical genotypes could be observed in individuals in a population.

Results

Confirming Maternity and Assigning Paternity

Mother-offspring relationships in the WGR and MNP populations were initially determined from field-based observations (*pers. comm.* J. Rachlow) and genetic analyses were used to confirm these observations. The number of alleles per locus ranged from one to four (Table 4.1.), with allelic richness of 2.7 and 2.5 alleles per locus, respectively, and observed heterozygosity of 0.39 and 0.43, respectively, for each population (described in Chapter 3). The estimated null allele frequencies (Table 4.1.) showed significant values for locus DB66 in both populations and it was the only locus to deviate from expected HW proportions. This locus was therefore excluded from all parentage assignment analyses. The PIC value is a measure of both the number of alleles and the distribution of their frequency. According to Mateescu *et al.* (2005), values < 0.30 represent uninformative markers, values between 0.30 and 0.60 moderately informative markers and values > 0.60 highly informative markers. Of the 12 microsatellite markers analysed in this study there were no highly informative loci, while nine loci were moderately informative and four loci were uninformative in each population (Table 4.1.). The mean PIC values for both populations were only just within the moderately informative range.

Individuals were classified into adult (>6 years), sub-adult (2.5 to 6 years) or calf (<2.5 years) age classes. Males and females were considered to be candidate parents if classified as adult at the time of sampling. This approach was a conservative estimate including more candidate parents than were actually available. Only young calves still associated with their mothers at the time of sampling were included in the analyses to be confident of the observed maternal relationship. There were 10 calves in the WGR population with 12 candidate fathers and 10 calves in the MNP population with 12 candidate fathers (three territorial males). A second set of analyses were performed with the assumption that neither parent was known and then 17 candidate mothers were included in each population.

MtDNA control region analysis showed incompatible haplotypes observed in five of the 10 known mother-offspring combinations in the WGR population (Table 4.3.). Of these five, three combinations also showed microsatellite homozygous mismatches at one or more loci. There were no mtDNA haplotype incompatibilities between known mothers and offspring in the MNP population (Table 4.4.), however this population only supports two haplotypes and this is likely to greatly reduce the chance of detecting incompatibilities.

Locus	Welgevonden Game Reserve			Matobo National Park		
	A	PIC	Nulls	A	PIC	Nulls
7B	3	0.521	+0.003	3	0.478	-0.049
7C	3	0.427	+0.019	3	0.464	-0.045
32A	2	0.346	-0.059	2	0.325	-0.024
AY138542	3	0.518	+0.039	3	0.571	-0.060
AY138545	2	0.374	+0.031	2	0.328	-0.052
BR6	4	0.429	+0.109	3	0.164	-0.040
DB1	2	0.215	+0.090	2	0.092	-0.017
DB44	3	0.233	+0.074	3	0.424	-0.081
DB49	3	0.579	+0.019	3	0.564	-0.009
DB66	3	0.412	+0.567	2	0.372	+0.450
Rh7	2	0.236	+0.144	2	0.364	-0.130
Rh8	3	0.148	-0.034	3	0.255	-0.008
Rh9	4	0.432	-0.011	2	0.246	+0.048
Mean	2.85	0.375		2.54	0.356	

Table 4.1.

The observed number of alleles (A), polymorphic informative content (PIC) and estimates of null allele frequencies for each microsatellite locus.

Critical Δ LOD values and the number of predicted and observed paternities were generated from allele frequency data simulations in order to assign (1) paternity for each calf with a known mother and (2) parentage with both parents unknown. These values (Table 4.2.) showed that a low proportion of paternities could be statistically resolved at either the 95% or 80% confidence level. The percentage of calves that simulation tests predicted could be assigned was greater than the observed assignments in both populations. This suggested that the number of paternal candidates may be incorrect, but repeated analyses using different numbers of paternal candidates (ranging from 2 to 12) did not bring the expected and observed number of assignments closer to parity. The combined probability of identity in both WGR and MNP (7.96×10^{-6} and 1.38×10^{-5} , respectively) were three to six orders of magnitude greater than the values reported in paternity studies of North Atlantic right whales (*Eubalaena glacialis*; Frasier *et al.* 2007) and grey seals (*Halichoerus grypus*; Worthington Wilmer *et al.* 1999). These relatively high values imply that matching genotypes may be possible in SWR populations due to the low levels of diversity and would therefore confound parentage assignments.

Assignment Test	Confidence Level	Critical Δ LOD		Predicted Paternities		Paternities Assigned	
		WGR	MNP	WGR	MNP	WGR	MNP
Paternity unknown	95%	1.57	1.90	64%	56%	20%	10%
	80%	0.00	0.00	92%	93%	40%	40%
Parentage unknown	95%	4.69	5.13	17%	10%	0%	0%
	80%	1.38	1.72	44%	33%	30%	20%

Table 4.2.

The critical delta log likelihood (Δ LOD) scores, predicted and observed number of paternities assigned at a 95% or 80% level of confidence generated from 10000 simulations.

Statistical analyses could assign few paternities in the WGR (Table 4.3.) and MNP (Table 4.4.) populations with statistical confidence. In the WGR population, paternity was assigned to one calf (10%) at the 95% confidence level and three calves (30%) at the 80% confidence level. These assignments are suspect, however, because the single calf-mother-father trio assigned with strict confidence had mismatched mtDNA haplotypes as well as homozygous mismatches at three of the microsatellite loci in the observed mother and calf. In the MNP population, paternity was assigned to one calf (10%) at the 95% confidence level and to six calves (60%) at the 80% confidence level, with no mtDNA haplotype mismatches in the observed mothers and calves, however, two of the calf-mother-father trio assigned at the 80% confidence level had homozygous mismatches at a microsatellite locus in the observed mother and calf.

The total exclusion probabilities calculated for WGR and MNP for the first parent (0.249 and 0.273, respectively) and second parent (0.055 and 0.064, respectively) suggested that paternal candidates and/or maternal candidates had similar genotypes. Furthermore, low LOD scores, despite the absence of allelic mismatches, indicated that common alleles were shared between the candidate parents and calves at most loci. Low Δ LOD scores revealed little difference between the most-likely and the second most-likely paternal candidate and it was not possible to statistically distinguish the true father. The genetic diversity displayed in SWR is not sufficient to assign paternity with statistical confidence, even for one known parent and the alleles inherited from that parent are taken into account in downstream analyses.

Offspring	Observed mother	Nm	LOD score	Assigned father	P _{excl} (first parent)	P _{excl} (second parent)	Nm	LOD score		
								1 st most-likely	2 nd most-likely	ΔLOD
W12 (3)	W10 (2)	0	0.88	W48	0.28	0.01	0	-0.33	-4.77	0.00
W14 (3)	W07 (3)	0	-5.05	W48	0.24	0.04	0	2.30	-5.53	0.61 ⁺
W19 (2)	W18 (2)	0	0.11	W48	0.27	0.00	0	0.82	-2.94	0.61 ⁺
W28 (1)	W27 (3)	3	-0.13	W48	0.27	0.12	0	3.38	-4.25	2.43 [*]
W29 (2)	W23 (2)	0	0.68	W15	0.57	0.06	0	0.72	-0.24	0.00
W36 (3)	W25 (2)	1	-4.64	W02	0.21	0.06	1	-2.16	-2.99	0.00
W39 (1)	W35 (2)	1	-1.18	W15	0.16	0.08	0	0.76	-1.61	0.00
W41 (3)	W03 (3)	0	-0.74	W17	0.22	0.03	0	0.72	-3.79	0.72 ⁺
W46 (3)	W44 (2)	0	0.41	W17	0.26	0.00	0	-2.26	-4.70	0.00
W49 (3)	W47 (3)	0	-7.38	W48	0.39	0.10	0	1.58	0.84	0.74

Table 4.3.

The LOD scores of the observed mother and most likely and second most likely paternal candidates for 10 calves (< 2 years) born in WGR. The probability of exclusion (P_{excl}; exclusion of the first and second parents), number of mismatched loci for each parent (Nm) and mtDNA control haplotypes in parentheses (with 1, 2 and 3 being SWR1, SWR2 and SWR3, respectively).

* and ⁺ ΔLOD scores exceed calculated critical ΔLOD score at 95% and 80% confidence, respectively.

Offspring	Observed mother	Nm	LOD score	Assigned father	P _{excl} (first parent)	P _{excl} (second parent)	Nm	LOD score		
								1 st most-likely	2 nd most-likely	ΔLOD
M01(1)	M43(1)	0	3.22	M33	0.17	0.06	0	0.12	-2.97	0.12 ⁺
M12(2)	M32(2)	0	2.37	M38	0.37	0.17	0	-2.73	-3.01	0.00
M16(1)	M13(1)	0	-3.20	M18	0.05	0.08	0	2.31	1.62	0.69 ⁺
M25(2)	M35(2)	0	-1.29	M04	0.33	0.00	0	-0.91	-5.89	0.00
M26(2)	M09(2)	0	-3.29	M18	0.12	0.00	0	2.29	1.77	0.52 ⁺
M34(2)	M36(2)	0	-5.09	M33	0.28	0.12	0	0.82	-3.08	0.82 ⁺
M39(1)	M28(1)	0	0.74	M23	0.33	0.02	0	1.16	-1.22	1.11 ⁺
M42(2)	M40(2)	0	-5.69	M20	0.22	0.01	0	2.85	2.34	0.51 ⁺
M46(2)	M10(2)	0	-5.88	M31	0.60	0.01	0	3.75	-0.28	3.75 [*]
M47(2)	M44(2)	0	-0.22	M33	0.41	0.00	1	-2.24	-5.87	0.00

Table 4.4.

The LOD scores of the observed mother and most likely and second most likely paternal candidates for 9 calves (< 2 years) born in MNP. The probability of exclusion (P_{excl}; exclusion of the first and second parents), number of mismatched loci for each parent (Nm) and mtDNA control haplotypes in parentheses (with 1 and 2 being SWR1 and SWR 2, respectively).

* and ⁺ ΔLOD scores exceed calculated critical ΔLOD score at 95% and 80% confidence, respectively.

Parentage analyses of all 12 microsatellite loci were repeated with both parents unknown (sex of the parent candidate known) in an attempt to resolve ambiguous maternal relationships. Parentage analyses were then also repeated for (1) paternity and (2) both parents unknown using only the nine moderately informative loci in each population. Loci that were uninformative, namely DB1, DB44, Rh7 and Rh8 in WGR and BR6, DB1, Rh8 and Rh9 in MNP, were discarded from analyses in order to provide greater resolution. All additional analyses, however, generated similarly low LOD and Δ LOD scores and probabilities of exclusion and did not improve the assignment of parentage in either SWR population.

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Maternal Relationships and the Identification of Calf-Swapping Events

Behavioural observations, as part of an ecological study of SWR in the Matobo National Park, Zimbabwe, identified a possible calf-swapping event between putative sisters (individuals M13 and M43; *pers. comm.* J. Rachlow). There were no mismatched mtDNA control region haplotypes between the mother-calf combinations (Table 4.5.) because all four individuals had haplotype SWR1, which was expected if the two adult females had the same mother. There were also no allelic mismatches at the microsatellite loci in either mother-calf combination. The LOD scores calculated for the two mother-calf combinations were -3.20 and 3.22, respectively, which according to Slate *et al.* (2000) indicated that the former is rejected as the true mother and the latter is approximately 20 times more likely to be the true mother than not the true mother. It was therefore not possible to confirm or refute this calf-swapping event.

The WGR population showed mismatched mtDNA control region haplotypes for five of the 10 observed mother-calf combinations and three of these also had homozygous mismatches at one or more microsatellite loci (Table 4.6.). With just two to three haplotypes (described in Chapter 3) supported in these populations, shared haplotypes do not necessarily confirm maternity but mismatches can be used to exclude maternity. The microsatellite loci mismatches could be due to genotyping error as a result of allelic dropout, null alleles or incorrect allele sizing but were confirmed with at least two independent PCR amplifications. The paucity of genetic diversity in SWR suggested that even a single allelic mismatch was noteworthy as most loci exhibited only common alleles. Parentage analyses, repeated for both parents unknown to identify the true mother of each calf, generated LOD scores that ranged from -2.75 to 2.08. Three of these were assigned at a relaxed confidence level (LOD score > 1.38; Table 4.2.), however, only one of these mother-calf combinations had compatible mtDNA haplotypes. It was therefore not possible to identify the true mothers of these five calves. The LOD scores for the five mother-calf combinations that did not show genetic mismatches ranged from -7.38 to 0.68 and inferences could not be made regarding the likelihood that the observed mother was the true mother. Despite the failure to identify the true mother of each calf, these data indicated unequivocally that calf-swapping events occur between adult female SWR.

Relationship	Individual	MtDNA	Microsatellite Markers											
			7B	7C	32A	AY138542	AY138545	BR6	DB1	DB44	DB49	Rh7	Rh8	Rh9
Mother	M13	SWR1	C / C	C / C	A / A	A / C	A / B	B / C	C / C	A / C	A / C	A / B	D / D	A / B
Calf	M16	SWR1	C / C	B / C	A / A	C / C	B / B	B / B	C / C	C / C	A / A	A / B	D / D	A / A
Mother	M43	SWR1	C / C	B / B	A / A	A / C	A / A	B / F	C / C	A / C	A / C	A / A	C / D	A / A
Calf	M01	SWR1	C / D	B / B	A / A	B / C	A / A	B / B	C / C	A / A	-	A / A	C / D	A / A

Table 4.5.

The mtDNA haplotypes and microsatellite marker alleles for two putative SWR sisters and calves that were observed to have swapped calves in the MNP population. A hyphen (-) indicates a locus that was not successfully genotyped for that individual.

Relationship	Individual	MtDNA	Microsatellite Markers											
			7B	7C	32A	AY138542	AY138545	BR6	DB1	DB44	DB49	Rh7	Rh8	Rh9
Mother	W27	SWR3	C / C	B / B	A / A	C / C	A / B	B / B	C / C	C / C	C / C	A / A	E / D	C / C
Calf	w28	SWR1	C / C	C / C	A / C	A / C	A / B	B / B	C / C	C / C	D / D	A / A	D / D	A / A
Mother	W25	SWR2	C / D	B / C	A / C	B / C	B / B	D / D	C / C	C / C	C / C	A / A	D / D	B / D
Calf	W36	SWR3	A / C	C / C	A / A	C / C	B / B	B / C	C / C	C / C	C / C	A / A	D / D	A / B
Mother	W35	SWR2	C / C	C / C	C / C	A / B	A / B	B / C	C / C	A / C	B / D	A / A	D / D	A / A
Calf	W39	SWR1	C / C	B / C	C / C	A / B	A / A	B / B	C / C	A / C	C / C	A / A	D / D	A / B

Table 4.6.

The mtDNA haplotypes and microsatellite marker alleles for three observed mother-calf combinations that had both mtDNA sequence mismatches and allelic mismatches (indicated in bold print) at one or more microsatellite loci in the WGR population.

Discussion

Contemporary free-ranging SWR populations are generally conserved in small populations on private game reserves across most of their historical range states, with more than 90% managed in “populations” with an average size of ~11 individuals. This strategy establishes fenced sanctuaries to protect individuals from poaching, however, dispersal and gene flow is restricted. These small and isolated populations are vulnerable to loss of diversity due to the effects of genetic drift and eventually to localised inbreeding (described in Chapter 3). As previously discussed, however, SWR populations do not exhibit signs of inbreeding depression (discussed in Chapter 1). A metapopulation management approach via the translocation of breeding males is recommended for the management of small and isolated populations in order to simulate gene flow and decrease subpopulation isolation (Foose 1987). The accurate identification of breeding males is vital for the successful implementation of this strategy because translocating males that have not contributed to the gene pool would squander valuable resources and disrupt the social structure of the population without increasing the N_e of the recipient population. In addition, improved understanding of the mating system and variance in reproductive success of males and females is crucial for making inferences regarding demography, genetic structure and selection in these populations.

The Statistical Challenges of Assigning Paternity

Since its introduction, the assignment of parentage in natural populations has proved to be an invaluable tool in the study of evolution and behavioural ecology, and forms a central component of the field of molecular ecology (Hughes 1998; Avise 1994). A multitude of empirical parentage studies using microsatellite markers have been reported in the literature and have been accompanied by a profusion of statistical methods for the analysis of biparentally inherited DNA data (Luikart & England 1999). These statistical methods, however, all have one vital requirement – sufficient DNA polymorphisms to resolve unknown parentage. In this study, however, parentage can not be assigned with statistical confidence due to the lack of genetic diversity displayed at hypervariable microsatellite markers in SWR.

CERVUS performs statistical analyses of parentage assignments taking into account a number of factors that could otherwise negatively affect the results (Marshall *et al.* 1998). The program performs simulations based on a particular number of candidate males in the population, individuals need not be typed at every locus and the program is robust with regards to genotyping errors. Version 3.0. makes use of the new likelihood equations of Kalinowski *et al.* (2007) that improve the probability of assigning paternity with statistical confidence and a greater number of assigned

paternities are expected in comparison with previous versions. These likelihood analyses are insensitive to high levels of relatedness, even when there are many half-siblings in populations and a polygynous mating system occurs.

A recent study by Garnier *et al.* (2001) was able to assign paternity to all the offspring in a population of black rhinoceroses using combined microsatellite and observational data sets collected over a 10 year period. Microsatellite loci displayed two to seven alleles per locus (mean of 4 alleles per locus) and a mean H_{Obs} of 0.73, which was sufficient to generate total exclusion values of 0.990 and 0.999 for one parent and two parents respectively. In comparison, this study of SWR revealed just two to four alleles per locus (mean of 2.7 alleles per locus) and a mean H_{Obs} of 0.41 that generated low probabilities of exclusion for the WGR and MNP populations for the first parent (0.25 and 0.27, respectively) and second parent (0.55 and 0.64, respectively). These probabilities clearly demonstrate the lack of resolution in the data sets as a direct result of low genetic diversity in SWR. Garnier *et al.* (2001) reported LOD scores for the most likely paternal candidate that ranged from 0.90 to 7.73, with an average of 2.84, and ΔLOD values that ranged from 0.34 to 7.73, with an average of 2.00. The resolution of their data set was sufficiently high to determine that one male sired more than half of the offspring and another two males sired >40% of the remaining offspring. This study provided genetic evidence to suggest that black rhinoceros males are polygynous. Furthermore, while females were observed to consort with several males before conception, seven females were revealed to have mated with the same male for up to four successive calves and only one female produced calves with three different males, suggesting a possible role for mate choice and/or sperm competition in black rhinos. In complete contrast, this study reveals LOD scores ranging from -7.38 to 3.22 (mean LOD of -1.03) for observed mothers and ranging from -2.73 to 3.75 (mean LOD of 0.65) for the most-likely paternal candidate. Only a small proportion of paternities or parent pairs could therefore be assigned (at a relaxed confidence level) and most of these assignments had mother-calf mtDNA haplotype or genotype mismatches. Parentage assignments were further confounded by evidence of calf-swapping in WGR. Parentage can be assigned with statistical confidence in black rhinoceroses because the species experienced a recent (during the 1970's to 1990's) and rapid decline in population numbers and has retained high levels of genetic diversity (Harley *et al.* 2005). In contrast, SWR underwent a prolonged bottleneck in the 1900's as well as subsequent founder events, although the lack of diversity appears to be an ancient characteristic of the lineage and does not reflect the recent species history. Inferences regarding the mating system and levels of reproductive skew in SWR cannot be made based on this microsatellite data set.

The functional monomorphism displayed at MHC *DQA* and *DQB* loci (described in Chapter 3) indicated that these genes are unlikely to be directly involved in a genetic component of mate choice. The caveat to this is that the mechanisms linking MHC to mate choice are complex (Penn & Potts 1999; 1998; Jordan & Bruford 1998) and have been demonstrated in a number of empirical studies including Atlantic salmon (*Salmo salar*; Landry *et al.* 2001) and ring-necked pheasants (*Phasianus colchicus*; Von Shantz *et al.* 1997; 1996; 1989). Captive SWR populations in zoos and captive-breeding programmes have a negative growth rate (discussed in Chapter 1) and anecdotal evidence has suggested that female mate choice may play a role in this reproductive failure. For example Patton *et al.* (1999) reported that a female SWR in captivity did not show signs of oestrus and did not mate with her male companion over a period of time, but then demonstrated an obvious interest in and mated with a newly introduced male. Single male and female SWR housed together in captivity seldom reproduce (Bertschinger 1994), suggesting that a choice in potential mate is required for successful reproduction. The SWR studbook shows that non-breeding SWR do occasionally start to reproduce when relocated to a new facility or when new opposite-sex individuals are introduced to the population (Ochs 1997). Only two MHC loci were characterised in this study, and it may therefore be of value to investigate diversity at additional MHC genes. Mate choice may, however, be based on behavioural or environmental factors, perhaps as subtle as hormonal or pheromonal cues (Kretzschmar *et al.* 2004). Alternatively, factors such as male-male competition determine mating success and negate female mate choice in SWR, a possibility suggested in Soay sheep (*Ovis aries*; Paterson & Pemberton 1997) and African buffalo (*Syncerus caffer*; Wenink *et al.* 1998).

Calf-Swapping in SWR: Evolutionary and Ecological Implications

This study presents unequivocal evidence of genetic mismatches between calves and their observed mother. These findings suggest that a degree of calf-swapping occurs in natural populations of SWR. Female SWR invest extensively in their offspring; this biological investment comprises a gestation of 16 months and a lactation period of 12 to 18 months (Owen-Smith 1988). Sophisticated mother-offspring recognition mechanisms have evolved in social species where misidentification errors are more likely to occur. In SWR it has been proposed that calf-swapping may be the result of artificially high population densities in small fenced reserves (*pers. comm.* J. Rachlow). The WGR population, founded between 1994 and 1996 with 40 individuals, had a population size of 50 in the 340 km² reserve at the time of sampling. The current population size, however, is estimated at only ~43 as 28 individuals were sold to alleviate increasingly high levels of aggression and fighting among males that resulted in five deaths between 1999 and 2006 (*pers. comm.* H. Kilian). WGR has

recently removed the boundary fences with neighbouring Marakele National Park and other private game reserves to allow wildlife free access across approximately 1500 km² of indigenous bushveld (*pers. comm.* H. Kilian), and this is likely to alleviate any density-related issues. Behavioural observations indicate a very strong maternal bond in SWR (*pers. comm.* K. Stratford, Director of Ongava Research Centre, Ongava Game Reserve) and it is therefore possible that calf-swapping only occurs at a very early age, before mother-offspring recognition imprinting takes place.

Calf-swapping in SWR is likely to have minor evolutionary implications. Individual fitness would not be compromised if all calves survive to sexual maturity, as suggested by low calf mortalities reported in free-ranging populations. This would be especially true if calf-swapping occurred between closely related females such as the putative sister pair in MNP. The ecological and management implications are, however, more considerable. The challenges of assigning paternity with such low levels of genetic diversity were further compromised by calf-swapping in this study, as the observed maternal relationships did not necessarily indicate true biological matrilineages. Together, these two factors currently limit improved understanding of the mating system and variance in male reproductive success in SWR, as well as the accurate identification of breeding males. Accurate parentage data would improve current management approaches, inform the effective stocking of new reserves and enable the identification of surplus males for limited trophy hunting.

Concluding Comments

This study has revealed that a metapopulation management strategy in SWR would merely serve to reshuffle the limited allelic diversity between populations. Furthermore, the challenges in accurately identifying breeding males for translocation are highlighted. Translocating non-breeding males would waste valuable resources and disrupt the social structure of the population without any of the associated genetic benefits. The one exception would be very small populations that consist effectively of family groups where the dominant male would need to be relocated periodically to prevent consanguinous matings.

The probability of identifying breeding males in large free-ranging populations could be improved by complementing genetic data with field-based pedigree reconstructions. This could include information regarding male territorial boundaries and the overlap of these territories with female home ranges, identifying males and females that consort during the females' oestrous, and the close observation of newly born calves to be certain of matrilineal relationships.

It is currently not possible to assign parentage in SWR with statistical confidence when using microsatellite data alone. Genetic markers that display greater levels of genetic diversity are required. Future research could return to the traditional techniques of minisatellite DNA fingerprinting (Jeffreys *et al.* 1985) or AFLP fingerprinting (amplified fragment length polymorphism; Vos *et al.* 1995) that sample genome-wide variation and are therefore more likely to provide the levels of resolution necessary for individual-based methods of parentage assignment.

Chapter 5

General Synthesis and Conclusions

Conservation biology is a multi-disciplinary endeavour, informed by research from a number of diverse fields. Genetic data are a critical component of an integrated approach that strives to ensure the persistence of threatened and endangered species. Genetic approaches have become an important component of the effective conservation and management of large mammals worldwide, for example North American bison (*Bison bison*; Marris 2009), Asian rhinoceros taxa (Fernando *et al.* 2006; Scott *et al.* 2004; Zschokke *et al.* 2003; Morales *et al.* 1997; Dinerstein & McCracken 1990) and African buffalo (*Syncerus caffer*; Van Hooft *et al.* 2002; 2000; Wenink *et al.* 1998) and elephant (*Loxodonta africana*; Nyakaana *et al.* 2002; Whitehouse & Harley 2001; Cumming *et al.* 1990). This study presents a genetic perspective on the recent population decline and recovery of Southern white rhinoceroses in southern Africa. The six key findings of this study are: (1) source and seeded SWR populations do not retain signatures of recent or historic genetic bottlenecks at neutral microsatellite loci, (2) reduced genetic diversity is not evident in seeded free-ranging SWR populations in southern Africa, (3) SWR lack functional diversity at two MHC genes and share most alleles at these loci with NWR, (4) managed populations of 100 or more individuals are critical for the long-term conservation of SWR, in order to reduce the effects of genetic drift and future loss of remaining diversity, (5) genetic diversity at microsatellite loci is not sufficient for accurate parentage analyses and therefore inferences regarding SWR mating systems, reproductive skew and mate choice can not be made based on this data set and finally (6) data from this study suggests that a degree of calf-swapping occurs between adult SWR females. This study has yielded novel, if unexpected, results and highlights the fact that inherent levels of genetic diversity ultimately determines the utility of molecular data as management and conservation tools.

Low levels of diversity at genetic markers in SWR are noteworthy; analyses reveal unequivocally that SWR populations are characterised by low variation at both neutral and adaptive DNA markers. Despite the general lack of diversity, there is no evidence of inbreeding depression or increased susceptibility to pathogens and infectious diseases in extant free-ranging populations. Furthermore, recently founded populations continue to increase in size across most of the species' historical range states. The observed data suggest that low levels of neutral and adaptive diversity may be an ancient characteristic of white rhinoceroses. A significant bottleneck event prior to 19th century decline may

have led to extensive purging of lethal recessive alleles and could account for a rhinoceros lineage that is, today, genetically depauperate but fit. The recent extirpation of SWR across the species' historical distribution range may, therefore, have had a negligible effect on the N_e of the remnant population in the Umfolozi Reserve. Functional monomorphism at adaptive MHC loci may reflect the retention of a high frequency allele through ancient and more recent bottlenecks, together with the concomitant loss of rare alleles via drift in increasingly small populations. The paucity of genetic diversity in contemporary SWR does not appear to be the result of recent anthropogenic decline and near extinction. One approach to test the hypothesis of an “ancient” bottleneck would be to carry out an ancient DNA study using skull material from museum specimens collected prior to the early 1900's from the Umfolozi region and across southern and central Africa; a limited number of specimens are currently held in museum collections. Population level studies of historical, archaeological and palaeontological species can now be carried out using innovative ancient DNA techniques (Leonard 2008) together with a recently developed bead-based sequencing technology (Margulies et al. 2005). For example, the poorly understood mass extinctions of the transition between the Pleistocene and Holocene Epochs can now be studied. MacPhee *et al.* (2005) reported that mtDNA diversity obtained from late Pleistocene to late Holocene *Ovibos* fossil samples recovered from across the holarctic distribution was greater than in contemporary tundra muskoxen (*Ovibos moschatus*) populations, and proposed that an ancient bottleneck during the Late Quaternary accounted for this difference. Similarly, Douglas (2006) showed that contemporary North American bison display reduced genetic diversity compared to extinct Pleistocene bison.

Established paradigms within conservation biology predict that population decline results in reduced genetic diversity and increased risks of inbreeding; this, in turn, can lead to reduced levels of individual and population fitness via inbreeding depression. Additionally, small populations are highly susceptible to any stochastic changes whether genetic, environmental or demographic. SWR represent a challenge to some of these paradigms; the taxon does not display reduced genetic diversity in seeded populations and, to date, manifests no evidence of inbreeding depression. It is therefore important to be informed by both the genetic and demographic histories of species when developing conservation initiatives. This combined approach would improve our understanding of species' responses to various pressures such as hunting/poaching, disease susceptibility and inbreeding, and ultimately their extinction risk. The study of a single species may lead to novel theoretical concepts and the conservation of just one umbrella species, such as rhinoceros taxa, inevitably results in the conservation of habitats and all biodiversity contained therein.

Extinction is a demographic process whereby one generation does not successfully give rise to the next, and demography is therefore a vital component of conservation management (Lacy 1988; Lande 1988). The continued decline of small populations worldwide is perpetuated by genetic, demographic and/or environmental factors that interact within positive feedback loops or “extinction vortices” (Gilpin & Soulé 1986). The primary cause of species extinction remains controversial in conservation biology. Lande (1988) argued that small populations are more likely to become extinct due to non-genetic deterministic factors, such as anthropogenic over-exploitation and destruction, modification or fragmentation of available habitat, long before stochastic genetic factors have an effect. Frankham (1995) reported experimental data that substantiated the pivotal role of inbreeding in increased rates of extinction and that naturally outbreeding endangered species may provide little warning of imminent extinction as a result of inbreeding. Lande (1998) acknowledged that conserving endangered species in small and/or fragmented populations would require integrated management approaches that incorporated all possible factors. Spielman *et al.* (2004) compared the average heterozygosities reported in 170 threatened species with that displayed by taxonomically related non-threatened species and showed that genetic diversity was reduced in 77% of the endangered taxa. Garner *et al.* (2005) reviewed microsatellite marker analyses of 108 mammalian species and also reported consistently reduced levels of heterozygosity in threatened species. These meta-analysis approaches reveal that genetic factors do affect populations before extinction.

Poaching remains the primary threat to the continued persistence of all rhinoceros taxa, despite modern conservation efforts, and the protection of these species must remain the focus. SWR populations successfully founded across most of the historic range states are again under severe pressure from poaching. In South Africa alone, more than 20 SWR were poached in the Kruger National Park between 2007 and 2008, and a further 12 SWR were killed in KwaZulu-Natal during 2008 (Gosling 2008). Poaching has also increased alarmingly in Zimbabwe where 70 black and white rhinoceroses have been killed in the lowveld conservancies since 2000 (Lekotjolo 2008). Added to this, at least 24 Indian rhinoceroses have been poached in Kaziranga National Park in Assam, India, between 2007 and 2008 (International Rhino Foundation, accessed 12th January 2009 [<http://www.rhinos-irf.org/en/art/178/>]). Subsistence poaching has been replaced by organised poaching to supply the demand of Far Eastern syndicates and possibly the recently more affluent Chinese market (International Rhino Foundation, accessed 6th January 2009 [<http://www.rhinos-irf.org/en/art/375/> and <http://www.rhinos-irf.org/en/art/437/>]).

Of the African rhinoceros taxa, SWR underwent a prolonged bottleneck in the 1900's and subsequent founder events, although the paucity of genetic diversity appears to be an ancient characteristic of the lineage rather than a reflection of the recent history of the species. Black rhinoceroses, in contrast, experienced a recent and rapid decline in population numbers (>96% from the 1970's to 1990's) but have retained relatively high levels of genetic diversity (Harley *et al.* 2005). A metapopulation management approach is therefore more appropriate for the conservation of extant fragmented black rhinoceros populations. SWR, however, are so genetically depauperate that a metapopulation management approach may not be sufficient to limit the effects of drift and potential inbreeding. The data from this study suggest that effective long-term strategies for the conservation of SWR require a shifting focus; a move from management via small populations and translocation programs to one that invests available resources in maintaining a network of larger populations across national parks and conservancies. This would be more likely to effectively mitigate the future loss of genetic diversity in this valuable and charismatic mega-herbivore.

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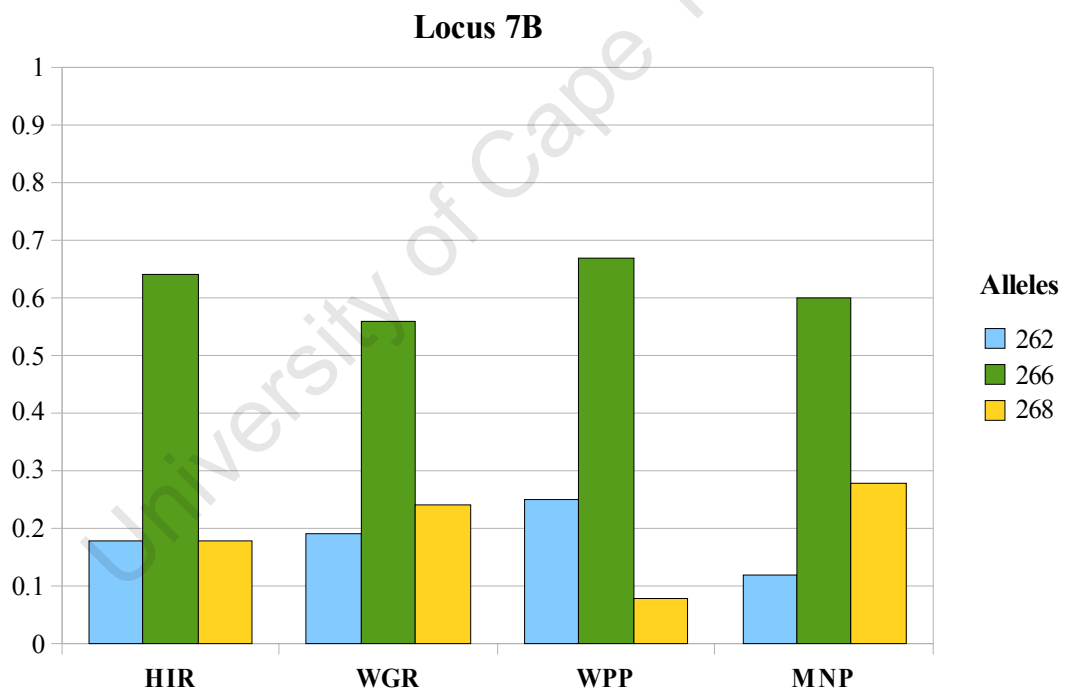
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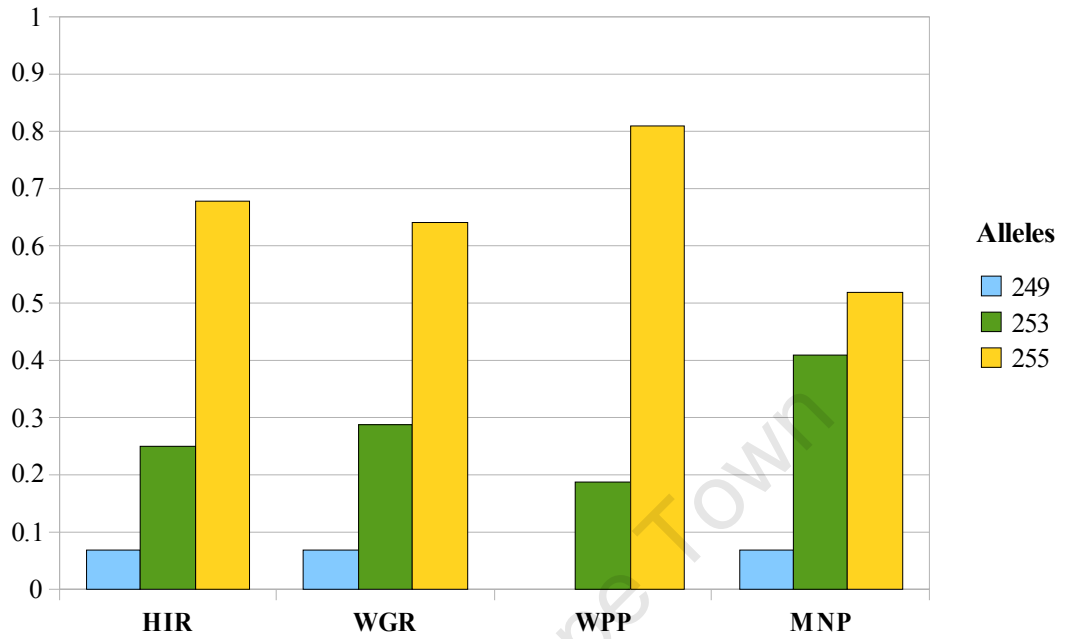
Appendix I

Graphs of Microsatellite Allele Frequencies

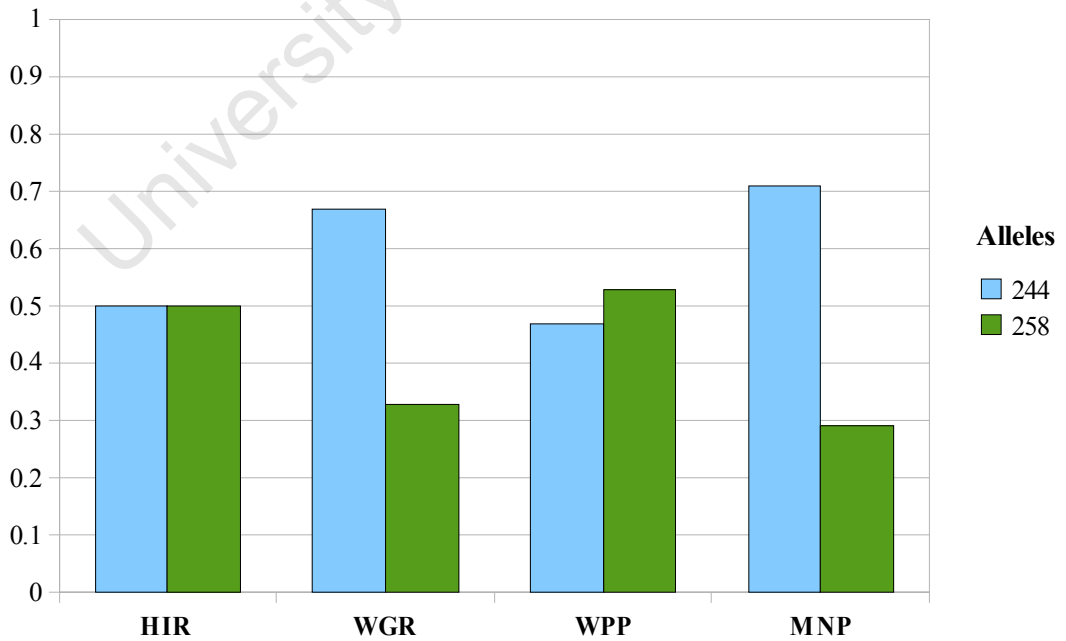
Allele frequency histograms for the source Hluhluwe Imfolozi Game Reserve (HIR) and three seeded populations, Welgevonden Private Game Reserve (WGR), Waterberg Plateau Park (WPP) and Matobo National Park (MNP) at all 13 microsatellite loci analysed.



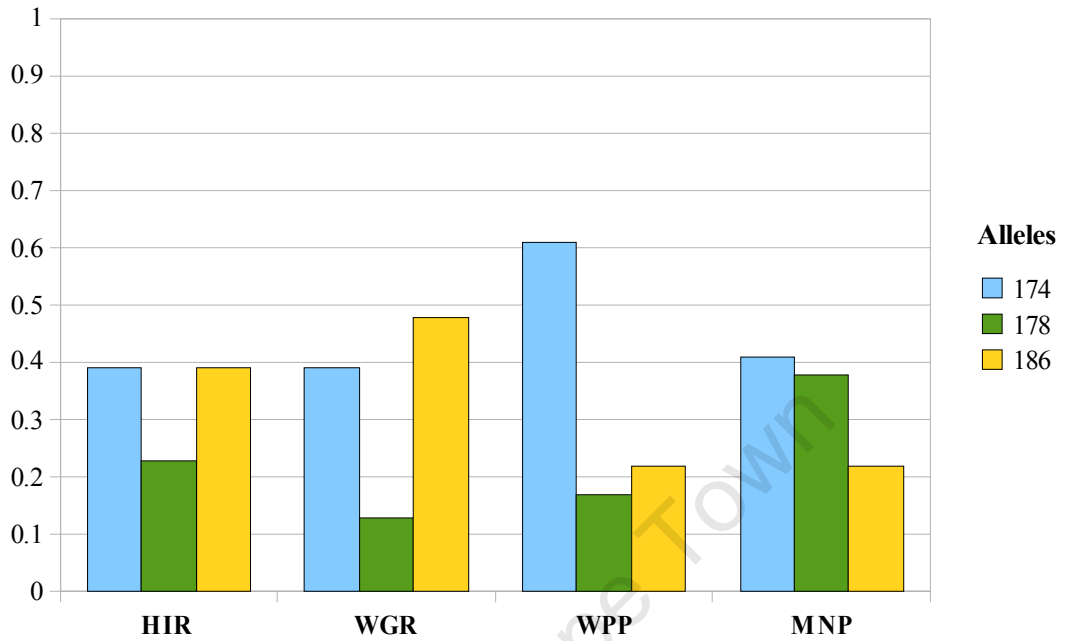
Locus 7C



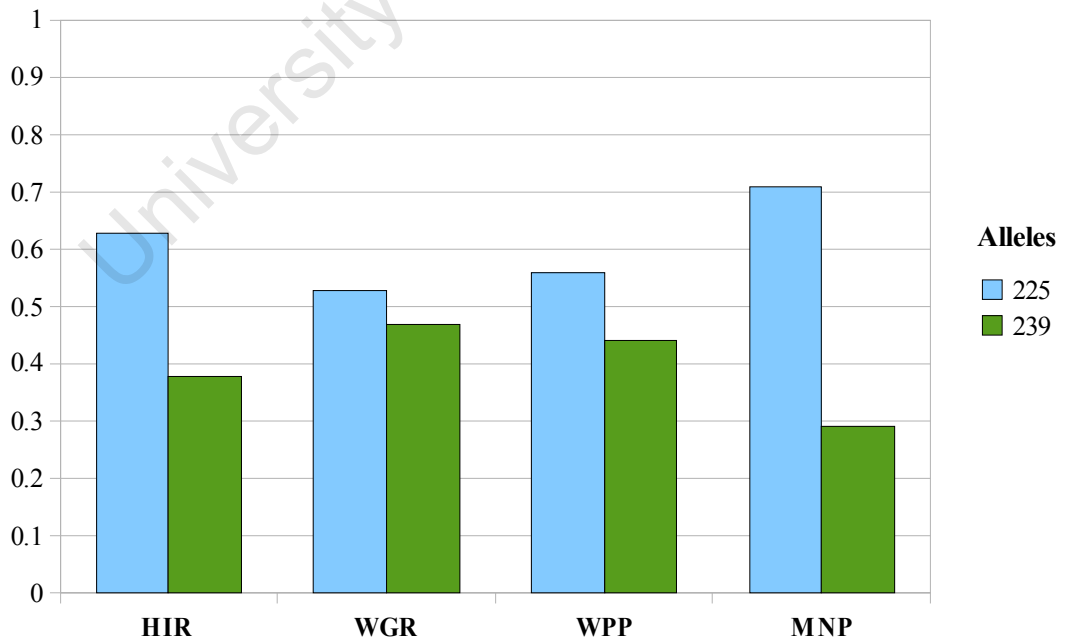
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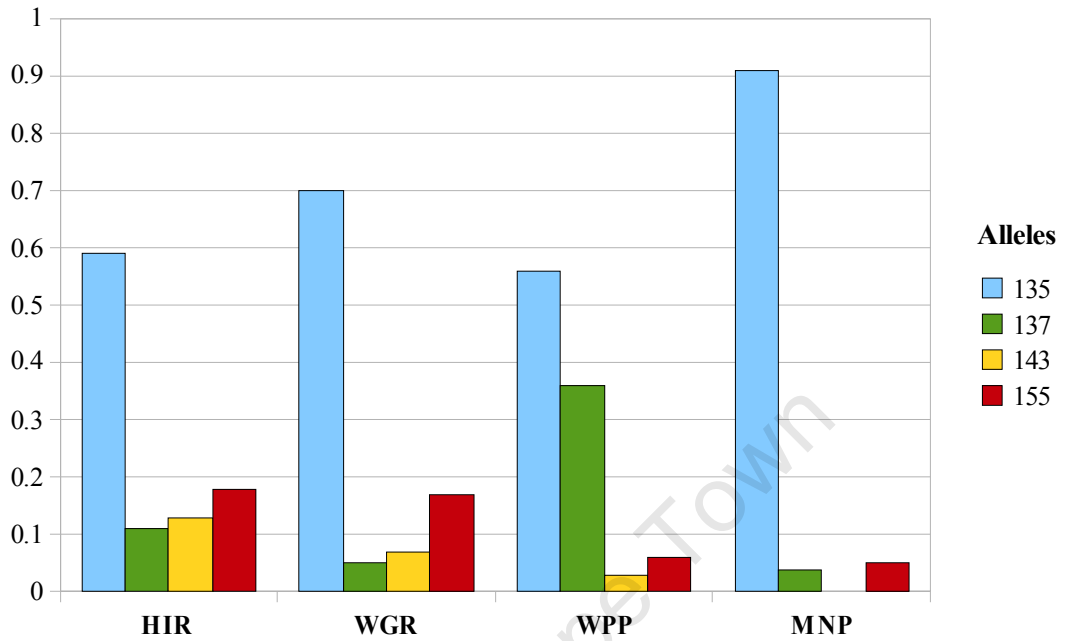
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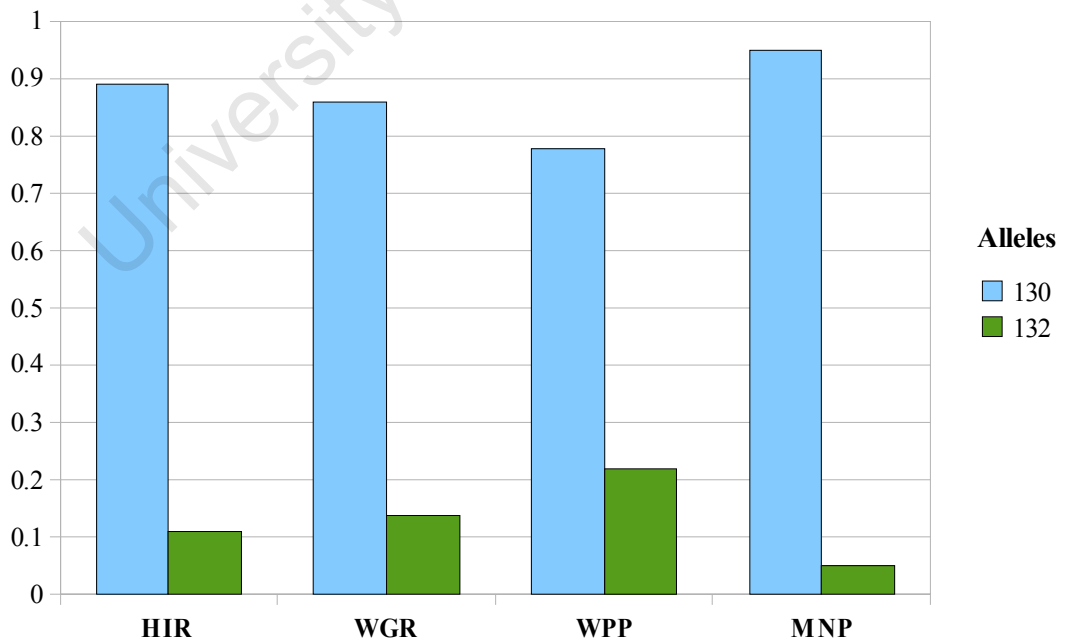
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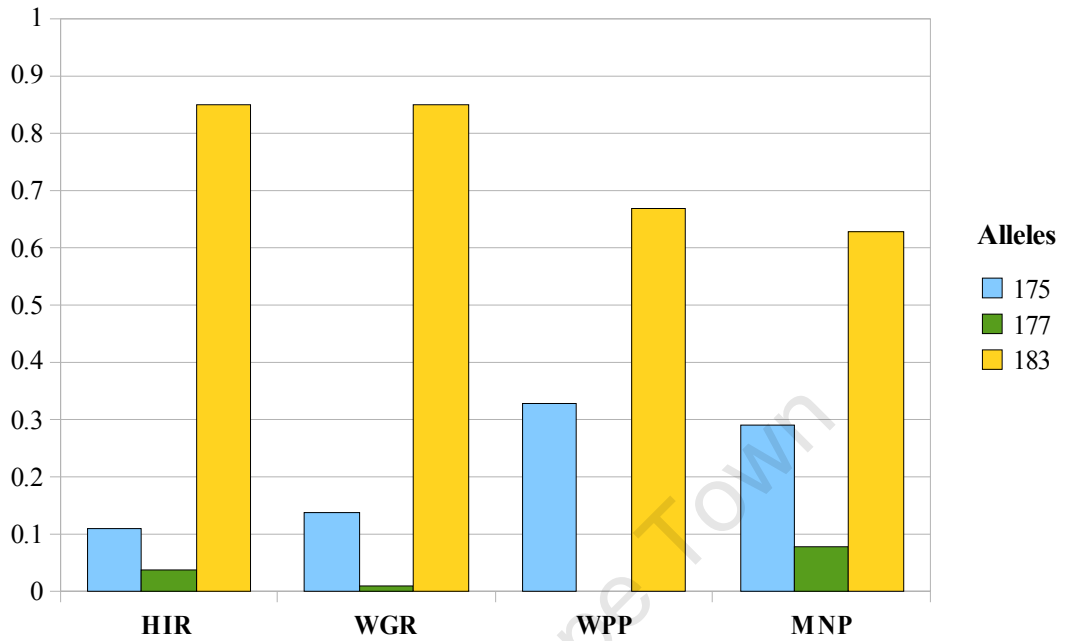
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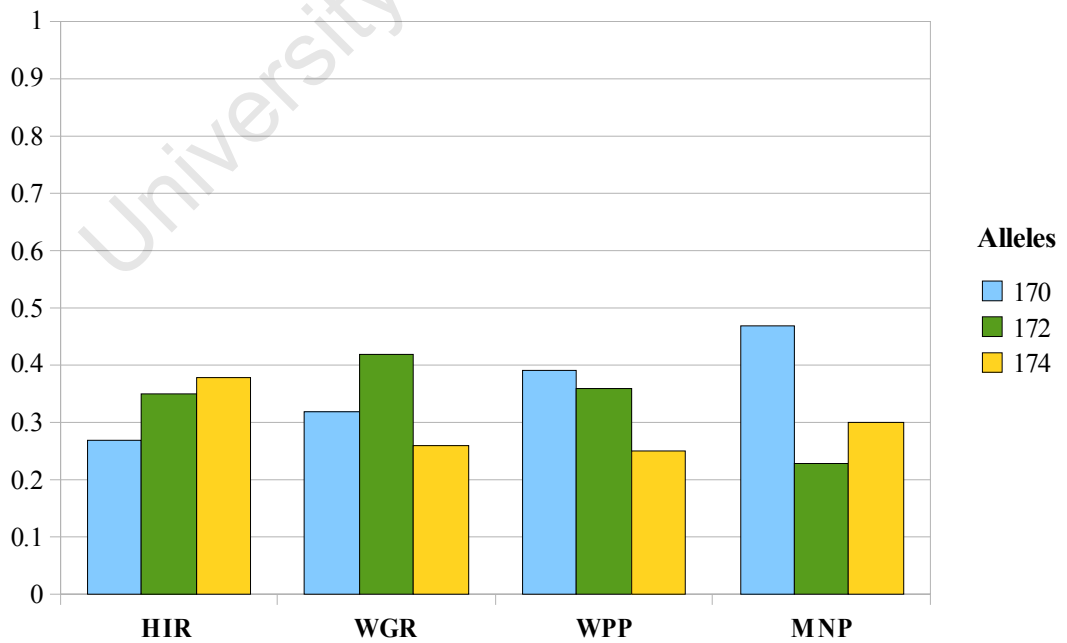
Locus DB1



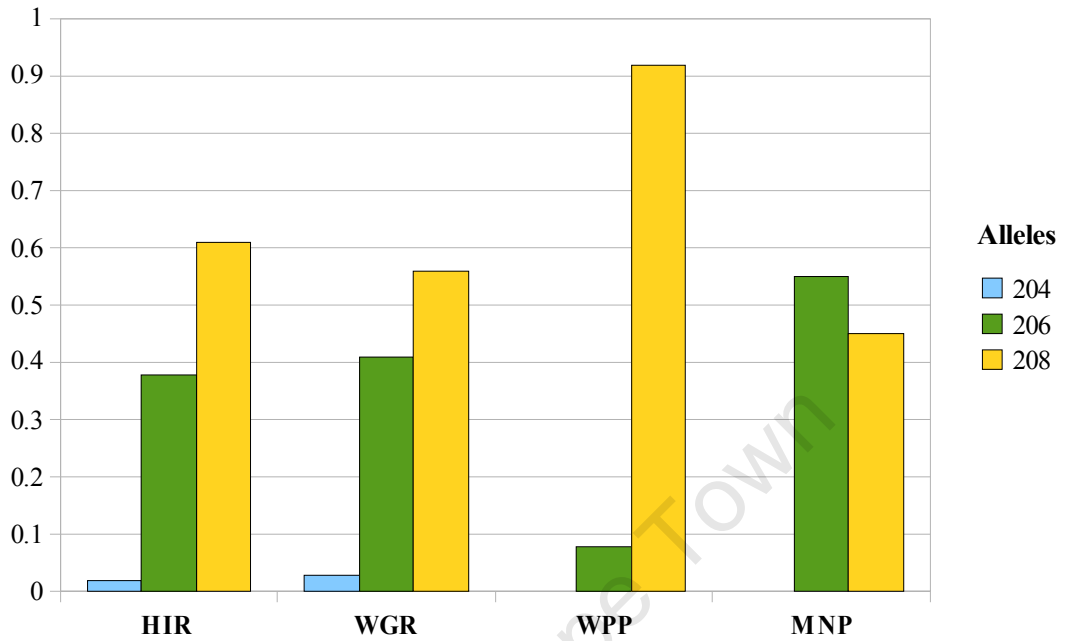
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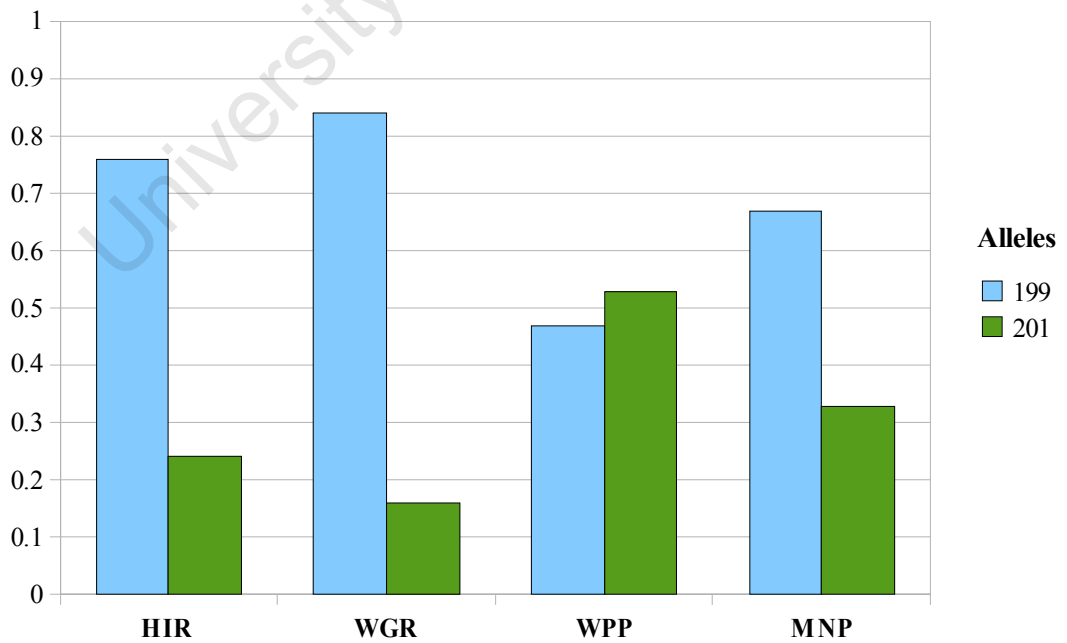
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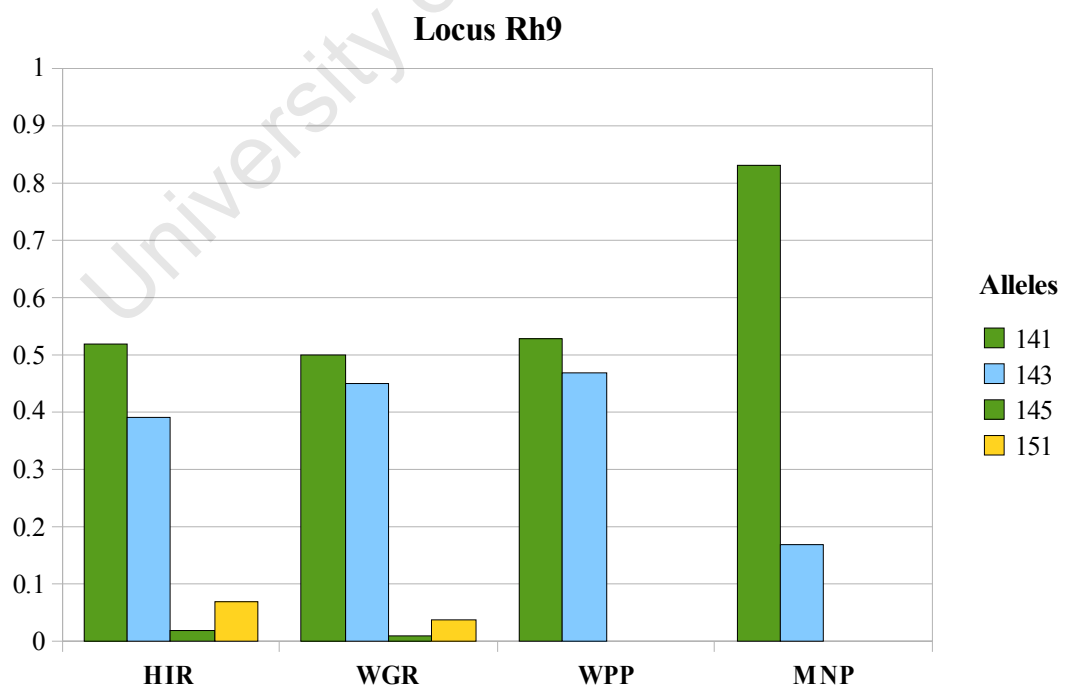
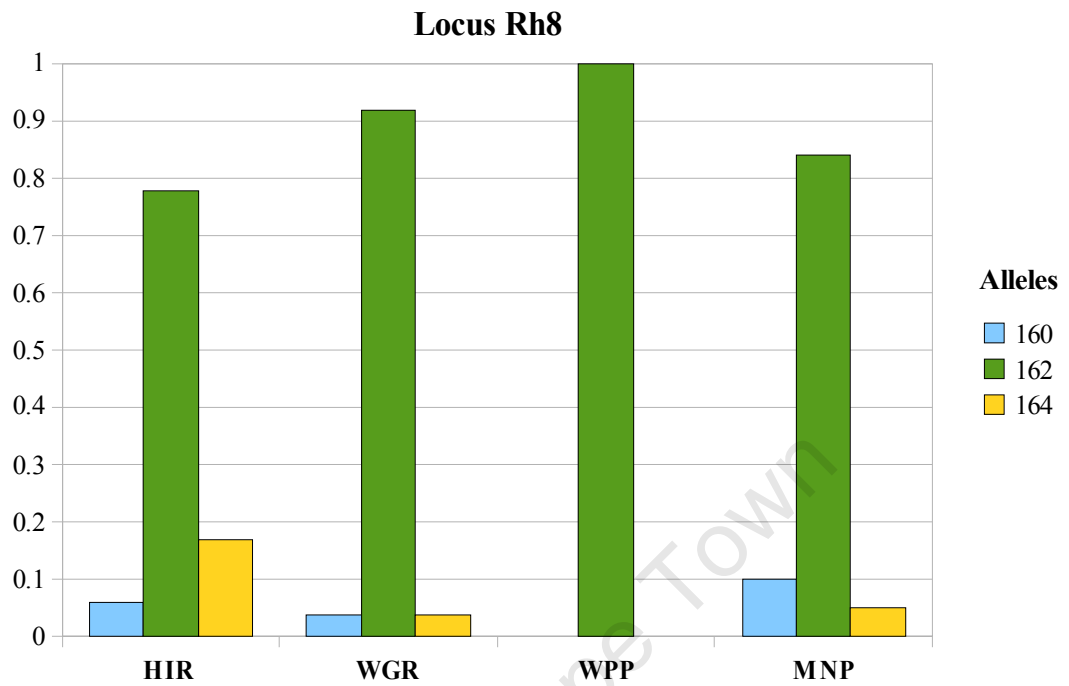


Locus DB66



Locus Rh7





Appendix II

MtDNA Control Region Haplotype Sequences

	10 20 30 40 50 60 70 80 90
SWR1	ACAATCGCCCCTTAAACCACAAAGCACCCTCACATGTAACACACCAGTATTAACATCATTTCCTTGCCACAAATGCAATCTGTACTTGC
SWR2
SWR3
SWR4
	100 110 120 130 140 150 160 170 180
SWR1	CACGTTACAAGTACATCTCGGGTATGTACATCGTGCATTACATTGTTTACCCCATGTCATATAAGCATGTACATTATATTATTATATTAC
SWR2
SWR3
SWR4T.....C.....
	190 200 210 220 230 240 250 260 270
SWR1	ATAACACATTAATAATTGTGATCGAGCATAAACACCCAAACACAGTACATGGATATCCATATCCTAAGCCTACTGTTGATCTTGCATAATA
SWR2
SWR3
SWR4
	280 290 300 310 320 330 340 350 360
SWR1	CATACTATTATTGATCGGACATACCCCATCCAAGTCAAAATCATTTCAGTCAACATGCGTATCACCACCAATATTCCGGCGCTTAATCAC
SWR2
SWR3
SWR4T.....T.....
	370 380 390 400 410 420 430 440 450
SWR1	CAAGCCGCGGAAATCATCAATCCTTCCACTTAATGCATCTGTTCTCGCTCCGGGCCATAACATGTGGGGTTCCTAGACCAGAAACTA
SWR2
SWR3
SWR4C.....C.....
	460 470 480 490 500 510 520 530 540
SWR1	TACCTGGCATCTGGTTCCTTACCTCAGGGCCATCTCACCTAAATCGCCATTCTTTCCCTCTTAAATAAGACATCTCGATGGACTAATGAC
SWR2
SWR3
SWR4
	550 560 570 580 590 600 610 620 630
SWR1	TAATCAGCCCATGATCACACATAAATGTGGTGTGCATTTGGTATCTTTTTTATTTGGGGATGCGTGTGACTCAGCTATGGCCGTCAA
SWR2
SWR3
SWR4A.....
	640 650 660 670 680 690 700 710 720
SWR1	AGGCCTTAACACAGTCAAGCAAATGTAGCTGGACTTAAATAGTACTATTCCCTCCGCATACCTTACCACATGGTGTTATTCAGTCAAT
SWR2
SWR3
SWR4
	730 740 750
SWR1	GGTTACAGGACATACGCATATACACGCATA
SWR2
SWR3
SWR4