

**THE UTILITY OF DNA MICROSATELLITE MARKERS IN CONSERVATION OF A NAMIBIAN
POPULATION OF THE BLACK RHINOCEROS (*DICEROS BICORNIS*)**

by

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ABSTRACT

Massive declines in the latter half of the 20th century left black rhinoceros populations perilously close to extinction with only approximately 3600 individuals remaining, in contrast to estimated census numbers of over 100,000 individuals in 1900. While *Diceros bicornis bicornis* is the second most abundant subspecies of black rhino, behind *D. b. minor*, relatively few genetic studies have focused on this taxon. I used polymorphic DNA microsatellite markers to describe population structure in this subspecies. Using these genetic markers, the goals of this study were: i) to characterize the genetic variability of *D.b. bicornis* individuals (n = 170) found in Etosha National Park, Namibia, using seven DNA microsatellite markers, ii) to determine if there is population structure within Etosha National Park, using both traditional Wright's F-statistics and a Bayesian model that requires no *a priori* population assignment, iii) to determine relatedness amongst founding and newly introduced black rhinoceroses held in private game farms throughout Namibia, using moment estimators of relatedness; and iv) to determine parentage of game farm rhinos with partial or no pedigree data available, using likelihood-based methods. I found that: i) the levels of genetic diversity in *D.b. bicornis* from Etosha National Park are consistent with previously published studies using microsatellite markers, and are relatively high; ii) the population structure within Etosha National Park is ambiguous using traditional F-statistics, but can be subdivided into three diagnosable clusters (western, central and eastern) using a Bayesian approach; iii) a relatively high degree of relatedness exists among founding members of game farm rhinos; and iv) assignments of parentage had greater success when there were fewer candidate parents and support the notion that black rhinoceroses are polygynous.

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CHAPTER I

GENERAL INTRODUCTION

Study Species

The black rhinoceros (*Diceros bicornis*) was once distributed across sub-Saharan Africa; however, widespread poaching and habitat loss in the 20th Century led to a precipitous decline in global population size and a marked range contraction (Fig. 1). Indeed this is one of the most dramatic declines of any megaherbivore attributable to habitat destruction and fragmentation brought on by human activities, in addition to, extensive poaching both for its highly desirable horns and bush meat. Historical records indicate that hundreds of thousands of black rhinos existed at any given time in the last two centuries, but since the 1970s numbers were reduced by 96% to a low population size estimate of ~2,400 individuals by 1998 (International Rhino Foundation, 2006). As a consequence of this decline, black rhinoceros populations have become increasingly isolated and herds on average are much smaller than those recorded historically. Today, most black rhinoceroses are located in four countries: South Africa, Namibia, Kenya and Zimbabwe and only five populations have been characterized as having greater than 100 animals or >50% of any subspecies (Emslie and Brooks, 1999). Through intensive census efforts at national and international levels, the current black rhinoceros population has been estimated to be approximately 3,600 individuals (International Rhino Foundation, 2006).

The black rhino is the third largest land mammal in Africa, behind only the African elephant (*Loxodonta africana*) and the white rhinoceros (*Ceratotherium simum*). It has a total body length of approximately 3.5 metres and an average weight of 1400 kilograms. Along with the white rhino, the black rhino has two prominent horns made from keratin with the front horn growing as long as 1.4 metres. These horns are highly prized by

poachers as trophies, are used in ceremonial daggers in such countries as Yemen, or used in medicinal therapies (e.g. aphrodisiacs). This poaching has led to the massive population declines and is the single greatest threat to the black rhino population at this time. Most black rhinos are purported to be solitary although several rhinos have been observed around a good feeding or watering site (P. Erb, pers. comm.). Black rhinos are territorial, a characteristic that may enhance genetic structuring (see below). To demarcate its territory an individual will stamp in its feces to deposit the odour with each footstep letting other animals know that they are within another's home range.

Four extant subspecies are currently recognized based on slight morphological distinctions and geographical distribution (Du Toit, 1987): *Diceros bicornis bicornis* (South-western), *D.b. minor* (South-eastern), *D.b. michaeli* (Eastern) and *D.b. longipes* (Western). *Diceros b. bicornis*, with a population of 1310 individuals, has a longer, straighter horn, is more adapted to arid climates and is found in Namibia, southern Angola, western Botswana and south-western South Africa (The International Rhino Foundation 2006). *Diceros b. minor* is the most abundant subspecies, with a wild population estimated to comprise 1770 individuals, and is mostly found in southern South Africa with smaller populations in Zimbabwe, Swaziland, southern Tanzania and Mozambique. *Diceros b. michaeli* has horn that is longer, more slender and more greatly curved horn than the two southern subspecies. This taxon has distinctive skin ridges on its sides and is reputedly more aggressive. The current wild populations of this subspecies, estimated to contain some 520 individuals, are found in Kenya, north-eastern South Africa and Tanzania. *Diceros b. longipes* is the rarest and most endangered subspecies with a putative population of 5 individuals in Cameroon only. Figure 1 illustrates the current and inferred historical distributions of all black rhinos in Africa.

Preservation of black rhinos will be aided by understanding their mating system and individual mate choice preferences in the wild to better manage the social dynamics of herds that are being culled and manage captive populations. Current knowledge of individual mate choice in the black rhino is limited, but it is known that males engage in courtship behaviours before mating that includes snorting and sparring with their horns (Joubert, 1971). Breeding pairs remain together for 2-3 days and sometimes even weeks during which they mate several times a day. Copulation bouts last approximately a half hour. However, copulations may be interrupted by a dominant male who will then proceed to mate with the female. Presumably this means that census population size and genetic effective population size may be quite different because of asymmetry in mating success among males – an important observation for maintenance of genetic diversity of this species.

The gestation period is 15 months with the cow giving birth to a single calf weighing approximately 35-50kg. Weaning occurs at around 2 years of age for the offspring. The mother and calf remain together post-weaning for an additional 2-3 years until the next calf is born. Sexual maturity is reached at 5 years of age for females and 7 years for males; however, in natural settings reproduction does not usually occur until females are approximately 10 years old and males 15 years (Goddard, 1966). Life expectancies of black rhinos range from 35-50 years. The long time between successive generations and other life history attributes imply that post population crash recovery might be very slow and that effective population sizes (N_e) may differ substantially from census population sizes, and have potentially profound implications for how we manage both wild and captive populations.

Although census numbers have increased over the last two decades, all subspecies are classified as either critically endangered or vulnerable (IUCN, 2006). Current

management policy strives to maintain these subspecies separately to preserve genetic integrity given that possible consequences of interbreeding taxa potentially including outbreeding depression as a result of a loss of locally adaptive features (Swart and Ferguson, 1997). For instance, *D. b. bicornis* is presumed to be able to tolerate arid desert conditions and the vegetation adapted to such an environment found in Namibia while *D. b. michaeli* is presumed to be adapted to a relatively more humid habitat and greener vegetation in eastern Africa. Thus, hybrid offspring from a mating between individuals of two subspecies may not help the conservation of the species if their phenotype is intermediate and thus ill suited for either parental environment. At this point, identifying and maintaining local populations of the different black rhinoceros subspecies is one goal of conservationists. Microsatellites are neutral, nuclear genetic markers that can be utilized to identify the genetic diversity of a given population and hoped to be reflective of the adaptive diversity present in the population (Bruford and Wayne, 1993).

Because of the rapid diminution of wild populations, the black rhino is of particular interest to scientists who wish to study how long-lived organisms respond to recent population bottlenecks, given that such bottlenecks are thought to reduce genetic diversity through genetic drift and increased inbreeding potential (Maruyama and Fuerst, 1985; Luikart and Cornuet, 1998). However, research to date suggests that black rhinos have suffered little to no such genetic consequences due to population bottlenecks (Harley *et al.*, 2005). Investigating the genetics of the black rhinoceros will provide insight into: i) the effect of population bottlenecks on genetic diversity of long-lived species, ii) the genetic structuring of an understudied and imperilled African taxon with marked social structure and high territoriality, iii) utility of high-resolution DNA markers in determining parentage

in known or partially known pedigrees of this species, and iv) patterns of relatedness amongst individuals residing within private sanctuaries.

Conservation Genetics

Conservation biology has been termed a crisis science because of the immediacy of the problem and need to make decisions based on imperfect data (Soulé, 1985). These crises are usually the result of large losses of population and/or habitat brought about by human activities, which threaten the survival of particular species. An important goal of conservation biology is to preserve the evolutionary potential of species by maintaining sufficient levels of genetic diversity so that they may respond to future environmental challenges. To aid this goal a newer branch of conservation biology has been developed, conservation genetics (Frankham, 1985). Conservation genetics encompasses both basic and applied approaches and uses a combination of ecology, molecular biology, population genetics, mathematical modeling and evolutionary systematics to provide a comprehensive picture of the distribution of genetic diversity within a species of conservation concern from the relationship among individuals within single populations to consideration of the evolutionary affinities among related species (see Frankham et al. 2004). Using the information derived from these studies, management techniques can be applied to preserve biological diversity in these organisms. Without using genetics, conservation efforts may be directed at the wrong population or waste valuable resources on a population that is not at risk for losing its biological diversity despite a population decline.

Ronald Fisher (1930), in what was to become known as his “Fundamental Theorem of Natural Selection” indicated that a population’s ability to respond to natural selection is proportional to the amount of genetic diversity present at the outset. This assertion is a

critical underpinning of management philosophy of endangered species; retaining genetic diversity presumably will increase the probability that a species or population segment thereof will have sufficient ability to adapt to future environmental changes precipitated by human activities. An important caveat is that assays of neutral genetic diversity as I have undertaken here in my data chapters may not accurately capture variation at loci that are of adaptive importance.

Fragmentation into small, isolated populations can lead to vulnerability due to environmental, demographic and genetic stochasticity (Gilpin and Soulé, 1986). For example, disease epidemics and natural catastrophes are environmental factors that can disproportionately threaten small populations. Demographic shifts can produce biased sex ratios and negatively impact mate recognition and acquisition (Gilpin and Soulé, 1986). Small populations are more susceptible to loss of genetic variability through inbreeding and/or genetic drift, which in turn may negatively affect individual fitness and reduce adaptability. Inbreeding depression can further reduce the effective population size (Saccheri *et al.*, 1998). These theoretical effects of habitat fragmentation require further assessment to see what practical implications, if any, there are for a long-lived species.

Many highly visible megafaunal species verge on extinction due to myriad man-made factors. At current rates of decline, several hallmark terrestrial mammal species such as the African elephant (*Loxodonta africana*), Asian elephant (*Elephas maximus*), river hippopotamus (*Hippopotamus amphibius*), cheetah (*Acinonyx jubatus*), African lion (*Panthera leo*), Siberian (*Panthera tigris altaica*) and Sumatran (*Panthera tigris sumatrae*) tigers, not to mention, all five rhinoceros species – black, white (*Ceratotherium simum*), Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran (*Dicerorhinus sumatrensis*) – will have no surviving member in a hundred year's time (IUCN, 2006). My

project focuses on evaluating patterns of genetic diversity of one such species, the black rhinoceros, and seeks to quantify genetic diversity across hierarchical scales to address a series of questions that are relevant to its conservation and presumably to other large mammals species with long generation times.

To help prevent the extinction of the black rhino, conservation biologists and wildlife researchers in Namibia have set up game parks and natural reserves to maintain swathes of natural habitat to house significant populations of *D.b. bicornis*. The founding populations of these game farms were established by translocating individual wild rhinos from locales surrounding Etosha National Park (geographical coordinates and distribution described in Chapter 3). However, lack of knowledge or resources may have meant that individuals were selected from within close proximity of each other. Thus, the possibility of high relatedness among these founding individuals exists because of the aforementioned high territoriality and finite dispersal distances in the arid Namibian desert where food and water sources are limited. In sum, the gene pool of the founder populations may not accurately have captured a representative diversity but this assertion remains to be tested. Translocations of new individuals into these various parks have taken place over the past 20 to 30 years to augment the genetic diversity contained within their borders. One goal of my thesis was to determine what impact, if any, these translocated individuals had on the overall genetic variability contained in populations of black rhinos within these parks.

A PubMed search reveals that most previous studies on the black rhinoceros have focused on the ecology and veterinary medicine of the species rather than on the genetics with only a handful studies focused on some aspects of genetic diversity of wild populations (see below). To derive a fuller picture of the genetic variability of this species, I decided to develop species-specific DNA microsatellite primers, in addition to those previously

published (Brown and Houlden, 1999; Cunningham *et al.* 1999), to assess the diversity present in different subspecies of the black rhinoceros in sub-Saharan Africa.

Genetics of the Black Rhinoceros

Past literature on the molecular diversity in black rhinos has shown a range in levels of genetic variation at the species level; thus, no consensus exists as yet. Allozyme data (Swart *et al.*, 1994; Swart and Ferguson, 1996) and DNA microsatellite studies (Brown and Houlden, 1999; Garnier *et al.*, 2001; Harley *et al.*, 2005) have shown that some wild black rhinoceros populations have maintained moderate to high levels of diversity; however, mitochondrial variation has been shown to be low relative to other large vertebrates (Ashley *et al.*, 1990; O’Ryan and Harley, 1993; O’Ryan *et al.*, 1994; Brown and Houlden, 2000). Levels of variation differ among subspecies with *D.b. minor* and *D.b. michaeli* found to have the highest levels of variation (Brown and Houlden, 1999; Harley *et al.*, 2005) although sample sizes were small with large error associated with diversity estimates. *Diceros b. longipes* is nearly extinct with only five individuals remaining in Cameroon (International Rhino Foundation, 2006); therefore, studies in this subspecies are lacking.

Despite the relatively large numbers of *D.b. bicornis* found in Namibia, this subspecies has also had remarkably limited genetic evaluation. Thus, one major focus of the current study was to characterize the variability in the largest sample size surveyed to date ($n = 170$) from this population using DNA microsatellites and to assess the presence or absence of genetic structuring within a large population enclosed within Etosha National Park.

DNA Microsatellite Molecular Markers

Microsatellites are rapidly evolving, non-coding sequences of DNA that are in the eukaryotic nuclear genome with base motifs 1-6 bp in length and repeats usually 6-30 units in length but occurring upwards of 100 times for some loci (Tautz and Renz, 1984; Tautz, 1993). Microsatellites are valuable for population genetic studies (Bruford and Wayne, 1993), as they are co-dominant Mendelian markers, provide reproducible results and are purported to be selectively neutral (Tautz and Renz, 1984). Due to their generally exceptionally high mutation rates and generally large numbers of alleles, microsatellites have found wide applicability in establishing genetic differentiation amongst populations (Paetkau *et al.*, 1995), DNA fingerprinting (Jeffreys and Pena, 1993), linkage analysis (Todd *et al.*, 1991), reconstruction of phylogeny (Bowcock *et al.*, 1994) and determination of parentage (Queller *et al.*, 1993). Due to their exceptional variability, microsatellites are particularly useful in determining recent evolutionary events such as population bottlenecks and sub-structuring due to rapid repopulation (Johnson *et al.*, 2003).

One potential difficulty with the metrics of differentiation employed for microsatellites arises from the possible mutational properties of microsatellite loci. Microsatellites appear to evolve according to a stepwise mutational model (SMM), where mutations result in the addition or deletion of one or more repeat units due to slippage (Weber and Wong 1993; Di Rienzo *et al.*, 1994). More complex patterns of variation, such as the presence of insertions/deletions in regions flanking the repeat array (Zardoya *et al.* 1996; Grimaldi and Crouau-Roy, 1997; Colson and Goldstein, 1999), nucleotide substitutions or insertion-deletions within the arrays (Estoup *et al.* 1995; Garza and Fremier, 1996; Angers and Bernatchez, 1997; van Oppen *et al.*, 2000), mutational biases related to array length (Weetman *et al.*, 2002) and allele size constraints (Garza *et al.*, 1995) indicate

that mutational patterns at microsatellite loci may deviate significantly from a strict SMM, adding unknown levels of bias to both frequency-based (F_{ST}) and size-based (R_{ST}) estimators of population differentiation. Size homoplasy of microsatellites may introduce a downward bias in estimates of population differentiation by making allelic distributions more similar than they would be under an infinite alleles model (Estoup and Cornuet, 1999). Uncertainty about the effects of mutational processes on the magnitude of differentiation is compounded by the wide variability in levels of polymorphism among individual microsatellite loci. Using simulations, Estoup *et al.* (2002) concluded that homoplasy is most prevalent when populations are large and mutation rates are high, conditions likely to characterize some microsatellite loci in historical black rhinoceros populations. The estimated slippage mutation rate of some microsatellite loci increases exponentially with the number of uninterrupted repeats (Brinkmann *et al.* 1998; Lai and Sun, 2003). However, highly polymorphic loci are often not observed in large mammalian species (DeWoody and Avise, 2000) suggesting that the number of uninterrupted repeats and mutation rates may be relatively low. Consistent with this, previous research looking at the development of microsatellite primers in the black rhino has yielded fewer than ten polymorphic loci (Brown and Houlden, 1999).

Population Structuring Methods: Traditional F-Statistics

In assessing population structure, ultimately we are interested in quantifying the statistical differences on allelic and genotypic frequencies that arise via the interaction of microevolutionary processes like genetic drift, mutation and selection (Wright, 1931; Wright, 1937). The impacts of each evolutionary force on populations depend on an

interplay of demography, natural history and dispersal among populations of a given species, which in turn may be influenced by human-caused habitat fragmentation. Evolution acts by redistributing genetic variation among different population levels, thereby establishing population genetic structure. In diploid species such as the black rhino, the simplest hierarchical organization is: i) individuals; ii) population; and iii) among populations within the species.

Analyzing the distribution of genetic diversity across these hierarchical levels can help us to infer which evolutionary forces are acting on the observed set of populations. Traditional population structuring methods are based on Wright's F-statistics (Wright, 1951) and their analogues (i.e. R_{ST} ; Slatkin, 1995). The extent of reduction in observed heterozygosity, which is discussed below, can be used to quantify the level of genetic differentiation between subpopulations. The understanding of these statistical measures requires the definition of terms. H_I = mean observed heterozygosity per individual within subpopulations. H_S = mean expected heterozygosity within random mating subpopulations. H_T = expected heterozygosity in random mating total population. Using these measures of heterozygosity (H), three hierarchical measures of F-statistics can be employed: F_{ST} , F_{IS} and F_{IT} .

F_{ST} relates to the fixation index and is equal to $(H_T - H_S)/H_T$. F_{ST} measures the mean reduction in H of a subpopulation relative to the total population due to genetic drift among these subpopulations. For a single diallelic locus, genetic differentiation among subpopulations can range from 0.0 indicating no differentiation to 1.0 where complete differentiation exists meaning subpopulations are fixed for different alleles. Wright (1978) suggests the following general guidelines for the interpretation of F_{ST} : i) 0.0 to 0.05 may be considered as indicating little genetic differentiation; ii) 0.05 to 0.15 indicates moderate

genetic differentiation; iii) 0.15 to 0.25 indicates great genetic differentiation; and iv) values of F_{ST} above 0.25 indicate very great genetic differentiation

The inbreeding coefficient, F_{IS} , which equals $(H_S - H_I)/H_S$, measures the mean reduction in H of an individual due to non-random mating within a subpopulation. The extent of genetic inbreeding within subpopulations can range from -1.0 (all individuals heterozygous) to $+1.0$ (no observed heterozygotes; Wright, 1951).

Finally, the overall fixation index, F_{IT} , which equals $(H_T - H_I)/H_T$, measures the mean reduction in H of an individual relative to the total population. F_{IT} combines contributions from non-random mating within groups (F_{IS}) and effects of random drift among demes (F_{ST}). The relationship between the three F-statistics is: $(1 - F_{IT}) = (1 - F_{IS})(1 - F_{ST})$.

F-statistics are indirect estimates of gene flow and population structure. Unfortunately, the mathematical model underlying the analyses relies on many biologically unrealistic, simplifying assumptions (Whitlock and McCauley, 1999). F-statistics are based on the island model where an infinite number of populations receive and distribute individuals at an equal rate, m , per generation and are of equal size, N . The migrating individuals are randomized and dispersed to other populations without regard for geographical structure; thus, yielding populations that are equally likely to receive and give migrants from all other populations. The island model also assumes that no selection or mutation exists, all populations persist indefinitely and an equilibrium has been reached between migration and drift (Whitlock and McCauley, 1999). Natural populations are likely to violate these assumptions and there is often limited quantitative information gained about dispersal by using gene frequency data in this manner (Whitlock and McCauley, 1999).

Population Structuring Methods: Bayesian Model

Traditional F-statistics are limited in their approach to population structure in that population assignments are determined prior to analysis sometimes based on quite arbitrary information derived from collecting provenances. Assignment tests provide an attractive alternative to these as they can be individual rather than population based and make no *a priori* assumptions about population structure. For example, a more robust analytical method using a Bayesian approach has been described (Pritchard *et al.*, 2000; Falush *et al.*, 2003). This new method does not require an *a priori* assignment of geography or population of origin and has been used to detect cryptic population structure in other large mammals such as wolverines (*Gulo gulo*; Cegelski *et al.*, 2003), Scandinavian lynx (*Lynx lynx*; Rueness *et al.*, 2003) and marten (*Martes americana*; Small *et al.*, 2003).

Pritchard *et al.* (2000) describe a model-based clustering method for using multilocus genotype data to infer population structure and assign individuals to populations based on allele frequencies at each locus. The model assumes there are K populations where K may be unknown at the outset. Individuals in the sample are probabilistically assigned to a population or jointly to two or more populations if their genotypes indicate that they are admixed. The model does not assume a particular mutation process and can be applied to DNA microsatellite markers. Applications of this Bayesian method include testing for the presence of population structure, assigning individuals to populations, studying hybrid zones, and identifying migrants and admixed individuals. This method can produce highly accurate assignments using modest numbers of loci such as those used in this study (Pritchard *et al.*, 2000; Falush *et al.*, 2003).

Objectives of the Study

In this thesis I investigated the levels and distribution of genetic variation in one subspecies, *Diceros bicornis bicornis*, found in Namibia. In Chapter 2, using DNA microsatellite markers I address the following questions: i) What levels of genetic variation currently exist within Etosha National Park in *D.b. bicornis*? ii) How do these levels compare with populations from two other subspecies, *D.b. minor* and *D.b. michaeli*? and iii) Given that habitat fragmentation has been extensive, is population structuring present at the geographic scale encompassed by the park and what are management implications if it does exist?

In Chapter 2, I hypothesize that: i) black rhinos found in Etosha National Park have relatively high levels of genetic diversity due to their long generation time associated with their long life spans compared to the relatively short timelines associated with the population bottleneck, and due to the introduction of individuals over the last two decades; ii) consistent with previously published studies, the Etosha National Park *D.b. bicornis* population maintains high levels of genetic diversity, but are lower than those found in *D.b. minor* and *D.b. michaeli* populations, and iii) population structure will be present within Etosha National Park and will be revealed using traditional F-statistics, recently developed Bayesian algorithms as encoded in the program STRUCTURE, or both.

To aid conservation efforts, several black rhinoceros game farms have been established within the last twenty years. Decisions on which animals to translocate to these farms were based on balancing numbers of mature and young animals and males to females. Because of the possibility raised above that closely related individuals may have been among the translocated individuals, genetic diversity in these game farms may not be as high as in natural populations. Thus, as the ultimate goal of these farms is to increase

population numbers, it is important to understand the social structure and mating system to better manage the species. This will help conservationists select more desirable individuals for future translocations. While this thesis does not attempt to evaluate individual sexually desirable traits in prolific reproducers, knowledge gained by determining who is producing the most offspring will allow farm managers to observe these individuals more closely to assist in the identification of those traits. Unfortunately, despite this, even within these man-made confines observing rhinos mate is nearly impossible due to the logistics. A general goal of this thesis is to evaluate the efficacy of microsatellites in studying a mammalian mating system. In Chapter 3, I address questions arising from black rhinoceros game farm management. Specifically, I hypothesize that: i) relatedness among founding black rhinos as measured by DNA microsatellite markers is higher than that of a naturally-derived population; and ii) parentage within game farms can be determined using microsatellites, which allows for construction of pedigrees.

The major findings from both chapters are summarized and discussed in Chapter 4.

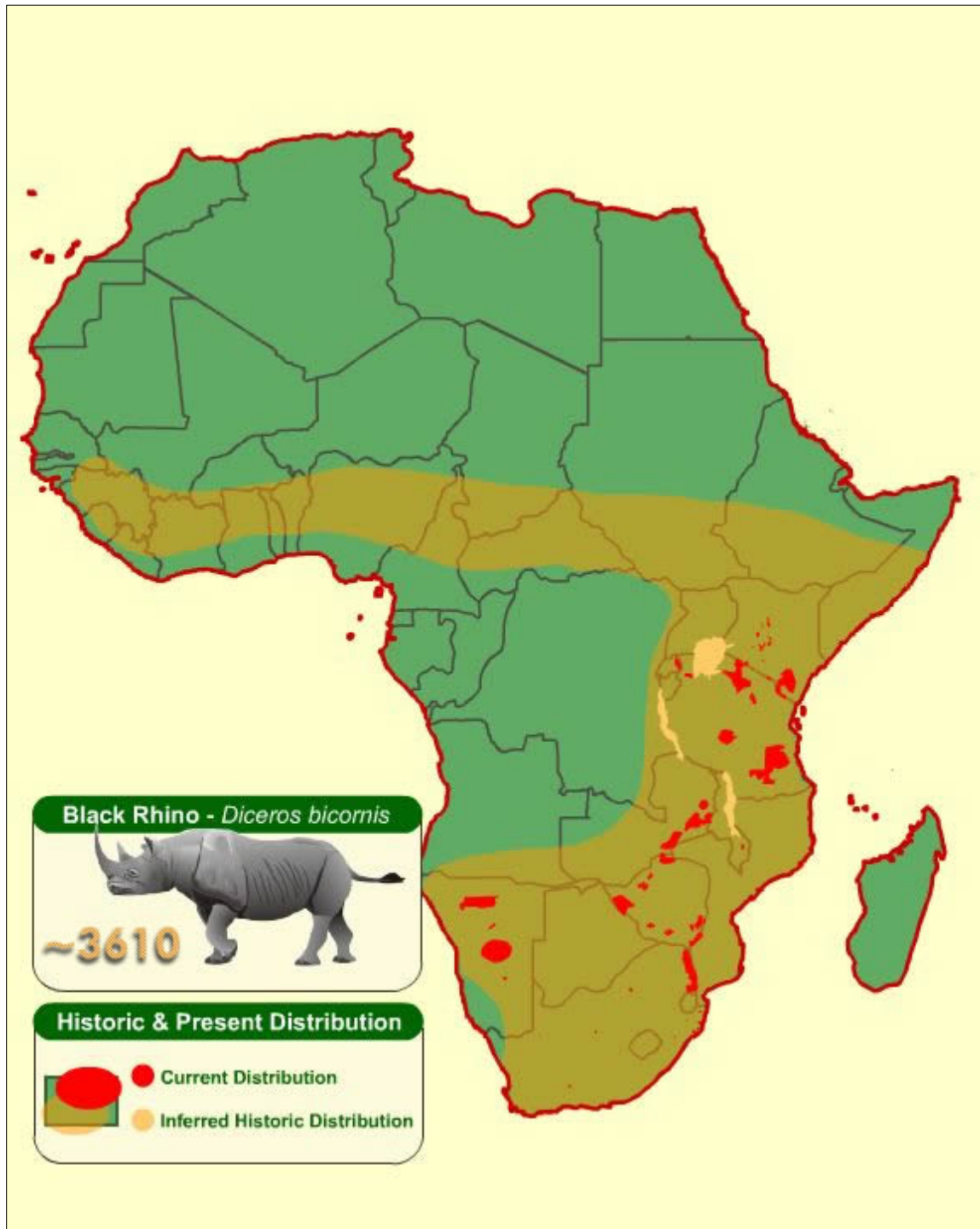


Figure 1. Map of current and historical distributions of black rhinos. (<http://www.rhinos-irf.org/rhinoinformation/blackrhino/popups/blackrhinodistrmap.htm>; International Rhino Foundation, 2006; used with permission).

CHAPTER 2
POPULATION STRUCTURE IN AND AROUND ETOSHA NATIONAL PARK

ABSTRACT

The black rhinoceros (*Diceros bicornis*) of sub-Saharan Africa, is comprised of four subspecies: *D.b. michaeli*, *D.b. minor*, *D.b. longipes* and *D.b. bicornis*. While the former two subspecies have been subject to a relatively large number of studies, research on the latter two is lacking. *Diceros b. longipes* is on the verge of extinction so meaningful studies are difficult, but *D.b. bicornis* is the second most common subspecies and has its largest population in Etosha National Park, Namibia. We conduct the largest population genetics study yet of individuals in this subspecies ($n = 170$) using seven polymorphic nuclear microsatellite loci. Measurements of genetic variability indicate a relatively high amount of diversity with expected heterozygosity of 0.483 (± 0.04 se) and an average number of alleles of 3.33 (± 1.6 se) despite the purported major population bottleneck that occurred over the past 50 years. These estimates are consistent with previously published studies with smaller sample sizes. Population structure within Etosha National Park was analyzed using traditional Wright's F-statistics and a Bayesian model, STRUCTURE. $F_{ST} = 0.15$ between the Western subpopulation and Central subpopulation, but $F_{ST} = 0.03$ between the Western and Eastern subpopulations implying population structure exists between the former subpopulations, but not the latter. No significant differences in F_{ST} were calculated between the Central and Eastern subpopulations. Thus, population structuring was evident using traditional methods, but results from the Bayesian approach suggested that three main clusters of individuals occur, which may be correlated with geographical barriers such as the distance between watering holes and the uninhabitable Etosha Pan.

INTRODUCTION

History of Black Rhinoceroses in Etosha National Park

Etosha National Park (ENP) is a 3500 km² semi-arid park in the northern region of Namibia. Historical accounts in the late 1700s indicate a contiguous population of the Southwestern black rhino throughout the country, although no direct census numbers exist from this period (Joubert, 1971). However, after 1900, the distribution of individuals changed north of Windhoek due to increasing human settlement (Joubert, 1971). Throughout the 20th century, and most prominently in the latter half, massive declines occurred due to poaching and other anthropogenic factors. As a result, efforts were made by the Namibian government to preserve this subspecies, one of which was to translocate individuals into ENP. Between 1970 and 1972, forty-three black rhinoceroses from within Namibia were captured using helicopters and transferred to previously rhinoceros-free ENP where they have become part of a well-established and viable population (Joubert, 1971).

The founding population of 43 black rhinos originated from five principal localities just outside of Etosha National Park including: farms in the Ugab Valley (n=10), the Otjihorongo Reserve (n=5), the immediate vicinity of the petrified forest west of Khorixas (n=11), farms to the southwest of Kamanjab (n=10) and in Kaokoland (n=6), an area adjoining the western boundary of ENP. The geographical range across the founding locales is very small and mature individuals would have been easily able to cover this entire range if permitted. One individual was also captured from the Erongo Mountains (Hofmeyr *et al.*, 1975).

After the initial introduction of the founding population in the early 1970s, new individuals were translocated into the park throughout the 1980s and 1990s and coexist with the descendants of the source population. Little is known about the genetic variability of the

individuals in this park. If the genetically effective population size (N_e) is small, then genetic diversity may decrease through genetic drift and inbreeding, which could constrain population growth. If this was indeed a problem, the introduction of new unrelated individuals into the park may mitigate the reduction of variability. The genetic profile of individuals who are candidates for translocation should be analyzed by biologists prior to introduction to ensure maximum diversity.

Other than anecdotal observations, no data exist to show if the individuals within Etosha National Park live in subpopulations or social groupings or remain one contiguous, panmictic population. Substructuring may lead to increases in genetic diversity across the population as a whole if drift leads to fixation of particular alleles in differing subpopulations. However, diminished diversity and increased inbreeding within each subpopulation would be expected in such a scenario. Knowing the population dynamics of these rhinos might aid conservationists in determining whether to protect the entire population. Or if they determine one or more particular subpopulations is of equal or greater interest based on increased genetic diversity, then efforts can be concentrated on preserving the subset of individuals. The driving force behind this effort in Etosha National Park is largely fiscal since they have expressed the inability to adequately manage the entire population in its current form indefinitely. Thus, the possibility of finding one subpopulation that retains all the genetic diversity of the entire population would allow the game managers to concentrate their limited resources.

The prioritization of protecting subpopulations within Etosha National Park is due to the lack of resources available in Namibia to securely manage the entire park at an adequately high level. Insufficient funding has driven the desire to characterize the areas

with the highest genetic diversity in black rhinos so that the park managers may dedicate themselves to these areas more fully (Erb, pers. comm.)

Based on field observations by employees at Etosha National Park, black rhinos appear to be restricted in their movements by the availability of drinking water and the uninhabitable, arid Etosha Pan. No known physiological studies have been conducted to assess the tolerance of black rhinos to water deprivation, but if they are similar to other desert-dwelling mammals, then individuals may not be able to survive without access to water for more than two or three days. An inadequate water supply within ENP prevents long-distance migrations and it is thought that black rhinos tend to establish site fidelity with regards to the natural and man-made watering holes. Although historically thought of as solitary animals, black rhinos may establish social clusters within ENP due to their need to remain close to a source of water (Erb, pers. comm.). The Etosha Pan, a 120 km long salt pan, covers approximately 25% of north-eastern Etosha National Park. The pan was originally a lake fed by the Kunene River; however, the course of the river changed thousands of years ago and the lake dried up making it uninhabitable since no source of year-round water exists. These physiological and geographical barriers to migration can impact the population structure of black rhinos by dictating movements. If individuals limited themselves to a region of Etosha National Park, then population structure could be established by lack of migrants, genetic drift and gene flow among subpopulations.

Detecting Population Structure

Allelic distributions change because of the interplay of evolutionary forces such as natural selection, genetic drift, mutation, gene flow and selective mating (Tallmon *et al.*, 2004). Demographically distinct populations (that is ones that are not connected via gene

flow) can be diagnosed through analysis of allelic distributions on a landscape. The magnitude of genetic exchange amongst populations influences whether they remain separated, fuse into successively larger pools or merge into a single population.

Traditionally, defining populations has been a subjective exercise and their designation has generally correlated with geographical proximity and morphological characteristics (Manel *et al.*, 2003). Molecular markers and novel individual-based analyses of genetic structure are increasingly valuable tools in aiding in the diagnoses and classification of populations.

Traditional approaches to assessing the degree of genetic structuring in populations have been in place since the mid 20th Century after the introduction of Wright's fixation indices and their various analogues (Wright, 1937; Wright, 1951) with many similar statistically analogous methods developed then (see Michalakis and Excoffier, 1996). Wright (1951) showed a direct relationship between his inbreeding statistic, F_{ST} , and the magnitude of genetic exchange between populations assuming an island model of migration (Wright, 1931). These indices provided a statistical basis for quantifying genetic variation between, among and within populations. However, F-statistics and their analogues have come under scrutiny because of the numerous assumptions that they make. F-statistics are based on the island model where an infinite number of populations receive and distribute individuals at an equal rate, m , per generation and are of equal size, N . The migrating individuals are randomized and dispersed to other populations without regard for geographical structure; thus, yielding populations that are equally likely to receive and give migrants from all other populations. The island model also assumes that no selection or mutation exists, all populations persist indefinitely and an equilibrium has been reached between migration and drift (Whitlock and McCauley, 1999). These assumptions may be violated under natural conditions and F_{ST} may not accurately reflect gene flow (Whitlock

and McCauley, 1999). Moreover, F-statistics also do not discriminate between historical and contemporary gene flow and dispersal; and therefore, cannot reveal recent demographic shifts in long-lived species.

Alternative methods of evaluating population structure have been developed recently that employ assignment tests based on individual genotypes. These tests assign individuals probabilistically to their most likely populations according to their multilocus genotype and also are asserted to estimate contemporary rates of gene flow and dispersal (Paetkau *et al.*, 1995; Waser and Strobeck, 1998). Assignment-based tests have been used in a number of studies including those for identifying likely source populations for immigrants and excluding unlikely ones (Cornuet *et al.*, 1999), identifying migrants (Rannala and Mountain, 1997), reassessing dispersal rates and patterns (Wilson and Rannala, 2003) and detecting admixture or hybridization (Pritchard *et al.*, 2000). The methodology behind many currently used individual-based assignment tests lies in the utilization of a Bayesian model (Rannala and Mountain, 1997; Cornuet *et al.*, 1999; Pritchard *et al.*, 2000; Manel *et al.*, 2002; Wilson and Rannala, 2003; Falush *et al.*, 2003; Manel *et al.*, 2003). Using this approach, each mixture individual – individuals who carry alleles which may have descended from >1 source population – is assigned to the baseline source population that is most likely, or for hybrid individuals the sources that are mostly likely for its parents. (Cornuet *et al.*, 1999; Luikart and England, 1999). The use of individual-based genetic assignment methods may provide an efficient and clearer alternative to traditional tests of isolation by distance for inferring patterns of dispersal.

Aims of the study

In this study, high-resolution DNA microsatellite markers were used to evaluate the following hypotheses: i) black rhinos found in Etosha National Park have relatively high levels of genetic diversity due to their long generation time associated with their long life spans compared to the relatively short timelines associated with the population bottleneck, and due to the introduction of individuals over the last two decades; ii) consistent with previously published studies, the Etosha National Park *D.b. bicornis* population maintains high levels of genetic diversity, but are lower than those found in *D.b. minor* and *D.b. michaeli* populations, and iii) population structure will be present within Etosha National Park and will be revealed using traditional F-statistics, recently developed Bayesian algorithms as encoded in the program STRUCTURE, or both.

MATERIALS AND METHODS

Study Site and Samples

The population structure of the black rhinoceros (*D.b. bicornis*) from Etosha National Park, Namibia was determined for 178 individuals across seven polymorphic DNA microsatellite loci. Tissue samples were collected between 1989 and 2000 as whole blood, serum or ear plugs. After genotyping, calves of known cows were removed from subsequent analyses to prevent an over representation of particular genotypes (n=8). Thus, my final sample size is 170 individuals. Movements of individuals within this park have been suggested to be influenced by the distribution of watering holes and the Etosha Pan (Figures 1 and 2).

Collection of *Diceros bicornis bicornis* tissue samples involved extensive fieldwork by African nationals throughout Namibia. These tissue samples, mostly in the form of blood and ear plugs, were frozen and shipped to the Queen's University Molecular Ecology Laboratory (QUMEL) in Kingston, Ontario, Canada. Samples in Etosha National Park were collected under the supervision of Peter K. Erb over the past decade. Tissue samples from two other subspecies, *D.b. minor* (n=48) and *D.b. michaeli* (n=29), were already present in the laboratory and obtained by Dr. Peter de Groot from South Africa and Kenya, respectively. In total, a collection of over 300 *D.b. bicornis* individuals were obtained; however, due to tissue degradation and difficulties with PCR application, only a subset of 170 individuals were retained for these analyses. Individuals each from the other two subspecies, *D.b. minor* and *D.b. michaeli*, were included for analysis to compare and contrast the genetic variability present. DNA was extracted from all tissue samples and amplified with seven polymorphic microsatellite loci retained. Analyses of population structure were undertaken with equal numbers of males and females.

DNA Extraction and Genotyping

Tissue samples were stored at -80°C in an EDTA buffer solution (Maniatis *et al.*, 1989) prior to extraction. Total genomic DNA was extracted using a DNEASY TISSUE EXTRACTION KIT[®] (QIAGEN) according to manufacturer's protocols and/or via a standard phenol/chloroform:isoamyl alcohol extraction (Maniatis *et al.*, 1989). Due to the possible degradation of samples, multiple extractions were performed for each individual. DNA samples were eluted into 10-150µL volumes in ddH₂O depending on starting amount of tissue extracted. Extracted samples were stored at -20°C until laboratory analyses began.

All seven primer pairs were optimized on a T-GRADIENT THERMOCYCLER[®] (Biometra) over a range of annealing temperatures (60–64°C). Microsatellite loci used for these analyses were previously characterized by Cunningham *et al.* (1999; BR4, BR6 and BR17) and by Brown and Houlden (1999; DB01, DB52 and DB66). One primer pair, 2B, was designed in-house (van Coeverden de Groot *et al.*, GenBank Accession No. AY606080). The PCR cocktail was comprised of 1 µL QIAGEN 10X reaction buffer containing 15 mM MgCl₂ (QIAGEN[®]), 2.5 mM each of dATP, dTTP, dCTP and dGTP, 0.05mM forward primer, 0.1mM reverse primer, 0.05mM end-labelled dATP forward primer (α -P33), 10ng DNA, 0.2U of *Taq* DNA polymerase (QIAGEN[®]) and sterile ddH₂O up to a total volume of 10 µL. Alternatively, a QIAGEN Multiplex PCR Kit[®] was used following manufacturer's protocols with loci grouped according to annealing temperature and base pair size. Amplification profile for all loci was initiated by a 3 minute denaturation phase at 94°C; followed by 35 cycles of 30 seconds denaturation at 94°C, 40 seconds annealing at 60-64° (see Chapter 3, Table 1) and 40 seconds extension at 72°C; final extension at 72°C was for 5 minutes. PCR products were electrophoresed through a 6%

polyacrylamide (PAA) gel using a LICOR 4100 AUTOMATED DNA SEQUENCER[®] (Licor). Amplifications were scored using GENEIMAGIR 4.05[®] software (Scanalytics, Inc.) and confirmed via visual inspection of each genotype. Alternatively, $\gamma^{33}\text{P}$ dATP was incorporated into the forward primer in an end-labelling reaction (Maniatis *et al.*, 1989) and was used in an optimized PCR reaction where the subsequent PCR products were electrophoresed on a vertical gel rig containing 6% PAA gel for 3-4 hours, blotted, vacuum dried and exposed to Kodak 100 Biomax MR film. After 24-48 hours of exposure, genotypes were scored manually. Known genotypes and size standards were run on each platform to ensure scoring accuracy and consistency. To ensure a reliable data set, precautions were taken to analyze a minimum of two extractions and two amplifications of each extraction to minimize mistyping due to DNA degradation, allelic dropout, PCR stutter or inter-observer error. Each individual's tissue was extracted and genotyped at least two times on separate occasions and the results were compared.

Data Analyses – Population Structure in Etosha National Park

PCR products were obtained via solo or multiplex reactions. Individuals that were retained for analyses contained a multilocus genotype across all seven loci or were missing data at only one locus. Separate analyses were also conducted for a dataset that included individuals that were missing genotypic data at two loci.

MICRO-CHECKER[®] (van Oosterhout *et al.*, 2004) was used to identify genotyping errors due to null alleles, short allele dominance (allelic dropout), scoring of strong stutter peaks or typographic errors. This software estimates the probability of the frequency of null alleles, and can adjust the allele and genotype frequencies of the amplified alleles to permit further population genetic analyses. The analyses performed by MICRO-CHECKER[®] can

discriminate between inbreeding and Wahlund effects, and Hardy-Weinberg deviations caused by null alleles (van Oosterhout *et al.*, 2004). If null alleles are present, then this software can estimate the null allele frequency and adjust the observed allele and genotype frequencies to account for this downward bias, and thus, will assume that the population is in Hardy-Weinberg equilibrium and permit further population genetic analyses.

Heterozygosity estimates, deviations from Hardy-Weinberg equilibrium and estimates of linkage equilibrium were calculated using POPGENE 1.32[®] (Yeh *et al.*, 1997). Microsatellite variation calculated using this software gave an unbiased estimate of expected heterozygosity using the formula of Nei and Roychoudhury (1974).

Traditional F-statistics were calculated using MICROSATELLITE TOOLKIT (Park, 2001). F_{ST} was calculated to determine levels of differences among populations of *D.b. bicornis* (n=27), *D.b. michaeli* (n=48) and *D.b. minor* (n=29) and among putative, predetermined subpopulations based on field observations and geographical locations where individuals were sampled within Etosha National Park. Population differentiation was also assessed using Fisher's exact test for allelic distributions.

Genetic Analysis Software

A Bayesian individual-based assignment test was implemented using the software STRUCTURE ver. 2.1 (Pritchard *et al.*, 2000, <http://pritch.bsd.uchicago.edu/software.html>; Falush *et al.*, 2003), which uses multilocus genetic data to infer population substructure using a model-based clustering analysis algorithm. The model assumes that each population is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned probabilistically to a population, or to two or more populations if their genotypes indicate they are admixed, based on estimated population of allele frequencies. The model

accounts for the presence of Hardy–Weinberg or linkage disequilibrium (for the total population) by introducing population structure and attempts to find population groupings that are not in disequilibrium (Pritchard et al., 2000). STRUCTURE does not assume a particular mutation process and can be applied across many commonly used genetic markers such as microsatellites, as long as they are unlinked. STRUCTURE also makes no *a priori* assumptions regarding population identity or affiliation. Primary focus of this type of analysis lies in the assignment of individuals to populations rather than determining population-wide statistics and structuring, while allowing for the presence of admixed individuals.

All individuals (n=166), except known first-order relatives, were included since no prior population structure was assumed for this analysis. STRUCTURE utilizes the multilocus genotype data to infer population structure and assign individuals to populations based on the notion that K populations exist where K can be from 1 to several populations (K need not be known previously). Many runs of various lengths were performed with different choices for the number of genetic clusters (K) represented by the individuals genotyped, testing values of K from 1 to 10. The maximum value of K was based on choosing a value higher than that seen by observational data, which suggested seven subpopulations. Clustering solutions of highest likelihood were obtained when the vast majority of genomic assignment was distributed over 1 to 5 clusters.

Five independent runs of $K = 1-10$ were performed at 10,000, 100, 000 and 1,000,000 burn-in periods and Markov Chain Monte Carlo (MCMC) repetitions using the F model (correlated allele frequencies / admixture). The optimal value of K is derived by choosing the lowest posterior probability, $\ln \Pr(X|K)$, where “ X ” denotes the genotype, and “ K ” is the value of K immediately preceding the value at which α asymptotes (Pritchard *et*

al., 2000). Analyses were conducted separately on males, females and on a pooled gender neutral population.

DOH (Brzustowski, 2002) was used to analyze the assignment of individuals from a known population to one of three or seven populations. Determination of three or seven populations was based on the observations of Etosha National Park's manager, Peter K. Erb (pers. comm.). While previously thought to be socially independent, recent field observations indicate that the black rhinoceros form social clusterings. These clusters are distributed around water holes, sources of vegetation, and other factors yet to be determined. Garnier *et al.* (2001) suggest that the black rhinoceros engages in a polygynous meeting system; however, at this time we cannot definitively suggest that individuals in Etosha National Park are clustering due to territoriality or other aspects of the reproductive system. Based on natural distributions of watering holes and potential geographic barriers, individuals found within this national park would theoretically be constrained to these population sites. DOH was used to see if individuals from a known population could be assigned back to their source population based on the allelic frequencies.

The validity of perceived groups – from field observations by park personnel and GPS data – was analyzed using an assignment test found in DOH (<http://www2.biology.ualberta.ca/jbrzusto/Doh.php>; Brzustowski, 2002), with all observational samples combined (n=170).

RESULTS

Traditional population analyses

Genotypic data were obtained for 170 individuals, and analysis of population structuring of *Diceros bicornis bicornis* black rhinoceroses within Etosha National Park based on an equal proportion of males and females.

Results from Fisher's exact test calculated in MICROSATELLITE TOOLKIT indicated significant differentiation existed amongst each of the three subspecies (Tables 1 and 2) or amongst putative subpopulations within Etosha National Park (Table 3). Quantification of population differentiation within Etosha National Park was suggestive of little to moderate population substructuring. All seven microsatellite loci retained for further analyses were polymorphic, with an average number of alleles per locus \pm one standard error of 3.33 ± 1.6 . Observed heterozygosity (H_o) was 0.518 ± 0.03 (Table 1). Null allele frequencies were calculated for each locus in MICROCHECKER and all loci showed that no null alleles were present.

Data for the other two subspecies, *D.b. minor* ($n = 48$) and *D.b. michaeli* ($n = 29$), were compared against a subset of *D.b. bicornis* ($n=27$) and showed an average number of alleles per locus of 6.11 ± 0.05 and 7.00 ± 0.03 , respectively. In *D.b. minor*, observed heterozygosity was 0.587 ± 0.02 while *D.b. michaeli* samples showed an observed heterozygosity of 0.672 ± 0.02 (Table 1).

Individuals retained for analyses of population structuring within Etosha National Park showed no departures from Hardy-Weinberg equilibrium after sequential Bonferroni corrections.

STRUCTURE analyses

Using the Bayesian clustering method found in STRUCTURE, the highest posterior probability for population structuring and number was found to differ amongst Etosha females versus Etosha males and the Etosha population as a whole. Results suggest that Etosha females are not partitioned, showing no structuring above $K = 1$ (Table 2). In contrast, Etosha males (Table 3) and the Etosha population as a whole are best modelled by a $K = 3$ (Table 4). Pritchard *et al.* (2000) suggest that the lowest and/or most biologically appropriate value should be considered in circumstances where K values are similar. Thus, population sub-structuring of black rhinoceroses within Etosha National Park as a whole is due to Etosha males.

Following the optimal assignment for K , each individual was assigned probabilistically to a source population with an inferred membership greater than or equal to 0.80 and/or 0.90. The 0.90 value was used when attempting to increase confidence if significant (i.e. assess the validity of an individual truly arising from its assigned source population); however, the 0.80 threshold value has been suggested as being appropriate for identifying general population trends in wildlife species (Rueness *et al.*, 2003).

Social clustering

Individuals in Etosha National Park were analyzed to see if they could be correctly assigned to their source social grouping. Based upon field observations by park rangers, black rhinos within this park tend to segregate into seven social clusters. However, the four most eastern groups are believed to co-mingle; thus, the park population may also be grouped into three clusters (Peter Erb, pers. comm.). DOH (Brzustowski, 2002) was used to assess the validity of either three or seven groupings, and to allocate individuals to their

correct cluster. With the original assumption of seven populations, the majority of individuals from population 1 (21/36) and 2 (6/8) were correctly assigned; however, a smaller proportion of individuals from populations 3 to 7 were successfully classified at a lower level (Table 5). Analysis of individuals and grouping populations 3 to 7 to yield three total sub-populations showed that 30 out of 36 individuals were correctly assigned to population 1, 6 out of 8 individuals were correctly assigned to population 2, while 25 out of 50 rhinos were assigned population 3 (Table 6). These results suggest that the black rhinoceroses in Etosha National Park more likely form three distinct subpopulations than seven, due to mixing of individuals in the eastern portion of this park.

DISCUSSION

Diceros bicornis bicornis is the second-most abundant subspecies of the black rhinoceros; however, past attempts to characterize this subspecies at a genetic level have been limited (Brown and Houlden, 1999; Cunningham *et al.*, 1999; Garnier *et al.*, 2001; Harley *et al.*, 2005). Similar to the other subspecies, *D.b. minor* and *D.b. michaeli*, a drastic decline in population numbers has ceased due to strong conservation efforts to preserve individuals within wild and captive populations. Knowledge of the genetic variability and structure of black rhinos in Etosha National Park can aid in the repopulation and recovery of this subspecies as I outline below.

Previous genetic studies on black rhinoceroses concerning population diversity of mitochondrial DNA markers (Ashley *et al.*, 1990; O’Ryan and Harley, 1993; O’Ryan *et al.*, 1994), allozymes (Merenlender *et al.*, 1989), proteins (Swart *et al.*, 1994; Swart and Ferguson, 1997) and microsatellites (Brown and Houlden, 1999; Cunningham *et al.*, 1999; Garnier *et al.*, 2001; Harley *et al.*, 2005) showed relatively high levels of genetic diversity. The relatively high levels of expected heterozygosity that I found in my study are consistent with previous microsatellite studies; however, these studies mainly focused on the *Diceros bicornis minor* and *D.b. michaeli* subspecies. My study characterizes genetic diversity of the largest sample size of the *D.b. bicornis* subspecies, to date.

Observed and expected heterozygosity were lower in the *D.b. bicornis* population compared to the other two subspecies, which is consistent with previous microsatellite studies using six of the same loci used in my study despite their small sample sizes, although no comment about power is included in their studies and they may be appropriate (Brown and Houlden, 1999; Cunningham *et al.*, 1999; Garnier *et al.*, 2001; Harley *et al.*, 2005). The relatively low genetic diversity found in my Namibian population could be a

reflection of a recent population bottleneck, with current numbers the result of subsequent population expansion. The signatures of bottlenecks can be detected in present day bottlenecks using newly developed coalescent models, but that analysis is beyond the scope of my thesis.

One cannot necessarily say that the lower heterozygosity is attributable to greater impact of a population bottleneck in *D.b. bicornis* in comparison to the other two subspecies. *Diceros b. bicornis* may, in fact, have always had lower diversity compared to their sister subspecies and my findings may simply reflect geographical isolation as this population was historically only found in Namibia, northern Angola and mid-southwestern South Africa and was not as free-ranging as its counterparts. Other explanations include the possibility that other subspecies exhibit differing social structures or mating system, or higher local population densities because of more favourable environments, factors which would influence effective population size. Indeed Harley *et al.* (2005) showed that the effective population size for *D.b. michaeli* is more than twice that of the other two subspecies. Anecdotal historical census numbers also indicate that *D.b. minor* and *D.b. michaeli* had larger population numbers over the past two centuries.

Detection of subpopulations in Etosha National Park using traditional F-statistics

F-statistics are often used to identify population structure and infer levels of gene flow between *a priori* defined populations based on population allele frequencies (Weir and Cockerham, 1984). However, problems exist with using F-statistics as the primary method of analysis for population structure in wildlife species. A major drawback is that individuals must be partitioned into predefined populations prior to analyses based often on nothing more than geographic provenance of haphazardly collected samples over multiple seasons.

In other words, researchers often impose population designations on species, which may actually have distributions that are more continuous than clustered (Manel et al. 2003). Another confound in the use of F-statistics occurs in situations of recent immigration. Recent immigrants alter the population level allele frequencies by either falsely increasing or decreasing the detectable level of differentiation between locales (Falush *et al.*, 2003). If recent immigration has occurred, then the allele frequencies would appear more similar and result in a decreased level of differentiation between neighbouring populations. Finally, F-statistics make explicit assumptions about the nature of migration (i.e. an island model) and the causes of differentiation (e.g. drift and gene flow) that simply are not met in real populations (Whitlock and McCauley, 1999).

Black rhinos in Etosha National Park showed no significant population structure using traditional Wright's F-statistics. Detectable differences were seen between pair-wise comparison of populations 1 (Western) vs. 2 (Central; $F_{ST} = 0.15$; Table 3). The levels detected are indicative of moderate genetic differentiation. However, no significant differentiation was detected between subpopulations 1 vs. 3 (Eastern; $F_{ST} = 0.03$) and the differentiation between 2 vs. 3 ($F_{ST} = 0.06$) was not significant; thus, the results suggest that this study does not have the resolution to detect the purported structure or no real population differentiation exists between subpopulations 2 vs. 3. Why subpopulation 1 is not significantly different from subpopulation 3, but is significantly different from subpopulation 2 is not entirely clear considering subpopulation 2 is in a geographically intermediary position and one would speculate that migrants from subpopulations 1 or 3 would have to pass through subpopulation 2 to mate with individuals in the other population. Whether this differentiation is real or a sampling artefact is unknown, but prompts the need for further studies. Knowledge of historical populations and subsequent

re-colonization will help clarify this picture to know if the population structure was driven by natural or man-made forces. These F_{ST} values suggest that gene flow amongst the predetermined subpopulations in Etosha National Park is ambiguous using traditional F-statistics.

Detection of subpopulations in Etosha National Park using a Bayesian model

Bayesian models estimate population structure without any *a priori* assumptions on an individual's population of origin, and because they are based on individual genotypes, this class of methods is purported to be more sensitive to detecting cryptic population structure (Pritchard *et al.*, 2000). In contrast to the F-statistic approach, the Bayesian-based assignment test encoding in STRUCTURE, identified population sub-structuring within Etosha NP. The dichotomy of results is probably attributable to the numerous assumptions I outlined in the Introduction that are often violated under natural conditions (Whitlock and McCauley, 1999) and that individual-based analyses are inherently more powerful. Importantly F-statistics also do not discriminate between historical and contemporary gene flow and dispersal (Whitlock and McCauley, 1999); therefore, do not provide adequate resolution for recent demographic shifts in long-lived species. The Bayesian approach mitigates some of these problems by using a maximum likelihood methodology to assign individuals to populations based on their multilocus genotypes alone with no assumptions about origins.

The existence of three subpopulations within Etosha National Park implied by my assignment tests appears to be mediated by males as females alone did not show any substructuring. No observations of sex-biased dispersal in rhinoceroses have been previously documented. However, African elephants are known to form matriarchal social

groups and force out juvenile bulls leading to a male-biased dispersal in this long-lived mammalian species (Clutton-Brock, 1989). In the south-western black rhino, the establishment and defence of territories could help explain the geographic distribution of males. Watering holes are scarce within this national park and may serve as valuable resources that males defend and stay close to. Vegetation may also be more abundant closer to water sources; thus, increasing the value of certain areas of the park. While rhinos are capable of traveling large distances, it may not be prudent for them to stray from known watering holes in this semi-arid park (Erb, pers. comm.). Females appear to form one panmictic population, which is perhaps mediated by the desire to find males in territories with higher resources. Thus, their dispersal within the park could have preceded sampling of individuals. In summary, the detected population structure appears to be mediated by male philopatry within Etosha National Park.

Conservation Implications

Conservation crises can be thought of as having five stages: 1) population decline, 2) crisis management, 3) stabilization at a low number, 4) precarious recovery and 5) sustained recovery (Linklater, 2003). The black rhinoceros populations within Etosha National Park and other parks and countries appear to have passed the most critical stages (1-3) and now show evidence of recovery due to recent conservation successes (Linklater, 2003). As Etosha National Park hosts the largest single population of *D.b. bicornis* individuals anywhere in its range, the protection of this area is of paramount importance to the survival of this subspecies. The conservation of the black rhinoceros is aided somewhat since this species is a member of the so-called 'charismatic megafauna' and much public attention can be focused on its preservation.

The conservation management unit (MU) is a fundamental element in developing a conservation management plan since it defines what to manage and protect. Depending on the scale and species involved, a MU may be at an individual, group, subpopulation or population level. Genetic information offers an objective means to delineate conservation units and provides a framework from which to develop and evaluate conservation priorities. The value of molecular markers in delineating breeding structures and evolutionarily important lineages is well-established (Avice, 1994).

The current approach to black rhino conservation in Etosha National Park black rhinos centres on a single large MU. The game managers at Etosha National Park endorsed this study to determine if it was feasible to concentrate their resources on a smaller region of the park, and thus, a subpopulation of rhinos, due to their lack of financial resources and the inability to adequately sustain the overall population in the future. If one subpopulation retained all the genetic variability of the entire population, then management decisions could be made on how best to sustain that particular geographical region and its residents.

My analyses suggest that the Bayesian-based approach can be used to delineate MUs that were unidentifiable using traditional F-statistics. Cryptic population structuring is present, with individuals diagnosed into Western, Central and Eastern groups. Knowledge of the spatial distribution of the genetic clusters is important for the effective conservation of these identified subpopulations, which are suggestive of site fidelity and movement between the sexes.

Perhaps a conservation approach to manage the black rhinoceros population within ENP would be to treat each of the three groups as a separate MU. Each MU would be more likely to be protected from factors such as poaching that could further reduce numbers of this subspecies. However, the close geographical proximity of each MU would likely

mitigate a potential widespread disease outbreak or natural disaster. Despite the substructuring that is evident, one or two migrants per generation would effectively ensure a homogenous population within ENP due to gene flow and neutralize the benefits of creating separate MUs. Establishing the black rhino population in Etosha National Park as separate management units or maintaining them as one population will be a risk:benefit endeavour.

My recommendation is to continue managing the current population as a single MU. Substantial supportive data are currently not available to suggest that the management of a single subpopulation over another would be beneficial for the long-term survival of the entire subspecies. The lack of financial resources would be further compounded by the cost of erecting fencing or other barriers to isolate and manage one subpopulation. Thus, maintaining the current conservation strategy within Etosha National Park would be a prudent, safe decision.

Table 1. Measures of genetic variability within samples of three subspecies, including Wright's inbreeding coefficient: *D.b. bicornis*, *D.b. michaeli* and *D.b. minor* populations. * indicates $P < 0.05$.

	n	H_o ± SE	H_E ± SE	F_{IS}
<i>D.b. bicornis</i>	27	0.518 ± 0.03	0.483 ± 0.07	-0.074
<i>D.b. michaeli</i>	29	0.672 ± 0.02	0.763 ± 0.05	0.034
<i>D.b. minor</i>	48	0.587 ± 0.02	0.608 ± 0.02	0.121*

Table 2. Pair-wise F_{ST} values amongst three black rhinoceros subspecies populations: *Diceros bicornis bicornis* (n = 27), *D.b. michaeli* (n = 29) and *D.b. minor* (n = 48). Bottom diagonals indicate F_{ST} values and significant values ($P < 0.05$) are indicated by “*”. Top diagonals indicate confidence intervals.

	<i>D.b. bicornis</i>	<i>D.b. michaeli</i>	<i>D.b. minor</i>
<i>D.b. bicornis</i>	-	0.02	0.02
<i>D.b. michaeli</i>	0.19*	-	0.02
<i>D.b. minor</i>	0.27*	0.17*	-

Table 3. F_{ST} values for 3 ‘subpopulations’ within Etosha National Park diagnosed *a priori* based according to field observations and geographical areas in which they were sampled. Bottom diagonals indicate F_{ST} values and significant values ($P < 0.05$) are indicated by “*”. Top diagonals indicate confidence intervals.

	<i>1</i>	<i>2</i>	<i>3</i>
<i>1</i>	-	0.02	0.02
<i>2</i>	0.15*	-	0.1
<i>3</i>	0.03*	0.06	-

Table 4. Determination of most likely population structuring amongst Etosha National Park females, males and with both sexes combined based on STRUCTURE output; bold values indicate most likely value of K and are significant ($P < 0.05$).

K	Females			Males			Both sexes		
	ln pr(X K)	Var ln pr(X K)	α	ln pr(X K)	Var ln pr(X K)	α	ln pr(X K)	Var ln pr(X K)	α
1	-1162.96	13.18	0	-1105.08	11.06	0.00	-2283.82	17.70	0.00
2	-1199.8	112.46	1.95	-1085.74	86.94	0.24	-2230.68	131.28	0.08
3	-1199.26	107.38	4.12	-1078.48	145.78	0.08	-2225.70	267.34	0.05
4	-1315.72	381.74	2.10	-1106.94	174.32	1.75	-2237.36	352.52	0.05
5	-1244.18	188.6	4.09	-1130.96	273.22	0.06	-2286.20	436.20	0.05
6	-1169.98	32.02	4.64	-1173.12	351.88	0.06	-2314.22	426.20	1.76

Table 5. Assignment of individuals from known populations to one of 7 populations based on DOH output. Original subpopulations are in rows along with total number of individuals sampled from that locale while putative assignments are in columns.

	1	2	3	4	5	6	7	Totals
1	21	0	1	6	3	4	3	36
2	0	6	2	0	0	0	0	8
3	2	5	13	2	0	0	1	23
4	1	0	0	6	1	1	1	10
5	2	0	0	2	1	1	0	6
6	3	0	0	1	2	1	0	7
7	0	0	0	2	0	0	3	5

Table 6. Assignment of individuals from known populations to one of 3 populations based on DOH output. Original subpopulations are in rows along with total number of individuals sampled from that locale while putative assignments are in columns.

	1	2	3	Total
1	30	0	6	36
2	0	6	2	8
3	13	12	25	50

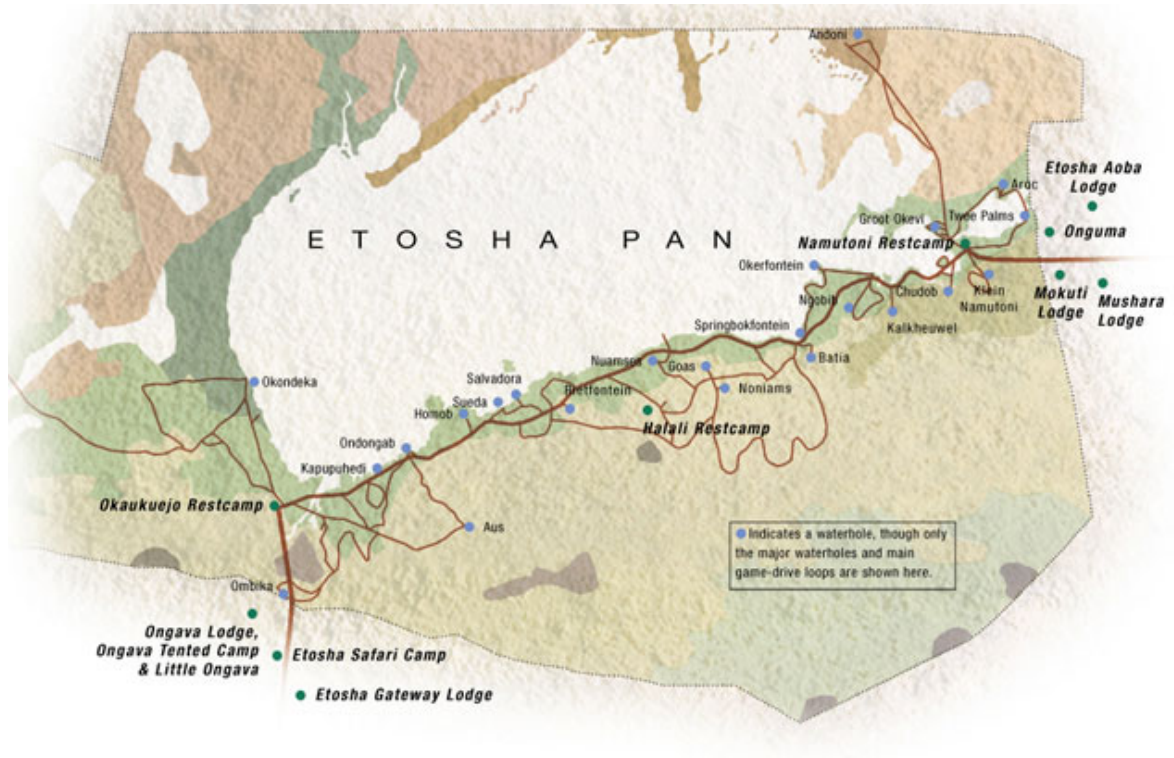


Figure 1. Map of eastern Etosha National Park showing the 120km long, arid, uninhabitable Etosha Pan. Waterholes are indicated by blue circles.
 (http://www.expertafrica.com/images/maps2007/Namibia-map-Etosha-National_Park.jpg)

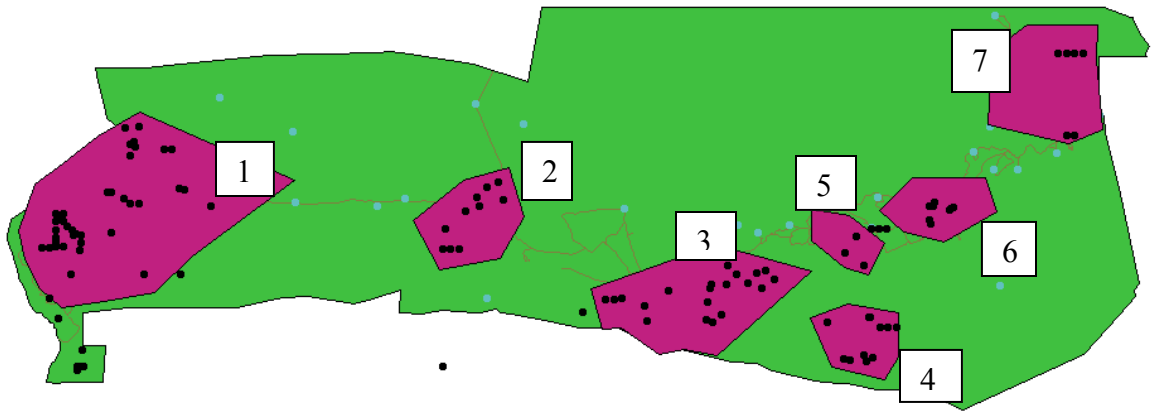


Figure 2. Map of seven black rhino sampling locations within Etosha National Park used to determine population structuring. Black dots indicate individual black rhino samples, blue dots reflect watering holes and pink polygons represent putative subpopulations.

CHAPTER 3
PEDIGREE AND RELATEDNESS OF NON-ETOSHA *D.B. BICORNIS*

ABSTRACT

I undertook analyses of the genetic relationships of black rhinos (*Diceros bicornis bicornis*) in Namibian game farms using a combination of pedigree and genetic data, and relatedness calculations for founding stock individuals are informative and can help inform management strategies involved in the future translocation of individuals. The determination and protection of highly fecund individuals should occur as those individuals will be invaluable in seeding the next generation. Current conservation strategies suggest incorporating as much genetic diversity as possible into founding stocks to preserve as much of the extant variability as possible. Using a multilocus genotype based on nine polymorphic microsatellite loci, we determined that relatively high levels of relatedness exist in the breeding program in Waterberg and Okosorong game farms. Despite these high levels of genetic similarity, I was able to determine the parentage of offspring using a likelihood-based methodology. Previous suggestions that the black rhino has a polygynous mating system is confirmed in these captive populations; however, caution must be taken to over-interpret this mating system and extend it to a wild population given that mate choice within a game park may be artificially driven with so few individuals of each sex.

INTRODUCTION

Black rhinoceros (*Diceros bicornis*) populations have undergone drastic declines over the last 100 years mostly due to anthropogenic factors such as poaching and habitat destruction. Current populations typically are small and highly fragmented, which contrasts to the nearly contiguous distribution of this species across sub-Saharan Africa at the start of the 20th Century. While current estimates indicate that numbers are increasing, active conservation efforts remain in place, in part, to minimize loss of genetic diversity within these populations. For many large megafauna *ex situ* conservation strategies representing the best hope for ensuring that these species persist reside with strategies for maintaining genetic diversity. Information that may be key includes detailed knowledge of pedigrees and genetic relatedness of individuals so that such species may be actively managed (Gilpin, 1991).

History of Game Farms

Recognizing the possibility of extinction of the *D.b. bicornis* subspecies of black rhinos in Namibia, several private sanctuaries were created in the 1970s to aid in its conservation. Of relevance to my study are game farms located in Waterberg, Okosorong and Hardap, all near Etosha National Park, Namibia, and all which have provided stock for reintroductions into the park.

Throughout the latter half of the 20th Century, wild black rhinos were captured and translocated into one of the respective game farms. Founding members in Waterberg were originally from Dolomietpunt, while those in Okosorong came from Kaross, both regions within Etosha National Park. The establishment of captive black rhino populations and their

continued success rely on successful matings and rearing of offspring. Recruitment of new rhinos that may be related to those previously acquired could result in matings between related individuals, especially if newly acquired rhinos derive from the same locales as those already in captivity. Thus, the possibility of inbreeding (and potentially the inbreeding depression that may accompany it) needs to be assessed within current populations of game parks in Namibia. Additionally, if we quantify relatedness and find it to be high within a particular game farm, then the selective movement of individuals between locales would be a logical recommendation.

Parentage Analysis Methods

Pedigree-based management practices can be used to identify important individuals based on mean relatedness to others to minimize inbreeding within the captive population, and thus, maximize diversity. Inferring paternity using highly polymorphic co-dominant markers, such as DNA microsatellites, has become the standard (e.g. Ritland, 1996; Marshall *et al.*, 1998) and an important complement to pedigree information. While each game farm may only have one or two bulls, recording paternity via observation is labour intensive. Field observations at these farms show cows in estrus mate with more than one bull, further confounding paternity.

In classical paternity testing, based on strict exclusion-based paternity assignment, a single mismatch at a locus is sufficient to rule-out the candidacy of a given male due to simple Mendelian inheritance (reviewed in Jones and Arden, 2003), even when the exclusion is due to sampling error. In practice, exclusion is prone to errors such as allelic dropout where an allele may fail to amplify during PCR, null alleles, mutation and inaccurate scoring, any of which can lead to a false-negative (Jones and Arden, 2003).

Parent:offspring is the relationship most affected by genotyping error. Hoffman and Amos (2005) have shown that an error rate of 0.0040 per allele can account for a rate of false paternity exclusion of slightly less than 10%, when lack of shared alleles at one locus is considered as reason for exclusion. However, exclusion-based parentage reconstruction is powerful when few candidate parents exist with a high number of polymorphic markers (Marshall *et al.*, 1998).

Due to these aforementioned limitations of exclusion-based assignment, recently likelihood-based methods have come to the forefront (Ritland, 1996; Marshall *et al.*, 1998). This class of methods integrate probabilities based on incomplete sampling, scoring error and population allelic frequencies to assign the most likely putative parent, even in the presence of related candidate parents (i.e. uncles, brothers, grandfathers, etc.). Likelihood techniques allocate offspring to parents either categorically or fractionally (reviewed in Jones and Arden, 2003). The two assignments differ in that, with categorical assignment, an offspring is wholly allocated to a particular male, whereas, in fractional assignment, an offspring is theoretically shared amongst compatible males. While fractional assignment is biologically impossible due to each offspring having only one true genetic father, categorical assignment likely overestimates the reproductive success of individuals with homozygous loci and underestimates reproductive success in individuals with many heterozygous loci (Devlin *et al.*, 1988).

Calculation of Relatedness

Determination of genetic relationships among individuals within and amongst populations plays an important role in pedigree construction (Ritland, 1996). Ideally, relationships can be derived from pedigree data; however, detailed knowledge of pedigree

information is not always feasible *in situ* and even as indicated above, challenging to determine in captive populations. Currently, two paradigms exist for determining relationships: likelihood approaches and moment estimators. Likelihood approaches determine the probability of a pair of individuals who fall into a particular familial grouping by analyzing molecular markers (Ritland, 1996; Marshall *et al.*, 1998). Moment estimators calculate relatedness between individuals by determining its probability with respect to identity-by-descent (Lynch, 1988), but were subject to sampling error as allele frequencies derived from sample sets were, in practice, too small to accurately reflect true population diversity. The latter approach was refined by Wang (2002 and 2004) and used in our study. This approach employs estimated allele frequencies based on the sample set and can withstand small sample sizes, changes in allele frequency distribution and inclusion of relatives in samples for estimating allele frequencies, which would otherwise skew the allelic proportions. Moment estimators approach relatedness by analyzing the similarity in genotypes and determine the variables associated with similarity based on sample population and actual relationships. When sampling effects are accurately accounted for, the observed similarity between individuals is a reflection of their relatedness (Wang, 2004).

Aims of the study

Ultimately, breeding success and viability of game farm populations depends on the quality of individuals reared. To minimize potentially harmful effects of inbreeding, the goal of a breeding program is to have unrelated, highly fecund individuals producing offspring.

The major hypotheses of this study were: i) Individuals from game farm captive populations exhibit high relatedness with samples of putative wild founder individuals, and

ii) rhinoceros microsatellites exhibit sufficient variability to accurately assign parentage using an assignment-based test when no or one parent is known.

MATERIALS AND METHODS

Samples and Study Sites

Genotyping of individuals was undertaken to determine or confirm parentage and relatedness across complete or partial (known cow-calf dyads) pedigrees. Samples originated from one known calf-cow-bull triad from the Hardap game farm (13.4°S, 20.0°W), and from cow-calf dyads with candidate bulls from Waterberg (14.5°S, 19.0°W) and Okosorongo (14.5°S, 19.4°W) game parks in Namibia. Known calf-cow dyads from Kaross (14.5°S, 19.4°W), which neighbours Okosorongo, were also analyzed; however, only a small subset of putative bulls could be assessed due to availability of tissue. Putative fathers were also eliminated based on other factors including the age of bull at time of conception of calves and geographical proximity to cows at conception (since translocations of mature bulls occur to minimize territoriality disputes).

The full pedigree of one bull-cow-calf triad from Hardap was known, but was also not retained for further analysis due to an inability to amplify the full multilocus genotype in the calf. However, no mismatches were observed with the known parents at the five loci that were amplified in the calf.

DNA Extraction and Genotyping

Samples from whole blood, serum and/or ear plug tissue were stored at -80°C in an EDTA buffer solution prior to extraction. Total genomic DNA was extracted using a DNEASY TISSUE EXTRACTION KIT[®] (QIAGEN) according to manufacturer's protocols and/or via a standard phenol/chloroform:isoamyl alcohol extraction (Maniatis *et al.*, 1989). Due to the possible degradation of samples, multiple extractions were performed for each individual. DNA samples were eluted into 10-150µL volumes in ddH₂O depending on

starting amount of tissue extracted. Extracted samples were stored at -20°C until laboratory analyses began.

All primer pairs were optimized on a T-GRADIENT THERMOCYCLER[®] (Biometra) over a range of annealing temperatures (60–64°C, Table 1). Nine microsatellite loci used for these analyses were previously characterized by Cunningham *et al.* (1999; BR4, BR6 and BR17) and by Brown and Houlden (1999; DB01, DB44, DB52 and DB66). Two primer pairs, 2B and 37D, were designed (van Coeverden de Groot *et al.*, GenBank Accession No. AY606080 and AY606083) to complement data from published primer pairs for later analyses. This pedigree study utilizes two additional primers (DB44 and 37D) from those employed in Chapter 2. These two primers were not able to be optimized for the large population-level dataset as seen in Chapter 2; however, given the smaller scope of this sub-study, I was able to amplify genotypes for the pedigree individuals at these two additional loci. The methods used were identical to those described in Chapter 2.

Data Analyses

When screening a large number of markers, some true father–calf mismatches are inevitable due to typing error, incomplete sampling and mutation. Pedigrees were assessed manually and using CERVUS v.2.0 (Marshall *et al.*, 1998), a likelihood-based approach to infer paternity with statistical confidence in natural populations. The effects of typing error, unsampled candidate males and missing genotypes are all incorporated into the program. Genotype frequencies were calculated using multiple random samples of 50 adults from Etosha National Park *D.b. bicornis* individuals (see Chapter 3) with an equal ratio of males:females and the pedigree analyses were run against each random sample to assess for deviations due to sampling. For Waterberg and Okosorong Game Parks, simulations were

run with 10,000 replications and with the assumption that 95% of the proportion of alleles within the population were sampled and again with 90% proportion. In other words, depending on the setting the software is assigned, it assumes that the dataset contains 90 or 95% of the total number of alleles found in a given population. These error rates were included to take into account novel alleles that were not detected during analysis. Typing error rates of both 0.01 and 0.05 were run to assess the probability of determining parentage with stringent vs. more realistic error rates. Parentage for three calves (BR199, BR288 and BR291) from Okosorong Game Park was unknown; thus, both cow and bull required identification. When both parents are unknown, CERVUS requires a two-step analysis: 1) run calves against the parent with the least number of candidates, and 2) if first parent can be assigned with confidence, then proceed by running analysis against putative second parents.

One key feature of CERVUS is that paternity can be assigned at any specified statistical confidence because of the simulation approach employed. Confidence of parentage for all analyses using CERVUS was determined at “strict” 95%, “relaxed” 80% or “most likely” levels. The “most likely” setting assigns paternity to one bull over another based on him being more genetically similar compared to another candidate bull. The “most likely” setting does not take into account whether it would be considered statistically significant at the 80% or 95% level.

The hypothesis that the CERVUS-assigned father is the true father was compared with the hypothesis that the CERVUS-assigned father was not the true father. The natural logarithm of likelihood ratios (LOD score) resulting from these analyses are added across loci. A LOD score of zero suggests that the putative father has an equal likelihood of being the father of a tested offspring as a randomly selected male. A positive LOD score implies that the alleged father is more likely to be the father of the offspring than a randomly

selected male. For example, an LOD of 3.0 means that the CERVUS-assigned father is about 20 times more likely than not to be the true father. A negative LOD score may occur if the alleged father and offspring share a particularly common set of alleles (Marshall *et al.*, 1998).

When comparing the two most-likely males, the logarithm of the ratio of likelihood ratios is designated as Δ , (equal to the difference in LOD scores). A critical value of Δ is determined by comparison to a distribution of Δ scores for cases where the most-likely male was the true father to a that where the most-likely male was assumed not to be the true father. If, for example, the criterion for Δ is set to give 95% confidence, CERVUS identifies the value of Δ such that 95% of Δ scores exceeding this value are obtained by true fathers. If the program fails to find this value of Δ , which is usually due to an insufficient power available for the markers used (Marshall *et al.*, 1998), the critical value of Δ is set to an arbitrary high value of 99.99. When a male fulfilling the 95% confidence criterion is assigned paternity of an offspring, we describe the father–offspring relationship as having a 95% confident paternity. Similarly, an 80% confident paternity is established when a male fulfills 80% of the confidence criterion.

Relatedness was calculated amongst all individuals within each park to determine the correct candidate male through identity-by-descent and to estimate the inbreeding potential between breeding pairs. Pairwise relatedness analyses were conducted using MER (Moment Estimate of Relatedness) v.3.0 (Wang, 2002; Wang, 2004), which utilizes Monte Carlo simulations to assess genotypes across all loci, and yields scores ranging from 1.0 (full identity to oneself) to 0.0 (indicating no relatedness), where 0.5 equals the mean relatedness within a given population. Therefore, relatedness amongst known parent-offspring dyads was expected at a 0.5 level. Calves exhibiting half-sibling, grandchild or

aunt/uncle relationships to other individuals were expected to yield a relatedness of 0.25.

Deviations from these expected values were seen when individuals could not be genotyped across all loci or a genotype mismatch occurred between a parent-offspring pair.

RESULTS

WATERBERG

Parentage

Six known calf-cow dyads, based on observational data, were analyzed against five putative bulls to determine paternity. These dyads included a three generation partial pedigree – grandmother (BR64), mother (BR47) and daughter (BR187). All individuals were fully genotyped across 9 polymorphic microsatellite loci except for one individual (BR64) who was missing data at one locus. All individuals were retained for further analyses. Number of alleles per locus ranged from 2 to 8, with a mean of 4.67 (Table 1). Based on the polymorphic information content (PIC), loci DB66, 2B and DB52 were the most informative while BR6 and BR4 were the least (Table 2). First-parent exclusionary power was 82%; however, the second-parent exclusionary power (i.e. the ability to exclude other males as potential fathers when the mother was known) is more relevant in this case and was 96%.

Varying the typing error (TE) rate between 0.01 and 0.05 yielded similar results; however, only two calves were unequivocally assigned to candidate bulls with “strict” 95% confidence under both simulation conditions (Table 3). Under the more stringent 0.01 typing error simulation, one other calf-bull dyad (BR43-BR75) revealed paternity at the 95% confidence level. Under both simulation conditions, one calf-bull pair (BR63-BR191) showed confidence at the relaxed 80% level, while two calves (BR55 and BR49) remained unassigned to any bull since their LOD scores relative to the most-likely male were negative.

These data indicate that one male sired offspring with the two inter-generational females – BR64 and BR47. However, he was not the sire of BR47, but rather her half-

sibling, BR43. No other male in this park appears to have sired more than one offspring with certainty.

Relatedness

Pairwise relatedness was estimated across all individuals within the Waterberg Game Park (Table 3). Mean relatedness amongst all pedigree individuals sampled (0.262 ± 0.22) was found to be significantly higher than expected in a population derived at random (one-tailed t-test, $P = 0.001$, $df = 5$). Amongst breeders (mom vs. mom, mom vs. dad, dad vs. dad) the degree of relatedness (0.225 ± 0.0) indicates approximately a half-sibling level of kinship and is also significantly different than in a random *D.b. bicornis* population (two-tailed t-test, $P = 0.002$, $df = 2$).

OKOSORONGO

Parentage

Seven known calf-cow dyads from Okosorong Game Park were genotyped across nine microsatellite loci and paternity was assessed against two putative males. Two calves were missing data at two loci, and one calf and one cow lacked a genotype at one locus; however, all individuals were retained for further analyses. Range of the number of alleles, mean number of alleles and loci exhibiting the greatest and least amount of variability according to PIC remained consistent with the individuals from Waterberg Game Park, which was to be expected since the founders of both game parks originated from the same locale (Table 5). However, first-parent exclusionary probability was slightly lower at 79%, while the second-parent exclusionary probability remained at 96%.

Paternity assignments at a strict 95% confidence level with a 0.01 typing error revealed that BR168 sired two offspring, while BR171 sired three calves (Table 6). Under this simulation model, two calves (BR89 and DbbA20) remained unassigned to either bull. However, by increasing the typing error to 0.05, all calves were assigned to a bull at least to the relaxed, 80% confidence interval (Table 6). Two calf-bull dyads (BR93-BR171 and DbbA11-BR171) consistently remained associated at the 95% confidence limit under either TE condition. At the 0.05 TE rate, one calf (DbbA20) was assigned to BR168.

Two calves – BR288 and BR291 – with unknown relationships to either a mother or father were sired by BR168 at the 95% confidence interval, while the remaining unknown calf, BR199, was assigned to this bull at an 80% confidence limit (Table 7).

Only one calf-cow-bull relationship – BR288 (unknown birth date), BR165 and BR168 – was determined to be correctly assigned with significance. BR165 (born 1984) gave birth to two other, known offspring – BR93 (born 1996) and DbbA12 (unknown birth date); thus, it remains to be seen whether BR165 is the true mother of BR288 based on known times she was pregnant or weaning her previous calves.

Overall, male reproductive success was not vastly different between the two males, BR168 and BR171. The former sired three offspring, while the latter sired four offspring out of a total of seven calves born within Okosorongu Game Park.

Relatedness

Pairwise relatedness estimates show that a high level of genetic similarity also exists within Okosorongu Game Park (Table 8). Mean relatedness amongst all pedigree individuals sampled (0.414 ± 0.20) was found to be significantly higher than expected in comparison to a random *D.b. bicornis* population (two-tailed t-test, $P = 0.0001$, $df = 5$).

Amongst breeders (mom vs. mom, mom vs. dad, dad vs. dad) the degree of relatedness (0.345 ± 0.0) indicates approximately a 2° level of kinship and is also significantly different than in a randomly generated *D.b. bicornis* population (one-tailed t-test, $P = 0.003$, $df = 2$).

DISCUSSION

Contributions of game farms and zoological parks to the recovery of endangered species are incalculable. Indeed, in many instances some species exist only in managed captive populations (e.g. species of birds on the Island of Guam). When managed appropriately, such *ex situ* populations serve as reservoirs and safe havens for individuals who might otherwise be susceptible to disease, injury or anthropogenic factors like poaching in the wild. Such active management requires detailed knowledge of mating histories and pedigrees, and when such data are imperfect, complementary genetic data.

The game parks in Namibia hosting *Diceros bicornis bicornis* individuals are faced with difficult challenges. Recruitment of individuals via translocations into these parks requires massive manpower, financial resources (typically not available), and is logistically complex. Black rhinos have been known to die in transport, and even when successfully moved, may require years before they are comfortable enough to reproduce (Hofmeyr *et al.*, 1975). Due to the rapid population decline over the latter half of the 20th Century, game farm managers perhaps did not have many options with regards to selection of individuals and could have simply relied on factors such as finding a desired balance of males-to-females. They may have also lacked the resources to acquire rhinos from vastly different locales. Individuals were mainly translocated from within the same region; thus, if black rhinos are indeed philopatric to some degree (see Chapter 2), then the probability of selecting related rhinos is increased. Familial relationships were not considered during the initial translocations, which led to a relatively high degree of relatedness amongst the founder populations in both Waterberg and Okosorong game parks.

Waterberg Game Farm

Relatively high levels of relatedness were discovered in the original breeders introduced into the Waterberg Game Farm. All founders were translocated from Dolomietpunt within Etosha National Park; thus, this finding is consistent with the notion that rhinos found within a particular locale or social grouping within the park show more genetic similarity than to their neighbours (Chapter 2). Kinship amongst combinations of cows and bulls (0.19) show a level that is consistent with individuals that have a relatedness intermediate between that of first cousins (0.13) and half-siblings (0.25). However, despite the high degree of relatedness, no evidence of heterozygote deficiency is yet seen in comparison to a large sample population in Etosha National Park (Chapter 2). Translocation of individuals to captive populations from the same locale within Etosha National Park has led to recruitment of rhinos that are more genetically similar than the current population in ENP. Genetic implications of acquiring individuals from one location were perhaps not fully known at the time of the translocations and were most likely done for practical purposes (i.e. to minimize costs by procuring as many rhinos as possible within a given time). If feasible, to mitigate the potential dangers of inbreeding depression, future rhinos should be carefully genetically screened and only distantly related rhinos allowed to breed with the existing population.

Five bulls are resident within Waterberg game farm, yet despite this small number it has been difficult to determine paternity of calves by field observations alone. Analyses using microsatellite markers confirm with 95% certainty the sire of three calves, while the paternity of another calf has been determined with 80% confidence and two other calves have been allocated to the “most likely” father. Two bulls, BR75 and BR191, each sired two offspring while other successful bulls included BR50 and BR70 with one calf each. BR73

was the only resident bull not found to have sired any calves. Obviously more field work is necessary to determine breeding success correlates in male black rhinos such as age, body size, dominance hierarchies, testosterone levels, territory defense, resource allocation, sperm competition. Previous studies have suggested that black rhinos are polygynous and our findings confirm this (Goddard, 1966; Adcock *et al.*, 1998; Garnier *et al.*, 2001); however, the mating system observed in my study may be an artefact of captive breeding as the mating choices for both males and females is vastly restricted in comparison to wild rhinos.

Okosorong Game Farm

Recruitment of all Okosorong Game Farm breeders occurred within Kaross, a region of Etosha National Park. As expected, the relatedness values amongst reproductively fertile individuals were significantly higher than that of a randomly generated population of *Diceros b. bicornis* population. The relatedness between cows and bulls (0.31 ± 0.3) was higher than that of a half-sibling relationship (0.25), although a high standard error precludes any statement beyond this. Similarly, as seen in the Waterberg game farm, no evidence yet exists to support a loss of heterozygosity in Okosorong due to the same factors as mentioned above.

With only two breeding males within this game farm, the dynamics of the black rhino mating system can be dissected more thoroughly, although obviously the degree to which this reflects wild populations is very much in question. Of ten known offspring, assignments tests imply paternity for 6/10 and 4/10 for potential sires BR168 (born 1987) and BR171 (born 1989), respectively. While the breeding success is nearly equal, a further look at the times of conception reveal a shift in mating success over time. Black rhinoceroses are sexually monomorphic, reach sexual maturity at 7 years of age with a

reproductive life of 30-35 years and a gestation period of 15-16 months (Goddard, 1966; Garnier *et al.*, 2001). Based on times of conception, BR171 would have only been a juvenile (approximate age of 5.5 years old) when the majority of BR168's matings occurred. However, the last 4 out of 5 calves born in Okosorongu are assigned to BR168, which corresponds to male BR171 entering adulthood. It is plausible that BR168 was incapable of acquiring a mate as a sub-adult either due to female rejection or because he was subordinate to BR171. A decline in BR171's fertility is not suspected at this time due to the long reproductive window for black rhinos (Goddard, 1966). Interestingly, while rhinos are capable of reproducing at 7 years of age, most males in the wild are not successful at this young age (Goddard, 1966; Garnier *et al.*, 2001). Thus, a unique breeding scenario was artificially created in this game farm by allowing only two relatively young males to be present since its inception. Whether either of these two would have had any mating success in the presence of a mature bull cannot be known.

Conservation Implications

Molecular tools can contribute greatly to the conservation of endangered wildlife, for example, by defining the distribution of genetic variation within and among conspecific populations and determining the genealogical relationships among closely related taxa (Frankham *et al.* 2004). I show how molecular data can complement pedigree data both of which can underpin both captive reproduction and wildlife management strategies.

Continued conservation success of black rhinoceroses will depend on genetic data, which will aid in the characterization of relatedness amongst breeding pairs. Minimizing introductions of related individuals via translocations into game parks or national parks reduces the risks of inbreeding depression.

Table 1. Characteristics of microsatellite markers used to assess parentage and relatedness across individuals in three separate game parks: Waterberg, Okosorongo and Kaross.

Locus	Repeat Motif	Size (bp)	T_a (°C)	No. of Alleles	Allelic Richness
2B	(CA) ₁₄	238-252	60	7	5.97
37D	(TG) ₆ (AG) ₁₁	163-165	60	2	2.00
BR4	(CA) ₁₉	142-150	60	5	4.84
BR6	(CA) ₁₅	234-258	62	5	4.49
BR17	(AT) ₆ (GT) ₁₈	123-133	62	2	2.00
DB01	(CA) ₁₄	121-131	60	4	4.00
DB44	(CA) ₄ G(CA) ₁₆	173-177	64	3	3.00
DB52	(CA) ₂₁	232-238	64	6	5.77
DB66	(CA) ₇ TA(CA) ₁₆	204-224	62	8	7.50

Table 2. Genetic characterization of adult *D.b. bicornis* individuals sampled within Waterberg Game Park. H_O = observed heterozygosity, H_E = expected heterozygosity, PIC = polymorphic information content, PE_1 and PE_2 = first and second parent exclusionary probabilities and Null freq = frequency of null alleles.

<i>Locus</i>	H_O	H_E	<i>PIC</i>	PE_1	PE_2
2B	0.717	0.699	0.662	0.299	0.485
37D	0.5	0.495	0.371	0.121	0.185
BR4	0.3	0.332	0.306	0.055	0.173
BR6	0.217	0.24	0.221	0.028	0.118
BR17	0.45	0.442	0.342	0.096	0.171
DB01	0.712	0.577	0.516	0.174	0.326
DB44	0.35	0.388	0.351	0.074	0.2
DB52	0.783	0.701	0.634	0.262	0.423
DB66	0.75	0.748	0.705	0.343	0.522
<i>Average</i>	0.531	0.514	0.456	0.810	0.962

Table 3. Estimates of pairwise relatedness amongst breeders and offspring within Waterberg Game Park. n = number of comparisons between individuals, SE = standard error.

	<i>n</i>	<i>Mean(R)</i>	<i>SE(R)</i>
Cow vs. Cow	6	0.206	0.25
Calf vs. Calf	15	0.288	0.17
Bull vs. Bull	10	0.282	0.26
Cow vs. Bull	20	0.187	0.27
Cow vs. Calf	23	0.301	0.21
Bull vs. Calf	30	0.301	0.20
<i>Average</i>	104	0.262	0.22

Table 4. Paternity assignments of *D.b. bicornis* calves born in Waterberg Game Farm against two typing error (TE) rates: 0.01 and 0.05. “*” indicates strict confidence at the 95% level, while “+” is reflective of a relaxed confidence level of 80%. Probability of each putative bull is shown in comparison against the next likeliest individual.

<i>Calf</i>	<i>Cow</i>	<i>Putative Bulls</i>	<i>TE = 0.01</i>		<i>TE = 0.05</i>		
			<i>LOD</i>	Δ	<i>Putative Bulls</i>	<i>LOD</i>	Δ
BR43	BR64	BR75*	2.97E+00	2.86E+00	BR75 ⁺	1.70E+00	9.95E-01
		BR50	1.08E-01	0.00E+00	BR50	7.09E-01	0.00E+00
BR47	BR64	BR50*	4.13E+00	4.13E+00	BR50*	2.68E+00	2.68E+00
		BR73	-1.69E+00	0.00E+00	BR70	-7.19E-02	0.00E+00
BR187	BR47	BR75*	3.32E+00	3.32E+00	BR75*	2.05E+00	1.92E+00
		BR50	-1.71E+00	0.00E+00	BR70	1.26E-01	0.00E+00
BR63	BR76	BR191 ⁺	2.19E-01	2.19E-01	BR191 ⁺	2.70E-01	2.70E-01
		BR75	-1.10E+00	0.00E+00	BR75	-1.07E+00	0.00E+00
BR55	BR76	BR70	-1.17E+00	0.00E+00	BR70	-8.62E-01	0.00E+00
		BR75	-4.27E+00	0.00E+00	BR75	-2.63E+00	0.00E+00
BR49	BR54	BR191	-1.41E+00	0.00E+00	BR191	-2.49E-01	0.00E+00
		BR73	-1.46E+00	0.00E+00	BR73	-5.29E-01	0.00E+00

Table 5. Genetic characterization of *D.b. bicornis* individuals sampled within Okosorongu Game Park. H_O = observed heterozygosity, H_E = expected heterozygosity, PIC = polymorphic information content, PE_1 and PE_2 = first and second parent exclusionary probabilities and Null freq = frequency of null alleles.

<i>Locus</i>	H_O	H_E	<i>PIC</i>	PE_1	PE_2
2B	0.688	0.693	0.658	0.295	0.48
37D	0.500	0.500	0.373	0.123	0.186
BR4	0.349	0.361	0.328	0.065	0.185
BR6	0.254	0.267	0.245	0.035	0.132
BR17	0.469	0.433	0.337	0.092	0.169
DB01	0.672	0.566	0.495	0.165	0.303
DB44	0.367	0.375	0.34	0.069	0.192
DB52	0.766	0.664	0.591	0.228	0.381
DB66	0.661	0.715	0.672	0.307	0.488
<i>Average</i>	0.525	0.508	0.449	0.789	0.956

Table 6. Paternity assignments of *D.b. bicornis* calves born in Okosorong Game Farm against two typing error (TE) rates: 0.01 and 0.05. “*” indicates strict confidence at the 95% level, while “+” is reflective of a relaxed confidence level of 80%. Probability of each putative bull is shown in comparison against the next likeliest individual.

Calf	Cow	Putative Bulls	TE = 0.01		TE = 0.05		
			LOD	Δ	Putative Bulls	LOD	Δ
BR91	BR170	BR168*	1.54E+00	1.54E+00	BR168 ⁺	1.35E+00	1.35E+00
		BR171	-4.14E-01	0.00E+00	BR171	-2.18E-01	0.00E+00
BR89	BR169	BR168	-2.83E-01	0.00E+00	BR168 ⁺	3.89E-01	3.89E-01
		BR171	-1.51E+00	0.00E+00	BR171	-4.25E-01	0.00E+00
BR93	BR165	BR171*	3.51E+00	3.51E+00	BR171*	2.58E+00	2.58E+00
		BR168	-5.05E+00	0.00E+00	BR168	-1.27E+00	0.00E+00
DbbA3	BR170	BR168*	9.55E-01	9.55E-01	BR168 ⁺	8.30E-01	7.46E-01
		BR171	-1.69E-01	0.00E+00	BR171	8.41E-02	0.00E+00
DbbA11	BR166	BR171*	1.53E-01	1.53E-01	BR171 ⁺	4.92E-01	4.92E-01
		BR168	-1.94E+00	0.00E+00	BR168	-8.05E-02	0.00E+00
DbbA12	BR165	BR171*	5.30E+00	5.30E+00	BR171*	3.81E+00	3.81E+00
		BR168	-7.90E+00	0.00E+00	BR168	-2.73E+00	0.00E+00
DbbA20	BR169	BR171	-1.19E+00	0.00E+00	BR168 ⁺	3.65E-01	3.65E-01
		BR168	-1.45E+00	0.00E+00	BR171	-4.17E-01	0.00E+00

Table 7. Assignment of three calves with unknown relationships to breeding males and females within Okosorongo Game Park assuming a typing error of 0.01. “*” indicates strict confidence at the 95% level, while “+” is reflective of a relaxed confidence level of 80%. Probability of each putative bull and cow is shown in comparison against the next likeliest individual.

<i>Calf</i>	<i>Putative Bull</i>	<i>LOD</i>	Δ	<i>Putative Cow</i>	<i>LOD</i>	Δ
BR199	BR168 ⁺	3.08E-01	3.08E-01	BR170*	1.37E+00	1.37E+00
	BR171	-4.86E-01	0.00E+00	BR169	-1.38E+00	0.00E+00
BR288	BR168*	1.63E+00	1.47E+00	BR165*	1.37E+00	1.32E+00
	BR171	1.57E-01	0.00E+00	BR170	5.20E-02	0.00E+00
BR291	BR168*	1.37E+00	1.37E+00	BR165 ⁺	5.88E-02	5.88E-02
	BR171	-1.04E-01	0.00E+00	BR169	-4.16E-01	0.00E+00

Table 8. Estimates of pairwise relatedness amongst cows, bulls and offspring within Okosorong Game Park.

	# of comparisons	Mean(R)	SD(R)
Cow vs. Cow	6	0.374	0.19
Calf vs. Calf	41	0.441	0.19
Bull vs. Bull	1	0.350	0.17
Cow vs. Bull	8	0.310	0.26
Cow vs. Calf	40	0.372	0.21
Bull vs. Calf	20	0.501	0.17
Average	116	0.414	0.20

CHAPTER 4: GENERAL DISCUSSION

The broad goal of my thesis was to determine the amount and geographic distribution of genetic variability present in the *Diceros bicornis bicornis* subspecies found within Etosha National Park and how such knowledge can contribute to the future conservation of this hallmark species and other megafauna that have undergone similar precipitous population declines. The specific goals and outcomes of this thesis have been discussed in the previous chapters. In brief, I attempted to characterize the genetic variability in the black rhinoceros and assess if there existed genetic structure within Etosha National Park or whether these populations might be characterized as panmictic. I also analyzed the genetic relationships amongst rhinos in Namibian game farms and explored how such information might be deployed to aid in captive breeding.

With few field observations, I was able to describe at least tentatively the likely social and mating interactions using only genetic tools. Black rhinoceroses appear to have sex-biased movement patterns and are not as solitary and transient as previously believed. Its polygynous mating system was found to be similar to Garnier *et al.*'s (2001) work.

Comparative studies

The paucity of genetic variation in other species due to population bottlenecks has been well-documented. Northern elephant seals, *Mirounga augustirostris*, and the African cheetah, *Acinonyx jubatus*, underwent massive population declines in the mid-1800's due to poaching and/or habitat destruction similar to the black rhinoceros. Bonnell and Selander (1974) documented a marked lack of remnant genetic variability in northern elephant seals using allozyme data, while O'Brien *et al.* (1983) showed similar findings in the cheetah.

Despite the low levels of variation noted in these two species they continue to persist with no further indications that their populations are declining. Lacy (1997) postulated that populations that remain small may become adapted to low levels of genetic variation and may maintain mechanisms to preserve adaptive variation even in small populations. While my study and previous studies show that *D.b. bicornis* maintains a relatively high level of genetic variation, the potential exists that its genetic diversity may decline due to further man-made or stochastic factors. Studies on other species such as red deer (*Cervus elaphus*; Coulson *et al.*, 1998), harbor seals (*Phoca vitulina*; Coltman *et al.*, 1998), greater horseshoe bats (*Rhinolophus ferrumequinum*; Rossiter *et al.*, 2001) and harp seals (*Phoca groenlandica*; Kretzmann *et al.*, 2006) have shown that although microsatellites are selectively neutral markers, they can be used as positively correlated with fitness (i.e. high levels of microsatellite diversity correspond to higher levels of fitness). Similarly, while black rhinos have undergone a massive population bottleneck, this species exhibits relatively high levels of genetic variability, and may still possess adequate adaptive variability and high levels of fitness. Thus, while the census population numbers of the black rhino are quite low, the species may have a good prognosis if current conservation strategies allow for its repopulation.

Difficulties encountered and possible solutions

Perhaps the single greatest obstacle to this work was the low quality of some of the tissue samples I received, which hindered the extraction and amplification of DNA. With a possible sample size of greater than 300 individuals, this study had the potential to be one of the largest of any endangered, large mammal. More specifically, this study would have characterized approximately one-quarter of all *D.b. bicornis* individuals alive today, and

thus provided a thorough survey of the neutral genetic diversity of this subspecies.

Unfortunately, the technology currently available to the African nationals who collected the samples is limited and I am simply thankful for the samples we were able to recover.

Despite a number of efforts to increase the number of microsatellite markers, the vast majority were either monomorphic, impossible to amplify consistently or produced unscorable PCR products. The power of my study could have been higher with more markers and/or if the current ones showed higher levels of polymorphism. Whether the relatively low levels of polymorphisms are natural or a result of a human-induced population bottleneck will not be conclusively determined without analyses of ancient samples (e.g. bone fragments, trophies, etc.) or via newer coalescent-based approaches to assessing population dynamics.

Future Directions

Aspects of my thesis could easily and profitably be expanded to more definitively answer the questions that I have proposed here. The first step would be to expand the number of polymorphic DNA microsatellite loci to give us a broader picture of the genetic diversity in this subspecies specifically and the species as a whole. Additional loci would permit us to determine pedigree and relatedness relationships at a more accurate level. With the advent of non-invasive sampling (i.e. fecal testing), the possibility exists for large-scale DNA studies with minimal behavioural disruptions (i.e. anxiety associated with the tranquilization process, recovery, etc.). Further sampling (or re-sampling in the case of degraded tissue) of *D.b. bicornis* individuals will yield a more complete picture of the genetic diversity within Etosha National Park. Similarly, genetic studies on the other extant

subspecies have not been undertaken yet with large sample sizes and these would allow for formal comparative analyses with *D.b. bicornis*.

Of particular note, with the determination of three subpopulations within Etosha National Park, it will be interesting to see if game managers attempt to increase, decrease or not change the amount of movement of individuals within the park. Given the relatively high levels of genetic diversity, a strategy of maintaining the current strategy would be appropriate. Some conservation strategies suggest that maintaining separate populations may help a species more than allowing integration to mitigate the spread of disease or other unforeseen natural disasters that could potentially decimate entire populations (Soulé, 1985; Linklater, 2003). However, given that the subpopulations within Etosha National Park are so geographically close, diseases or natural disasters would like affect all rhinos irrespective of their subpopulation.

One concern is the admixture of different subspecies, which could potentially result in outbreeding depression. While *D.b. bicornis* lives in a semi-arid environment and is perceived to have locally adapted traits (Joubert, 1971), it remains to be seen if the other subspecies or hybrids could cope in this harsher climate. At this time, however, effective population sizes appear adequate to permit maintaining the subspecies separately (Harley *et al.*, 2005).

To further assess the breeding capabilities in the game park rhinos, analyses of other correlates to fitness such as age, body size, sex hormone levels, sperm competition, etc. should be undertaken. Body size measurements would be a relatively simple method of determining if that plays a role in reproductive success with respect to dominance, territoriality and female mate choice. While logistically difficult in such a large organism, it

would be interesting to see if sperm competition occurs in females if they undergo multiple matings.

These future projects would enhance our understanding of the black rhinoceros and will aid in conservation efforts and management strategies to protect this species.

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