GENETIC DIVERSITY OF TWO SUBPOPULATIONS OF BLACK RHINOCEROS,
DICEROS BICORNIS BICORNIS, AT ADDO ELEPHANT NATIONAL PARK,
SOUTH AFRICA
by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Environmental Science and Policy

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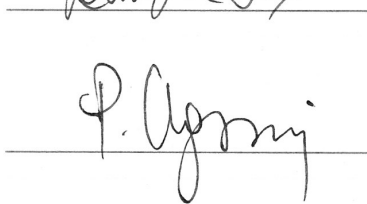George Mason University
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Genetic Diversity of Two Subpopulations of Black Rhinoceros, *Diceros bicornis bicornis,* at Addo Elephant National Park, South Africa

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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DEDICATION

This thesis is dedicated to my husband, John Hallett. Thank you for your infinite encouragement, support and culinary achievements.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Addo Elephant National Park .... AENP
African Rhino Specialist Group... AfRSG
International Union for Conservation of Nature...IUCN
Mitochondrial DNA ....mtDNA
Mitochondrial DNA D-loop Haplotype 1 ....Hap1
Mitochondrial DNA D-loop Haplotype 2 ....Hap2
Polymerase chain reaction ....PCR
South African National Parks ....SANParks
Southern African Development Community -
Regional Program for Rhino Conservation.. SADC RPRC

ABSTRACT

GENETIC DIVERSITY OF TWO SUBPOPULATIONS OF BLACK RHINOCEROS, *DICEROS BICORNIS BICORNIS*, AT ADDO ELEPHANT NATIONAL PARK, SOUTH AFRICA

Sarah E. Josway, M.S.

George Mason University, 2014

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Due to international initiatives to increase the efficacy of conservation programs, the critically endangered black rhinoceros is making a tenuous recovery from significant population declines (Emslie & Brooks, 1999). However, as this species has slow maturation and reproduction rates, maximizing the productivity and genetic health of remaining populations is crucial to conserving this iconic animal. Assessing genetic diversity of a threatened population using mitochondrial DNA (mtDNA), a maternally inherited molecular marker, can elucidate the genetic viability of populations and subsequently inform conservation strategies (Frankham, Ballou & Briscoe, 2010). Using mtDNA this study assessed the genetic diversity of two subpopulations of the southwestern arid subspecies of black rhinoceros, *Diceros bicornis bicornis* (*D. b. bicornis*), at Addo Elephant National Park (AENP), South Africa. A total of 112 noninvasively collected fecal samples were assayed including 87 samples from the

Nyathi subpopulation, 24 samples from the Addo subpopulation and one sample collected outside of the park. Identities were assigned to the samples when possible. Only two mtDNA D-loop haplotypes were identified. Low genetic diversity of this marker could indicate that the population is at risk of inbreeding (Frankham, Ballou & Briscoe, 2010), which could have serious implications for small populations like the *D. b. bicornis* at AENP. This study also described a new set of primers that amplify the polymorphic region between the two haplotypes identified at AENP, providing a new tool for managers at the park to monitor the genetic variation within the subpopulations. Although additional genetic testing is recommended to further characterize the genetic diversity of these subpopulations of *D. b. bicornis*, the data reported in this study provide critical information to the managers at AENP about the potentially limited genetic variation in this important population.

INTRODUCTION

Description
The black rhinoceros (rhino), *Diceros bicornis,* is an iconic species that was driven to the

brink of extinction in the 1990s by poaching, habitat destruction and human incursion

(Dublin & Wilson, 1998). Beginning in the 1960s, the species experienced a population

decline of 97%, reaching a low of 2,410 individuals in 1995 (Emslie & Knight, 2012).

The precipitous decline – driven largely by demand for rhinoceros horn for medicinal and

ornamental purposes – was exacerbated by poverty and civil unrest throughout rhino

range states (Emslie & Brooks, 1999). Black rhinoceros have started to recover slowly

due to international initiatives to reduce demand for rhino horn and increasingly effective

management strategies that prioritize genetic health and viability at a metapopulation

level (Emslie & Knight, 2012; Emslie, 2012; Dublin & Wilson, 1998). Despite these

efforts, the recovery remains tenuous (Emslie & Knight, 2012). Due to slow rates of

maturation and reproduction of the black rhinoceros, understanding the genetic diversity

of wild populations will aid conservation managers in optimizing propagation and

conservation efforts, maximizing breeding success and ensuring the genetic viability of

remaining populations.

In collaboration with South African National Parks (SANParks) and scientists at

George Mason University and Chicago's Lincoln Park Zoo, this study investigated the

genetic variation of the southwestern arid subspecies of black rhinoceros, *Diceros*

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bicornis bicornis (D. b. bicornis), at Addo Elephant National Park (AENP), South Africa. The goals of this research were to: (1) assess the genetic diversity of two subpopulations of *D. b. bicornis* at AENP and (2) provide AENP managers with an objective tool to better monitor and manage the subpopulations to maximize productivity and health.

Conservation Status
There are five extant species of rhinoceros. The two African species are the black rhinoceros (*Diceros bicornis*) and the white rhinoceros (*Ceratotherium simum*). The three Asian species are the Indian rhinoceros (*Rhinoceros unicornis*), Sumatran rhinoceros (*Dicerorhinus sumatrensis*) and Javan rhinoceros (*Rhinoceros sondaicus*) (Emslie, 2012). The five extant rhinoceros species have been listed under CITES Appendix I since 1977, which bans international commercial trade in rhinoceros parts (Emslie & Brooks, 1999). Since 1996, the black rhinoceros species has been listed as critically endangered on the IUCN *Red List of Threatened Species* (Emslie, 2012).

In 1986, four specific units for conservation within the black rhinoceros species were delineated based on geographical and ecological data rather than genetic data (Anonymous, 1987). These conservation units were later adopted as subspecies designations (du Toit, 2006). The western subspecies, *Diceros bicornis longipes*, was officially declared extinct in 2011, leaving three extant subspecies (Emslie, 2012). The eastern subspecies, *Diceros bicornis michaeli,* and the south-central subspecies, *Diceros bicornis minor,* are both listed as critically endangered. The southwestern arid subspecies, *Diceros bicornis bicornis,* is listed as vulnerable (Emslie, 2012).

Range

The current ranges of the remaining wild black rhinoceros populations span eastern and southern Africa with 98.6% of Africa's black rhinos conserved in four major range states: South Africa, Kenya, Zimbabwe and Namibia (Figure 1) (Emslie, 2012). These areas were home to 4,880 black rhinoceros at the end of 2010, and since 1995 have had an average annual population growth rate of 4.8% (Emslie & Knight, 2012). Although this growth rate is near the continent-wide conservation objective of 5% per annum for each subspecies, biological management failures along with increasing poaching pressures have significantly slowed the rate of recovery (Emslie, 2012). In the face of such failures, increasing the success of the remaining populations is critical.

The historical range of the *D. b. bicornis* subspecies included Namibia, Angola, Botswana and South Africa (Emslie & Brooks, 1999). This subspecies currently occurs in Namibia with smaller populations in South Africa, which as of 2013 supported 8.9% of the total subspecies (Figure 1) (Knight, Balfour & Emslie, 2013; Downie & Mavrandonis, 2013).

Threats

The black rhinoceros has been exploited for consumptive purposes – ornamental and medicinal uses – since the early 1900's (Dublin & Wilson, 1998). In Asia, rhinoceros horn has been used for centuries in traditional Chinese medicine to treat a variety of ailments that range from fever to cancer (du Toit, 2006). Although clinical studies fail to support any of the reputed pharmacological properties of rhinoceros horn, China, South Korea and Vietnam remain large consumers of rhino horn products for medicinal uses (Emslie & Brooks, 1999). In Yemen rhinoceros horn is used to make jambiyas,

ceremonial daggers that serve as symbols of status (Emslie & Brooks, 1999). Despite

national bans on trade and use initiated by international non-governmental organizations,

lack of enforcement in Yemen continues to threaten the black rhinoceros with recent

reports indicating an increasing trend in rhinoceros horn consumption (Vigne & Martin,

2008; Vigne, Martin & Okita-Ouma, 2007).

While poaching for consumptive use is currently the primary threat to black

rhinoceros (Emslie & Knight, 2012), lack of political will, declining funds, poverty and

habitat degradation in rhinoceros range states also seriously threaten the perpetuation of

the species (Reid et al., 2007; Emslie & Brooks, 1999). Therefore, it is imperative to

maximize the productivity and health of remaining populations. Due to the slow

maturation and reproductive cycles of the black rhinoceros, prioritizing genetic viability

of wild populations through cooperative international initiatives is key to achieving this

goal.

Management

Management of Black Rhinoceros

The current global approach to rhinoceros conservation has led to the precarious recovery

of the black rhinoceros in Africa by bolstering the efficacy of field management

strategies through increased information dissemination, better support allocation to

priority populations and the concentration on genetic-based management at a

metapopulation level for each subspecies (du Toit, 2006). The long history of black

rhinoceros management in the four major range states - Kenya, Zimbabwe, South Africa

and Namibia - illustrates the multi-faceted nature of the crisis and the necessity for

cooperation at all levels, from local public/private collaborations to multiparty global initiatives (Emslie & Brooks, 1999).

The current conservation philosophy provides a top-down organizational scheme emphasizing international coordination to manage rhinoceros at a metapopulation level and to increase the efficacy of law enforcement efforts (du Toit, 2006). The top-down scheme begins with continent-wide conservation goals set by the IUCN Species Survival Commission African Rhino Specialist Group (AfRSG) and regional implementation recommendations by the Southern African Development Community Regional Program for Rhino Conservation (SADC RPRC) (Emslie & Brooks, 1999; du Toit, 2006). The conservation objectives set by AfRSG – which include growing metapopulations of each subspecies at 5% per year while maintaining genetic diversity – provide a coherent and standardized framework from which nations can tailor action plans to their own economic and cultural needs. In addition to adaptive management at the metapopulation level, all current national plans prioritize annual reporting, coordination at regional and continental levels and law enforcement (Adcock, 2009; du Toit, 2006; Knight, Balfour & Emslie, 2011; KWS, 2012). Finally, non-governmental organizations play critical roles throughout all levels of black rhinoceros management by spearheading international education and awareness initiatives and by providing locals with economic incentives to protect rhinoceros (du Toit, 2006).

The AfRSG and SADC RPRC conservation objectives for black rhinoceros explicitly call for the preservation of genetic diversity of populations within each metapopulation (du Toit, 2006). Maintaining genetic diversity provides small populations

a buffer against potentially devastating stochastic demographic (biased sex ratios), environmental and genetic factors (Gilpin & Soulé, 1986). This is especially relevant to black rhinoceros, when founding populations are generally composed of only twenty individuals (Emslie & Brooks, 1999). At AENP the Addo and Nyathi subpopulations boasted only twenty and 26 rhinoceros, respectively, including calves at the completion of this study. These subpopulations were established without genotyping the individuals, and thus the genetic diversity of these subpopulations currently is unknown. Genotyping the individual rhino will provide SANParks managers with information critical to making translocation and breeding decisions.

As early as 1989, maintenance of genetic diversity within metapopulations was a priority of continental and regional conservation strategies (Cumming, du Toit & Stuart, 1990; Brooks, 1989; KWS, 1993). Techniques such as genetic fingerprinting used to assess the genetic diversity of a population are not just limited to the identification of individual rhino. Such techniques can also determine the parentage of calves and elucidate mating strategies within a population, thus identifying important demographic trends (Garnier, Bruford & Goossens, 2001). In addition to the utility of genetic techniques for single populations, a compiled DNA database of all African black rhinoceros has the potential to lead to better-informed translocation decisions and to an increased ability to identify and track poached horn across national boarders (Emslie & Brooks, 1999).

Management of *D. b. bicornis* in South Africa
The most recent national strategy for the conservation of *D. b. bicornis* in South Africa

called for the growth of the metapopulation of *D. b. bicornis* to 260 individuals by 2020

(Knight, Balfour & Emslie, 2013). The South Africa Rhino Biodiversity Management

Plan 2011-2020 specified a long-term goal of expanding the metapopulation to 500 *D. b.*

bicornis including one population of at least 100 individuals. To meet these goals, the

authors presented six key components of the plan: accurate population monitoring;

adequate staffing; protection from poaching and raids of horn caches; maintenance of

financial security; cooperation and coordination of all stockholders from private parties to

NGOs and SADC; and biological management. The biological management strategy

highlighted the importance of prioritizing genetic diversity through translocations and a

harvesting regime that allows for the hunting of excess adult bulls (Knight, Balfour &

Emslie, 2013; Downie & Mavrandonis, 2013). The first goal of this research – to assess

the genetic diversity in the subpopulations of *D. b. bicornis* at AENP – supports the

objectives of the conservation organizations in South Africa and international parties by

contributing to the currently limited knowledge about the genetic diversity of the

metapopulation of this subspecies.

Molecular Studies of *D. b. bicornis*
To date, there are fewer than 25 published studies that have examined genetic diversity in

black rhinoceros and only ten investigating genetic diversity in the southwestern

subspecies, *D. b. bicornis*. As molecular technologies advanced during the 1990s, early

markers used to estimate genetic diversity (e.g. allozyme markers and restriction

fragment length polymorphisms) have been replaced by lower-cost and more precise

methods (e.g. sequencing of nuclear and mitochondrial loci and microsatellite fingerprinting) (Frankham, Ballou & Briscoe, 2010). In addition to being more informative, sequencing and microsatellite methods can be used on noninvasively collected samples such as fecal and hair samples, which is advantageous when working with rare or endangered species (Frankham, Ballou & Briscoe, 2010).

The molecular studies on *D. b. bicornis* populations have reported conflicting amounts of genetic diversity depending on the molecular method (Table 1). Early molecular studies identified high genetic diversity in *D. b. bicornis* using allozyme markers (Swart et al., 1994; Swart & Ferguson, 1997), which identify differences in proteins from invasively collected tissues such as blood or organ tissue and estimate diversity at only a few loci (Frankham, Ballou & Briscoe, 2010). In contrast, restriction length fragment polymorphism (RFLP) analysis indicated low diversity within this subspecies (O'Ryan, Flamand & Harley, 1994). RFLP analysis identifies sequence differences between individuals by comparing the sizes of DNA fragments produced by restriction enzyme digestion (Frankham, Ballou & Briscoe, 2010). In this study, RFLP analysis was conducted using mitochondrial DNA (mtDNA) (O'Ryan, Flamand & Harley, 1994), a genetic marker that evolves rapidly and has a high copy rate, making it ideal for the estimation of genetic diversity in many endangered species (Frankham, Ballou & Briscoe, 2010). Using this method, only one mtDNA haplotype was identified in five *D. b. bicornis* from Namibia (O'Ryan, Flamand & Harley, 1994).

Studies using microsatellite markers generally identified moderate levels of diversity compared to other subspecies (see: (Karsten et al., 2011; Harley et al., 2005; de

Groot et al., 2011; Brown & Houlden, 1999; Anderson-Lederer, 2013). Microsatellites are tandem repeats of short sequences of nuclear DNA that, due to their highly polymorphic nature, abundance and sensitivity, are often used to assess genetic diversity and mating strategies in populations (Schlotterer, 2004). In 2011, the largest genetic study of *D. b. bicornis* reported a moderate amount of diversity (de Groot et al., 2011). Based on additional analyses, the authors concluded that the population at Etosha National Park, Namibia likely did not lose significant genetic diversity due to the population bottleneck that occurred in the mid-1900s (de Groot et al., 2011). Interestingly, spatial analyses indicated that dispersal distances of females within the population were limited to 25 km, whereas males exhibited no spatial genetic autocorrelation. This finding was the first evidence of female-limited dispersal behavior in rhino, which could have important implications for the management of *D. b. bicornis* influencing translocation decisions and carrying capacity calculations (de Groot et al., 2011).

To date, only one study has assessed genetic diversity in this subspecies by sequencing the D-loop region of the mtDNA (Anderson-Lederer, Linklater & Ritchie, 2012). Results from this study indicated low genetic diversity and identified only a single haplotype (Anderson-Lederer, Linklater & Ritchie, 2012). The authors suggested the low genetic diversity may not reflect the true diversity of the subspecies and that the result was more likely due to the small number of samples available for the study (O'Ryan, Flamand & Harley, 1994; Anderson-Lederer, Linklater & Ritchie, 2012).

In summary, molecular studies reported conflicting amounts of genetic diversity in *D. b. bicornis* (Table 1). By assessing the genetic diversity of two subpopulations of *D. b. bicornis* at AENP, this study sought to expand the limited knowledge about the genetic variation in the metapopulation of this subspecies and provide critical information to AENP managers about the genetic health and viability of this important population of rhinoceros.

D. b. bicornis subpopulations at AENP, South Africa

In the 1990s, three subpopulations of *D. b. bicornis* were established at AENP in different sections of the park, the Nyathi, Addo and Darlington sections. The founding members of these subpopulations were wild black rhinoceros that were translocated from Namibia and Augrabies National Park, South Africa. Since then, the Nyathi and Addo subpopulations have experienced growth with the births of over twenty offspring. The Nyathi subpopulation was also supplemented with bulls from Etosha National Park, Namibia and the Mountain Zebra National Park, South Africa. At the time of sample collection for this study, there were 26 *D. b. bicornis* in the Nyathi subpopulation and twenty individuals in the Addo subpopulation (Freeman et al., 2014).

To identify rhinoceros, AENP managers developed a system of marking each individual between the ages of two and four with specific ear notching patterns as well as noting other physical marks (Figure 2) (Freeman et al., 2014). The camera trap photos are used to assign identities to fecal samples left near the trap during the previous night.

The environmental conditions in the Nyathi and Addo sections of AENP differ in a number of important ways that could potentially impact the growth and success of the

rhino subpopulations (Freeman et al., 2014). For example, the Nyathi section is 14,000 ha

with abundant vegetation while the Addo section is 11,500 ha with more limited

vegetation sources. Due to the territorial behavior of black rhino, which base their home

ranges on resource distribution, the total area and browse availability of management

sections could significantly impact subpopulation success (Adcock, 1994). The Nyathi

and Addo sections of the park also differ in biotic stressors such as the abundance of

predators and elephants, as well as the level of anthropogenic activity (Freeman et al.,

2014). Freeman et al. (2014) identified longer inter-calving intervals in Addo females

compared with Nyathi females, and the authors suggested that this finding may indicate

how different levels of biotic stressors can impact reproductive physiology in wild black

rhino. These biotic stresses may also contribute to reported differences in sleep patterns

between rhino in the Nyathi and Addo sections (Santymire, Meyer & Freeman, 2012). By

investigating how these two subpopulations differ genetically, this present study aimed to

add to these data on environmental impacts to provide AENP managers a more detailed

picture of the factors that impact reproductive success in wild rhino in the park.

Objective
The primary objective of this study was to investigate the genetic diversity of the Nyathi

and Addo subpopulations of *D. b. bicornis* at Addo Elephant National Park, South Africa.

The genetic diversity of a small population such as the *D. b. bicornis* at AENP can

indicate whether the population is genetically healthy or at risk of inbreeding, which can

negatively impact the population (Frankham, Ballou & Briscoe, 2010). Two molecular

methods, mitochondrial DNA sequencing and microsatellite analysis, were explored with

the fecal samples collected in 2010 from the rhino residing within the two sections of AENP. These methods were chosen based on their reliability, low cost, sensitivity and utility in analyzing genetic diversity from noninvasively collected samples (Frankham, Ballou & Briscoe, 2010) such as the fecal samples collected from AENP. As both molecular methods were utilized successfully to assess genetic diversity in other *D. b. bicornis* populations (see: Karsten et al., 2011; Harley et al., 2005; de Groot et al., 2011; Cunningham, Harley & O'Ryan, 1999; Brown & Houlden, 1999; Anderson-Lederer, Linklater & Ritchie, 2012), results from this study add to the current assessment of genetic diversity in the subspecies.

The goals of this research were to: (1) assess the genetic diversity of two subpopulations of *D. b. bicornis* at AENP and (2) to provide AENP managers with an objective tool to better monitor and manage the subpopulations to maximize productivity and health.

Hypothesis
In the two subpopulations of *D. b. bicornis* at AENP, a moderate level of genetic diversity was expected. Specifically, a moderate level of diversity as indicated by multiple mtDNA D-loop haplotypes and a moderate degree of heterozygosity using ten microsatellite markers was expected. This hypothesis was based on two lines of evidence:

(1) moderate levels of genetic diversity were identified in *D. b. bicornis* populations in Namibia, the origin of the founders of the AENP populations

(see: Karsten et al., 2011; Harley et al., 2005; de Groot et al., 2011; Anderson-Lederer, 2013) and

(2) this study includes samples representing eleven putative mtDNA D-loop haplotypes (Tables 3 and 4). A previous study identified only one haplotype in four *D. b. bicornis* using the same method of DNA sequencing (Anderson-Lederer, Linklater & Ritchie, 2012).

MATERIALS AND METHODS

Sample Collection

A total of 178 rhinoceros fecal samples were collected noninvasively at AENP between January and December 2010. When possible, the identity of the individual that deposited the sample was recorded during collection. Identities were determined using ear notch patterns and distinguishing physical markings such as scars or horn deformations. Samples were collected in the field when defecation was observed or at camera trap locations, sites of high rhinoceros activity with motion activated cameras (ScoutGuard 550V and ScoutGuard SG550, HCO, Norcross, GA, USA; and Wildview STC-TGL3IR, Grand Prairie, TX, USA). In the mornings at camera trap locations, fresh fecal samples in view of the camera were collected and stored in plastic bags. A subsample was placed in 5mL plastic tubes and preserved in lysis buffer (Longmire, Maltbie & Baker, 1997) at room temperature. Date, location and time of collection were recorded. The identity of the rhinoceros that defecated was determined from the digital photos taken by the camera. If no identity or descriptive label such as "subadult" could be assigned, samples were labeled "unknown." During sample collection, other information including environmental and geographic data were recorded. Samples were heat treated according to USDA permits for importation to the United States.

DNA Extraction

DNA was extracted from each sample using the QIAamp® DNA Stool Mini Kit

following manufacturers protocols (QIAGEN GMBH, Hilden, Germany). DNA samples

were stored in 50uL elution buffer at –20° C. Dilutions of 1:1, 1:5, 1:100, 1:500, and

1:100 were made with sterile water for optimization.

Mitochondrial DNA D-loop Analysis

Final Data Set

The final data set for the mtDNA D-loop analysis included visually confirmed samples

for each individual, if available, and all unknown samples from calves, subadults, and

adults (Tables 3 and 4). Names of the rhinoceros were coded to protect confidential

information. Maternal groups are assigned. First generation offspring born within a

maternal group and their second generation offspring were assigned an letter code. Adult

bulls were coded as Male with a number. When multiple samples were available, three

different fecal replicates per individual were analyzed. The final sample set included a

total of 112 samples from AENP rhino with 87 fecal samples from the Nyathi section, 24

fecal samples from the Addo section, and one sample collected outside of the park.

PCR Amplification and Sequencing

A 491 base pair length region of the mitochondrial DNA D-loop was amplified from total

DNA with primers mt16502H (5′-TTTGATGGCCCTGAAGTAAGAACCA-3') (Moro

et al., 1998; Brown & Houlden, 1999) and mt15996L (5′-

TCCACCATCAGCACCCAAAGC-3') (Campbell et al., 1995). Polymerase chain

reaction (PCR) was performed in 20 ul reactions with 2 ul GeneAmp 10X PCR Buffer, 2

mM MgCl, 200 uM of each dNTP, 0.5 uM of the forward and reverse primers, 1.25 units

of hot start AmpliTaq Gold DNA Polymerase (Life Technologies, Carlsbad, CA), and 2 ul template DNA. Polymerase chain reaction was performed with the following thermal cycling steps: 95°C for eleven min, 39 cycles of 95°C for 30s, 60°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 10 min. After confirmation of amplification with gel electrophoresis, PCR products were sent to Macrogen USA (Rockville, MD) for PCR product purification and sequencing in both the forward and reverse directions.

To troubleshoot samples that failed to amplify with the mt16502H and mt15996L, new primers were designed in Primer3 that spanned the polymorphic region of the two haplotypes identified from the first round of sequencing (Untergrasser et al., 2012; Koressaar & Remm, 2007). The new primers, DbbDLoopF (5'-TGTGAGTACATCCCGGGTATG-3') and DbbDLoopR (5'-TGGTGGTGATATGCGTGTTG-3'), produce a 245 base pair product. DbbDLoop is 21 bases long and starts at position 121 in the 491 base pair product produced by the mt16502H and mt15996L primers. DbbDLoopR is twenty bases long and starts at the 365 position. PCR, gel electrophoresis, and sequencing were performed as previously described for the mt16502H and mt15996L primers.

Data Analysis
Nucleotide sequences were edited and aligned in Geneious (Drummond et al., 2011). MEGA 5.1 was used to calculate pairwise distances and standard deviation and create a maximum likelihood phylogenetic tree (Tamura et al., 2011). The tree was edited in Figtree (Rambaut, 2012). A statistical maximum parsimony haplotype network was created in Network 4.610 (Bandelt, Forster & Röhl, 1999).

For the analysis, 40 sequences were downloaded from Genbank including four *D. b. bicornis* sequences (accession numbers JN593091 - JN593094) (Anderson-Lederer, Linklater & Ritchie, 2012), 21 *D. b. michaeli* sequences (accession number JN593090) (Anderson-Lederer, Linklater & Ritchie, 2012), (accession numbers AF187834 and AF187835) (Brown & Houlden, 2000), (accession numbers AY742830 and AY742831) (Fernando et al., 2006), (accession numbers FJ227483 - FJ227498) (Muya et al., 2011), and eleven *D. b. minor* sequences (accession number JN593089) (Anderson-Lederer, Linklater & Ritchie, 2012), (accession numbers AF187825 - AF187833) (Brown & Houlden, 2000), and (accession numbers AY742832 and AY742833) (Fernando et al., 2006). Additionally, four sequences from *Ceratotherium simum simum, C. s. simum,* (accession numbers AF187836 - AF187839) were included to represent an outgroup (Brown & Houlden, 2000).

Microsatellite Fingerprinting Analysis

Final Data Set
The final data set for the microsatellite analysis had a three-tiered prioritization plan based on importance to the specific goals of the research. The first priority list, which included three samples per individual, would provide a general picture of genetic diversity of rhino at AENP with reasonable confidence in the genotypes assigned per individual. The second priority list contained all unidentified samples. The third list, which included all remaining samples, would verify the sample identification methods used in sample collection at AENP.

PCR Amplification and Troubleshooting
Ten previously published microsatellite loci (Table 2) were chosen for use in this study

based on number of alleles, prior use in *D. b. bicornis* populations and primer specificity

as determined by blasting the forward and reserve primer sequences in the Genbank.

Primers were ordered and tested by performing PCRs in 20 ul reactions with 0.5 uM

concentration of the forward and reverse primers, 2 ul template DNA, 2 ul GeneAmp

10X PCR Buffer, 2 mM MgCl, 200 uM of each dNTP, 1.25 units of hot start AmpliTaq

Gold DNA Polymerase (Life Technologies, Carlsbad, CA). PCR products were

electrophoresed on 1.5% agarose gels to visualize length polymorphisms among

individuals and to verify PCR conditions.

The amplification of nuclear DNA from the fecal samples using these

microsatellite primers produced inconsistent and poor quality results. To improve

amplification several alternate methods were explored including: testing different

concentrations of individual and combined PCR additives such as BSA, Betaine, and

DMSO; altering the concentration of MgCl; testing different dilutions of the template

DNA including 1:5, 1:10, 1:100, and 1:1000; and varying the PCR conditions including

decreasing the annealing temperature, increasing the number of cycles, and using a

touchdown protocol. These optimization steps failed to produce results of high enough

quality and consistency to proceed with the fingerprinting analysis. As such, no results

were included in this manuscript.

RESULTS

The mtDNA D-loop sequence was determined for 112 samples from a total of 178 black

rhinoceros fecal samples from AENP, South Africa. Two unique haplotypes were

identified with ten polymorphic sites. The first haplotype (Hap1) identified in 100 of the

112 samples was previously reported by Anderson-Lederer et al. (2012). Hap1 was

identified in 76 of the 87 samples sequenced from the Nyathi section, 23 of the 24

samples sequenced from the Addo section, and one sample collected outside the park.

The second haplotype (Hap2) was identified in eleven samples from the Nyathi section

and one sample from the Addo section.

In the Nyathi section, the samples identified as Hap2 included four samples from

B1, five samples from C4, one sample from A1a, and one unknown (Table 3). Of the

seven samples genotyped from C4, five were Hap2, whereas two were Hap1. B1, C4, and

A1a are all members of different maternal lineages in which siblings exhibited the Hap1

haplotype. For example, the only available sample from A1a was Hap2, whereas A1a's

mother, A1, sister, A1b, and two of A1b's calves were Hap1. In the C maternal lineage, C

and three of her offspring (C3, C2, and C5) were Hap1, whereas C4 had both Hap1 and

Hap2 samples. In the B maternal lineage, all available samples from B2 and B3 were

Hap1. Four samples from their sibling B1 were Hap2. Two of the three adult bulls in the

Nyathi section, Male1 and Male2, were also identified as Hap1. No samples were

available for Male3, the third adult bull. Of the 59 samples lacking assigned identities, only one was Hap2.

In the Addo section, 23 of the 24 samples were Hap1 (Table 4). These samples were from D1 and D2 in the D maternal lineage, E1 in the E maternal lineage, F1 and F in the F maternal lineage, and from two of the three adult bulls, Male5 and Male7. The only sample identified as Hap2 was from the third adult bull, Male6. All of the fifteen samples without assigned identities were Hap1.

The final alignment included 112 sequences with a sequence length of 491 bp. There were ten polymorphic sites between the two haplotypes identified in this study with an average pairwise distance of 3.4% ± 1.4% (Figure 3). There was an average pairwise distance between Hap2 and *D. b. michaeli* of 4.6% ± 1.1% and between Hap2 and *D. b. minor* of 1.1% ± 0.5%. There was an average pairwise distance between Hap1 and *D. b. minor* of 3% ± 1.24% and between Hap 1 and *D. b. michaeli* of 5.3% ± 1.7%.

To better characterize the relationship between Hap2 and other sequences in the final alignment, a maximum likelihood tree was constructed (Figure 4). The 100 Hap1 sequences clustered with the four *D. b. bicornis* sequences from Etosha National Park, Namibia identified by Anderson-Lederer et al. (2012). The twelve samples genotyped with Hap2 in this study clustered within the *D. b. minor* clade.

Seven distinct haplotypes were identified for *D. b. minor.* For *D. b. michaeli,* thirteen haplotypes were identified. The network analysis determined that the samples from this study genotyped with Hap2 were more similar to *D. b. minor* haplotypes, while Hap1 clustered with the four *D. b. bicornis* samples sequenced by Anderson-Lederer et al. (2012) (Figure 5).

DISCUSSION

Based on: (1) the moderate levels of genetic diversity identified in the source populations in Namibia of the *D. b. bicornis* at AENP (see: Karsten et al., 2011; Harley et al., 2005; de Groot et al., 2011; Anderson-Lederer, 2013) and (2) the far greater number of samples sequenced in this study than in a previous study that identified only one haplotype in four *D. b. bicornis* (Anderson-Lederer, Linklater & Ritchie, 2012), one would expect to find a moderate amount of genetic diversity in the *D. b. bicornis* at AENP. However, only a limited amount of genetic diversity was identified at the mtDNA D-loop locus. Unfortunately, the microsatellite analyses failed to produce quality or reliable results, leaving the two distinct mtDNA D-loop haplotypes as the only estimates of genetic diversity within this population.

Of the 112 fecal samples sequenced from the two subpopulations at AENP, twelve exhibited a previously uncharacterized haplotype, Hap2. Eleven of the Hap2 samples had assigned identities, whereas one was an "unknown" sample. In the Nyathi section, 76 of the 87 samples were Hap1 and eleven samples were Hap2. In the Addo subpopulation, only one sample of the 24 assayed was Hap2. The identification of only two mtDNA haplotypes in the Nyathi and Addo sections of AENP reflects lower levels of genetic diversity than expected.

This low level of genetic diversity may indicate that the AENP population is at risk of inbreeding. Inbreeding can increase the exposure of the population to deleterious alleles, thus potentially reducing fitness through many mechanisms (Frankham, Ballou & Briscoe, 2010). Inbreeding depression, the decline in reproductive fitness caused by inbreeding, poses a significant threat to many wild populations of threatened taxa such as black rhinoceros and may contribute to extinction risk (Brook et al., 2002).

Previous studies on the genetic diversity of *D. b. bicornis* have produced conflicting results that vary with molecular method. The two previous studies that assessed genetic diversity of *D. b. bicornis* using mtDNA identified low variation (O'Ryan, Flamand & Harley, 1994; Anderson-Lederer, Linklater & Ritchie, 2012). Restriction fragment length polymorphisms on mitochondrial DNA in five individuals from Namibia yielded only one haplotype (O'Ryan, Flamand & Harley, 1994). In four individuals from Etosha National Park, Namibia, a single mtDNA D-loop haplotype was identified using sequencing techniques (Anderson-Lederer, Linklater & Ritchie, 2012).

In contrast to the low genetic diversity in mtDNA found at AENP, moderate levels of genetic diversity were identified in other populations of *D. b. bicornis* using alternative molecular markers. Using allozymes, observed heterozygoties of 0.053 were reported in six individuals (Swart et al., 1994) and 21 individuals from Namibia (Swart & Ferguson, 1997). In 53 *D. b. bicornis* from Namibia and South Africa, an expected heterozygozity of 0.505 was determined using microsatellite markers (Harley et al., 2005). Similarly, microsatellite analyses of 144 individuals from Etosha National Park, Namibia exhibited a mean expected heterozygosity of 0.51 (de Groot et al., 2011).

An earlier study found a comparably low level of genetic diversity in *D. b. minor* from Kwa-Zulu-Natal (KZN) Province, South Africa (Anderson-Lederer, Linklater & Ritchie, 2012). Sequencing the mtDNA control region from 65 *D. b.* minor yielded only a single haplotype. In contrast, Anderson-Lederer et al. (2012) also identified high variation in mtDNA control region sequences in *D. b. minor* from Zimbabwe, with six distinct haplotypes from eleven individuals. The authors postulated two hypotheses for the fixed mtDNA control region haplotype in the KZN population: (1) demographic decline; and alternatively, (2) "long-term demographic separation, historically small population sizes and local adaptation." The contrasting hypotheses present a conundrum for conservation managers. Reversing demographic decline caused by fragmentation or other anthropogenic factors would involve introducing new haplotypes, whereas the same action could lead to outbreeding depression in stable populations with a fixed haplotype. The authors recommended additional genotyping to determine the best course of management for the KZN population (Anderson-Lederer, Linklater & Ritchie, 2012).

Therefore, if the low genetic diversity of the population at AENP is a result of demographic decline, introducing new haplotypes would be recommended to avoid inbreeding depression (Anderson-Lederer, Linklater & Ritchie, 2012). Alternatively, if the low genetic diversity at AENP was caused by local adaption and long-term separation of populations, the introduction of new haplotypes to the AENP population could lead to outbreeding depression (Anderson-Lederer, Linklater & Ritchie, 2012), resulting in reduced fitness through dilution of ecologically advantageous alleles (Frankham, Ballou & Briscoe, 2010). Because the population at AENP was established relatively recently in

the 1990s, it is more likely that the low genetic diversity in the subpopulations is due to demographic decline. Importantly, as the offspring within these subpopulations near sexual maturity, managers should consider strategies to avoid inbreeding including translocating animals between different sections of the park or exchanging rhinoceros with other parks that conserve *D. b. bicornis.*

Due to severe bottlenecking of the *D. b. bicornis* subspecies, significant genetic variation may have been lost (Emslie & Brooks, 1999). The slow rates of mutation to regenerate lost diversity require that every effort be made to identify and preserve current levels of genetic diversity, while preventing further losses (Frankham, Ballou & Briscoe, 2010). Thus, determining the best course of management for *D. b. bicornis* at AENP calls for further genetic testing in order to better estimate the genetic variation within this population. As efforts in this study failed to assess genetic diversity using microsatellite analysis on the fecal samples from AENP, using alternative samples such as tissue or blood samples with the microsatellite primers might be more successful and yield greater insights about the genetic health of this population.

The implications of Hap2
Sequencing identified two mtDNA haplotypes in the AENP population, and phylogenetic analyses were utilized to further characterize the relationship of the haplotypes to each other and to haplotypes from other subspecies. These analyses included calculating pairwise distances, constructing a maximum likelihood tree, and creating a maximum parsimony haplotype network. An average pairwise distance of 3.4% ± 1.4% between the two haplotypes was identified at AENP. Interestingly, this distance was smaller than the

pairwise distances between both haplotypes and *D. b. minor*. Additionally, Hap2

clustered within the *D. b. minor* sequences in both the maximum likelihood tree and the

maximum parsimony haplotype network (Figures 4 and 5).

There are multiple explanations for these results. First, there is a *D. b. minor*

individual that currently is undetected within the boundaries of AENP. Beginning in

1961, *D. b. michaeli* individuals were translocated to the park (Freeman et al., 2014).

However, remaining individuals were translocated to Tanzania as AENP prioritized the

management of the *D. b. bicornis* subspecies (Emslie & Brooks, 1999). A single

reference that reported the presence of *D. b. minor* rhinoceros at AENP was published

over three decades ago (de Vos & Braack, 1980). Additionally, Hap2 samples were found

in both the Nyathi and Addo sections of the park, which are isolated from each other.

Therefore, it is extremely unlikely that there is an undetected rhino of a different

subspecies within AENP.

An alternative explanation is that the Hap2 samples are indicative of a past

hybridization event between the two subspecies *D. b. minor* and *D. b. bicornis*. As

subspecies designations were based on ecological and geographic parameters rather than

genetic, taxonomical data (du Toit, 2006), hybridization is a possibility. In fact, Swart

and Ferguson (1997) suggested that *D. b. bicornis* and *D. b. minor* likely originated from

a single, ancestral population and should be considered part of a "genetic continuum"

rather than distinct subspecies.

To further explore this hypothesis, it is prudent to review the taxonomic history

and classification of black rhino subspecies as discussed by Rookmaaker (2011) and

summarized here. In the mid 1900's the first extensive study of black rhinoceros taxonomy reported seventeen different subspecies distinguished by skull morphological characters (Zukowsky, 1965). Just two years later, another study described seven distinct subspecies of black rhino (Groves, 1967). Rookmaaker and Groves (1978) published a detailed taxonomic review of *D. b. bicornis* and concluded that the subspecies went extinct around 1850. The paper suggested that even though the ranges of *D. b. bicornis* and *D. b. minor* were in close proximity to each other, *D. b. chobiensis* was more closely related to *D. b. bicornis* (Rookmaaker & Groves, 1978). However, the authors postulated that interbreeding between *D. b. bicornis* and *D. b. minor* likely occurred due to the proximity of the ranges.

Although the published data supported at least six extant subspecies of black rhinoceros, the proceedings of the African Rhino Workshop held in 1986 in Cincinnati, OH announced that conservation efforts for black rhinoceros would focus on four conservation units that were based on ecological niches:

(I)   "the southwestern populations in Namibia;

(II)   the southern-central populations extending from Natal through Zimbabwe and Zambia into southern Tanzania;

(III)   the eastern populations in Kenya and northern Tanzania;

(IV)   the north-western populations extending from the horn of Africa to Central African Republic and Cameroun (Anonymous, 1987)."

These ecotype designations persist, and conservation plans have since presented these ecotype designations as subspecies with the southwestern ecotype known as *D. b.*

bicornis, the south-central known as *D. b. minor*, the eastern ecotype known as *D. b. michaeli*, and the western ecotype known as *D. b. longipes* (Emslie & Brooks, 1999).

A decade later, molecular studies shed more light on the complicated relationship of these subspecies ultimately challenging the rationale for the four units of conservation for the black rhinoceros species. Based on their findings of low levels of mtDNA diversity between the subspecies, O'Ryan, Flamand, and Harley (1994) concluded that it would be unlikely that interbreeding between the subspecies would lead to outbreeding depression or have significant fitness impacts. The authors questioned the subspecies designations that were based on ecotypes and suggested that the conservation units within the black rhinoceros species should be reexamined (O'Ryan, Flamand & Harley, 1994).

In 2005, a study examined the population differentiation between 121 individuals of the three extant subspecies using nine microsatellite markers (Harley et al., 2005). The authors reported that based on the moderate level of population differentiation between the subspecies, interbreeding would be unlikely to lead to outbreeding depression. They cautioned that if current effective population sizes or levels of diversity within the subspecies begin to fall, managers should consider interbreeding as a means to prevent the significant loss of genetic diversity (Harley et al., 2005).

More recently, Groves revisited the controversy (Groves & Grubb, 2011). After reviewing the current literature, Groves and Grubb presented eight subspecies classifications: *D. b. longipes, D. b. brucii, D. b. ladoensis, D. b. minor, D. b. michaeli, D. b. bicornis, D. b. chobiensis,* and *D. b occidentalis.* The authors emphasized that the *D. b. bicornis* subspecies went extinct years ago, and lamented the current use of the

subspecies name. The rhino from Namibia that are currently classified as *D. b. bicornis* would, under their classification scheme, be known as *D. b. occidentalis* (Groves & Grubb, 2011).

The implications of the discrepancy between taxonomic classifications and subspecies designations based on ecotypes are profound (Rookmaaker, 2011). Current management strategies and the allocation of limited resources are entirely based on the separate management of the ecotype subspecies (Emslie & Brooks, 1999), which ultimately may lead to the loss of precious genetic diversity in the black rhinoceros species (Rookmaaker, 2011). Rookmaaker (2011) adamantly advocated for the immediate adoption of the subspecies designations described by Groves and Grubb (2011). He acknowledged that readdressing subspecies classifications in black rhinoceros would lead to significant challenges, but he remained optimistic about the capacity for stakeholders to come together with the shared aim of preserving the genetic diversity of all of the subspecies of this iconic animal (Rookmaaker, 2011).

This controversy highlights the complications of interpreting the presence of Hap2 in the AENP population. The fact that this haplotype clusters more closely with haplotypes from *D. b. minor* than with *D. b. bicornis* raises questions about the origin of this haplotype. Perhaps Hap2 is indeed a *D. b. bicornis* haplotype or, alternatively, it could be evidence of a past hybridization event between the two ecotypes. In either case, this finding requires further genetic investigation with microsatellite analysis and additional sequencing to identify individuals with the novel haplotype, to characterize the origin of Hap2, and to assess the implications.

Sample misidentification

The distribution of the Hap2 samples within the subpopulations conflicts with the expectation that all members of a single maternal lineage would exhibit a single haplotype. In the Nyathi section, the eleven samples that were identified as Hap2 came from rhino in three different maternal lineages in addition to one unknown sample. The Hap2 rhino, B1, A1a, and C4, all have siblings or mothers identified as Hap1. For example, in the B maternal lineage, B1 exhibited Hap2 while two of B1's siblings, B2 and B3, were Hap1. Discordant haplotype patterns were not just identified within maternal lineages, but also within one individual. Of the seven samples genotyped for C4, five were Hap2 and two were Hap1. As mitochondrial sequences are inherited from mother to offspring with little recombination (Frankham, Ballou & Briscoe, 2010), multiple mtDNA haplotypes within a maternal lineage and within an individual are highly improbable, so these discordant patterns are likely due to sample misidentification.

During sample collection in 2010, identities were assigned to fecal samples based on visual confirmation in the field or the analysis of camera trap photos. For samples with assigned identities that were questionable, camera trap photos were reevaluated when available. These photos highlighted the challenges of assigning identities to fecal samples collected at camera traps. As the camera traps were set up near areas of high rhino traffic, multiple rhino may have potentially defecated in the same area. Additionally, some of the images were grainy due to low light levels, making it difficult to accurately identify rhino based on ear notch patterns and other unique physical characteristics.

This result reiterates the importance of developing a reliable method of identifying samples from specific individuals – a process critical to ensuring accuracy

when collecting samples for genetic or hormonal studies and general monitoring of the subpopulations for demographic changes including births and deaths. Camera trap photos are a valuable resource for collection of noninvasive samples or behavioral data, because they allow for reexamination if identification errors are suspected as they were in this study.

Summary
Low genetic diversity at the mtDNA D-loop locus was identified in two subpopulations of *D. b. bicornis* at Addo Elephant National Park, South Africa. This finding may indicate that the population is at risk of inbreeding. Further genetic testing using other markers such as microsatellites will yield a more comprehensive picture of the genetic variation of this population. Based on these results, it is recommended that managers consider strategies to avoid inbreeding within the subpopulations by translocating animals between different sections of the park or exchanging adult males with other parks that conserve *D. b. bicornis*.

The identification of a mtDNA D-loop haplotype, Hap2, that clustered more closely with sequences from *D. b. minor* warrants further study to characterize the origin of this haplotype. This finding could be evidence of a past hybridization event between the two subspecies. More in depth genetic analysis of the two haplotypes would aid managers in interpreting the presence of Hap2 at AENP. In light of the black rhinoceros subspecies designation controversy, it is also recommended to explore subspecies boundaries within this species by comparing sequences of protein coding genes and non-

coding regions of nuclear DNA of Hap1 and Hap2 samples from AENP with sequences from the other subspecies, *D. b. minor* and *D. b. michaeli.*

This study produced a novel set of primers designed to more specifically and effectively amplify the polymorphic region between the two haplotypes identified in the AENP population. As these primers had a higher success rate in the samples collected at AENP, managers in the park could utilize this new tool in the future, the second goal of this study.

At AENP, there are banked blood samples and ear notch tissue from most of the adult rhinoceros in the population. Based on the results of this study, it is recommended that these blood and tissue samples be used to establish a genetic database for the subpopulations in the park. DNA should be extracted from these samples, sequenced at the mitochondrial DNA D-loop locus and fingerprinted with microsatellite markers described in this study. This comprehensive genetic database would allow researchers to assign identities to the fecal samples collected for this study and for future studies with confidence. As recommended by Emslie and Brooks (1999), the database could also be compiled with genetic data from other parks to inform translocation decisions and management strategies for the *D. b. bicornis* metapopulation.

The analysis of the blood and tissue samples with microsatellite markers would also further characterize the genetic diversity in the two subpopulations and the origin of the novel haplotype, Hap2. Additionally, these data could be used to explore the mating strategies within each subpopulation, identify the fathers of new calves, and investigate whether there is a diversity in reproductive success among males (see (Garnier, Bruford

& Goossens, 2001), which could provide critical information to SANParks managers for

future translocation and management decisions in order to maximize productivity and

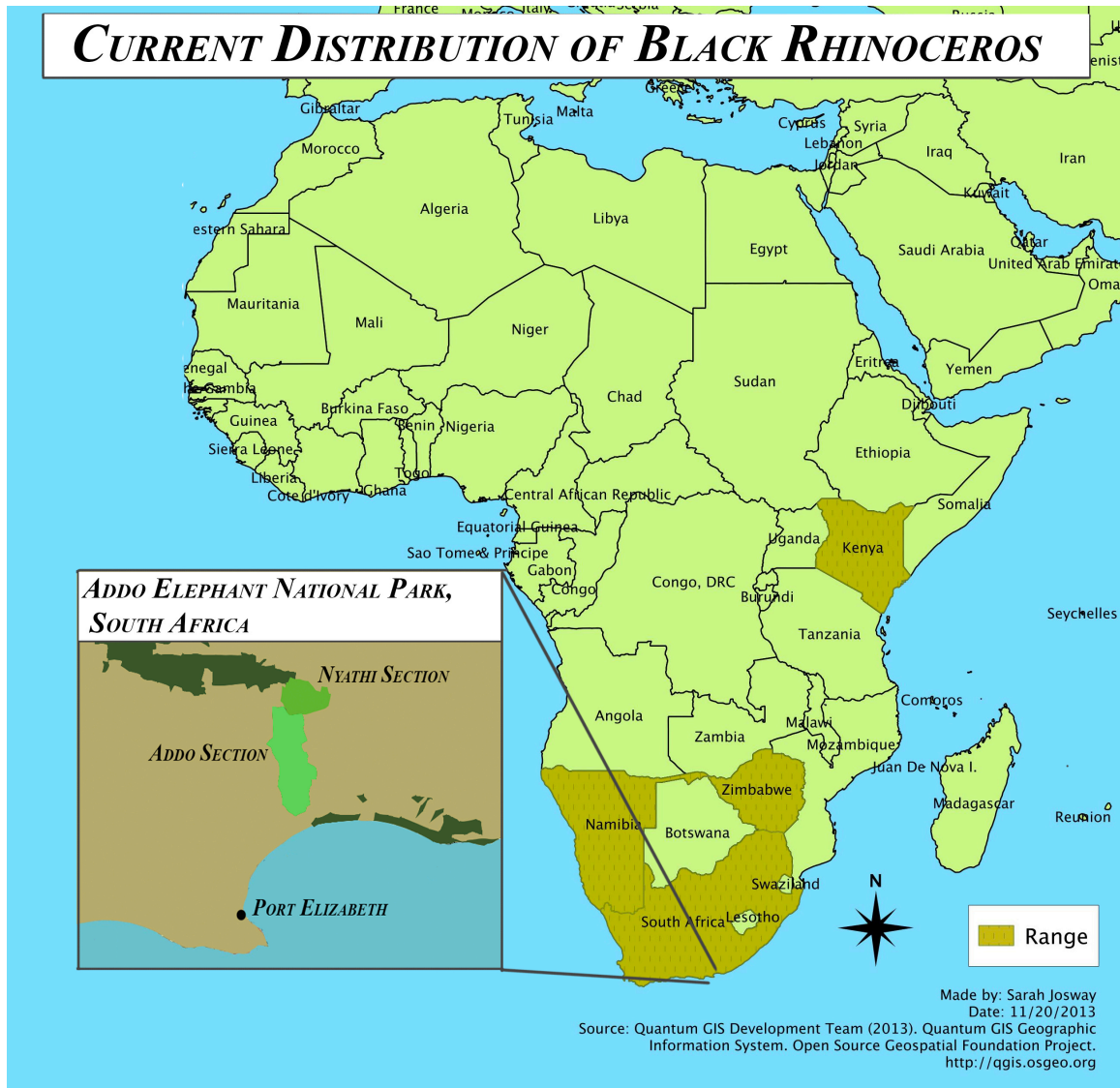health of the *D. b. bicornis* at AENP.

Figure 1. Distribution map of black rhinoceros.
The current range of black rhinoceros spans South Africa, Kenya, Zimbabwe, and Namibia (Emslie, 2012). Inset shows Addo Elephant National Park, South Africa (SANParks, 2014).

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Figure 2. Camera trap photo of a subadult with ear notches (right) and calf (left).

Figure 3. Alignment of two AENP mtDNA D-loop haplotypes.
This alignment shows the ten polymorphic sites between the previously identified
mtDNA haplotype (Hap1) and the novel haplotype (Hap2). The top sequence in each row
is the consensus sequence. The alignment was made in Geneious (Drummond et al.,
2011).
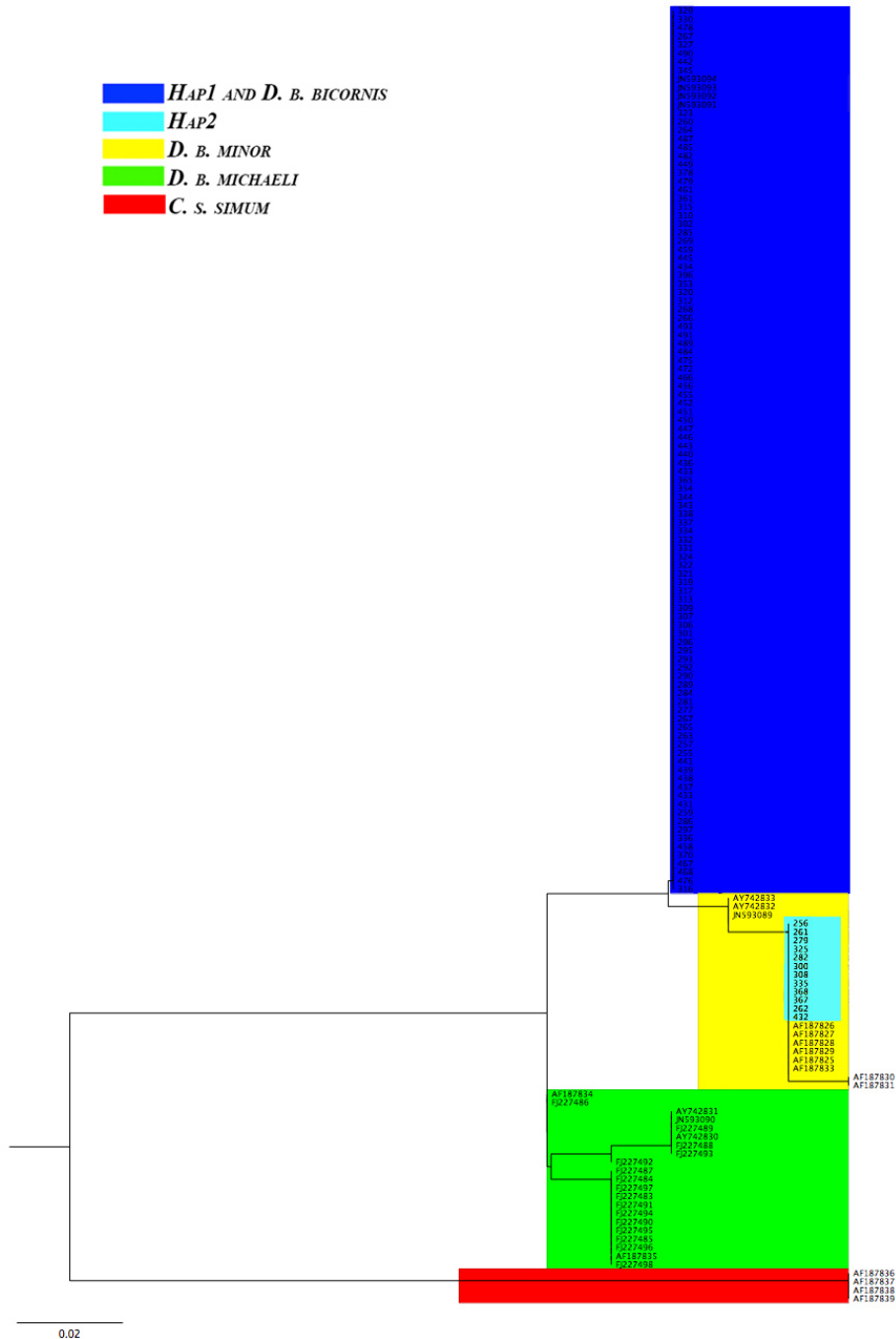
Figure 4. Maximum likelihood tree of African rhinoceros species.
Sequences from *C. s. simum* were used as an outgroup. Accession numbers are shown for
all sequences downloaded from NCBI. Samples from this study are designated with the
sample number. This tree was made using maximum likelihood with bootstrap support in
MEGA (Tamura et al., 2011) and edited in FigTree (Rambaut, 2012)
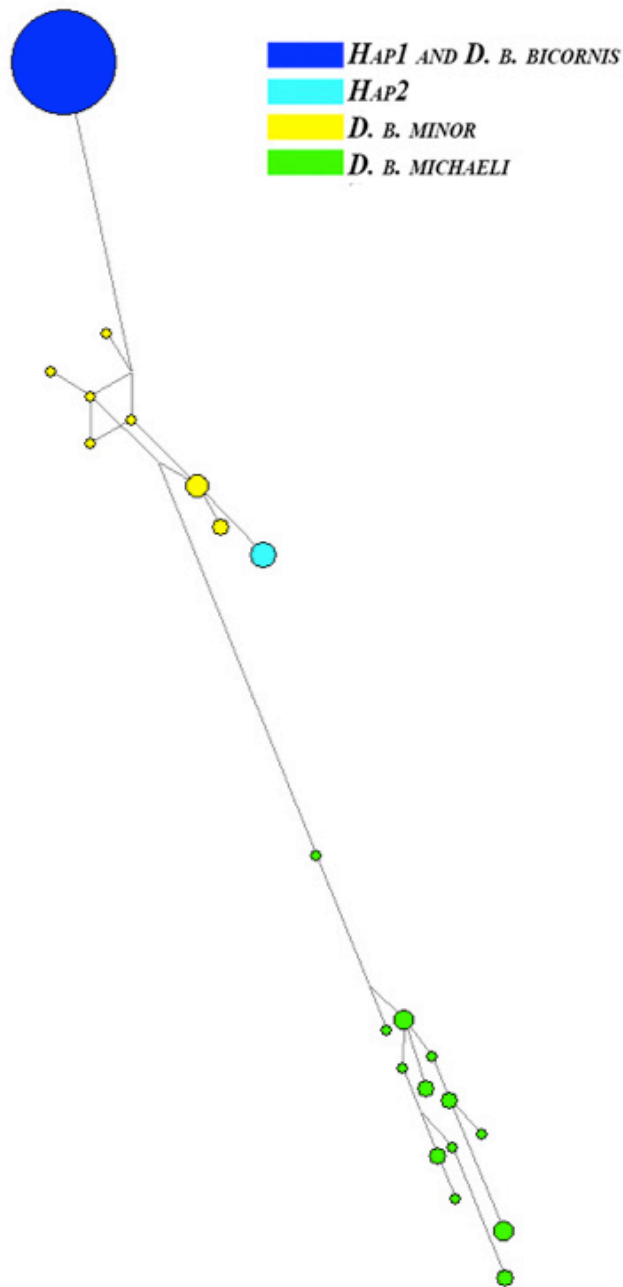
Figure 5. Statistical maximum parsimony haplotype network for black rhinoceros subspecies.
The Hap2 haplotype clustered with the *D. b. minor* haplotypes. In comparison to *D. b. minor* and *D. b. michaeli*, the *D. b. bicornis* subspecies showed limited genetic diversity at the mtDNA D-loop locus. This haplotype network was made in Network (Bandelt, Forster & Röhl, 1999).

Table 1. Studies examining genetic diversity within *D. b. bicornis*.

Adapted from (Anderson-Lederer, 2013).

Reference	Molecular Marker	Sites/loci	N	Sample Origin	~He	~Ho	mtDNA Haplotypes
(Swart et al., 1994)	Allozyme (plasma and red blood cells)	30 (6 polymorphic)	6	Namibia	0.013	0.053	-
(Swart & Ferguson, 1997)	Allozyme (plasma and red blood cells)	30 (6 polymorphic)	21	Namibia	-	0.053	-
(O'Ryan, Flamand & Harley, 1994)	RFLP (mtDNA)	16 (3 polymorphic)	5	Namibia	-	-	1
(Brown & Houlden, 1999)	Microsatellites	11	7	Unknown	0.686	-	-
(Cunningham, Harley & O'Ryan, 1999)	Microsatellites	5	72	Unknown	0.580	-	-
(Harley et al., 2005)	Microsatellites	9	53	Namibia	0.505	0.523	-
(Karsten et al., 2011)	Microsatellites	10	4	Namibia	0.43	0.46	-
(de Groot et al., 2011)	Microsatellite	9	144	Namibia	0.51	0.52	-
(Anderson-Lederer, Linklater & Ritchie, 2012)	mtDNA D-loop sequence		4	Namibia			1
(Anderson-Lederer, 2013)	Microsatellites	10	4	Namibia	0.52	-	-

Table 2. Microsatellite primers used in this study.

Locus	Repeat Motif	Primer Name	Sequence	Size range (bp)	Alleles	Reference
AF129734	$(ca)_{12}$	AF129734-F	ATCTTCCTCAGCA ATAAGG	237–251	2	(Nielsen et al., 2008)
		AF129734-R	ATCATCAGAGTTT CCAGTTC			
AY606078	$(gt)_{13}gca(tg)_3$	AY606078-F	GATCAGTAACACC AAAGTCC	237–243	2	(Nielsen et al., 2008)
		AY606078-R	AGTGAAGACAGAA GGATCAC			
BlRh2B	$(CA)_{19}$	BlRh2B-F	CCCTTTTCTCCCTT TATCTAG	239–253	8	
		BlRh2B-R	ATACTGTGAAATC CTGTTCC			(de Groot et al., 2011)
BlRh37D	$(TG)_6(AG)_{17}$	BlRh37-F	CCACTCAGAATGA GAAATGG	163–165	3	
		BlRh37D-R	TCTCCCTACTTAAT CCCACC			(de Groot et al., 2011)
BR4	$(CA)_{19}$	BR4-F	CCCCTAAATTCTA GGAACAC	143–147	6	(Cunningham, Harley & O'Ryan, 1999)
		BR4-R	CCAAAGACCACCA GTAATTC			
BR6	$(CA)_{15}$	BR6-F	TCATTTCTTTGTTC CCCATAGCAC	134–156	4	(Cunningham, Harley & O'Ryan, 1999)
		BR6-R	AGCAATATCCACG ATATGTGAAGG			
BR17	$(AT)_6(GT)_{18}$	BR17-F	ACTAGCCCTCCTTT CATCAG	123–133	2	(Cunningham, Harley & O'Ryan, 1999)
		BR17-R	GCATATTGTAAGT GCCCCAG			
DB01	$(CA)_{14}$	DB01-F	AGATAATAATAGG ACCCTGCTCCC	120–130	4	
		DB01-R	GAGGGTTTATTGT GAATGAGGC			(Brown & Houlden, 1999)
DB44	$(CA)_4g(CA)_{16}$	DB44-F	GGTGGAATGTCAA GTAGCGG	174–178	4	
		DB44-R	CTTGTTGCCCCATC CCTG			(Brown & Houlden, 1999)
DB66	$(CA)_7ta(CA)_{16}$	DB66-F	CCAGGTGAAGGGT CTTATTATTAGC	204–224	8	
		DB66-R	GGATTGGCATGGA TGTTACC			(Brown & Houlden, 1999)

Table 3. Nyathi subpopulation.

	Name	F1	F2	F3	Sex	Birth - Death	Samples Genotyped/Total Samples	Haplotype
*	A				F	1981-2006		
		A1			F	1991	1/1	Hap1
			A1a		M	2004	1/1	Hap2
			A1b		F	1998	3/3	Hap1
				A1b1	F	2006	4/9	Hap1
				A1b2	F	2008	1/1	Hap1
			A1c		F	2008	1/1	Hap1
*	B				F	1993		
		B1			M	2000	4/9	Hap2
		B2			M	2004	4/4	Hap1
		B3			M	2006	2/2	Hap1
*	C				F	1991	3//11	Hap1
		C1			F	1998		
			C1a		F	2007	1/1	Hap1
		C2			M	2000	1/1	Hap1
		C3			F	2002	1/1	Hap1
		C4			M	2005	7/8	Hap1(2samples) Hap2(5samples)
		C5			M	2008	3/7	Hap1
*	**Male1**				M	1992	4/10	Hap1
*	**Male2**				M	1994	3/11	Hap1
	Male3				M	1981		
	Adult						1/1	Hap1
	Subadult						3/3	Hap1
	Calf						3/4	Hap1
	Young						3/3	Hap1
	Unknown						33/48	Hap1(32samples) Hap2(1sample)
	Total						87/144	Hap1(76samples) Hap2(11samples)

represented maternal lineage

Table 4. Addo subpopulation.

	Name	F1	F2	Sex	Birth	Genotyped Samples/ Total Samples	Haplotype
*	D			F			
		D1		M	2003	3/4	Hap1
		D2		F	2005	1/1	Hap1
*	E			F			
		E1		M	2008	1/1	Hap1
*	F			F		1/1	Hap1
		F1		F	2002	4/4	Hap1
	Male4			M	Died 2010		
*	Male5			M		1/1	Hap1
*	Male6			M		1/1	Hap2
*	Male7			M	2003	1/1 (labeled as MR/LR/LL)	Hap1
	Adult					0/1	
	Subaadult					1/1	Hap1
	Calf					1/1	Hap1
	Unknown					9/12	Hap1
	Total					24/32	Hap1(23samples) Hap2(1sample)

* *represented maternal lineage*

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