

CONSERVATION AND POPULATION GENETICS OF AFRICAN AND ASIAN
RHINOCEROS

BY

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DISSERTATION

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ABSTRACT

There are five living species of rhinoceros inhabiting Africa and Asia: black rhinoceros (*Diceros bicornis*), white rhinoceros (*Ceratotherium simum*), Indian rhinoceros (*Rhinoceros unicornis*), Javan rhinoceros (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*). Anthropogenic activities, such as poaching and habitat disruption, have led to steep declines in the population size of all rhinoceros species, placing them in danger of extinction. The development of genetic markers for assessment of diversity at neutral and adaptive loci can be used to address a number of questions that will aid in the conservation of rhinoceros populations both *ex situ* and in the wild. In order to evaluate genetic diversity in rhinoceros populations, I investigated three research questions that will contribute substantially to the conservation and management of rhinoceros species.

(1) Accurate estimates of population size are often difficult to obtain for rhinoceros species that are elusive or prefer dense habitat. Knowing the precise number of individuals in an area is essential for managers to develop and implement conservation plans that address the issues facing a particular population. To enable the use of molecular methods for censusing of rhinoceros populations 29 novel Sumatran rhinoceros microsatellites and 17 novel black rhinoceros microsatellites were characterized from next generation sequencing data for use with low quality DNA extracted from non-invasively collected fecal samples. A subset of these markers is sufficient for identification of individuals based on P_{ID} and $P_{ID(sib)}$ values. Through a series of optimization steps I was able to show that these markers can be successfully used to obtain genotypes from fecal samples. These markers are of particularly importance for Sumatran rhinoceros populations since the reported number of individual has been difficult to accurately estimate and drastically overstated. Studies aimed at implementing these markers for estimating census size in wild rhinoceros populations are ongoing.

(2) The Sumatran rhinoceros, once widespread across Southeast Asia, now consists of ca. 100 individuals primarily found in three isolated populations on the island of Sumatra. No studies have examined the population genetic structure of Sumatran rhinoceros using techniques beyond mitochondrial restriction mapping analysis. Given the requirement for substantial management of the remaining Sumatran rhino populations in the wild and in *ex situ* breeding facilities, more information regarding their genetic status needs to be available. I used mitochondrial DNA sequences from modern and archival museum samples to assess genetic

diversity and structure. Among all samples, haplotype diversity was high; samples identified as being members of the subspecies *D. s. sumatrensis* formed a cluster containing ten haplotypes. The number of haplotypes and the haplotype diversity among the museum samples of *D. s. sumatrensis* were higher than in the modern samples even after rarefaction, suggesting that genetic diversity has been lost as the population has declined. Microsatellite data from the modern samples indicated low diversity and showed the presence of three distinct genetic clusters associated with geographic barriers to gene flow within the modern population. Continual isolation of the extant populations without management intervention will likely result in further loss of genetic diversity.

(3) Adaptive loci within the immune system possess crucial information about the ability of a population to resist infectious pathogens. Toll-like receptors (TLR) bind pathogen-specific molecules and initiate both innate and adaptive immune responses, and thus may be of particular relevance to conservation geneticists and management authorities. I sequenced gene regions coding for the extracellular domain of eight TLR loci in eastern black (*D. b. michaeli*), south-central black (*D. b. minor*), and southern white (*C. s. simum*) rhinos from North American zoos and *ex situ* breeding facilities. Additionally, mitochondrial control region haplotypes were sequenced for all individuals and multi-locus genotypes were obtained for the black rhinos. Overall, diversity was very low at TLR and mitochondrial loci among white rhinos. Black rhinos exhibited higher levels of diversity at the TLR loci than white rhinos. Between subspecies, the south-central black rhino was less diverse than the eastern black rhino at the TLR genes; however, they share some haplotypes at all TLR loci. Mitochondrial haplotypes and microsatellite genotypes support strong differentiation between the two studied subspecies. Unique TLR haplotypes and differentiation at mitochondrial and microsatellite loci between the black rhinoceros subspecies were identified, supporting the continued management of the taxa as two separate conservation units. Limited variation in the TLR genes of the African rhinos, especially the white rhinoceros, suggests that the evolutionary potential of the immune system is limited. Future management efforts and breeding programs for rhinoceros species should seek to preserve immune system diversity.

“Don’t be too timid and squeamish about your actions. All life is an experiment. The more experiments you make the better.” – Ralph Waldo Emerson

For the future.

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION and LITERATURE REVIEW	1
White rhinoceros (<i>Ceratotherium simum</i>)	1
Black rhinoceros (<i>Diceros bicornis</i>).....	5
Indian rhinoceros (<i>Rhinoceros unicornis</i>)	8
Javan rhinoceros (<i>Rhinoceros sondaicus</i>).....	9
Sumatran rhinoceros (<i>Dicerorhinus sumatrensis</i>)	11
Rhinoceros phylogenetics	12
Research objectives.....	13
Figures.....	15
CHAPTER 2: DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS FROM NEXT GENERATION SEQUENCING FOR UTILIZATION IN LOW QUALITY DNA FROM DUNG.....	25
Abstract	25
Introduction.....	26
Methods and Materials.....	29
Samples	29
Black rhinoceros marker design	30
Sumatran rhinoceros marker design	30
Microsatellite molecular characterization	32
Optimization for amplification in fecal samples	33
Fecal genotyping error rate analysis.....	36
Results.....	37
Black rhino marker design and characterization	37
Sumatran rhino marker design and characterization	38
Optimization for fecal analysis.....	39
Fecal genotyping error rate.....	41
Discussion.....	43
Tables.....	48
CHAPTER 3: GENETIC STRUCTURING AND REDUCED DIVERSITY OF SUMATRAN RHINOCEROS (<i>DICERORHINUS SUMATRENSIS</i>)	63
Abstract	63
Introduction.....	64
Methods and Materials.....	67
Samples	67
Sample preparation and DNA extraction	68
PCR amplification	68
Mitochondrial control region sequencing and analysis	69
Microsatellite genotyping and analysis	70
Results.....	71
Mitochondrial control region analysis.....	71
Microsatellite analysis	72
Discussion.....	73

Tables and Figures	77
CHAPTER 4: TOLL-LIKE RECEPTOR DIVERSITY IN AFRICAN RHINOCEROS SPECIES	
.....	89
Abstract	89
Introduction.....	90
Methods and Materials.....	93
Samples	93
TLR primer design	93
TLR and mitochondrial DNA amplification and sequencing	94
TLR analysis.....	94
Mitochondrial control region analysis.....	96
Microsatellites	96
Results.....	97
TLRs	97
Mitochondrial control region.....	99
Microsatellites	100
Discussion	100
Tables and Figures	104
LITERATURE CITED	119
Appendix A. Black rhino microsatellite genotype data used for assessment of variability	139
Appendix B. Sumatran rhino microsatellite genotype data used for assessment of variability..	140
Appendix C. Microsatellite genotypes of 13 modern Sumatran rhinoceros individuals	141
Appendix D. Mitochondrial control region sequence alignment for modern and museum	
Sumatran rhinoceros samples	142
Appendix E. Mitochondrial control region sequence alignment for two subspecies of black	
rhinoceros	145
Appendix F. Mitochondrial control region sequence alignment for white rhinoceros	148
Appendix G. Microsatellite genotypes for two subspecies of black rhinoceros.....	151
Appendix H. Inferred TLR1 amino acid sequence for white and black rhinoceros	152
Appendix I. Inferred TLR2 amino acid sequence for white and black rhinoceros.....	156
Appendix J. Inferred TLR3 amino acid sequence for white and black rhinoceros.....	160
Appendix K. Inferred TLR4 amino acid sequence for white and black rhinoceros	164
Appendix L. Inferred TLR5 amino acid sequence for white and black rhinoceros.....	168
Appendix M. Inferred TLR6 amino acid sequence for white and black rhinoceros.....	172
Appendix N. Inferred TLR7 amino acid sequence for white and black rhinoceros	176
Appendix O. Inferred TLR10 amino acid sequence for white and black rhinoceros	181

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

All extant members of the family Rhinocerotidae are charismatic megafauna that have experienced range wide population declines and are targeted species for management through wildlife conservation programs. There are five living species of rhinoceros: black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) in Africa; and Indian rhinoceros (*Rhinoceros unicornis*), Javan rhinoceros (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) in Asia. High levels of poaching and habitat destruction have been the cause of considerable population declines in all rhinoceros species, resulting in the persistence of only remnant populations that are often small and fragmented with subsequently restricted gene flow (Linklater 2003; Scott 2008; Guerier et al. 2012). All rhinoceros taxa are considered to be in danger of extinction (Scott 2008; CITES 2010), with the exception of the southern white rhinoceros subspecies (*C. s. simum*), which is listed as threatened under the Endangered Species Act and included in Appendix II of the Convention on International Trade in Endangered Species (CITES) as a taxon in need of regulated trade. Such decreases in population size can greatly reduce the amount of genetic diversity present in a species and negatively affect long-term population survival (Frankham 1996; van Coeverden de Groot et al. 2011). Molecular tools can be implemented in the conservation management of these species by providing information on genetic diversity and population structure within and among populations; estimating total and effective population sizes; assessing population viability; elucidating historical and contemporary gene flow patterns; determining mating systems; and identifying unique evolutionary lineages (DeYoung & Honeycutt 2005).

White rhinoceros (*Ceratotherium simum*)

The white rhinoceros (*Ceratotherium simum*), currently the most numerous rhinoceros species, has two designated subspecies, the northern white rhino (*C. s. cottoni*) and the southern white rhino (*C. s. simum*). Conflicting evidence on the level of divergence between the subspecies has left their taxonomic standing in question. Limited variation between the white rhinoceros subspecies was reported by Merenlender and others (1989) based on analysis of allozymes, leading them to suggest that the white rhino subspecies may represent populations

from two extremes of a previously contiguous range. However, classification as distinct subspecies has been supported by two mitochondrial restriction map studies showing divergences of 4% and 1.4% between northern and southern white rhinos compared to differences of 7% and 4.5%, respectively, between black and white rhinos (George et al. 1983; George et al. 1993). Morales and Melnick (1994) found additional support for the subspecies designation, suggesting that the two populations had diverged into evolutionarily independent lineages approximately one million years ago. A recent analysis of mitochondrial (D-loop, 12S, and NADH) and nuclear (*amelogenin*) loci with morphological data indicated substantial divergence (0.75 to 1.4 million years ago) between the subspecies, leading to the suggestion that they should be reclassified as separate species (Groves et al. 2010). Current whole genome analysis is being undertaken to resolve their taxonomic standing and provide a wealth of much needed genetic information for these taxa (Ryder et al. 2015). As of now, management efforts continue to treat the two populations as separate and evolutionarily unique subspecies of *Ceratotherium simum*.

Only three individuals of the critically endangered northern white rhinoceros subspecies (*C. s. cottoni*) remain. The northern white rhino historically occurred in parts of Central African Republic, Chad, South Sudan, and Uganda (Hillman-Smith et al. 1986; Emslie & Brooks 1999) (Figure 1.1). The three remaining individuals consist of an older male and two females that are believed to be unable to reproduce naturally; they currently reside in Ol Pejeta Conservancy in Kenya where they are under constant armed protection (Emslie et al. 2013). Efforts to save this subspecies are now looking toward cryopreservation of living cells and artificial reproductive techniques (Ryder et al. 2015). The southern white rhino subspecies was numerous and widespread in the 1800s, distributed mainly south of the Zambezi River across present-day South-eastern Angola, Botswana, Mozambique, Eastern Namibia, South Africa, Swaziland, South-western Zambia, and Zimbabwe (Cumming et al. 1990; Emslie & Brooks 1999) (Figure 1). By the late 1800s the southern white rhinoceros was thought to be extinct as a result of overhunting and habitat loss. However, a small remnant population of between 20 and 100 individuals, from which the current population originated, was discovered in what is today the KwaZulu-Natal province of South Africa (Groves 1972). Protection and conservation efforts allowed for rapid recovery to a current estimated population size of more than 20,000 (Emslie et al. 2013; Labuschagne et al. 2013), with South Africa containing more than 90% of the

individuals. Smaller populations founded through reintroductions are present in former range states of: Botswana, Mozambique, Namibia, Swaziland, Zambia, and Zimbabwe, and also in countries outside of the historic range: Kenya and Uganda (Emslie & Brooks 1999; Emslie et al. 2013). A recent increase in poaching has put many white rhinoceros populations at risk (Emslie et al. 2013; Harper et al. 2013), especially those that are small and isolated.

Despite the successful recovery of the of southern white rhinoceros, populations are still limited in size and lack gene flow with each other; thus, maintenance of genetic diversity is a primary goal of conservation efforts (Frankham 2005). Two published papers aimed to develop polymorphic microsatellites for the white rhinoceros: Florescu and others (2003) identified 5 polymorphic microsatellite loci with 2-3 alleles per locus, and Hou and colleagues (2012) identified 27 loci for the white rhino of which none were polymorphic. The high number of monomorphic loci and the low number of alleles per variable locus noted by both Florescu et al. (2003) and Hou et al. (2012) indicates that white rhinoceros are characterized by low genetic diversity. Nielsen and colleagues (2008) redesigned primers for the 5 previously published white rhino microsatellite markers (Florescu et al. 2003), in addition to 16 loci characterized in black rhinoceros. Of the 21 redesigned markers 16 were polymorphic in white rhinos ($H_O = 0.436$) and allele size ranges were non-overlapping for black and white rhinos at seven loci, allowing for differentiation between the African species. Due to the dearth of microsatellite loci characterized in white rhinos many studies have relied on a panel of markers originally developed in black, Indian, and Sumatran rhinos (Scott 2008; Coutts 2009; Guerier et al. 2012; Harper et al. 2013), although heterozygosity tends to be considerably lower when heterospecific microsatellite loci are used (Scott 2008).

Studies on southern white rhinoceros populations examining allozymes, microsatellites, mitochondrial DNA, and the major histocompatibility complex have all reported low levels of genetic diversity (Merenlender et al. 1989; O'Ryan & Harley 1993; Scott 2008; Coutts 2009; Guerier et al. 2012). A combination of molecular loci was used to assess whether populations seeded through translocations exhibit reduced genetic diversity in comparison to their source population. Individuals from the original source population of Hluhluwe-iMfolozi National Park in South Africa and three seeded populations were analyzed; an average observed heterozygosity of 0.44 (ranging from 0.39 to 0.46), limited mtDNA control region haplotype diversity, and functional monomorphism at the MHC loci indicated low overall genetic diversity (Coutts 2009).

Coutts (2009) concludes that there is no evidence of reduced diversity between recently translocated populations and the source population; however, the presence of differentiation between the seeded populations is likely a function of genetic drift and lack of gene flow. Small populations, as exemplified by the white rhinoceros, are particularly susceptible to loss of genetic diversity at adaptive and neutral loci through drift in the absence of gene flow (Alcaide & Edwards 2011; Guerier et al. 2012). Similarly low levels of heterozygosity ($H_O = 0.393$ and $H_O = 0.342$, respectively) have been estimated in studies characterizing diversity by using suites of microsatellite loci designed across species (Scott 2008; Harper et al. 2013). In addition, Guerier and colleagues (2012) used behavioral observations coupled with genotypes from a panel of microsatellite markers to estimate local diversity ($H_O = 0.46$), assign parentage, and create a pedigree to aid in management and conservation of the white rhinoceros population on the Ongava Game Reserve, Namibia. Several studies have found that southern white rhinoceros populations do not show genetic signatures of recent population bottlenecks; thus, historic levels of genetic diversity were likely low prior to recent bottlenecks (Scott 2008; Coutts 2009). Low levels of variability present at microsatellite and mitochondrial markers suggest that white rhinos may be genetically depauperate genome wide.

Other studies have aimed to characterize genetic markers and develop methods for forensic purposes (e.g., assignment of white rhinoceros products to populations of origin, or individual identification). Peppin et al. (2010) characterized markers for the co-amplification of a zinc finger (ZF) protein intron in both X and Y chromosomes that differ in size by 7 base pair in African rhinos and allow for genetic sexing of various specimen types. Harper and others (2013) developed an extraction method to obtain DNA of sufficient quality and quantity from rhinoceros horn for microsatellite genotyping. Individual DNA profiles were then generated using 22 previously published microsatellite loci designed across rhinoceros species, as well as a marker for sex determination (Peppin et al. 2010). These loci proved capable of assigning individual identity to specimens and were tested on paired blood and horn or hair samples (Harper et al. 2013). In an effort to provide additional markers for identification of the region from which white rhinoceros products originated, 10 single nucleotide polymorphisms (SNPs) were characterized (Labuschagne et al. 2013); observed heterozygosity was low, ranging from 0.05 to 0.37 across the SNP loci. Investigation into the patterns of diversity at adaptive loci in

white rhinos may provide critical insight into the dynamics of populations that are subject to intensive management for conservation purposes.

Black rhinoceros (*Diceros bicornis*)

Prior to 1960 black rhinoceros populations numbered well over 100,000 and occupied a large range throughout Africa (Emslie 2012) (Figure 1.2). Between 1960 and the mid-1990s the total population of black rhinos had declined by more than 95% as a result of poaching and habitat alteration (Harley et al. 2005; Metzger et al. 2007; van Coeverden de Groot et al. 2011); the current population is estimated to be ~5,000 individuals. Seven subspecies of the black rhinoceros were described by Groves (1967): *D. b. bicornis*, *D. b. chobiensis*; *D. b. minor*, *D. b. michaeli*, *D. b. brucii*, *D. b. ladeonsis*, and *D. b. longipes*. However, relationships among the extant subspecies (*D. b. bicornis*, *D. b. minor*, and *D. b. michaeli*) are unresolved. Mitochondrial restriction maps of *D. b. minor* and *D. b. michaeli* showed relatively low sequence differentiation (0.29%) and a divergence time of less than 100,000 years (Ashley et al. 1990). Additional mtDNA restriction maps from samples attributed to *D. b. minor*, *D. b. bicornis*, and *D. b. michaeli* were monomorphic within recognized subspecies and showed low amounts of sequence differentiation between any pair of subspecies (0.4%) (O’Ryan et al. 1994). Limited genetic distance among black rhino subspecies may indicate that they are not unique evolutionary lineages (Ashley et al. 1990; O’Ryan et al. 1994), but rather, represent populations along a geographic cline (Swart & Ferguson 1997). However, Brown and Houlden (2000), analyzing a portion of the mitochondrial control region, found a reciprocally monophyletic relationship between *D. b. minor* and *D. b. michaeli* with a nucleotide divergence of 2.6%. The divergence time between the two lineages was estimated to be 0.93 – 1.3 million years. Other studies reported a mitochondrial control region haplotype network with a distinct pattern of divergence among the black rhinoceros subspecies (Anderson-Lederer et al. 2012), moderate genetic differentiation ($F_{ST} > 0.25$) estimated using microsatellite loci (Harley et al. 2005), and evidence of variation in chromosomal morphology between *D. b. minor* and *D. b. michaeli* (Houck et al. 1995). Despite differences in the reported level of variation it is typically suggested that recognized subspecies should be managed as separate entities as long as feasible (O’Ryan et al. 1994; Brown & Houlden 2000; Harley et al. 2005). Overall, no consensus has been reached as to

what extent the three extant black rhinoceros subspecies represent distinct and evolutionarily important lineages.

Microsatellite markers are an important tool for conservation genetics and have been widely used in studies of black rhinoceros populations. Polymorphic microsatellites for the black rhinoceros have been described by (Brown & Houlden 1999) (N = 11) and (Cunningham et al. 1999) (N = 3). These microsatellite markers were used to obtain genotypes from fecal samples, which, when coupled with behavioral observations, indicated that black rhinos may exhibit a polygynous mating system (one male mates with multiple females) with high variance in reproductive success among males (Garnier et al. 2001). Nielsen and others (2008) redesigned primers for the microsatellite loci characterized by Brown and Houlden (1999) and other microsatellite sequences previously submitted to GenBank for black rhinoceros, in addition to five previously published white rhinoceros microsatellite loci (Florescu et al. 2003). Of the redesigned markers 12 were polymorphic in black rhinos ($H_O = 0.322$) and seven loci can be used to differentiate between African species (Nielsen et al. 2008). A panel of microsatellites ($H_O = 0.365$) and genetic sexing markers to be used for individual identification of black rhinos has been developed (Peppin et al. 2010; Harper et al. 2013). Genetic markers beyond mitochondrial control region and microsatellites have not yet been implemented in black rhino conservation research.

The subspecies *D. b. bicornis* is estimated to have a population of 1,920 individuals mostly in Namibia, the current and historic major range state. As few as 90 individuals may have persisted in Namibia at the population's lowest point (van Coeverden de Groot et al. 2011); however, protection of populations and frequent translocations within the country have resulted in the increasing population trend (van Coeverden de Groot et al. 2011). Individuals from Namibia's Etosha National Park (ENP) black rhinoceros population (*D. b. bicornis*), which has grown substantially, are commonly translocated to seed or supplement additional populations (van Coeverden de Groot et al. 2011). Van Coeverden de Groot and others (2011) characterized baseline population genetic data for ENP and Waterberg Plateau Park (partially founded by ENP individuals); they found a mean observed heterozygosity of 0.51, limited population structuring, no signature of a recent bottleneck, and evidence of sex biased dispersal (limited female dispersal). Overall, Waterberg Plateau Park retains a majority of the alleles present in the source population (87%) (van Coeverden de Groot et al. 2011). Other studies have found similar level

of heterozygosity in *D. b. bicornis* using microsatellite loci: $H_O = 0.46$ (Karsten et al. 2011), $H_O = 0.40$ (Scott 2008), and $H_O = 0.52$ (Harley et al. 2005).

The *D. b. michaeli* population reached a low of fewer than 400 individuals in the 1990s and is currently the most endangered of the extant black rhino subspecies, with approximately 740 individuals remaining in Kenya (major range state), northern Tanzania, and South Africa (out of range) (Emslie & Brooks 1999; Muya et al. 2011). *D. b. michaeli* exhibits the highest overall levels of genetic variability at mitochondrial ($h = 0.73$) and microsatellite loci ($H_O = 0.7$), despite the history of substantial population decline (Muya et al. 2011). Genetic structuring among subpopulations of *D. b. michaeli* in Kenya is limited (except for the isolated Masai Mara population which underwent an extended and more severe bottleneck than other populations); absence of structure among populations is potentially due to translocations that maintain gene flow and reduce genetic drift (Muya et al. 2011). Lack of genetic partitions suggests that current management efforts aimed at maintaining distinct “montane” and “lowland” populations are unnecessary and may have undesired implications on future population structure and diversity (Muya et al. 2011). Among 12 subpopulations levels of diversity varied considerably ($H_O = 0.48$ to 0.8 ; $h = 0.48$ to 0.93), which could be a reflection of each population’s unique demographic history (Muya et al. 2011). Other studies have consistently found higher levels of variation in the *D. b. michaeli* subspecies than in the other black rhino subspecies when examining both microsatellite diversity (e.g., $H_O = 0.54$, Karsten et al. 2011; $H_O = 0.73$, Harley et al. 2005; $H_O = 0.57$, Scott 2008) and mitochondrial haplotype diversity (e.g., $h = 0.952$, Anderson-Lederer et al. 2012).

The subspecies *D. b. minor*, with 2,220 individuals, has the largest population size of any black rhino subspecies. The population is primarily restricted to South Africa, where it was reestablished through translocations from two surviving populations of 110 total individuals in Hluhluwe-iMfolozi Park and Mkhuze Game Reserve in the KwaZulu-Natal (KZN) province (Emslie & Brooks 1999; Okita-Ouma et al. 2007; Karsten et al. 2011). An additional population of 425 rhinos remained in Zimbabwe in the early 1990s and continues to serve as an important population for the conservation of this subspecies (Emslie 2012). Despite its relatively large size, multiple studies have demonstrated low genetic diversity in the *D. b. minor* KZN population (Harley et al. 2005; Karsten et al. 2011; Anderson-Lederer et al. 2012). Lack of genetic diversity has been found using both microsatellite loci ($H_O = 0.38$, Karsten et al. 2011; $H_O = 0.32$, Nielsen

et al. 2008) and the mitochondrial control region ($H = 1$; Anderson-Lederer et al. 2012). In contrast, a recent study indicated the presence of higher microsatellite diversity ($H_0 = 0.52$) and seven mitochondrial haplotypes within the Zimbabwean black rhino population (Kotzé et al. 2014). There is no evident structure among the populations within the KZN province (Swart & Ferguson 1997; Karsten et al. 2011); however, there is structuring between Zimbabwe populations founded by South African and those founded by local individuals (Kotzé et al. 2014). It is unclear if the lack of diversity in the South African population is a result of the bottleneck, although other studies of black rhinos suggest that recent bottlenecks are not responsible for current patterns of genetic diversity (e.g., (Swart et al. 1994; van Coeverden de Groot et al. 2011), or if this population has been historically isolated from other lineages (Karsten et al. 2011; Anderson-Lederer et al. 2012). Suggestions for management (e.g. genetic supplementation) of black rhinoceros populations with low genetic diversity vary across studies. Since the black rhinoceros occur almost exclusively in remnant populations (Hillman-Smith & Groves 1994; Moehlman et al. 1996), genetic monitoring and assessment of diversity is needed for successful conservation planning and management implementation.

Indian rhinoceros (*Rhinoceros unicornis*)

The Indian rhinoceros (also known as the greater one horned rhino) had an estimated historic population size of hundreds of thousands of individuals, inhabiting the region from Northern Pakistan to Northwestern Myanmar (Laurie 1978; Dinerstein & McCracken 1990; Zschokke & Baur 2002) (Figure 1.3). Habitat loss through land clearing and fragmentation resulted in large scale population decline, which was further exacerbated by poaching pressure (Zschokke et al. 2011). The current estimated population size is approximately 3,250 individuals (Emslie et al. 2013), with populations confined to reserves in three Indian states (Assam, Uttar Pradesh, and West Bengal) and the Himalayan foothills of Southern Nepal (Laurie et al. 1983; Foose & van Strien 1997). Assam's Kaziranga National Park and immediately surrounding areas contain one of the main populations of about 2700 individuals (Merenlender et al. 1989; Zschokke et al. 2011; Emslie et al. 2013), increased from an estimated low of 20 individuals in the early 1900s (Laurie et al. 1983). The main population in Nepal (in the Chitwan Valley) was reduced to as few as 60 – 80 individuals in the 1960s, but since the early 2000s has fluctuated

between 400 and 500 individuals depending on poaching pressure (Zschokke et al. 2011; Emslie et al. 2013). No individuals have been moved between the two remaining main populations which are naturally isolated from each other, thus preventing gene flow (Zschokke et al. 2011).

Limited population level genetic studies have been conducted on the Indian rhinoceros. Eleven polymorphic microsatellite loci were designed by (Zschokke et al. 2003); a panel of these microsatellite loci can be used to differentiate between the Nepal and Assam populations. Evidence of significant genetic differentiation between the populations was found by mitochondrial control regions haplotypes that are restricted to specific populations and a relatively high F_{ST} value ($F_{ST} = 0.202$) at microsatellite loci; based on these measures it is possible to assign individuals to their population of origin with high confidence (Zschokke et al. 2011). It is suggested that in the future crossing or translocation of individuals between Indian and Nepal should be avoided, and that the populations should be managed separately to maintain genetic distinctiveness (Zschokke & Baur 2002; Zschokke et al. 2011). Genetic diversity in the Assam population was high despite a severe bottleneck (Assam, $H_o = 0.57$; Nepal, $H_o = 0.43$) (Zschokke et al. 2011). Retention of genetic variation in the Indian rhino populations may be a result of their previously large population size (prior to the 1950s), long generation time, and recentness of the bottleneck, which may have been less severe than originally reported (Dinerstein & McCracken 1990; Zschokke et al. 2011). Contrarily, Scott 2008 observed heterozygosity of 0.34 in Indian rhinos using a panel of markers characterized in four rhinoceros species (black, white, Indian, and Sumatran); with Indian rhinoceros species specific microsatellites the observed heterozygosity was 0.51 (Scott 2008). Microsatellites have been successfully implemented in non-invasively collected wild Indian rhino dung samples; through this research management recommendations were put forth (Das et al. 2015), suggesting the potential utility of fecal samples for conservation genetics work in other rhinoceros species.

Javan rhinoceros (*Rhinoceros sondaicus*)

Historically ranging from Northern India through Bangladesh and Indochina to the Indonesian islands of Java and Sumatra (Figure 1.4), the Javan rhinoceros was once so abundant that it was considered an agricultural pest (Ramono et al. 1993; Fernando et al. 2006). The population decreased in size due to land use changes combined with pressure from sport hunting

and poaching (Fernando et al. 2006). In Java's Ujung Kulon National Park a population size of 25 was estimated in the late 1960s, increasing to approximately 50 by the 1980s (Ramono et al. 1993), but this population is now estimated to contain approximately 60 rhinos (Jong 2016). Three subspecies of Javan rhinoceros have been recognized: *R. s. inermis* (extinct) in Bangladesh, India, and Myanmar; *R. s. annamiticus* (extinct) in Cambodia, Laos, Thailand, and Vietnam; and *R. s. sondaicus* in Indonesia, Malaysia, and Thailand (Rookmaaker 1980; Groves & Leslie 2011; Brook et al. 2012). The subspecies *R. s. annamiticus* was thought to be extinct in the mainland until poached parts were found at a market in 1988, leading to the rediscovery of a population of 10-15 individuals in Cat Tien, Vietnam (Groves 1995; Fernando et al. 2006; Brook et al. 2012); however, this subspecies was confirmed extinct in 2010 after the last individual was found shot in Vietnam (Brook et al. 2012; Emslie et al. 2013). Estimating population size and conducting genetic studies of this species is difficult due to its low population density, cryptic nature, and a lack of well-established population monitoring efforts.

The Javan rhinoceros has been the focus of very few population - or species - level studies; of those that have been conducted, only two incorporate genetic markers. In one study, genetic analysis of portions of the mitochondrial genome suggested that the Vietnamese and Javan populations were as divergent as subspecies described in other rhinoceros species (Fernando et al. 2006). The subspecies are estimated to have shared a common ancestor 300,000 to 2 million years ago, which is consistent with the biogeographic history of the region where sea level fluctuations resulted in periods of connection and disconnection between the Sunda Islands and the mainland (Fernando et al. 2006). Since the two subspecies had been geographically separated, with distinct evolutionary trajectories and genetic differences in the mitochondrial genome, Fernando and colleagues (2006) suggested that they comprised distinct evolutionarily significant units. The second study used genotypes and genetic sex data from dung samples collected during a field survey of Cat Tien National Park, Vietnam in 2009 – 2010 to confirm that all specimens were from the same individual found dead in 2010 (Brook et al. 2012). Using 16S rRNA barcoding markers, bacterial diversity profiles were generated for fecal samples collected from 2003 – 2006 and 2009 – 2010; this methodology suggested that two Javan rhinos were sampled in the earlier survey and only a single individual was sampled during the latter survey (Brook et al. 2012). Additional molecular genetic methods and markers are needed in

order to produce more accurate census estimates and improve population monitoring capabilities for the surviving subspecies in Java.

Sumatran rhinoceros (*Dicerorhinus sumatrensis*)

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) was once distributed across Southeast Asia into the foothills of the Himalayan Mountains (Figure 1.5), but habitat loss combined with poaching has resulted in substantial decreases in population size (Scott et al. 2004; Zafir et al. 2011). The current population is estimated to be less than 100 individuals with a decreasing trend (Havmøller et al. 2016). There are two extant subspecies; *D. s. harrissoni* occurring in three populations in Indonesian Borneo (N = 15) and *D. s. sumatrensis* found in three national parks on the Indonesian island of Sumatra (< 100 individuals) (Groves & Kurt 1972; van Strien et al. 2008; Emslie et al. 2013). The species was recently declared extinct in Peninsular Malaysia (IRF 2016) and in the wild of Malaysian Borneo (Havmøller et al. 2016). A third subspecies, *D. s. lasiotis*, is likely extinct, but unconfirmed reports suggest the possibility of a population in Myanmar (van Strien et al. 2008). To optimize conservation efforts thorough surveys need to be conducted to determine the presence or absence of rhinoceros populations throughout its range in Sumatra.

There is a paucity of genetic studies of Sumatran rhinoceros populations. A publication by Scott and colleagues (2004) optimized 10 polymorphic microsatellite loci in the Sumatran rhinoceros; no published studies have utilized these markers for research on Sumatran rhinoceros populations. Using a suite of 24 microsatellite loci characterized across rhinoceros species, 23 Sumatran rhino samples were genotyped and an observed heterozygosity of 0.380 was reported (Scott 2008). However, when using microsatellite markers designed in conspecifics on Sumatran individuals an observed heterozygosity of 0.529 was obtained (Scott 2008). Earlier studies on the Sumatran rhino utilized mitochondrial DNA by restriction mapping to assess population differentiation and to identify conservation units (Amato et al. 1995; Morales et al. 1997). These studies agree that low levels of genetic differentiation occur between populations of *D. s. sumatrensis* from the island of Sumatra and the Malay Peninsula (Amato et al. 1995; Morales et al. 1997). Furthermore, they found that higher levels of sequence divergence exist between populations representing the subspecies *D. s. harrissoni* and populations representing the

subspecies *D. s. sumatrensis* (Amato et al. 1995; Morales et al. 1997). Yet, conflicting suggestions about whether the subspecies were distinct enough to be managed as one or two conservation units arose from these studies. It is imperative for the future survival of the Sumatran rhinoceros to gain a better understanding of this species genetic diversity, to confirm the proper number of management units by way of genome wide analyses, and to integrate molecular techniques into monitoring of populations.

Rhinoceros Phylogenetics

Relationships within the family Rhinocerotidae have been inferred using morphological (e.g. number of horns) (Simpson 1945; Loose 1975), geographic (Pocock 1945; Groves 1983), and molecular data (Morales & Melnick 1994; Xu & Arnason 1997; Tougard et al. 2001; Orlando et al. 2003; Willerslev et al. 2009). Due to incongruent topology among studies of the five extant species and the extinct woolly rhinoceros (*Coelodonta antiquitatis*), the existence of a hard polytomy has been proposed, which would imply that multiple branching events occurred simultaneously (Willerslev et al. 2009). Commonly accepted sister taxa relationships within the rhinoceros phylogeny are as follows: African species are placed in the subtribe Dicerotina, Indian and Javan rhinoceros within the subtribe Rhinocerotina, with Sumatran and extinct woolly rhinos forming the clade *Dicerorhinus* (Morales & Melnick 1994; Xu & Arnason 1997; Tougard et al. 2001; Orlando et al. 2003; Willerslev et al. 2009). There has been no consensus reached about where the Sumatran rhinoceros lineage falls in relation to the other lineages. Studies that sequenced mitochondrial genes reported conflicting relationships; placement of the *Dicerorhinus* lineage closest to the African rhinoceros clade (Hsieh et al. 2003), placement of the *Dicerorhinus* lineage with the other Asian rhinos (Tougard et al. 2001; Orlando et al. 2003), or placing the *Dicerorhinus* lineage basal to all other extant rhinoceros species (Fernando et al. 2006). Even when complete mitogenomes were analyzed there was no resolution of relationships within the Rhinocerotidae family, as topologies varied across tree building methodologies and assessment of individual mitochondrial genes (Willerslev et al. 2009). Additionally, the independent analysis of sequences from mitochondrial or nuclear genes among four rhinoceros species (*C. simum*, *D. bicornis*, *R. unicornis*, and *D. sumatrensis*) produced inconsistencies in topology; when a

combined dataset was assessed the *Dicerorhinus* lineage was placed most closely to the African rhinoceros species and the Indian rhinoceros was the most basal lineage (Steiner & Ryder 2011).

Lack of comprehensive genomic studies has resulted in unresolved relationships among rhinoceros species and subspecies. The estimated time of divergence between the African and Asian rhinoceros lineages is 26 million years ago (mya) (Tougard et al. 2001). Estimates of divergence between black and white rhinoceros lineages have varied based on methodology; when using mtDNA restriction maps 3.4 mya was estimated (O'Ryan & Harley 1993) as compared to 17 mya when using portions of the mitogenome (Tougard et al. 2001) or 15 mya when estimated using full mitochondrial genome sequences (Willerslev et al. 2009). Other estimated divergence times between rhinoceros lineages are Indian-Javan, 13 mya and woolly-Sumatran, 20 mya (Willerslev et al. 2009). More data, ideally from the nuclear genome, is needed to resolve relationships among rhinoceros lineages and to properly assess populations or regions that comprise important conservation units.

Research Objectives

All rhinoceros species are listed as threatened or endangered under the Endangered Species Act and have been included in Appendix I or II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) since 1977. Through ESA and CITES regulations rhinoceros species have been afforded protection against commercial trade of rhino products (i.e., horn) and other actions that endanger populations; yet poaching and habitat loss continue to be a major threat to conservation (CITES 2010; Emslie et al. 2013). The development of genetic markers for assessment of diversity at neutral and adaptive loci can be used to answer a number of questions that will ultimately aid in the conservation of rhinos populations both *ex situ* and in the wild. In order to comprehensively evaluate genetic diversity in rhinoceros populations, three research objectives, which will contribute substantial knowledge to the conservation and management of rhinoceros species, were identified:

(1) Accurate estimates of population size are often difficult to obtain for rhinoceros species that are elusive or prefer dense habitat. Knowing the true number of individuals in an area is essential for managers to develop and implement conservation plans that address the issues facing a particular population. To encourage the use of molecular methods for censusing

of rhinoceros populations, novel microsatellites were characterized from next generation sequencing data. These markers were designed specifically for use with low quality DNA extracts from non-invasively collected fecal samples. In particular, since the reported number of individual Sumatran rhinos has been drastically overstated and difficult to accurately estimate markers were designed for implementation on wild populations in Sumatra. Additionally, estimates of wild black rhino population size can be difficult to accurately approximate owing to their cryptic nature and preference for dense habitat; therefore, markers allowing for censusing of black rhinos populations from fecal samples were designed. Due to availability of black rhino fecal samples from individuals in North American zoos, success of these markers in amplifying genotypes from low quality DNA could be assessed.

(2) No studies have examined the population genetic structure of Sumatran rhinoceros using analyses beyond mitochondrial restriction mapping techniques (Amato et al. 1995; Morales et al. 1997). Yet, assessing population wide diversity and structure within this species using sequence or genotype data is important for conservation efforts. Multilocus genotypes from the newly characterized microsatellite markers (objective 1) and mitochondrial control region haplotypes were used to investigate diversity and structuring within the existing Sumatran rhino population. Furthermore, changes in genetic diversity and population structure over time were assessed through incorporation of high quality DNA samples from recently living individuals and DNA of degraded nature from museum bone specimens.

(3) Beyond the neutral markers typically used to assess diversity in population genetic studies, genes involved in the immune system can provide information relevant to conservation efforts. Most studies of immunogenetics in wildlife species focus on the major histocompatibility complex; however, other gene suites, such as the Toll-like receptors (TLRs) of the innate immune system, have been shown to be important in studies of threatened and endangered species. Toll-like receptor genes code for proteins that are a crucial part of the innate immune system; thus, nucleotide diversity in these genes is critical for wild populations to defend against pathogens. If immune system diversity is low, a population may not be able to resist pathogens and long term viability will be impacted. Genetic diversity of eight TLR genes were characterized in black and white rhinos from North American zoos and *ex situ* breeding facilities. Knowledge of individual and population level variation at the TLR loci can be used in conservation planning, particularly for translocations and *ex situ* breeding programs.

Figures

Figure 1.1. Map showing the range of the white rhinoceros.

This map of the continent of Africa shows the approximate historic and current distributions of white rhinoceros (*Ceratotherium simum*). Areas shown in yellow are the historic range, and regions encircled in red are locations currently occupied. The white rhinoceros subspecies, northern white rhino (*C. s. cottoni*) and southern white rhino (*C. s. simum*), ranges are denoted. The northern white rhino had a historical distribution disjunct from that of the southern subspecies, occurring in parts of Central African Republic, Chad, South Sudan, and Uganda. The remaining individuals currently reside in Ol Pejeta Conservancy in Kenya (outside of their historic range). The southern white rhino subspecies was numerous and widespread in the 1800s, distributed mainly south of the Zambezi River across present-day South-eastern Angola, Botswana, Mozambique, Namibia, South Africa, Swaziland, South-western Zambia, and Zimbabwe. Current populations are found in all historic range states and also in Kenya and Uganda, outside of the historic range (not shown). The map and image were modified from the International Rhinoceros Foundation (www.rhinos.org).

Figure 1.1. Cont.

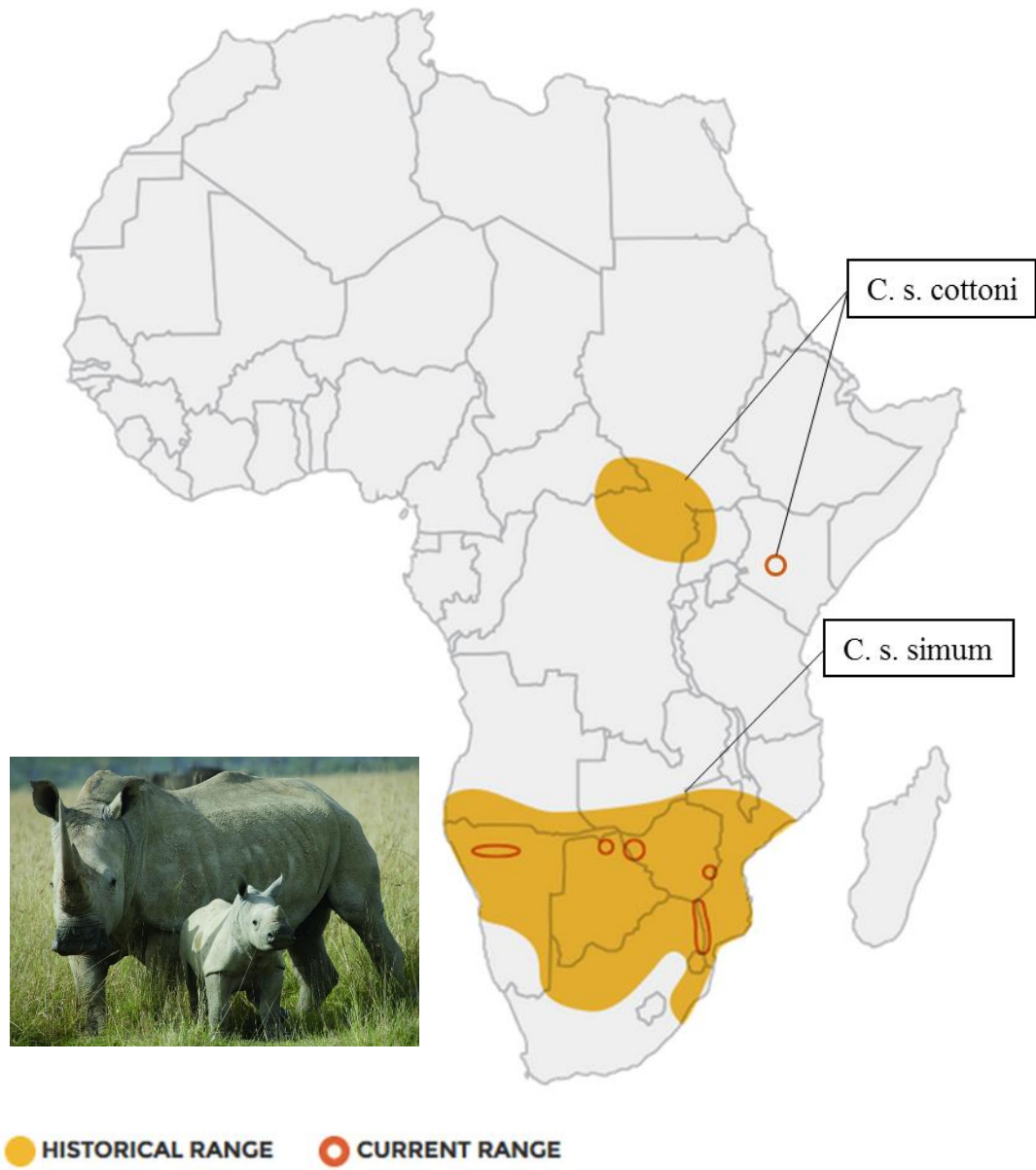


Figure 1.2. Map showing the range of the black rhinoceros.

This map of the continent of Africa shows the approximate historic and current distributions of black rhinoceros (*Diceros bicornis*). Areas shown in yellow are the historic range, and regions encircled in red are locations currently occupied. The black rhinoceros population is comprised of three recognized subspecies, eastern black rhino (*D. b. michaeli*), south-central black rhino (*D. b. minor*) and south-western black rhino (*D. b. bicornis*). The eastern black rhinoceros has populations in Kenya and Tanzania. The south-central black rhinoceros mainly occurs in South Africa with additional populations occurring along the eastern portion of the African continent in Botswana, Malawi, Swaziland, Tanzania, Zambia, and Zimbabwe. The south-western black rhinoceros is restricted to Namibia. The map and image were modified from the International Rhinoceros Foundation (www.rhinos.org).

Figure 1.2. Cont.

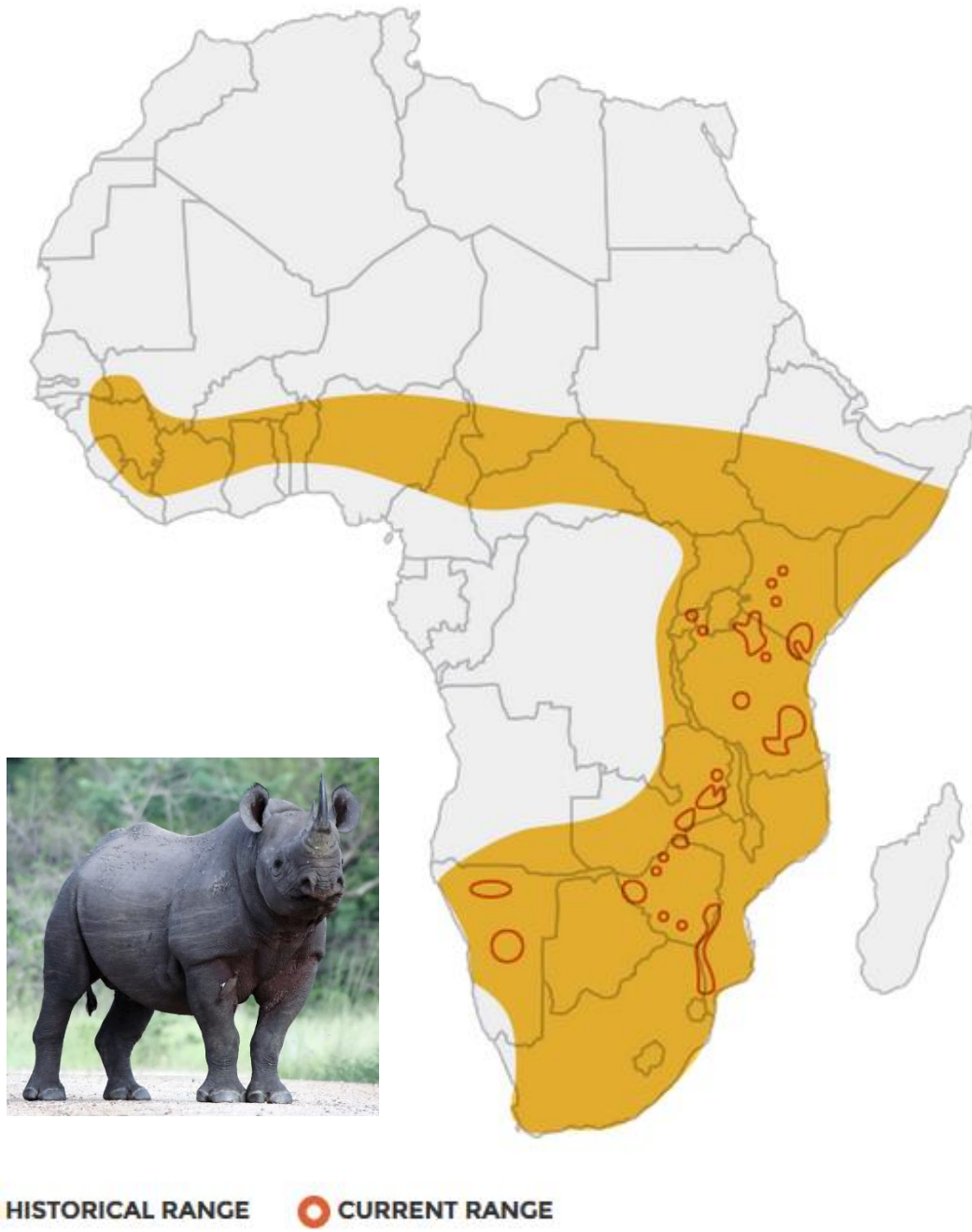


Figure 1.3. Map showing the range of the Indian rhinoceros.

This map of a portion of the continent of Asia shows the approximate historic and current distributions of Indian rhinoceros (*Rhinoceros unicornis*). Areas shown in yellow are the historic range, and regions encircled in red are locations currently occupied. Indian rhinos formerly inhabited the region from Northern Pakistan to Northwestern Myanmar. The current populations confined to reserves in three Indian states (Assam, Uttar Pradesh, and West Bengal) and the Himalayan foothills of Southern Nepal. The map and image were modified from the International Rhinoceros Foundation (www.rhinos.org).

Figure 1.3. Cont.

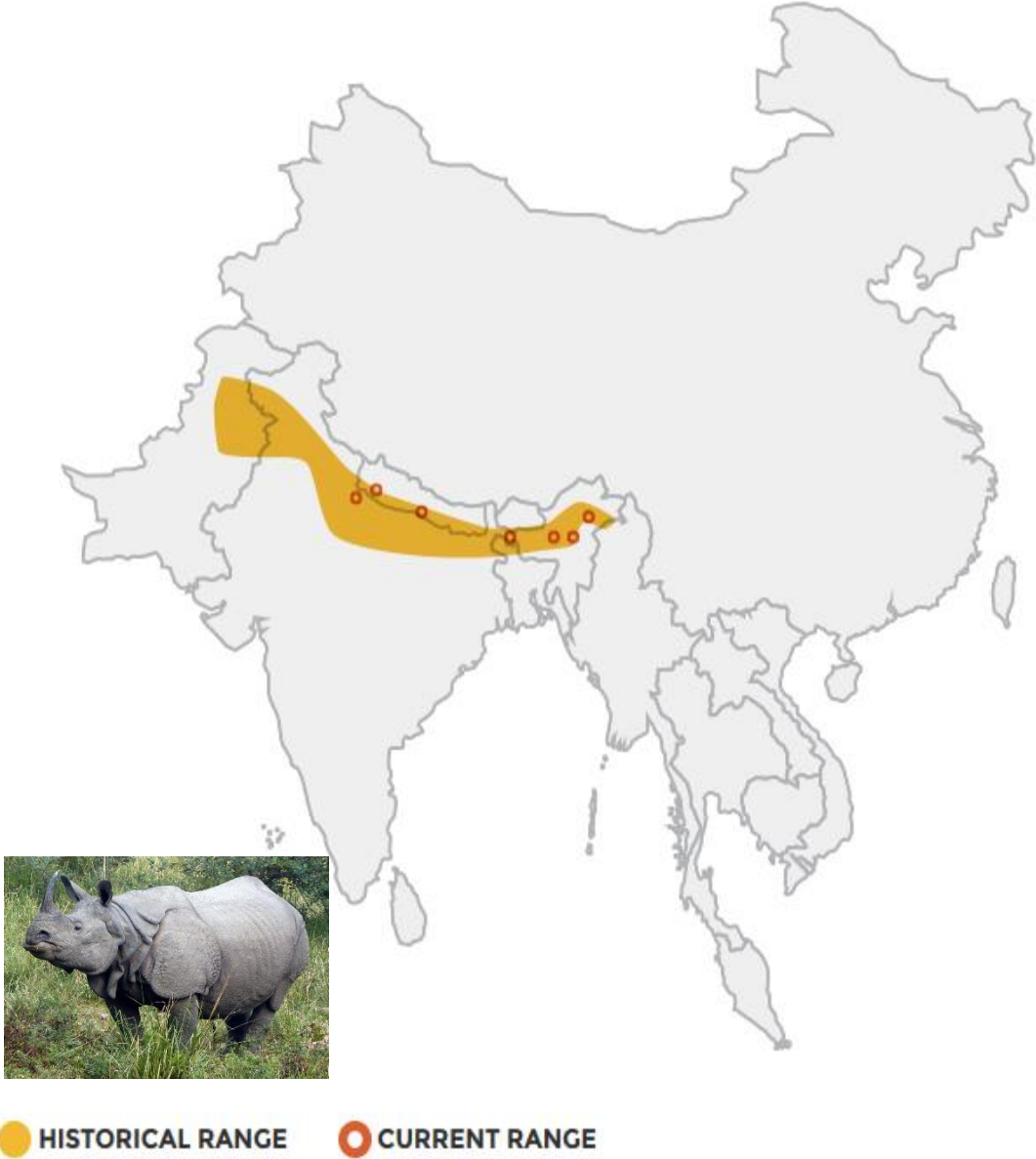


Figure 1.4. Map showing the range of the Javan rhinoceros.

This map of a portion of the continent of Asia shows the approximate historic and current distributions of Javan rhinoceros (*Rhinoceros sondaicus*). Areas shown in yellow are the historic range, and regions encircled in red are locations currently occupied. The Javan rhinoceros historically ranged from Northern India through Bangladesh and Indochina to the Indonesian islands of Java and Sumatra. The current population is isolated to Java's Ujung Kulon National Park. The map and image were modified from the International Rhinoceros Foundation (www.rhinos.org).

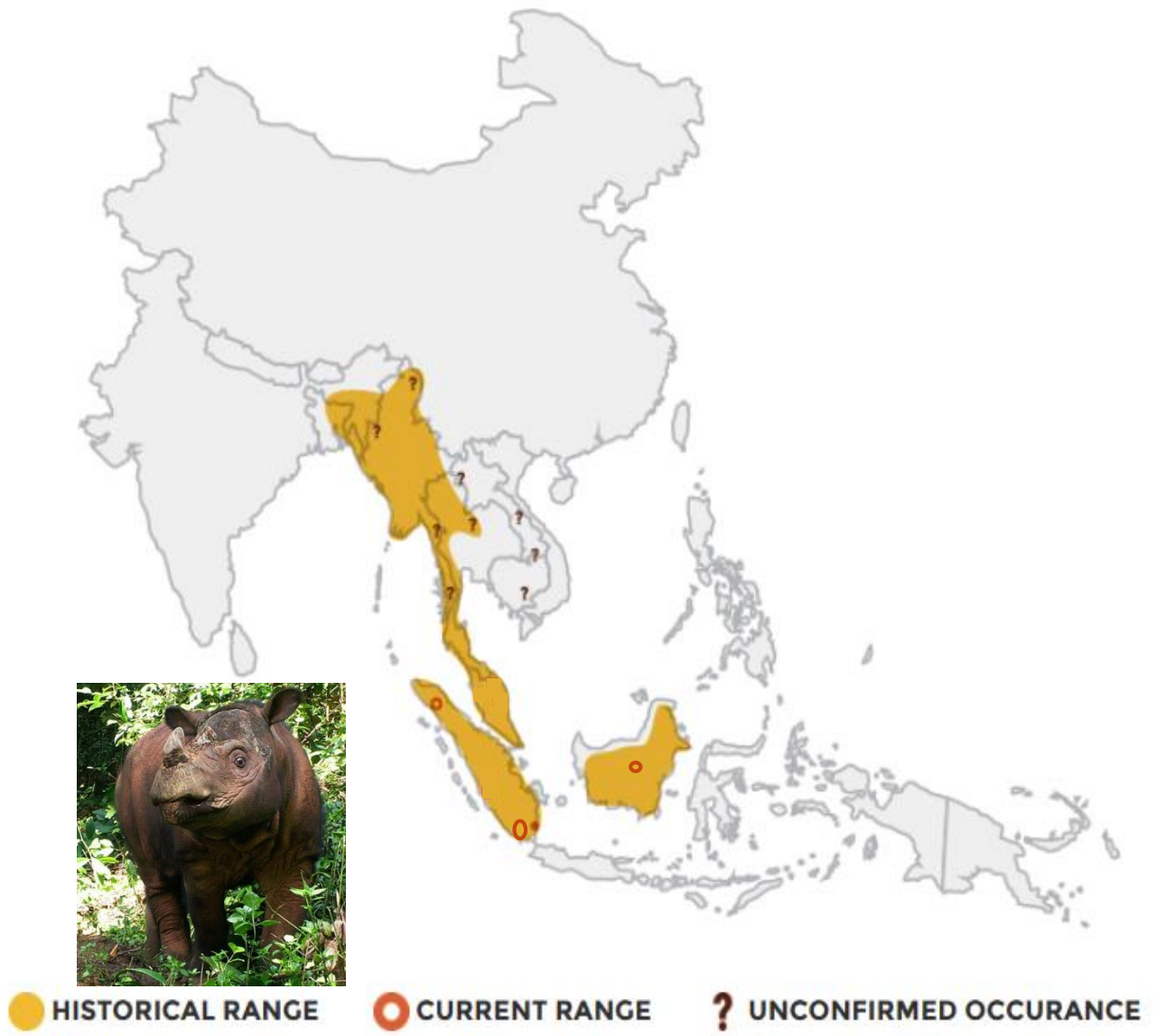
Figure 1.4. Cont.



Figure 1.5. Map showing the range of the Sumatran rhinoceros.

This map of a portion of the continent of Asia shows the approximate historic and current distributions of Sumatran rhinoceros (*Dicerorhinus sumatrensis*). Areas shown in yellow are the historic range, regions encircled in red are locations currently occupied, and locations with question marks denote uncertain population status (putatively extinct). The Sumatran rhinoceros was once distributed across Southeast Asia into the foothills of the Himalayan Mountains. There are two extant subspecies; *D. s. harrissoni* occurs only in Indonesian Borneo, and *D. s. sumatrensis* is restricted to the island of Sumatra in Indonesia. The map and image were modified from the International Rhinoceros Foundation (www.rhinos.org).

Figure 1.5. Cont.



CHAPTER 2. DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS FROM NEXT GENERATION SEQUENCING FOR UTILIZATION IN LOW QUALITY DNA FROM DUNG

Abstract

Accurate estimates of population size are often difficult to obtain for rhinoceros species that are elusive or occupy dense habitat. Knowing the precise number of individuals in an area is essential for managers to develop and implement conservation plans that address the issues facing a particular population. Despite their importance, population estimates for Sumatran and black rhinoceros are often challenging to calculate or subject to detection biases; therefore, we expect that implementing molecular methods utilizing DNA from non-invasively collected fecal samples will substantially improve current techniques. From Roche 454 sequencing data of one black rhinoceros sample, 17 novel black rhinoceros microsatellites were characterized. These markers were successfully amplified across two black rhinoceros subspecies: the south-central black rhino ($A = 2.5$; $H_O = 0.43$) and the eastern black rhino ($A = 3.4$; $H_O = 0.39$). Two Sumatran rhinoceros samples were sequenced using the Illumina MiSeq v3 platform; due to limited sample quantity and potential lack of genome wide diversity in this species, a novel bioinformatics pipeline was developed to scan the sequencing databases for putatively polymorphic loci. Using this new methodology 29 novel polymorphic microsatellites were characterized ($A = 2.4$; $H_O = 0.30$). A subset of these markers is sufficient for identification of individuals based on P_{ID} and $P_{ID(sib)}$ values of < 0.001 for black rhinos and < 0.0001 for Sumatran rhinos. Through a series of optimization steps I demonstrated that these markers used to successfully generate genotypes from fecal samples. Genotyping success rate in black rhinoceros fecal samples ranged from 56.6% to 91.7% with allelic dropout rate ranging from 6.8 – 11.7 % and false alleles from 0 – 3.2% depending on the amplification conditions. These microsatellite markers, used from molecular censusing, will serve an important role in conservation of rhino species, particularly the Sumatran rhinoceros, for which the reported number of individuals has been drastically overstated and is extremely difficult to accurately estimate.

Introduction

Accurate census estimates are important for the assessment of long-term viability and development of management goals for critically endangered rhinoceros species. Overestimation of population size can be particularly problematic as it may result in inadequate protection and poor management of remaining individuals. Traditional methods of censusing rhinoceros populations include aerial or vehicular surveys, camera trapping, and visual identification of individuals (Brockett 2002; Mulama & Okita 2002; Metzger et al. 2007; Stein et al. 2010; Hariyadi et al. 2011). When individuals are not located in open habitat or are elusive, as is the case for black and Sumatran rhinoceros, these techniques may be ineffective and prone to detection biases that can result in inaccurate estimates (Brockett 2002; Mulama & Okita 2002; Stein et al. 2010; Hariyadi et al. 2011). In addition, traditional surveying methods are expensive to execute and some (e.g., capture and release sampling or biopsy darting) have the potential to cause injury or elevated stress levels; therefore, they may not be ideal for use with threatened or endangered species.

By contrast, molecular methods that utilize non-invasively obtained dung samples can be employed to estimate population size while avoiding handling or even direct observation of individuals (Kohn et al. 1999; Bellemain et al. 2005; Brook et al. 2012). Furthermore, the accuracy of census estimates can be improved by the systematic collection of dung (e.g., sweeps or line transect schemes) from across a species habitat for genetic analysis. Systematic sampling schemes can substantially increase the proportion of a population represented in a dataset compared to alternative detection methods (Zhan et al. 2006; Arrendal et al. 2007; Guschanski et al. 2009; Gray et al. 2013). Census surveys that incorporate a genetic component allow for the study of population dynamics over time (Guschanski et al. 2009) and permit greater insight into population processes, including: paternity, mating systems, and levels of inbreeding. A combined management approach including non-invasive molecular methods and traditional monitoring can be particularly powerful for conservation efforts (Bischof & Swenson 2012).

For many rare or elusive mammals, fecal material may be the most readily available source for genetic studies. Dung contains DNA from the host species in epithelial cells that are shed during defecation (Reed et al. 1997; Wilson et al. 2003). Fecal samples collected while fresh and under ideal environmental conditions may contain DNA in adequate quality and

quantity for genetic analysis (Wilson et al. 2003; Okello et al. 2005; Fernando et al. 2006; Arrendal et al. 2007). However, DNA from feces is sometimes degraded, present in small quantities, and is likely to contain inhibitors or contaminants (Taberlet et al. 1999; Ishida et al. 2011a, 2012). Degraded or low quality DNA can be more challenging to amplify by polymerase chain reaction (PCR) than DNA from blood, tissue or other intact samples. However, rate of amplification success in degraded or low quality DNA (e.g., dung samples, museum specimens, or forensic materials) can be improved by shortening the targeted amplicon length to < 200 bp (Butler et al. 2003; Ishida et al. 2011a, 2012; Brandt et al. 2013b).

Due to their high rates of evolution, microsatellite loci are widely used to address questions in wildlife management and conservation research (van Coeverden de Groot & Boag 2004; Knowles et al. 2009; Ishida et al. 2012; Brandt et al. 2013a), and they have been included in many studies of rhinoceros species (Guerier et al. 2012; Karsten et al. 2011; Muya et al. 2011; Scott et al. 2004; van Coeverden de Groot et al. 2011; Zschokke et al. 2011). Among other applications, microsatellites can be used to assign individual identity to samples, elucidate patterns of gene flow, determine levels of population differentiation, and estimate relatedness (Selkoe & Toonen 2006). However, there are few studies that have utilized microsatellites in dung samples from wild rhinoceros populations for genetic analyses (Garnier et al. 2001; Brook et al. 2012). The use of dung for genetic studies may have been avoided due to the potential for lack of repeatability or high genotyping error rate (i.e., allelic dropout and false alleles), often a result of a shortage of markers that are reliable when implemented on low quality DNA from dung (Taberlet et al. 1999; Cunningham et al. 2001; Ishida et al. 2011a; Guerier et al. 2012; Ishida et al. 2012; Brandt et al. 2013b). Despite these possible drawbacks, the rate of dung genotyping success can be improved when microsatellite markers and amplification protocols are specifically designed for use on non-invasively collected samples (Ishida et al. 2012). Furthermore, other studies have demonstrated that microsatellites can be used to effectively estimate population census size from fecal DNA of various wildlife species (Kohn et al. 1999; Bellemain et al. 2005; Mowry et al. 2011; Bonesi et al. 2013; McCarthy et al. 2015).

To improve the chances of accurately estimating rhinoceros population sizes using dung, a panel of microsatellite markers that can identify unique multilocus genotypes must be developed for the specific rhinoceros species of interest. The currently available microsatellite markers for black rhinos have produced limited results when used on dung samples

(Cunningham et al. 2001), and for Sumatran rhinos only a few species specific markers have been developed (Scott et al. 2004). Both of these species would benefit from improved methods for accurately estimate population sizes; therefore, markers targeting short amplicons that have been characterized specifically for genotyping of dung DNA are necessitated. The chances of characterizing a large panel of polymorphic microsatellites that can be used to assign individual identity have improved with recent developments in molecular methods. Advances include the increasing accessibility and decreasing cost of next-generation sequencing (NGS) platforms (e.g., Roche 454 FLX Titanium and Illumina Genome Analyzer) that allow for large-scale identification of genetic markers in species of interest (Csencsics et al. 2010; Saarinen & Austin 2010; Brandt et al. 2013b). NGS methods provide rapid and effective means for identification of hundreds to thousands of candidate polymorphic microsatellite loci in any species for which DNA is available (Castoe et al. 2010; Lepais & Bacles 2011; Brandt et al. 2013b).

This study aimed to use NGS technology to characterize species specific polymorphic microsatellite markers implemented using DNA from fecal samples for molecular censusing studies of rhinoceros populations. I focus on black and Sumatran rhinos, two species for which accurate census estimates are difficult to obtain but are required for adequate management. For the black rhinos I used Roche 454 shotgun sequencing data and a standard bioinformatics pipeline to characterize polymorphic microsatellite and assess their success rate when used with DNA from fecal samples. Due to the potential for low genetic diversity in the Sumatran rhinoceros caused by drift and inbreeding in small, isolated populations, an alternative approach was developed. A bioinformatics routine was designed to aligns multiple copies of microsatellite loci and identify those in which variation could be identified. This novel methodology was tested using Illumina sequencing data from two high quality Sumatran rhino DNA samples. This procedure allowed for the exclusion of many potentially monomorphic loci before conducting any laboratory genotyping; thereby, saving time, research funds, and valuable sample. The utility of the microsatellites designed in this study for use in wild populations will be tested by using black rhino fecal samples collected on Namibian game farms to establish census estimates, and Sumatran rhinoceros markers will be tested *in situ* through a collaboration with local Indonesian researchers at the Eijkman Institute.

Methods and Materials

Samples

Endorsement of the proposed research was obtained from the Association of Zoos and Aquariums Rhino Advisory Group and Rhino Research Council, as required for the collection of samples from rhinoceros individuals held *ex situ* in North America. Whole blood samples were obtained from 17 black rhinoceros (*D. b. michaeli*, N = 9; *D. b. minor*, N = 8) (Table 2.1). A total of eight high quality Sumatran rhinoceros samples were collected: 2 whole blood samples from individuals at the Cincinnati Zoo, 2 previously isolated DNA samples from San Diego Zoo Institute for Conservation Research, and four samples of whole blood or tissue from Sumatran rhino individuals collected from Sumatran or Peninsular Malaysia within the past 30 years (Table 2.2). Whole blood samples from North American zoos and research institutions were collected during routine veterinary care; samples were stored in EDTA tubes to prevent clotting and kept refrigerated until DNA isolation (< 1 week from time of collection). DNA was isolated from whole blood or tissue samples using Qiagen DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's recommended protocol. All research was conducted with IACUC approval (protocol # 15053). CITES/ESA Permit 14US84465A/9, CITES COSE Permit 12US757718/9, and appropriate CITES and foreign required export permits were used for specimens imported from international collaborators.

For all 17 black rhino and 2 Sumatran rhino samples from North American zoos, paired fecal samples from the same individual were collected (Table 2.1 & 2.2). Fresh fecal samples were collected by veterinary staff using a sterile collection instrument (e.g., wooden tongue depressor) to scrape approximately 2mL from the exterior of each sample into a collection tube. Samples were stored briefly (less than 3 days) at 4°C, until shipment to the University of Illinois at Urbana-Champaign. Upon arrival, 10 – 12 mL of a 20% DMSO salt saturated solution was added to each collection tube; samples were subsequently stored at -20°C. To compare the impact of DNA extraction methods on fecal genotyping success rate, DNA was isolated from each dung sample using the manufacturer's recommended protocol and a modified protocol for the QIAmp DNA Stool Kit (QIAGEN) as described below.

Black Rhinoceros Marker Design

Total genomic DNA isolated from one black rhino tissue sample, subspecies *D. b. michaeli*, was submitted to the University of Illinois at Urbana-Champaign Biotechnology Center for library preparation and shotgun sequencing on the Roche 454 Genome Sequencer FLX + platform. Sequence data were screened for di-, tri-, tetra-, penta-, and hexanucleotide microsatellite motifs, each with a minimum of 8 tandem repeats, in MSATCOMMANDER 1.0.8 (Faircloth 2008). Flanking primer pairs were designed with stringent criteria using the PRIMER3 (Rozen & Skaletsky 2000) interface in MSATCOMMANDER to meet the following criteria: amplification of a target product in the 75 to 150 bp size range (inclusive of the two primer lengths), optimal length of 20 base pair (range 18 to 22 base pair), optimal melting temperature of 60.0°C (range of 58.0°C to 62.0°C), optimal GC content of 50%, inclusion of at least 1 bp GC clamp, low self or pair complementarity and a maximum end stability of 8.0 (Faircloth 2008). Once designed, a number of quality checks were implemented before selection of primer pairs for testing. To prevent amplification of multiple non-target loci two steps were taken to ensure the uniqueness of the primer sequences: 1) a Perl script was written to search each primer sequence against the entire 454 generated sequence database and 2) primer sequences were searched against the non-redundant BLAST database. Any primers showing evidence of being part of a repetitive element (e.g. LINEs or SINEs) or that closely matched sequences of human DNA (a potential contaminating factor) were removed from further consideration.

Sumatran Rhinoceros Marker Design

High quality total genomic DNA samples from two individual Sumatran rhinoceros (Dsu-33 and Dsu-35), both wild caught on the island of Sumatra and subsequently held *ex situ* in zoos in North America, were submitted to the University of Illinois at Urbana-Champaign Biotechnology Center for library preparation and sequencing on the Illumina MiSeq v3 platform. Each Sumatran rhino sample was given a unique identifying barcoding tag before being pooled for sequencing. For the reads obtained, the following bioinformatics methodology was developed by Dr. Kai Zhao to identify variable microsatellite loci for which high quality primer pairs could be designed and tested. Paired-end reads with overlapping sequence from each individual were

merged using FLASH 1.2.8. A program, SSRSCAN, was written in C to take large genome-scale, unassembled high-throughput sequences and returns microsatellite-containing reads for subsequent analysis. In this program we used relaxed criteria for extraction of reads containing microsatellite motifs, thus filtering uninformative reads out of the working databases. For our purposes SSRSCAN selected reads with di-, tri- and tetra-nucleotide motifs containing at least four repeats. Reads that contained short tandem repeats in the first or last 50 nucleotides were eliminated, and to ensure a sufficient flanking region for primer design was present the full sequence read was required to be at least 120 bp in length.

Our next step was to search among the microsatellite containing reads for potentially polymorphic loci. A Python-based script was written to combine all reads containing microsatellites from both rhinos into one database and subsequently remove the microsatellite motif from each read, leaving a set of flank-pairs (i.e., a pair of flanks from the same original read, one from either side of the microsatellite motif). A MegaBLAST pair-wise analysis, requiring 99% sequence identity and an ungapped alignment, was completed to identify matching flank-pair sequences. The sequences of matching flank-pairs were aligned and those containing microsatellite motifs with a differing number of repeats were retained. Within each alignment the read with the longest minimal flank was chosen as the representative sequence.

The representative sequences of potentially polymorphic loci were further analyzed in MSATCOMMANDER 1.0.8. Sequences were again screened for di-, tri-, and tetra-, microsatellite motifs, this time with a minimum of 6 tandem repeats. Primers were designed in MSATCOMMANDER through an interface with PRIMER3 software to meet the following criteria: amplification of a target product in the 75 to 150 bp size range (inclusive of the two primer lengths), optimal length of 20 base pair (range 18 to 22 base pair), optimal melting temperature of 60.0°C (range of 58.0°C to 62.0°C), optimal GC content of 50%, inclusion of at least 1 bp GC clamp, low self or pair complementarity and a maximum end stability of 8.0 (Faircloth 2008).

Once the set of potential loci were identified a number of quality checks and screening criteria were implemented before selection of primer pairs for testing in the laboratory. To determine if the designed primer pairs would produce amplicons of varying size (as expected at a polymorphic locus), the IPCRESS program was used to run *in silico* PCR. Each primer pair was computationally “amplified” against the joined paired-end sequencing databases from Dsu-33

and Dsu-35. IPCRESS identified “amplicons” that would potentially be produced from each individual during PCR with no priming mismatches, one priming mismatch, and two priming mismatches. Primer sets that showed the potential to produce only one amplicon or amplicons of more than four varying lengths in the *in silico* PCR step were removed from further consideration. Additionally, loci that exhibited broad size ranges (more than 20 bp difference between alleles) were eliminated to prevent potential non-specific amplification. Remaining primer sequences and full amplicon sequences were searched against the non-redundant BLAST database. Any locus showing evidence of being part of a repetitive element (e.g. LINEs or SINEs), or that closely match sequences of human DNA (a potential contaminating factor) were screened out.

Microsatellite Molecular Characterization

The primer pairs identified in the previous step which were most likely to amplify and be polymorphic after quality checks were tested in the laboratory. DNA extracts from the high quality blood samples of 6 Sumatran rhinos or 17 black rhinos (comprised of representatives from two subspecies, *D. b. michaeli* and *D. b. minor*) were used to assess amplification success and variability at the novel loci. PCR products were fluorescently labeled using M13-tailed forward primers (TGTAACGACGGCCAGT) (Boutin-Ganache et al. 2001). Use of an M13 tailed primer is often helpful with genotyping as it reduces cost while increasing the length of the amplicon and reducing stutter peaks (Schuelke 2000; Boutin-Ganache et al. 2001). A primer mix consisting of 8.5uM reverse primer, 0.6uM of M13 tailed forward primer, and 8.5uM of fluorescently labeled M13 forward primer was used for PCR. Primer pairs were initially tested by PCR performed in a 10uL reaction mixture that included: 2mM MgCl₂, 200uM of each dNTP (Applied Biosystems Inc. [ABI]), 1x PCR buffer, and 0.4 units/ul final concentration of AmpliTaq Gold DNA Polymerase (ABI). Negative PCR controls were included with each PCR amplification. A step down PCR algorithm was used with an initial 95°C for 10 min; cycles of 15 sec at 95°C; followed by 30 sec at 60°C, 58°C, 56°C, 54°C, 52°C (2 cycles at each temperature) or 50°C (last 30 cycles); and 45 sec at 72°C; and a final extension of 30 min at 72°C.

PCR amplification success was examined using a 1% agarose gel stained with ethidium bromide. Samples with amplicons present of the expected size range were genotyped by capillary electrophoresis on the ABI 3730XL genetic analyzer at the University of Illinois at Urbana-Champaign Biotechnology Center. Fragments were assessed to determine if the markers produced readable peaks and if they were variable using GeneMapper Version 3.7 software. Microsatellite variability was evaluated by number of alleles per locus (A), expected heterozygosity (H_E), and observed heterozygosity (H_O). In the black rhinoceros samples diversity indices were calculated for all individuals together and separately for each subspecies. Probability that the characterized markers would be useful in establishing individual identity was calculated by P_{ID} and $P_{ID(sib)}$ (Waits et al. 2001) for each marker as well as total P_{ID} and $P_{ID(sib)}$ values for all markers in CERVUS v3.0.7 (Kalinowski et al. 2007).

Optimization for Amplification in Fecal Samples

An initial round of marker testing using dung DNA isolated using the manufacturer's extraction protocol and amplified with the standard procedure (detailed above) failed to produce visible amplicons. Therefore, a series of alternative protocols were tested to identify the best conditions for genotyping DNA from rhinoceros fecal samples. Since only low concentrations of DNA were detected in the fecal extracts by the Qubit 2.0 Fluorometer (Invitrogen) (Table 2.8), an initial optimization of the QIAmp DNA Stool Kit protocol was conducted. The following modifications were made to the QIAmp DNA Stool Kit protocol: each fecal sample was thoroughly homogenized in 20% DMSO salt saturated buffer by vortexing for 5 minutes; the initial sample volume was increased to 800ul; samples were digested overnight in 1mL of ASL buffer and 1mg of proteinase K at 56°C; vortex times throughout were increased (especially for the InhibitEx step which was vortexed for 5 minutes); and final elution was done twice with 50ul of elution buffer each time and a minimum 30 minute incubation at room temperature. DNA concentrations were measured again using the Qubit 2.0 Fluorometer for the modified extraction protocol; concentrations were compared to those obtained with the standard protocol.

To test for the presence of rhinoceros DNA in the fecal extracts and to check for cross contamination between samples an approximately 450 base pair fragment of the mitochondrial control region was amplified in the paired blood and fecal samples. Amplification was completed

using previously published primers (Campbell et al. 1995; Moro et al. 1998) and the following mixture in 20ul reactions with final concentrations of: 0.4uM of each forward and reverse primer, 0.2mM of each dNTP, 1x PCR buffer, 2mM MgCL₂, and 0.4 units of AmpliTaq Gold DNA polymerase. The PCR algorithm for all mitochondrial control region reactions was: initial denaturation at 95°C for 9:45 min; 3 cycles of 20 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 56°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 54°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 52°C, 30 sec at 72°C; 22 cycles of 20 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C; and a final extension at 72°C for 7 min. Mitochondrial PCR products that produced clear, single amplicons of the expected size on an ethidium bromide stained agarose gel were enzymatically purified (Hanke & Wink 1994) using an Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP) reaction. Purified PCR products were Sanger sequenced in both directions using the BigDye Terminator System (ABI), and resolved on an ABI 3730XL capillary sequencer at the University of Illinois at Urbana-Champaign Biotechnology Center. Resulting sequences were trimmed, concatenated, and edited in the software SEQUENCHER (Gene Codes Corporation) and compared between fecal and blood samples from the same individual.

Using the modified extraction protocol there was a substantial increase in total DNA yield and quantity of host specific DNA. Given these outcomes, all subsequent optimization steps used DNA isolated following the modified protocol. Combinations of the following conditions were tested to maximize genotyping success of microsatellite loci in rhinoceros fecal samples:

- 1) The Zymo OneStep™ PCR Inhibitor Removal Kit (Zymo) was used to reduce concentration of PCR inhibitors commonly present in DNA isolated from fecal samples. For a subset of individuals, the total final elution volume of three separate DNA extracts from the same sample was treated with Zymo OneStep™ PCR Inhibitor Removal Kit according to the recommended manufacturer's protocol twice, once, and untreated.

- 2) To assess if the presence of PCR inhibitors was preventing amplification reactions were carried out with full undiluted DNA, 1:2 dilutions, and 1:10 dilutions.
- 3) Two different Taq enzymes were tested: AccuPrime Taq DNA polymerase (Invitrogen), selected for its proofreading and high fidelity characteristics, and AmpliTaq Gold DNA polymerase (ABI), selected for its sensitivity, specificity, and evidence of previous success in amplifying microsatellites in fecal samples.
- 4) Different concentrations of MgCl₂ were assessed for amplification success rate and specificity. Varying final concentrations of 1.5mM, 2.0mM, 2.5mM, 3.0mM, 4.0mM, and 5.0mM of MgCl₂ were tested.
- 5) Touchdown PCRs were selected for testing due to their usefulness in preventing or reducing the incidence of non-specific amplification by optimizing specificity in initial primer binding and increasing final product yield (Korbie and Mattick 2008). Two touchdown thermal profiles were tested; one included annealing temperatures from 66 – 56°C and the second included annealing temperatures from 60 – 50°C.
- 6) Given that the microsatellites being amplified were all less than 200 bp in length, the time necessary for adequate elongation was expected to be short. Length of the elongation step during PCR was varied to be 30 seconds, 10 seconds, or 5 seconds.
- 7) To find the balance between amplification specificity and quantity of target amplicons produced the total number of cycles and the number of cycles completed at each annealing temperature were varied. Success with 3, 4, and 5 cycles at each annealing temperature and a total of 45 or 60 cycles was evaluated.
- 8) When using DNA from fecal samples it is possible that the region targeted for amplification will not be represented in the reaction due to low concentration of host DNA and the small volume of isolate used for PCR. To maximize the amount of host DNA present in each amplification increasing volumes of DNA template of 1ul, 2ul,

and 4ul were tested in reactions with final volumes of 10ul, 20ul, and 40ul, respectively.

Fecal Genotyping Error Rate Analysis

Using the combination of conditions that resulted in the highest rate of amplification, fecal DNA from black rhinos was genotyped. An initial round of testing was completed on three fecal samples at 16 of the microsatellite loci with a final MgCl₂ concentration of 3.0mM. The full set of 17 black rhino fecal DNA samples was genotyped twice, with differing final MgCl₂ concentrations of 1.5mM or 2.5mM. Each DNA extract was purified once using the Zymo OneStep™ PCR Inhibitor Removal Kit prior to amplification. Genotyping was carried out by PCR amplification with fluorescently labeled M13 forward primer mixes (see above) for all markers. The PCR conditions were as follows: 40uL reaction mixture that included final concentrations of: 3.0, 2.5, or 1.5mM MgCl₂, 200uM of each dNTP (Applied Biosystems Inc. [ABI]), 1ug/ul of bovine serum albumin (BSA; New England BioLabs Inc.), 1.6 units of AmpliTaq Gold DNA Polymerase (ABI), 1X PCR Buffer II (ABI), and 4ul of template DNA. Negative and positive (blood DNA) PCR controls were included with each PCR amplification. A touchdown PCR algorithm was used with an initial denaturation at 95°C for 10 min; cycles of 15 sec at 95°C, followed by 15 sec at 66°C, 64°C, 62°C, 60°C, 58°C (4 cycles at each temperature) or 56°C (last 25 cycles), and 10 sec at 72°C; and a final extension of 30 min at 72°C. PCR amplification success was checked on a 1% agarose gel stained with ethidium bromide. Samples with amplicons present in the expected size range were genotyped by capillary electrophoresis on the ABI 3730XL genetic analyzer at the University of Illinois at Urbana-Champaign Biotechnology Center and subsequently genotypes were scored using GeneMapper Version 3.7 software. Genotypes obtained from fecal samples were compared to those from blood DNA; instances of allelic dropout and false allele rate, which produce incorrect genotypes, were recorded.

Given the lack of fecal sample availability for Sumatran rhinos, an assessment of genotyping accuracy was not possible. However, two fecal samples provided by Cincinnati Zoo were used to evaluate potential genotyping success. One sample from Dsu-28 was considered low quality due to post mortem collection and frozen storage followed by repeated freeze thaw

cycles; a second sample from Dsu-44 was considered high quality due to fresh collection and immediate storage in 20% DMSO salt saturated buffer. Six markers were tested three times on each of the fecal samples; genotypes from blood samples of the same individuals were also scored. The remaining 23 markers were each genotyped once in the fecal samples; at the time of genotyping, blood from Dsu-44 was not available; however, genotypes from the dam and sire of this individual were collected. Genotyping was carried out using the following PCR conditions with fluorescently labeled M13 forward primers (see above) for markers: 10 or 20uL reaction mixture that included: 4mM MgCl₂, 200uM of each dNTP (Applied Biosystems Inc. [ABI]), 1ug/ul of bovine serum albumin (BSA; New England BioLabs Inc), 1X PCR Buffer II (ABI), 1.0 unit final concentration of AmpliTaq Gold DNA Polymerase (ABI), and 1 or 2ul of template DNA. Negative and positive (blood DNA) controls were included with each PCR amplification. The same step down PCR algorithm that is detailed above for black rhino fecal amplifications was used for the Sumatran rhino fecal genotyping. PCR amplification success was checked on a 1% agarose gel stained with ethidium bromide. Samples with amplicons of the expected size range were genotyped by capillary electrophoresis on the ABI 3730XL genetic analyzer at the University of Illinois at Urbana-Champaign Biotechnology Center and subsequently genotyped using GeneMapper Version 3.7 software.

Results

Black rhino marker design and characterization

Roche 454 shotgun sequencing of a black rhinoceros sample generated a total of 23,511 reads, with an average length of 556 bp. Microsatellite motifs with eight or more repeats were identified in 427 reads, thus 1.8% of the total reads contained the targeted repeat regions. Primers met the stringent design requirements for 75 of the microsatellite containing loci. After quality checks of the primer sequences were complete, a set of 65 high quality markers were identified for further testing, of which 48 were evaluated for variability. A total of 17 of the loci were variable and produced genotyping peak patterns that could be reliably scored. In a combined dataset including members of both subspecies, the average number of alleles per locus was 4.2 and ranged from 2 to 8 (Table 2.3). Loci *Dibi3* and *Dibi22* were monomorphic within the

subspecies *D. b. minor* while loci *Dibi48* and *Dibi51* were monomorphic within *D. b. michaeli*; each of these loci was found to be polymorphic in the other subspecies (Table 2.4). The average expected heterozygosity across all 17 markers was 0.55; the average observed heterozygosity was 0.41, and overall F_{IS} value across the markers was 0.26 (Table 2.3). Within the *D. b. michaeli* subspecies the average number of alleles per locus was 3.4 and ranged from 1 to 6. The average expected heterozygosity within *D. b. michaeli* was 0.48; the average observed heterozygosity was 0.39, and overall F_{IS} value across the markers was 0.22 (Table 2.4). Within the *D. b. minor* subspecies the average number of alleles per locus 2.5 and ranged from 1 to 5. The average expected heterozygosity within *D. b. minor* was 0.44; the average observed heterozygosity was 0.43 (Table 2.4). The overall F_{IS} value across the markers was 0.02. Cumulative P_{ID} and $P_{ID(sib)}$ suggest that a subset of these markers can be used to confidently distinguish individual identity in both subspecies at a $p < 0.001$ level (Table 2.5).

Sumatran rhino marker design and characterization

The availability of high molecular weight DNA for the Sumatran rhinoceros enabled the completion of NGS sequencing this species. A total of 30,556,224 sequencing reads were obtained, with individual Dsu-33 producing 16,813,030 reads (average length of 410 bp) and individual Dsu-35 producing 13,743,194 reads (average length of 440 bp). After paired-end sequences were joined databases of 7,399,098 reads for Dsu-33 and 5,993,320 reads for Dsu-35 were created. A total of 176,357 reads (2.4%) from Dsu-33 and 167,849 reads (2.8%) from Dsu-35 were found to contain microsatellite motifs with four or more repeat units by SSRSCAN. Of the loci containing microsatellite motifs, 861 potentially polymorphic loci were identified. Suitable priming regions were identified for 229 of the potentially polymorphic microsatellite loci. After IPCRESS and quality checking a final set of 55 potentially polymorphic loci remained for testing in the laboratory.

Of the 55 loci identified as potentially polymorphic, 53 produced amplicons with a single band present in the expected size range for at least 2 DNA samples in an initial PCR. Further, 29 of the markers produced 2 or more alleles across the 6 tested samples (Table 2.6). The average number of alleles per locus was 2.4 and ranged from 2 to 4. The average expected heterozygosity across all 29 loci was 0.45, and the average observed heterozygosity was 0.30. (Table 2.6) The

overall F_{IS} value across the markers was 0.44. Cumulative P_{ID} and $P_{ID(sib)}$ suggest that a subset of these markers can be used to confidently distinguish individual identity at the $p < 0.0001$ level (Table 2.7).

Optimization for fecal analysis

The modified DNA extraction protocol resulted in substantial increase in DNA yield from fecal samples compared to the manufacturer protocol (Table 2.8). All DNA isolates from fecal samples were sequenced for a short portion of the mtDNA control region. Resulting sequences show no differences in haplotypes between fecal and blood samples collected from the same individual and no evidence of secondary peaks that may be indicative of contamination. This confirmed the presence of rhinoceros DNA in all of the fecal samples and the lack of cross contamination between samples. Given the increase in DNA concentration and positive amplification of mitochondrial haplotypes, all further PCRs were conducted with DNA isolated using the modified protocol.

During DNA extraction using the modified protocol co-extraction of increased amounts of plant and microbial DNA and inhibitors, along with a suspected increase in host rhinoceros DNA, is likely to have occurred. To combat the presence of PCR inhibiting compounds, commonly from plant materials, the Zymo OneStep™ PCR Inhibitor Removal Kit was used to reduce concentration of PCR inhibitors commonly present in DNA extract from fecal samples. Samples treated once showed improved amplification success compared to untreated samples; however, there was no evident improvement between samples treated once and samples treated twice. Given the increased loss of DNA template with each successive Zymo OneStep™ PCR Inhibitor Removal treatment, one cleanup was determined to be optimal. To further assess the presence of inhibitors, DNA extracts were tested in PCR amplification at full concentration (undiluted), at a 1:2 dilution, and at a 1:10 dilution. There was no improvement of amplification with increased dilution suggesting that inhibition of PCR is not a concern for downstream applications; therefore, all subsequent reaction were carried out with undiluted DNA extract as template.

The next step in optimizing the PCR was testing different enzymes and adjusting the final concentration of magnesium chloride ($MgCl_2$). A series of reactions were set up to compare the

specificity and amplification success rate of AccuPrime Taq to AmpliTaq Gold in DNA from fecal samples. Both enzymes were tested on four dung DNA samples at two microsatellite markers, and with three MgCl₂ concentrations (1.5mM, 2.5mM, and 4mM). Overall, the AccuPrime Taq performance was inferior compared to AmpliTaq Gold, showing higher rates of non-specific binding and stronger amplification of extraneous bands. For both enzymes increased concentrations of MgCl₂ resulted in higher rates of extraneous banding.

Increasing concentrations of MgCl₂ in PCRs, when using DNA from fecal samples as template, produced two contradictory outcomes: higher rate of extraneous banding from non-specific primer binding and increased amplification rate of the targeted region. Such extraneous banding can be particularly problematic if amplification of a non-target region occurs close to, or within, the size range of the expected amplicon; thus, potentially producing false alleles. Throughout the optimization process for the black rhino microsatellites various concentrations of MgCl₂ were tested, ranging from 1.5mM to 5.0mM. In order to retain the desired effect of improved amplification rate of targeted loci while eliminating the non-specific amplification byproduct of high MgCl₂ concentrations annealing temperature ranges and length of elongation steps were adjusted. Touchdown thermal cycles were implemented for all reactions; of the two thermal profiles with annealing temperature ranges of 60 – 50°C or 66 – 56°C with step downs of 2°C, the 66 – 56°C range reduced the extent of extraneous banding. This range of annealing temperatures was tested with elongation times of 30 seconds, 15 seconds, 10 seconds and 5 seconds. A substantial reduction in extraneous banding was observed with the elongations times less than 30 seconds; however, no distinct difference was seen between 10 and 5 second elongations.

In a final series of optimization steps the number of cycles at each annealing temperature, the total number of cycles, and total reaction volume were varied. Initially 3 or 5 cycles at each touchdown temperature from 66 – 58°C were tried with a total of 45 cycles; no observable difference in rate of amplification was noted. Remaining touchdown thermal cycles were conducted with 4 cycles at each annealing temperature from 66 – 58°C. A total of either 45 or 60 of cycles was completed; while 60 cycles appeared to increase the strength of the amplicon it also caused increased primer dimer and stronger patterns of extraneous banding. A total of 45 cycles, or fewer, was considered optimal for all further PCRs. The last modification tested was increasing the total PCR volume from 10ul to 20ul or 40ul. The largest overall improvement of

any adjusted parameter was seen with increased reaction volume. A reaction volume of 40ul with 4ul of DNA template produced the most positive amplifications.

For all amplification reactions using microsatellite markers in black rhinoceros fecal DNA samples the following parameters were used: extraction of DNA using the modified Qiagen protocol, one treatment with the Zymo OneStep™ PCR Inhibitor Removal Kit according to manufacturer's recommended protocol, undiluted DNA, and AmpliTaq Gold enzyme. The thermal profile used was an initial denaturation at 95°C for 10 min; cycles of 15 sec at 95°C, followed by 15 sec at 66°C, 64°C, 62°C, 60°C, 58°C (4 cycles at each temperature) or 56°C (25 cycles), and 10 sec at 72°C; and a final extension of 30 min at 72°C. An initial subset of three fecal DNA samples was tested at 16 loci with a final concentration of 3.0mM MgCl₂. Due to some extraneous banding complete runs including all 17 black rhino fecal DNA samples were tested twice with the final concentration of MgCl₂ of 1.5mM and 2.5mM.

Fecal genotyping error rate

Given that amplification of microsatellites in fecal samples appears to be highly impacted by final MgCl₂ concentration, an initial round of testing using three fecal DNA samples across 16 loci with a final concentration of 3.0mM MgCl₂ was completed. Overall, genotypes were obtained for 91.7% of the loci among all samples; 86.4% of the obtained genotypes were correct with a total allelic dropout rate of 6.8% (excluding loci with no amplification) and no false alleles (Tables 2.9 & 2.10). Thus, multilocus genotypes showed high amplification success rate across the loci with moderate occurrence of allelic drop out and no false alleles. However, due to extraneous banding at multiple loci close to the expected size range of the targeted amplicon lower MgCl₂ concentrations were used for further testing on all black rhino fecal DNA samples.

Overall, out of 16 loci amplified in black rhinoceros fecal samples with a final concentration of 2.5mM of MgCl₂ the average number of loci successfully genotyped per sample was 10.2. Five of the samples showed amplification success at ≤ 50% of test microsatellite loci. Total amplification success rate across all markers and individuals was 64.0%; within sample rate of amplification ranged from 12.5% (2 out of 16 loci) to 100% (Table 2.11). The proportion of correct single locus genotypes was 75.9% when compared to those obtained from matched blood samples (Table 2.11); however, there was a wide variation in genotyping error between

samples. Overall error rates across all loci in all individuals were 10.6% incidence of allelic dropout and 3.2% false allele rate (Table 2.11). Allelic dropout rate varied by locus ranging from 0% (*Dibi3*) to 50% (*Dibi34*) (Table 2.12). False allele rate per locus ranged from 0% (at 11 loci) to 33% (*Dibi34*); a total of 5 loci showed evidence of false alleles (Table 2.12). High values for allelic dropout and false alleles were skewed by one locus (*Dibi34*) which exhibited poor amplification; when *Dibi34* is excluded from consideration the highest rate of allelic dropout was 20% (*Dibi25*) and the highest rate of false alleles was 16.7% (*Dibi15*) (Table 2.12).

Overall, out of 16 loci amplified in black rhinoceros fecal samples with a final concentration of 1.5mM of MgCl₂ the average number of loci successfully genotyped per sample was 9.1. Six of the samples showed amplification success rates of $\leq 50\%$ of loci. Total amplification success rate across all markers and individuals was 56.6%; within sample rate of amplification ranged from 0% (0 out of 16 loci) to 88% (14 out of 16 loci) (Table 2.13). Two markers (*Dibi24* and *Dibi34*) failed to amplify alleles in any sample. The proportion of correct single locus genotypes was 75.3% when compared to those obtained from matched blood samples (Table 2.13). Across all markers and loci there was an 11.7% rate of allelic dropout and 1.3% false allele rate (Table 2.13). Allelic dropout rate per locus varied across loci from 3.9% (*Dibi9*) to 25% (*Dibi25* and *Dibi56*), and false allele rate per marker ranged from 0% (at 10 loci) to 4.6% (*Dibi22*); a total of 4 loci showed evidence of false alleles (Table 2.14).

The Sumatran rhino high quality fecal sample (Dsu-44) showed no evidence of allelic dropout or false alleles at the six loci for which both blood and dung DNA were genotyped in any of the three repeated amplifications. The low quality sample (Dsu-28) showed reduced rates of successful amplification over three rounds of amplification at the same six loci, producing amplicons eight times (out of 18 reactions). In four of these amplifications the correct genotype was present, one amplification showed allelic dropout, and in three amplifications (all at the same loci) a false allele was present. At the other 23 markers Dsu-44 produced genotypes for 22 loci in one round of amplification; based on comparison to parental genotypes there appears to be no evidence of unexpected alleles or allelic. Dsu-28 produced genotypes for six out of the additional 23 loci, three of the genotypes are the same as those obtained in blood samples while there is evidence of allelic dropout at the other three. Since the Sumatran fecal samples were amplified prior to completion of all troubleshooting parameters discussed above, a majority of

the reactions were only tested in a final reaction volume of 10ul; success rate of the lower quality sample would likely be improved with increased reaction volume.

Discussion

Large sequence databases produced by next generation sequencing platforms have been used to identify and develop high numbers of informative microsatellite loci for many species (Castoe et al. 2010; Csencsics et al. 2010; Saarinen & Austin 2010; Lepais & Bacles 2011; Brandt et al. 2013b). Using two NGS platforms and unique bioinformatics pipelines I was able to characterize microsatellite markers that will ultimately be used for censusing populations of two species of endangered rhinoceros from non-invasively collected fecal samples. Through Roche 454 sequencing of a black rhinoceros sample 17 polymorphic microsatellites were characterized; initial results suggest these markers can be successfully implemented to amplify DNA from fecal samples. For the Sumatran rhinoceros a novel bioinformatics pipeline was developed to scan large sequencing databases, containing one or more individuals, for putatively polymorphic loci. From Illumina MiSeq databases of two Sumatran rhinos 29 polymorphic microsatellites were identified and characterized. This study found that microsatellite loci designed to amplify short target regions (< 200) specifically for use in low quality, degraded DNA sources can be characterized by employing various bioinformatics techniques on NGS databases.

Previous studies have found that among black rhino subspecies, *D. b. michaeli* is the most diverse and *D. b. minor* is the least diverse (Harley et al. 2005; Scott 2008; Karsten et al. 2011). Unexpectedly, observed heterozygosity calculated from the 17 variable microsatellite loci characterized in this study was slightly higher among the *D. b. minor* individuals ($H_O = 0.43$) compared to the *D. b. michaeli* individuals ($H_O = 0.39$); although the average number of alleles per locus was higher in the *D. b. michaeli* samples ($A = 3.4$) than in the *D. b. minor* sample ($A = 2.5$). Additional samples would be needed to further assess the diversity within each subspecies. To confidently determine individual identity ($p < 0.001$) using estimates of P_{ID} and $P_{ID(sib)}$ (Waits et al. 2001) between 8 and 14 loci, respectively, are needed for *D. b. michaeli* and between 9 and 15 loci, respectively, are needed for *D. b. minor* individuals. It may be possible to achieve individual identity using fewer loci, if the most informative markers for each subspecies are used. The novel microsatellite markers designed here amplified successfully in both subspecies

and have the capacity for individual identification; as such, they can be widely implemented in conservation genetic studies of black rhinoceros populations.

The Sumatran rhinoceros is one of the most endangered mammalian species; yet, due to a lack of available high quality samples very little genetic research has been conducted on this species. The Sumatran rhinoceros, which is likely to exhibit low levels of genetic diversity as a result of genetic drift and potential inbreeding during persistence in small, isolated populations (Frankham 2005; Jamieson 2015), was used as a study species for the validation of a novel bioinformatics pipeline. This pipeline scans genomic data for putatively polymorphic loci, which can reduce the amount of time, money, and sample expended in the lab during characterization of microsatellite markers. By comparing the expected amplicon length of microsatellite loci within and between Illumina sequencing databases for two Sumatran rhinos hundreds of likely polymorphic markers were identified without lab work. When the best 55 putatively polymorphic markers were tested on six Sumatran rhino samples 29 were found to amplify consistently and to be variable. As expected, the Sumatran rhino exhibited low levels of diversity across the markers ($A = 2.4$; $H_O = 0.30$) and a high fixation index ($F_{IS} = 0.44$), indicating potential inbreeding or subpopulation structuring within the genotyped samples. To confidently determine individual identity ($p < 0.001$) using estimates of P_{ID} and $P_{ID(sib)}$ (Waits et al. 2001) between 10 and 17 loci, respectively, are needed. The number of markers required to identify individuals will vary based on the composition of the population being surveyed; to prevent underestimation of abundance more markers will need to be implemented when populations have lower diversity which results in individuals sharing multi-locus genotypes (Taberlet and Luikart 1999; McKelvey & Schwartz 2004). Development of microsatellite markers that amplify short amplicons, and are therefore likely to be successful in genotyping from fecal samples (Butler et al. 2003; Ishida et al. 2012; Brandt et al. 2013b), is immensely important for conservation by allowing for censusing studies and biological surveys of Sumatran rhinos.

Several previous studies have successfully estimated population size by genotyping DNA from fecal samples (Kohn et al. 1999; Bellemain et al. 2005; Mowry et al. 2011; Bonesi et al. 2013; McCarthy et al. 2015). There are, however, many technical considerations to be made when working with fecal samples given the propensity for amplification and genotyping errors resulting from the degraded nature of the host DNA and the presence of non-target DNA and PCR inhibitors (Taberlet & Luikart 1999; Taberlet et al. 1999; McKelvey & Schwartz 2004). It

is clear from this study that amplification success rate and genotyping error are associated with sample quality, marker design criteria, and PCR protocols. By optimizing each of these components I was able to validate the use of microsatellites in DNA samples from rhino feces. Markers or samples that failed consistently despite optimization protocols (e.g., *Dibi34*) are likely to low quality and should not be included in censusing studies (Taberlet & Luikart 1999). The parameters that appeared to most impact amplification success in the rhinoceros samples were reaction volume and MgCl₂ concentration. Magnesium chloride concentration in PCR alters the activity and specificity of Taq polymerase (Williams 1989; Harris and Jones 1997). Increased concentrations of MgCl₂ are often found to improve the success of amplification in reactions with low target DNA copy number; however, high concentrations of MgCl₂ can result in non-specific binding and the amplification of extraneous product (Williams 1989; Harris and Jones 1997). To combat the problems associated with varying MgCl₂ concentrations annealing temperatures and cycle lengths must be adjusted to prevent extraneous banding or weak amplification. Poor amplification or genotyping error can result in overestimation of population size (Waits & Leberg 2000; Creel et al. 2003; McKelvey & Schwartz 2004); therefore, it is essential to limit these sources of error when possible.

Since non-invasive studies are prone to the incorporation of multiple samples representing the same individual (Taberlet & Luikart 1999; McKelvey & Schwartz 2004) it is important to implement a panel of markers with relatively low rates of false alleles and allelic dropout. In the black rhino fecal samples genotyping errors from allelic dropout were more common than errors caused by the presence of false alleles, which is consistent with previous studies (Lucchini et al. 2002; McKelvey & Schwartz 2004). Rates of amplification and genotyping success vary widely in the published literature, for example genotyping of North American river otter scat samples yielded a 24% success rate (Mowry et al. 2011) while analysis of mountain gorilla feces generated over 98% success rate (Guschanski et al. 2009). For the black rhinoceros fecal samples in this study amplification success rate, calculated as the proportion of samples for which a genotype could be scored, ranged from 56.6% to 91.7% depending on the PCR conditions; amplification success rate decreased as MgCl₂ concentration was reduced. Similarly rates of allelic dropout and false allele genotyping error varied greatly by marker and sample. For the black rhinos the rate of allelic dropout ranged from 6.8 – 11.7 % and false alleles from 0 – 3.2%; higher concentrations of MgCl₂ tended to result in lower rates of

allelic dropout but higher rates of false alleles. Other studies have reported similar or worse performance of microsatellites in fecal samples, e.g., 12% dropout rate in wolverine (*Gulo gulo*) feces (Hedmark et al. 2004) and 18% dropout in wolf (*Canus lupus*) scats (Lucchini et al. 2002). When implementing these markers for population censusing of black rhinos it will be essential to use a multi-tube (Taberlet et al. 1996) or a modified multi-tube approach (Frantz et al. 2003; Paetkau 2003) to reduce the instance of mis-identifying two samples from the same individual as unique. Amplification of each sample multiple times prevents incorrectly assigning a genotype that has been impacted by allelic dropout or false alleles. In the wolverine study by Hedmark and colleagues (2004) it was noted that after amplifying each sample three times all multi-locus genotypes, determined by consensus, were correct compared to reference genotypes from tissue / blood samples. Further analysis of these novel markers through a multi-tube or modified multi-tube approach will provide an accurate estimate of allele scoring error rates per locus.

The black rhinoceros fecal genotyping project is being done in conjunction with the Namibian Ministry of Environment and Tourism and Namibian rhinoceros managers to reach the goal of censusing populations using non-invasively collected samples. Development of polymorphic microsatellite loci that reliably amplify in fecal samples from *ex situ* black rhinos is the first step in reaching the larger project goal. The studies in progress on Namibian black rhinoceros samples are designed to answer the following main questions: how reliable are the novel microsatellite markers for genotyping of fecal samples collected from wild animals and does sampling scheme impact census estimates / which sampling scheme provides the most accurate census estimate. To validate the utility of these markers in censusing of Namibian black rhinoceros populations, three sample collection schemes are ongoing. The first sampling scheme will be used to further assess amplification success and error rates in the Namibian black rhino subspecies (*D. b. bicornis*); for this paired blood and fecal samples have been collected from anesthetized wild animals that are undergoing routine medical procedures / vaccinations / ear notching. The second collection design involves anti-poaching units that routinely track individual animals; these fecal samples will be collected immediately after defecation and will be used to assess how well the markers amplify in fresh wild samples. The third sample set will be come from a private game farm with a known population size. These samples will be collected by guides during game drives and by wildlife managers out in the field; they will represent the most likely scenario under which fecal samples will be collected for censusing efforts. Most

often these samples will have been exposed to conditions in the field for a short period of time (\leq 48 hours) before collection and which animal they are from will not be known. From this third sample set we will assess if genotyping of fecal samples provides an accurate census estimate when compared to the known number of animals on the reserve.

Similarly, the Sumatran rhinoceros fecal genotyping project is being done in conjunction with Indonesian collaborators who have already begun testing the microsatellites in fecal samples collected from wild populations. They have produced preliminary results suggesting that genotypes can be successfully obtained for population censusing. The need for better methods to survey and census populations of Sumatran rhinos has become abundantly clear recently. A population estimate of about 200 individuals has been reported since 2009; however, an updated estimate finds no more than 100 individuals persist across the Sumatran range (Havmøller et al. 2016). It is also noted that the current number contains a large amount of uncertainty due to a lack of population data for many regions and are generally considered “best estimates.” Since management decisions for all rhinoceros species are based on census values and surveys of suitable habitat it is crucial to have reliable methods for estimating population size.

Given the high success rate observed using low coverage genomic shotgun sequences to design potentially polymorphic loci for the Sumatran rhinoceros it is reasonable to assume that this approach can be implemented in other species. Similarly to the Sumatran rhino, the Javan rhinoceros population has persisted in low numbers for many generations with fewer than 65 individuals estimated to remain (Jong 2016). It is likely that these remaining individuals will exhibit some degree of loss of genetic diversity. Further, it is very difficult to gain access to high quality samples, and there are no Javan rhinos held in zoos or *ex situ* breeding facilities. With a set of Javan rhino bones obtained from various international museums, we intend to conduct Illumina HiSeq sequencing and identify potentially polymorphic markers using the bioinformatics pipeline discussed here. Overall, this study has resulted in panels of microsatellite markers that will be useful in estimating population census size and informing managers about the genetic diversity and status of these endangered species.

Tables

Table 2.1. Black rhinoceros sample information.

Lab ID	Subspecies	Specimens	Institution	Sex	Birth Year	Studbook Number	Sire	Dam
Dbi-870	<i>michaeli</i>	Blood/dung	Cincinnati Zoo	M	2002	870	488	397
Dbi-294	<i>michaeli</i>	Blood/dung	Oklahoma City Zoo	F	1999	294	169	190
Dbi-490	<i>michaeli</i>	Blood/dung	Oklahoma City Zoo	M	1995	490	301	53
Dbi-664	<i>michaeli</i>	Blood/dung	Lincoln Park Zoo	M	1997	664	377	213
Dbi-362	<i>michaeli</i>	Blood/dung	Lincoln Park Zoo	M	1986	362	259	202
Dbi-935	<i>michaeli</i>	Blood/dung	Lincoln Park Zoo	F	2008	935	636	677
Dbi-683	<i>michaeli</i>	Blood/dung	Cleveland Metroparks Zoo	F	1993	683	Wild	Wild
Dbi-904	<i>michaeli</i>	Blood/dung	Cleveland Metroparks Zoo	F	2003	904	457	683
Dbi-957	<i>michaeli</i>	Blood/dung	Cleveland Metroparks Zoo	M	2012	957	435	904
Dbi-718	<i>minor</i>	Blood/dung	Fossil Rim Wildlife Center	F	1999	718	401	462
Dbi-667	<i>minor</i>	Blood/dung	White Oaks Conservation Center	M	1997	667	522	410
Dbi-521	<i>minor</i>	Blood/dung	White Oaks Conservation Center	M	1999	521	378	410
Dbi-669	<i>minor</i>	Blood/dung	White Oaks Conservation Center	F	2005	669	401	462
Dbi-847	<i>minor</i>	Blood/dung	Disney Animal Kingdom	F	2000	847	670	486
Dbi-873	<i>minor</i>	Blood/dung	Disney Animal Kingdom	M	2001	873	670	574
Dbi-868	<i>minor</i>	Blood/dung	Fossil Rim Wildlife Center	M	2001	868	465	411
Dbi-0022	<i>minor</i>	Blood/dung	Fossil Rim Wildlife Center			Unassigned		

Table 2.2. Sumatran rhinoceros sample information.

Lab ID	Specimen Type	Name	Institution	Sex	Birth Year	Studbook Number	Location of Origin
Dsu-33	DNA	Rami	San Diego Zoo (ICR)	F	1991	33	Sumatra
Dsu-35	DNA	Tanjung	San Diego Zoo (ICR)	M	1980	35	Sumatra
Dsu-28	Blood/dung	Ipuh	Cincinnati Zoo	M	1980	28	Sumatra
Dsu-29	DNA	Emi	Royal Ontario Museum	F	1988	29	Sumatra
Dsu-63	DNA	Merah	Royal Ontario Museum	F	1980	19	Malay Peninsula
Dsu-64	DNA	Minah	Royal Ontario Museum	F	1987	15	Malay Peninsula
Dsu-66	DNA	Panjang	Royal Ontario Museum	F	1983	13	Malay Peninsula
Dsu-44	Blood/dung	Harapan	Cincinnati Zoo	M	2007	44	Captive born

Table 2.3. Characterization of genetic diversity in black rhinoceros microsatellites.

Locus	Repeat Motif	Primer Sequence (5' - 3')	A	Size Range (bp)	H _E	H _O	F _{IS}
<i>Dibi3</i>	AC(8)	F: GTCAGGCTTGGGTGTGTAAC R: TTGGGCAAGTGGTGGGTTAG	3	145 - 155	0.12	0.12	-0.02
<i>Dibi5</i>	AC(8)	F: CAGAGTGACCAGGGTGTGTC R: ATCCTTCTCCAGTGCCTGTG	2	170 - 172	0.51	0.53	-0.01
<i>Dibi9</i>	AC(13)	F: GCTCTGCCAACTTCCTCTTC R: GGTGTGTGACATGGCATCAG	2	134 - 136	0.40	0.29	0.27
<i>Dibi15</i>	AC(19)	F: GACATGACAGAGACGGGAGG R: AGGCTGTGCTTCTTGGAGAG	6	135 - 157	0.71	0.41	0.43
<i>Dibi22</i>	AC(8)	F: CTGCCGGTTATTCACGATGG R: GTCTTCAGGCTTACACACCC	2	166 - 170	0.40	0.18	0.57
<i>Dibi23</i>	CG(9)	F: TTACGTCCGAGAAAGCCTGG R: CAAACCGTTGCTTCTTTGTGAG	2	133 - 135	0.22	0.12	0.48
<i>Dibi24</i>	AC(12)	F: TGGCCTCCTTAAAGAACAGC R: TGACAGTGGGTTGGCTAAGC	7	130 - 142	0.85	0.77	0.10
<i>Dibi25</i>	AC(13)	F: GACAGATTCCTTGGGCACAC R: GCAACAGACAACAGTAGGGC	6	146 - 162	0.76	0.65	0.15
<i>Dibi26</i>	AC(10)	F: GAATAACTCAGTTTGGGCGC R: TGCATTCTCAGTGCCCAC	7	157 - 179	0.71	0.29	0.59
<i>Dibi27</i>	AC(10)	F: AACCTTACCACAGCCTCTCC R: ACTGACAGATGTGGGACCTG	4	168 - 174	0.75	0.53	0.30
<i>Dibi32</i>	AC(10)	F: TAATGCCCTCAGAGTCCACC R: AACAGCCTAAGTGTCCATCAG	7	166 - 182	0.81	0.53	0.35
<i>Dibi34</i>	AC(19)	F: GATGCCCGGAGAAATGATGC R: TGCTGGTTCATCGTTCACAAG	5	136 - 144	0.65	0.47	0.28
<i>Dibi48</i>	AC(8)	F: ACCAGATCTACCAACCTGCC R: AAGCTGGCTGTGGAGAGAAG	2	132 - 134	0.37	0.12	0.69
<i>Dibi49</i>	AC(11)	F: TAGCCCAGGGTCAATCTTCC R: TGAGTGTCCCTGTGCAGAAC	8	165 - 185	0.88	0.77	0.14
<i>Dibi50</i>	AC(13)	F: GGGTGATGTTTAAAGCCTCACC R: AAGATTGGCATTGGATGTTAGC	3	147 - 151	0.35	0.29	0.16
<i>Dibi51</i>	AC(10)	F: AGAAGCCTCCTCTGCAGATC R: CCTTAGCTTACTCTCACTGCC	2	150 - 152	0.26	0.29	-0.14
<i>Dibi56</i>	AT(8)	F: TCTCCACAGCCAGTCTTTCC R: GTAAACATGCTCCTGACACATC	3	162 - 166	0.66	0.59	0.11

A is the mean number of alleles per locus.

H_E is the mean expected heterozygosity.

H_O is observed heterozygosity.

F_{IS} is the average deviation from Hardy-Weinberg proportions.

Table 2.4. Genetic diversity in black rhinoceros subspecies at 17 novel microsatellite loci.

Locus	<i>D. b. michaeli</i>				<i>D. b. minor</i>			
	A	H _E	H _O	F _{IS}	A	H _E	H _O	F _{IS}
<i>Dibi3</i>	3	0.22	0.22	-0.03	1	0.00	0.00	-
<i>Dibi5</i>	2	0.52	0.44	0.16	2	0.53	0.63	-0.21
<i>Dibi9</i>	2	0.11	0.11	-	2	0.53	0.50	0.07
<i>Dibi15</i>	5	0.64	0.56	0.14	2	0.23	0.25	-0.08
<i>Dibi22</i>	2	0.53	0.33	0.38	1	0.00	0.00	-
<i>Dibi23</i>	3	0.31	0.11	0.65	2	0.13	0.13	-
<i>Dibi24</i>	5	0.74	0.78	-0.06	3	0.68	0.75	-0.12
<i>Dibi25</i>	6	0.86	0.78	0.10	3	0.57	0.50	0.13
<i>Dibi26</i>	4	0.53	0.11	0.80*	4	0.64	0.50	0.23
<i>Dibi27</i>	4	0.60	0.44	0.26	3	0.63	0.63	0.00
<i>Dibi32</i>	6	0.72	0.67	0.08	2	0.53	0.38	0.30
<i>Dibi34</i>	4	0.65	0.56	0.15	3	0.34	0.38	-0.11
<i>Dibi48</i>	1	0.00	0.00	-	2	0.53	0.25	0.55
<i>Dibi49</i>	4	0.73	0.78	-0.07	5	0.81	0.75	0.08
<i>Dibi50</i>	3	0.45	0.33	0.27	2	0.23	0.25	-0.08
<i>Dibi51</i>	1	0.00	0.00	-	2	0.46	0.63	-0.40
<i>Dibi56</i>	3	0.60	0.44	0.27	3	0.66	0.75	-0.15

A is the mean number of alleles per locus.

H_E is the mean expected heterozygosity.

H_O is observed heterozygosity.

F_{IS} is the average deviation from Hardy-Weinberg proportions

*statistically significant, $p < 0.05$.

Table 2.5. Estimates of probability of identity (P_{ID}) and probability of identity between siblings ($P_{ID(sib)}$) with total P_{ID} and $P_{ID(sib)}$ among genotypes within black rhino subspecies.

Locus	<i>D. b. michaeli</i>				<i>D. b. minor</i>			
	P_{ID}	Total P_{ID}	$P_{ID(sib)}$	Total $P_{ID(Sib)}$	P_{ID}	Total P_{ID}	$P_{ID(sib)}$	Total $P_{ID(Sib)}$
<i>Dibi3</i>	0.64	0.6439	0.81	0.8091	1.00	1.0000	1.00	1.0000
<i>Dibi5</i>	0.38	0.2435	0.60	0.4835	0.38	0.3790	0.60	0.5987
<i>Dibi9</i>	0.81	0.1964	0.90	0.4348	0.38	0.1421	0.59	0.3555
<i>Dibi15</i>	0.21	0.0414	0.50	0.2175	0.63	0.0902	0.80	0.2841
<i>Dibi22</i>	0.38	0.0155	0.59	0.1292	1.00	0.0902	1.00	0.2841
<i>Dibi23</i>	0.53	0.0082	0.74	0.0951	0.79	0.0709	0.89	0.2523
<i>Dibi24</i>	0.14	0.0011	0.44	0.0414	0.21	0.0150	0.49	0.1228
<i>Dibi25</i>	0.06	0.0001	0.36	0.0150	0.28	0.0042	0.56	0.0681
<i>Dibi26</i>	0.30	<0.0001	0.58	0.0086	0.21	0.0009	0.50	0.0342
<i>Dibi27</i>	0.24	<0.0001	0.53	0.0046	0.24	0.0002	0.52	0.0177
<i>Dibi32</i>	0.15	<0.0001	0.45	0.0020	0.38	0.0001	0.60	0.0106
<i>Dibi34</i>	0.20	<0.0001	0.50	0.0010	0.49	<0.0001	0.71	0.0075
<i>Dibi48</i>	1.00	<0.0001	1.00	0.0010	0.38	<0.0001	0.59	0.0045
<i>Dibi49</i>	0.16	<0.0001	0.44	0.0005	0.10	<0.0001	0.40	0.0018
<i>Dibi50</i>	0.38	<0.0001	0.63	0.0003	0.63	<0.0001	0.80	0.0014
<i>Dibi51</i>	1.00	<0.0001	1.00	0.0003	0.42	<0.0001	0.64	0.0009
<i>Dibi56</i>	0.27	<0.0001	0.53	0.0002	0.22	<0.0001	0.50	0.0004

P_{ID} is the probability of identity.

$P_{ID(sib)}$ is the probability of identity between siblings.

Table 2.6. Characterization of genetic diversity in Sumatran rhinoceros microsatellites.

Locus	Primer Sequence (5' - 3')	A	Size Range (bp)	H _E	H _O	F _{IS}
<i>Disu542</i>	F: AAACACTACAGGCACGTACAGC R: TTGAGAGATGAGGTGCGGTC	2	128 - 130	0.20	0.20	--
<i>Disu501</i>	F: TGGCCACATCTTCAGCATTAAAG R: GCACCTAACACAGTTACAGGC	2	155 - 157	0.47	0.60	-0.33
<i>Disu556</i>	F: GCCAATTAAATCTACCTGCCAC R: GCCAAGACTCAAACCCAGG	2	168 - 174	0.25	0.25	--
<i>Disu863</i>	F: GAAGCTGTATGTCCGGATGC R: GCTAAACAGACCTTCCTCAGAG	2	162 - 166	0.36	0.40	-0.14
<i>Disu448</i>	F: CAGGTTTCGTTACTGCAGGAC R: TCTGGTGACCTGAGATGCAC	2	154 - 156	0.20	0.20	--
<i>Disu201</i>	F: TGGAGAGAATTTTCAGACATGGG R: CTAGCCCAAGATCCATTGGC	2	156 - 158	0.53	0.00	1.00
<i>Disu847</i>	F: AAAGTCGCCTCTCACACACC R: TCAGAGCCTCCTTGTAAGCG	2	138 - 140	0.20	0.20	--
<i>Disu393</i>	F: AGTGAGCAAGGGAATGTGTG R: GGGTGCTGTCTCTTGATTGG	2	155 - 157	0.36	0.40	-0.14
<i>Disu733</i>	F: TGGCACAGAGACACCCATG R: TCTGTGGTGGTAGCTGTGAC	2	151 - 159	0.36	0.00	1.00
<i>Disu149</i>	F: GAGCGTGCATGGTAGTTTCC R: GGTTCTCATAGCAGACGGAG	4	160 - 162	0.73	1.00	-0.43
<i>Disu783</i>	F: CCTTGCCTTGCCTTCAATCC R: CCATCCTTTCTCCTACACAGAC	3	126 - 134	0.51	0.60	-0.20
<i>Disu050</i>	F: CTCCACATTCAGCAAACCTTC R: CCAGGCAGTGATGACTCTAC	3	160 - 166	0.51	0.20	0.64
<i>Disu748</i>	F: CCTTGATTGGTGGGTTCCC R: AGAGAGAGCGCACGTGTG	3	106 - 116	0.64	0.80	-0.28
<i>Disu476</i>	F: AAACAGGGAAACAAGGTGCG R: GACTGCGCCCTTTCTGTTAG	3	162 - 174	0.60	0.80	-0.39
<i>Disu151</i>	F: CATTGTGCTCGCTACGCAG R: CTAGGTGTCAAGAGCCAGGG	2	135 - 137	0.36	0.00	1.00
<i>Disu127</i>	F: CCACCACCACCATGCATAG R: CATTTGCTCCCATGCTGAAG	2	162 - 164	0.36	0.00	1.00
<i>Disu098</i>	F: GCTAGGAGAGGGTGTGGAC R: TGGTAGCCTTGCCTCTTTCC	4	98 - 126	0.78	0.20	0.76
<i>Disu582</i>	F: TCTGTGGTGGTAGCTGTGAC R: TGGCACAGAGACACCCATG	2	144 - 152	0.36	0.00	1.00
<i>Disu100</i>	F: TGTGGACTTGTTCATATATGGGC R: TTCATCCATGCTGTCACAAATG	2	120 - 122	0.36	0.40	-0.14
<i>Disu480</i>	F: CCTGCCTTCTAGTCCTGTGG R: AGCAAGCAGGATCAGGAAGG	2	112 - 116	0.47	0.20	0.60

Table 2.6. Cont.

Locus	Primer Sequence (5' - 3')	A	Size Range (bp)	H_E	H_O	F_{IS}
<i>Disu593</i>	F: CCACGTCCCAGGTCAAGAG R: AGCTGTTCCCTGGTGGCTC	3	164 - 166	0.56	0.20	0.67
<i>Disu487</i>	F: TATCATGTCCACAAGCACGCG R: GTCTTCTTCACGACAGCACC	2	148 - 160	0.20	0.20	--
<i>Disu545</i>	F: TGTTGTCCAAGCTGTGTCTG R: TGGCAGCTGGTACCTAACAG	2	148 - 150	0.20	0.20	--
<i>Disu076</i>	F: TTCCAGCCGCTCTTATGACC R: TCATGTGCTTATTGGCCATCTG	2	125 - 129	0.53	0.00	1.00
<i>Disu269</i>	F: CAAGACCACACCTGCTTGTC R: ACTCACTCATCACCCAGCC	3	115 - 152	0.60	0.33	0.50
<i>Disu261</i>	F: AAACCATACGCGGGAGAAGG R: GAAGGGAAGATCATGCAGGAG	2	150 - 166	0.60	0.33	0.50
<i>Disu071</i>	F: TTGAGATGCATTGCCGTGG R: CCATGGTTTCTGCATCGTGG	3	168 - 172	0.73	0.33	0.60
<i>Disu033</i>	F: TCTGGATACCTGAGGCTTGAC R: ACTGGCATCACTTCTTTCCC	2	152 - 164	0.53	0.00	1.00
<i>Disu138</i>	F: GGGACACATGACTCCTCTTATC R: CCACTCCACCTTATACTACCAC	2	167 - 169	0.53	0.00	1.00

A is the mean number of alleles per locus.

H_E is the mean expected heterozygosity.

H_O is observed heterozygosity.

F_{IS} is the average deviation from Hardy-Weinberg proportions.

Table 2.7. Estimates of probability of identity (P_{ID}) and probability of identity between siblings ($P_{ID(sib)}$) with total P_{ID} and $P_{ID(sib)}$ values among genotypes of Sumatran rhinoceros.

Locus	P_{ID}	Total P_{ID}	$P_{ID(sib)}$	Total $P_{ID(sib)}$
<i>Disu542</i>	0.69	0.6886	0.83	0.8322
<i>Disu501</i>	0.42	0.2924	0.65	0.5378
<i>Disu556</i>	0.63	0.1855	0.80	0.4298
<i>Disu863</i>	0.51	0.0953	0.72	0.3088
<i>Disu448</i>	0.69	0.0656	0.83	0.2569
<i>Disu201</i>	0.39	0.0253	0.61	0.1558
<i>Disu847</i>	0.69	0.0174	0.83	0.1297
<i>Disu393</i>	0.51	0.0089	0.72	0.0932
<i>Disu733</i>	0.51	0.0046	0.72	0.0669
<i>Disu149</i>	0.18	0.0008	0.47	0.0311
<i>Disu783</i>	0.34	0.0003	0.61	0.0188
<i>Disu050</i>	0.34	0.0001	0.61	0.0114
<i>Disu748</i>	0.26	<0.0001	0.53	0.0060
<i>Disu476</i>	0.29	<0.0001	0.55	0.0033
<i>Disu151</i>	0.51	<0.0001	0.72	0.0024
<i>Disu127</i>	0.51	<0.0001	0.72	0.0017
<i>Disu098</i>	0.14	<0.0001	0.44	0.0007
<i>Disu582</i>	0.51	<0.0001	0.72	0.0005
<i>Disu100</i>	0.51	<0.0001	0.72	0.0004
<i>Disu480</i>	0.42	<0.0001	0.65	0.0002
<i>Disu593</i>	0.38	<0.0001	0.59	0.0001
<i>Disu487</i>	0.69	<0.0001	0.83	0.0001
<i>Disu545</i>	0.69	<0.0001	0.83	0.0001
<i>Disu076</i>	0.41	<0.0001	0.63	0.0001
<i>Disu269</i>	0.30	<0.0001	0.58	<0.0001
<i>Disu261</i>	0.38	<0.0001	0.59	<0.0001
<i>Disu071</i>	0.23	<0.0001	0.50	<0.0001
<i>Disu033</i>	0.41	<0.0001	0.63	<0.0001
<i>Disu138</i>	0.41	<0.0001	0.63	<0.0001

P_{ID} is the probability of identity.

$P_{ID(sib)}$ is the probability of identity between siblings.

Table 2.8. DNA concentration in black rhinoceros fecal isolates using various extraction protocols.

Sample	Kit Protocol (ng/ul)	Modified Protocol (ng/ul)
Dbi-294	0.335	92
Dbi-362	--	84.6
Dbi-664	--	70.4
Dbi-957	--	56.6
Dbi-847	7.76	56.4
Dbi-667	1.4	97.6
Dbi-683	--	29.6
Dbi-669	0.378	62.8
Dbi-490	0.224	67
Dbi-521	1.19	90
Dbi-904	--	9.44
Dbi-873	9.3	19.1
Dbi-935	--	55.52
Dbi-870	10.6	28.6

Table 2.9. Fecal genotyping success and error rates by sample with final MgCl₂ concentration of 3.0mM per amplification reaction.

Sample	Amplification success (%)	Correct Genotypes (%)	Correct Alleles (%)	ADO (%)	FA (%)
Dbi-718	16/16 (100)	16/16 (100)	32/32 (100)	0/32 (0)	0/32 (0)
Dbi0022	15/16 (93.8)	12/15 (80.0)	27/30 (90.0)	3/30 (10.0)	0/30 (0)
Dbi-868	13/16 (81.3)	10/13 (76.9)	23/26 (88.5)	3/26 (11.5)	0/26 (0)
Overall	44/48 (91.7)	40/44 (86.4)	82/88 (93.2)	6/88 (6.8)	0/88 (0)

ADO is allelic drop out.

FA is false alleles.

Table 2.10. Fecal genotyping success and error rates by microsatellite locus with final MgCl₂ concentration of 3.0mM per amplification reaction.

Locus	Individuals Genotyped (%)	Correct Genotypes (%)	Correct Alleles (%)	ADO (%)	FA(%)
<i>Dibi3</i>	3/3 (100)	3/3 (100)	6/6 (100)	0/6 (0)	0/6 (0)
<i>Dibi5</i>	3/3 (100)	2/3 (66.7)	5/6 (83.3)	1/6 (16.7)	0/6 (0)
<i>Dibi9</i>	3/3 (100)	2/3 (66.7)	5/6 (83.3)	1/6 (16.7)	0/6 (0)
<i>Dibi15</i>	3/3 (100)	3/3 (100)	6/6 (100)	0/6 (0)	0/6 (0)
<i>Dibi22</i>	2/3 (66.7)	2/2 (100)	4/4 (100)	0/4 (0)	0/4 (0)
<i>Dibi24</i>	3/3 (100)	3/3 (100)	6/6 (100)	0/6 (0)	0/6 (0)
<i>Dibi25</i>	3/3 (100)	1/3 (33.3)	4/6 (66.7)	2/6 (33.3)	0/6 (0)
<i>Dibi26</i>	3/3 (100)	3/3 (100)	6/6 (100)	0/6 (0)	0/6 (0)
<i>Dibi27</i>	3/3 (100)	2/3 (66.7)	5/6 (83.3)	1/6 (16.7)	0/6 (0)
<i>Dibi32</i>	3/3 (100)	3/3 (100)	6/6 (100)	0/6 (0)	0/6 (0)
<i>Dibi34</i>	2/3 (66.7)	2/2 (100)	4/4 (100)	0/4 (0)	0/4 (0)
<i>Dibi48</i>	3/3 (100)	3/3 (100)	6/6 (100)	0/6 (0)	0/6 (0)
<i>Dibi49</i>	3/3 (100)	2/3 (66.7)	5/6 (83.3)	1/6 (16.7)	0/6 (0)
<i>Dibi50</i>	2/3 (66.7)	2/2 (100)	4/4 (100)	0/4 (0)	0/4 (0)
<i>Dibi51</i>	3/3 (100)	3/3 (100)	6/6 (100)	0/6 (0)	0/6 (0)
<i>Dibi56</i>	2/3 (66.7)	2/2 (100)	4/4 (100)	0/4 (0)	0/4 (0)

ADO is allelic drop out.

FA is false alleles.

Table 2.11. Fecal genotyping success and error rates by sample with final MgCl₂ concentration of 2.5mM per amplification reaction.

Sample	Amplification success (%)	Correct Genotypes (%)	Correct Alleles (%)	ADO (%)	FA (%)
Dbi-718	15/16 (93.8)	13/15 (86.7)	28/30 (93.3)	2/30 (6.7)	0/30 (0)
Dbi-667	15/16 (93.8)	14/15 (93.3)	29/30 (96.7)	1/30 (3.3)	0/30 (0)
Dbi-521	14/16 (87.5)	10/14 (71.4)	24/28 (85.7)	3/28 (10.7)	1/28 (3.6)
Dbi-669	11/16 (68.8)	10/11 (90.9)	21/22 (95.5)	1/22 (4.6)	0/22 (0)
Dbi-847	9/16 (56.3)	6/9 (66.7)	15/18 (83.3)	3/18 (16.7)	0/18 (0)
Dbi-873	3/16 (18.8)	1/3 (33.3)	4/6 (66.7)	2/6 (33.3)	0/6 (0)
Dbi-870	8/16 (50.0)	6/8 (75.0)	12/16 (75.0)	2/16 (12.5)	2/16 (12.5)
Dbi-294	15/16 (93.8)	12/15 (80.0)	26/30 (86.7)	3/30 (10.0)	1/30 (3.3)
Dbi-490	14/16 (87.5)	12/14 (85.7)	26/28 (93.8)	2/28 (7.1)	0/28 (0)
Dbi-664	16/16 (100)	14/16 (87.5)	29/32 (90.1)	2/32 (6.3)	1/32 (3.1)
Dbi-362	10/16 (62.5)	8/10 (80.0)	18/20 (90.0)	1/20 (5.0)	1/20 (5.0)
Dbi-935	10/16 (62.5)	7/10 (70.0)	17/20 (85.0)	2/20 (10.0)	1/20 (5.0)
Dbi-683	5/16 (31.3)	2/5 (40.0)	7/10 (70.0)	2/10 (20.0)	1/10 (10)
Dbi-904	2/16 (12.5)	2/2 (100)	4/4 (100)	0/4 (0)	0/4 (0)
Dbi-957	8/16 (50.0)	6/8 (75.0)	13/16 (81.3)	1/16 (6.3)	2/16 (12.5)
Dbi0022	10/16 (62.5)	4/10 (40.0)	13/20 (60.0)	6/20 (30.0)	1/20 (5.0)
Dbi-868	9/16 (56.3)	5/9 (55.6)	14/18 (77.8)	4/18 (22.2)	0/18 (0)
Overall	174/272 (64.0)	132/174 (75.9)	300/348 (86.2)	37/348 (10.6)	11/348 (3.2)

ADO is allelic drop out.

FA is false alleles.

Table 2.12. Fecal genotyping success and error rates by microsatellite locus with final MgCl₂ concentration of 2.5mM per amplification reaction.

Locus	Individuals Genotyped (%)	Correct Genotypes (%)	Correct Alleles (%)	ADO (%)	FA(%)
<i>Dibi3</i>	11/17 (64.7)	11/11 (100)	22/22 (100)	0/22 (0)	0/22 (0)
<i>Dibi5</i>	12/17 (70.6)	10/12 (83.3)	22/24 (91.7)	2/24 (8.3)	0/24 (0)
<i>Dibi9</i>	15/17 (88.2)	12/15 (80.0)	27/30 (90.0)	3/30 (10.0)	0/30 (0)
<i>Dibi15</i>	15/17 (88.2)	8/15 (53.3)	22/30 (73.3)	3/30 (10.0)	5/30 (16.7)
<i>Dibi22</i>	13/17 (76.5)	11/13 (84.6)	23/26 (88.5)	2/26 (7.7)	1/26 (3.9)
<i>Dibi24</i>	12/17 (70.6)	8/12 (66.7)	20/24 (88.3)	4/24 (16.7)	0/24 (0)
<i>Dibi25</i>	10/17 (58.8)	5/10 (50.0)	14/20 (70.0)	4/20 (20.0)	2/20 (10.0)
<i>Dibi26</i>	15/17 (88.2)	12/15 (80.0)	27/30 (90.0)	3/30 (10.0)	0/30 (0)
<i>Dibi27</i>	8/17 (47.1)	6/8 (75.0)	14/16 (87.5)	2/16 (12.5)	0/16 (0)
<i>Dibi32</i>	11/17 (64.7)	8/11 (72.7)	18/22 (81.8)	3/22 (13.6)	1/22 (4.6)
<i>Dibi34</i>	3/17 (17.7)	0/3 (0)	1/6 (16.7)	3/6 (50.0)	2/6 (33.3)
<i>Dibi48</i>	13/17 (76.5)	11/13 (84.6)	24/26 (92.3)	2/26 (7.7)	0/26 (0)
<i>Dibi49</i>	9/17 (52.9)	6/9 (66.7)	15/18 (83.3)	3/18 (16.7)	0/18 (0)
<i>Dibi50</i>	6/17 (35.3)	5/6 (83.3)	11/12 (91.67)	1/12 (8.33)	0/12 (0)
<i>Dibi51</i>	10/17 (58.8)	9/10 (90.0)	19/20 (95.0)	1/20 (5.0)	0/20 (0)
<i>Dibi56</i>	11/17 (64.7)	10/11 (90.9)	21/22 (95.5)	1/22 (4.6)	0/22 (0)

ADO is allelic drop out.

FA is false alleles.

Table 2.13. Fecal genotyping success and error rates by sample with final MgCl₂ concentration of 1.5mM per amplification reaction.

Sample	Amplification success (%)	Correct Genotypes (%)	Correct Alleles (%)	ADO (%)	FA (%)
Dbi-718	13/16 (81.3)	12/13 (92.3)	25/26 (96.2)	1/26 (3.9)	0/26 (0)
Dbi-667	9/16 (56.5)	5/9 (55.6)	14/18 (77.8)	3/18 (16.7)	1/18 (5.6)
Dbi-521	12/16 (75.0)	9/12 (75.0)	21/24 (87.5)	2/24 (8.33)	1/24 (4.2)
Dbi-669	10/16 (62.5)	8/10 (80.0)	18/20 (90.0)	2/20 (10.0)	0/20 (0)
Dbi-847	11/16 (68.8)	7/11 (63.3)	18/22 (81.8)	4/22 (18.2)	0/22 (0)
Dbi-873	5/16 (31.3)	3/5 (60.0)	8/10 (80.0)	2/10 (20.0)	0/10 (0)
Dbi-870	8/16 (50.0)	4/8 (50.0)	11/16 (68.8)	4/16 (25.0)	1/16 (6.3)
Dbi-294	14/16 (87.5)	13/14 (92.9)	27/28 (96.4)	1/28 (3.6)	0/28 (0)
Dbi-490	14/16 (87.5)	14/14 (100)	28/28 (100)	0/28 (0)	0/28 (0)
Dbi-664	14/16 (87.5)	13/14 (92.9)	27/28 (96.4)	1/28 (3.6)	0/28 (0)
Dbi-362	9/16 (56.3)	5/9 (55.6)	14/18 (77.8)	4/18 (22.2)	0/18 (0)
Dbi-935	8/16 (50.0)	6/8 (75.0)	14/16 (87.5)	2/16 (12.5)	0/16 (0)
Dbi-683	1/16 (6.3)	0/1 (0)	1/2 (50.0)	1/2 (50.0)	0/0 (0)
Dbi-904	0/16 (0)	--	--	--	--
Dbi-957	7/16 (43.8)	4/7 (57.1)	10/14 (71.4)	3/14 (21.4)	1/14 (7.1)
Dbi0022	10/16 (62.5)	8/10 (80.0)	18/20 (90.0)	2/20 (10.0)	0/20 (0)
Dbi-868	9/16 (56.3)	5/9 (55.6)	14/18 (77.8)	4/18 (22.2)	0/18 (0)
Overall	154/272 (56.6)	116/154 (75.3)	268/308 (87.0)	36/308 (11.7)	4/308 (1.3)

ADO is allelic drop out.
FA is false alleles.

Table 2.14. Fecal genotyping success and error rates by microsatellite locus with final MgCl₂ concentration of 1.5mM per amplification reaction.

Locus	Individuals Genotyped (%)	Correct Genotypes (%)	Correct Alleles (%)	ADO (%)	FA(%)
<i>Dibi3</i>	13/17 (76.5)	11/13 (84.6)	24/26 (92.3)	2/26 (7.7)	0/26 (0)
<i>Dibi5</i>	11/17 (64.7)	9/11 (81.8)	20/22 (90.9)	2/22 (9.1)	0/22 (0)
<i>Dibi9</i>	13/17 (76.5)	12/13 (92.3)	25/26 (96.2)	1/26 (3.9)	0/26 (0)
<i>Dibi15</i>	10/17 (58.8)	7/10 (70.0)	17/20 (85.0)	3/20 (15.0)	0/20 (0)
<i>Dibi22</i>	11/17 (64.7)	10/11 (90.9)	20/22 (90.9)	1/22 (4.6)	1/22 (4.6)
<i>Dibi24</i>	0/17 (0)	--	--	--	--
<i>Dibi25</i>	12/17 (70.6)	5/12 (41.7)	17/24 (70.8)	6/24 (25.0)	1/24 (4.2)
<i>Dibi26</i>	10/17 (58.8)	9/10 (90.0)	19/20 (95.0)	1/20 (5.0)	0/20 (0)
<i>Dibi27</i>	12/17 (70.6)	7/12 (58.3)	19/24 (79.2)	5/24 (20.8)	0/24 (0)
<i>Dibi32</i>	12/17 (70.6)	9/12 (75.0)	20/24 (83.3)	3/24 (12.5)	1/24 (4.2)
<i>Dibi34</i>	0/17 (0)	--	--	--	--
<i>Dibi48</i>	13/17 (76.5)	10/13 (76.9)	23/26 (88.5)	2/26 (7.7)	1/26 (3.9)
<i>Dibi49</i>	7/17 (41.2)	5/7 (71.4)	12/14 (85.7)	2/14 (14.3)	0/14 (0)
<i>Dibi50</i>	11/17 (64.7)	10/11 (90.1)	21/22 (95.5)	1/22 (4.6)	0/22 (0)
<i>Dibi51</i>	10/17 (58.8)	8/10 (80.0)	18/20 (90.0)	2/20 (10.0)	0/20 (0)
<i>Dibi56</i>	10/17 (58.8)	5/10 (50.0)	15/20 (75.0)	5/20 (25.0)	0/20 (0)

ADO is allelic drop out.

FA is false alleles.

CHAPTER 3. GENETIC STRUCTURING AND REDUCED DIVERSITY OF SUMATRAN RHINOCEROS (*DICERORHINUS SUMATRENSIS*)

Abstract

The Sumatran rhinoceros, once widespread across Southeast Asia, now consists of ca. 100 individuals largely restricted to three isolated populations on the island of Sumatra. No studies have examined the population genetic structure of Sumatran rhinoceros using analyses beyond mitochondrial restriction mapping techniques. Given the requirement for substantial management of the remaining Sumatran rhino populations in the wild and in *ex situ* breeding facilities, more information regarding their genetic status needs to be available. Here, mitochondrial control region sequences from individuals representing the modern population ($N = 13$), were used to estimate current levels of diversity. To assess changes in genetic diversity over time, mitochondrial control region haplotypes from archival museum samples ($N = 25$) were sequenced. Overall, a total of 17 mitochondrial control region haplotypes were identified with high haplotype diversity ($h = 0.90$). All samples identified as *D. s. sumatrensis*, the subspecies with the largest population size, formed a single cluster containing ten haplotypes. Of the ten haplotypes, three were shared between modern and museum samples, two were unique to the modern sample set, and five were restricted to the museum sample set. Genetic diversity has been lost as the population size decreased as evident by the presence of more haplotypes and higher haplotype diversity in the *D. s. sumatrensis* museums samples ($H = 8$; $h = 0.9$) than in the modern samples ($H = 5$; $h = 0.74$). Additionally, microsatellite genotypes from the modern samples indicated low diversity ($A = 2.8$; $H_O = 0.28$). Analysis of genetic structure suggested the presence of three distinct genetic partitions consisting of individuals from the Malay Peninsula and two distinct groups within the island of Sumatra. It appears that the observed genetic differentiation is associated with geographic barriers to gene flow present in the population historically. Continued isolation of small populations within the island of Sumatra will probably result in further loss of genetic diversity; this information, provided by genetic analysis, is required to make informed management decisions.

Introduction

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) was once distributed across Southeast Asia into the foothills of the Himalayan Mountains, but habitat loss coupled with poaching and population isolation resulted in substantial decreases in population size (Scott et al. 2004; Zafir et al. 2011; Havmøller et al. 2016). The current population is estimated to consist of fewer than 100 individuals (Nardelli 2014; Havmøller et al. 2016) occupying less than 1% of its former range (Dinerstein 2011) (Figure 3.1). Subspecies *D. s. harrissoni*, restricted to the island of Borneo, is comprised of an estimated 15 individuals in Indonesian Borneo. The population formerly found in the Malaysian state of Sabah, now only occurs ex-situ and consists of three individuals (Groves & Kurt 1972; van Strien et al. 2008; Emslie et al. 2013). After extensive surveys found no sign of wild Sumatran rhinos in Malaysian Borneo, the population was declared extinct in the wild in 2015 (Havmøller et al. 2016). The last two wild individuals from this population, both females, were captured in 2011 and 2014 and added to the breeding program. The second extant subspecies, *D. s. sumatrensis*, is found in isolated populations on the island of Sumatra in Indonesia (< 100 individuals). The remaining *D. s. sumatrensis* individuals comprise three populations in national parks (Gunung Leuser, Way Kambas, and Bukit Barisan Selatan); six individuals comprise the *ex situ* breeding program for this subspecies. A third subspecies *D. s. lasiotis* has been declared extinct from its range states of India, Bhutan, Bangladesh, and Myanmar.

In the past two decades the total population size of this species has decreased by more than 50% (Pusparini et al. 2015; Foundation 2016). Despite intense planning and implementation of various management efforts the drastic decline in Sumatran rhino populations has not been stemmed. Due to the number of problems plaguing the remaining Sumatran rhinoceros populations a series of management strategies were outlined during the Sumatran Rhino Crisis Summit (Havmøller et al. 2016) and in the Bandar Lampung Declaration (IUCN 2013). One key issue that has precipitated further planning of management efforts for this species was the realization that reported estimates of population size have been inaccurate compared to the actual numbers of individuals. There still remains a large amount of uncertainty in the census estimates of Sumatran rhino populations due to inadequate counting techniques. However, since more realistic population estimates have been put forth, management strategies have been revisited and

a number of key actions delineated. The main actions, as outlined by Havmøller and colleagues (2015), include placement of Rhino Protection Units where there are breeding populations, intensive management zones that include protection and monitoring, and enhancement of *ex situ* breeding programs. These actions are all in the process of being implemented; however, there is additional room for improvement as funding and technology advance.

The breeding program for Sumatran rhinos is of particular importance but has only recently started resulting in the production of offspring. Artificial insemination techniques have led to the production of offspring in white rhinos (Hildebrandt et al. 2007; Hermes et al. 2009b). Such artificial reproductive technologies may become crucial components of survival for the Sumatran rhino species (Goossens et al. 2013), but they have not yet proven successful for Sumatrans in *ex situ* breeding programs.. The entire *ex situ* breeding program consists of nine rhinos. There are three individuals representing *D. s. harrissoni*; two females that produce eggs but exhibit severe reproductive tract pathology and a male that produces low quality sperm. Thus, while experts continue to pursue options and conduct research, it is unlikely that this subspecies will successfully produce offspring through natural mating. An additional six *D. s. sumatrensis* individuals, three females and three males, are part of the breeding program. However, only one of the three females has produced offspring, and all three of the males are closely related to each other. A major concern for the future of the *ex situ* breeding program as a whole is the high incidence of severe reproductive pathology in females causing infertility (Roth 2006; Hermes et al. 2009a), This is particularly evident in the remaining Malaysian females, of whom, more than 50% are affected (Havmøller et al. 2016). These conditions may become an issue in Indonesia as population sizes decrease to the point that breeding events become rare; lack of natural mating opportunities result in an increase of reproductive conditions that lead to infertility (Hermes et al. 2007; Hermes et al. 2009a). Despite the effort to protect naturally breeding population and bolster *ex situ* breeding programs, Sumatran rhino populations continue to decline and may become extinct before conservation efforts are fully implemented

Another major component of the Sumatran Rhino Crisis Summit and the Bandar Lampung Declaration is the decision to manage the entire remaining Sumatran rhino population, inclusive of both subspecies, as a single metapopulation. While this strategy has not yet been put into action, the national governments of Malaysia and Indonesia are prepared for collaboration. There is currently a deficit of genetic information that can be used to evaluate whether the

populations of the island of Sumatra and Borneo truly represent one or two conservation units. Earlier studies on the Sumatran rhino utilized mitochondrial restriction mapping data to assess population differentiation and to identify conservation units (Amato et al. 1995; Morales et al. 1997). Amato et al. (1995) suggested that the subspecies populations were not different enough to represent separate conservation units. However, Morales et al. (1997) found low genetic divergence between the populations on the island of Sumatra (0.3% haplotype sequence divergence) and higher divergence, which was enough to justify management as separate evolutionary lineages, between the Borneo and other populations (1.0% haplotype sequence divergence). The methods used in these previous studies provide relatively little information on true levels of differentiation between populations. Furthermore, they do not necessarily reflect the patterns and relationships that may be seen when using nuclear genetic markers. Since results from mitochondrial and nuclear genomes can be incongruent (Roca et al. 2005; Ishida et al. 2011b) it is important to consider both for conservation management planning. Despite of the paucity of genetic data for this species a management strategy that treats all Sumatran populations as one unit has been implemented (Goossens et al. 2013). Interbreeding of these two distinct subspecies of Sumatran rhinoceros may results in the loss of a genetically unique evolutionary lineages and has the potential to result in outbreeding depression or loss of local adaptations (Allendorf et al. 2001; Edmands 2007). For future conservation and management of Sumatran rhinos, given their critically endangered status, decreasing population trend, and the small, isolated nature of remain populations, it is crucial to determine the current genetic status of the extant population.

Understanding the population genetics of endangered species can be of tremendous benefit to conservation management planning and implementation. A number of important factors can be addressed through genetic information, yet to date, little genetic research has been published on Sumatran rhinos. In addition to the two studies that used mitochondrial markers, Scott and colleagues (2004) optimized 10 polymorphic microsatellite loci in the Sumatran rhinoceros; no published studies have utilized these markers for research on Sumatran rhinoceros populations. Of primary importance in the Bandar Lampung Declaration is the condition that Sumatran rhino populations should be monitored frequently and intensively through collaborative efforts to detect population trends and inform future management decisions. Genetic analyses are of paramount importance for population monitoring and can provide

estimates of population differentiation, census size, identification of conservation units, phylogenetic relationships, assessment of the impact of isolation and inbreeding on population fitness, and details about population histories. In order to successfully incorporate genetic monitoring of endangered species into management plans, it is first necessary to know the current genetic status of the species.

Here I report, for the first time, on the genetic diversity of Sumatran rhinoceros populations using both nuclear microsatellite and mitochondrial makers. The overall goal of this research was to elucidate how patterns of diversity have changed over time as the Sumatran rhinoceros population has declined and to determine whether there is structure within the largest extant subspecies, *D. s. sumatrensis*. To determine this I assessed mitochondrial haplotype diversity in a set of samples representing the modern Sumatran rhino population and compared it to mitochondrial haplotype diversity identified in a set of archival Sumatran rhino bone samples obtained from museums. Additional microsatellite analyses were conducted on the modern samples to identify patterns of diversity and subpopulation structuring in order to make informed management decisions.

Methods and Materials

Samples

To represent the “modern” Sumatran rhinoceros population tissue or blood samples were obtained from 15 individuals alive within the past 30 years (Table 3.1). Whole blood samples were collected from two Sumatran rhinoceros at the Cincinnati Zoo during routine veterinary care; samples were collected in EDTA tubes to prevent clotting and kept frozen or refrigerated until DNA isolation (< 1 week from time of collection). Other samples of whole blood or tissue were kept frozen at -20°C after collection until the time of extraction (Table 3.1). To represent the “historic” population 28 Sumatran rhino bone samples from ca. 1860 – 1940 were collected from numerous museums in North American and Europe (Table 3.2). DNA from four museum samples was extracted prior to importation; DNA from all other historic samples was isolated after arrival at the University of Illinois at Urbana-Champaign. Specimen were imported from international collaborators under CITES/ESA Permit 14US84465A/9 and CITES COSE Permit

12US757718/9. Endorsement for the proposed rhinoceros research was obtained from the Association of Zoos and Aquariums, and all work was conducted with IACUC approval (protocol # 15053).

Sample preparation and DNA extraction

DNA was isolated from whole blood or tissue samples using Qiagen DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's recommended protocol. Sample preparation and DNA extraction for museum specimens were completed in a designated ancient DNA laboratory facility at the University of Illinois at Urbana-Champaign. Bones were surface decontaminated by submersion in bleach for 5 minutes, followed by three rinses in DNA-free ddH₂O, and a final rinse in isopropanol. Samples were then dried in a UV-crosslinker for a minimum of 10 minutes or until completely dry. Approximately 0.2g of each bone was crushed into small pieces or a fine powder using a mortar and pestle in a designated drilling hood and collected in a sterile 15mL centrifuge tube. All surfaces in the drilling hood and equipment were sterilized between samples with 10% bleach and/or DNA-Off followed by at least 10 minutes of exposure to UV light. Crushed samples were incubated for 24 – 48 hours in 4ml of extraction buffer (0.5M EDTA, 33.3mg/ml Proteinase K, 10% N-lauryl sarcosine) at 37°C. A negative extraction control was included with each set of samples. The extraction solution containing digested sample was concentrated to approximately 250ul using Amicon centrifuge tubes with a 30K molecular weight filter. Remaining undigested bone fragments were kept at 4°C for future extractions. Concentrated digest was put through the QIAquick PCR Purification kit (QIAGEN) two times and eluted in a final volume of 60ul.

PCR Amplification

An approximately 450 base pair fragment of the mitochondrial control region was amplified by polymerase chain reaction (PCR) in the modern samples using previously published primers (Campbell et al. 1995; Moro et al. 1998) and the following mixture in 10ul reactions with final concentrations of: 0.4uM of each forward and reverse primer, 0.2mM of each dNTP (Applied Biosystems Inc. [ABI]), 1x PCR buffer, 1.5 - 2mM MgCl₂, and 0.4 units of AmpliTaq

Gold DNA polymerase (ABI). Given the fragmented nature of ancient DNA, novel primers (F: TGATTTGACTTGGATGGGGTA and R: TTGAGATACACCCCGCTATG) were designed to amplify a 218 bp region of the Sumatran rhino mitochondrial control region that is internal to the region amplified in the modern samples. Amplification by PCR used the following mixture in 20ul reactions with final concentrations of: 0.3uM of each forward and reverse primer, 0.4mM of each dNTP (New England Biolabs [NEB]), 1x PCR buffer, 5mM MgCl₂, and 0.75 units of Platinum Taq DNA polymerase (INVITROGEN). The PCR algorithm for all mitochondrial control region reactions was: initial denaturation at 95°C for 9:45 min; 3 cycles of 20 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 56°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 54°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 52°C, 30 sec at 72°C; followed by 22 additional cycles with 50°C annealing and a final extension at 72°C for 7 min.

In addition, eighteen unpublished microsatellite loci developed in Sumatran rhinos (*Disu542*, *Disu501*, *Disu556*, *Disu863*, *Disu448*, *Disu201*, *Disu847*, *Disu393*, *Disu733*, *Disu149*, *Disu783*, *Disu50*, *Disu748*, *Disu476*, *Disu151*, *Disu127*, *Disu89*, and *Disu582*) were amplified in the modern samples. As described by Ishida et al. 2012, PCR products were fluorescently labeled using M13-tailed forward primers (TGTAACGACGGCCAGT). A primer mix consisting of 8.5uM reverse primer, 0.6uM of M13 tailed forward primer, and 8.5uM of fluorescently labeled M13 forward primer was used for PCR. Primer pairs were amplified by PCR performed in a 10 uL reaction mixture that included final concentrations of: 2mM MgCl₂, 200uM of each dNTP (Applied Biosystems Inc. [ABI]), 1x PCR buffer, and 0.4 units of AmpliTaq Gold DNA Polymerase (ABI). Negative PCR controls were included with each PCR amplification. A step down PCR algorithm was used with an initial 95°C for 10 min; cycles of 15 sec at 95°C; followed by 30 sec at 60°C, 58°C, 56°C, 54°C, 52°C (2 cycles at each temperature) or 50°C (last 30 cycles); and 45 sec at 72°C; and a final extension of 30 min at 72°C.

Mitochondrial Control Region Sequencing and Analysis

Mitochondrial PCR products with clear, single amplicons of the expected size on an ethidium bromide stained agarose gel were enzymatically purified (Hanke & Wink 1994) using an Exonuclease I and shrimp alkaline phosphatase (ExoSAP) reaction. Purified PCR products

were Sanger sequenced in both directions using the BigDye Terminator System (ABI), and resolved on an ABI 3730XL capillary sequencer at the University of Illinois at Urbana-Champaign Core Sequencing Facility. Resulting sequences were trimmed, concatenated, and edited in the software SEQUENCHER (Gene Codes Corporation). Control region sequences from both modern and museum samples were trimmed to be the same length. Samples were grouped as modern or museum for initial analyses; further categorization into geographic region of origin was done within the museum sample set for additional analyses. The DNAsp v5 (Librado & Rozas 2009) software was also used to estimate basic diversity indices, haplotype (h) and nucleotide (π) diversity. Due to the unequal sample size between the museum and modern samples sets, where possible rarefaction was completed using HP-RARE v1.0 (Kalinowski 2005). Control region sequences were used to generate a median-joining network using the software NETWORK version 4.6.1 (Bandelt et al. 1999).

Microsatellite Genotyping and Analysis

PCR amplification success of microsatellite loci was checked on a 1% agarose gel stained with ethidium bromide. Samples that successfully amplified were genotyped on an ABI 3730XL Genetic Analyzer and scored using GENEMAPPER v3.7 software (ABI). Microsatellite variability was assessed using the following parameters calculated by FSTAT, v2.9.3.2 (Goudet 1995), GENEPOP, v4.0 (Raymond & Rousset 1995), and GenAlEx, v6.1 (Peakall & Smouse 2006; Peakall & Smouse 2012): number of alleles per locus, expected heterozygosity, and observed heterozygosity. F_{IS} values, estimating the reduction of heterozygosity due to non-random mating, were calculated for all microsatellite loci in GENEPOP, v.4.0. Linkage disequilibrium between pairs of loci using a log-likelihood ratio statistic was calculated with FSTAT. Exact tests (Guo & Thompson 1992) were performed in GENEPOP to determine whether each microsatellite locus within each population was in Hardy-Weinberg equilibrium. Probability that the markers could distinguish individual identity was calculated by P_{ID} and $P_{ID(sib)}$ (Waits et al. 2001) for each marker as well as total P_{ID} and $P_{ID(sib)}$ values for all markers in CERVUS, v3.0 (Marshall et al. 1998; Kalinowski et al. 2007).

Bayesian clustering in STRUCTURE v2.3.3 (Pritchard et al. 2000) was used to assess patterns of genetic partitioning among Sumatran rhinos. Four models with varying assumptions

regarding individual ancestry and relatedness among populations were implemented. The four models considered were: 1) admixture with correlated allele frequencies; 2) admixture with independent allele frequencies; 3) no admixture with correlated allele frequencies; and 4) no admixture with independent allele frequencies. Each model was run three times for values of $K = 1$ through $K = 6$ with 1 million Markov chain Monte Carlo steps and a burn in of 100,000 steps. The most likely number of population clusters (K) was evaluated by examining species biology and through two *ad hoc* methods in STRUCTURE HARVESTER (Earl & vonHoldt 2012): ΔK (Evanno et al. 2005) and log probability of data, $\ln P(D)$ (Pritchard et al. 2000). A factorial correspondence analysis (FCA) was completed in GENETIX, v4.02.2 (Belkhir et al. 1996-2004) to further assess the overall relationship across individuals in the population.

Results

Mitochondrial Control Region Analysis

A total of 26 (93%) of the museum specimens yielded DNA of sufficient quality for PCR amplification and sequencing. From the 15 modern samples 13 were included in control region analysis. After alignment and trimming of priming sequences 177 bp of mitochondrial control region was used for analysis. Among all samples combined a total of 17 distinct haplotypes (designated as Ds1 – Ds17) were identified with 36 mutations detected, haplotype diversity was 0.90, and nucleotide diversity was 0.040 (Figure 3.2; Table 3.3).

A median joining networks was generated to assess the relationships across the control region haplotypes. Haplotypes grouped by geographic region of origin, showing differentiation between subspecies. *D. s. harrissoni* and *D. s. sumatrensis* formed clusters by subspecies that were separated by five mutations (Figure 3.2). Individuals carrying haplotypes Ds1 – Ds10 mainly originated from populations of subspecies *D. s. sumatrensis* in Sumatra and Peninsular Malaysia. Additionally, all samples of unknown origin were identified as having haplotypes within the Ds1 – Ds10 subcluster; thus, they fall within known variation of *D. s. sumatrensis*. Haplotypes Ds11 and Ds 12 were found in samples from Myanmar and Laos, respectively, representing the extinct subspecies *D. s. lasiotis*. Most Bornean individuals from the subspecies *D. s. harrissoni* had haplotypes Ds13 – Ds17. In one instance a museum sample recorded as

being collected from Borneo carried haplotype Ds1, which is a common haplotype in the subspecies *D. s. sumatrensis*. There was no other evidence of haplotypes being shared among subspecies.

The modern dataset, containing only samples from the subspecies *D. s. sumatrensis*, had a total of five distinct haplotypes identified ($h = 0.74$; $\pi = 0.022$) (Table 3.3). An estimate of historic haplotype diversity was calculated by excluding museum samples from the Bornean subspecies (*D. s. harrissoni*) and the mainland subspecies (*D. s. lasiotis*). The remaining museum dataset ($N = 17$) contained a total of 8 distinct haplotypes ($h = 0.90$; $\pi = 0.032$) (Table 3.3). Three haplotypes (Ds1, Ds8, and Ds9) were found in both the modern and museum samples sets. Two haplotypes (Ds4 and Ds5) were found only in the modern sample set and 5 haplotypes (Ds2, Ds3, Ds6, Ds8, and Ds10) were restricted to the museum samples (Figure 3.3). To account for unequal samples size between modern and museum datasets rarefaction analysis was used. After rarefaction of the museum dataset to 13 samples an estimated 7.2 haplotypes were be found.

Microsatellite Analysis

Multilocus genotypes for 18 microsatellite loci were obtained from 13 individuals representing the modern population. No linkage disequilibrium at microsatellite loci was detected after correction for multiple comparisons ($p < 0.0003$). Two-tailed tests for departure from Hardy-Weinberg equilibrium indicated significant deviation at six loci ($p < 0.05$). The average number of alleles per locus was 2.8 and ranged from 2 to 5. Overall mean observed heterozygosity was low ($H_o = 0.28$) compared to expected heterozygosity ($H_E = 0.50$), and fixation index values were high overall ($F_{IS} = 0.44$).

Genetic partitioning across the modern Sumatran rhino individuals was examined with STRUCTURE. Ad hoc methods to determine the number of partitions provided support for a varying number of clusters, with a minimum of 2 and a maximum of 4. The best supported K value using the ΔK method was $K = 2$, regardless of model assumptions. When estimating the most likely number of genetic partitions based on $\text{LnP}(D)$ values $K = 3$ was found for models assuming independent allele frequencies, and $K = 4$ was best supported in models assuming correlated allele frequencies. To further identify the most likely number of genetic partitions we

used the guideline put forth by Pritchard and colleagues (2000) that information regarding the geography of the study area must also be taken into consideration when assessing the potential number of genetic clusters identified by STRUCTURE. Important differences in clustering patterns were identified when the value of K was raised from K = 2 to K = 3, corresponding to the biogeography of region inhabited by Sumatran rhinos. However, when the K value was further increased to K = 4 no additional clusters were apparent. At K = 3, genetic distinctiveness between rhinos from the island of Sumatra and the Malay Peninsula was evident (Figure 3.4), in addition a clear partition was observed within individuals from Sumatra. A factor correspondence analysis conducted using the software GENETIX supported the genetic partitions estimated by STRUCTURE (Figure 3.5).

Discussion

This study is the first to investigate genetic diversity across historic and modern Sumatran rhino populations. High amplification and sequencing success rate (93%) for a 218 bp portion of the mitochondrial control region was observed in the museum bone specimens collected between 1860 and 1941. Thus, this study shows that museum specimens can be a valuable source of information on the diversity present in historic rhino populations; other studies have likewise successfully used museum samples as a proxy for historic genetic diversity (Leonard et al. 2005; Tsangaras et al. 2012). Such specimens may be of particular importance when there is limited availability of samples representing the modern population or when extant populations are very small. In the case of the Sumatran rhinoceros, fewer than 100 individuals are estimated to occur in the wild, and an additional nine Sumatran rhinos are held in *ex situ* breeding facilities (Havmøller et al. 2016). Using archival Sumatran rhinoceros specimens allowed for the evaluation of range wide historic genetic diversity and the comparison to modern levels of diversity in a species that has experienced large scale declines.

Across a combined dataset including all Sumatran rhinoceros specimens (modern and museum) high haplotype diversity was detected ($H = 17$, $h = 0.90$, $\pi = 0.04$). When sampling was restricted to specimens that were collected from Sumatra or peninsular Malaysia or clustered with known *D. s. sumatrensis* individuals in the network, haplotype diversity in the museum specimens was high ($H = 8$, $h = 0.90$, $\pi = 0.03$) in comparison to the modern *D. s. sumatrensis*

samples ($H = 5$, $h = 0.74$, $\pi = 0.02$). Diversity within the museum sample set was higher ($H = 7.2$) than in the modern set even after rarefaction to adjust for differing sample size. Three of the five haplotypes present in the modern population were also identified in museum specimens (Ds1, Ds7, and Ds9); there were 2 haplotypes (Ds4 and Ds5) restricted to the modern population and 5 (Ds2, Ds3, Ds6, Ds8, and Ds10) restricted to the historic sample set. The 2 haplotypes restricted to the modern population occur in low frequency and are likely missing from the historic samples due to limited sampling; thus, they are unlikely to be the result of recent mutations. There has been a substantial loss of genetic diversity in the mitochondrial genome as the population has experienced significant declines. While other species that exhibit present day low genetic diversity have historic populations with similarly low diversity (koalas, Tsangaras et al. 2012; Tasmanian devils, Miller et al. 2011), this is not case for the Sumatran rhinoceros. The recent decline in Sumatran rhino populations, which has caused wide spread local extinctions and loss of subspecies, also resulted in decreased genetic diversity.

The substantial number of mutations were found between subspecies mitochondrial control region haplotypes; thus, corroborating previous studies that have shown differentiation and genetic structure between Sumatran rhino subspecies (Amato et al. 1995; Morales et al. 1997). During the last glacial maximum when sea levels were low, peninsular Malaysia and the island of Borneo were connected by the Sunda shelf (Heaney 1991; Morales et al. 1997; Leonard et al. 2015); despite this connectivity it has been suggested that a semiarid corridor and river basins may have prevented gene flow between these land masses during this time (Morley & Flenley 1987; Morales et al. 1997). The presence of a common *D. s. sumatrensis* haplotype in a *D. s. harrissoni* individual indicates that historically mitochondrial haplotypes may have been shared between subspecies. Without analysis of additional historical samples and the modern *D. s. harrissoni* individuals we are unable to resolve if haplotypes are often shared among subspecies; though, considering the currently small population size and results from earlier studies it is unlikely. It is also possible that the record for the sample of interest was incorrect or that the sequence was the result of contamination as is common when working with ancient DNA. Despite physical separation for at least ten thousand years I find, in agreement with previous reports (Amato et al. 1995; Morales et al. 1997), that mitochondrial haplotypes cannot be used to differentiate between individuals from the Malay Peninsula and the islands of Sumatra. The pattern of genetic relatedness observed across Sumatran rhinos, with populations

from Sumatra and Peninsular Malaysia being more closely related to each other than they are to populations on Borneo, is similar to patterns among a wide range of species inhabiting the Sunda shelf region (Leonard et al. 2015).

The current strategy of managing Sumatran rhinoceros populations aims to combine the subspecies into one conservation unit (Havmøller et al. 2016). This management plan may drastically alter the genetic composition of the extant populations. Nuclear microsatellite loci were used to assess levels of genetic diversity within *D. s. sumatrensis*. This subspecies exhibited low diversity ($A = 2.8$; $H_O = 0.28$), as may be expected when populations are small and isolated for multiple generations. Low diversity may be the result of processes such as drift and inbreeding, which are common in small populations that have limited or no opportunity for gene flow (Frankham 2005; Jamieson 2015). Deviations from Hardy-Weinberg equilibrium at 6 loci and a high fixation index value ($F_{IS} = 0.44$) are suggestive of subpopulation structuring within the sample set. Due to a lack of available high quality samples from the subspecies *D. s. harrissoni* analysis was restricted to the subspecies *D. s. sumatrensis*.

Genetic clustering techniques show three distinct partitions, correlating to groups consisting of peninsular Malaysian individuals and two clusters within the Sumatran island individuals. Based on the biogeographic history of the Sunda Shelf region, in which Sumatra and the Malay Peninsula have been isolated for about ten thousand years, differentiation between these populations at microsatellite loci is expected. These landmasses, currently separated by the narrow Malacca Strait, were connected during the Pleistocene and separated after the last glacial maximum (Heaney 1991; Morales et al. 1997; Leonard et al. 2015). Since the separation of Sumatra and Peninsular Malaysia, variation at microsatellite loci, likely driven by drift, has accumulated resulting in notable genetic distinction between the populations.

There is also evidence of strong differentiation among the rhinos occupying the island of Sumatra. Genetic partitions appear to correspond to populations from east and west Sumatra, which are separated by the Barisan Mountains. The Barisan Mountains, running the full length of Sumatra north to south, are a volcanic arc that has been active for millions of years (Morales et al. 1997); thus, they represent a long term barrier to gene flow. Continued isolation of small populations within the island of Sumatra will probably result in further loss of genetic diversity. To prevent further decline in genetic diversity and to increase natural mating opportunities,

conservation efforts should focus on bolstering connectivity between populations that were historically joined.

Genetic studies can provide powerful data that is imperative for sound management of endangered species (Luo et al. 2010; Jamieson 2015; McCartney-Melstad & Shaffer 2015); yet, implementation in conservation planning is limited (Frankham 2010; Keller et al. 2015). One key question that can be readily addressed with the help of genetics tools is the assessment of management units and unique evolutionary lineages (Crandall 2009; Schwartz 2009; Oliver et al. 2014). With strong evidence of genetic structuring within the subspecies *D. s. sumatrensis*, even within the populations on the island of Sumatra, coupled with the biogeographic history of the region it is expected that between subspecies genetic differentiation will be substantial. Nonetheless, current management plans aim to join the subspecies for treatment as one unit; this will effectively eliminate genetic differences and merge two potentially unique evolutionary lineages (Allendorf et al. 2001). Recent discovery of approximately 15 individuals in three populations in Indonesian Borneo gives hope for recovery of the subspecies *D. s. harrissoni* independent of hybridization with the *D. s. sumatrensis* individuals. Further, in March of 2016 a young female was captured in Indonesian Borneo for *ex situ* breeding purposes (Howard 2016). Information, such as that presented here, detailing the genetic structure within subspecies and changes in genetic diversity over time in this species as a whole should be considered to advise best management practices. Given the critically endangered status of the Sumatran rhinoceros and the serious need for conservation efforts to be improved, more genetic studies at the population should be conducted.

This study is the first to include archival Sumatran rhinoceros specimens as a way to determine historic levels of genetic diversity across the species. Using mitochondrial control region sequences from these samples, coupled with specimens from recently living rhinos, I find evidence supporting the management of *D. s. harrissoni* and *D. s. sumatrensis* as distinct conservation units. Before subspecies are interbred, levels of differentiation and estimates of divergence dates between the populations should be further investigated. Without intervention to stem further population decline and efforts to boost reproductive rates within subspecies the Sumatran rhinoceros will continue to head towards extinction.

Tables and Figures

Table 3.1. Sample information for Sumatran rhinos representing the modern population.

Lab ID	Specimen Type	Name	Sender	Sex	Birth Year	Studbook Number	Location of Origin
Dsu-28	Blood	Ipuh	Cincinnati Zoo	M	1980	28	Sumatra
Dsu-33	DNA	Rami	San Diego Zoo (ICR)	F	1980	33	Sumatra
Dsu-35	DNA	Tanjung	San Diego Zoo (ICR)	M	1980	35	Sumatra
Dsu-29	DNA	Emi	Peter de Groot	F	1988	29	Sumatra
Dsu-63	DNA	Merah	Peter de Groot	F	1980	19	Peninsular Malaysia
Dsu-64	DNA	Minah	Peter de Groot	F	1987	15	Peninsular Malaysia
Dsu-66	DNA	Panjang	Peter de Groot	F	1983	13	Peninsular Malaysia
Ratu	Skin	Ratu	Peter de Groot	F	2000	46	Sumatra
TomFoose	Skin	--	Peter de Groot	--	--	Wild	Sumatra
24	Blood	--	Peter de Groot	--	--	Unk	Sumatra
25	Blood	Dusun	Peter de Groot	F	1980	12	Peninsular Malaysia
126	Muscle	Mahato	Peter de Groot	F	1980	24	Sumatra
128	Muscle	--	Peter de Groot	--	--	Unk	Sumatra
4273	Muscle	--	Peter de Groot	--	--	Wild	Sumatra
34965	Blood	Barakas	Peter de Groot	F	1980	25	Sumatra

-- indicates information is unavailable.

Table 3.2. Sample information for archival museum Sumatran rhinos representing the historical population.

Sample Number	Tissue Type	Institution	Collection Location	Collection Year
539	Bone	National Museum of Natural History of the Netherlands	Borneo	1896
4947	Bone	National Museum of Natural History of the Netherlands	Sumatra	1941
19594	Bone	National Museum of Natural History of the Netherlands	Sumatra	1860
19595	Bone	National Museum of Natural History of the Netherlands	Sumatra	1883
19596	Bone	National Museum of Natural History of the Netherlands	Sumatra	1880
19-0311	Bone	Palaeontological Museum Munich	Borneo	1903
1908/571	Bone	Palaeontological Museum Munich	Borneo	1908
190312	Bone	Palaeontological Museum Munich	Borneo	1903
56616	Bone	Natural History Museum of Bern	Sumatra	Unk
56618	Bone	Natural History Museum of Bern	Sumatra	Unk
1880-1233	Tissue	National Museum of Natural History (Paris)	Unk	Unk
1902-308	Tissue	National Museum of Natural History (Paris)	Unk	Unk
1903-329	Bone	National Museum of Natural History (Paris)	Unk	Unk
USNM198854	Bone	National Museum of Natural History - Smithsonian	Borneo	1914
USNM199551	Bone	National Museum of Natural History - Smithsonian	Borneo	1912
USNM102076	Bone	National Museum of Natural History - Smithsonian	Borneo	1900
1500	Bone	Natural History Museum Vienna	Unk	1884
3082	Bone	Natural History Museum Vienna	Unk	1910
4294	Bone	Natural History Museum Vienna	Unk	1873
7529	Bone	Natural History Museum Vienna	Unk	1920
8173	Bone	Natural History Museum Vienna	Laos	1904
29566	Bone	Natural History Museum Vienna	Sumatra	Unk
29567	Bone	Natural History Museum Vienna	Sumatra	Unk
29568	Bone	Natural History Museum Vienna	Unk	Unk
AMNH4-54763	DNA	American Museum of Natural History	Myanmar	1924
AMNH5-81892	DNA	American Museum of Natural History	Malaysia	1933
AMNH6-173576	DNA	American Museum of Natural History	Sumatra	Unk
AMNH7-54764	DNA	American Museum of Natural History	Myanmar	1924

Table 3.3. Genetic diversity of the Sumatran rhinoceros individuals at the mitochondrial control region.

Sample Set	N	H	<i>h</i>	π
Modern <i>D. s. sumatrensis</i>	13	5	0.74	0.02
Museum <i>D. s. sumatrensis</i>	17 (13)	8 (7.2)	0.90	0.03
All museum	26	15	0.95	0.04
All	39	17	0.90	0.04

N is the number of samples.

H is the number of observed haplotypes.

h is haplotype diversity.

π is nucleotide diversity.

Rarefied values are shown in parentheses.

Table 3.4. Genetic diversity of the modern Sumatran rhinoceros individuals at 18 novel microsatellite loci.

Locus	A	F_{IS}	H_E	H_O	P_{ID}	Total P_{ID}	P_{ID(sib)}	Total P_{ID(sib)}
<i>Disu542</i>	2	-0.091	0.212	0.231	0.65	0.6542	0.81	0.8115
<i>Disu501</i>	2	-0.063	0.508	0.538	0.38	0.2493	0.60	0.4879
<i>Disu556</i>	2	0.529	0.518	0.250	0.38	0.0939	0.60	0.2907
<i>Disu863</i>	3	0.040	0.480	0.462	0.35	0.0326	0.61	0.1762
<i>Disu448</i>	2	0.520	0.471	0.231	0.40	0.0131	0.62	0.1100
<i>Disu201</i>	2	0.842*	0.471	0.077	0.40	0.0053	0.62	0.0687
<i>Disu847</i>	4	0.445	0.545	0.308	0.30	0.0016	0.56	0.0387
<i>Disu393</i>	2	-0.200	0.323	0.385	0.52	0.0008	0.73	0.0281
<i>Disu733</i>	3	1.000*	0.537	0.000	0.31	0.0003	0.57	0.0160
<i>Disu149</i>	4	0.048	0.726	0.692	0.14	<0.0001	0.44	0.0070
<i>Disu783</i>	3	0.318	0.668	0.462	0.20	<0.0001	0.48	0.0033
<i>Disu050</i>	3	0.865*	0.551	0.077	0.33	<0.0001	0.57	0.0019
<i>Disu748</i>	3	0.104	0.428	0.385	0.41	<0.0001	0.65	0.0012
<i>Disu476</i>	3	0.286	0.532	0.385	0.29	<0.0001	0.57	0.0007
<i>Disu151</i>	2	0.442	0.271	0.154	0.58	<0.0001	0.77	0.0005
<i>Disu127</i>	3	0.514*	0.465	0.231	0.38	<0.0001	0.62	0.0003
<i>Disu098</i>	5	0.665*	0.725	0.250	0.14	<0.0001	0.44	0.0001
<i>Disu582</i>	3	1.000*	0.542	0.000	0.31	<0.0001	0.57	0.0001
Overall	2.83	0.440	0.499	0.284	--	--	--	--

A is the mean number of alleles per locus.

F_{IS} is the average deviation from Hardy-Weinberg proportions.

*statistically significant, $p < 0.05$.

H_E is the mean expected heterozygosity.

H_O is observed heterozygosity.

P_{ID} is the probability of identity.

P_{ID(sib)} is the probability of identity between siblings.

Figure 3.1. Map of the current distribution of Sumatran rhinoceros populations.

This map shows land area belonging to Malaysia, including northern Borneo and the Malay Peninsula, highlighted in light red around the edges, and Indonesian regions, including southern Borneo and the island of Sumatra, highlighted with light yellow around the edges. Approximate locations of the confirmed Sumatran rhinoceros populations, indicated in green, consist of three national parks on the island of Sumatra and one region of Indonesia Borneo. Regions with recently extirpated populations in Malaysian Borneo, the Malay Peninsula and the island of Sumatra are shown in red. This map was edited from IUCN and National Geographic (www.iucnredlist.org).



Figure 3.2. Median joining network of mitochondrial control region haplotypes from modern and museum Sumatran rhinoceros samples.

Each circle in the network represents one of the 17 unique mitochondrial control region haplotypes detected. Hash marks between haplotypes indicate the occurrence of mutations. Circle sizes are proportional to the number of rhinos carrying each haplotype and are color coded by sampling location. Individuals carrying haplotypes Ds1 – Ds10 mainly originated from populations in Sumatra (orange) and Peninsular Malaysia (red), and are members of the subspecies *D. s. sumatrensis*. All samples of unknown origin (yellow) were identified as having haplotypes within the Ds1 – Ds10 subcluster; thus, they fall within known variation of *D. s. sumatrensis* individuals. Haplotypes Ds11 and Ds 12 were found in samples from Myanmar (gray) and Laos (blue), respectively, representing the subspecies *D. s. lasiotis*. Bornean individuals (green) from the subspecies *D. s. harrissoni* had haplotypes Ds13 – Ds17, with the exception of one sample potentially mislabeled as being from Borneo which had haplotype Ds1.

Figure 3.2. Cont.

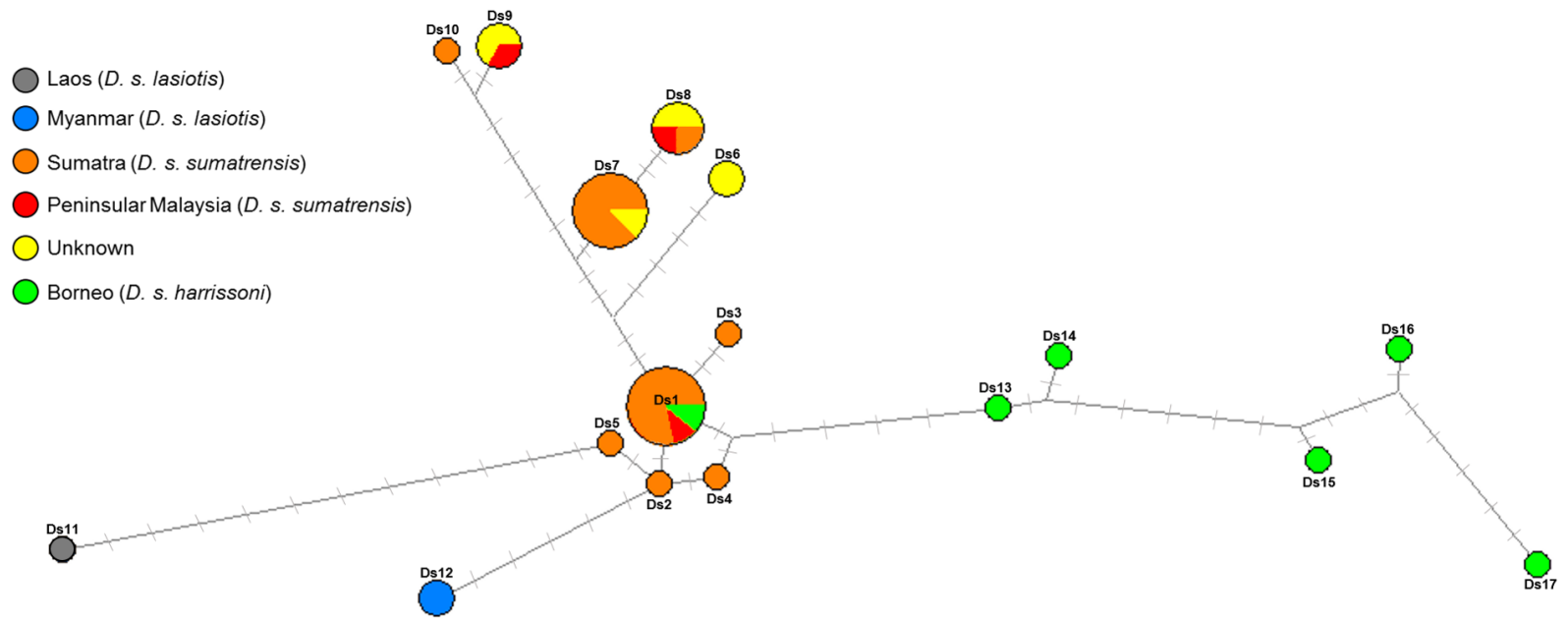


Figure 3.3. Median joining networks of mitochondrial control region haplotypes for individuals within the putative *D. s. sumatrensis* subcluster separated into modern and museum groups.

Each circle in the network represents a distinct mitochondrial control region haplotype; each hash mark indicates a mutation. Circles representing haplotypes are proportional to the number of rhinos carrying each haplotype and are color coded by sampling location: Sumatra (orange), Peninsular Malaysia (red), and unknown (yellow). Three haplotypes, **Ds1**, **Ds7**, and **Ds9**, were identified in both modern and museum samples (names shown in bold). Haplotypes **Ds4** and **Ds5** were only identified in modern samples while **Ds2**, **Ds3**, **Ds6**, **Ds8**, and **Ds10** were only found in museum samples. There were a total of 12 mutations among haplotypes in modern samples and 19 mutations among haplotypes in museum samples. The Borneo museum specimen with haplotype **Ds1** was excluded from this analysis.

Figure 3.3. Cont.

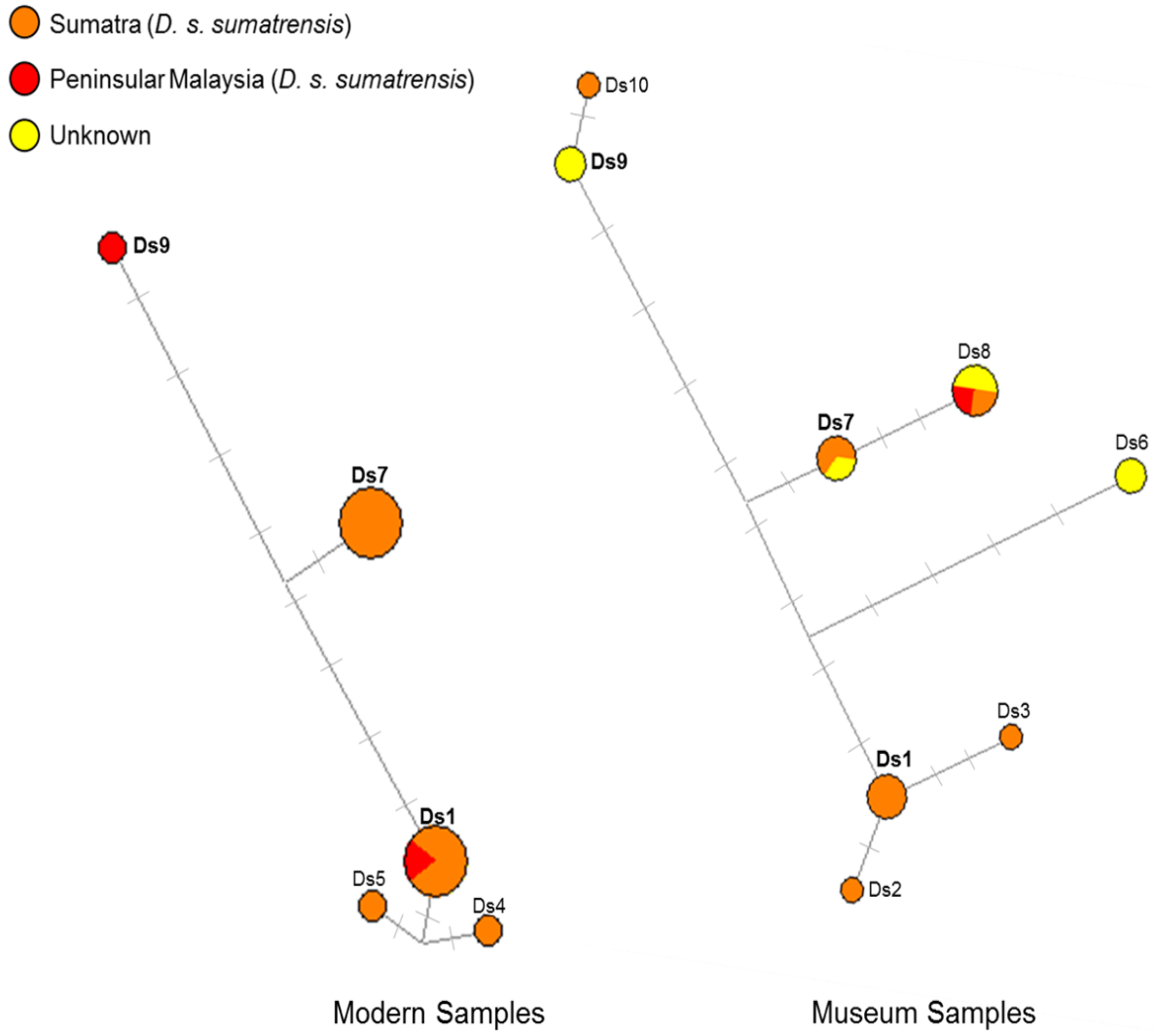


Figure 3.4. Genetic partitioning within modern Sumatran rhinoceros samples.

STRUCTURE analyses using multilocus genotypes from 18 microsatellite in 13 Sumatran rhinoceros samples representing the modern population. Software settings assumed admixture between populations and correlated allele frequencies. Ad hoc methods to determine the number of partitions provided support for a varying number of clusters, with a minimum of 2 and a maximum of 4. The best supported K value using the ΔK method was $K = 2$ and $\text{LnP}(D)$ supported $K = 4$. To further identify the most likely number of genetic partitions the geography of the study area and biology of the species was considered to best determine the correct number of genetic clusters identified by STRUCTURE. Given the biology and biogeography of Sumatran rhinos clustering patterns at $K = 3$ were informative, while $K = 4$ did not provide any additional information. At $K = 3$, genetic distinctiveness between rhinos from the island of Sumatra (red and blue) and the Malay Peninsula (green) was evident, in addition a clear partition was observed within individuals from Sumatra.

Figure 3.4. Cont.

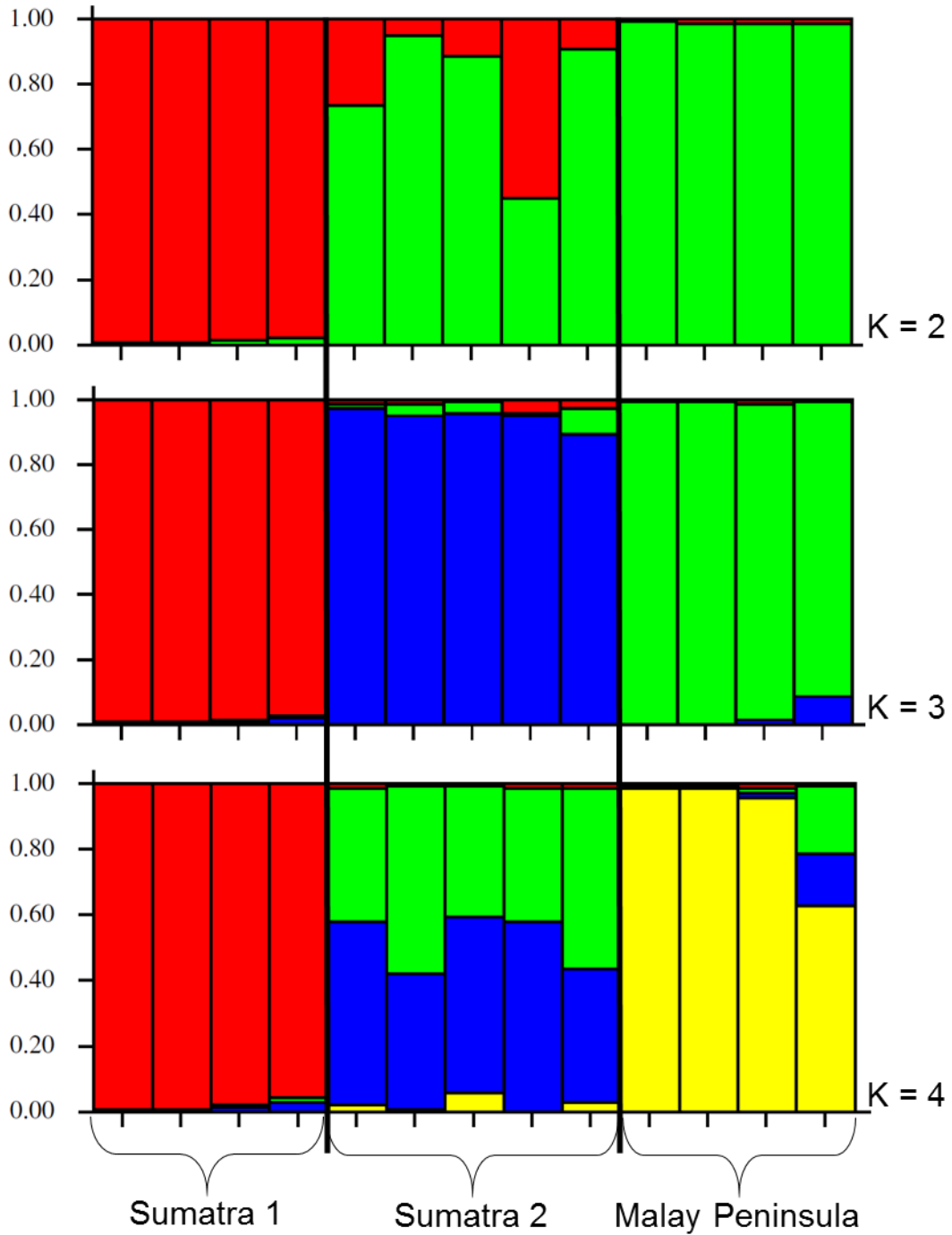
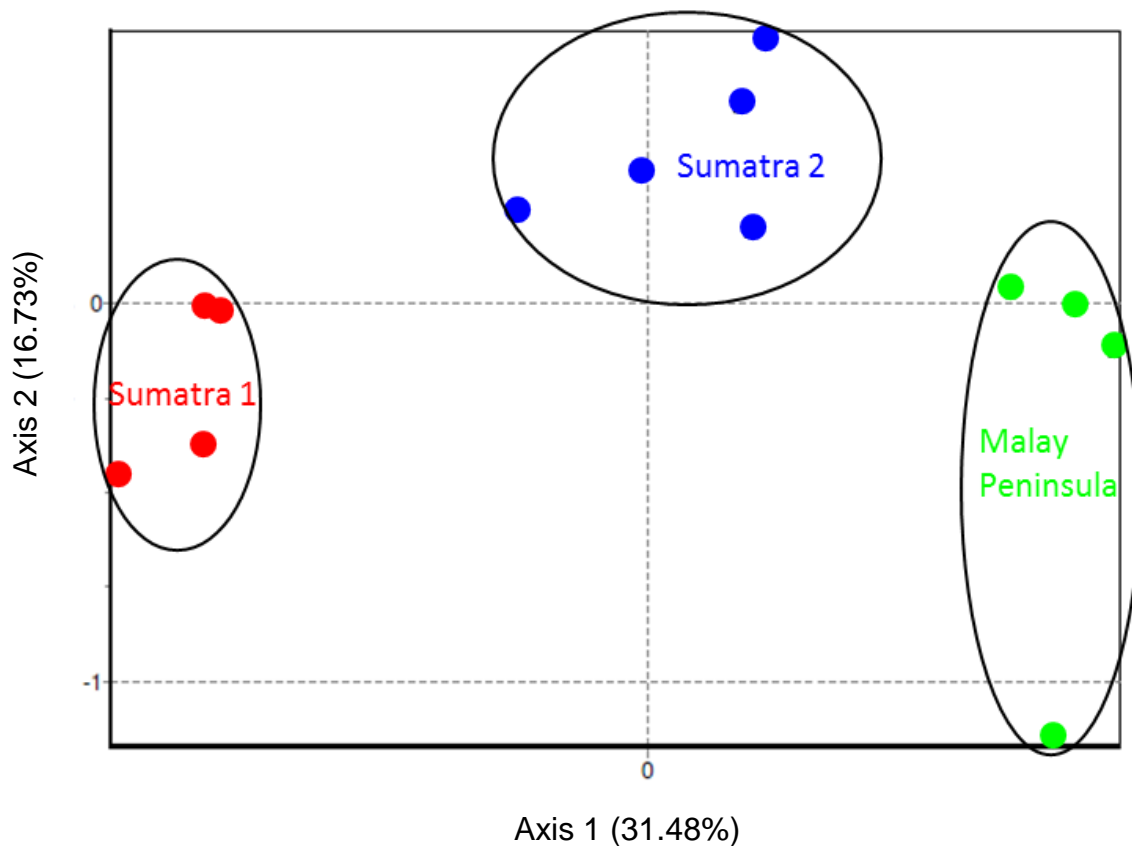


Figure 3.5. Genetic clustering within modern Sumatran rhinoceros samples.

Multivariate factorial correspondence analysis, implemented in the program GENETIX, used 18 microsatellite loci genotyped in 13 Sumatran rhinoceros samples representing the modern population. The multilocus genotype of each individual is represented by a circle on the graph. Individuals were colored based on the STRUCTURE cluster they were primarily assigned to: Sumatra 1 (red), Sumatra 2 (blue), and Malay Peninsula (green). Three genetic clusters were identified, with two distinct groups forming within the island of Sumatra and a group containing individuals from the Malay Peninsula. Variation explained along Axes 1 and 2 was 31.48% and 16.73%, respectively.



CHAPTER 4. TOLL-LIKE RECEPTOR DIVERSITY IN AFRICAN RHINOCEROS SPECIES

Abstract

Neutral loci (e.g., microsatellites) have commonly been used in wildlife genetic studies to assess levels of genetic diversity and structure. Unlike neutral markers, adaptive loci possess crucial information about the ability of a population to adapt to emerging challenges or environmental change. In particular, adaptive loci within the immune system provide insight about the ability of a population to resist infectious pathogens. Toll-like receptors (TLR) bind pathogen-specific molecules and initiate both innate and adaptive immune responses, and thus may be of particular relevance to conservation geneticists and management authorities. To examine the diversity of TLRs in highly endangered rhinos, I sequenced the gene regions coding for the extracellular domain of 8 TLR loci in eastern black (N = 12), south-central black (N = 11), and white (N = 26) rhinos from North American zoos and *ex situ* breeding facilities. Additionally, mitochondrial control region haplotypes were sequenced for all individuals and multi-locus microsatellite genotypes were obtained for the black rhinoceros samples. Overall, TLR diversity was very low among white rhinos with a total of five SNPs (three nonsynonymous; two synonymous) and 13 haplotypes identified, and only two control region haplotypes ($h = 0.50$) were detected. The eastern black rhino subspecies was most diverse subspecies containing five mitochondrial haplotypes ($h = 0.78$), moderate microsatellite loci diversity ($A = 5$; $H_O = 0.57$), and 22 SNPs (12 nonsynonymous; 10 synonymous) comprising 28 TLR haplotypes. The south-central black rhino subspecies had five mitochondrial haplotypes ($h = 0.80$), modest microsatellite loci diversity ($A = 3.3$; $H_O = 0.49$), and 18 SNPs (11 nonsynonymous; 7 synonymous) comprising 19 TLR haplotypes. Some haplotypes were shared by the subspecies at all TLR loci, but based high genetic differentiation ($F_{ST} = 0.27$), monophyletic mitochondrial haplotype clades, and the presence of unique TLR haplotypes between the black rhinoceros subspecies, continued management of the taxa as two separate conservation units is supported. Limited variation in the TLR genes of the African rhinos, particularly the white rhinoceros, suggests that the evolutionary potential of the immune system is limited. Future management and breeding programs for rhinoceros species should seek to preserve immune system diversity.

Introduction

Conservation genetic studies assessing variation in wildlife species have been largely reliant on diversity estimates derived from putatively neutral markers, such as microsatellites. While neutral markers can be highly informative, adaptive gene loci can be used to better understand a species evolutionary potential (Hedrick 1999; Holderegger et al. 2006; Kirk & Freeland 2011). The diversity present at immune system genes (i.e., genes responsible for recognizing invading pathogens and mediating subsequent immune responses) may be of particular interest since they are expected to evolve more quickly than other genes due to parasite mediated selection (Alcaide & Edwards 2011). High levels of diversity in the immune genes of natural populations have been associated with defense against and resistance to infection by pathogens and parasites (Villasenor-Cardoso & Ortega 2011; Grueber et al. 2013); thus, potentially improving long term survival of a species. When a population becomes highly fragmented, decreases in size, or experiences a bottleneck, as is common in rare, threatened, and endangered species, the diversity of genes that protect against pathogens may be reduced through genetic drift (Sommer 2005; Bos et al. 2008; Ujvari & Belov 2011). The impact of infection on populations of endangered species, especially those with low genetic diversity, is of major concern, as some may not persist if faced with an emerging pathogen (O'Brien & Evermann 1988; Daszak et al. 2000; Acevedo-Whitehouse & Cunningham 2006; Smith et al. 2006).

The major histocompatibility complex (MHC) has dominated studies of adaptive diversity in non-model wildlife species due to commonly high levels of variability (Acevedo-Whitehouse & Cunningham 2006; Tschirren et al. 2011; Grueber et al. 2012). Despite the importance of MHC genes in studies of diversity and disease resistance, technical issues associated with efficient and reliable genotyping (e.g., amplification of artifacts and pseudogenes) complicate analysis (Babik 2010; Grueber et al. 2012). Additionally, MHC genes do not account for all of the genetic variability that plays a role in pathogen/parasite resistance (Acevedo-Whitehouse & Cunningham 2006); thus, essential variation is present in other candidate immune genes. As such, there is need for increased research on other immune system genes (e.g., Toll-like receptors) in wildlife populations. Toll-like receptors (TLRs) comprise a multigene family of pattern recognition receptors (PRRs) that are critical to the functioning of

both the innate and adaptive immune systems (Akira et al. 2001; Pasare & Medzhitov 2005; Alcaide & Edwards 2011; Grueber et al. 2012).

TLRs are type I integral transmembrane glycoproteins characterized structurally by a leucine-rich repeat (LRR) region in the extracellular domain (which is directly involved in pathogen recognition), a transmembrane region, and a Toll-IL-1 receptor domain (TIR) (Medzhitov et al. 1997; Akira & Takeda 2004; Bergman et al. 2012). As PRRs, each TLR recognizes specific conserved pathogen-associated molecular patterns (PAMPs; Janeway 1989; Medzhitov 2001) (Table 4.1). After ligand binding of the PAMP to the extracellular domain of the TLR, an intracellular signaling cascade initiates innate and subsequently adaptive immune responses (Janeway 1989; Akira et al. 2001; Medzhitov 2001; Akira & Takeda 2004; Akira et al. 2006; Uematsu & Akira 2008; Barreiro et al. 2009; Bergman et al. 2010; Alcaide & Edwards 2011; Tschirren et al. 2011). In most mammals, at least 10 members of the TLR family have been identified, each responding to a unique set of ligands/agonists (Akira & Takeda 2004). Recent studies have shown TLR loci to be informative in investigations of diversity in species of conservation concern (Grueber et al. 2012; Grueber et al. 2013; Hartmann et al. 2014; Cui et al. 2015a; Cui et al. 2015b; Grueber et al. 2015; Morris et al. 2015), and can be helpful in addressing a number of conservation issues including: planning translocations and *ex situ* breeding programs, identifying conservation units, assessing the impact of a bottleneck, and quantifying disease susceptibility (Ujvari & Belov 2011).

High levels of poaching and habitat destruction have caused considerable population declines in all rhinoceros species, resulting in categorization of threatened or endangered (Scott 2008; CITES 2010). The southern white rhino (*Ceratotherium simum simum*) is currently the most abundant of any rhinoceros subspecies. Southern white rhinos were numerous and widespread in the 1800s, distributed mainly south of the Zambezi River (Cumming et al. 1990; Emslie & Brooks 1999). By the late 1800s the southern white rhinoceros was thought to be extinct; however, a small remnant population of 20 to 100 individuals, from which the current population originated, was discovered in what is today the KwaZulu-Natal province of South Africa (Groves 1972). Protection and conservation efforts allowed for rapid recovery to a current estimated population size of more than 20,000 (Emslie et al. 2013; Labuschagne et al. 2013), with more than 90% of the individuals occurring in South Africa. Two published papers aiming to develop polymorphic microsatellites for the white rhinoceros found a high number of

monomorphic loci and a low number of alleles per variable locus (Florescu et al. 2003; Hou et al. 2012); thus, indicating that white rhinos are characterized by low genetic diversity. Low levels of diversity in the white rhino has been further corroborated by studies using allozymes, microsatellites, and mitochondrial DNA (Merenlender et al. 1989; O'Ryan & Harley 1993; Scott 2008; Coutts 2009; Guerier et al. 2012). Many southern white rhinoceros populations are limited in size and lack gene flow with other populations, thus maintenance of genetic diversity is a major goal of conservation efforts (Frankham 2005).

In comparison, historic population size estimates for the black rhinoceros (*Diceros bicornis*) were well over 100,000 prior to 1960 (Emslie 2012). Between 1960 and the mid-1990s the total population of black rhinos had declined by more than 95% as a result of poaching and habitat alteration (Harley et al. 2005; Metzger et al. 2007; van Coeverden de Groot et al. 2011); the current population is estimated to be ~5,000 individuals. The black rhinoceros occurs almost exclusively in remnant populations, which is a major concern for the persistence of the species and individual populations (Hillman-Smith & Groves 1994; Moehlman et al. 1996); genetic monitoring and assessment of diversity is needed for successful conservation planning and management implementation. The eastern black rhino (*D. b. michaeli*) population, reaching a low of < 400 individuals, is now the most endangered subspecies, with 740 individuals remaining primarily in Kenya (Emslie & Brooks 1999; Muya et al. 2011). The south-central black rhino (*D. b. minor*) subspecies, with 2,220 individuals restricted to South Africa and Zimbabwe, has the largest population size of any of the subspecies. The current *D. b. minor* population was established through translocations from two small populations of 110 total individuals in South Africa and 425 individuals in Zimbabwe (Emslie & Brooks 1999; Okita-Ouma et al. 2007; Karsten et al. 2011; Kotzé et al. 2014). Previous genetic studies have established that the eastern black rhino exhibits the highest overall levels of genetic variability while the south-central black rhino shows the lowest level of diversity at mitochondrial and microsatellite loci (Harley et al. 2005; Scott 2008; Karsten et al. 2011; Muya et al. 2011; Anderson-Lederer et al. 2012). Both subspecies of black rhinoceros are currently managed in active *ex situ* breeding programs in North American zoos and research institutions.

Here, I characterize TLR diversity in African rhinoceros species. In this study I: 1) designed primers to amplify extracellular domain orthologs of eight TLR genes across African rhinoceros species, 2) assessed levels of single nucleotide polymorphisms across a suite of Toll-

like receptor genes in *ex situ* white rhinoceros and in two subspecies of black rhinoceros, and 3) determined whether diversity levels and intra-taxa relationships seen at TLR genes are consistent with those observed using other genetic marker types. Information derived from these important immune genes will serve as a critical tool for conservation and management of these species.

Methods and Materials

Samples

Endorsement for the proposed rhinoceros research was obtained from the Association of Zoos and Aquariums, which is necessary for collection of samples from rhinoceros individuals held *ex situ* in North America. Whole blood or DNA samples were obtained from 26 southern white rhinoceros and 23 black rhinoceros (*D. b. michaeli*, N = 12; *D. b. minor*, N = 11) (Table 4.2). All whole blood samples were collected during routine veterinary care; samples were collected in EDTA tubes to prevent clotting and kept refrigerated until DNA isolation (< 1 week from time of collection). All work was conducted with IACUC approval (protocol # 15053). DNA was isolated from whole blood samples using Qiagen DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's recommended protocol

TLR Primer Design

Complete mRNA sequences of TLR genes identified in the sequenced white rhinoceros genome were accessed from GenBank (accession numbers: TLR1: XM_004418976, TLR2: XM_004420930, TLR3/variant1: XM_004428765, TLR3/variant 2: XM_004428766, TLR4/variant1: XM_004423370, TLR4/variant2: XM_004423371, TLR5: XM_004439536, TLR6: XM_004418978, TLR7: XM_004435114, TLR10: XM_004418975). White rhinoceros sequences were aligned to TLR genes sequences available from closely related species (e.g., horse, *Equus caballus*) using MUSCLE (Edgar 2004) in the software MEGA6 (Tamura et al. 2013). TLR gene sequences from humans were included as well to avoid designing primers that would potential amplify non-target human DNA. Using PRIMER3 (Rozen & Skaletsky 2000) rhinoceros-specific primers were designed as closely as possible to the extracellular LRR-N-

terminal (LRRNT) domain of the longest exon of TLRs 1, 2, 3, 4, 5, 6, 7, and 10; this primer placement allowed for the sequencing of a majority of functionally relevant sites involved in pathogen recognition and dimerization.

TLR and Mitochondrial DNA Amplification and Sequencing

Novel TLR primer sets (Table 4.3) and an fragment of the mitochondrial control region (~450 bp) were amplified by polymerase chain reaction using the following reaction mixture with final concentrations of: 0.4 μ M of each forward and reverse primer, 0.2mM of each dNTP (Applied Biosystems Inc., [ABI]), 1x PCR buffer, 1.5 - 2mM MgCL₂, and 0.4 units of AmpliTaq Gold DNA polymerase (ABI). The PCR algorithm used for the TLR genes was as follows: initial denaturation at 95°C for 9:45 min; with 16 cycles of 20 sec at 94°C, 30 sec at 64°C down to 56°C (1°C decrease every 2 cycles), 3 min at 72°C; followed by 18 additional cycles with 56°C annealing and a final extension at 72°C for 7 min. The PCR algorithm for the mitochondrial control region was: initial denaturation at 95°C for 9:45 min; 3 cycles of 20 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 56°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 54°C, 30 sec at 72°C; 5 cycles of 20 sec at 94°C, 30 sec at 52°C, 30 sec at 72°C; followed by 22 additional cycles with 50°C annealing and a final extension at 72°C for 7 min.

TLR and mitochondrial PCR products that produced clear, single bands of the expected size on an ethidium bromide stained agarose gel were enzymatically purified using an Exonuclease I and shrimp alkaline phosphatase (ExoSAP) reaction (Hanke & Wink 1994). Purified PCR products were Sanger sequenced in both directions using the BigDye Terminator System (ABI), and purified and resolved on an ABI 3730XL genetic analyzer at the University of Illinois at Urbana-Champaign Biotechnology Center. Internal sequencing primers were used as required to obtain high quality chromatograms across the region of interest.

TLR Analysis

Resulting sequences were trimmed, concatenated, and edited in the software SEQUENCHER (Gene Codes Corporation). Single nucleotide polymorphisms (SNPs) in the

TLR genes were visually confirmed, and International Union of Pure and Applied Chemistry ambiguity codes were used where the chromatogram showed double-peaks typical of a heterozygote. Sequences for each individual within a taxon were aligned after initial editing and SNP locations were recorded. Subsequently, chromatograms for each individual were examined to verify correct identity at each SNP; putative SNPs that occurred at low frequency were reamplified and resequenced to avoid overestimation of diversity due to errors during amplification or sequencing. No SNP data was missing for individuals included in analysis.

Within each species haplotypes were inferred using PHASE 2.1 (Stephens et al. 2001) in DNAsp v5 (Librado & Rozas 2009). Markov chain Monte Carlo options were set to 1000 iterations, 10 thinning intervals, and 100 burn-in iterations, all other parameters were set at default values. The DNAsp software was also used to estimate basic polymorphism statistics, haplotype (h) and nucleotide (π) diversity, and identify non-synonymous and synonymous SNP loci in each TLR locus. NCBI ORF Finder was used to identify open reading frames and verify each SNP as synonymous or non-synonymous. Tests of Hardy-Weinberg equilibrium (HWE) and expected (H_E) and observed heterozygosity (H_O) of each TLR locus were calculated using the GenAIEx v6.501 software (Peakall & Smouse 2006; Peakall & Smouse 2012). To estimate HWE , H_E , and H_O each haplotype identified in an individual was assigned a letter code and used as a genotype. To examine the relationship among TLR haplotypes across the different rhinoceros species, we constructed median-joining (MJ) networks using the software NETWORK v4.6.1 (Bandelt et al. 1999) for each TLR.

Tajima's D test statistic (Tajima 1989) was calculated using DNAsp to identify potential deviations from neutrality within each of the study taxa. Negative Tajima's D values are often indicative of purifying selection or a population expansion following a bottleneck, while a positive Tajima's D value may indicate balancing selection or a population contraction. In addition, for each TLR gene the McDonald-Kreitman statistic (McDonald & Kreitman 1991) was calculated; since this test requires sequence from multiple species, samples were grouped by species to achieve similar sample sizes. McDonald-Kreitman identifies signatures of positive selection by comparing the ratio of fixed and polymorphic synonymous and non-synonymous mutations between species.

To characterize the structure of rhinoceros TLR proteins, including the locations of the LRRs, LRRNT, LRR-C-terminal (LRRCT), and Toll-IL-1 receptor (TIR) domains, the white

rhinoceros amino acid sequences from GenBank (accession numbers: TLR1: XP_004419033.1, TLR2: XP_004420987.1, TLR3/variant2: XP_004428823.1, TLR4/variant2: XP_004423428.1, TLR5: XP_004439593.1, TLR6: XP_004419035.1, TLR7: XP_004435171.1, TLR10: XP_014642233.1) were analyzed in LRRFINDER (Offord et al. 2010; Offord & Werling 2013). A diagrammatic structure of each white rhinoceros TLR protein based on the LRRFINDER results was created, and the portion of the protein represented by inferred amino acid sequence (translated from nucleotide sequence generated in this study) was identified. To determine if any SNPs disrupted conserved LRR motifs, the location of each non-synonymous mutation was located in the inferred amino acid sequence alignment of white and black rhinoceros TLRs in MEGA6.

Mitochondrial Control Region Analysis

Sequences of mitochondrial control region haplotypes were aligned in SEQUENCHER, and haplotype diversity indices were calculated for each taxon in the DNAsp software. Control region sequences were used to generate a median-joining network using the software NETWORK. To confirm relationships among taxa a maximum-likelihood tree was generated in MEGA6 using Indian rhinoceros (*Rhinoceros unicornis*) sequence as an outgroup. One thousand bootstrap replicates were completed to estimate node support, and all other parameters were set at default values.

Microsatellites

To further analyze the differentiation between black rhinoceros subspecies, ten unpublished microsatellite loci developed in black rhinos, *Dibi15*, *Dibi24*, *Dibi25*, *Dibi26*, *Dibi27*, *Dibi32*, *Dibi34*, *Dibi49*, *Dibi50*, and *Dibi56*, were genotyped. PCR primers were tagged for fluorescence detection and amplified using a touchdown profile as previously described in by Ishida and others (2012). Samples were genotyped on an ABI 3730XL genetic analyzer and scored using GENEMAPPER software (ABI). Diversity indices and F-statistics were calculated using GENEPOP and FSTAT (Raymond & Rousset 1995; Rousset 2008). Bayesian clustering in STRUCTURE v2.3.3 (Pritchard et al. 2000) was used to assess patterns of genetic partitioning

between black rhino subspecies. Four models with varying assumptions regarding individual ancestry and relatedness among populations were implemented. The four models considered were: 1) admixture with correlated allele frequencies; 2) admixture with independent allele frequencies; 3) no admixture with correlated allele frequencies; and 4) no admixture with independent allele frequencies. Each model was run three times for values of $K = 1$ through $K = 5$ with 1 million Markov chain Monte Carlo steps and a burn in of 100,000 steps. The most likely number of population clusters (K) was evaluated in STRUCTURE HARVESTER (Earl & vonHoldt 2012) through the estimation of two *ad hoc* values: ΔK (Evanno et al. 2005) and log probability of data, $\ln P(D)$ (Pritchard et al. 2000).

Results

TLRs

The extracellular domains of eight TLR genes were successfully sequenced in 46 to 49 individuals of white ($N = 26$) and black rhinoceros ($N = 20 - 23$) (GenBank accession numbers: Pending) (Table 4.4). Across the TLR genes between 1306 bp (TLR3) and 1922 bp (TLR7) of sequence was obtained. No alignments among or within taxa showed frame-shift or in-del mutations, and there were no more than two peaks per site in each chromatogram; thus, no evidence of pseudogenation was found. The sequenced portion of TLR6 exhibited the least diversity with no SNPs in any of the study taxa. TLR1 was monomorphic in white and eastern black rhinos, while TLR10 was monomorphic in white and south-central black rhinos. TLRs 2 and 5 were monomorphic in white rhinoceros but polymorphic in both black rhinoceros subspecies.

White rhinos exhibited low TLR diversity based on number of detected SNPs and haplotypes, haplotype diversity, and nucleotide diversity. A total of five SNPs of which three were non-synonymous were present across all loci in the white rhinoceros (Table 4.4). A single unique haplotype was detected at five TLR loci (TLR1, TLR2, TLR5, TLR6, and TLR10) in the white rhino. At loci with more than one haplotype, diversity ranged from 0.31 (TLR4) to 0.50 (TLR7) and nucleotide diversity ranged from 0.0002 (TLR4) to 0.0003 (TLR3 and TLR7) (Table 4.5). The eastern black rhinoceros had the highest overall levels of diversity across TLR loci

with 22 SNPs (12 non-synonymous) identified (Table 4.4). The number of haplotypes detected per gene ranged from 1 (TLR1 and TLR6) to eight (TLR4). Haplotype diversity at loci with more than one haplotype ranged from 0.16 (TLR10) to 0.81 (TLR4) and nucleotide diversity ranged from 0.0001 (TLR10) to 0.0016 (TLR4) (Table 4.5). Diversity in the south-central black rhino was slightly lower; a total of 18 SNPs (11 non-synonymous) were detected (Table 4.4). In the south-central black rhino the number of haplotypes detected per gene ranging from one (TLR6, and TLR10) to four (TLR4 and TLR5). Haplotype diversity at loci with more than one haplotype ranged from 0.37 (TLR1) to 0.66 (TLR5) and nucleotide diversity ranged from 0.0003 (TLR1 and TLR7) to 0.0017 (TLR5) (Table 4.5).

All TLR loci were in Hardy-Weinberg equilibrium with the exception of TLR7 in the eastern black rhinoceros. Mean TLR H_O and H_E varied by taxa with eastern black rhino having the highest values ($H_O = 0.37$; $H_E = 0.42$), followed by the south-central black rhinoceros ($H_O = 0.31$; $H_E = 0.36$), and white rhinoceros having the lowest values ($H_O = 0.14$; $H_E = 0.16$) (Table 4.6). No haplotypes were shared by black and white rhinos; thus, haplotypes were restricted by species as shown by median joining networks for each TLR gene (Figure 4.1). The number of mutations separating white rhinoceros haplotypes from black rhinoceros haplotypes was between 1 (TLR5) and 12 (TLR10) (Figure 4.1). The black rhino subspecies haplotypes were shared at all loci; however, with the exception of TLR6 (where only one haplotype is detected) haplotypes distinct to at least one of the subspecies were detected (Figure 4.1). Most black rhinoceros haplotypes formed tight clusters and were separated by one or two mutations.

A positive and significant Tajima's D value ($D = 2.084$) was found at TLR5 in the south-central black rhinoceros group (Table 4.5), suggesting the presence of balancing selection or a recent population contraction. Ten out of fifteen Tajima's D estimates had positive, but statistically insignificant, values. Using the McDonald-Kreitman statistic the ratio of synonymous and non-synonymous SNPs fixed between and polymorphic within white and black rhinoceros species was compared. Evidence of negative selection at TLR4, which contained a significant excess of non-synonymous polymorphisms within species ($G = 5.269$, $p = 0.0217$), was identified. Estimates of McDonald-Kreitman statistic were non-significant for all other loci (Table 4.5).

Published sequences for the white rhinoceros were used to estimate the expected protein structure of TLRs in the African rhinoceros species (Figure 4.2). Between 14 (TLR4) and 24

(TLR7) LRR regions were recognized as having database matches or were significantly identified per protein sequence. Amino acid sequence was inferred for a majority of the LRRs (85%) present in the extracellular domains for the sequenced TLR genes. The proportion of LRRs represented by inferred amino acid sequence varied by TLR gene, ranging from 65% (TLR3) to 100% (TLR10) (Figure 4.2). With the exception of one A to T SNP variant in TLR1 of the south-central black rhino, there were no disruptions in the expected highly conserved 11 residue (LxxLxLxxNxL) or 12 residue (LxxLxLxxCxxL) portions of the LRRs identified for each rhinoceros TLR gene. Four south-central black rhinos contained this SNP mutation resulting in a change from asparagine to isoleucine at amino acid position 58 of the reference sequence; one individual was homozygote for the isoleucine variant while the other three were heterozygote.

Mitochondrial control region

We successfully amplified 415 bp of mtDNA control region in 26 white rhinos, 11 south-central black rhinos, and 10 eastern black rhinos. Two haplotypes were identified in the white rhinoceros samples; overall haplotype (h) and nucleotide (π) diversity were low at 0.50 (SD \pm 0.025) and 0.00467 (SD \pm 0.00023), respectively (Table 4.6). A total of 10 haplotypes were found among black rhinoceros samples; for each black rhinoceros subspecies five distinct haplotypes were identified. Both subspecies had relatively high haplotype and nucleotide diversity; for the south-central subspecies $h = 0.80$ (SD \pm 0.041) and $\pi = 0.00696$ (SD \pm 0.00046) and for the eastern subspecies $h = 0.78$ (SD \pm 0.061) and $\pi = 0.0092$ (SD \pm 0.00099) (Table 4.6). One haplotype, identified in a single the eastern black rhino sample, had not been previously reported (GenBank accession number: Pending). Black rhino mitochondrial haplotypes form clusters that correspond to subspecies designations (Figure 4.3a). The two black rhino subspecies haplotype clusters were distinguished from each other by 12 mutations; black and white rhinoceros haplotype groups are separated by 53 mutations (Figure 4.3a). Likewise, maximum likelihood analysis showed three monophyletic clades comprised of white rhino, eastern black rhino, and south-central black rhino; all clades were support by high bootstrap values (Figure 4.3b).

Microsatellites

Complete multilocus microsatellite genotypes were obtained for 11 eastern black rhinos and 11 south-central black rhinos; one additional eastern black individual was genotyped at 8 out of 10 microsatellite loci. Within the eastern subspecies, the average number of alleles per locus (A) was 5.0 and observed and expected heterozygosity were 0.57 and 0.65, respectively (Table 4.6). Within the south-central subspecies, the average number of alleles per locus was 3.3 and observed and expected heterozygosity were 0.49 and 0.54, respectively (Table 4.6). No significant linkage disequilibrium was detected between loci, and all loci were in Hardy-Weinberg equilibrium after Bonferroni adjustment for multiple comparison. The pairwise F_{ST} value of 0.274 between subspecies was high and statistically significant, suggesting strong genetic differentiation between the groups. Both ΔK and $\ln P(D)$ methods for determining the most likely number of genetic clusters from STRUCTURE outputs supported $K = 2$ for all four models, correlating to subspecies groupings, regardless of which model parameters were chosen (Figure 4.4).

Discussion

Here I characterized baseline levels of variation in TLR loci of the white and black rhinoceros; both species have gone through population bottlenecks and are currently threatened or endangered. We found that the southern white rhinoceros has low variation at TLR loci, exhibiting monomorphism at five genes, in comparison to south-central and eastern black rhinoceros subspecies. A profound lack of genetic diversity has been previously reported in studies of mitochondrial and microsatellite loci in white rhinoceros (Merenlender et al. 1989; O'Ryan & Harley 1993; Florescu et al. 2003; Scott 2008; Coutts 2009; Guerier et al. 2012; Hou et al. 2012). Similarly low diversity has now been detected across adaptive loci (i.e., gene with evolutionary potential). Previous studies have suggested that this reduced level of genetic variation is not the result of the recent population bottleneck (Scott 2008; Coutts 2009), but may be due to a historic population event. Similarly low levels of diversity across TLR genes in other threatened and endangered species has been reported (Tasmanian devils, Cui et al. 2015a; New

Zealand robins, Grueber et al. 2012); however, other species of conservation concern exhibit higher levels of diversity than those reported in rhinoceros (Cui et al. 2015b).

Overall diversity in black rhinoceros subspecies was higher than that observed in white rhinoceros. Estimates of diversity at TLR and microsatellite loci in this study followed the previously published patterns suggested by mtDNA and microsatellite markers (Harley et al. 2005; Scott 2008; Karsten et al. 2011; Anderson-Lederer et al. 2012). At these two marker types the eastern black rhinoceros subspecies exhibited higher levels of diversity (TLRs: $H = 3.63$ and $h = 0.44$; microsatellite: $A = 5.0$ and $H_O = 0.57$) than the south-central black rhinoceros subspecies (TLRs: $H = 2.38$; $h = 0.38$; microsatellite: $A = 3.3$ and $H_O = 0.49$). However, at the mitochondrial control region the south-central black rhinoceros ($h = 0.80$ and $\pi = 0.00696$) showed slightly higher haplotype diversity but lower nucleotide diversity than was observed in the eastern black rhinoceros ($h = 0.78$ and $\pi = 0.0092$). The two subspecies do not share any mitochondrial control region haplotypes and show high levels of differentiation based on microsatellite analysis, but share haplotypes at all TLR loci. Shared TLR haplotypes have been reported in different species of recently diverged pipet species (Gonzalez-Quevedo et al. 2015) and are potentially the result of incomplete lineage sorting or balancing selection. Despite shared TLR haplotypes, high estimates of population differentiation support the current management strategy of maintaining the subspecies as separate conservation units.

Pathogen mediated selection is expected to shape diversity at immune-associated genes through rapid co-evolution between microbes and the immune system (Haldane 1949). Such selective pressures presumably enable the immune system to retain diversity and mount adequate defenses against novel and resurging pathogens. The level of diversity at immune system genes, as presented in the rhinoceros species here, may be critical for consideration when developing management plans for recovering populations. For example, some populations of black and white rhinoceros are experiencing mortality from emerging tick borne pathogens, including *Theileria bicornis* and *Babesia bicornis* (Nijhof et al. 2003; Penzhorn et al. 2008; Govender et al. 2011; Obanda et al. 2011; Otiende et al. 2014; Otiende et al. 2015). Those populations with high levels of diversity at TLRs may be more likely to mount an adequate immune response when a novel pathogen emerges and, as a result, have improved long long-term fitness and survival. Availability of immune gene diversity data can be used by managers to ensure that *ex situ* populations which are part of breeding programs do not go through another bottleneck by

ensuring that all TLR alleles are retained in future generations (Cui et al. 2015a). Using this approach breeding programs could determine which mating pairs would be of most value to the population's disease resilience, while preventing mating between genetically incompatible individuals (e.g., those with low and similar immune diversity) (Glatston 2001; Ujvari & Belov 2011). In a similar sense immune system diversity data could provide crucial insight for genetic rescue through translocation of individuals between populations. In this case individuals with high immunogenetic diversity could be identified and moved to supplement populations exhibiting low or differing diversity (Tallmon et al. 2004).

No evidence of strong selection was detected across TLR loci in the African rhinoceros species; however, potential balancing selection is detected at one locus (TLR5) in the south-central black rhino subspecies. Given the species history and current small population size, drift may drive allele frequency variation, especially over short timescales (Gonzalez-Quevedo et al. 2015). As such, drift may cause fixation of immune system genes resulting in ineffective responses to pathogens and decreased fitness (Frankham 2005; Alcaide & Edwards 2011). Previous studies have identified varying patterns of selection acting on TLR genes, including: positive directional selection (Nakajima et al. 2008; Wlasiuk et al. 2009; Wlasiuk & Nachman 2010; Tschirren et al. 2012), balancing selection (Ferrer-Admetlla et al. 2008; Fisher et al. 2011), and, most commonly, purifying selection (Yilmaz et al. 2005; Barreiro et al. 2009; Mukherjee et al. 2009; Shen et al. 2012; Fornuskova et al. 2013). However, there is evidence suggesting that drift is a major force shaping diversity at TLR loci in recently bottlenecked or expanding populations (Grueber et al. 2013; Hartmann et al. 2014; Quemere et al. 2015). Further studies are needed to improve our understanding of how evolutionary forces influence TLR variation in wildlife species.

In conclusion, this study demonstrated the importance of incorporating adaptive genes, especially those representing the immune system, into conservation genetics research. Knowing the levels and patterns of variation present at adaptive loci provides researchers and managers a fundamental understanding of total species diversity. Furthermore, when Grueber and colleagues (2015) assessed the relationship between individual multilocus heterozygosity at microsatellites and TLR heterozygosity in 10 bird species they found no association. Therefore, heterozygosity estimates obtained from neutral markers may not accurately predict diversity at TLR loci (Grueber et al. 2015). Overall, using *ex situ* white and black rhinoceros populations in North

America I found that TLR genes show unique patterns of diversity by species and subspecies. This diversity is worth further investigation in order to gain a more complete understanding of the driving processes and the resulting patterns. Breeding programs and conservation efforts will benefit from using this knowledge when striving to maintain immune system diversity in threatened and endangered species.

Tables and Figures

Table 4.1. Toll-like receptors and their ligands.

Receptor	Cellular Location	Ligands	Pathogen Type	Signaling Pathway	Subfamily
TLR1	Surface	Triacyl lipopeptides	Bacteria	MyD88	2
TLR1/2*	Surface	Peptidoglycans Lipoproteins	Bacteria	MyD88	2
TLR2	Surface	Glycolipids Porins Lipoproteins/peptides Zymosan Heat Shock Protein 70 Many others	Bacteria Fungi Host	MyD88	2
TLR3	Endosome	Double stranded RNA	Viruses	TRIF	3
TLR4	Surface Endosome	Lipopolysaccharides Heat shock proteins Several viral proteins Taxol	Bacteria Host Virus Plant	MyD88 TRAM	4
TLR5	Surface	Flagellin	Bacteria	MyD88	5
TLR6/2*	Surface	Lipopeptides	Bacteria (<i>Mycoplasma</i>)	MyD88	2
TLR7	Endosome	Single stranded RNA Imidazoquinoline	Viruses Synthetic compounds	MyD88	9
TLR8	Endosome	Single stranded RNA Synthetic agonists	Viruses Synthetic compounds	MyD88	9
TLR9	Endosome	CpG containing DNA	Bacteria DNA viruses	MyD88	9
TLR10	Surface				2

Adapted from Akira et al. 2001; Akira and Takeda 2004; Akira et al. 2006; Uematsu and Akira 2008

*Form a heterodimer

Table 4.2. Black and white rhinoceros sample information.

Lab ID	Species	Subspecies	Sender	Sex	Birth Year	Studbook Number	Sire	Dam
Dbi-870	<i>Diceros bicornis</i>	<i>michaeli</i>	Cincinnati Zoo	M	2002	870	488	397
Dbi-125	<i>Diceros bicornis</i>	<i>michaeli</i>	San Diego Zoo (ICR)	F	1955	125	W	W
Dbi-124	<i>Diceros bicornis</i>	<i>michaeli</i>	San Diego Zoo (ICR)	M	1957	124	W	W
Dbi-294	<i>Diceros bicornis</i>	<i>michaeli</i>	Oklahoma City Zoo	F	1999	294	169	190
Dbi-490	<i>Diceros bicornis</i>	<i>michaeli</i>	Oklahoma City Zoo	M	1995	490	301	53
Dbi-664	<i>Diceros bicornis</i>	<i>michaeli</i>	Lincoln Park Zoo	M	1997	664	377	213
Dbi-362	<i>Diceros bicornis</i>	<i>michaeli</i>	Lincoln Park Zoo	M	1986	362	259	202
Dbi-935	<i>Diceros bicornis</i>	<i>michaeli</i>	Lincoln Park Zoo	F	2008	935	636	677
Dbi-683	<i>Diceros bicornis</i>	<i>michaeli</i>	Cleveland Metroparks Zoo	F	1993	683	W	W
Dbi-904	<i>Diceros bicornis</i>	<i>michaeli</i>	Cleveland Metroparks Zoo	F	2003	904	457	683
Dbi-957	<i>Diceros bicornis</i>	<i>michaeli</i>	Cleveland Metroparks Zoo	M	2012	957	435	904
Dbi-718	<i>Diceros bicornis</i>	<i>minor</i>	Fossil Rim Wildlife Center	F	1999	718	401	462
Dbi-667	<i>Diceros bicornis</i>	<i>minor</i>	White Oaks Conservation Center	M	1997	667	522	410
Dbi-521	<i>Diceros bicornis</i>	<i>minor</i>	White Oaks Conservation Center	M	1999	521	378	410
Dbi-770	<i>Diceros bicornis</i>	<i>minor</i>	White Oaks Conservation Center	M	2000	770	522	402
Dbi-669	<i>Diceros bicornis</i>	<i>minor</i>	White Oaks Conservation Center	F	2005	669	401	462
Dbi-847	<i>Diceros bicornis</i>	<i>minor</i>	Disney Animal Kingdom	F	2000	847	670	486
Dbi-873	<i>Diceros bicornis</i>	<i>minor</i>	Disney Animal Kingdom	M	2001	873	670	574
Dbi-392	<i>Diceros bicornis</i>	<i>minor</i>	San Diego Zoo (ICR)	F	1986	392	W	W
Dbi-471	<i>Diceros bicornis</i>	<i>minor</i>	San Diego Zoo (ICR)	M	1982	471	W	W
Dbi-868	<i>Diceros bicornis</i>	<i>minor</i>	Fossil Rim Wildlife Center	M	2001	868	465	411
Dbi-0022	<i>Diceros bicornis</i>	<i>minor</i>	Fossil Rim Wildlife Center	--	--	--	--	--
Csi-559	<i>Ceratotherium simum</i>	<i>simum</i>	Lion Country Safari	M	1968	559	W	W
Csi-1400	<i>Ceratotherium simum</i>	<i>simum</i>	Lion Country Safari	F	2001	1400	685	624
Csi-1142	<i>Ceratotherium simum</i>	<i>simum</i>	Lion Country Safari	F	1996	1142	625	624
Csi-624	<i>Ceratotherium simum</i>	<i>simum</i>	Lion Country Safari	F	1980	624	562	565
Csi-1054	<i>Ceratotherium simum</i>	<i>simum</i>	Lion Country Safari	F	1995	1054	685	566
Csi-1451	<i>Ceratotherium simum</i>	<i>simum</i>	Lion Country Safari	M	1973	1451	W	W
Csi-1150	<i>Ceratotherium simum</i>	<i>simum</i>	Lion Country Safari	F	1996	1150	180	182
Csi-1548	<i>Ceratotherium simum</i>	<i>simum</i>	Lion Country Safari	F	2008	1548	1451	1172
Csi-1223	<i>Ceratotherium simum</i>	<i>simum</i>	White Oaks Conservation Center	F	1988	1223	W	W
Csi-1390	<i>Ceratotherium simum</i>	<i>simum</i>	White Oaks Conservation Center	F	2001	1390	1222	1226
Csi-1228	<i>Ceratotherium simum</i>	<i>simum</i>	White Oaks Conservation Center	M	1990	1228	W	W
Csi-1495	<i>Ceratotherium simum</i>	<i>simum</i>	Lowry Park Zoo (Tampa)	F	2004	1495	W	W
Csi-1497	<i>Ceratotherium simum</i>	<i>simum</i>	Lowry Park Zoo (Tampa)	F	2004	1497	W	W
Csi-1498	<i>Ceratotherium simum</i>	<i>simum</i>	Lowry Park Zoo (Tampa)	F	2004	1498	W	W
Csi-1380	<i>Ceratotherium simum</i>	<i>simum</i>	Lowry Park Zoo (Tampa)	M	1996	1380	W	W
Csi-916	<i>Ceratotherium simum</i>	<i>simum</i>	Indianapolis Zoo	M	1988	916	558	566

Table 4.2. Cont.

Lab ID	Species	Subspecies	Sender	Sex	Birth Year	Studbook Number	Sire	Dam
Csi-533	<i>Ceratotherium simum</i>	<i>simum</i>	Indianapolis Zoo	F	1979	533	390	391
Csi-1381	<i>Ceratotherium simum</i>	<i>simum</i>	Indianapolis Zoo	F	1995	1381	W	W
Csi-1235	<i>Ceratotherium simum</i>	<i>simum</i>	Disney Animal Kingdom	F	1999	1235	279	391
Csi-1224	<i>Ceratotherium simum</i>	<i>simum</i>	Disney Animal Kingdom	F	1992	1224	W	W
Csi-1403	<i>Ceratotherium simum</i>	<i>simum</i>	Disney Animal Kingdom	M	2002	1403	1216	1219
Csi-1276	<i>Ceratotherium simum</i>	<i>simum</i>	Fossil Rim Wildlife Center	F	1999	1276	618	612
Csi-612	<i>Ceratotherium simum</i>	<i>simum</i>	Fossil Rim Wildlife Center	F	1979	612	562	566
Csi-391	<i>Ceratotherium simum</i>	<i>simum</i>	Fossil Rim Wildlife Center	F	1968	391	W	W
Csi-1222	<i>Ceratotherium simum</i>	<i>simum</i>	Fossil Rim Wildlife Center	M	1990	1222	W	W
Csi-2070	<i>Ceratotherium simum</i>	<i>simum</i>	Fossil Rim Wildlife Center	--	--	--	--	--

-- indicates information in unavailable.

Table 4.3. PCR and sequencing primers for eight African rhinoceros TLR genes and a mitochondrial control region fragment.

Gene	Primer Sequence (5' - 3')	Amplicon Length (bp)
TLR1	F: AGCAGGTCTCACCCATGTTC R: AATGGATTGTTCCCTGCTTT	1479
TLR2	F: ACGGCAGCTGTGAAAAGTCT R: AGGATCTGGTCCAGTGCYTG	1511
TLR3	F: CTCCAGGGTGTTCATGCA R: CATCACTGGAAGCCATGAT	1430
TLR4	F: GGAGCACTTGGACCTTTYCA R: GGCCACACCAGGAATRAAGT	1624
TLR5	F: CGTTTCTGCAACCTCACYCA R: GCTGAGTCCCCTTAATGCAG	1497
TLR6	F: CTGCCACCAGAAACCAAAGT R: ACCCTCYACCACATCACTTG	1515
TLR7	F: TGGACTGCACAGACAAGCAT R: CTTGAGGCTSCTGGAACAGT	2036
TLR10	F: TGCCAGAAGAAAGGGAATTG R: CACAAGTACACCGGAATGGA	1515
Control Region	F ¹ : TCCACCATCAGCACCCAAAGC R ² : TTTGATGGCCCTGAAGTAAGAACCA	~500

¹Previous published by Campbell et al. 1995.

²Previous published by Moro et al. 1998.

Table 4.4. Polymorphisms in TLR genes of *ex situ* white and black rhinoceros populations

Gene	Length (bp)	Taxon	N	SNPs	Syn:Nonsyn	NT	AA	Position
TLR1	1377	White Rhino	26	0	--	--	--	--
		Eastern Black Rhino	11	0	--	--	--	--
		South-central Black Rhino	11	1	0:1	A/T/W	Asn/Ile	18
TLR2	1397	White Rhino	26	0	--	--	--	--
		Eastern Black Rhino	10	3	2:1	C/Y	Ser	146
						C/Y	Phe	1205
						A/M	Asp/Ala	1231
		South-central Black Rhino	11	1	1:0	C/T/Y	Phe	1205
TLR3	1306	White Rhino	26	1	0:1	A/C/M	Ile/Leu	642
		Eastern Black Rhino	9	5	1:4	C/T/Y	Thr/Ile	109
						C/M	His/Pro	237
						A/G/R	Leu	530
						G/R	His/Arg	820
						A/G/R	Lys/Glu	894
		South-central Black Rhino	11	4	1:3	C/T/Y	Thr/Ile	109
						C/T/Y	Pro/Gln	508
						A/G/R	Leu	530
						A/G/R	Lys/Glu	894
G/R	Lys/Glu					243		
TLR4	1507	White Rhino	26	3	1:2	G/R	His/Arg	592
		Eastern Black Rhino	12	6	3:3	T/Y	Asn	989
						T/C/Y	Pro	53
						A/G/R	Arg	278
						T/C/Y	Asn	785
						G/R	Gln/Arg	790
		South-central Black Rhino	11	6	3:3	T/C/Y	Tyr/His	1107
						T/C/Y	Thr/Ile	1321
						T/Y	Pro	53
						A/G	Arg	278
C/Y	Thr/Met					451		
Eastern Black Rhino	12	6	3:3	T/C/Y	Asn	785		
				C/Y	Tyr/His	1107		
				T/C/Y	Thr/Ile	1321		

Table 4.4. Cont.

Gene	Length (bp)	Taxon	N	SNPs	Syn:Nonsyn	Nt	AA	Position
TLR5	1370	White Rhino	26	0	-	-	-	-
		Eastern Black Rhino	11	4	1:3	T/G/K	Ala/Ser	475
						C/S	Met/Ile	588
						A/G/R	Pro	1047
						A/R	Met/Ile	1101
		South-central Black Rhino	11	5	2:3	C/T/Y	Thr/Met	92
						T/G/K	Ala/Ser	475
						C/S	Met/Ile	588
						A/G/R	Ser	705
						A/G/R	Pro	1047
TLR6	1401	White Rhino	26	0	--	--	--	--
		Eastern Black Rhino	12	0	--	--	--	--
		South-central Black Rhino	11	0	--	--	--	--
TLR7	1922	White Rhino	26	1	1:0	A/G/R	Gly	1289
		Eastern Black Rhino	9	3	3:0	A/G	Leu	638
						C/T/Y	Asp	893
						C/T/Y	Ser	1658
		South-central Black Rhino	10	1	0:1	T/G/K	Tyr/Asp	528
TLR10	1393	White Rhino	26	0	--	--	--	--
		Eastern Black Rhino	12	1	0:1	A/R	Arg/Asp	1230
		South-central Black Rhino	11	0	--	--	--	--

bp is the number of nucleotide bases.

N is the sample size.

SNPs is the number of single nucleotide polymorphisms in the sequence.

Syn:Nonsyn is the number of synonymous SNPs and the number of non-synonymous SNPs.

Nt is the nucleotide base present at the SNP position.

AA is the inferred amino acid residue at the SNP location.

-- values were not calculated due to lack of SNP diversity.

Table 4.5. Diversity indices for TLR genes in ex situ white and black rhinoceros populations

Gene	Taxon	H	<i>h</i>	H _o	H _E	π	Tajima's D
TLR1	White Rhino	1	--	--	--	--	--
	Eastern Black Rhino	1	--	--	--	--	--
	South-central Black Rhino	2	0.37	0.27	0.35	0.0003	0.593
TLR2	White Rhino	1	--	--	--	--	--
	Eastern Black Rhino	4	0.61	0.70	0.58	0.0009	-0.260
	South-central Black Rhino	2	0.42	0.36	0.40	0.0003	0.895
TLR3	White Rhino	2	0.43	0.54	0.43	0.0003	1.281
	Eastern Black Rhino	4	0.53	0.44	0.49	0.0010	-0.390
	South-central Black Rhino	3	0.65	0.73	0.62	0.0014	1.878
TLR4	White Rhino	4	0.31	0.31	0.40	0.0002	-1.027
	Eastern Black Rhino	8	0.81	0.67	0.78	0.0016	1.525
	South-central Black Rhino	4	0.40	0.18	0.38	0.0008	-0.917
TLR5	White Rhino	1	--	--	--	--	--
	Eastern Black Rhino	5	0.74	0.64	0.70	0.0011	0.992
	South-central Black Rhino	4	0.66	0.73	0.63	0.0017	2.084*
TLR6	White Rhino	1	--	--	--	--	--
	Eastern Black Rhino	1	--	--	--	--	--
	South-central Black Rhino	1	--	--	--	--	--
TLR7	White Rhino	2	0.50	0.31	0.49	0.0002	1.661
	Eastern Black Rhino ¹	4	0.70	0.33	0.66	0.0005	0.427
	South-central Black Rhino	2	0.53	0.20	0.50	0.0003	1.565
TLR10	White Rhino	1	--	--	--	--	--
	Eastern Black Rhino	2	0.16	0.17	0.15	0.0001	-0.681
	South-central Black Rhino	1	--	--	--	--	--

H is the number of observed haplotypes.

h is haplotype diversity.

H_o is observed heterozygosity.

H_E is expected heterozygosity.

π is nucleotide diversity.

* statistically significant ($p < 0.05$).

¹Deviates from Hardy-Weinberg Equilibrium.

-- diversity indices were not calculated due to presence of single haplotype.

Table 4.6. Diversity indices for microsatellite loci and the mitochondrial control region in ex situ white and black rhinoceros populations.

Taxon	Mitochondrial Control Region			Microsatellites				
	H	<i>h</i>	π	N	A	H _O	H _E	F _{IS}
White Rhino	2	0.50	0.00467	--	--	--	--	--
Eastern Black Rhino	5	0.78	0.00920	12	5.0	0.57	0.65	0.136
South-central Black Rhino	5	0.80	0.00696	11	3.3	0.49	0.54	0.090

H is the number of observed haplotypes.

h is haplotype diversity.

π is nucleotide diversity.

A is the mean number of alleles per locus.

H_O is observed heterozygosity.

H_E is the mean expected heterozygosity.

F_{IS} is the average deviation from Hardy-Weinberg proportions.

-- no microsatellite data for white rhino.

Figure 4.1. Median-joining networks for eight TLR genes in African rhinoceros.

Median-joining networks of African rhinoceros TLR haplotypes. Each circle represents a unique TLR haplotype, and hash marks indicate the number of nucleotide differences between haplotypes. Circles representing haplotypes within each locus are proportional to the number of rhinos carrying each haplotype. Haplotypes found in white rhinoceros are represented with white circles, haplotypes carried by eastern black rhinos are represented in gray, and haplotypes detected in south-central black rhinos are shown in black. White rhinos were monomorphic at five TLR genes. The number of mutations separating white rhinoceros haplotypes from black rhinoceros haplotypes was between 1 (TLR5) and 12 (TLR10). The black rhino subspecies haplotypes were shared at all loci; however, with the exception of TLR6 (where only one haplotype is detected) haplotypes distinct to at least one of the subspecies were detected. Most black rhinoceros haplotypes formed tight clusters and were separated by one or two mutations. There were no shared haplotypes between white and black rhinoceros with a mean of 6.5 nucleotide differences between two species.

Figure 4.1. Cont.

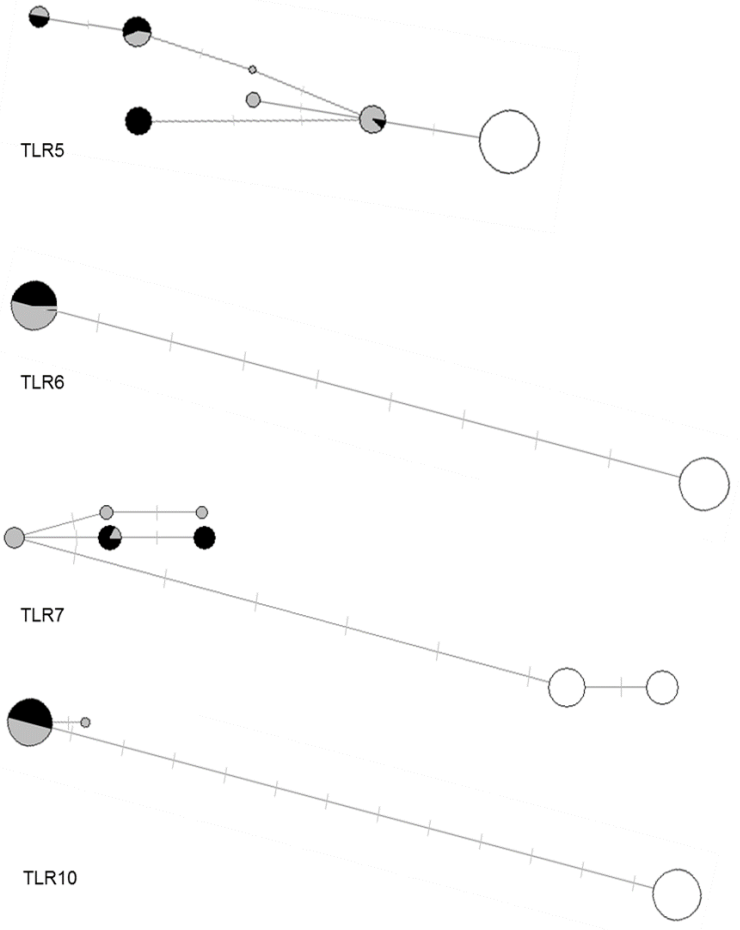
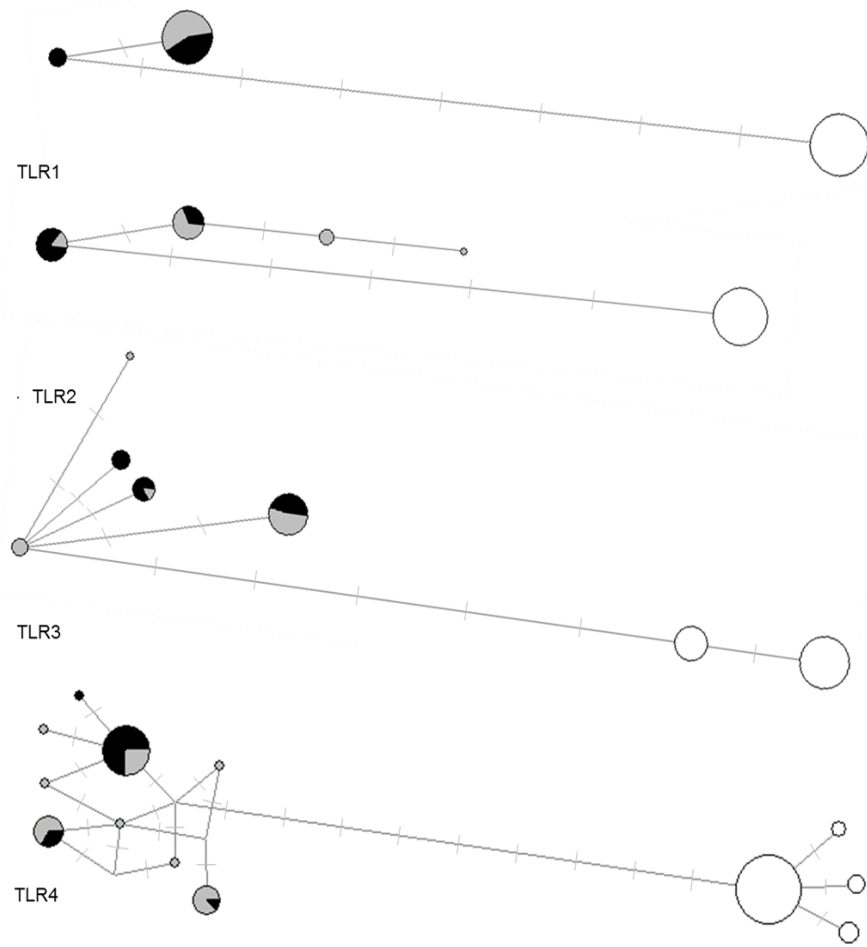


Figure 4.2. Schematic representation showing the protein structure of eight TLRs in white rhinoceros.

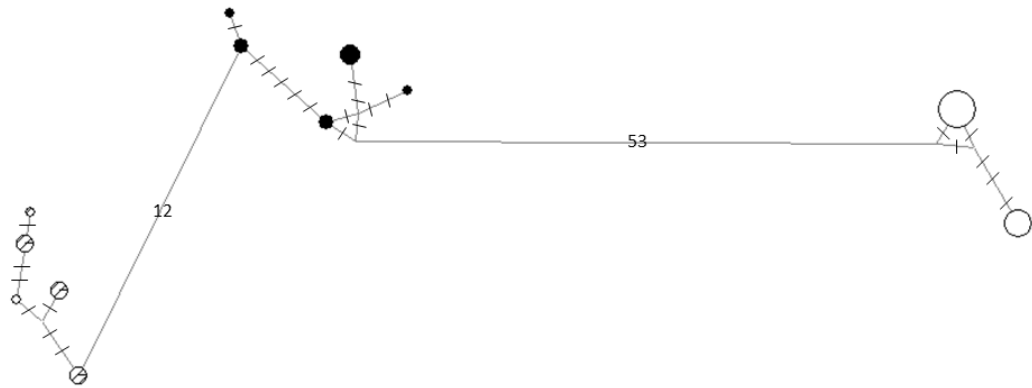
Protein structure of eight white rhinoceros TLR loci as identified by LRRFinder. For each protein the recognized LRRs (narrow gray rectangles), LRR_CT (gray ovals) and TIR (large gray rectangle) are shown. Between 14 (TLR4) and 24 (TLR7) LRR regions were recognized as having database matches or were significantly identified per protein sequence ($E < 0.05$). The regions of inferred amino acid sequence generated in this study are shown between the arrows and represent a majority of the LRRs in each protein. Generated nucleotide sequence was used to infer amino acid sequence for an average of 85% of the LRRs in a TLR, ranging from 65% of expected LRRs in TLR3 to 100% of expected LRRs in TLR10. An average of 1459 bp of sequence was obtained per locus, ranging from 1306 bp (TLR3) to 1922 bp (TLR7).

Figure 4.3. Relationship between mitochondrial control region haplotypes of white rhinoceros and two subspecies of black rhinoceros held in North American zoos and research institutions.

(A) Median-joining network of African rhinoceros mitochondrial control region haplotypes. Hash marks indicate the number of nucleotide differences between haplotypes, numbers are listed for haplotypes separated by more than 10 nucleotide mutations. Circles represent haplotypes are with sizes proportional to the number of rhinos carrying each haplotype. Three haplotypes, shown in white, were detected in white rhinoceros ($h = 0.50$; $\pi = 0.00467$). Five haplotypes were identified in the eastern black rhino (black) samples ($h = 0.78$; $\pi = 0.0092$) and five were found among south-central black rhino (gray) samples ($h = 0.80$; $\pi = 0.00696$). No haplotypes were found to overlap between species or subspecies; there were 12 variable sites between haplotypes in the black rhinoceros subspecies. (B) Maximum-likelihood analysis of mitochondrial control region haplotypes represented by 415 bp of nucleotide sequence in African rhinoceros species; Indian rhinoceros control region haplotypes were included for rooting purposes. High bootstrap values are reported for all nodes. A strongly supported separation of black rhinoceros subspecies was present, with all haplotypes forming clades corresponding to subspecies.

Figure 4.3. Cont.

A.



B.

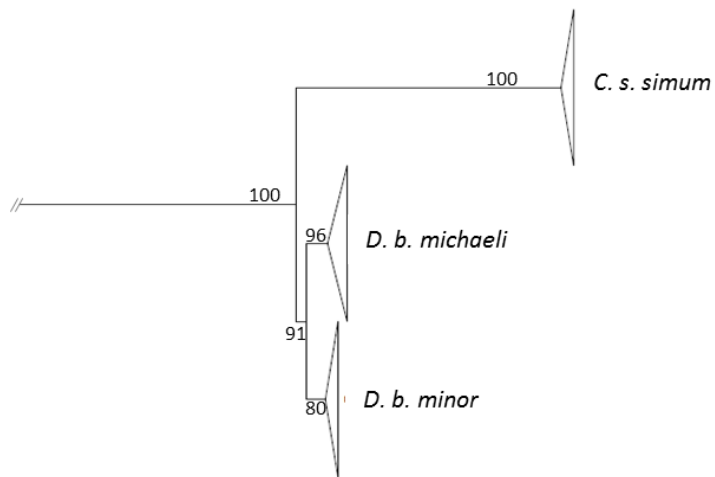
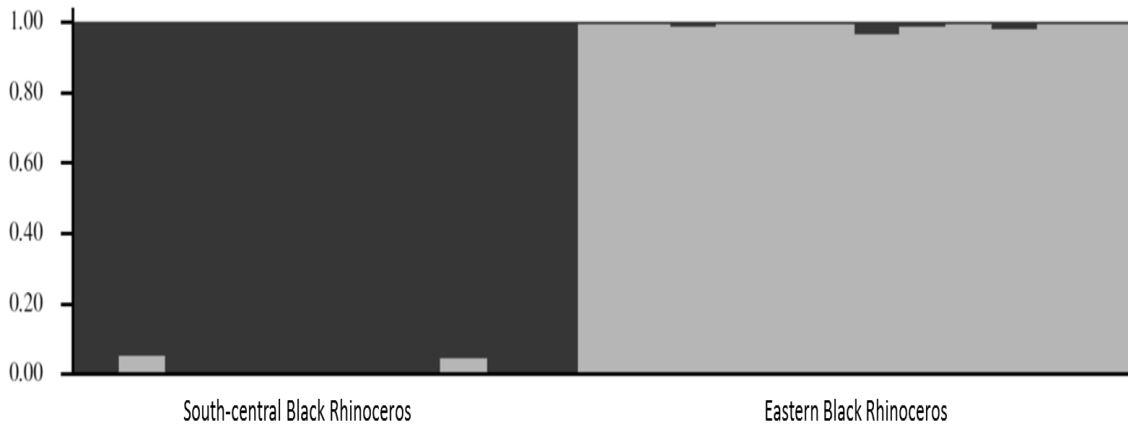


Figure 4.4. Genetic structuring and differentiation of white rhinoceros and two subspecies of black rhinoceros held in North American zoos and research institutions.

STRUCTURE analysis using 10 microsatellite loci genotyped in south-central black rhinos and eastern black rhino subspecies. Regardless of model assumptions (admixture with correlated allele frequencies shown) individuals subdivided into genetic clusters corresponding to subspecies, and *ad hoc* methods of estimating the most likely number of genetic clusters support $K = 2$ (shown). Genetic distinctiveness between south-central rhinos (black) and eastern black rhinos (gray) was evident.



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Appendix A. Black rhino microsatellite genotype data used for assessment of variability

South-Central Black Rhinoceros Samples

	Dbi-718		Dbi-667		Dbi-521		Dbi-669		Dbi-847		Dbi-873		Dbi0022		Dbi-868	
<i>Dibi3</i>	153	153	153	153	153	153	153	153	153	153	153	153	153	153	153	153
<i>Dibi5</i>	170	172	170	170	170	170	170	172	170	172	170	172	172	172	170	172
<i>Dibi9</i>	134	136	134	136	136	136	134	134	134	136	134	134	134	136	136	136
<i>Dibi15</i>	149	149	135	149	149	149	149	149	149	149	149	149	149	149	135	149
<i>Dibi22</i>	170	170	170	170	170	170	170	170	170	170	170	170	170	170	170	170
<i>Dibi23</i>	133	133	133	133	133	135	133	133	133	133	133	133	133	133	133	133
<i>Dibi24</i>	136	140	140	142	136	140	136	140	140	142	140	140	136	142	136	136
<i>Dibi25</i>	150	150	150	154	154	154	146	150	150	150	150	150	146	150	150	154
<i>Dibi26</i>	177	177	167	175	167	167	177	177	167	177	177	179	177	177	175	177
<i>Dibi27</i>	170	172	170	174	172	174	172	172	170	172	172	172	170	174	172	172
<i>Dibi32</i>	176	176	178	178	176	178	176	176	178	178	176	178	176	176	176	178
<i>Dibi34</i>	138	142	142	144	142	142	138	142	142	142	142	142	142	142	142	142
<i>Dibi48</i>	134	134	132	132	132	132	134	134	132	134	132	134	134	134	132	132
<i>Dibi49</i>	177	183	165	165	181	185	177	183	183	185	177	177	183	185	177	185
<i>Dibi50</i>	147	147	147	147	147	147	147	147	147	147	147	149	147	149	147	147
<i>Dibi51</i>	150	152	150	150	150	150	150	152	150	152	150	152	150	152	150	150
<i>Dibi56</i>	164	166	164	164	162	164	164	166	162	162	162	164	162	164	164	166

Eastern Black Rhinoceros Samples

	Dbi-870		Dbi-294		Dbi-490		Dbi-664		Dbi362		Dbi-935		Dbi-683		Dbi-904		Dbi-957	
<i>Dibi3</i>	153	155	153	153	153	153	153	153	145	153	153	153	153	153	153	153	153	153
<i>Dibi5</i>	170	170	172	172	170	172	172	172	170	170	170	170	170	172	170	172	170	172
<i>Dibi9</i>	136	136	134	136	136	136	136	136	136	136	136	136	136	136	136	136	136	136
<i>Dibi15</i>	147	157	151	151	157	157	149	151	151	157	151	157	151	151	151	155	151	151
<i>Dibi22</i>	166	166	170	170	166	170	166	170	170	170	166	166	170	170	166	170	166	166
<i>Dibi23</i>	133	135	133	133	133	133	133	133	131	131	133	133	133	133	133	133	133	133
<i>Dibi24</i>	134	138	134	138	138	138	130	134	134	134	134	138	132	142	134	142	132	134
<i>Dibi25</i>	146	150	154	158	150	150	146	158	154	162	162	162	150	152	150	152	152	162
<i>Dibi26</i>	167	167	167	167	167	167	167	167	167	167	157	159	165	165	165	165	167	167
<i>Dibi27</i>	168	170	168	168	168	168	172	174	170	170	168	174	168	170	168	168	168	168
<i>Dibi32</i>	166	168	168	168	170	182	168	174	166	168	168	168	166	176	166	168	166	166
<i>Dibi34</i>	140	144	136	144	144	144	144	144	142	142	142	144	136	142	136	144	144	144
<i>Dibi48</i>	132	132	132	132	132	132	132	132	132	132	132	132	132	132	132	132	132	132
<i>Dibi49</i>	171	175	165	175	175	175	173	175	173	175	171	173	173	175	171	173	171	171
<i>Dibi50</i>	149	149	147	151	147	147	147	147	147	147	147	149	147	149	147	147	147	147
<i>Dibi51</i>	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150
<i>Dibi56</i>	164	164	166	166	166	166	162	166	164	164	164	166	166	166	162	166	164	166

Appendix B. Sumatran rhino microsatellite genotype data used for assessment of variability

		Sumatran Rhinoceros Samples									
		Dsu-28		Dsu-29		Dsu-66		Dsu-64		Dsu-63	
Microsatellite Loci	Disu542	128	130	128	128	128	128	128	128	128	128
	Disu501	155	157	155	157	155	155	155	157	155	155
	Disu556	174	174	168	174	174	174	174	174	000	000
	Disu863	162	162	162	162	162	166	162	162	162	166
	Disu448	154	156	156	156	156	156	156	156	156	156
	Disu201	156	156	156	156	158	158	158	158	156	156
	Disu847	138	138	138	138	138	138	138	140	138	138
	Disu393	155	157	155	157	155	155	155	155	155	155
	Disu733	151	151	151	151	151	151	159	159	151	151
	Disu149	160	162	160	168	160	162	162	166	160	162
	Disu783	126	128	126	128	126	126	126	126	126	134
	Disu50	160	166	164	164	160	160	160	160	160	160
	Disu748	110	116	106	116	106	116	106	116	106	106
	Disu476	172	172	172	174	172	174	162	172	172	174
	Disu151	135	135	137	137	135	135	135	135	135	135
	Disu127	164	164	162	162	162	162	162	162	162	162
	Disu98	126	126	122	126	98	98	104	104	104	104
	Disu582	144	144	144	144	144	144	152	152	144	144
	Disu100	120	122	120	122	120	120	120	120	120	120
	Disu480	112	112	116	116	116	116	112	116	116	116
	Disu593	164	166	164	164	166	166	164	164	166	166
	Disu487	160	160	160	160	160	160	160	160	148	160
	Disu545	148	148	148	148	148	148	148	148	148	150
	Disu76	129	129	NA		125	125	125	125	NA	
	Disu269	115	134	NA		152	152	152	152	NA	
	Disu261	150	150	NA		166	166	150	166	NA	
Disu71	168	172	NA		168	168	170	170	NA		
Disu33	152	152	NA		164	164	164	164	NA		
Disu138	169	169	NA		169	169	167	167	NA		

Appendix C. Microsatellite genotypes of 13 modern Sumatran rhinoceros individuals

		Dsu-28	Dsu-29	Dsu-66	Dsu-64	Dsu-63	Dsu-TF	Dsu-126	Dsu-128	Dsu-147	Dsu-4273	Dsu-24	Dsu-25	Dsu-34965	
Microsatellite Loci	Disu542	128 130	128 128	128 128	128 128	128 128	128 128	128 128	128 128	128 128	128 128	128 130	128 128	128 130	
	Disu501	155 157	155 157	155 155	155 157	155 155	155 157	157 157	155 157	155 157	157 157	155 155	155 155	155 157	
	Disu556	174 174	168 174	174 174	174 174	000 000	174 174	168 168	168 168	168 174	168 174	168 168	174 174	168 168	
	Disu863	162 162	162 162	162 166	162 162	162 166	162 162	162 164	162 164	162 164	164 164	162 162	162 162	162 164	
	Disu448	154 156	156 156	156 156	156 156	156 156	154 156	154 154	154 154	156 156	156 156	154 156	156 156	154 154	
	Disu201	156 156	156 156	158 158	158 158	156 156	156 156	156 158	158 158	156 156	156 156	156 156	156 156	158 158	156 156
	Disu847	138 138	138 138	138 138	138 140	138 138	138 146	146 146	146 146	138 138	138 138	146 146	138 148	138 146	
	Disu393	155 157	155 157	155 155	155 155	155 155	155 155	155 157	155 157	155 155	155 157	155 155	155 157	155 155	155 155
	Disu733	151 151	151 151	151 151	159 159	151 151	151 151	000 000	149 149	151 151	000 000	149 149	151 151	149 149	151 151
	Disu149	160 162	160 168	160 162	162 166	160 162	168 168	166 168	166 168	162 168	168 168	168 168	162 162	160 168	
	Disu783	126 128	126 128	126 126	126 126	126 134	126 126	134 134	134 134	126 128	128 128	128 134	126 126	128 134	
	Disu50	160 166	164 164	160 160	160 160	160 160	160 160	164 164	164 164	164 164	164 164	164 164	160 160	164 164	
	Disu748	110 116	106 116	106 116	106 116	106 106	116 116	116 116	116 116	116 116	116 116	116 116	106 116	116 116	
	Disu476	172 172	172 174	172 174	162 172	172 174	172 172	172 172	162 172	162 162	172 172	174 174	172 172	172 172	
	Disu151	135 135	137 137	135 135	135 135	135 135	135 137	135 135	135 135	135 137	135 135	135 135	135 135	135 135	
Disu127	164 164	162 162	162 162	162 162	162 162	164 164	162 164	158 164	162 162	162 162	162 162	162 162	162 162		
Disu98	126 126	122 126	98 98	104 104	104 104	104 118	000 000	122 122	122 122	122 122	122 122	98 104	122 122		
Disu582	144 144	144 144	144 144	152 152	144 144	144 144	142 142	142 142	144 144	144 144	142 142	144 144	142 142		

Appendix D. Mitochondrial control region sequence alignment for modern and museum Sumatran rhinoceros samples

Set	#	Sample	Nucleotide																																								
			T	G	T	A	C	G	A	T	C	A	A	T	A	A	T	A	T	A	A	T	G	T	A	C	T	A	T	G	C	A	A	T	A	T	A	A	T				
Modern Samples	1	Dsu-Ratu (Sumatra)			
	2	Dsu-35 (Sumatra)			
	3	Dsu-28 (Sumatra)		
	4	Dsu-29 (Sumatra)		
	5	Dsu-34965 (Sumatra)	C		
	6	Dsu-33 (Sumatra)		
	7	Dsu-126 (Sumatra)	C		
	8	Dsu-TomFoose (Sumatra)	C		
	9	Dsu-24 (Sumatra)	C		
	10	Dsu-128 (Sumatra)	C	
	11	Dsu-4273 (Sumatra)	
	12	Dsu-64 (Peninsular Malaysia)	C	
	13	Dsu-66 (Peninsular Malaysia)	
Museum Samples	14	Dsu-539 (Borneo)	C		
	15	Dsu-4947 (Sumatra)	
	16	AMNH6-173576 (Sumatra)	C
	17	AMNH5-81892 (Peninsular Malaysia)	C
	18	Dsu-19594 (Sumatra)
	19	Dsu-19595 (Sumatra)
	20	Dsu-19596 (Sumatra)	C
	21	AMNH4-54763 (Burma)
	22	Dsu19-0311 (Borneo)	C
	23	Dsu1908-571 (Borneo)
	24	Dsu-190312 (Borneo)	C
	25	AMNH7-54764 (Burma)
	26	Dsu-56616 (Sumatra)
	27	Dsu-56618 (Sumatra)
	28	Dsu-1902-308 (Unk)	C
	29	Dsu-1903-329 (Unk)	C
	30	Dsu-199551 (Borneo)
	31	Dsu-102076 (Borneo)
	32	Dsu-1500 (Unk)	C
	33	Dsu-3082 (Unk)	C
	34	Dsu-4294 (Unk)	C
	35	Dsu-7529 (Unk)	C
	36	Dsu-8173 (Loas)
	37	Dsu-29566 (Sumatra)	C
	38	Dsu-29567 (Sumatra)	C
	39	Dsu-29568 (Unk)	C

Appendix G. Microsatellite genotypes for two subspecies of black rhinoceros

South-Central Black Rhinoceros Samples

	Dbi-718	Dbi-667	Dbi-521	Dbi-770	Dbi-669	Dbi-847	Dbi-873	Dbi-392	Dbi-471	Dbi0022	Dbi-868
<i>Dibi15</i>	149 149	135 149	149 149	149 149	149 149	149 149	149 149	149 149	149 149	149 149	135 149
<i>Dibi24</i>	136 140	140 142	136 140	142 142	136 140	140 142	140 140	140 140	134 140	136 142	136 136
<i>Dibi25</i>	150 150	150 154	154 154	150 150	146 150	150 150	150 150	146 150	150 150	146 150	150 154
<i>Dibi26</i>	177 177	167 175	167 167	177 177	177 177	167 177	177 179	177 177	167 179	177 177	175 177
<i>Dibi27</i>	170 172	170 174	172 174	170 170	172 172	170 172	172 172	168 172	168 172	170 174	172 172
<i>Dibi32</i>	176 176	178 178	176 178	178 178	176 176	178 178	176 178	178 178	176 178	176 176	176 178
<i>Dibi34</i>	138 142	142 144	142 142	142 142	138 142	142 142	142 142	142 142	142 144	142 142	142 142
<i>Dibi49</i>	177 183	165 165	181 185	181 183	177 183	183 185	177 177	177 183	177 183	183 185	177 185
<i>Dibi50</i>	147 147	147 147	147 147	149 151	147 147	147 147	147 149	149 149	147 147	147 149	147 147
<i>Dibi56</i>	164 166	164 164	162 164	162 164	164 166	162 162	162 164	162 164	162 162	162 164	164 166

Eastern Black Rhinoceros Samples

	Dbi-870	Dbi-125	Dbi-124	9	Dbi-294	Dbi-490	Dbi-664	Dbi362	Dbi-935	Dbi-683	Dbi-904	Dbi-957
<i>Dibi15</i>	147 157	151 161	151 159	151 151	151 151	157 157	149 151	151 157	151 157	151 151	151 155	151 151
<i>Dibi24</i>	134 138	134 138	134 134	130 134	134 138	138 138	130 134	134 134	134 138	132 142	134 142	132 134
<i>Dibi25</i>	146 150	158 162	150 150	146 160	154 158	150 150	146 158	154 162	162 162	150 152	150 152	152 162
<i>Dibi26</i>	167 167	159 167	169 169	165 167	167 167	167 167	167 167	167 167	157 159	165 165	165 165	167 167
<i>Dibi27</i>	168 170	170 172	168 168	168 170	168 168	168 168	172 174	170 170	168 174	168 170	168 168	168 168
<i>Dibi32</i>	166 168	166 174	168 180	168 168	168 168	170 182	168 174	166 168	168 168	166 176	166 168	166 166
<i>Dibi34</i>	140 144	144 148	140 142	144 144	136 144	144 144	144 144	142 142	142 144	136 142	136 144	144 144
<i>Dibi49</i>	171 175	173 173	165 175	000 000	165 175	175 175	173 175	173 175	171 173	173 175	171 173	171 171
<i>Dibi50</i>	149 149	147 147	147 147	147 149	147 151	147 147	147 147	147 147	147 149	147 149	147 147	147 147
<i>Dibi56</i>	164 164	164 166	164 166	000 000	166 166	166 166	162 166	164 164	164 166	166 166	162 166	164 166

