MICROSATELLITE VARIABILITY IN FOUR CONTEMPORARY RHINOCEROS SPECIES: IMPLICATIONS FOR CONSERVATION

By

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Abstract

Rhinoceroses are arguably the most endangered of all large mammal taxa. In order for ongoing conservation of this species to be successful, it is crucial to accurately characterize the remaining genetic diversity for all rhinoceros species.

To do this, I optimized a *standard* suite of 24 taxon-specific rhinoceros microsatellite loci. These loci have the power to provide the most comprehensive estimate of comparative microsatellite genetic diversity within and among the four extant African and Asian rhinoceros genera.

These loci were further used to evaluate the comparative influences of rhinoceros species versus microsatellite taxonomic origin as predictors of rhino microsatellite diversity, and finally to examine the evolutionary relationships between extant rhino taxa.

The African black *michaeli* rhino subspecies had the highest level of microsatellite genetic variability of all available rhinos, while southern white and Indian rhinos were the least variable rhinos. These findings also suggested that species and taxonomic origin of microsatellite loci were both significant predictors of microsatellite heterozygosity in rhinoceroses.

A weak association between the Sumatran and black rhinos was found with a D_{LR} neighbour-joining tree.

The *standard* loci were able to assign unique genotypes to all available rhinos as well as differentiate between all rhino species by correctly assigning individual rhinos back to their respective populations.

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General Introduction

The current biodiversity crisis has impacted many species spanning a wide range of taxa. However, it is clear that some groups are disproportionately affected by human actions such as habitat alteration and degradation, as well as poaching and overhunting. Because of long gestation time, low population recruitment, and large range requirements, large mammals have demonstrably higher extinction risks than their smaller counterparts (Cardillo *et al.* 2005; Garner *et al.* 2005). Rhinoceroses are emblematic of this with all five extant rhinoceros species considered threatened (Amin *et al.* 2006b; CITES 2007; IUCN 2007).

The most critically endangered rhinoceros is the northern white (*Ceratotherium simum cottoni*); with less than 20 individuals remaining, it is arguably the most critically endangered living large mammal. The Sumatran (*Dicerorhinus sumatrensis*) and Javan (*Rhinoceros unicornis*) rhino species are running a close second, having populations of less than 300 and 60 individuals respectively (Dierenfeld *et al*. 2006; Fernando *et al*. 2006; IRF 2008). The precarious status of rhino populations has prompted numerous regional and global conservation initiatives that feature intensive population protection and monitoring (Conway and Goodman 1989; Berger and Cunningham 1992, 1994 and 1997; Walpole *et al.* 2001; Amin *et al.* 2006a; Mills *et al.* 2006); native habitat preservation (Rachlow *et al.* 1999; Fernando *et al.* 2006), along with captive and *in situ* breeding programs (Dean and Bos 2006; Foose and Wiese 2006; Hermes *et al.* 2006; Roth 2006; Pluháček *et al.* 2007).

Comprehensive management plans for any species of conservation concern should include plans for maintaining existing genetic diversity, both to ensure ability to adapt to changing environments and to preserve the possibility of future speciation (Lande 1988; Lacy 1997; Amos and Balmford 2001; Allendorf *et al.* 2008). Thus, conservation efforts for all surviving rhinoceros taxa can be enhanced by accurate and detailed estimates of genetic variability. My research goal is to optimize a comprehensive suite of species-specific microsatellite DNA markers that I will use to generate estimates of genetic diversity for all four extant rhinoceros genera. These markers will provide insight into *in situ* rhinoceros population characteristics such as mating system structure, recruitment and dispersal, as well as contribute to captive breeding programs where it is crucial to accurately characterize relatedness among potential breeders.

An Overview of Extant Rhinocerotidae:

Based on large body size (>1000kg) and diet, Rhinoceroses (excluding *Dicerorhinus*), elephants, hippopotami and giraffes comprise the four extant families of megaherbivores (Owen-Smith 1988). Rhinocerotidae (rhinoceroses), along with Equidae (horses) and Tapiridae (tapirs) form the order Perissodactyla (uneven-toed ungulates) (Guérin 1982; Pitra and Veits 1999). The extant Rhinocerotidae includes four genera that encompass five species and eleven subspecies (Orlando *et al.* 2003; IRF 2008). There are two African species, each within a monotypic genus: the white rhinoceros (*Ceratotherium simum*) and the black rhinoceros (*Diceros bicornis*). White rhinos include two extant subspecies: southern (*simum*) and northern (*cottoni*) white rhinos (Houck *et al.* 1994; Kellner *et al.* 2000; Hermes *et al.* 2005). There are possibly four extant black rhinoceros subspecies (Harley *et al*. 2005). The three verified black rhino

subspecies include the south-western *bicornis*, south-central *minor* and eastern *michaeli* black rhinos (Ashley *et al.* 1990; Swart and Ferguson 1997; Harley *et al.* 2005; IRF 2008). It is not known if the fourth, north-western black rhino species (*longipes*) has been extirpated, because political instability has prevented an accurate census of any remaining individuals in Cameroon (IRF 2008). The remaining two rhinoceros genera include three Asian rhino species: the Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran rhinos (*Dicerorhinus sumatrensis*). Of the Asian rhinos, only the Sumatran and Javan species have recognized subspecies which include the western (*sumatrensis*) and the eastern (*harrissoni*) Sumatran rhinos and the Indonesian (*sondaicus*) and Vietnamese (*annamiticus*) Javan rhinos (Amato *et al.* 1995; Morales *et al.* 1997; Fernando *et al.* 2006). Recent studies dispute whether or not the Assam and Nepalese Indian rhino populations represent two distinct subspecies (Zschokke and Baur 2002; Zschokke *et al.* 2003; Pluháček *et al.* 2007).

Systematics of Contemporary Rhinoceroses:

The systematic relationships between rhinoceros species have been inferred using morphological, behavioural, geographical and, to a lesser degree, genetic data (Loose 1975; Groves 1983; Merenlender *et al.* 1989; Morales and Melnick 1994; Cerdeño 1995; Swart and Ferguson 1997; Xu and Arnason 1997; Tougard *et al.* 2001; Orlando *et al.* 2003; Fernando *et al.* 2006). The most accepted topology places the African rhino genera as sister taxa relative to all other rhinos based simply on their geographic distribution (Groves 1983; Merenlender *et al.* 1989; Xu and Arnason 1997; Tougard *et al.* 2001) (Figure 1.0). Fossil evidence along with genetic analyses comparing the entire 12S rRNA gene (*12S*)(840-975bp) and fragments of the cytochrome *b* (*cyt b*) (total 688bp) regions

of mitochondrial DNA (mtDNA) for all rhino taxa species (Morales and Melnick 1994; Cerdeño 1995; Tougard *et al.* 2001; Geraads 2005), as well as the entire nucleotide sequence of the mitochondrial genomes (16,832 bp) of white and Indian rhinos (Xu *et al.* 1996; Xu and Arnason 1997), support a divergence time between the Asian *Rhinoceros* genus and African rhinoceros lineages of approximately 22-26mya.

The African Black and White Rhino as Sister Taxa:

Of the two African genera the white rhinoceros, the largest of all rhino species, solely inhabit grassland savannahs and are unique in that they are the only true grazing rhinos (Codron *et al.* 2007) with a supportive ridge of hyper-developed neck muscles and ligaments and no prehensile lip (Owen-Smith 1988). It is unclear whether this is an ancestral or derived trait related to their specific feeding habits (Owen-Smith 1988). White rhinos form both temporary and persistent social groups, with sociality being unique to African rhinoceros species (Mukinya 1973; Shrader and Owen-Smith 2002).

The African black rhinoceroses are smaller than white rhinos, have prehensile lips used for mixed-browse feeding (Codron *et al.* 2007), inhabit a variety of terrains extending from grasslands and savannahs to tropical bushlands and jungles, and also form social groups (Mukinya 1973; Ashley *et al*. 1990; Berger 1994; Brown and Houlden 1999; Cunningham *et al*. 1999; Tatman *et al.* 2000; Walpole *et al.* 2001; White *et al.* 2007). Morphological traits common to both African genera include two horns, relatively smooth grey skin, and absence of canine and secondary incisor teeth (I_2) (Simpson 1945; Owen-Smith 1987; Tougard *et al.* 2001). Sequence analyses of the *12S* and *cytochrome b* regions of mtDNA support the notion that the African genera are sister taxa (Morales and Melnick 1994; Tougard *et al.* 2001; Orlando *et al.* 2003).

The Indian and Javan Rhinoceros Sister Species:

There are also two Asian rhinoceros genera: *Rhinoceros and Dicerorhinus*. Although the Indian rhinoceros is larger than the Javan rhinoceros, these two species are grouped into the single *Rhinoceros* genus based on a combination of geography and similar morphologic characteristics such as single horns, semi-prehensile lips, heavy folds of riveted armor-like skin, tusks formed from highly developed secondary incisor and canine teeth, as well as various skull and nasal synapomorphies (Owen-Smith 1988; Tougard *et al.* 2001; Fernando *et al.* 2006). Both *Rhinoceros* species combine browsing and grazing feeding habits (Steinheim *et al.* 2005; Wegge *et al.* 2006) and inhabit a variety of habitats ranging from dense lowland forests to open flood-plains (Merenlender *et al.* 1989; Zschokke *et al.* 2003; Fernando *et al.* 2006). Phylogenetic analyses based on mtDNA data, including the entire sequence of the rhinoceros *12S* gene as well as partial regions of *cyt b* and D-loop (total 805bp) genes, support the grouping of the Javan and Indian rhinos into a single genus (Orlando *et al.* 2003; Fernando *et al.* 2006).

Where do the Sumatran Rhinos Belong?

In contrast to the sister relationships of the black and white African rhinos and the Asian *Rhinoceros* genus, the placement of *Dicerorhinus* has long puzzled taxonomists because its morphology is very different from all other contemporary rhino taxa. Sumatran rhinos, thought to be the most primitive extant species, retain ancestral morphological characteristics such as small stature, anterior dentition and a coat of long brown hair not present in any other extant rhinos (Guérin 1982; Owen-Smith 1988; Orlando *et al*. 2003). Comparisons of DNA sequence data from portions of both the *12S* and *cyt b* mitochondrial genes of contemporary Asian and African rhino taxa to ancient

fossil DNA sequences support this hypothesis, suggesting that the Sumatran rhino is the most closely related extant rhinoceros species to the ancient wooly rhinoceros (*Coelodonta antiquititas*) (Cerdeño 1995; Orlando *et al.* 2003; Zainal-Zahari *et al.* 2005). These findings led to an early hypothesis suggesting the Sumatran rhinos are a separate lineage from all other rhinoceros taxa (Guérin 1982; Prothero and Schoch 1989; Cerdeño 1995; Orlando *et al.* 2003) (Figure 1.0). An alternate hypothesis suggests that Sumatran rhinos are most closely related to the African genera because these combined species retain two horns and lack highly developed secondary incisors and canines (Simpson 1945; Loose 1975; Morales and Melnick 1994) (Figure 1.0). Recent genetic studies have done little to resolve the placement of the Sumatran rhino. On the one hand, restriction mapping of the *12S* region of mtDNA indicates a relationship between the Sumatran and African *Diceros* species (Morales and Melnick 1994). Alternatively, research that compared direct sequence data from fragments of the *12S* and *cyt b* mtDNA regions among all extant rhinoceros genera indicated an association between the Sumatran rhino and the other Asian rhino species (Tougard *et al.* 2001), supporting a third taxonomic hypothesis that the Sumatran rhinos are a sister taxon to the Asian *Rhinoceros* genus (Groves 1983; Tougard *et al.* 2001). A study of fast evolving microsatellite variation (Hedrick 1999; Neff and Gross 2001; Côté *et al*. 2002) among extant rhinoceros taxa may provide a higher level of genetic resolution to help corroborate mtDNA evidence regarding the evolutionary affinities of the Sumatran rhino relative to other rhino species (Richard and Thorpe 2001).

Why are Rhinoceroses Endangered?

The Convention on International Trade in Endangered Species of Wild Fauna and

Flora (CITES) considers all rhinoceroses to be Appendix I endangered species (those in the greatest danger of extinction), except for the white rhino populations of South Africa and Swaziland, which are indexed as Appendix II animals (CITES 2007). Appendix II removes the aforementioned southern white rhinos from the most endangered category and allows limited trade of these rhinos within their respective countries for farming or trophy hunting purposes (CITES 2007). The World Conservation Union (IUCN) considers all rhino species "at risk", classifying black, Javan and Sumatran rhinos as critically endangered, Indian rhinos as endangered and white rhinos as near threatened but still warranting protective measures (IUCN 2007).

The primary threat to all rhinoceros taxa is still poaching for their horns (Leader-Williams *et al.* 1990; Berger 1994; Swart and Ferguson 1997; Amin *et al.* 2003; Bollongino *et al.* 2003; Hsieh *et al.* 2003). Asian medical texts, particularly the Chinese pharmacopoeia, prescribe powdered rhino horn for a variety of ailments ranging from congestion to impotence, and these beliefs are unfortunately still found in current scientific literature (Gray 1985; But *et al.* 1990 and 1991; Costa-Neto 1999 and 2004). Rhino horn and skin are also prized for use in ornamental armor and weaponry in a variety of African and Asian cultures (Emslie 2004; Rookmaaker 2005), and rhinoceros meat has been identified as a product in studies characterizing bush meat harvests (Baldus 2001; Draulans and Van Krunkelsven 2002; Santiapillai and Wijeyamohan 2003; Bulte and Damania 2005; Wato *et al.* 2006).

The second major threat to rhinos, particularly the Asian taxa, is habitat loss, alteration and fragmentation (Flynn and Abdullah 1984; Sukumar 1991; Amato *et al.* 1995; Rabinowitz 1995; Kinnaird *et al.* 2003; Dierenfeld *et al.* 2006; Dixon *et al.* 2007;

Honnay and Jacquemyn 2007). Ongoing human population growth and associated activity is rapidly disturbing preferred Asian rhino habitats. Land is cleared for farming or logging and the dense forest favored by the Asian rhinoceroses is either fragmented or destroyed (Flynn and Abdullah 1984; Kinnaird *et al.* 2003).

Ongoing conservation efforts for these species would be greatly assisted if the relative impacts of recent and contemporary events on genetic diversity of remaining populations were quantified (Amos and Balmford 2001; Linklater 2003). Since the recent histories of these taxa differ in important ways that may lead to different genetic outcomes, I will now summarize the recent histories of each species.

How Recent Events May Have Shaped Contemporary African Rhino Genetic Diversity

Prior to the late 1800s, hundreds of thousands of rhinos existed across all of western, central and southern Africa (Western and Vigne 1985; Owen-Smith 1988; Conway and Goodman 1989; Ashley *et al.* 1990; Berger and Cunningham 1992; Swart *et al.* 1994; Harley *et al.* 2005; Amin *et al.* 2006b) (Figure 1.1). Within a very short time period many of these animals had been killed and by 1894, it was thought that the African southern white rhino had been extirpated. Fortunately, this was not true and in 1895 a small population of southern white rhinos was discovered in Natal, South Africa (Western and Vigne 1985; Conway and Goodman 1989). It is these few rhinos (<100) that founded the current population comprised of more than 13,000 contemporary free-ranging and captive white rhinos, presently the most abundant rhinoceros species (IRF 2008). The largest free-ranging population of southern white rhinos resides in Kruger National Park, South Africa and is estimated to include between 5,000 and 8,000 rhinos (Peter Buss, pers. comm. 2007), with smaller populations scattered throughout South

Africa, Botswana, Kenya, Mozambique, Namibia, Swaziland, Tanzania, Zambia and Zimbabwe (IRF 2008) (Figure 1.2). This population increase implies a species in sustained recovery (Linklater 2003). However, the effect of this bottleneck (and subsequent expansion) is unknown. The magnitude of the southern white rhino population bottleneck might have resulted in a large loss of allelic diversity simply because rarer alleles would have been lost during the rapid decline in this taxon's numbers (England *et al.* 2003). Loss of genetic diversity also may have been exacerbated through increased inbreeding and increased genetic drift common to small populations – effects similar to populations exhibiting founder effects – leading to a reduction in heterozygosity (Hedrick *et al.* 2001; Hawley *et al.* 2006).

Until 1963 there were approximately 1,300 northern white rhinos located in the Sudan, Uganda and Garamba National Park in the Democratic Republic of Congo (DRC) (Hiliman-Smith 1990) (Figure 1.1). Survival of the northern white rhino has been opposite to that of its southern counterpart. By 1976 there were less than 600 northern white rhinos, further reduced to 15 individuals by 1983 (Hiliman-Smith 1990). Between 1984 and 1990 northern white rhinos were heavily protected and subsequently there were an additional 11 births in Garamba, but due to civil unrest in the DRC, northern white rhinos could no longer be effectively protected, with the last aerial census in 2005 revealing only four remaining individuals (IRF 2008) (Figure 1.2). These events would have likely impacted the expected genetic diversity of the northern white rhinos, particularly by decreasing allelic diversity, similar to the southern white rhinos.

All subspecies of black rhinos have undergone population bottlenecks similar to those of the southern white rhinos but with a less dramatic recovery. Prior to 1930, there

were an estimated 100,000 black rhinos ranging from Sierra Leone; east through to Somalia and south through to the eastern-most cape of South Africa (Western and Vigne 1985; Ashley *et al.* 1990) (Figure 1.1). By 1970, black rhino numbers were reduced to ~ 65,000 and by 1987 over 96% of black rhinos had been killed with the remaining 2,400 distributed in remnant populations of each subspecies (Hrabar and du Toit 2005; Metzger *et al.* 2007). Through extreme protective measures adopted in the last two decades, black rhino numbers have started to increase with the current census estimate of more than 3,500 suggesting that this species may be in recovery (Linklater 2003). The majority of the survivors are south-central *minor* (*n=1800*) and south-western *bicornis* (*n=1300*) subspecies. South Africa (*n=1177*) and Zimbabwe (*n=536*) have the most *minors* with the remainders in small fragmented populations in Botswana, Malawi, Mozambique, Swaziland, Tanzania and Zambia (IRF 2008). All *bicornis* reside in Namibia, primarily in Etosha National Park, except for ~70 individuals that dwell in South Africa (IRF 2008). The majority of the ~500 eastern black *michaeli* subspecies inhabit Kenya (*n=439*), with small populations in South Africa and Tanzania (IRF 2008). In contrast to the other three black rhino subspecies, the north-western *longipes* has fared less well, with the only probable survivors comprising a population of less than 10 individuals in Chad (IRF 2008). It is not unreasonable to assume that significant genetic diversity may have been lost to the black rhinos during these severe bottlenecks.

How Recent Events may have Shaped Contemporary Asian Rhino Genetic Diversity

The greater one-horned Indian rhino has had a history similar to that of the southern white rhinos with a recent short-lived bottleneck followed by a relatively rapid recovery (Merenlender *et al.* 1989; Dinerstein and McCracken 1990; Linklater 2003).

Historically, Indian rhinos have favored grasslands and river flood plains in a distribution from Pakistan, India and Nepal, through to China, Bhutan and Myanmar (Merenlender *et al.* 1989; Dinerstein and McCracken 1990; Ali *et al.* 1999) (Figure 1.1). Similar to the southern white rhinos in Africa, the Kaziranga Indian rhinos were reduced to a small breeding population by 1900 (Merenlender *et al.* 1989; Zschokke and Baur 2002). By the early 1960's, the Nepalese Indian rhino population had also been poached down to 21-28 individuals with total Indian rhinos encompassing less than 200 individuals (Merenlender *et al.* 1989; Dinerstein and McCracken 1990). Efforts of the Indian and Nepalese governments have seen the Indian rhino increase to > 2,500 composed of two populations in the Royal Chitwan National Park, Nepal (*n=800*) and the Kaziranga National Park in Assam, India (*n=1700*) (Zschokke *et al.* 2003) (Figure 1.2). Although these increases are impressive, these populations remain under threat, and there has been a reported increase in Indian rhino poaching over the last two years, with an estimated loss of 5% of the Nepalese rhinos in the last two years (Foose and Wiese 2006; IRF 2008). If recent history alone shaped contemporary rhino genetic diversity, the southern white and Indian rhinos might be expected to retain similar levels of genetic variability to each other.

In contrast to the other three rhino species, the Javan and Sumatran rhinos are perilously close to extinction with all animals confined to two single populations (Javan) or a larger number of small fragmented populations (Sumatran) (Amato *et al.* 1995; Morales *et al.* 1997; Fernando *et al.* 2006). In the past, both of these species numbered in the thousands, ranging from Bhutan, Laos and Myanmar through to China, Vietnam, Thailand, Peninsular Malaysia and the islands of Sumatra and Java (Groves 1983; Amato *et al.* 1995; Rabinowitz 1995; Morales *et al.* 1997; Agil *et al.* 2006; Fernando *et al.* 2006;

Earl of Cranbrook and Piper 2007) (Figure 1.1). Prior to the contemporary threats of poaching and habitat fragmentation, numbers were high enough for both species to be considered agricultural nuisances (Amato *et al.* 1995; Rabinowitz 1995). There are presently fewer than 275 Sumatran and 60 Javan rhinos (Flynn and Abdullah 1984; Agil *et al.* 2006; Fernando *et al.* 2006; IRF 2008). Although there are more Sumatran rhinos than Javan rhinos, Sumatran rhinos exist in extremely small, highly fragmented populations, preventing interaction among the majority of individuals and subsequently reducing both potential breeding success and gene flow (Côté *et al.* 2002; Kraaijeveld-Smit *et al.* 2005; Dixon *et al*. 2007). Conversely, all of the Indonesian Javan rhinos live in a single population in the Ujung Kulon National Park (Indonesia) while an unknown number of Vietnamese Javan rhinos exist in the Cat Tien National Park (Vietnam). This distribution allows for greater interaction between individuals, significantly increasing the chance of mating opportunities relative to the Sumatran rhinos (Fernando *et al.* 2006). Although only the Sumatran rhino, and not the Javan rhino, is assayed in this study, I would expect genetic diversity for both of these species to be low given the small number of survivors. For the Sumatran rhino, low levels of genetic variability may be further compromised because their populations are small and severely fragmented leading to reduced genetic exchange, vulnerability to genetic drift as well as minimal mating opportunities and limited recruitment (Redeker *et al.* 2006).

Rhinoceros Conservation and Management

Rhinoceros conservation efforts have focused on two initiatives: 1) protection of specific threatened *in situ* populations and 2) augmentation of population numbers through captive breeding (Leader-Williams and Alborn 1988; Berger 1996; Pukazhenthi

and Wildt 2004; Regan *et al.* 2005; Foose and Wiese 2006; Pluháček *et al.* 2007). Conservation programs under the first initiative include physical protection of individual populations with armed guards, rhino dehorning and remote monitoring of individuals with satellite and radio-collars. The corporal protection of wild rhinoceros populations is dangerous but has had the single-most positive impact on rhinoceros conservation to date (Ashley *et al.* 1990; Linklater 2003; Amin *et al.* 2006ab; Hilborn *et al.* 2008). The dramatic increase in southern white rhinos was in large part attributable to such intense protection. Despite its successes, this strategy is difficult and expensive because rhinos are often dispersed over wide geographical areas making individual monitoring and protection impossible (Hiliman-Smith 1990; Leader-Williams *et al.* 1990; Hilborn *et al.* 2006; Talukdar *et al.* 2007). Many rhinos also exist in politically unstable regions where their protection is both dangerous and of low national priority (Draulans and van Krunkelsven 2002; Linklater 2003; Amin *et al.* 2006). It is important to note that, in the past, rhinos were managed on a population basis without significant consideration of overall species conservation and, particularly, the maintenance of intraspecies genetic diversity (Western 1982; Rachlow *et al.* 1999; Tatman *et al.* 2000; Walpole 2001; Walpole *et al.* 2002; Mills *et al.* 2006; Rice and Jones 2006).

Although rhino dehorning has been implemented in a few areas with high poaching threats or where protection is difficult (Berger and Cunningham 1994) it has met with mixed success because the impacts to the rhinos are controversial. Support for dehorning is based on the premise that it prevents poaching as well as reduces intraspecific mortality from horn wounds incurred during fights (Berger and Cunningham 1994). The practice was thought to leave the calves of dehorned mothers vulnerable to

predation but this has not been conclusively supported (Berger *et al.* 1993; Berger and Cunningham 1994; Lindeque and Erb 1996; Brain *et al.* 1999). Another criticism suggests that horn size is a measure of sexual selection or social dominance and dehorning may affect rhinoceros social structures thus negatively influencing overall survival (Berger and Cunningham 1997; Garnier 2001). This notion has been countered by showing that rhino horns have a wide size-range and therefore are unlikely to be a measure of evolutionary significance (Lindeque and Erb 1996).

While radio-collars or radio-tags have been used to track a variety of species ranging from fish and reptiles through to large mammals such as wild dogs and bears (Keck 1994; Bethke *et al.* 1996; Mills and Gorman 1997; Jepsen *et al.* 2001), this approach to remote monitoring has met with limited success in rhinos (Linklater 2003; White *et al.* 2007). The main problems include false transmission rates, ineffective design thus allowing the collars to slip off, and injury to rhinos by the collar themselves (Alibhai *et al.* 2001; Dinerstein *et al.* 2001). In addition, the attachment of radio collars is an invasive technique that requires tranquilization, which can lead to reduced fertility rates and in some cases death (Alibhai *et al.* 2001; Dinerstein *et al.* 2001; Linklater 2006).

The difficulties associated with *in situ* preservation have led to significant captive breeding efforts (Santiago and Caballero 2000; Bulte and Damania 2005; Dean and Bos 2006; Foose and Wiese 2006; Pluháček *et al.* 2007) focusing on captive behavioural research and reproductive technologies that will enhance captive breeding success (Hearne and Swart 1991; Houck *et al.* 1994; Kellner *et al.* 2000; Linklater 2003 and

2006; Roth *et al.* 2004; Dierenfeld *et al.* 2006, Hermes *et al.* 2006; McDougall *et al.* 2006; Swaisgood *et al.* 2006).

There have been many successful captive births of white, black and Indian rhinos and more recently three births of captive-bred Sumatran rhinos after no successes despite 100 years of effort (Roth *et al.* 2004; Zainal-Zahari *et al.* 2005; IRF 2008). Against this apparent success however, are less encouraging data for white, black and Indian rhinos which show initial captive breeding successes leading to subsequent losses of fecundity after the birth of a first or second calf (Carlstead *et al.* 1999; Pukazhenthi and Wildt 2004; Regan *et al.* 2005; Hermes *et al.* 2006; Roth 2006; Swaisgood *et al.* 2006).

Although it is clear that both *in situ* preservation strategies and captive breeding programs have had positive short-term impacts for some rhino taxa, ongoing conservation efforts can be significantly augmented with estimates of variables that encroach upon microevolutionary processes; these include estimates of recurrent dispersal in non-threatened populations, quantification of mating system structure and reproductive variance among sexes in focal populations, through to accurate descriptions of, and reasons for, the distribution of genetic variation within and among extant rhinoceros. At a minimum, the above require improved, relatively fast evolving, high resolution genetic markers such as microsatellites to examine intrapopulation rhino demographies.

Genetic Techniques Applied to Conservation Biology

Recent genetic advances have broad application to conservation biology (Hedrick and Miller 1992; Hedrick 2001 and 2004; DeSalle and Amato 2004), including, but not limited to, DNA restriction site mapping, Restriction and Amplified Fragment Length

Polymorphisms (RFLPs and AFLPs) of whole or partial genomic DNA and complete sequencing of DNA. Of particular interest to recent conservation research are Polymerase Chain Reaction (PCR) based techniques that can rapidly produce millions of copies of targeted DNA sequences (Hedrick 2004) allowing researchers to utilize minute, ancient or degraded samples that would not otherwise contain enough genetic material for more traditional techniques.

RFLPs are techniques that utilize restriction endonuclease cut site variants within mtDNA, total genomic DNA (gDNA) or amplified target sequences to examine genetic variability within populations and species (Morales and Melnick 1994; Morales *et al.* 1997; Ali *et al.* 1999). These markers have the capability for high genetic resolution in many species, but have shown little to no intraspecific variation in rhinos (Ashley *et al*. 1990; Morales and Melnick 1994; O'Ryan *et al.* 1994).

Various PCR techniques have been used to isolate genetic markers similar to RFLPs. These include Single Strand Conformation Polymorphisms (SSCPs), Amplified Fragment Length Polymorphisms (AFLPs) (Bonin *et al.* 2007; Kellner *et al.* 2000), Random Amplified Polymorphic DNA (RAPDs) (Shankaranarayanan *et al.* 1997) and Microsatellite Associated Sequence Amplification (MASA) (Ali *et al.* 1999; Kapur *et al.* 2003). SSCPs, AFLPs and RAPDs are useful for generating comparisons between nuclear and mitochondrial organelles while MASAs are generally confined to nuclear DNA regions. In rhinos, these markers have the same analytical pitfalls associated with traditional RFLPs that may lead to inaccurate or underestimated levels of genetic variability (Ashley *et al.* 1990; Hedrick and Miller 1992; Hedrick 2004) along with a low

rate of reproducibility that makes them potentially unreliable tools for conservation genetics research.

Genetic Tools Applied to Conservation Biology

Mitochondrial DNA (mtDNA) is the non-recombining maternally inherited (in vertebrates) portion of the genome that has proved useful in determining phyletic and phylogeographical relationships among species (Cerdeño 1995; Friesen *et al.* 1996; Fernando *et al.* 2000; Derry *et al.* 2003). Additionally the mutation rates have been well-defined for different regions of mtDNA allowing estimates of divergence among evolutionary lineages (Douzery and Catzeflis 1995). Due to a high rate of nucleotide substitution (5-10 times greater than nuclear DNA) (Hedrick and Miller 1992; Douzery and Catzeflis 1995; Fernando *et al.* 2000), mtDNA variability in many taxa, including zooplankton, birds and fish , is often high enough for comparing intrapopulation variation (Billington and Hebert 1991; Friesen *et al.* 1996; Derry *et al.* 2003). However, mtDNA variability can be too low for intrapopulation resolution in many large mammal species (Ashley *et al.* 1990; Amato *et al.* 1995; Hedrick 2001; Tougard *et al.* 2001; Vidya *et al.* 2005; Oliveira *et al.* 2006). Nevertheless, the utility of mtDNA assays is particularly evident for studies that include fossils, bone, hair shafts or heavily degraded tissues. Most mammalian cells have one nucleus but multiple mitochondria; the aforementioned tissue types may not retain any nuclear material but may have trace amounts of mitochondria from which mtDNA can be isolated (Amin *et al.* 2003; Bollongino *et al.* 2003; Hsieh *et al.* 2003; Orlando *et al.* 2003; Broquet *et al.* 2007).

Increased numbers of recent conservation genetics studies have surveyed variable number tandem repeat (VNTR) nuclear DNA markers such as mini- and microsatellites

or Single Nucleotide Polymorphisms (SNPs) (Paetkau and Strobeck 1994; Van Hooft *et al.* 2000; Kim *et al*. 2004; Hsu *et al*. 2006; Mitrovski *et al*. 2007; Nielsen *et al.* 2007). Microsatellites, are ubiquitous throughout most genomes, codominant, multi-allelic, highly reproducible, easily isolated and useful for poor samples that may contain degraded DNA (Neff and Gross 2001; Oliveira *et al.* 2006; Broquet *et al.* 2007). The high resolution of microsatellites has been useful for assigning parentage, pedigree reconstruction, quantifying population structure and dispersal, delineating mating systems, phylogenetic estimation and identification of population bottlenecks (Paetkau *et al.* 1995; Hedrick *et al.* 2001; Kalinowski and Hedricks 2001; Richard and Thorpe 2001; Whitehouse and Harley 2001; Hawley *et al*. 2006; Redeker *et al.* 2006; Takezaki *et al.* 2007; Webley *et al.* 2007). These types of markers have also been used to measure gene flow, genetic drift and metapopulation structures as well as for defining Evolutionarily Significant Units (ESUs) and conservation Management Units (MUs) (Paetkau *et al.* 1995 and 1997; Luikart *et al.* 1998; Hedrick 1999; Amos and Balmford 2001; Hedrick 2001; Côté *et al.* 2002; Garner *et al.* 2005; Kraaijeveld-Smit *et al.* 2005; Palsbøll *et al.* 2006).

Rhinoceros Conservation Genetics

There is no extensive comparative estimate of genetic variability within and among all rhinoceros taxa and until this is completed the evaluation of the impacts of different recent and contemporary histories of each taxon on genetic diversity is not possible. In general, rhino genetics studies include too few taxa, too few samples, or differing genetic markers that prevent broad comparative inferences of variability among taxa. The net results are conflicting and incomplete estimates of genetic variability. To

illustrate this, I highlight genetic studies of rhinos using allozymes, mtDNA and, more recently, microsatellite polymorphism.

Although allozyme data have been collected for several rhinoceros taxa including black rhinos (Merenlender *et al.* 1989; Swart and Ferguson 1997), white rhinos (Merenlender *et al.* 1989) and Indian rhinos (Merenlender *et al.* 1989; Dinerstein and McCracken 1990) the results are contradictory leading to no clear picture of comparative allozyme variation in Rhinocerotidae. Initial allozyme studies suggested that black, white and Indian rhinos are genetically depauperate and undifferentiated at the selected loci (Merenlender *et al.* 1989). Subsequent studies implied that black and Indian rhinos had high levels of allozyme diversity (Dinerstein and McCracken 1990; Swart and Ferguson 1997). It is possible that small sample sizes (<10 individuals/taxa) biased the initial estimates downward (Merenlender *et al.* 1989). The picture is further confounded by the misrepresentation of allozyme variability in the latter two studies, with the exclusion of monomorphic loci in the calculation of heterozygosity estimates. Of the 30+ allozyme loci surveyed in each study, only six were polymorphic in black rhinos (Swart and Ferguson 1997) and nine were polymorphic in Indian rhinoceros (Dinerstein and McCracken 1990) in studies having very different sample sizes (Black rhinos *n=235*; Swart and Ferguson 1997 and Indian rhinos *n=23*; Dinerstein and McCracken 1990). Despite the apparent increased variability of *bicornis* and *minor*, these recognized black rhino subspecies were undifferentiated at these loci (Swart and Ferguson 1997).

The characterization of variation in mitochondrial DNA of rhinoceros species has been more extensive than allozymes ranging from early RFLP studies of whole mtDNA (Ashley *et al.* 1990; Morales and Melnick 1994; O'Ryan *et al.* 1994), through restriction

site polymorphism studies of D-Loop and *12S* sequence (Morales *et al*. 1997), to more recent sequence polymorphism comparisons of the entire *12S* gene (Tougard *et al.* 2001; Fernando *et al.* 2006), and fragments of the mitochondrial D-Loop (Fernando *et al.* 2006) and cytochrome *b* regions (Tougard *et al*. 2001).

The two early whole mtDNA RFLP studies suggested limited variation in the black rhino subspecies (Ashley *et al.* 1990; O'Ryan *et al.* 1994). In 23 *minor* and *michaeli* rhinos, only three RFLP mtDNA haplotypes were identified (Ashley *et al.* 1990) and the authors concluded these two taxa did not represent distinct subspecies. A subsequent study of 33 black rhinos (5 *bicornis*, 27 *minor* and 1 *michaeli*) distinguished three haplotypes, one unique haplotype for each subspecies (O'Ryan *et al.* 1994).

A broader study utilizing 78 restriction sites contained within a 1.6kB fragment of the total ribosomal mtDNA region (*12S, Valine, tRNA and 16S*) assessed variation between 28 black rhinos (*bicornis* and *minor*), four southern white rhinos, 14 Indian rhinos and four Sumatran rhinos (2 Borneo and 2 Malaysian) with the horse (*Equus caballus*) as an outgroup. Low variation was shown in this region with only seven distinguished haplotypes; a single haplotype for each taxon (Morales and Melnick 1994).

A subsequent study of polymorphism at 22 restriction sites in the fast evolving mtDNA D-Loop region (1550 bp) of 15 Sumatran rhinos from four geographic populations revealed increased polymorphism in the Sumatran rhinos with four unique haplotypes, one for each population (Morales *et al.* 1997). Any inference that Sumatran rhinos are more genetically variable than other rhino taxa is premature, as the early studies of whole mtDNA used different restriction enzymes (Ashley *et al.* 1990, O'Ryan

et al. 1994) than those used in latter assays of targeted sequence variation (Morales and Melnick 1994; Morales *et al.* 1997).

A study of mtDNA variation in Javan rhinos differentiated Indonesian from Vietnamese rhinos by a single haplotype within the *12S* mtDNA gene (Tougard *et al.* 2001; Fernando *et al.* 2006). Two fragments totaling 805bp of the mitochondrial D-loop region revealed three additional haplotypes: two specific to the Indonesian and one specific to the Vietnamese subspecies of Javan rhinos (Fernando *et al.* 2006). Similar to other mtDNA assays, comparative genetic variation between Javan and other rhino taxa is premature because the available data sets are derived from different methods, sample sizes and DNA loci.

Similar to the surveys of mtDNA variation, no broad comparison of genetic diversity using nuclear DNA markers exists for all Rhinocerotidae. Although a number of studies have reported the optimization of microsatellites in black (Brown and Houlden 1999; Cunningham *et al.* 1999; Nielsen *et al.* 2007), white (Florescu *et al.* 2003; Nielsen *et al.* 2007), Indian (Zschokke *et al.* 2003), and Sumatran rhinos (Scott *et al.* 2004), only diversity estimates within a single taxon or small subset of rhino taxa were simultaneously reported.

More recently, comparative surveys of multiple rhinoceros taxa using microsatellite data have been conducted but are limited to a single study of genetic variability within three black rhino subspecies (Harley *et al.* 2005) and a small study of microsatellite diversity between the African black and white rhinos (Nielsen *et al.* 2007). A comparative survey of genetic variability including both Asian and African rhinoceros taxa is still needed.

Research Goals and Hypotheses

There are seven specific goals and hypotheses for this thesis research listed as follows*:*

1. Optimize a *standard* suite of rhinoceros-specific microsatellite loci that can be used to assess microsatellite genetic variability in all rhinoceros species.

2. Provide the most accurate and comprehensive estimate of microsatellite genetic diversity within and among the four African and Asian rhinoceros genera to date.

3. Examine the hypothesis that recent population bottlenecks have similarly impacted the genetic diversity of all rhinoceros species.

4. Evaluate the comparative influences of microsatellite taxonomic origin and rhinoceros species as predictors of rhino microsatellite diversity.

5. Use microsatellite data to examine the evolutionary relationship of Sumatran rhinos to other extant rhino taxa.

6. Examine the forensic utility of the *standard* loci by evaluating the power of these loci to assign unique genotypes to all rhinos.

7. Examine the forensic utility of the *standard* loci by investigating the power of these loci to differentiate among rhinoceros species as well as correctly assign individual rhinos to their respective populations.

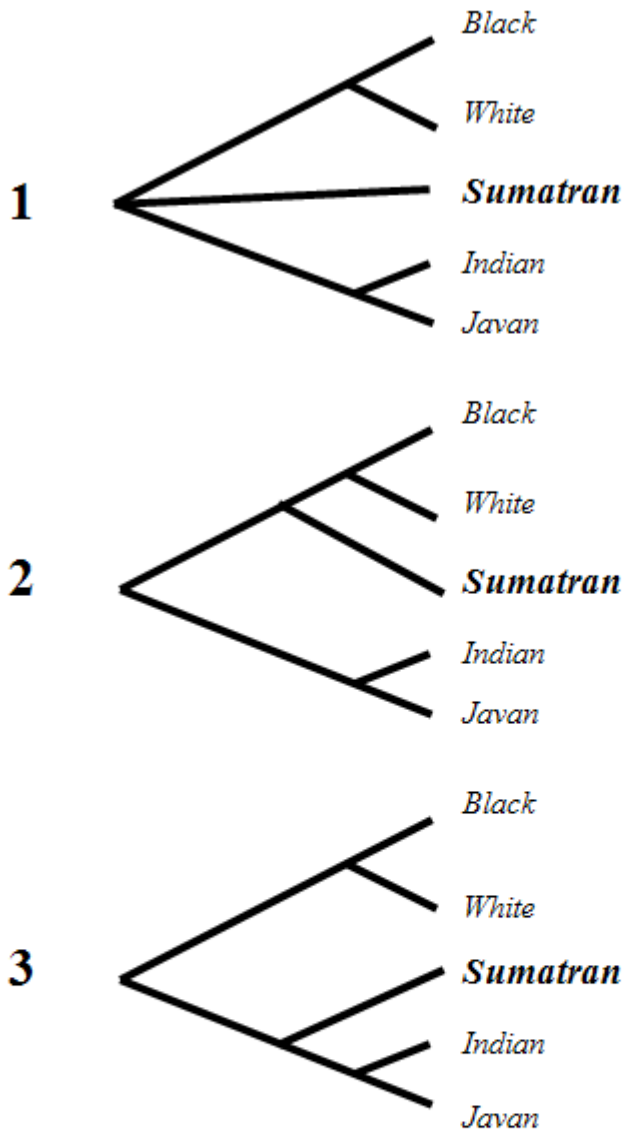

Figure 1.0: Three hypotheses regarding the systematic placement of Sumatran rhinos (*Dicerorhinus)* with other extant rhinoceros genera. Sumatran rhinos as: 1) a separate lineage from all other rhinos (Guérin 1982; Prothero and Schoch 1989; Cerdeño 1995; Orlando *et al.* 2003); 2) a sister taxon to the African genera based on horn number and dental synapomorphies (Simpson 1945; Loose 1975; Morales and Melnick 1994) and 3) a sister taxon to the Asian Rhinoceros genus based on geographical distribution (Groves 1983, Tougard *et al.* 2001).

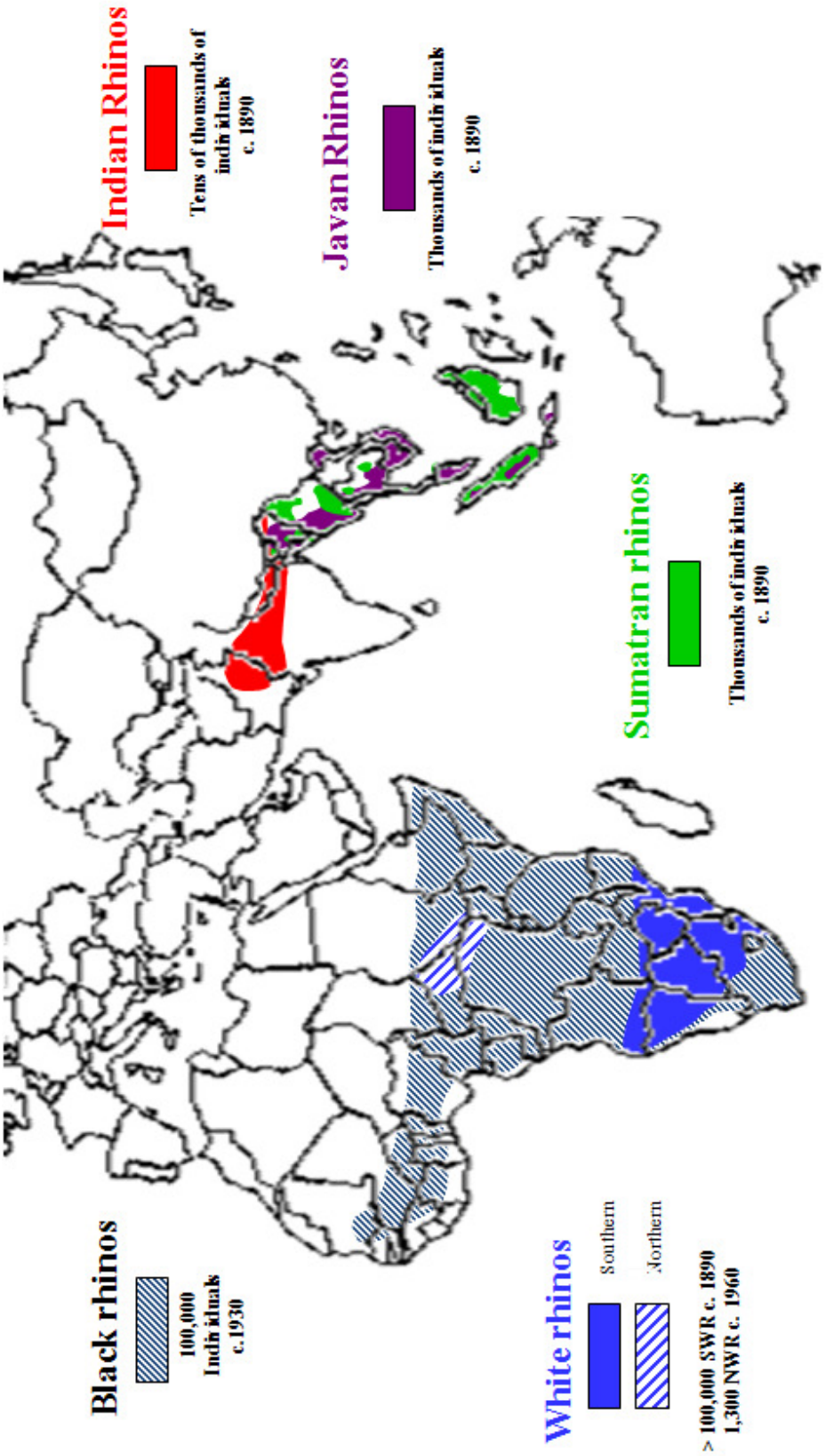

Figure 1.1: Historical distribution and numbers for five extant rhinoceros species. Numbers and distribution were taken from the International Rhino Foundation Statistics pages (IRF 2008).

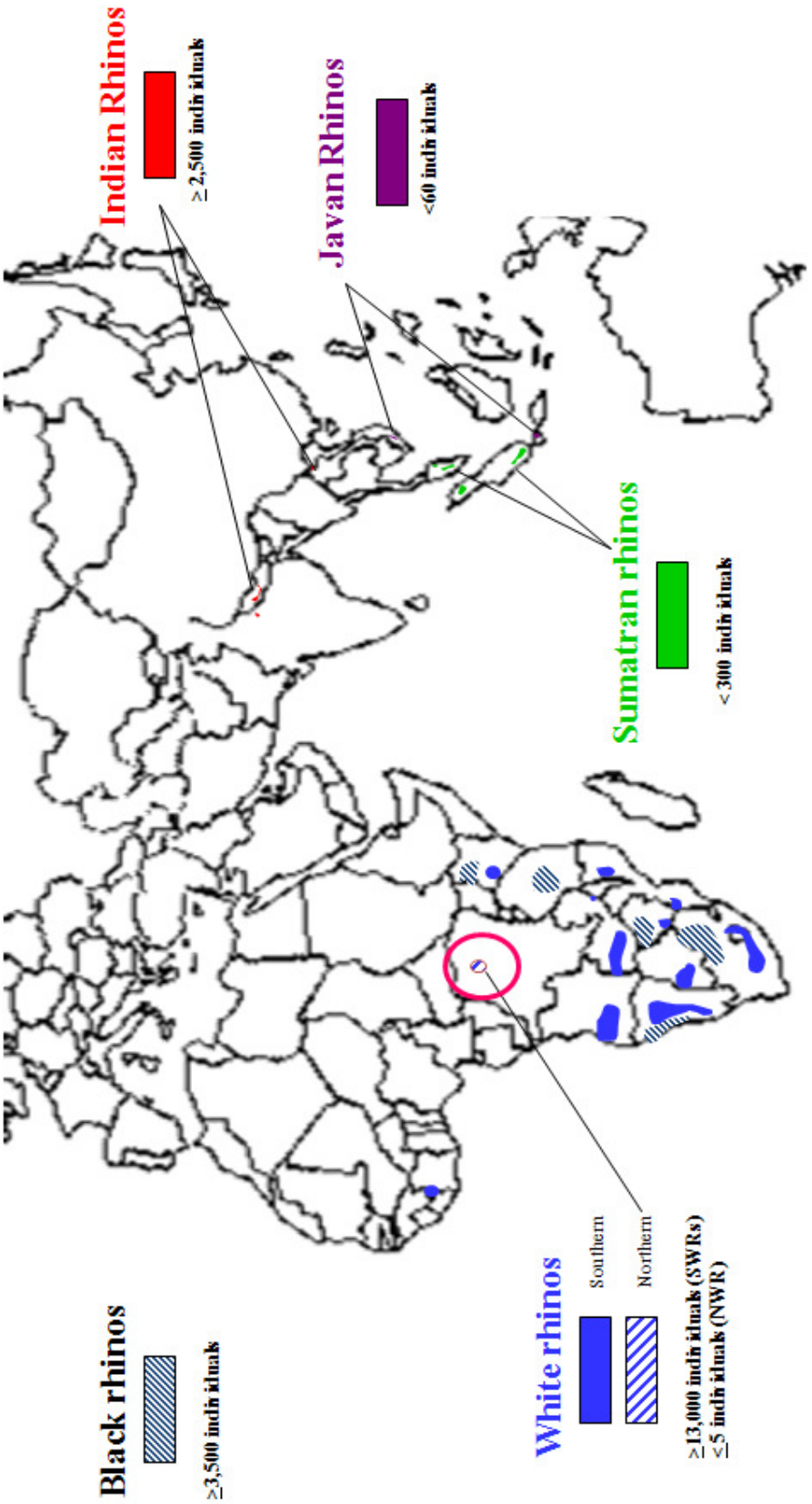

Figure 1.2: Contemporary distribution and numbers of five extant rhinoceros species. Numbers and distribution were taken from the International Rhino Foundation Statistics pages (IRF 2008). (IRF 2008).

Chapter 2: Methods and Materials

Sample Compilation

The 210 rhinoceros samples used in this research were collected from international zoo, game-park, sanctuary and national park rhinoceros populations by a variety of people (Appendix I). These samples include seven subspecies belonging to one of four rhinoceros genera: Black rhinos *(Diceros*), White rhinos *(Ceratotherium*), Indian rhinos *(Rhinoceros*) and Sumatran rhinos (*Dicerorhinus*).

The black rhino samples (*n=104*) comprise individuals from three of the four extant subspecies. The south-eastern black rhino (*D. b. bicornis*) subset (*n=31*) contains only individuals from Etosha National Park (Namibia), while both the south-central (*D. b. minor*) and eastern (*D. b. michaeli*) black rhino subsets (*n=48* and *n=25* respectively) are comprised of individuals from a variety of international zoos and game parks (Table 2.0; Appendix I). Unless otherwise specified (Appendix I), all samples were originally sourced from wild populations.

The white rhinoceros sample set (*n=65*) includes both southern (*C.s. simum*) and northern subspecies (*C.s. cottoni*). The southern white rhinos (*n=59*) include individuals from the single original post-bottleneck population at Hhluhluwe-iMfolozi Park in KwaZulu-Natal (South Africa), founders of the Waterberg National Park rhinoceros population (Namibia) and white rhinos from the Metro Toronto Zoo (Canada). Except for one white rhino born at the Metro Toronto Zoo, all white rhinos originated from the remnant population of southern white rhinos at Natal, South Africa (Appendix I). The northern white rhinos (*n=6*) include individuals from the Sudan, Dvůr Králové (Czech Republic) and the Henry Dourly Zoo (USA) (Table 2.0; Appendix I).

Indian rhino samples (*n=18*) include animals from zoos as well as the two largest free-ranging populations of Indian rhinos at the Royal Chitwan National Park (Nepal) and Kaziranga National Park (India) (Table 2.0; Appendix I). All Indian rhinos used in this study originated from either the Assam or Chitwan wild populations (Appendix I).

Only the western subspecies of Sumatran rhinoceros (*D.s. sumatrensis*) is represented here as there are no individuals from the eastern Borneo subspecies (*D. s. harrissoni*) in captivity. This sample set (*n=23*) includes samples from the Cincinnati Zoo (USA), and from two *in situ* breeding facilities: Way Kambas National Park (Indonesia) and the Sungai Dusun Rhinoceros Sanctuary (Peninsular Malaysia) (Table 2.0; Appendix I). All rhinos except for Andalas were wild-caught in either Indonesia or Peninsular Malaysia (Appendix I)

Genomic DNA Isolation

Since tissue type and quality (Table 2.0) varied between individuals, three different genomic DNA (gDNA) isolation methods were used. For high quality tissue (e.g. well-preserved, fresh ear-plugs) a standard phenol: chloroform extraction (Maniatis *et al.* 1982) was used. For whole blood and/or plasma, the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, USA; cat# 69506) was used, and for all badly degraded and/or organ tissue, the Purelink™ Genomic DNA Mini Kit (Invitrogen Corporation, Carlsbad, 92008, USA; cat#K1820-20) was used. All gDNA was initially eluted in 200µL of 0.5X TE (Tris·EDTA) and quantified using a Biochrom Ultrospec 2100 Pro (Biochrom Ltd., Cambridge Science, Cambridge, UK; cat#802112-21) for subsequent concentration (vacuum concentration) or dilution (with sterile ddH_20) to working stocks of 5ng/µL. Genomic DNA was qualified via agarose gel electrophoresis: 5µL of undiluted stock gDNA combined with 5µL of OG loading dye for each sample were run out at 5V/cm on

0.8% agarose gels in 1X TBE (1X Tris·Borate·EDTA) buffer and post-stained in 1X TBE containing 1µL/mL Ethidium Bromide (EtBr). Gels were visualized by UV transillumination.

Optimization of a Standard Suite of Rhino Microsatellite Loci

A starting set of candidate rhinoceros specific microsatellite loci were selected from all previously published rhino microsatellite loci (Brown and Houlden 1999; Cunningham *et al.* 1999; Florescu *et al.* 2003; Zschokke *et al.* 2003; Scott *et al.* 2004) or were derived from clones generated out of partial gDNA microsatellite libraries produced in the laboratory (Florescu *et al.* 2003; Scott *et al.* 2004) (Appendix II). In order to negate a potential ascertainment bias, loci were only selected and optimized for final analyses if three criteria were met: 1) loci must contain between 12 and 28 dinucleotide repeats in their taxon of origin; 2) loci must be polymorphic in their taxon of origin (*i.e.* black rhino loci must amplify two or more alleles within the black rhino subset, white rhino loci within the white rhino subset *etc.*); and 3) loci must amplify DNA from all heterospecific rhinoceros taxa (either mono- or polymorphically).

Stage One of Microsatellite Optimization

To begin, an initial combination of 83 rhinoceros specific microsatellite loci with between 12 and 28 dinucleotide repeats in their taxon of origin (Appendix II) was selected for optimization using PCR to amplify six high quality conspecific gDNA samples. These loci included both published and novel microsatellite loci isolated from one of four rhinoceros species: black *minor*, southern white, Indian or Sumatran rhinos (Appendix II). All PCR products were generated using a Biometra T-Gradient Thermocycler and subsequently visualized via agarose gel electrophoresis. Conditions

for each PCR optimization were as follows: 6ng of template DNA, 1X QIAGEN PCR buffer (Tris-HCl pH 8.7, KCl, $(NH_4)SO_4$, 15mM $MgCl_2$), 1mM dNTPs, 1µM forward primer, 1µM reverse primer, 0.05U *Taq* DNA polymerase and sterile ddH_2O to a final volume of 10µL. Cycling parameters for each PCR included a gradient of annealing temperatures (T_m) ranging from 55-65˚C. An initial denaturing cycle of three minutes at 94˚C was followed by 35 cycles of 94˚C for 15 seconds, annealing T_m for 30 seconds, 72˚C for 30 seconds and a final 10 minute extension at 72˚C. Products were run out at 3V/cm on 2% agarose gels and post-stained in 1X TBE containing 1µL/mL [20mg/mL] EtBr before UV transillumination. Loci that did not produce a single, clear PCR product within the expected size range (based on published conditions or known clone fragment sizes) after this optimization process were deemed unsuitable for further analyses and discarded. For those loci that did amplify viable PCR amplicons, the optimal annealing temperature for each locus was selected based on the temperature condition that produced the clearest and brightest single-band PCR product.

Stage Two of Microsatellite Optimization

In step two, all loci that appeared to amplify the expected microsatellite product during the first screen were assayed for allelic variation in number of base pair repeats (bp) in conspecific rhinoceros samples. In contrast to the first screening procedure I used, high-resolution 6% polyacrylamide (PAA) gels to obtain single base separation which is not possible with agarose gels. I employed two different techniques to visualize and score PCR products. The first technique utilized standard PCR processes with the addition of γ^{33}P-radioactively labeled primers, visualized through manual autoradiography (exposure to Kodak Biomax MR™ X-ray film). Primers were manually

labeled using T4 polynucleotide kinase (T4 PNK) to replace the terminal phosphate with a $\gamma^{33}P^{-}$radioactively-labeled dATP (10µM forward primer, 1X forward reaction buffer [50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA], 10µCi γ^{33}P-dATP, 5U T4 PNK, 2.4µL ddH_2O/20 reactions; incubated at 37˚C for 30 minutes followed by enzyme inactivation at 65˚C for 10 minutes). In order to correctly size alleles, a commercial DNA ladder (Invitrogen 10bp DNA ladder; catalogue # 10821-015) was also end-labeled with γ^{33}P and run alongside the microsatellite PCR products. The second amplification technique employed LI-COR infrared technology (LI-COR 4200 Global IR^2 System™, LI-COR Inc., Lincoln NB, USA) and a competitive PCR protocol that contained both locus specific primers as well as a third fluorescent M13 Forward (M13F) universal primer (Sequence: 5'-CACGACGTTGTAAAACGAGC-3') (LI-COR Inc. M13F-700IRD cat#4200-20, M13F-800IRD cat#4000-20B). All forward primers used for the LI-COR platform were synthesized with the LI-COR M13 universal sequence preceding the unique locus-specific microsatellite primer sequence. Fluorescent PCR products were subsequently visualized via computer generated electrophoretograms and specialized genotyping software (Gene ImagIR™ 4.05, Scanalytics Inc.) and sized using a commercially prepared 50-350bp size standard ladder (LI-COR cat# 829-05343/4). PCR conditions for both platforms were identical to those determined during the original optimization in stage one with the addition of either 0.5µM of radio-labeled forward primer or 0.25µM of M13F fluorescent primer per reaction (Appendix III). Those loci that were polymorphic within their species of origin were selected for further assessment in heterospecific taxa.

Stage Three of Microsatellite Optimization

In the third step of locus optimization, the criterion that loci must amplify microsatellite DNA in heterospecific rhinoceros taxa was assessed. To complete this, four samples from each of the heterospecific rhinoceros taxa were amplified using the locus-specific PCR conditions optimized during the conspecific assays in steps one and two.

The definitive suite of rhinoceros microsatellite loci that satisfied all three selection criteria were defined as the *standard* set and used to assess microsatellite genetic variation across all 210 rhinoceros samples.

Multiplex PCR Optimization

In order to increase efficiency and reduce genotyping error, I attempted to combine multiple loci into multiplex PCR reactions that amplified several loci concurrently. All multiplexes were optimized to the LI-COR 4200 IR^2 platform. I used the QIAGEN Multiplex Kit (cat# 206143) to optimize multiplexes where each PCR reaction contained: 5ng/μL template DNA per locus, 5μL 2X QIAGEN Multiplex PCR Master Mix™ (PCR buffer, 6mM $MgCl_2$, dNTPs and HotStar™ *Taq* polymerase), 1X primer cocktail (mix of 1μM of each unique forward and reverse primer), 1.0μM M13F-IRD700/800IRD primer, and sterile ddH_2O up to a final volume of 15μL. All multiplex PCRs were generated using a Biometra T-Gradient or Biometra UNOII cycler. Cycling conditions for all multiplexes were as follows: 95˚C for 15 minutes followed by 35 cycles of 94˚C for 1.0 minute, 55˚C for 1.5 minutes, 72˚C for 1.5 minutes and a final extension of 72˚C for 15 minutes.

Estimating Genotyping Errors

I estimated my genotyping error for the *standard* set of loci using two methods. First, I used two known parent and offspring groups (WR-Bull/Pistol/Shaboola and SR-Ipuh/Emi/Andalas) from within the sample set to quantify locus specific genotyping error in my sample set. Each set of pedigree gDNA was PCR amplified three separate times using the original gDNA extractions and new PCR cocktails.

For my second error estimate, I re-extracted gDNA from 10 randomly selected rhinos (DBB1, DBB3, DBMIN5, DBMIN39, DBMIC8, DBMIC17, SWR5, SWR34, SWR55 and SR2; Appendix I) and repeated the PCR amplifications of these samples for of all loci in the *standard* set of rhinoceros microsatellite loci using fresh PCR cocktails for each locus or multiplex. The genotypes from the second set of gDNA extractions were compared to the original data I collected to provide an overall estimate of error for each locus and across all loci.

Estimating Allelic Dropout, Potential Stutter Error and Null Alleles:

To determine the likelihood of allelic dropout, potential stutter error and null alleles in the genotypes of all 210 rhinoceros samples for my *standard* set of microsatellite loci, I used the computer program MICRO-CHECKER (Van Oosterhout *et al.* 2004). Allelic dropout is a phenomenon that occurs where the smaller allele of a heterozygous individual is preferentially amplified with complete failure to amplify the larger allele, and is often an effect of low template DNA concentration (Miller *et al.* 2002). Stutter error is an effect of *Taq* polymerase slippage during PCR amplification that can add a faint band up and above the largest allele band (Van Oosterhout *et al.* 2004). This additional band if read incorrectly will give a false heterozygous genotype.

Null alleles are often indicative of a mutation in the priming region (where the primers bind to the template DNA) of one chromosomal copy of a locus, so that only one allele is amplified, giving a false homozygous genotype (Van Oosterhout *et al.* 2004).

Allele sizes for dinucleotide microsatellites repeats are typically expected to be separated in series of two base pair skips (e.g. 200, 202, 204 etc.). MICRO-CHECKER also looks for genotypes that may contain incorrectly sized alleles.

Microsatellite Genetic Variability within Rhinoceros Taxa

Individual and species mean observed (H_o) and mean expected (H_e) heterozygosity (H_e; Nei 1978) as well as average numbers of alleles per locus were computed for each rhinoceros taxon for the *standard* set of microsatellite loci. I tested for significant differences between cumulative *Ho* and *He* for each taxon individually using paired *t*-tests (Zar 1984). I also calculated allelic richness (standardized mean number of alleles per locus) for each taxon relative to the smallest sample size (northern white rhinos: *n=6)* using FSTAT v. 2.9.3 (Goudet 1995). Putative private alleles as well as overall allele frequencies for each locus were calculated for each rhino subspecies using GENEPOP v. 4.0 (Raymond and Rousset 1995). Pairwise estimates of Linkage Disequilibrium (LD) (Hill and Robertson 1968) for the *standard* loci were estimated for each of the seven rhino taxa using the program ARLEQUIN v. 3.1 (Excoffier *et al.* 2005) with a forecasted Markov chain length of 100,000 and 1000 dememorization steps followed by sequential Bonferroni corrections for multiple comparisons (Rice 1989).

Comparative Microsatellite Genetic Variability among Rhino Taxa

To evaluate the hypothesis that all rhinoceros taxa are equally diverse, I used individual *Ho* averaged across all 24 *standard* microsatellite loci. All available

rhinoceros subspecies were tested (except for the northern white rhinos) using a least squares one way Analysis of Variance (ANOVA) (Zar 1984). The northern white rhinos were dropped from this analysis due to the small available sample size ($n=6$). Tukey-Kramer HSD tests for comparisons between all pairs, as well as Student t-tests for each pair of rhino species were used to look for significant differences of individual *Ho* averaged across all *standard* loci (Zar 1984). Tests for heteroscedasticity (unequal variance among species estimates) as well as one-way ANOVA, Tukey-Kramer HSD and Student t-tests were computed using the program JMP 7 (SAS Inc.).

I also examined the contribution of taxonomic origin of microsatellites (*i.e.* whether they were conspecifically or heterospecifically derived) and species as predictors of microsatellite variability in rhinos. For this I calculated individual observed heterozygosity estimates for all members of a taxon for six conspecific loci and then (for the same individuals) six heterospecific loci. The heterospecific loci were six of BR06, BR37D, WR7C, WR12F, IR10, IRI2, SR268 and SR281 (with the conspecific pair removed for the corresponding study taxon). As an example, in the case of the white rhino heterospecific loci set, polymorphism data at WR7C and WR12F were omitted from the above eight loci. I then computed Fixed Model (I) and Random Model (II) two-way ANOVAs of standard least squares to determine the relative importance of species versus locus origin as predictors of microsatellite diversity in rhinos (JMP 7). For this analysis, I only included diversity estimates for those rhino taxa from which the microsatellite loci used in this study were derived (black *minor*, southern white, Indian and Sumatran rhinos). To present the above analyses in a more qualitative way I compared population *Ho* estimates for black *minor*, southern white, Indian and Sumatran

rhinos using the six conspecific loci and their six respective heterospecific loci along with population *Ho* derived from the *standard* set of 24 microsatellite loci.

Analyzing the Power of the Standard Microsatellite Loci

In order to evaluate the ability of my *standard* loci set to discriminate among individuals, I calculated the Probability of Identity (P_{ID}) for each rhino subspecies. P_{ID} is the probability that two randomly selected individuals from a population will have identical genotypes across all loci (Paetkau and Strobeck 1997). A large P_{ID} means that the loci do not have the power to discriminate among members of the same subspecies.

At a coarse level, I explored if my *standard* loci would discriminate among all seven rhinoceros study taxa. I first calculated frequency-based population matrices of inter-individual genetic distance (Paetkau *et al.* 1995) for each pair of rhinoceros taxa using the program GenAlEx v. 6.0 (Peakall and Smouse 2006) to see if individual rhinos were correctly assigned back to their respective subspecies. Expected genotype frequencies for each locus were calculated (assuming random mating), multiplied across all loci and log-transformed to give a log likelihood estimate for each population (Paetkau *et al.* 1995). Each specimen was assigned to the population with the highest log-likelihood value. In each case, population allele frequencies were calculated by excluding the sample to be assigned as recommended (Peakall and Smouse 2006).

In order to determine if my *standard* microsatellite loci improved the ability to assign rhinos to their respective subspecies when compared to analyses involving loci derived from a single taxon alone, I conducted a series of Principle Coordinates Analyses (PCAs) (Peakall and Smouse 2006). Pairwise inter-individual genetic distances (Smouse and Peakall 1999) were computed for all rhino taxa. To further determine if all loci

included in my *standard* set should be included in comparative analyses, I calculated PCAs from genotypes collected from smaller randomly selected sets of microsatellite loci derived from the *standard* set.

Microsatellite Polymorphism and Rhinoceros Systematics

Finally, to reveal the systematic relationships between rhino taxa in this study, I generated unrooted neighbour-joining trees (Saitou & Nei 1987) from interpopulation assignment (D_{Lr}) (Paetkau *et al.* 1997), D_s 'standard' (Nei 1972), D_m 'minimum' (Nei 1972) D_a improved (Nei *et al.* 1983), $D\mu^2$ (Goldstein *et al.* 1995), D_{sw} (Shriver et al. 1995) and mean shared allele D_{msa} distances. All distance matrices and tree files were created from raw genotype data using JEANS (Brzustowski 2002) and viewed in TREEVIEW (Page 1996). I evaluated support for the topologies by analyzing 1000 replicate matrices created by resampling the individuals with replacement (bootstrapping) and calculating the number of times as a percentage each node was recovered in all replicates.

Table 2.0: Samples sizes, origin and tissue type for rhinoceros specimens. *N* refers to the total number of samples and *n* refers to the number of samples from each location. For further details see Appendix 1.

Subspecies	*N*	*n*	Origin	Tissue Type
Black rhinos				
D. b. bicornis	*31*	31	Etosha National Park, Namibia	Ear Plugs, Blood
D. b. minor	*48*	1	Caldwell Zoo, USA	Kidney
		4	Denver Zoo, USA	Blood, Liver
		43	Hluhluwe-iMfolozi National Park, SA	Blood, DNA
D. b. michaeli	*25*	4	Berlin-West Zoo, Germany	Earplugs, Blood
		1	Busch Gardens Tampa Bay, USA	Heart
		1	Cincinnati Zoo, USA	Earplug
		1	Denver Zoo, USA	Blood, Plasma
		1	Detroit Zoo, USA	Liver
		6	Dvůr Králové, Czech Republic	Earplugs, Organ
		1	Granby Zoo, USA	Liver
		1	Kansas City Zoo, USA	Heart
		1	London Zoo, UK	Earplug
		1	Los Angeles Zoo, USA	Blood
		4	Tsavo National Park, Kenya	Earplugs
White Rhinos				
Southern	*59*	25	Hluhluwe-iMfolozi National Park, SA	Ear Plugs, Blood, Plasma
		24	KwaZulu-Natal, SA	Ear Plugs, Blood, Plasma
		5	Metro Toronto Zoo, Canada	Blood, Skin, Saliva
		25	Waterberg National Park, Namibia	Ear Plugs, Blood
Northern	*6*	3	Dvůr Králové, Czech Republic	Blood
		1	Henry Dourly Zoo, USA	Ear Plugs
		2	Shambe, Sudan	Ear Plugs
Indian Rhinos	*18*	2	Metro Toronto Zoo, Canada	Skin
		2	Los Angeles Zoo, USA	Heart, Liver
		8	Royal Chitwan National Park, Nepal	Blood
		6	Kaziranga National Park, India	Blood
Sumatran Rhinos	*23*	1	Bronx Zoo, USA	Blood
		1	Los Angeles Zoo, USA	Liver
		3	Cincinnati Zoo, USA	Blood, Hair
		7	Sungai Dusun, Peninsular Malaysia	Blood
		10	Way Kambas National Park, Indonesia	Blood
		1	Poached Rhino Carcass, Indonesia	Liver

Chapter 3: Microsatellite Optimization Results

Microsatellite Locus Selection and Optimization

Of the total 83 rhinoceros microsatellite loci that were assayed, 52 loci amplified polymorphic products in their taxon of origin (Appendix III). Of these 52 loci, 33 loci - nine black rhino loci, six white rhino loci, six Indian rhino loci and 12 Sumatran rhino loci - met all three selection criteria: each locus contained between 12 and 28 dinucleotide repeats, was polymorphic in the species of origin and amplified viable PCR products in all rhino taxa examined (Appendix III).

A *standard* set of 24 loci (six loci derived from each of the *minor*, southern white, Indian and Sumatran rhinos) was chosen based on the limiting number of loci available to each of the white and Indian rhinoceros taxa (*n=6 each*), while the best six black and Sumatran rhino loci were subsequently chosen for reliability and clarity of PCR amplification products (Table 3.0).

The six black rhino loci were optimized along with one Sumatran rhino loci into two multiplex PCRs (Table 3.1). Several forward primers were synthesized with LI-COR fluorescence tags (Table 3.1) in order to take advantage of both wavelengths of infrared detection for loci that amplified similarly sized PCR fragments and could not otherwise be resolved in a multiplex.

The first multiplex (CSRT 1) contained four black rhino microsatellite loci (BR06, DB44, BlRh2B and BlRh37D) and the second multiplex (CSRT 2) contained two black rhino and one Sumatran rhino microsatellite locus (DB01, DB52 and SR 262) (Table 3.1).

There was no evidence of linkage disequilibrium between loci after testing by multiple Fisher's exact tests (Slatkin 1994) followed by sequential Bonferroni correction (Rice 1989). These tests were conducted using the program ARLEQUIN v. 3.1 (Excoffier *et al.* 2005) with a forecasted Markov chain length of 100,000 and 1000 dememorization steps.

Genotyping Error Estimates

Genotyping errors from all sources were considered to be low. Both known rhinoceros pedigrees were successfully validated at all 24 *standard* rhinoceros microsatellite loci (Appendix IV). Pedigree samples were PCR amplified for all 24 loci three separate times using the original gDNA extractions and new PCR cocktails. Calves were consistently matched to their respective parents.

Genomic DNA was successfully re-extracted for 10 samples selected from all taxa (DBB1, DBB3, DBMIN5, DBMIN39, DBMIC8, DBMIC17, SWR5, SWR34, SWR55 and SR2). The same genotypes as the original template DNA were reproduced $\geq$ 95% when PCR amplifications were repeated for each locus using the new gDNA. While individual allele sizes did not change, a heterozygous individual instead showed a homozygous genotype, suggesting allelic dropout may have occurred. Together, these error checking methods suggest a low level of genotyping error in the extraction and amplification protocols used.

Allelic Dropout, Potential Stutter Error and Null Alleles

There was no significant evidence for null alleles, stutter error or allelic dropout in the genotypes collected for the *standard* microsatellite loci set.

All microsatellite loci surveyed contained dinucleotide motifs so the expectation was that all allele sizes would be separated in a series of two base pair length skips. A potential single base pair skip was identified at locus IR10 where five *michaeli* individuals (DBMIC4, DBMIC5, DBMIC7, DBMIC11, and DBMIC20) appeared to contain an allele sized at 197bp when all other *michaeli* specimens contained only alleles sized 196, 198 and 200bp. PCR amplifications of these individuals at microsatellite locus IR10 were repeated three separate times using new PCR cocktails and in each instance each sample maintained the 197bp allele, with DBMIC5 consistently presenting the genotype 197/196. These results suggest that the genotypes for these specimens are correct but may warrant further investigation.

Table 3.0: Taxon specific microsatellite characteristics for definitive set of loci used to assess microsatellite variability among four rhinoceros study species. BR - black rhinos, WR = white rhinos, IR = Indian rhinos, SR = Sumatran rhinos. Origin refers to rhino species to which loci were derived. DBMIN - black *minor*, SWR - southern white rhinos.

	SPECIES	BR	WR	IR	SR
LOCUS ID	Origin	*(n=25+33+49)*	*(n=59+6)*	*(n=18)*	*(n=23)*
BlRh2B	DBMIN	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
BlRh37D	DBMIN	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
BR06	DBMIN	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
DB01	DBMIN	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
DB44	DBMIN	POLYMORPHIC	POLYMORPHIC	MONOMORPHIC	POLYMORPHIC
DB52	DBMIN	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
WR7B	SWR	POLYMORPHIC	POLYMORPHIC	MONOMORPHIC	POLYMORPHIC
WR7C	SWR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
WR12F	SWR	POLYMORPHIC	POLYMORPHIC	MONOMORPHIC	POLYMORPHIC
WR32A	SWR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
WR32F	SWR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
WR35A	SWR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
IR10	IR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
IR12	IR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
IR14	IR	MONOMORPHIC	MONOMORPHIC*	POLYMORPHIC	POLYMORPHIC
IR22	IR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
RH3	IR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
RH5	IR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
SR54	SR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
SR63	SR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
SR74	SR	POLYMORPHIC	MONOMORPHIC	MONOMORPHIC	POLYMORPHIC
SR262	SR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
SR268	SR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC
SR281U	SR	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC	POLYMORPHIC

**Fixed differences for each subspecies*

Table 3.1: Details of *standard* 24 microsatellite loci for used for estimates of rhinoceros genetic diversity in available taxa. *Origin* = taxonomic origin of microsatellite locus; *Motif* = microsatellite repeat motif; T_m = Optimal annealing temperature of primers; and *Source* = Original source of characterized microsatellite loci.

Locus	Origin	Motif	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'- 3')	T_m (°C)	Source
BlRh2B	Black rhino (*minor*)	$(CA)_{19}$	(M13) CCCTTTTCTCCCTTTATCTAG	ATACTGTGAAATCCTGTTCC	†55	*QUMEL unpublished*
BlRh37D	Black rhino (*minor*)	$(TG)_{23}$	(M13) CCACTCAGAATGAGAAATGG	TCTCCCTACTTAATCCCACC	†55	*QUMEL unpublished*
BR06	Black rhino (*minor*)	$(CA)_{15}$	(800) TCATTTCTTTGTTCCCCATAGCAC	AGCAATATCCCACGATATGTGAAGG	†55	*Cunningham et al. 1999*
DB01	Black rhino (*minor*)	$(CA)_{14}$	(700) AGATAATAATAGGACCCTGCTCCC	GAGGGTTTATTGTGAATGAGGC	‡55	*Brown and Houlden 1999*
DB44	Black rhino (*minor*)	$(CA)_4g(CA)_{16}$	(800) GGTGGAATGTCAAGTAGCGG	CTTGTTGCCCCATCCCTG	†55	*Brown and Houlden 1999*
DB52	Black rhino (*minor*)	$(CA)_{21}$	(M13) CATGTGAAATGGACCGTCAGG	ATTTCTGGGAAGGGGCAGG	‡55	*Brown and Houlden 1999*
WR7B	Southern white rhino	$(GT)_{20}$	CCTCTGTGATTAAGCAAGGC	ATGAACAGGAAGGAAGACGC	62	*Florescu et al. 2003*
WR7C	Southern white rhino	$(CT)_{14}$	AAACAGGTCTTGATTAGTGC	TGAACTCTGATGGAAATGAG	62	*Florescu et al. 2003*
WR12F	Southern white rhino	$(CA)_{14}$	(M13) AAGCAGCCAGCCTAGGACAC	GGAGCTCTCCCATATGGTCG	62	*Florescu et al. 2003*
WR32A	Southern white rhino	$(CA)_{15}$	(M13) CAGTCCTGCTGCATAAATCTC	GCAGTACAGCTAGAATCACC	62	*Florescu et al. 2003*
WR32F	Southern white rhino	$(CA)_{17}$	CTGGAAATGGAAACCCGAAC	GCAACATCCATCGGACTGTC	62	*Florescu et al. 2003*
WR35A	Southern white rhino	$(CA)_{20}$	AGCCTGCTTTGCTGCCTTGC	AGGTGCACACATCCCACTCG	62	*QUMEL unpublished*
IR10	Indian rhino	$(CA)_{22}$	CAGTGAGGAAGATTGGTTGC	CCTGACTCACACATCACCAG	62	*QUMEL unpublished*
IR12	Indian rhino	$(CA)_{18}$	GAATGCTGATCATTTAGTGAC	GGGTCCAGTTGAGATATCAC	62	*QUMEL unpublished*
IR14	Indian rhino	$(GT)_{15}$	CCTAGTAGTCAACGGCAAGG	TGGACTCTTGCATAGGCTCC	62	*QUMEL unpublished*
IR22	Indian rhino	$(CA)_{22}$	ATGGTGGAAGAAGTGCAGCC	ACTTCTGTGTCTCTAGCGCC	62	*QUMEL unpublished*
RH3	Indian rhino	$(TC)_{8tg}(TC)_{7cctg}(TC)_{4tg}(TC)_{16}$	(M13) TGTGTGGAGCACATCAGTCTTC	CCAGGGACCCGTGAGGAT	58	*Zschokke et al. 2003*
RH5	Indian rhino	$(TG)_{15}$	(M13) CCCATTAGAGGCTGTAGAGTAATATC	GGACTCTAAACTCCAGGGTCAC	58	*Zschokke et al. 2003*
SR54	Western Sumatran rhino	$(CA)_{26}$	CAATATCCAGGCTTCCAGG	CTGTTTACTGTTATCGATGCTC	62	*Scott et al. 2004*
SR63	Western Sumatran rhino	$(AC)_{19}$	CTTGAGCAGAGTAGAATTTGG	CTCTGTATCCACCTCATTCC	62	*Scott et al. 2004*
SR74	Western Sumatran rhino	$(CA)_{19}$	CAGCACAATGTTTGGCACTTG	TTGGAGTCTTATGTCACCACC	62	*Scott et al. 2004*
SR262	Western Sumatran rhino	$(TG)_{28}$	(800) CTGCCTTAACAACTGAACTGC	TGGAGGTTATCTCATGCCAC	‡55	*QUMEL unpublished*
SR268	Western Sumatran rhino	$(CA)_{25}$	GTTTATACTATGCCCTGCAC	GGATGCTACCGAATAGATTG	62	*QUMEL unpublished*
SR281U	Western Sumatran rhino	$(GT)_{23}$	AGGTGATTAGGGAATTGCTGG	TTCTTCTGTCCTGGCATTGC	62	*Scott et al. 2004*

(M13)Addition of M13F sequence (5'-CACGACGTTGTAAAACGAGC-3') at the beginning of unique primer

(700)/(800) - synthesized with 700IRD/800IRD Licor fluorescent tag

†Multiplex PCR CSRT1; ‡Multiplex PCR CSRT2

Chapter 4: Results of Genetic Variability Comparisons

Genetic Variability Estimates

All 210 available rhinoceros specimens (Appendix I) were successfully amplified at all 24 microsatellite loci.

Mean *Ho* among rhino taxa did not exhibit significant heteroscedasticity ($p > 0.05$) as evidenced by four tests for variance among means: O'Brien (O'Brien 1979), Brown-Forsythe (Brown and Forsythe 1974), Levene (Levene 1960) and Bartlett (Bartlett and Kendall 1946). Observed heterozygosity (*Ho*) values averaged across all 24 microsatellite loci differed significantly among the rhino study taxa and ranged from a high of 0.573 ($SE \pm 0.018$) in the *michaeli* rhinos to a low of 0.338 ($SE \pm 0.023$) in the Indian rhinos (Table 4.0). A one-way analysis of variance (ANOVA) of averaged *Ho* for available rhino taxa was significant ($p<0.0001$) (Table 4.1). For Tukey-Kramer HSD and Student t-tests of mean *Ho*, the northern white rhinos were not included due to small sample size ($n=6$). These tests indicated that mean *Ho* for black *michaeli* rhinos was significantly higher than Indian ($p<0.0001$), *bicornis* ($p<0.001$), southern white ($p<0.01$) and Sumatran rhinos ($p<0.01$). Black *minor* rhino mean *Ho* was significantly higher than Indian ($p<0.0001$), Sumatran ($p=0.0002$), southern white ($p<0.0001$) and *bicornis* rhinos ($p<0.001$). Black *bicornis* rhino mean *Ho* was significantly higher than the Indian rhinos ($p<0.05$) and southern white rhino mean *Ho* was significantly higher than the Indian rhinos ($p<0.05$).

Average number of alleles per locus ranged from a high of 5.3 in the *michaeli* and Sumatran rhinos to a low of 2.3 in the northern white rhinos (Table 4.0). Standardized

allelic richness ranged from a high of 3.9 alleles per locus (*michaeli*) to a low of 2.2 alleles per locus in the southern white rhinos (Table 4.0).

Putative private alleles for each species ranged from a high of 44 in the Sumatran rhinos to a low of 6 in the *bicornis* rhinos (Table 4.0). Mean *Ho* and mean *He* were compared for each rhinoceros taxon. Paired t-tests (Zar 1984) indicated significant differences between mean *Ho* and mean *He* for each of the *michaeli* ($p < 0.05$), southern white ($p < 0.05$), Indian ($p = 0.001$) and Sumatran rhinos ($p < 0.0001$) (Table 4.0).

The Effects of Species and Locus Origin on Rhinoceros Genetic Diversity

As tested by both the Fixed and Random Model two-way ANOVAs, both species and locus origin (con- or heterospecific) were significant predictors of microsatellite variability at $\alpha < 0.05$ (Table 4.1A). Although only ~ 13% of the variation in rhino microsatellite diversity is explained by species and locus origin, species effects explain more than twice the amount of variation as does locus origin (9.4% versus 3.7%; Table 4.1B) The interaction effect of species $*$ locus origin was insignificant in both analyses, exemplified by the finding that for all rhino species, mean *Ho* estimates were consistently higher for conspecific loci versus heterospecific loci (Table 4.2).

For all four taxa, conspecific microsatellite loci yield higher population *Ho* estimates compared to heterospecific loci, with the increase varying from 12.5% in the white rhinos to 19.4% in the Indian rhinos. The rank order of population variability remains similar for estimates of *Ho* taken from conspecific loci and the *standard* set of 24 loci (Table 4.3), with only the rank positions of the white and Indian rhinos reversing for estimates of *Ho* taken from heterospecific loci and compared to the *standard* set of 24 microsatellite loci (Table 4.2).

Power of Microsatellites for Identifying Rhino Individuals and Populations

Probability of Identity (P_{ID}) (Paetkau *et al.* 1995) was calculated for each rhino taxon using the *standard* set of 24 microsatellite loci (Table 4.0) to test the ability of the these loci to assign unique genotypes to each rhino. The chance of randomly selecting two individuals from a population with identical 24 locus genotypes ranges from a low P_{ID} of 1.94×10^{-22} in the DBMIC to a high P_{ID} of 1.45×10^{-11} in the SWR, still a very low probability. A complementary analysis to check for duplicate multi-locus genotypes was conducted using GENEPOP v. 4.0 (Raymond and Rousset 1995), which found all genotypes to be unique. Consistent with the above analyses, all rhinoceros subspecies were correctly assigned back to their respective populations based on allele frequency-based individual log-likelihoods using all 24 microsatellite loci (GenAlEx v. 6.0, Peakall and Smouse 2006) (Appendix V and VI).

Principle Coordinate Analyses

A multivariate Principle Coordinates Analysis (PCA) of all rhinoceros samples based on the *standard* set of 24 microsatellite loci showed complete differentiation between all rhinoceros taxa except for the *bicornis* and *minor* black rhinos (Figure 4.0).

The majority of rhinoceros conservation genetics studies that have used microsatellite loci to make statements regarding population structure and current status of genetic diversity have relied on a single set of six to nine black rhino microsatellite loci (Brown and Houlden 1999; Cunningham *et al.* 1999; Garnier *et al.* 2001, Harley *et al.* 2005). To compare such an approach with the one used here, individual PCAs were computed for the four sets of six taxon-specific loci making up the 24 *standard* loci used here. Similarly, PCAs were produced for a set of six loci from all taxa (BlRh37D,

WR7C, WR12F, IR10, IR12, and SR268), a set of nine randomly selected loci (DB01, DB44, DB52, WR32F, WR35A, WR12F, IR14, IR22, SR54, and SR63) and a further set of 12 randomly selected microsatellite loci (BlRh2B, BlRh37D, BR06, WR7B, WR7C, WR12F, IR10, IR12, IR14, SR262, SR268 and SR281) to investigate how the number of loci and their taxonomic origin affect attempts to differentiate rhino species.

For all sets of six taxon-specific microsatellite loci PCA differentiation of rhinoceros was less than that obtained with the *standard* set of rhino loci (Figures 4.1-4.4). The six black rhino loci could be used to differentiate white and black (*bicornis* only) rhinos from all other rhinos (Figure 4.1); white rhino loci differentiated the three black rhino subspecies from all other rhinos (Figure 4.2); the Indian rhino loci differentiated the southern white rhinos as well as grouping together the three black rhino subspecies, but pooled the northern white, Indian and Sumatran rhinos (Figure 4.3); and the Sumatran rhino loci differentiated the white and Indian rhinos but pooled the black and Sumatran rhinos (Figure 4.4).

Similarly, randomly selected subsets of six, nine and 12 loci from the *standard* set failed to differentiate all rhino taxa. The PCA of six randomly selected loci could be used to differentiate the southern white and the black *bicornis* and *minor* rhino subspecies but pooled the remaining taxa (Figure 4.5). The PCA with nine randomly selected loci revealed a similar level of taxon differentiation to the six-locus (multi-taxa loci) PCA and was able to differentiate each white rhino subspecies, but grouped together the three black rhino subspecies and could not differentiate the two Asian taxa (Figure 4.6). The 12-locus PCA revealed a moderate to high level of taxon differentiation and could be

used to differentiate between all available rhino taxa, but still grouped all of the black rhino subspecies together (Figure 4.7).

Using Microsatellite Data to Examine the Systematic Placement of Sumatran Rhinos

Only assignment distance (D_{Lr}) and mean shared allele (D_{msa}) distance neighbour-joining trees supported the sister relationships of the two white rhino and three black rhino taxa (Figure 4.8). The D_{Lr} tree supports a Sumatran-Black rhino versus a Sumatran-Indian rhino affinity while the D_{msa} tree supports a Sumatran Rhino, Black rhino and Indian-White rhino trichotomy. The Sumatran-Black rhino association is only weakly supported by the microsatellite data, with only 10.6% of the 1000 D_{Lr} bootstrap replicates recovering this association (Figure 4.8).

Table 4.0: Microsatellite variability in four rhinoceros species (including seven subspecies) using the *standard* suite of 24† microsatellite loci. *n* = number of individuals; *Ho* = average observed heterozygosity (± *SE*); *He* = average expected heterozygosity (± *SE*) (Nei 1987); and *N* = average number of alleles per locus. Allelic richness is the mean number of alleles per locus standardized for the smallest population (Northern white rhinos: *n=6*) and private alleles are those that are unique to a respective subspecies. P_{ID} = probability of identity (Paetkau and Strobeck 1997).

Species	*n*	*Ho* (± *SE*)	*He* (± *SE*)	*N*	*Allelic richness*	*Private alleles*	P_{ID}
Black rhinos							
D. b. bicornis	31	0.401 ± 0.018	0.455 ± 0.016	3.9	2.7	6	4.30×10^{-13}
D. b. minor	48	0.477 ± 0.014	0.507 ± 0.010	4.6	3.1	14	5.36×10^{-15}
D. b. michaeli	25	0.573 ± 0.005*	0.635 ± 0.012	5.3	3.9	17	1.94×10^{-22}
White rhinos							
Southern	59	0.342 ± 0.003*	0.388 ± 0.005	2.7	2.2	10	1.45×10^{-11}
Northern	6	0.368 ± 0.031	0.338 ± 0.007	2.3	2.3	16	1.99×10^{-14}
Indian rhinos	18	0.338 ± 0.023*	0.428 ± 0.013	4.0	2.9	22	1.65×10^{-15}
Sumatran rhinos	23	0.380 ± 0.021*	0.605 ± 0.090	5.3	3.8	44	1.61×10^{-21}

† Suite of loci includes six loci isolated from each target species: BR2B, BR37D, BR06, DB01, DB44, DB52 , WR7B, WR7C, WR12F, WR32A, WR32F, WR35A, IR RH3, IR RH5, IR10, IR12, IR14, IR22, SR54, SR63, SR74, SR262, SR268 AND SR281

** Two-tailed t-test indicates significant difference ($p<0.05$) between observed and expected heterozygosities.*

Table 4.1: One-way ANOVA comparing mean observed heterozygosity values for all available rhinoceros species.

Source	df	Sum of Squares	Mean Squares	F ratio	Prob > F
Species	5	1.2705	0.2541	26.4856	< 0.0001
Error	198	1.9000	0.0096		
Total	203	3.1701			

Table 4.2: A) Both species and locus origin are significant predictors of rhinoceros microsatellite diversity at 0.05 significance. The Sums of Squares (SS), F value (F) and probability (P) of these effects is shown. The interaction between species and locus origin is insignificant at 0.05. Fixed Effects (Model I) and Random Effects (Model II) results were the same. B) Percentage of variance explained by species and locus origin effects (Two-Way ANOVA, Model II) showing species is a greater predictor of microsatellite diversity in rhinoceroses.

A)

Source	df	Sum of Squares	F ratio	Prob > F
Species	3	0.9671	9.9852	< 0.0001
Locus origin	1	0.2060	6.3819	0.0121
Species x Locus	3	0.0523	0.5401	0.6552

B)

Source	Percent of Total
Species	9.4
Locus origin	3.7
Residual variance	86.9
Total	100

Table 4.3: Average observed heterozygosities (*Ho* ± SE) and ranked order of population diversity for the four rhinoceros taxa that microsatellite loci were isolated from, using either six conspecific loci,six heterospecific loci or all 24 *standard* loci. *Rank* refers to the ranked order of taxon population diversity where 1 is the highest level of diversity and 4 is the lowest level of diversity for each respective set of microsatellite loci.

Taxon	Conspecific loci *(n=6)*	Heterospecific loci* *(n=6)*	Standard Loci *(n=24)*
Black rhinos (*minor*)	*Ho* = 0.635 ± 0.018 *Rank = 1*	*Ho* = 0.448 ± 0.007 *Rank = 1*	*Ho* = 0.477 ± 0.014 *Rank = 1*
White rhinos (southern)	*Ho* = 0.475 ± 0.017 *Rank = 4*	*Ho* = 0.350 ± 0.032 *Rank = 3*	*Ho* = 0.342 ± 0.003 *Rank = 3*
Indian rhinos	*Ho* = 0.509 ± 0.051 *Rank = 3*	*Ho* = 0.315 ± 0.013 *Rank = 4*	*Ho* = 0.338 ± 0.023 *Rank = 4*
Sumatran rhinos	*Ho* = 0.529 ± 0.036 *Rank = 2*	*Ho* = 0.399 ± 0.014 *Rank = 2*	*Ho* = 0.380 ± 0.021 *Rank = 2*

**Heterospecific loci include (Species dependent):*
Black rhino (*minor*) loci: BlRh 2B and BlRh 37D
White rhino (southern) loci: WR 7C and WR 12F
Indian rhino loci: IR 10 and IR 12
Sumatran rhino loci: SR 268 and SR 281

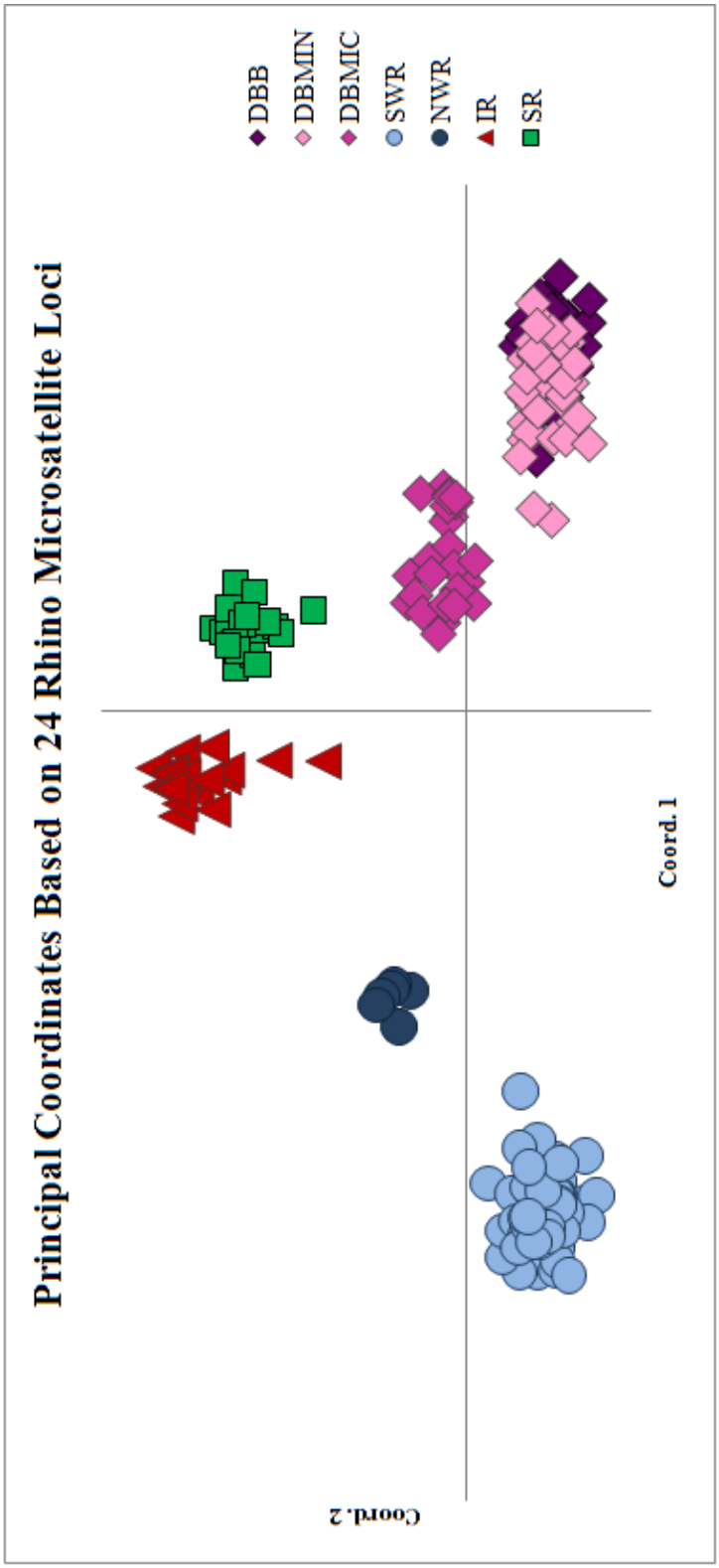

Figure 4.0: Complete differentiation between black, white, Indian and Sumatran rhinos using 24 microsatellite loci comprised of 6 loci isolated from each of white, black, Indian and Sumatran rhinos. A Principal Coordinates Analysis of four (4) rhinoceros species including 3 subspecies of black rhinos: DBB = *D.b.bicornis (n=31)*, DBMIN = *D.b.minor (n=48)* and DBMIC = *D.b.michaeli (n=25)*; 2 subspecies of white rhino: SWR = southern white rhinos *(n=59)* and NWR = northern white rhinos *(n=6)*; Indian rhinos (IR)*(n=18)* and Sumatran rhinos (SR) *(n=23)* with 63.90% of variation explained by the first two axes.

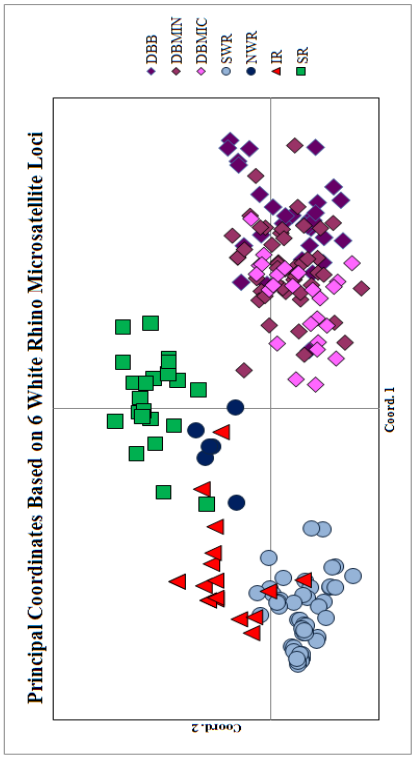

Figure 4.1: Differentiation between rhinoceros taxa based on 6 microsatellite loci isolated from black rhinos. A PCA of four (4) species of rhinoceros: 3 subspecies of black rhinos: DBB = *D.b.bicornis*, DBMIN = *D.b.minor* and DBMIC = *D.b.michaeli*; 2 subspecies of white rhino: SWR = southern white rhinos and NWR = northern white rhinos; Indian rhinos (IR) and Sumatran rhinos (SR) with 56.19% of variation explained by the first two axes.

Figure 4.2: Differentiation between rhinoceros taxa based on 6 white rhino microsatellite loci. A PCA of four (4) species of rhinoceros: 3 subspecies of black rhinos: DBB = *D.b.bicornis*, DBMIN = *D.b.minor* and DBMIC = *D.b.michaeli*; 2 subspecies of white rhino: SWR = southern white rhinos and NWR = northern white rhinos; Indian rhinos (IR) and Sumatran rhinos (SR) with with 57.16% of variation explained by the first two axes.

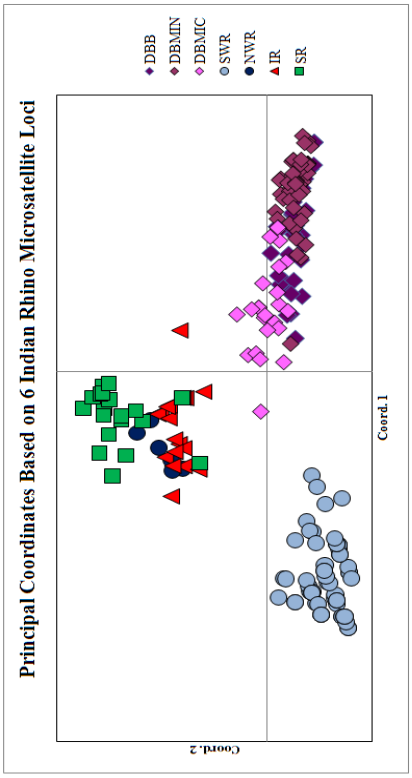

Figure 4.3: Differentiation between rhinoceros taxa based on 6 microsatellite loci isolated from Indian rhinos. A PCA of four (4) species of rhinoceros: 3 subspecies of black rhinos: DBB = *D.b.bicornis*, DBMIN = *D.b.minor* and DBMIC = *D.b.michaeli*; 2 subspecies of white rhino: SWR = southern white rhinos and NWR = northern white rhinos; Indian rhinos (IR) and Sumatran rhinos (SR) with 64.02% of variation explained by first two axes.

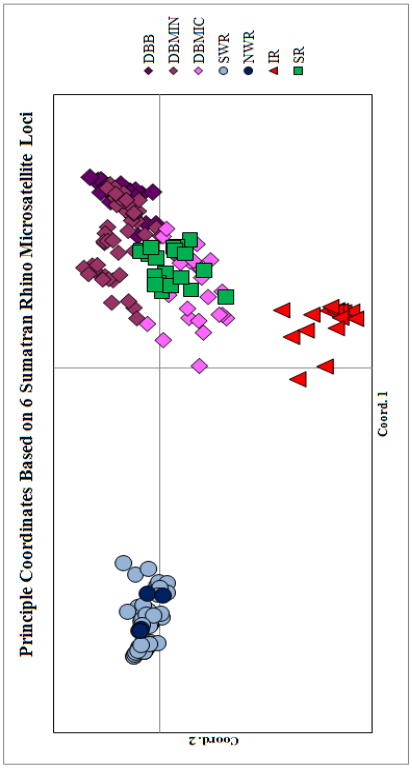

Figure 4.4: Differentiation between rhinoceros taxa based on 6 SUmatran rhino microsatellite loci. A PCA of four (4) species of rhinoceros: 3 subspecies of black rhinos: DBB = *D.b.bicornis*, DBMIN = *D.b.minor* and DBMIC = *D.b.michaeli*; 2 subspecies of white rhino: SWR = southern white rhinos and NWR = northern white rhinos; Indian rhinos (IR) and Sumatran rhinos (SR) with 67.82% of variation explained by the first two axes.

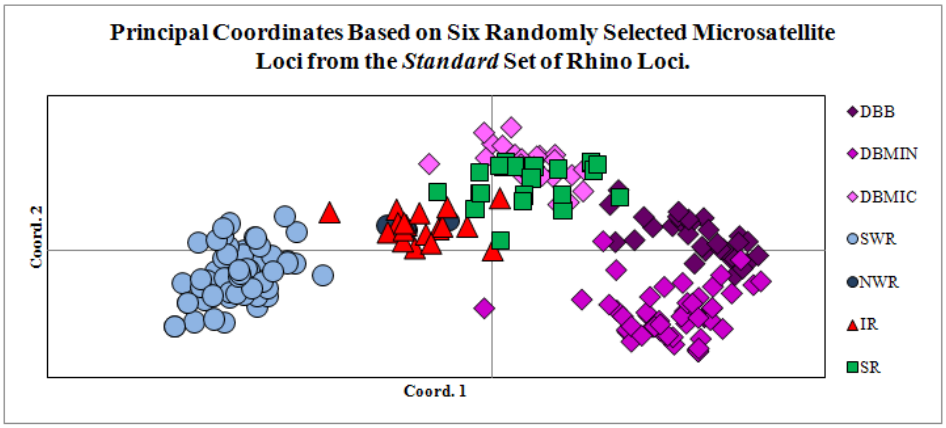

Figure 4.5: Differentiation between rhinoceros subspecies using 6 randomly selected microsatellite loci (BlRh37D, WR7C, WR12F, IR10, IR12 and SR268) taken from the *standard* set of 24 loci. A PCA of four (4) species of rhinoceros: 3 subspecies of black rhinos: DBB = *D.b.bicornis*, DBMIN = *D.b.minor* and DBMIC = *D.b.michaeli*; 2 subspecies of white rhino: SWR = southern white rhinos and NWR = northern white rhinos; Indian rhinos (IR) and Sumatran rhinos (SR) with 56.75% of variation explained by the first two axes.

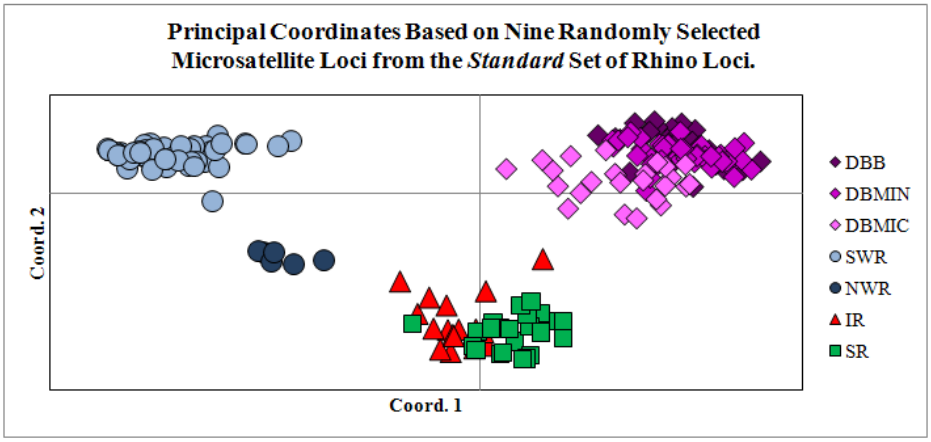

Figure 4.6: Differentiation between rhinoceros subspecies using 9 randomly selected loci (DB01, DB44, DB52, WR32F, WR35A, WR12F, IR14, IR22, SR54, and SR63) taken from the *standard* set of 24 loci. A PCA of four (4) species of rhinoceros: 3 subspecies of black rhinos: DBB = *D.b.bicornis*, DBMIN = *D.b.minor* and DBMIC = *D.b.michaeli*; 2 subspecies of white rhino: SWR = southern white rhinos and NWR = northern white rhinos; Indian rhinos (IR) and Sumatran rhinos (SR) with 64.87% of variation explained by the first two axes.

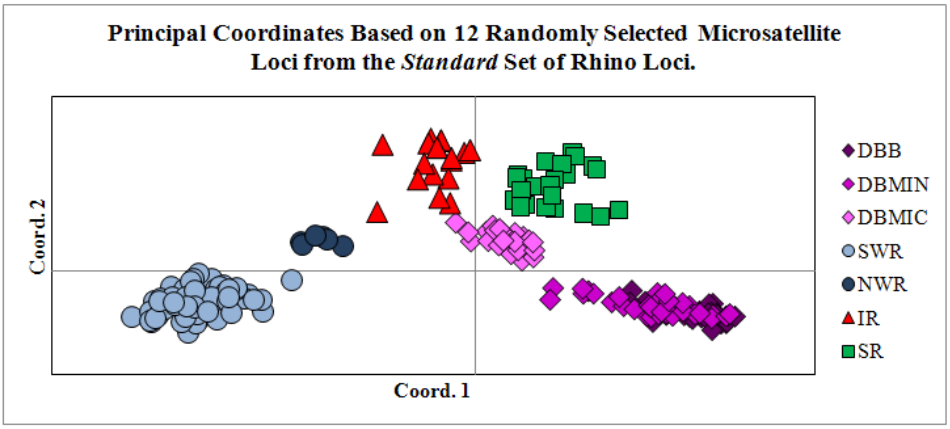

Figure 4.7: Differentiation between rhinoceros subspecies using 12 randomly selected loci (BlRh2B, BlRh37D, BR06, WR7B, WR7C, WR12F, IR10, IR12, IR14, SR262, SR268 and SR281) taken from the *standard* set of 24 loci. A PCA of four (4) species of rhinoceros: 3 subspecies of black rhinos: DBB = *D.b.bicornis*, DBMIN = *D.b.minor* and DBMIC = *D.b.michaeli*; 2 subspecies of white rhino: SWR = southern white rhinos and NWR = northern white rhinos; Indian rhinos (IR) and Sumatran rhinos (SR) with 60.60% of variation explained by the first two axes.

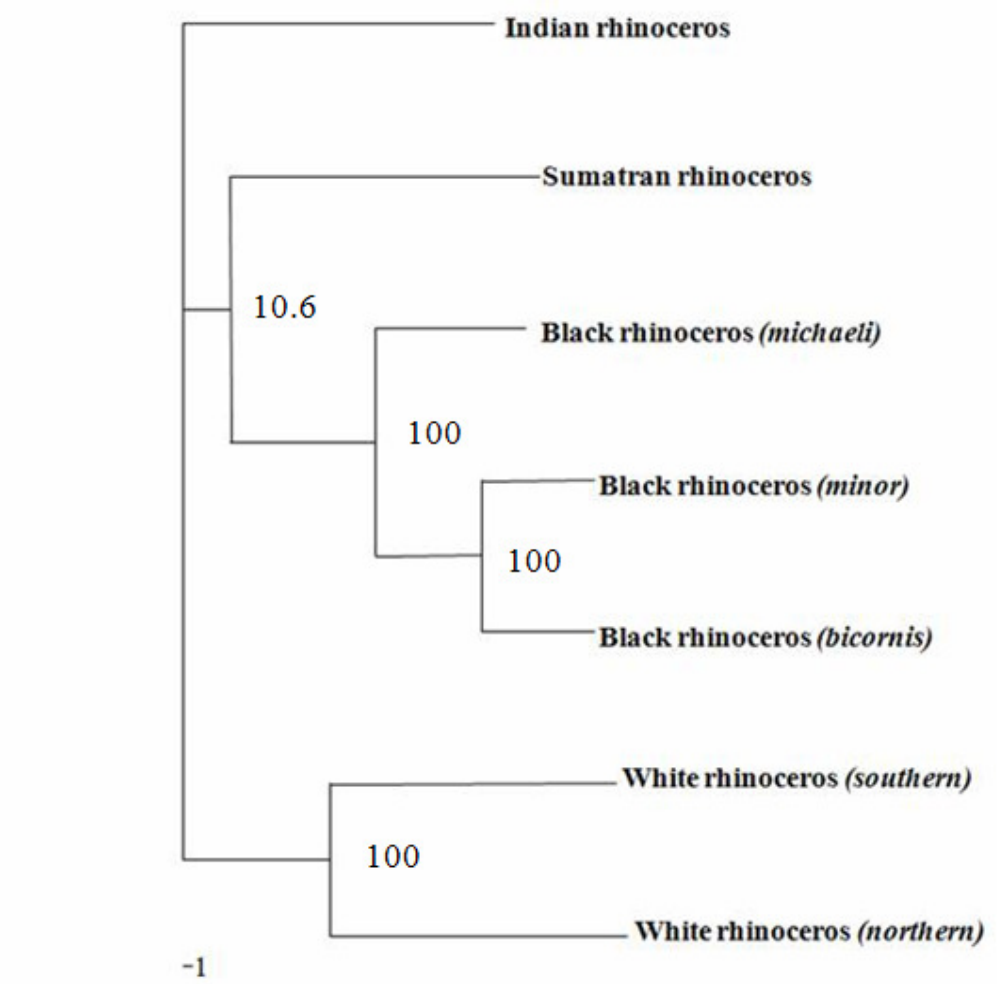

Figure 4.8: An un-rooted neighbour-joining tree (Saitou and Nei 1987) of pairwise assignment distances D_{LR} (Paetkau *et al.* 1997) from 24 *standard* rhinoceros microsatellite loci, showing (weak) affinity between the Asian Sumatran rhinos and the African black rhinos (Simpson 1945; Loose 1975; Morales and Melnick 1994) . Shown to the right of the nodes are percentage of 1000 bootstrap replicates that recover shown topology. All nodes were recovered 100% of the time except for the Sumatran – black rhino node which was recovered in only 10.6% of replicates. D_{LR} branch lengths are shown.

Chapter 5: Discussion

Summary of Results

After optimizing a *standard* set of 24 rhinoceros microsatellite loci (Objective 1) I was able to provide the most comprehensive estimate of microsatellite genetic diversity within and among African and Asian rhinoceros species to date (Objective 2). Assays of these *standard* microsatellite loci showed that the African black *michaeli* rhino subspecies, the most endangered of all recognized extant black rhino subspecies, had the highest level of microsatellite genetic variability, while southern white and Indian rhinos, taxa that are in sustained recovery from recent bottlenecks, had the lowest levels of microsatellite diversity. My findings suggest that this pattern of genetic diversity in rhinos is not explained by recent bottlenecks (Objective 3). I found that species and microsatellite locus origin – if the locus was conspecifically or heterospecifically derived - were significant predictors of microsatellite heterozygosity in the four extant rhino genera (Objective 4). My genealogical analyses using microsatellite data showed a weak association between the Sumatran and black rhinos to the exclusion of the white rhinos (Objective 5). Using the *standard* microsatellite loci, unique genotypes were assigned to all available rhinos (Objective 6) as well as correctly assigning all rhinos to their respective populations (Objective 7) suggesting these loci are powerful forensic tools for investigating rhinos. I will discuss the implications of my major findings below and conclude with important conservation outputs of this work.

Sample Compilation

Although it is always preferable to obtain sufficient samples from a single population to investigate the genetic structure of a species, this is often impossible for

highly endangered species such as the rhinoceros. First, the process of sampling is often invasive and potentially harmful to those remaining individual rhinos. Second, for solitary species such as the Sumatran rhino, sampling is only possible if the animals can be found, which is often very difficult. My rhinoceros sample set represents the largest available collection of multi-species rhinoceros specimens. While many samples were collected from zoos and parks around the world, unless otherwise noted (Appendix I), all of the black, white and Indian rhinos originated from single remnant populations. All of the southern white and Indian rhino samples are from similar temporal scales, collected during the late 1970s and early 1980s. Similarly, the black rhino samples represent individuals collected during the 1990s until present. All Sumatran rhino samples (except *Andalas*) were collected throughout peninsular Malaysia and Indonesia and include only breeding aged animals.

Microsatellite Genetic Diversity among Rhinoceros Species

Assays of these *standard* microsatellite loci showed that the African black *michaeli* rhino subspecies, the most endangered of all recognized extant black rhino subspecies, had the highest level of microsatellite genetic variability; evidenced by the highest mean heterozygosity, the highest average number of alleles per locus, and the highest allelic richness among all seven available rhino taxa. These results support prior research comparing microsatellite diversity among black rhino subspecies (Harley *et al.* 1995). Conversely, the southern white rhinos, which have been the most successful of all extant rhinos in recovering from recent, severe population bottlenecks, have retained low levels of microsatellite diversity among the available rhino taxa with the second lowest mean heterozygosity as well as the least allelic richness. These results agree with

previous reports that white rhinos show low observed heterozygosity for both AFLP and microsatellite data (Kellner *et al.* 2001; Florescu *et al.* 2003), while disputing a smaller, more recent, study that suggested prior estimates of black and white rhino microsatellite diversity were incorrect (Nielsen *et al.* 2007). A similar pattern of genetic variability exists within the subset of Asian rhino taxa where the species that is considered to be the most threatened of all extant rhinos (*Sumatran rhino*) retains a higher level of microsatellite genetic diversity relative to the species that appears to have recovered from a recent population bottleneck (*Indian rhino*). The Sumatran rhino has an average observed heterozygosity, similar to previous estimates of microsatellite diversity in this species (Scott *et al.* 2004), an average number of alleles per locus equal to that of the black *michaeli* rhinos (*5.3*) as well as allelic richness comparable to the *michaeli* rhinos (3.8:3.9), which have the highest allelic diversity of all available rhinoceros taxa. In contrast, all genetic diversity estimators for the Indian rhino taxa are lower than those of the Sumatran taxa and mean observed heterozygosity in the Indian rhinos is the lowest of all rhinoceros species. These findings corroborate initial estimates of Indian rhino microsatellite variability (Zschokke *et al.* 2003). Of further interest is the comparative microsatellite diversity of the two African white rhino subspecies. As mentioned previously, the southern white rhinos make up the majority of extant rhinos with more than 13,000 individuals, while there are less than 20 northern white rhinos remaining globally. These few northern white rhinos have both a higher mean observed heterozygosity as well as higher allelic diversity than their southern counterparts.

These comparative estimates of genetic variability suggest that further investigation is needed to understand the relationship between genetic diversity and

demographic histories of rhinos. Microsatellite DNA regions are often referred to as neutral markers in that they do not code for fitness traits (Luikart 1998; Neff and Gross 2001) suggesting that they may not always reflect how events such as bottlenecks or inbreeding may have impacted a population's genetic fitness (Hedrick and Miller 1992; Hedrick 1999; Gaudeul *et al.* 2004; Hedrick 2004). For rhinos, and white rhinos in particular, analyses of hypervariable DNA regions such as the Major Histocompatibility Complex (MHC) genes that code for a measurable trait linked to fitness may be useful in augmenting and evaluating the levels of genetic diversity indicated by microsatellites (Garrigan and Hedrick 1993; Hedrick *et al.* 2001; Bonin *et al.* 2007). Current research debates the comparative values of microsatellite versus MHC genetic data (Zachos *et al.* 2006), however a recent trend has emerged that loss of diversity is often positively correlated between microsatellite and MHC regions (Hedrick *et al.* 2001; Aguilar and Garza 2006).

Genetic diversity estimates, particularly reduced heterozygosity, of various taxa have often been linked to their respective bottlenecks (Ashley *et al.* 1990; Swart *et al.* 1994; Houlden *et al.* 1996; Comps *et al*. 2001; Whitehouse *et al*. 2001). It is the severity and duration of a bottleneck that may lead to reduced diversity. A short, severe bottleneck will likely reduce allelic diversity but not heterozygosity (England *et al*. 2003) and it is the subsequent inbreeding and genetic drift common to small populations that will reduce the overall heterozygosity (Nei *et al.* 1975). Here, I suggest that it is not recent bottlenecks that have structured current rhinoceros genetic diversity. Instead, it is more likely that historical (evolutionary) events have impacted contemporary rhinoceros microsatellite genetic diversity. For available rhinoceros taxa, except for southern white

and Indian rhino species, heterozygosity is comparable to other threatened megaherbivores such as Asian elephants (*Elephus maximus*)(Vidya *et al.* 2005), as well as non-threatened closely related perissodactyls such as horses (*Equus caballus*) (Plante *et al.* 2007). Although heterozygosity is relatively lower in the southern white and Indian rhinos, it is still equivalent to other non-threatened ungulates such as the fallow deer (*Dama dama dama*) and giraffe (*Giraffa camelopardalis*) (Brown *et al.* 2007; Webley *et al.* 2007) suggesting that lowered heterozygosity *per se* is not necessarily evidence of an inbred, genetically depauperate population or of a population bottleneck as it has been previously suggested in rhinos (Merenlender *et al.* 1989; Dinerstein and McCracken 1990; Moehlman *et al*. 1996; Côté *et al.* 2002).

In a study similar to this one, genetic diversity in wild rabbits, a species that has never suffered a population bottleneck in its native range, was examined using 29 microsatellite loci (Gage *et al.* 2006), and showed a similar level of heterozygosity (*0.450*) to the mean H_o of combined rhino taxa (*0.456*). This lends support to the hypothesis that it is contemporary events and not recent bottlenecks that may have shaped the genetic architecture of extant rhino populations. The reduced allelic richness may be an indicator of past bottlenecks or subsequent inbreeding effects (Nei *et al*. 1975; England *et al.* 2003) or the increased susceptibility of small populations to genetic drift (England *et al.* 2003; White and Searle 2007; Willi *et al*. 2007). However, without access to historical samples, this is impossible to test at this time. For similar reasons, tests for bottleneck signatures were not generated because there are no significant demographic data for all of the available rhinos, making these types of analyses difficult (Spencer *et al.* 2000).

Predictors of Rhinoceros Genetic Variability

Clearly not all rhinos are equally genetically variable. In what I believe to be the most unbiased estimate of microsatellite genetic diversity in rhinos to date, I have shown that the African black *michaeli* rhinos are significantly more variable than the southern white and Indian rhino species for 24 microsatellite loci. This was supported when the rank order of mean *Ho* remained the same across rhino taxa for both conspecific loci versus the *standard set* of microsatellite loci.

There has been an influx of research into developing standardized sets of highly variable microsatellite markers for use as forensic tools or measurements of genetic diversity for cross-species amplifications of closely related species (Hsu *et al.* 1993; Primmer *et al.* 1996; Moazami-Goudarzi *et al.* 1997; Van Hooft *et al.* 2000; Maudet *et al.* 2004). While many of these studies have effectively isolated microsatellites that can differentiate among species based on unique allele sizes, ascertainment biases that may subsequently influence the accuracy of genetic diversity estimates for a taxon are sometimes ignored (Kim *et al.* 2004; Maudet *et al.* 2004; Pertoldi *et al.* 2005; Zachos *et al.* 2006; Nielsen *et al.* 2007).

To prevent ascertainment bias, I used three criteria to optimize loci for the *standard* set of rhinoceros microsatellites: 1) loci must contain between 12 and 28 dinucleotide repeats in their taxon of origin; 2) loci must be polymorphic in their taxon of origin; and 3) loci must amplify DNA from all heterospecific rhinoceros taxa regardless of polymorphism (Chapter 2). By removing ascertainment biases that may influence either intra- or interspecies genetic diversity estimates this *standard* set of loci should

provide the most accurate picture of microsatellite genetic diversity among contemporary rhinoceros taxa (Neff and Gross 2001; Kim *et al.* 2004).

The first microsatellite locus selection criterion was invoked because it has been suggested that the length of a microsatellite repeat is correlated with its mutation rate (Nauta and Weissing 1996; Neff and Gross 2001). By choosing loci that contain repeats of similar sizes, I assumed that mutations rates, and indirectly allelic diversity, would be comparable among loci. The second selection condition required that loci must be polymorphic in their taxon of origin. This criterion in and of itself does not remove ascertainment bias and instead might actually introduce bias, however monomorphic loci are uninformative for most genetic analyses such as pedigree validations, characterizing population structures or assigning unique genetic identities, and therefore are subsequently excluded from analyses (Oliveira *et al.* 2006). It is the third selection criterion, that loci must amplify microsatellites in all rhino taxa regardless of polymorphism that, hopefully, removes bias introduced by the second criterion. Because the *standard* loci only need to be polymorphic in their species of origin, loci that are monomorphic in alternate rhino species are still included in analyses of microsatellite diversity within and among rhinoceros taxa. These combined selection criteria allow for the most unbiased estimates of interspecific rhinoceros microsatellite diversity as well as characterizing various subsets of loci that will best inform intraspecific analyses such as pedigree validation that require highly polymorphic microsatellite loci.

Of the 24 *standard* rhinoceros microsatellite loci all but three loci (12.5%) (DB44: *imperfect* repeat [$(CA)_4g(CA)_{16}$]; WR7C: *compound* repeat [$(CT)_{14}(AT)_{11}$]; and RH3: interrupted repeat [$(TG)_{22}(TC)_7cctg(TC)_4tg(TC)_{16}$]) contained *perfect* microsatellite

repeats (Appendix II). It would have been preferable to use only loci with *perfect* repeat motifs as it has been shown that different microsatellite motifs may have different evolutionary mechanisms and therefore may bias estimates of genetic diversity (Oliveira *et al.* 2006; Broquet *et al.* 2007), however no other available rhino microsatellite loci met all three selection criteria and contained *perfect* microsatellite repeats (Appendices II and III).

Rhinocerotidae Systematic Implications

I used the *standard* 24 rhino microsatellite loci to produce a D_{LR} neighbour-joining tree that weakly supported a polyphyletic African taxon and sister Sumatran – Black relationship adding support to the hypothesis that Sumatran rhinos are more closely related to the African rhinos than to the other Asian rhinos (Simpson 1945; Groves 1983; Morales and Melnick 1994). These data should be viewed with caution; there has been much debate regarding the use of microsatellites to create phyletic trees (Takezaki and Nei 1996; Richard and Thorpe 2001; Kalinowski 2005). Research has suggested that the various methods of calculating genetic distances from small sample sizes using microsatellite data can produce very different results (Nei and Roychoudhury 1974; Chakraborty and Tateno 1976). Along with the fact that the D_{Lr} tree was the only tree that didn't completely separate the African rhino genera, the D_{Lr} method makes no *a priori* assumptions of microsatellite evolution when calculating genetic distances (Paetkau *et al.* 1997).

Conservation Implications

The utility of this *standard* set of microsatellite loci for rhinoceros conservation and management strategies is clear. For conservation strategies to be successful they

must not only consider species demographies but genetic factors as well (Spielman *et al.* 2004).

The power of this *standard* set of rhinoceros microsatellite loci to identify individuals or populations of rhinos is high. The probability of assigning a unique genotype to any rhinoceros is extremely high (P_{ID} for all available taxa $\leq 1.45 \times 10^{-11}$) and considering that combined extant rhino taxa represent less than 20,000 individuals, these *standard* microsatellite loci have the potential to assign unique genotypes to all contemporary rhinos. Additionally, for all available rhino taxa, the *standard* loci were able to unequivocally assign individual rhinos to their respective populations using frequency-based population assignments (Paetkau *et al.* 1995) (Appendix V). For black rhinos only, these assignments are supported by prior population assignments (using Fisher's exact tests) among all black rhino subspecies (Harley *et al.* 2005). Further support for the power of the *standard* set of rhinoceros microsatellite loci is provided by a principle coordinates analysis based on all 24 microsatellite loci (Figure 4.0). These loci were able to differentiate between all available rhinoceros taxa with the exception of the black rhino *bicornis* and *minor* subspecies. It is important to note that a minimum of 12 loci from the *standard* set were needed to differentiate the majority of available rhinoceros taxa (Figures 4.1 - 4.7) and it was only by using either the conspecific black rhino microsatellite loci alone (Figure 4.1) or at least 12 microsatellite loci from multiple rhino origins (Figure 4.7) that any level of differentiation was seen between the black rhino subspecies. This supports the hypothesis that locus origin will impact estimates of diversity and that whenever microsatellites from a target organism are unavailable,

measures should be taken to ensure enough loci are being used in analyses of genetic diversity as well as tests to see how loci origin may impact genetic diversity estimates.

Rhinos are one of the largest; most critically endangered and yet poorly understood large mammal taxa. Genetic data collected by my *standard* set of rhinoceros microsatellite loci will be a big step towards better characterizing the demographies of these animals.

Before effective management of *in situ* rhinoceros populations can be accomplished, it is necessary to have an accurate census of how many rhinos are contained within a population and how that population is structured (Nicholls *et al.* 1996; Shea 1998; Garnier *et al.* 2001; Linklater 2003; Garner *et al.* 2005; Harley *et al.* 2005). As I mentioned in the introduction, it is both expensive and difficult to physically monitor individual rhinos. Furthermore, traditional mark-recapture or aerial census techniques are highly invasive and have failed to accurately estimate sizes of known populations of rhinos (Goddard 1967; Peter Buss pers. comm. 2007). This is particularly evident for black rhinos, a non-gregarious rhino taxon that often hides in brush-filled territory and is therefore often overlooked during aerial census (Goddard 1967; Peter Buss pers. comm. 2007). With the improved techniques for the extraction of gDNA from faecal matter (Fernando *et al.* 2006), my *standard* microsatellites can potentially be used to generate unique genotypes from the non-invasive sampling of rhino dung middens (Broquet *et al.* 2007). By themselves, these genotypes will improve upon traditional census counts, as well as identify patterns of dispersal and range territories for individual rhinos. To develop a more comprehensive picture of rhinoceros population structure including mating systems and individual recruitment, these unique genotypes could be

complemented by faecal hormone assays that can identify the approximate age and sex of rhinos (Agil *et al.* 2006; Linklater 2003).

Rhinos are often translocated between parks and zoos to increase stocks or more importantly to prevent localized inbreeding depression (Hearne and Swart 1991; Linklater 2006). This has usually been done in a random way without any understanding of how a population is structured with regard to mating systems, sex-bias or metapopulation structure (Linklater 2003 and 2006). These *standard* microsatellite loci will provide invaluable data towards characterizing genetic architecture and social structures in both captive and *in situ* rhino populations.

Ongoing and Future Research

I am currently using these *standard* rhino microsatellite loci to assign paternity and characterize the population structure and genetic diversity within an entirely closed population of southern white rhinos in Thaba Tholo (South Africa) where there has been no translocation of rhinos to or from this population. This research is particularly interesting because the entire demography of this rhinoceros population has been documented since the park's inception (births, deaths, sex-ratios etc.). This park has not artificially managed its population and instead has allowed the rhinos to develop their own mating system structure. This system is now being questioned because of a skewed sex bias towards females, leaving fewer potential fathers and thus potentially increasing the effects of inbreeding.

I am also using these *standard* loci to assay genetic diversity in the remaining Javan rhino taxa relative to other rhino species. This is an example of a study with no available conspecific markers, where it is particularly critical to generate an unbiased

estimate of genetic diversity in a small remnant population of individuals ($\leq$ 60) so that maximum genetic diversity can be retained.

A final study to determine the dispersal patterns and reduced gene flow in highly fragmented populations of Sumatran rhinos is forthcoming. These *standard* rhino loci will be used to track dispersal by generating unique genotypes from DNA extracted from faecal samples of geographically distributed known rhino dung middens.

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Table AI.a: Details of south-western black rhinoceros (*Diceros bicornis bicornis*) samples. *Sample ID* refers to the number assigned to the sample during this study, *QUMEL ID* refers to unique laboratory identification and source population refers to the birth origin of the rhinoceros.

Sample ID	*QUMEL ID*	*Origin of Tissue Sample*	*Source Population*	*TISSUE TYPE*	*Collector*
DBB1	IMP1	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB2	IMP2	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB3	IMP3	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB4	IMP4	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB5	IMP5	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB6	IMP6	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB7	IMP7	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB8	IMP8	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB9	IMP9	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB10	IMP10	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB11	IMP11	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB12	IMP13	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB13	IMP14	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB14	IMP15	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB15	IMP16	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB16	IMP17	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB17	IMP18	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB18	IMP19	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB19	IMP20	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB20	IMP21	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB21	IMP26	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB22	IMP28	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB23	IMP29	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB24	IMP30	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB25	IMP31	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB26	IMP32	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB27	IMP33	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB28	IMP36	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB29	IMP38	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB30	IMP39	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb
DBB31	IMP40	Etosha National Park, Namibia	Namibia	Ear Plug	Peter Erb

Table AI.b: Details of south-central black rhinoceros (*Diceros bicornis minor*) samples. *Sample ID* refers to the number assigned to the sample during this study, *QUMEL ID* refers to unique laboratory identification and source population refers to the birth origin of the rhinoceros.

Sample ID	*QUMEL ID*	*Origin of Tissue Sample*	*Source Population*	*TISSUE TYPE*	*Collector*
DBMIN1	CHIZ1	Hwange National Park, Zimbabwe	Zimbabwe	Plasma	Peter van Coeverden de Groot
DBMIN2	CHIZ2	Hwange National Park, Zimbabwe	Zimbabwe	Blood	Peter van Coeverden de Groot
DBMIN3	CHIZ4	Hwange National Park, Zimbabwe	Zimbabwe	Blood	Peter van Coeverden de Groot
DBMIN4	CHIZ5	Hwange National Park, Zimbabwe	Zimbabwe	Liver	Peter van Coeverden de Groot
DBMIN5	CHIZ6	Hwange National Park, Zimbabwe	Zimbabwe	Liver	Peter van Coeverden de Groot
DBMIN6	CHIZ7	Hwange National Park, Zimbabwe	Zimbabwe	Blood	Peter van Coeverden de Groot
DBMIN7	CHIZ10	Hwange National Park, Zimbabwe	Zimbabwe	Blood	Peter van Coeverden de Groot
DBMIN8	CHIZ11	Hwange National Park, Zimbabwe	Zimbabwe	Blood	Peter van Coeverden de Groot
DBMIN9	CHIZ12	Hwange National Park, Zimbabwe	Zimbabwe	Plasma	Peter van Coeverden de Groot
DBMIN10	CHIZ13	Hwange National Park, Zimbabwe	Zimbabwe	Blood	Peter van Coeverden de Groot
DBMIN11	CHIZ14	Hwange National Park, Zimbabwe	Zimbabwe	Heart	Peter van Coeverden de Groot
DBMIN12	CHIZ15	Hwange National Park, Zimbabwe	Zimbabwe	Heart	Peter van Coeverden de Groot
DBMIN13	CHIZ17	Hwange National Park, Zimbabwe	Zimbabwe	Liver	Peter van Coeverden de Groot
DBMIN14	CHIZ19	Hwange National Park, Zimbabwe	Zimbabwe	Heart	Peter van Coeverden de Groot
DBMIN15	MAT3	Hwange National Park, Zimbabwe	Zimbabwe	Plasma	Peter van Coeverden de Groot
DBMIN16	MAT4	Hwange National Park, Zimbabwe	Zimbabwe	Kidney	Peter van Coeverden de Groot
DBMIN17	MAT7	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN18	MAT16	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN19	MAT18	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN20	MAT19	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN21	MAT20	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN22	MAT21	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN23	SEN1	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN24	SEN3	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN25	SEN4	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN26	MIN9119	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN27	MIN9201	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN28	MIN9215	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN29	MIN9216	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN30	MIN9218	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN31	MIN9223	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN32	MIN9224	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN33	MIN9225	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN34	MIN9226	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN35	MIN9227	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN36	MIN9229	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN37	MIN9230	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN38	MIN9231	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN39	MIN9233	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN40	MIN9238	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN41	MIN9240	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN42	MIN9242	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN43	MIN9243	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN44	MIN9245	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN45	MIN9246	Hwange National Park, Zimbabwe	Zimbabwe	DNA	Peter van Coeverden de Groot
DBMIN46	HNP1	iMfolozi-Hluhluwe, South Africa	Natal, SA	DNA	Peter van Coeverden de Groot
DBMIN47	HNP2	iMfolozi-Hluhluwe, South Africa	Natal, SA	DNA	Peter van Coeverden de Groot
DBMIN48	HNP3	iMfolozi-Hluhluwe, South Africa	Natal, SA	DNA	Peter van Coeverden de Groot

Table AI.c: Details of eastern black rhinoceros (*Diceros bicornis michaeli*) samples. *Sample ID* refers to the number assigned to the sample during this study, *QUMEL ID* refers to unique laboratory identification and source population refers to the birth origin of the rhinoceros.

Sample ID	*QUMEL ID*	*Origin of Tissue Sample*	*Source Population*	*TISSUE TYPE*	*Collector*
DBMIC1	GEORGE	Busch Gardens Tampa Bay, FL USA	Kenya	Heart	Peter van Coeverden de Groot
DBMIC2	PETE	Denver Zoo, CO USA	Kenya	Blood, Plasma	Peter van Coeverden de Groot
DBMIC3	MIC3819	Detroit Zoo, MI USA	Kenya	Liver	Peter van Coeverden de Groot
DBMIC4	MIC3485	Granby Zoo, QUE Canada	Kenya	Liver	Peter van Coeverden de Groot
DBMIC5	MIC3530	Kansas City Zoo, KS USA	Kenya	Heart	Peter van Coeverden de Groot
DBMIC6	MIC3674	Los Angeles Zoo, CA USA	Kenya	Blood	Peter van Coeverden de Groot
DBMIC7	MIC101	Tsavo National Park, Kenya	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC8	MIC103	Berlin-West Zoo, Germany	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC9	MIC104	Tsavo National Park, Kenya	Kenya	Blood	Katherina Kellner, Petra Kretzschmar
DBMIC10	MIC105	Berlin-West Zoo, Germany	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC11	MIC106	Berlin-West Zoo, Germany	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC12	MIC107	Berlin-West Zoo, Germany	Berlin-West	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC13	MIC108	Tsavo National Park, Kenya	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC14	MIC109	Dvůr Králové, Czech Republic	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC15	MIC110	Cincinatti Zoo, OH USA	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC16	MIC111	Dvůr Králové, Czech Republic	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC17	MIC112	Dvůr Králové, Czech Republic	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC18	MIC113	Mosman Taronga Zoo, Australia	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC19	MIC114	Dvůr Králové, Czech Republic	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC20	MIC115	Dvůr Králové, Czech Republic	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC21	MIC116	Dvůr Králové, Czech Republic	Dvůr Králové	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC22	MIC117	Dvůr Králové, Czech Republic	Dvůr Králové	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC23	MIC118	Dvůr Králové, Czech Republic	Kenya	Organ	Katherina Kellner, Petra Kretzschmar
DBMIC24	MIC119	London Zoo, United Kingdom	Kenya	Ear Plug	Katherina Kellner, Petra Kretzschmar
DBMIC25	MIC120	Tsavo National Park, Kenya	Kenya	Placenta	Katherina Kellner, Petra Kretzschmar

Table AI.d: Details of southern white rhinoceros (*Ceratotherium simum simum*) samples. *Sample ID* refers to the number assigned to the sample during this study, *QUMEL ID* refers to unique laboratory identification and source population refers to the birth origin of the rhinoceros.

Sample ID	*QUMEL ID*	*Origin of Tissue Sample*	*Source Population*	*TISSUE TYPE*	*Collector*
WR1	Yeki	Metro Toronto Zoo (MTZ), Canada	MTZ	Skin, Blood	Graham Crawshawe
WR2	Bull	Metro Toronto Zoo (MTZ), Canada	Natal, SA	Skin, Blood	Graham Crawshawe
WR3	Shaboola	Metro Toronto Zoo (MTZ), Canada	MTZ	Skin, Blood	Graham Crawshawe
WR4	Pistol	Metro Toronto Zoo (MTZ), Canada	Natal, SA	Skin, Blood	Graham Crawshawe
WR5	Bender	Metro Toronto Zoo (MTZ), Canada	Natal, SA	Saliva	Graham Crawshawe
WR6	WR 001	iMfolozi-Hluhluwe, South Africa	Natal, SA	Blood	Katherina Kellner, Petra Kretzschmar
WR7	WR 002	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR8	WR 005	iMfolozi-Hluhluwe, South Africa	Natal, SA	DNA	Katherina Kellner, Petra Kretzschmar
WR9	WR 006	KwaZulu-Natal, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR10	WR 010	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR11	WR 011	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR12	WR 014	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR13	WR 015	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR14	WR 016	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR15	WR 022	iMfolozi-Hluhluwe, South Africa	Natal, SA	Blood	Katherina Kellner, Petra Kretzschmar
WR16	WR 023	KwaZulu-Natal, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR17	WR 028	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR18	WR 030	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR19	WE 031	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR20	WE 032	KwaZulu-Natal, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR21	WR 033	iMfolozi-Hluhluwe, South Africa	Natal, SA	Blood	Katherina Kellner, Petra Kretzschmar
WR22	WR 037	iMfolozi-Hluhluwe, South Africa	Natal, SA	Blood	Katherina Kellner, Petra Kretzschmar
WR23	WR 045	KwaZulu-Natal, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR24	WR 046	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR25	WR 047	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR26	WR 048	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR27	WR 049	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR28	WR 051	iMfolozi-Hluhluwe, South Africa	Natal, SA	Blood	Katherina Kellner, Petra Kretzschmar
WR29	WR 052	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR30	WR 057	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR31	WR 058	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR32	WR 061	iMfolozi-Hluhluwe, South Africa	Natal, SA	Blood	Katherina Kellner, Petra Kretzschmar
WR33	WR 062	iMfolozi-Hluhluwe, South Africa	Natal, SA	DNA	Katherina Kellner, Petra Kretzschmar
WR34	WR 063	iMfolozi-Hluhluwe, South Africa	Natal, SA	Ear Plug	Katherina Kellner, Petra Kretzschmar
WR35	17	Waterberg National Park, Namibia	Natal, SA	DNA, blood	Peter Erb
WR36	18	Waterberg National Park, Namibia	Natal, SA	Blood	Peter Erb
WR37	19	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR38	20	Waterberg National Park, Namibia	Natal, SA	Blood	Peter Erb
WR39	21	Waterberg National Park, Namibia	Natal, SA	Blood	Peter Erb
WR40	22	Waterberg National Park, Namibia	Natal, SA	Blood	Peter Erb
WR41	23	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR42	24	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR43	25	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR44	26	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR45	27	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR46	28	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR47	29	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR48	30	Waterberg National Park, Namibia	Natal, SA	DNA	Peter Erb
WR49	31	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR50	32	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR51	33	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR52	34	Waterberg National Park, Namibia	Natal, SA	Blood	Peter Erb
WR53	35	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR54	36	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR55	37	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR56	38	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR57	39	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR58	40	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb
WR59	41	Waterberg National Park, Namibia	Natal, SA	Ear Plug	Peter Erb

Table AI.e: Details of northern white rhinoceros (*Ceratotherium simum cottoni*) samples. *Sample ID* refers to the number assigned to the sample during this study, *QUMEL ID* refers to unique laboratory identification and source population refers to the birth origin of the rhinoceros.

Sample ID	*QUMEL ID*	*Origin of Tissue Sample*	*Source Population*	*TISSUE TYPE*	*Collector*
NWR1	90	Shambe, Sudan	Sudan	Ear Plug	Katherina Kellner, Petra Kretzschmar
NWR2	91	Shambe, Sudan	Sudan	Ear Plug	Katherina Kellner, Petra Kretzschmar
NWR3	92	Dvůr Králové, Czech Republic	Sudan	Blood	Katherina Kellner, Petra Kretzschmar
NWR4	93	Dvůr Králové, Czech Republic	Sudan	Ear Plug	Katherina Kellner, Petra Kretzschmar
NWR5	94	Dvůr Králové, Czech Republic	Dvůr Králové	Ear Plug	Katherina Kellner, Petra Kretzschmar
NWR6	Dinka	Henry Dourly Zoo, NB USA	Sudan	Blood	Peter van Coeverden de Groot

Table AI.f: Details of Indian rhinoceros (*Rhinoceros unicornis*) samples. *Sample ID* refers to the number assigned to the sample during this study, *QUMEL ID* refers to unique laboratory identification and source population refers to the birth origin of the rhinoceros.

Sample ID	*QUMEL ID*	*Origin of Tissue Sample*	*Source Population*	*TISSUE TYPE*	*Collector*
IR1	Patrick	Metro Toronto Zoo, ON Canada	India	Skin	Graham Crawshawe
IR2	Indira	Metro Toronto Zoo, ON Canada	India	Skin	Graham Crawshawe
IR3	CERC1	Kaziranga National Park, Assam, India	India	Blood	Prithiviraj Fernando
IR4	CERC2	Kaziranga National Park, Assam, India	India	Blood	Prithiviraj Fernando
IR5	301	Kaziranga National Park, Assam, India	India	Blood	Katherina Kellner, Petra Kretzschmar
IR6	302	Kaziranga National Park, Assam, India	India	Ear Plug	Katherina Kellner, Petra Kretzschmar
IR7	303	Kaziranga National Park, Assam, India	India	Ear Plug	Katherina Kellner, Petra Kretzschmar
IR8	304	Kaziranga National Park, Assam, India	India	Ear Plug	Katherina Kellner, Petra Kretzschmar
IR9	306	Kaziranga National Park, Assam, India	India	Ear Plug	Katherina Kellner, Petra Kretzschmar
IR10	4279	Los Angeles Zoo, CA USA	India	Heart	Prithiviraj Fernando
IR11	4288	Los Angeles Zoo, CA USA	India	Liver	Prithiviraj Fernando
IR12	5002	Royal Chitwan National Park, Nepal	Nepal	Blood	Prithiviraj Fernando
IR13	201	Royal Chitwan National Park, Nepal	Nepal	Blood	Prithiviraj Fernando
IR14	209	Royal Chitwan National Park, Nepal	Nepal	Plasma	Prithiviraj Fernando
IR15	218	Royal Chitwan National Park, Nepal	Nepal	Liver	Prithiviraj Fernando
IR16	219	Royal Chitwan National Park, Nepal	Nepal	DNA	Prithiviraj Fernando
IR17	220	Royal Chitwan National Park, Nepal	Nepal	DNA	Prithiviraj Fernando
IR18	221	Royal Chitwan National Park, Nepal	Nepal	DNA	Prithiviraj Fernando

Table AI.g: Details of western Sumatran rhinoceros (*Dicerorhinus sumatrensis sumatrensis*) samples. *Sample ID* refers to the number assigned to the sample during this study, *QUMEL ID* refers to unique laboratory identification and source population refers to the birth origin of the rhinoceros.

Sample ID	*QUMEL ID*	*Origin of Tissue Sample*	*Source Population*	*TISSUE TYPE*	*Collector*
SR1	Rapunzel	Bronx Zoo, NY USA	Malaysia	Blood	Terri Roth
SR2	Ipuh	Cincinatti Zoo and Botanical Garden, OH USA	Malaysia	Blood	Terri Roth
SR3	Emi	Cincinatti Zoo and Botanical Garden, OH USA	Malaysia	Blood	Terri Roth
SR4	Andalas	Cincinatti Zoo and Botanical Garden, OH USA	Cincinatti Zoo	Hair	Terri Roth
SR5	126	Way Kambas National Park, Indonesia	Indonesia	Blood	Juan-Carlos Morales
SR6	128	Way Kambas National Park, Indonesia	Indonesia	Blood	Juan-Carlos Morales
SR7	138	Way Kambas National Park, Indonesia	Indonesia	Blood	Juan-Carlos Morales
SR8	147-6	Poached Sample, Indonesia	Indonesia	Organ	Tom Foose, Nico van Strien
SR9	4270	Way Kambas National Park, Indonesia	Indonesia	Blood	Juan-Carlos Morales
SR10	4273	Way Kambas National Park, Indonesia	Indonesia	Blood	Juan-Carlos Morales
SR11	34965	Los Angeles Zoo, CA USA	Malaysia	Liver	Terri Roth
SR12	2	Way Kambas National Park, Indonesia	Indonesia	Blood	Muhammad Agil
SR13	21	Way Kambas National Park, Indonesia	Indonesia	Blood	Muhammad Agil
SR14	24	Way Kambas National Park, Indonesia	Indonesia	Blood	Muhammad Agil
SR15	25	Way Kambas National Park, Indonesia	Indonesia	Blood	Muhammad Agil
SR16	61	Sungai Dusun Rhinoceros Conservation Centre, Malaysia	Malaysia	Blood	Aidi Mohammad
SR17	62	Sungai Dusun Rhinoceros Conservation Centre, Malaysia	Malaysia	Blood	Aidi Mohammad
SR18	63	Sungai Dusun Rhinoceros Conservation Centre, Malaysia	Malaysia	Blood	Aidi Mohammad
SR19	64	Sungai Dusun Rhinoceros Conservation Centre, Malaysia	Malaysia	Blood	Aidi Mohammad
SR20	65	Sungai Dusun Rhinoceros Conservation Centre, Malaysia	Malaysia	Blood	Aidi Mohammad
SR21	66	Sungai Dusun Rhinoceros Conservation Centre, Malaysia	Malaysia	Blood	Aidi Mohammad
SR22	67	Sungai Dusun Rhinoceros Conservation Centre, Malaysia	Malaysia	Blood	Aidi Mohammad
SR23	Ratu	Way Kambas National Park, Indonesia	Indonesia	Blood	Muhammad Agil

Table AII: Origin and motif structure for all rhinoceros species-specific microsatellite loci screened for use in this study. *Origin* refers to the subspecies that each locus was derived from.

Origin	Locus	Motif
D. b. minor	BlRh1B	$(GA)_{10}$
D. b. minor	BlRh1C	$(GT)_{13}$
D. b. minor	BlRh2B	$(CA)_{19}$
D. b. minor	BlRh27A	$(CA)_{14}$
D. b. minor	BlRh11B	$(CA)_{16}$
D. b. minor	BlRhR11C	$(AC)_{18}(TC)_{17}$
D. b. minor	BlRh 14C	$(AC)_{22}$
D. b. minor	BlRh 14D	$(GA)_3g(GA)ag(GA)_{28}$
D. b. minor	BlRh 14E	$(TG)_{10}ca(TG)_4cg(TG)_5tc(TG)_4cn(TG)_{13}tc(TG)_3ac(TG)_{17}$
D. b. minor	BlRh37D	$(AG)_{17}$
D. b. minor	BR04*	$(CA)_{19}$
D. b. minor	BR06*	$(CA)_{15}$
D. b. minor	BR17*	$(AT)_6(GT)_{18}$
D. b. minor	DB01**	$(CA)_{14}$
D. b. minor	DB05**	$(AC)_{13}$
D. b. minor	DB23**	$(CA)_{12}$
D. b. minor	DB30**	$(AC)_{21}$
D. b. minor	DB 44**	$(CA)_4g(CA)_{16}$
D. b. minor	DB 52**	$(CA)_{21}$
D. b. minor	DB 66**	$(CA)_7ta(CA)_{16}$
C. s. simum	WR 4B	$(CA)_4cg(CA)_6ct(CA)_2ct(CA)_4ct(CA)_3cc(CA)_4ct(CA)_3$
C. s. simum	WR 6A	$(AG)_7aa(AG)_5ggagaa(AG)_7gg(AG)_{11}gg(AG)_2gg(AG)_5(GT)_6$
C. s. simum	WR 7B†	$(GT)_{20}$
C. s. simum	WR 7C†	$(CT)_{14}(AT)_{11}$
C. s. simum	WR 9A	$(AG)_2ac(AG)_{32}$
C. s. simum	WR 9D	$(CT)_{19}gc(CT)_4$ and $(TCTG)_8$
C. s. simum	WR 9E	$(CT)_{24}$
C. s. simum	WR 9F	$(GT)_6gcac(GT)_5g(GT)_{11}$
C. s. simum	WR 9H	$(AG)_{30}$
C. s. simum	WR 10A	$(TC)_{11}$
C. s. simum	WR 12A	$(CT)_{35}$
C. s. simum	WR 12C	$(TG)_{20}$
C. s. simum	WR 12E	$(TG)_{18}$
C. s. simum	WR 12F†	$(CA)_{14}$
C. s. simum	WR 12G	$(TC)_{12}tg(TC)_{12}tg(TC)tg(TC)_6$
C. s. simum	WR 12J	$(GA)_{10}ca(GA)_2ca(GA)_7$

C. s. simum	WR 32A†	$(CA)_{15}$
C. s. simum	WR 32E	$(GT)_{13}$
C. s. simum	WR 32F†	$(CA)_{17}$
C. s. simum	WR 34A	$(TG)_{20}$
C. s. simum	WR 35A	$(AC)_{20}$
R. unicornis	IR 10	$(CA)_{22}$
R. unicornis	IR 11 (90)	$(CA)_{16}$
R. unicornis	IR 12 (52)	$(CA)_{18}$
R. unicornis	IR 13 (111)	$(GT)_{15}$
R. unicornis	IR 14 (107)	$(GT)_{15}$
R. unicornis	IR 15 (40)	$(GT)_4tt(GT)_{21}$
R. unicornis	IR 22	$(CA)_{22}$
R. unicornis	IR 34	$(CA)_{24}$
R. unicornis	IR 121	$(CA)_{25}$
R. unicornis	IR 133	$(CA)_{21}$
R. unicornis	IR 135	$(GT)_{23}$
R. unicornis	IR 145	$(CA)_{24}$
R. unicornis	IR 151	$(TG)_{25}$
R. unicornis	IR 167	$(GT)_{19}$
R. unicornis	IR 181	$(CA)_{21}$
R. unicornis	IR 188	$(GT)_{25}$
R. unicornis	RH1◊	$(TG)_{13}$
R. unicornis	RH2◊	$(GT)_{36}$
R. unicornis	RH3◊	$(TC)_8tg(TC)_7cctg(TC)_4tg(TC)_{16}$
R. unicornis	RH4◊	$(AC)_{22}$
R. unicornis	RH5◊	$(TG)_{15}$
R. unicornis	RH8◊	$(TG)_{22}(AG)_2anaca(GA)_{28}ca(GA)_3ca(GA)_9cg(TA)_5$
R. unicornis	RH10◊	$(GT)_{24}(GC)_7$
R. unicornis	RH11◊	$(CA)_{23}$
D. s. sumatrensis	SR IIIA‡	$(GT)_{21}$
D. s. sumatrensis	SR IIIB‡	$(GT)_{22}ttcc(GT)_{15}ttcc(GT)_7$
D. s. sumatrensis	SR IVA	$(CA)_{24}$
D. s. sumatrensis	SR 54‡	$(CA)_{26}$
D. s. sumatrensis	SR 55	$(CA)_{25}$
D. s. sumatrensis	SR 63‡	$(AC)_{19}$
D. s. sumatrensis	SR 70	$(GT)_{17}$
D. s. sumatrensis	SR 71‡	$(CA)_{21}$
D. s. sumatrensis	SR 74‡	$(CA)_{19}$
D. s. sumatrensis	SR 191‡	$(CA)_{21}$
D. s. sumatrensis	SR 261‡	$(CT)_6(CA)_{22}$
D. s. sumatrensis	SR 262	$(TG)_{28}$

D. s. sumatrensis	SR 263	$(CA)_{21}$
D. s. sumatrensis	SR 267	$(CA)_{29}$
D. s. sumatrensis	SR 268	$(CA)_{25}$
D. s. sumatrensis	SR 275‡	$(CA)_{22}$
D. s. sumatrensis	SR 281‡	$(GT)_{23}$
D. s. sumatrensis	SR 283	$(TG)_{20}$

** Cunningham et al. 1999*

*** Brown and Houlden 1999*

† Florescu et al. 2003

‡ Scott et al. 2004

◊ Zschokke et al. 2003

Unmarked loci are unique to this study.

Table AIII: Primer sequences and annealing temperatures for microsatellite loci carried over from the first optimization step of the *standard* set of rhinoceros specific microsatellite loci. *Tm* refers to the optimal annealing temperature for the PCR reaction.

Locus	Forward Primer Sequence (5' – 3')	Reverse Primer Sequence (5' – 3')	*Tm* (˚C)
BlRh2B (M13F)	CCCTTTTCTCCCTTTATCTAG	ATACTGTGAAATCCTGTTCC	58
BlRh27A	CAACAAAGTGGGTATAGAGG	TGGCATCTTTTAAACCTGATC	58
BlRh11B	TCACAACAGCCAAGGTATGG	TAATGCCCTCCAGGATTGTC	62
BlRh37D (M13F)	CCACTCAGAATGAGAAATGG	TCTCCCTACTTAATCCCACC	58
BR04 -700IRD	CCCCTAAATTCTAGGAACAC	CCAAAGACCACCAGTAATTC	62
BR06 -800IRD	TCATTTCTTTGTTCCCCATAGCAC	AGCAATATCCCACGATATGTGAAGG	62
BR17 (M13F)	ACTAGCCCTCCTTTCATCAG	GCATATTGTAAGTGCCCCAG	62
DB01 -700IRD	AGATAATAATAGGACCCTGCTCCC	GAGGGTTTATTGTGAATGAGGC	62
DB 44 -800IRD	GGTGGAATGTCAAGTAGCGG	CTTGTTGCCCCATCCCTG	62
DB 52 (M13F)	CATGTGAAATGGACCGTCAGG	ATTTCTGGGAAGGGGCAGG	58
DB 66 (M13F)	CCAGGTGAAGGGTCTTATTATTAGC	GGATTGGCATGGATGTTACC	55
WR 7B	CCTCTGTGATTAAGCAAGGC	ATGAACAGGAAGGAAGACGC	62
WR 7C	AAACAGGTCTTGATTAGTGC	TGAACTCTGATGGAAATGAG	62
WR 10A	CCTGGGTCTTGCTTCTAACC	CTAGAGGCAATCCACGGTTC	62
WR 12A	TCAAGACTCTAGTGGGATAAACAC	AGGGAAGCAGGTTCTGGAGT	62
WR 12F (M13F)	AAGCAGCCAGCCTAGGACAC	GGAGCTCTCCCATATGGTCG	62
WR 32A (M13F)	CAGTCCTGCTGCATAAATCTC	GCAGTACAGCTAGAATCACC	62
WR 32E	TCACAACAGCCAAGGTATGG	TAATGCCCTCCAGGATTGTC	62
WR 32F	CTGGAAATGGAAACCCGAAC	GCAACATCCATCGGACTGTC	62
WR 34A	CCAAATACTAAGGGAATCACC	GTTTAGTATATACCCTGGTGG	62
WR 35A	AGCCTGCTTTGCTGCCTTGC	AGGTGCACACATCCCACTCG	62
IR 10	CAGTGAGGAAGATTGGTTGC	CCTGACTCACACATCACCAG	62
IR 11 (90)	CATCCATCACCTCACATAGTTAC	GCATGGCGACTACGATTAAC	62
IR 12 (52)	GAATGCTGATCATTTAGTGAC	GGGTCCAGTTGAGATATCAC	62
IR 13 (111)	TGGGTACACTGGGTGACTG	CAGGTAGAAGGAATTACAACCC	62
IR 14 (107)	CCTAGTAGTCAACGGCAAGG	TGGACTCTTGCATAGGCTCC	62
IR 15 (40)	GCCAGGTCTTTGTTGGTCTC	GTTCATCACGCGGTGTTAAC	62
IR 22	ATGGTGGAAGAAGTGCAGCC	ACTTCTGTGTCTCTAGCGCC	62
IR 121	ATGTGAGGGGGACTAAGAGG	AATGCAATGCTGCCCAGAC	62
IR 181	TTCCTGATTGACAGCAGAAGG	CACCTGACTCACACATCACC	62
IR 188	CAATGCAATGCTGCCCAGAC	ACCTCTTTCACACAGACCCG	62
RH2 (M13F)	GACTTCAAACTTCGCAGCAGCAATC	GCCCTAGACCTGGAAATAACC	62
RH3 (M13F)	TGTGTGGAGCACATCAGTCTTC	CCAGGGACCCGTGAGGAT	58
RH4 (M13F)	CAAAATGTGGGTTTTGTGAGC	GACGAGCTTTGTTTGAATGC	58
RH5 (M13F)	CCCATTAGAGGCTGTAGAGTAATATC	GGACTCTAAACTCCAGGGTCAC	58
SR IIIA	GGCGAAAGGTAAGAGCAGC	GCTTCTTTCCGAGGATCTGG	62

SR IIIB	GCCAGCCACCTTCCTCAATG	TTCATAGACGACGAATGCCTATG	62
SR IVA	GTGGAAACAACTTAAGTGTCC	GCATAATGCCTTCAAGGTCC	62
SR 54	CAATATCCAGGCTTCCAGG	CTGTTTACTGTTATCGATGCTC	62
SR 55	TCGTGGTGATGGATGCAGAC	TGAGCCAGTGATGTGAGGAG	62
SR 63	CTTGAGCAGAGTAGAATTTGG	CTCTGTATCCACCTCATTCC	62
SR 70	CTCTGTATCCACCTCATTCC	CTTGAGCAGAGTAGAATTTGC	62
SR 71	ATCATCTCTCTCACACAGACC	CAACGCTGCACAGACTTCAC	62
SR 74	CAGCACAATGTTTGGCACTTG	TTGGAGTCTTATGTCACCACC	62
SR 191	TGTAATGTAAAGCACAGTGAC	GACGTGTATATTGCAAAGTG	62
SR 261	CTGCTGGCCTGTAGATTGC	CTCCCTGAGCAGTAACTATCC	62
SR 262 (M13F)	CTGCCTTAACAACTGAACTGC	TGGAGGTTATCTCATGCCAC	62
SR 263	GCATCTAGTGTCCAGCATGG	CCATCACCTCGCATAGTTACC	62
SR 267	TGTTCTCCAGCATCAGCAGG	CAGTCACATTGAGGGTCAGG	62
SR 268	GTTTATACTATGCCCTGCAC	GGATGCTACCGAATAGATTG	62
SR 275	GGACTTAGAACCAGGCAATC	GTCTTGATGCCTGCATTCTG	62
SR 281	AGGTGATTAGGGAATTGCTGG	TTCTTCTGTCCTGGCATTGC	62

(M13F) is the addition of the sequence 5'- CACGACGTTGTAAAACGAGC -3' to the beginning of the locus specific forward primer.

700IRD are locus specific forward primers tagged with the Licor IRD 700 fluorescent dye

800IRD are locus specific forward primers tagged with the Licor IRD 800 fluorescent dye

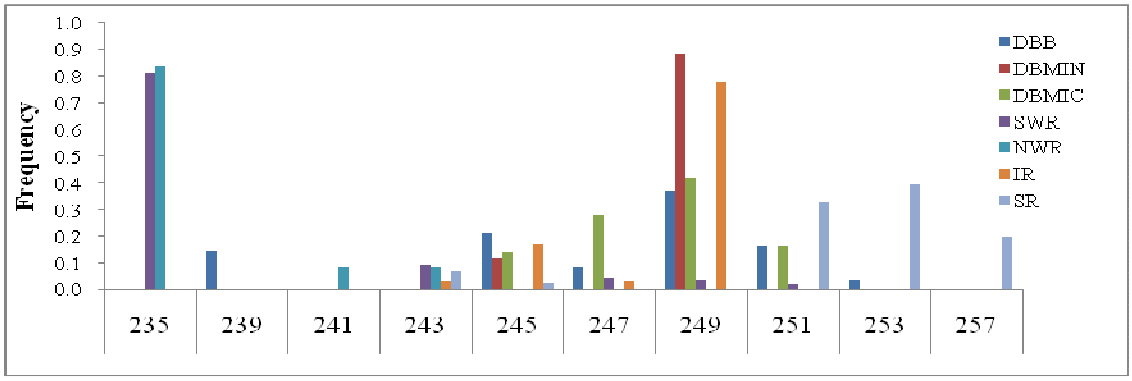
Table AIV.a: Raw genotype data for known white rhinoceros pedigree for *standard* set of microsatellite loci. All genotypes are by locus and measured in base pairs.

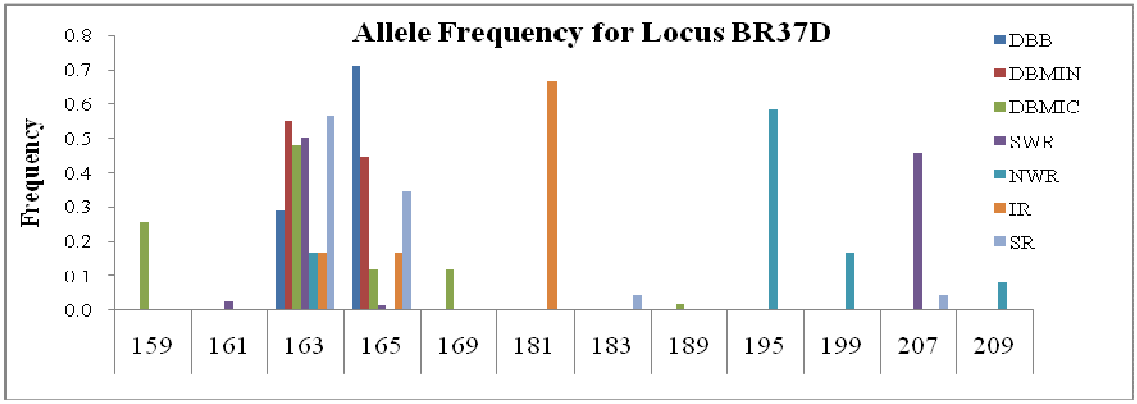
		Loci and Genotypes											
Sample		**BlRh2B**		**BlRh37D**		**BR06**		**DB44**		**DB01**		**DB52**	
Bull	**Sire**	235	235	207	163	140	132	182	182	128	128	236	236
Pistol	**Dam**	243	235	207	163	140	132	182	182	128	128	238	236
Shaboola	**Calf**	235	235	207	163	140	132	182	182	128	128	238	236
		WR7B		**WR7C**		**WR12F**		**WR32A**		**WR32F**		**WR35A**	
Bull	**Sire**	268	268	256	254	265	251	234	232	242	232	156	156
Pistol	**Dam**	268	266	254	254	251	251	234	232	242	228	156	156
Shaboola	**Calf**	268	266	254	254	251	251	234	232	242	228	156	156
		IRRH3		**IRRH5**		**IR10**		**IR12**		**IR14**		**IR22**	
Bull	**Sire**	207	207	213	213	202	202	175	175	222	222	187	187
Pistol	**Dam**	207	205	213	213	202	200	175	175	222	222	187	187
Shaboola	**Calf**	207	207	213	213	202	200	175	175	222	222	187	187
		SR54		**SR63**		**SR74**		**SR262**		**SR268**		**SR281**	
Bull	**Sire**	161	159	189	189	172	172	104	104	181	181	224	212
Pistol	**Dam**	161	159	191	189	172	172	104	104	181	181	224	212
Shaboola	**Calf**	161	159	191	189	172	172	104	104	181	181	224	212

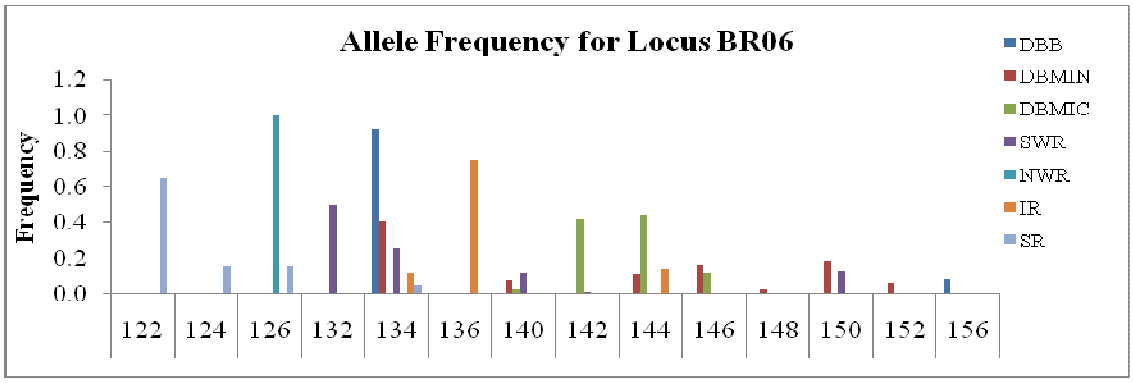
Table AIV.b: Raw genotype data for known Sumatran rhino pedigree for *standard* set of microsatellite loci. All genotypes are by locus and measured in base pairs.

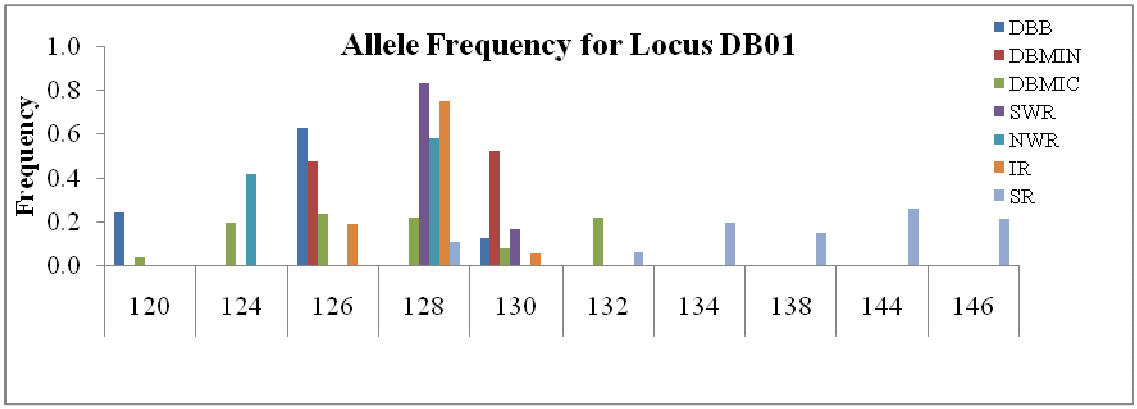
		Loci and Genotypes											
Sample		**BlRh2B**		**BlRh37D**		**BR06**		**DB44**		**DB01**		**DB52**	
Ipuh	**Sire**	257	251	165	163	126	122	194	194	146	146	234	234
Emi	**Dam**	257	251	165	163	126	122	196	196	138	134	234	228
Andalas	**Calf**	257	251	165	163	126	122	196	194	146	138	234	228
		WR7B		**WR7C**		**WR12F**		**WR32A**		**WR32F**		**WR35A**	
Ipuh	**Sire**	268	268	264	264	245	245	226	226	328	242	154	154
Emi	**Dam**	268	268	264	264	245	245	236	226	326	242	150	150
Andalas	**Calf**	268	268	264	264	245	245	226	226	326	242	154	150
		IRRH3		**IRRH5**		**IR10**		**IR12**		**IR14**		**IR22**	
Ipuh	**Sire**	213	213	209	203	200	200	195	193	224	224	203	197
Emi	**Dam**	213	197	203	203	200	200	195	193	224	224	211	197
Andalas	**Calf**	213	213	203	203	200	200	193	193	224	224	197	197
		SR54		**SR63**		**SR74**		**SR262**		**SR268**		**SR281**	
Ipuh	**Sire**	189	189	201	201	172	172	128	114	201	201	228	228
Emi	**Dam**	189	187	201	199	176	172	128	128	201	201	228	228
Andalas	**Calf**	189	189	201	199	176	172	128	128	201	201	228	228

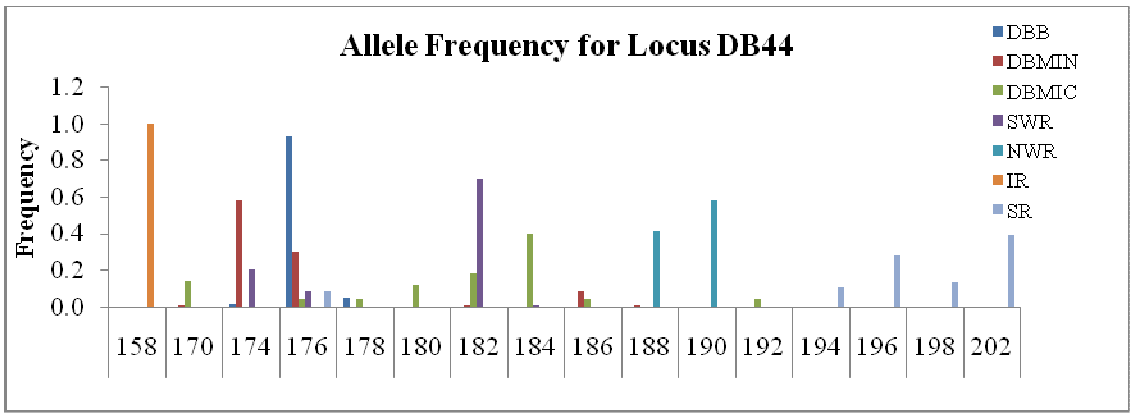
Allele frequencies by locus and species. X-axis shows allele size in base pairs. Black rhinos: DBB = *bicornis*, DBMIN = *minor*, DBMIC = *michaeli*; White rhinos: SWR = Southern white, NWR = Northern white; IR = Indian and SR = Sumatran rhinos.










Allele Frequency for Locus DB44
Frequency
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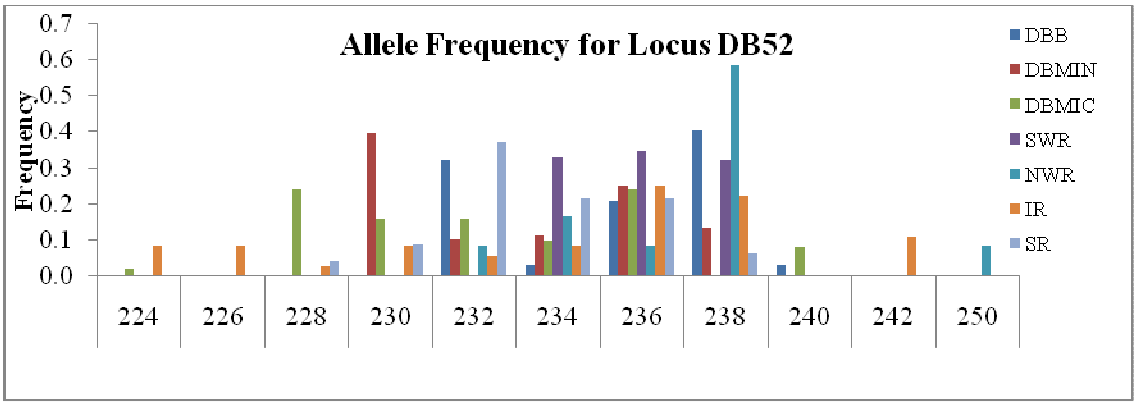
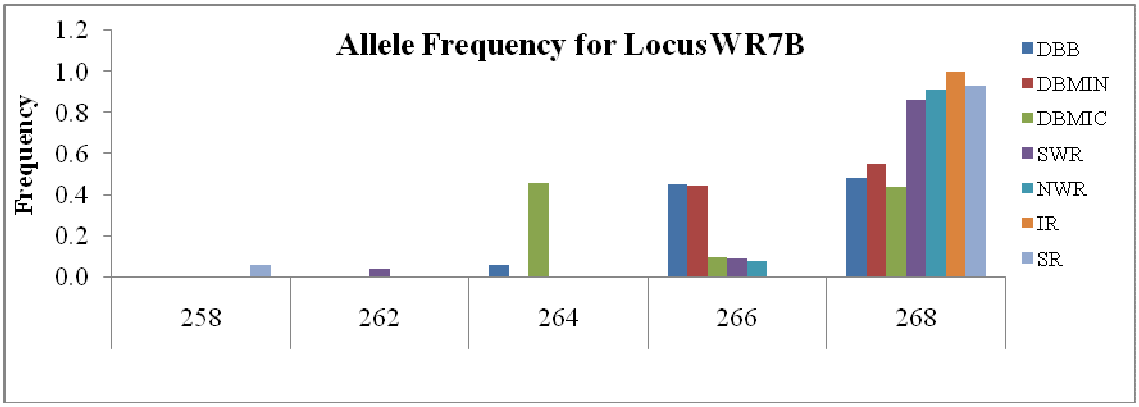
1.0
0.8
0.6
0.4
0.2
0.0
158 170 174 176 178 180 182 184 186 188 190 192 194 196 198 202
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR


Allele Frequency for Locus DB52
Frequency
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
224 226 228 230 232 234 236 238 240 242 250
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR


Allele Frequency for LocusWR7B
Frequency
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1.0
0.8
0.6
0.4
0.2
0.0
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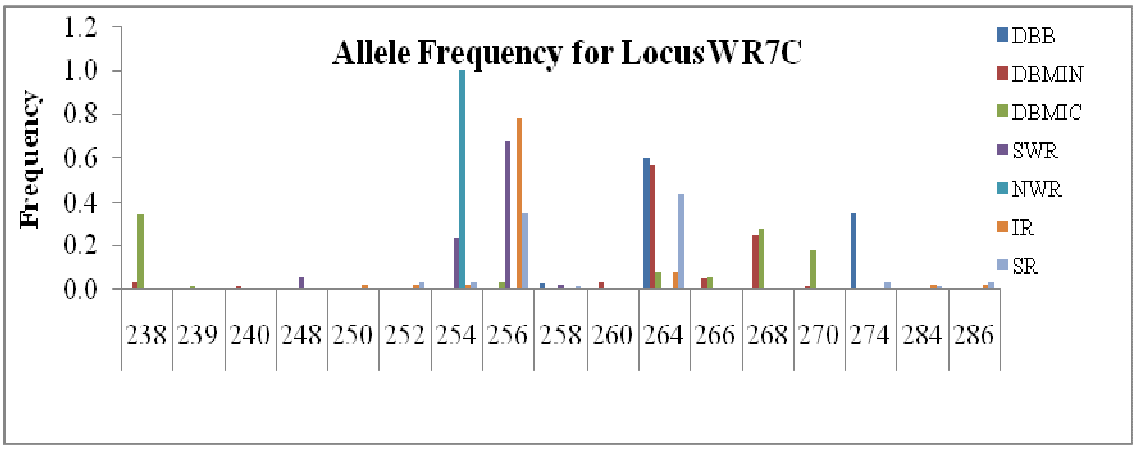
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR


Allele Frequency for LocusWR7C
Frequency
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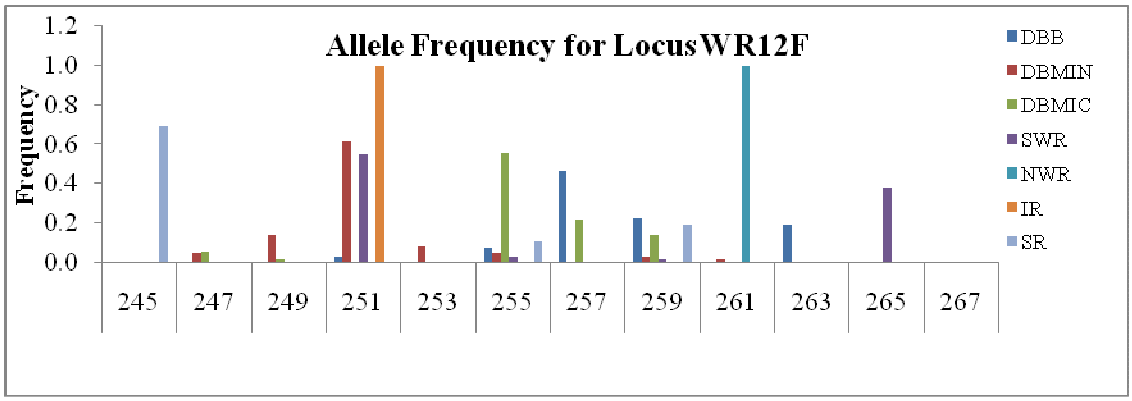
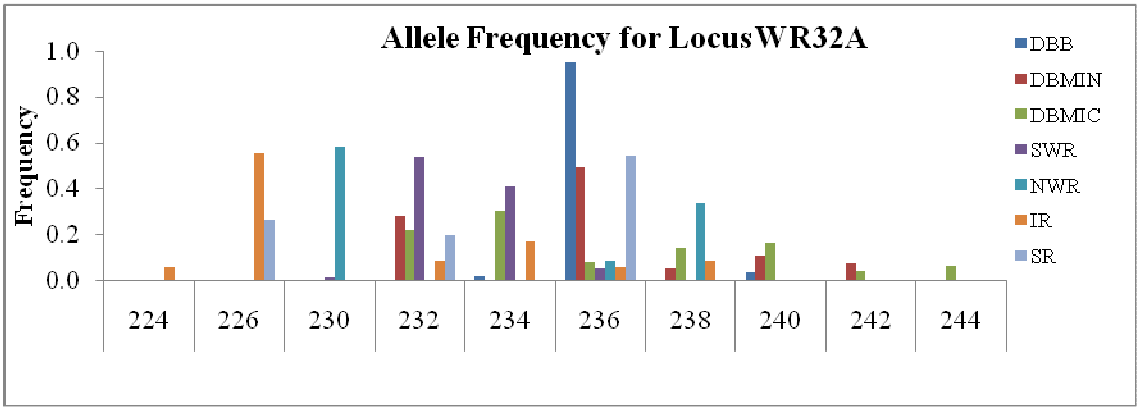
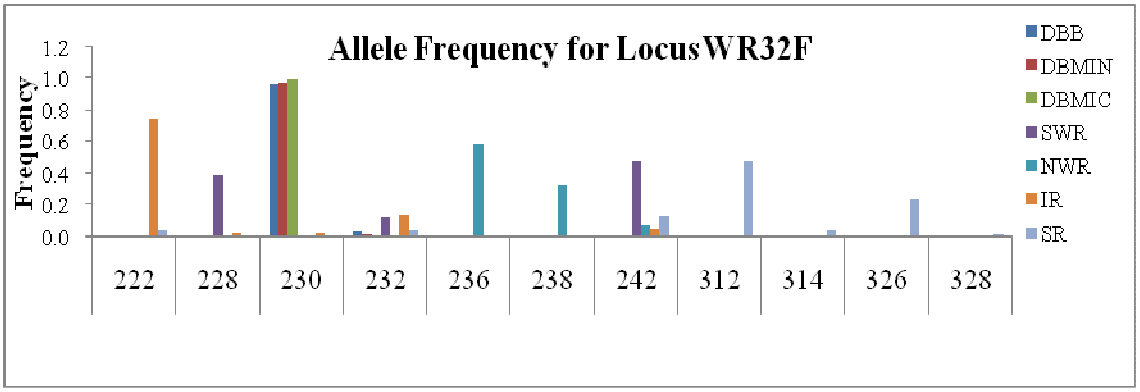
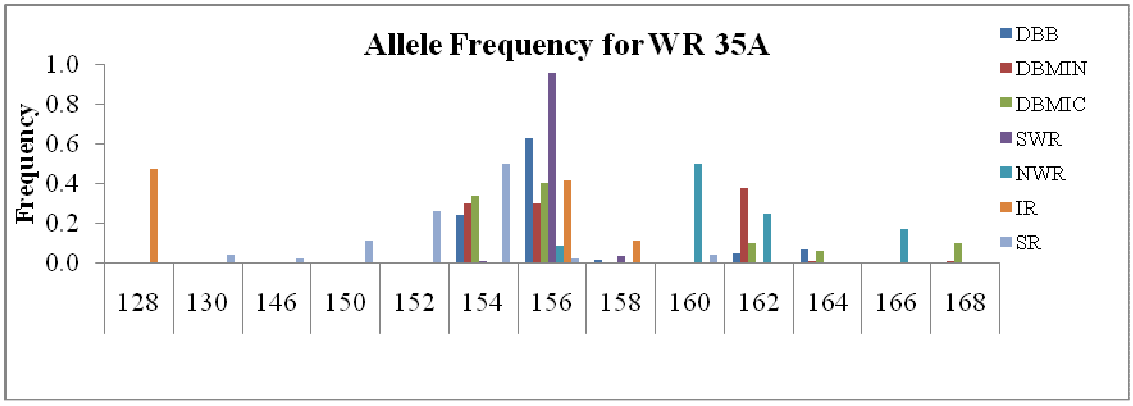
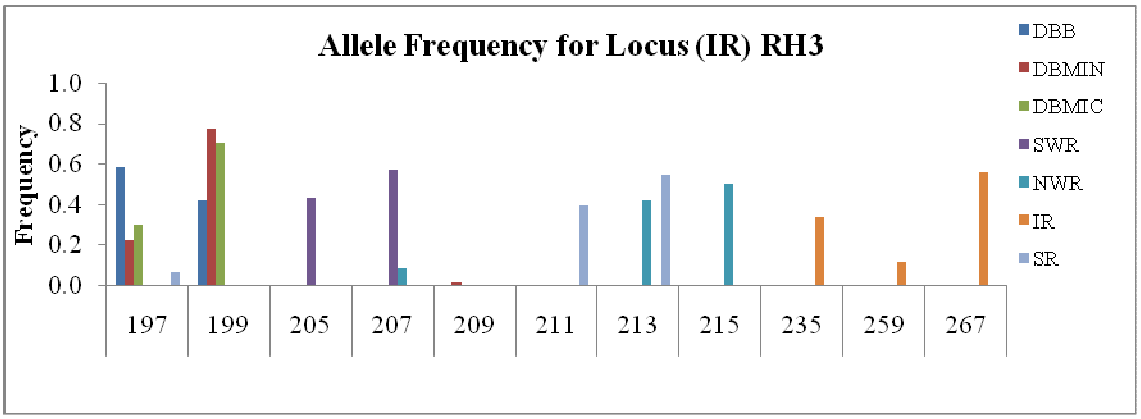
1.0
0.8
0.6
0.4
0.2
0.0
238 239 240 248 250 252 254 256 258 260 264 266 268 270 274 284 286
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

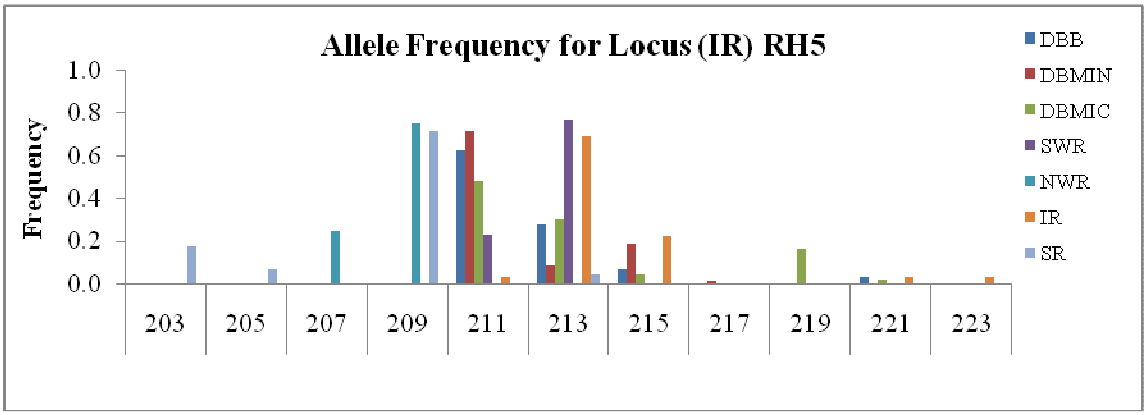

Allele Frequency for LocusWR12F
Frequency
1.2
1.0
0.8
0.6
0.4
0.2
0.0
245
247
249
251
253
255
257
259
261
263
265
267
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

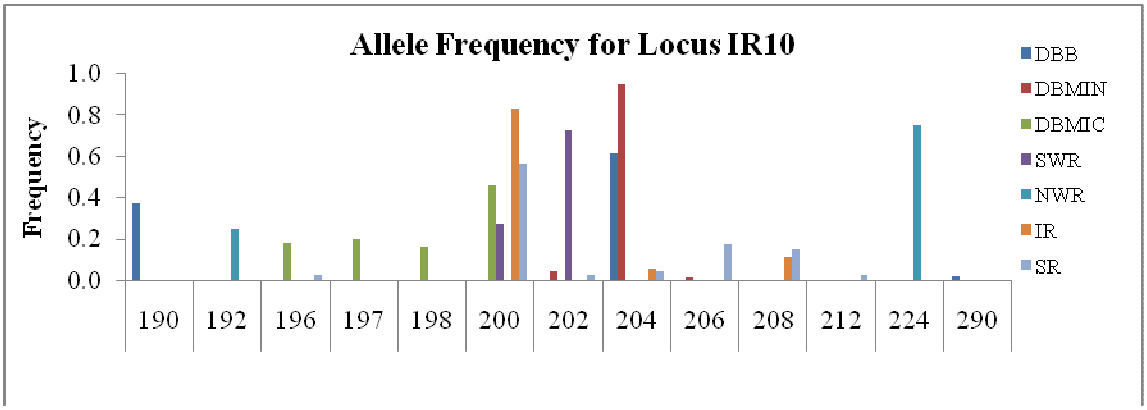

Allele Frequency for LocusWR32A
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
224
226
230
232
234
236
238
240
242
244
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

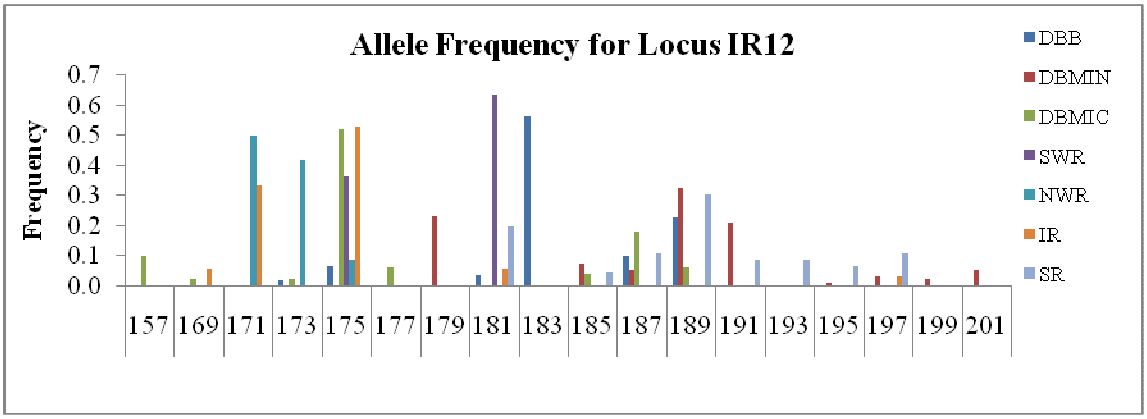

Allele Frequency for LocusWR32F
Frequency
1.2
1.0
0.8
0.6
0.4
0.2
0.0
222
228
230
232
236
238
242
312
314
326
328
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

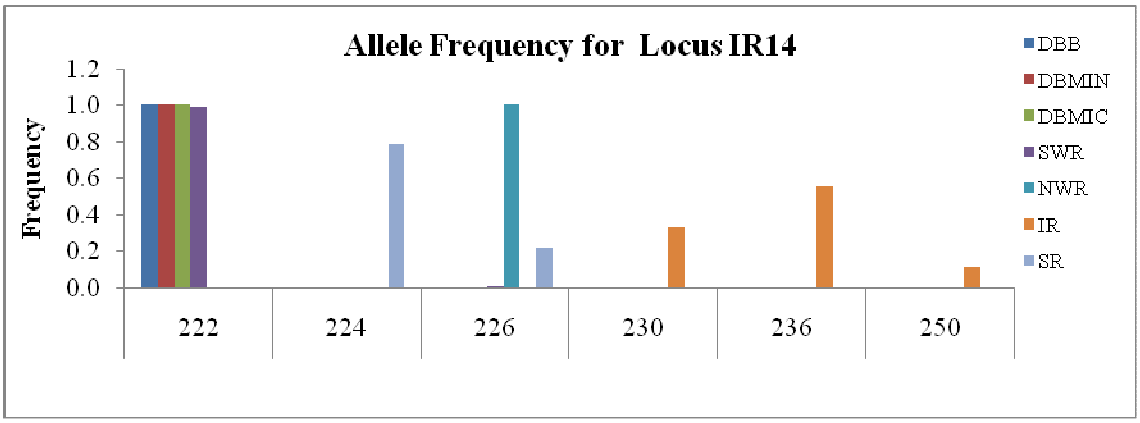

Allele Frequency for WR 35A
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
128
130
146
150
152
154
156
158
160
162
164
166
168
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

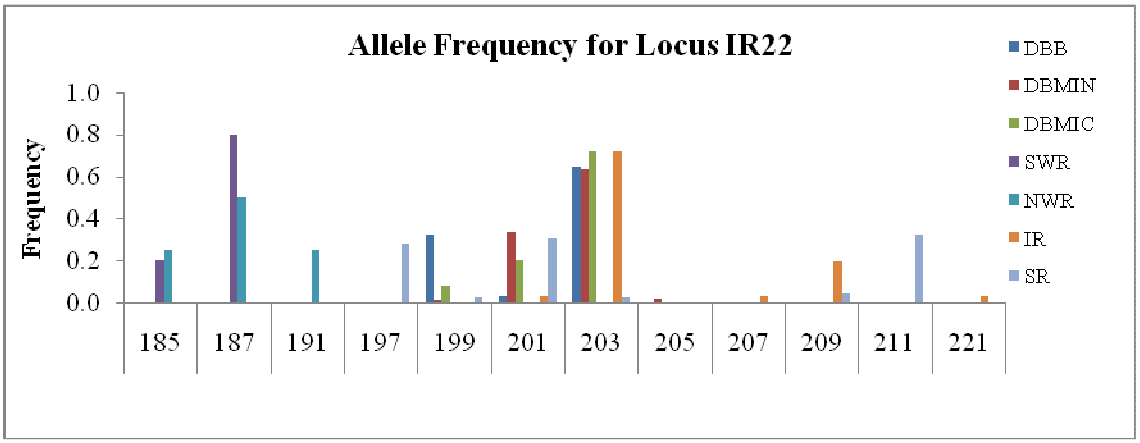

Allele Frequency for Locus (IR) RH3
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
197
199
205
207
209
211
213
215
235
259
267
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

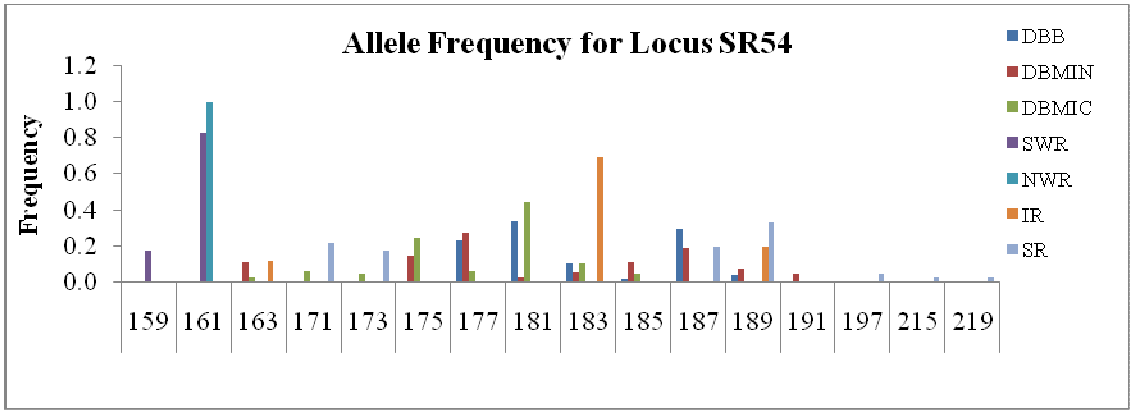

Allele Frequency for Locus (IR) RH5
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
203
205
207
209
211
213
215
217
219
221
223
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

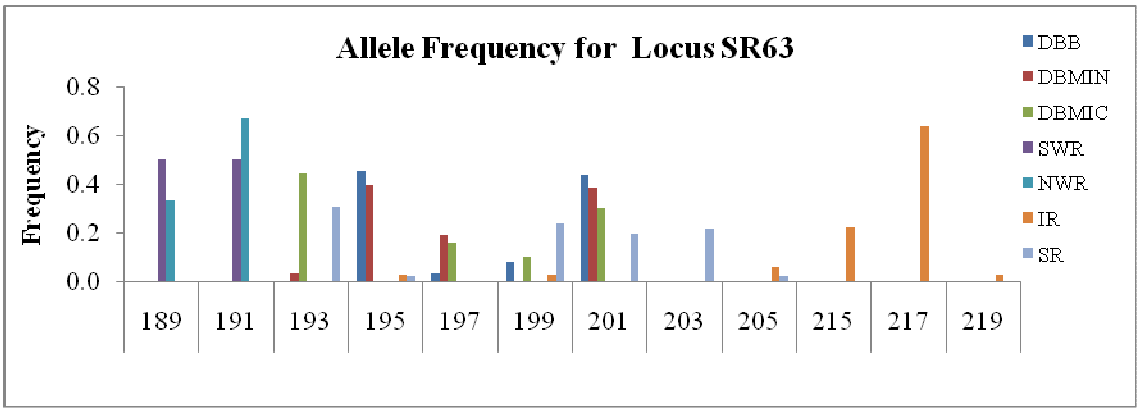

Allele Frequency for Locus IR10
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
190
192
196
197
198
200
202
204
206
208
212
224
290
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

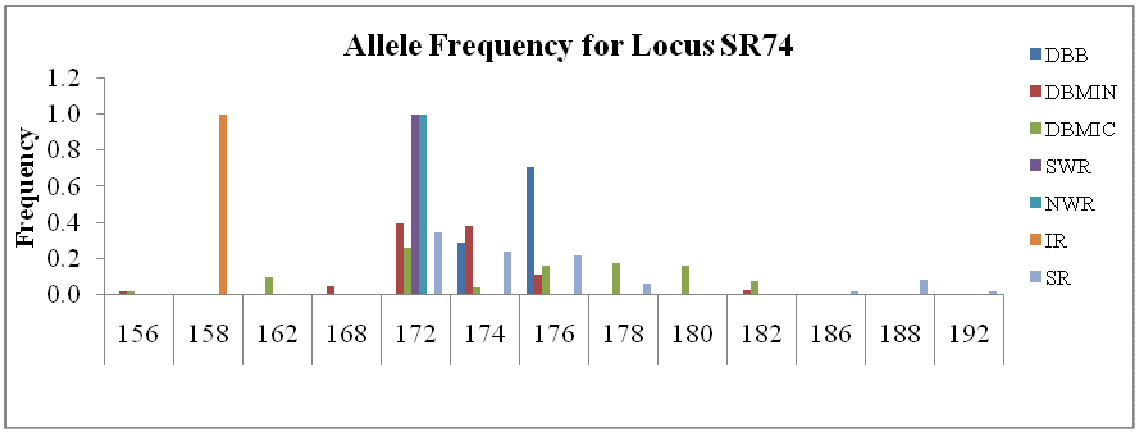

Allele Frequency for Locus IR12
Frequency
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
157
169
171
173
175
177
179
181
183
185
187
189
191
193
195
197
199
201
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

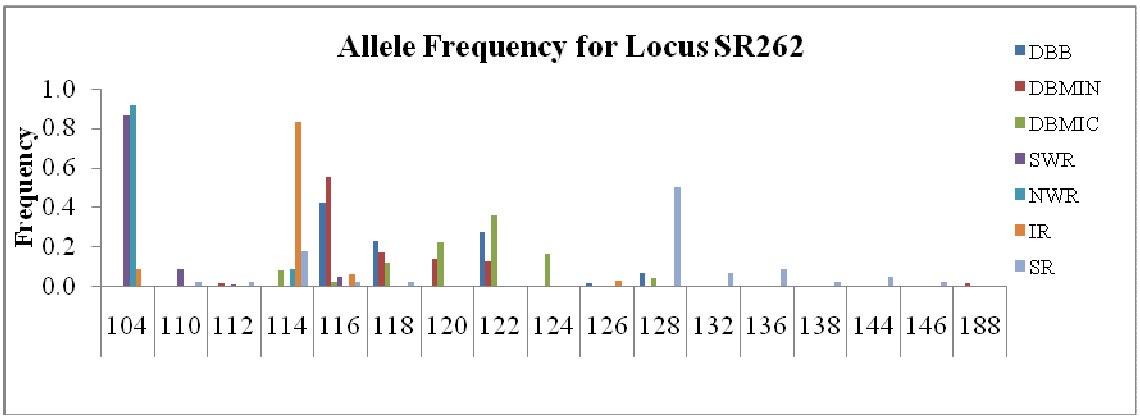

Allele Frequency for Locus IR14
Frequency
1.2
1.0
0.8
0.6
0.4
0.2
0.0
222
224
226
230
236
250
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

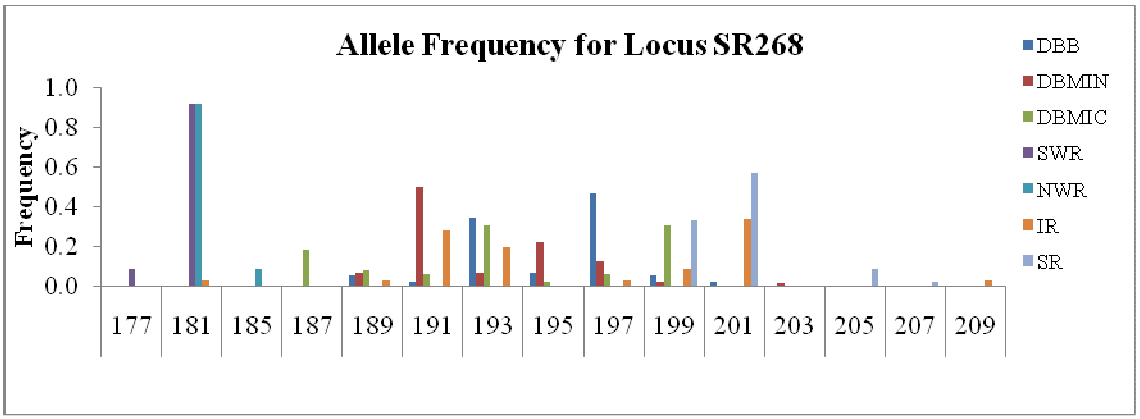

Allele Frequency for Locus IR22
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
185
187
191
197
199
201
203
205
207
209
211
221
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

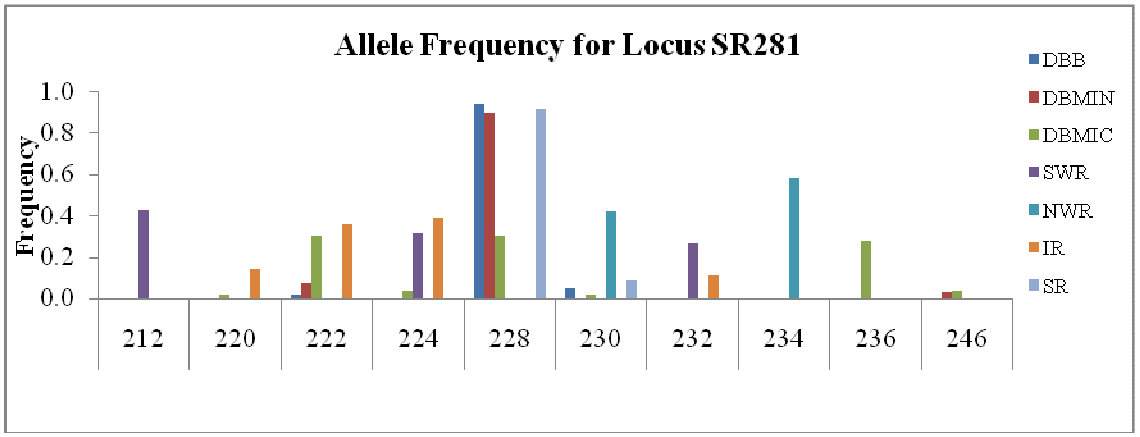

Allele Frequency for Locus SR54
Frequency
1.2
1.0
0.8
0.6
0.4
0.2
0.0
159
161
163
171
173
175
177
181
183
185
187
189
191
197
215
219
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR


Allele Frequency for Locus SR63
Frequency
0.8
0.6
0.4
0.2
0.0
189
191
193
195
197
199
201
203
205
215
217
219
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

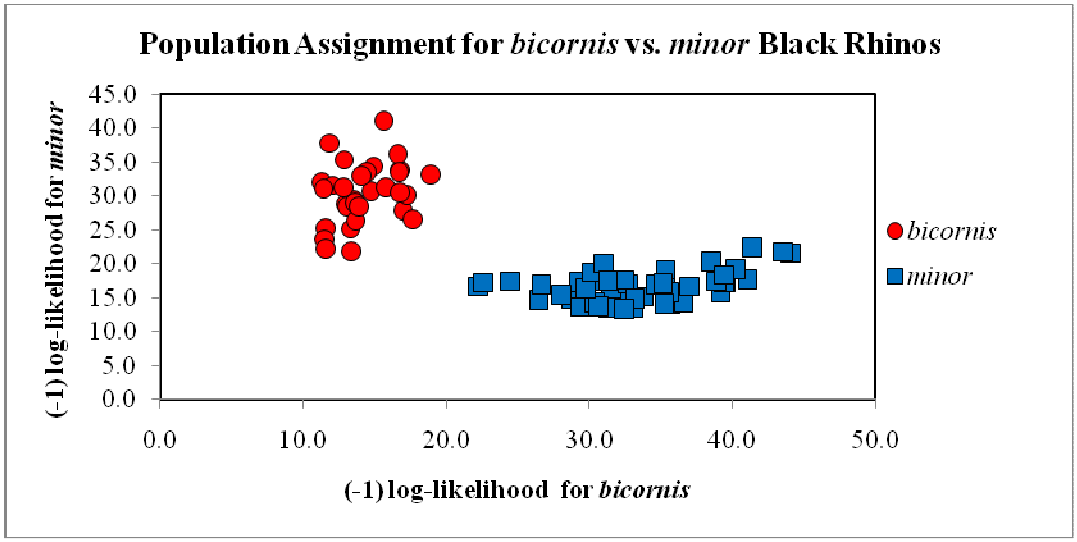

Allele Frequency for Locus SR74
Frequency
1.2
1.0
0.8
0.6
0.4
0.2
0.0
156 158 162 168 172 174 176 178 180 182 186 188 192
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

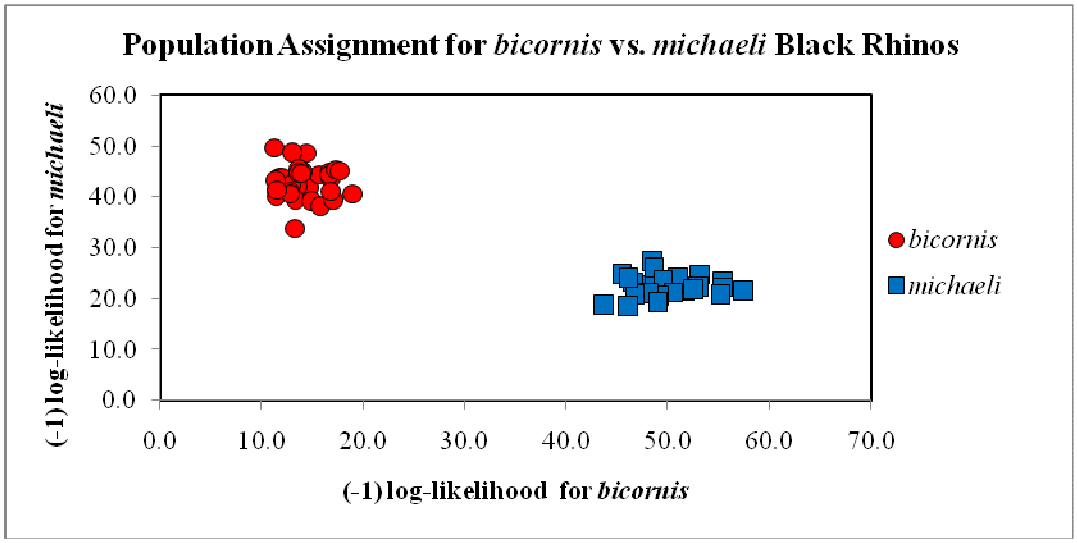

Allele Frequency for Locus SR262
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
104 110 112 114 116 118 120 122 124 126 128 132 136 138 144 146 188
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

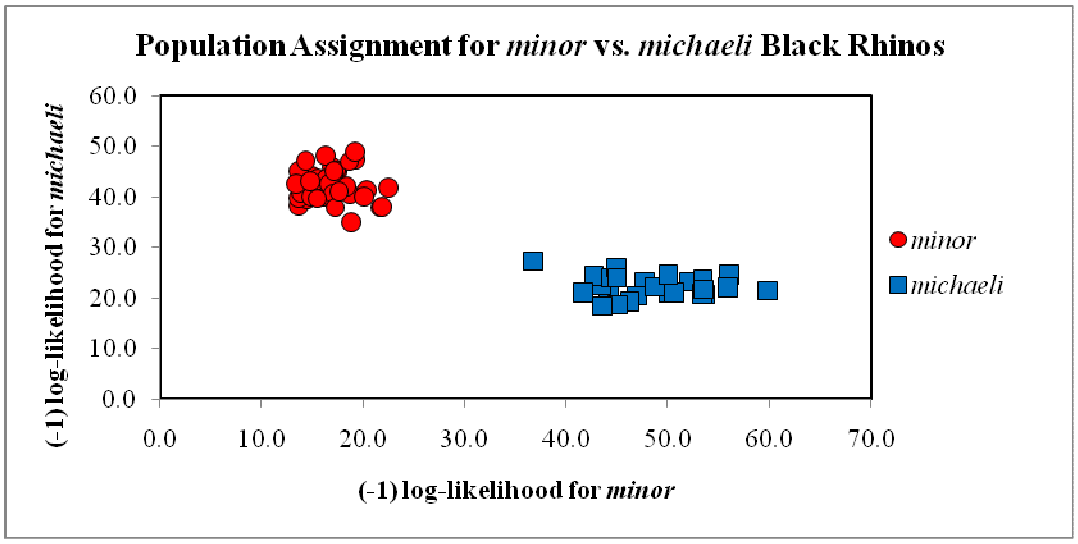

Allele Frequency for Locus SR268
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
177 181 185 187 189 191 193 195 197 199 201 203 205 207 209
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

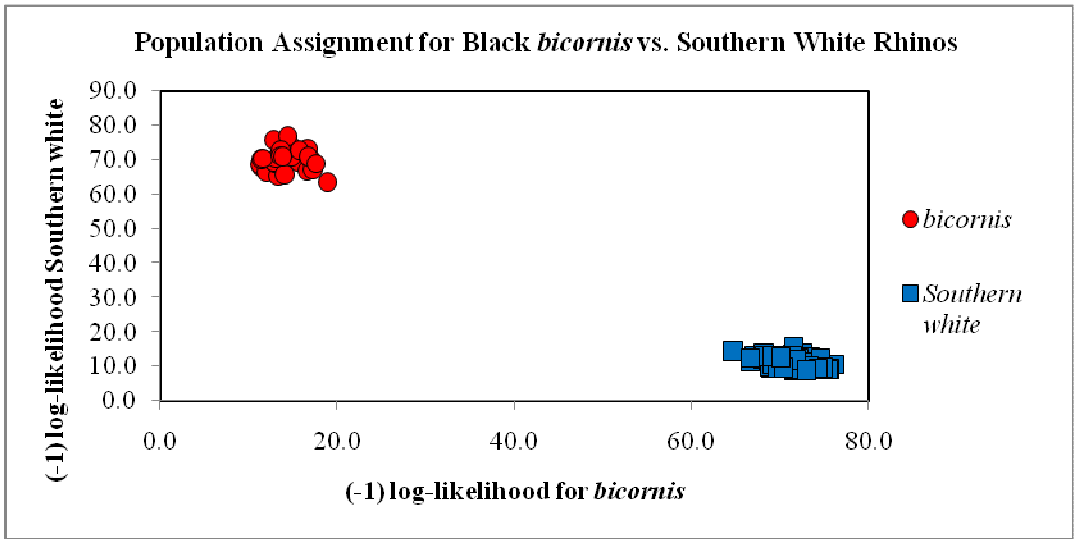

Allele Frequency for Locus SR281
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
212 220 222 224 228 230 232 234 236 246
DBB
DBMIN
DBMIC
SWR
NWR
IR
SR

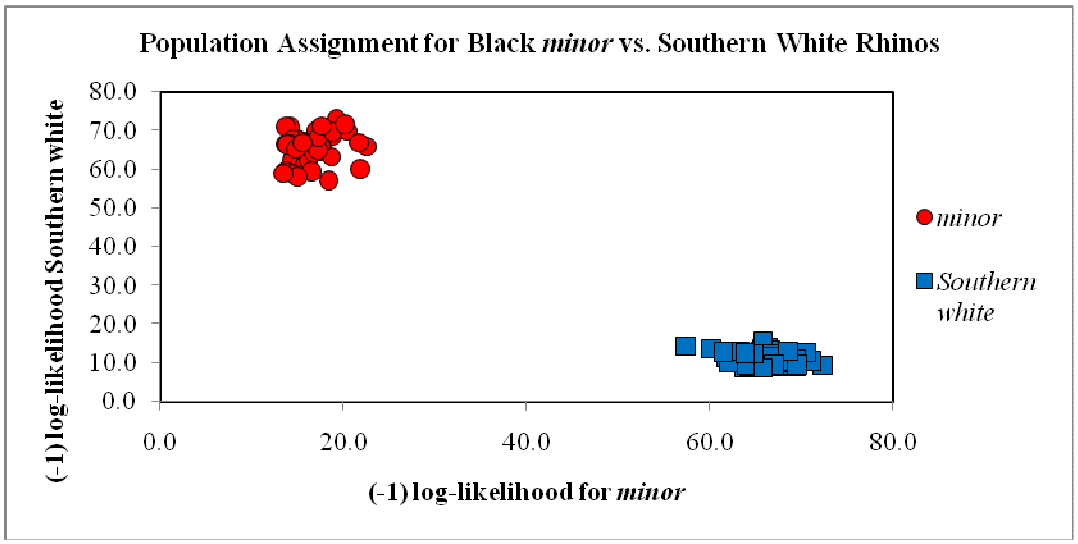
Frequency based population assignments (Paetkau *et al.* 1997) for each pair of available rhino taxa based on the *standard* set of rhino microsatellite loci (*n = 24*). Expected genotypes are calculated for each locus (assuming random mating), multiplied across all loci, log transformed to give a log-likelihood value. Axis values represent positive log-likelihood values (negative log-likelihood multiplied by -1). Individuals are assigned to a population with the highest negative log-likelihood value.

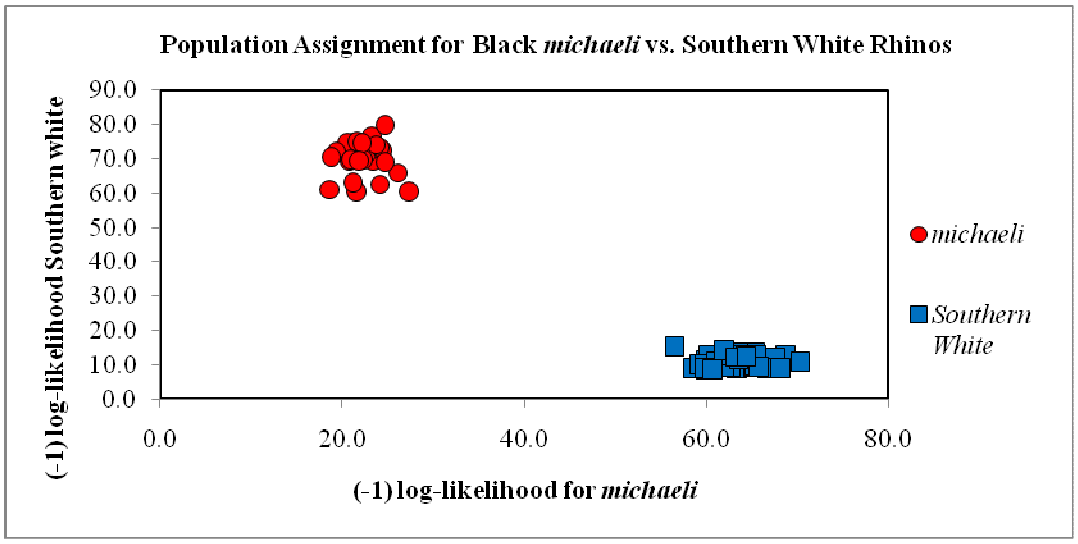


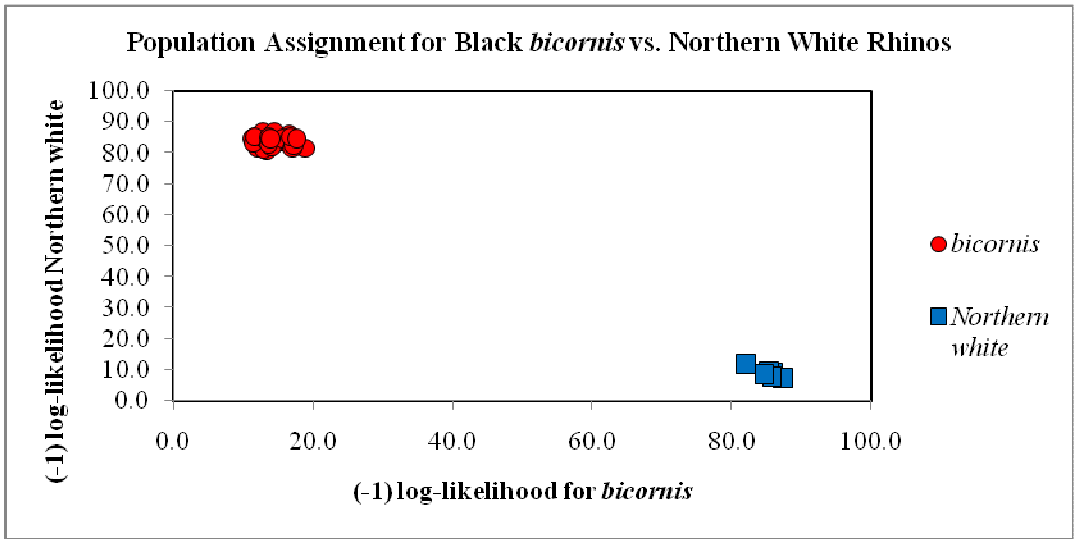


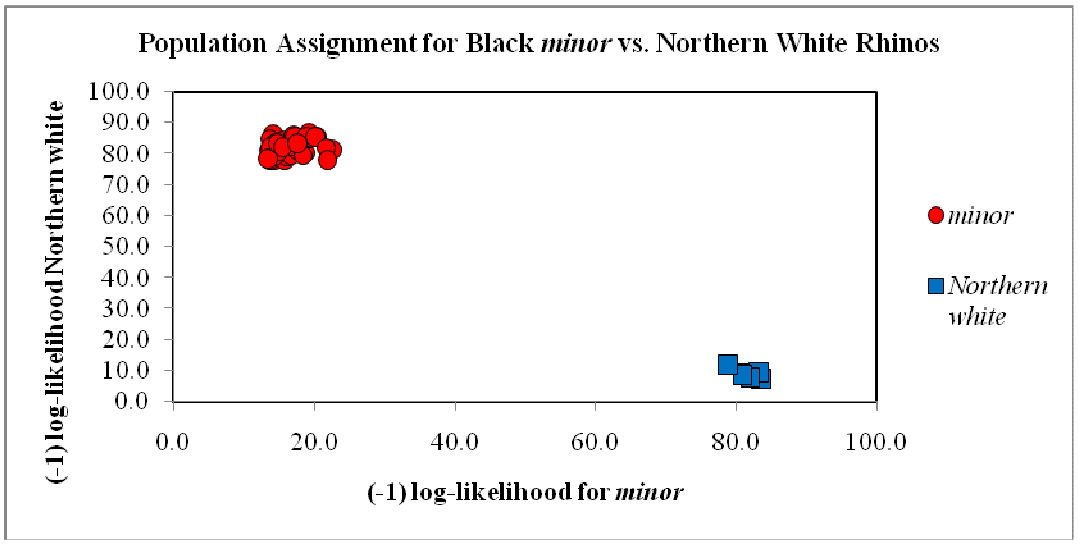

Population Assignment for *minor* vs. *michaeli* Black Rhinos
(-1) log-likelihood for *michaeli*
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
(-1) log-likelihood for *minor*
minor
michaeli

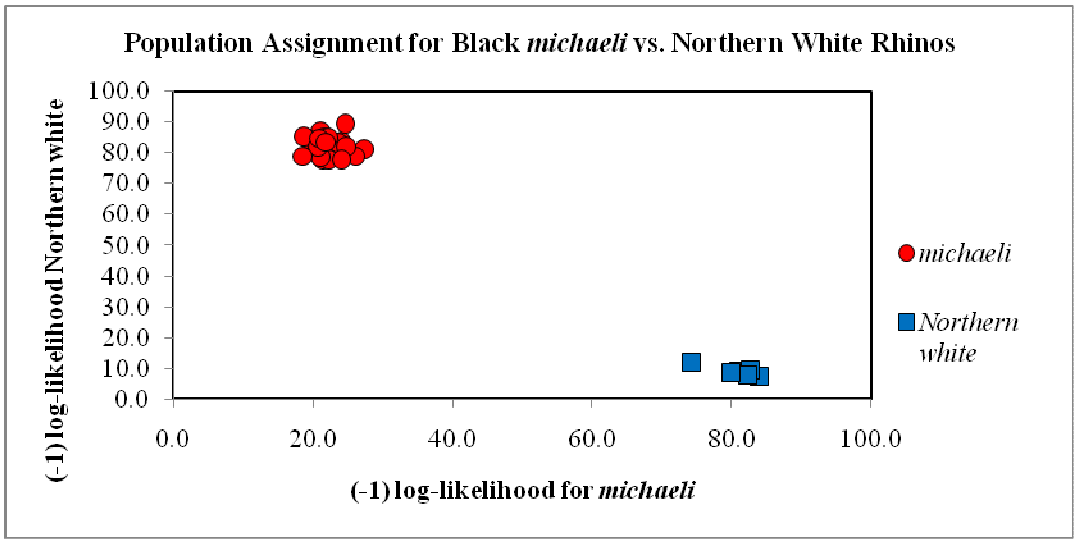

Population Assignment for Black *bicornis* vs. Southern White Rhinos
(-1) log-likelihood Southern white
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
(-1) log-likelihood for *bicornis*
bicornis
Southern white

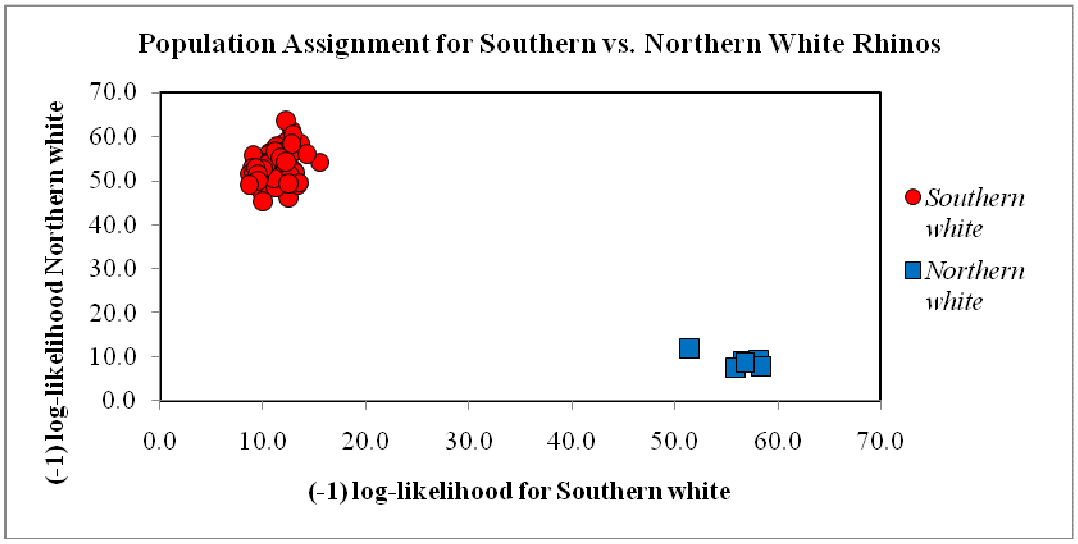

Population Assignment for Black *minor* vs. Southern White Rhinos
(-1) log-likelihood Southern white
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
(-1) log-likelihood for *minor*
minor
Southern white

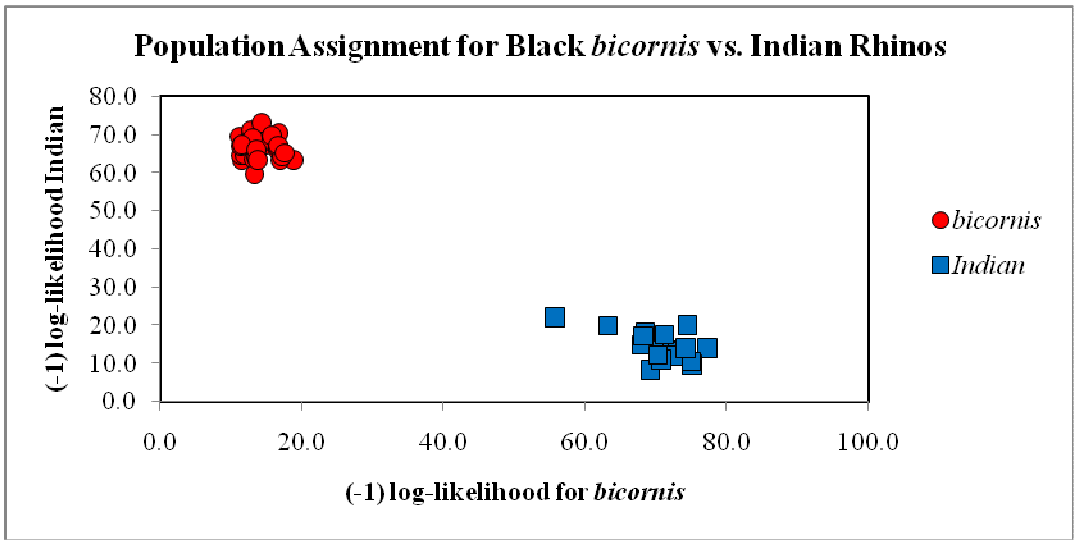

Population Assignment for Black *michaeli* vs. Southern White Rhinos
(-1) log-likelihood Southern white
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
(-1) log-likelihood for *michaeli*
michaeli
Southern White

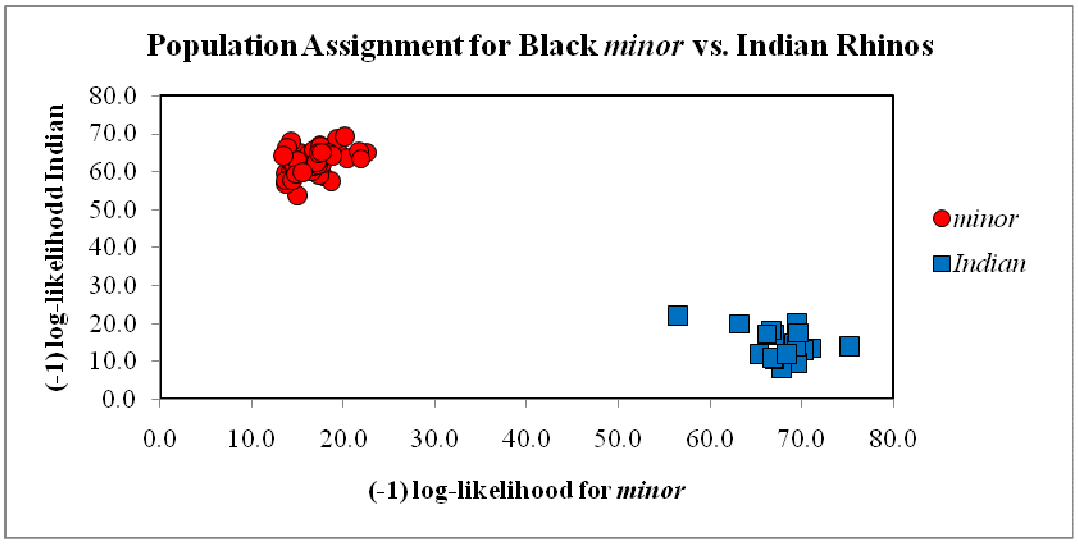

Population Assignment for Black *bicornis* vs. Northern White Rhinos
(-1) log-likelihood Northern white
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
(-1) log-likelihood for *bicornis*
bicornis
Northern white

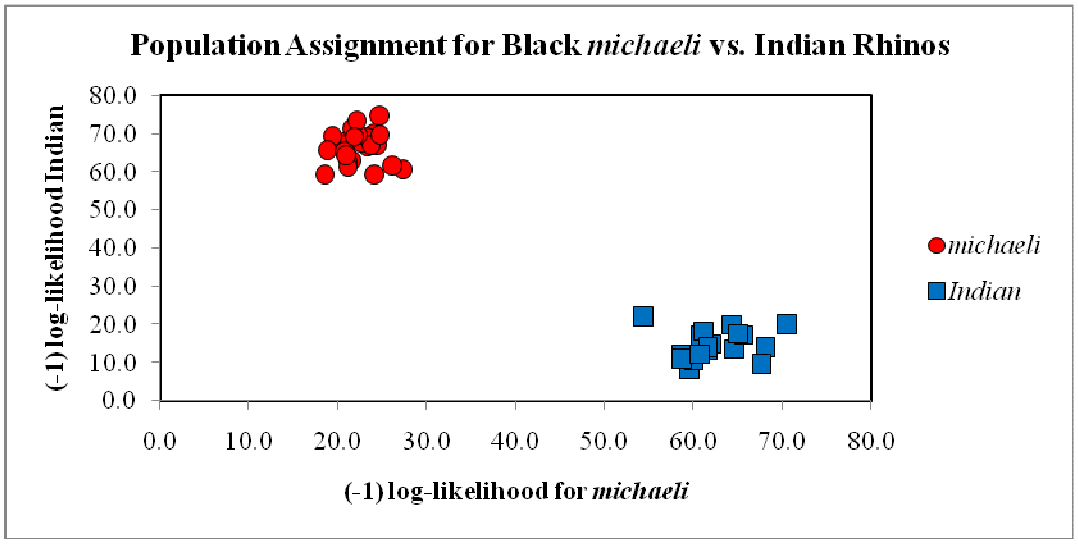

Population Assignment for Black *minor* vs. Northern White Rhinos
(-1) log-likelihood Northern white
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
(-1) log-likelihood for *minor*
minor
Northern white

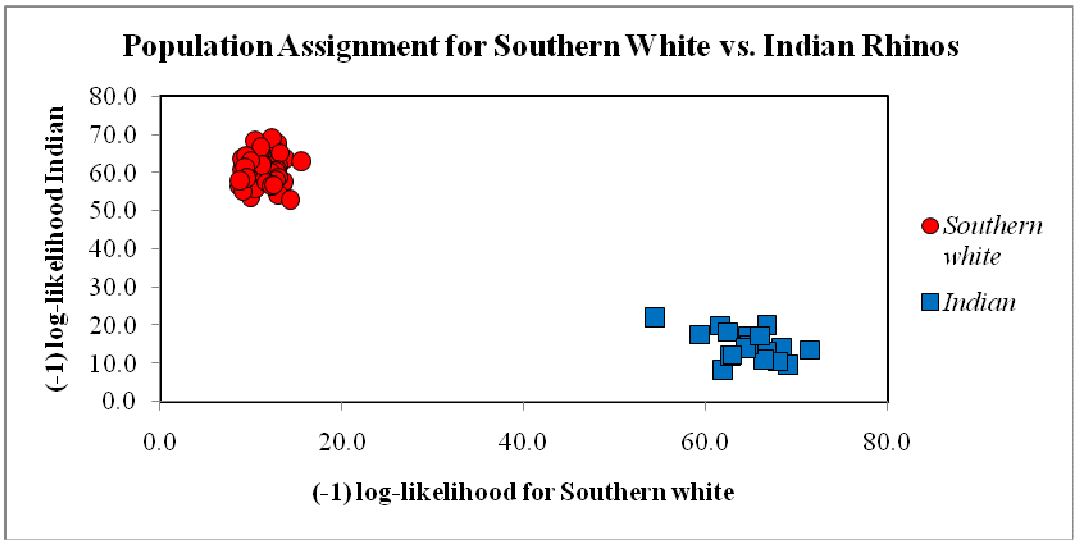

Population Assignment for Black *michaeli* vs. Northern White Rhinos
(-1) log-likelihood Northern white
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
(-1) log-likelihood for *michaeli*
michaeli
Northern white

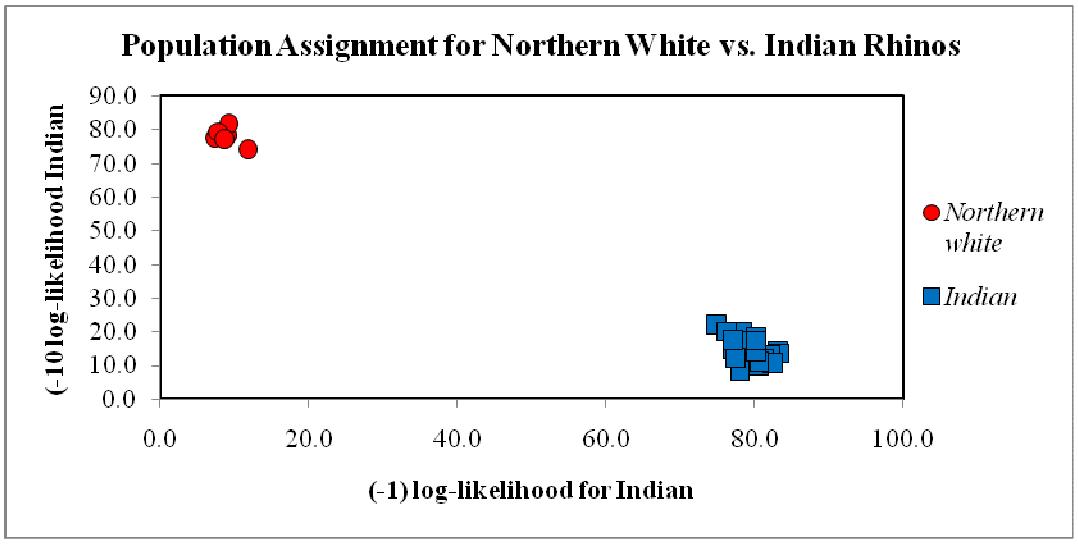

Population Assignment for Southern vs. Northern White Rhinos
(-1) log-likelihood Northern white
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
(-1) log-likelihood for Southern white
Southern white
Northern white

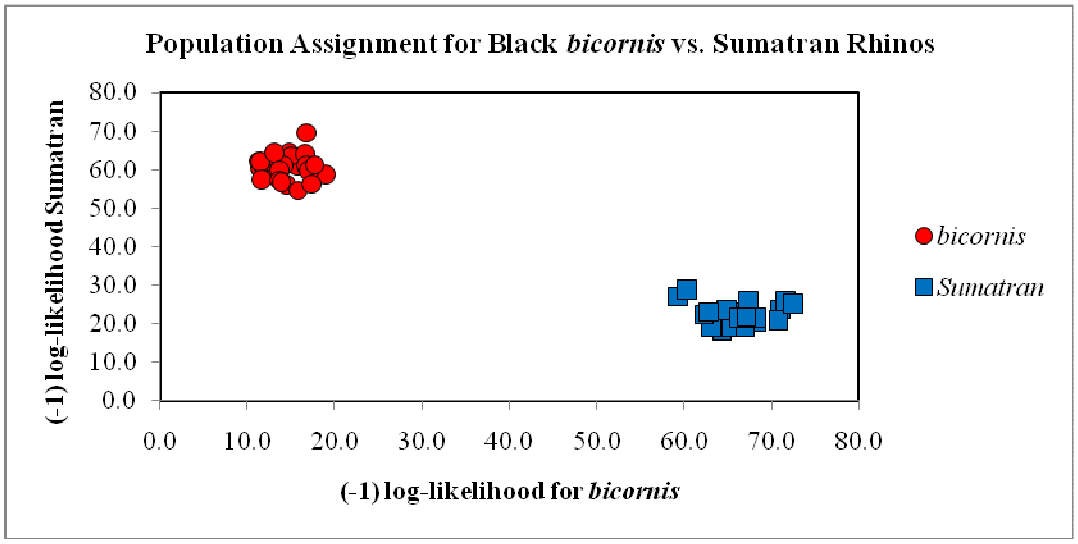

Population Assignment for Black *bicornis* vs. Indian Rhinos
(-1) log-likelihood Indian
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
(-1) log-likelihood for *bicornis*
bicornis
Indian

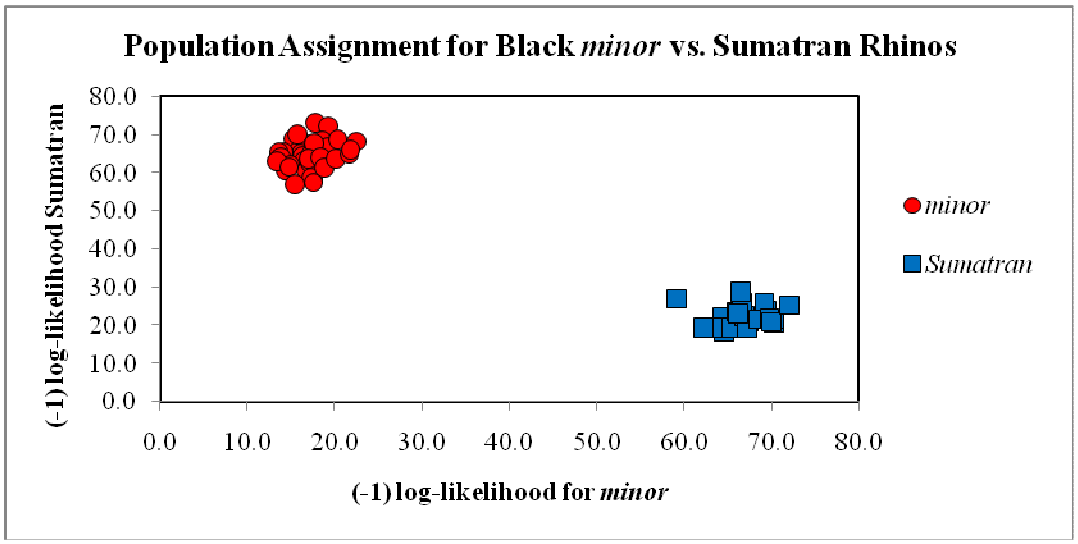

Population Assignment for Black minor vs. Indian Rhinos
(-1) log-likelihodd Indian
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
(-1) log-likelihood for minor
minor
Indian

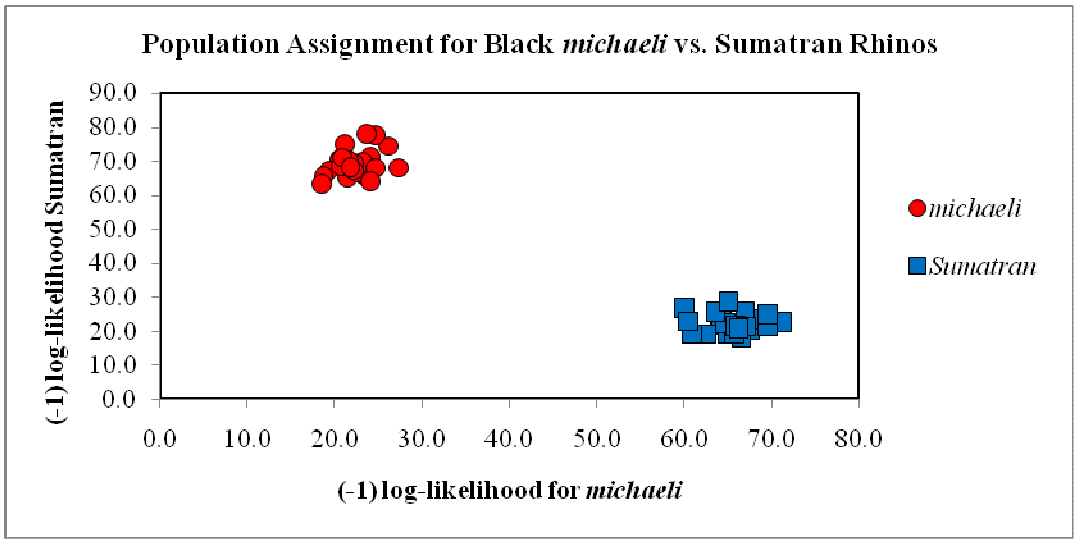

Population Assignment for Black michaeli vs. Indian Rhinos
(-1) log-likelihood Indian
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
(-1) log-likelihood for michaeli
michaeli
Indian

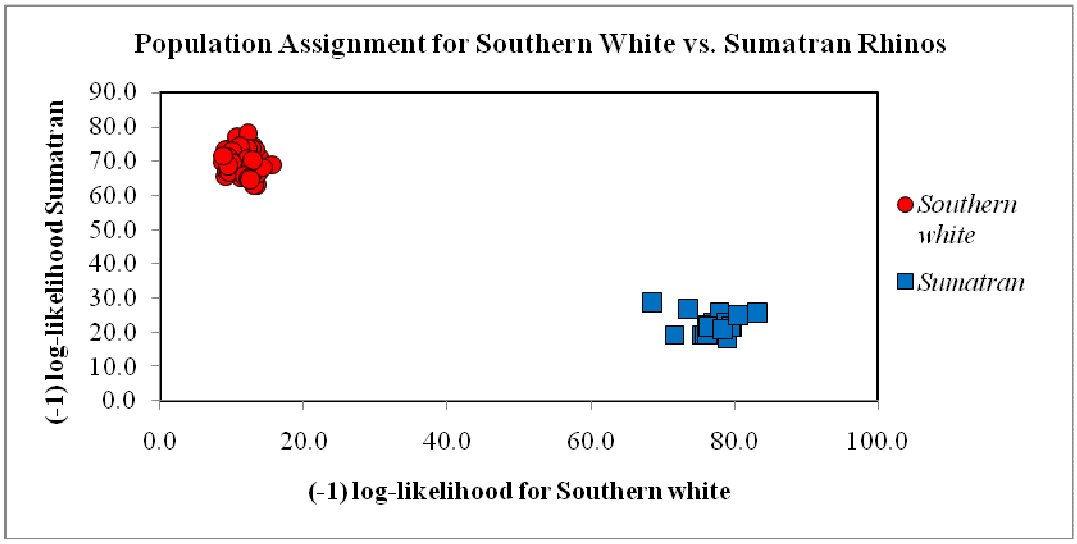

Population Assignment for Southern White vs. Indian Rhinos
(-1) log-likelihood Indian
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
(-1) log-likelihood for Southern white
Southern white
Indian

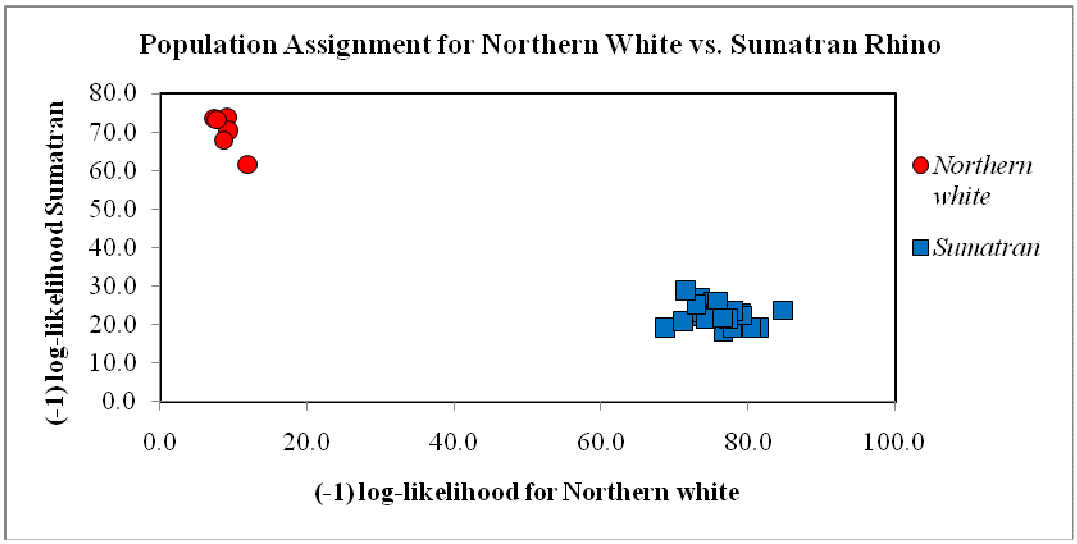

Population Assignment for Northern White vs. Indian Rhinos
(-10) log-likelihood Indian
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
(-1) log-likelihood for Indian
Northern white
Indian

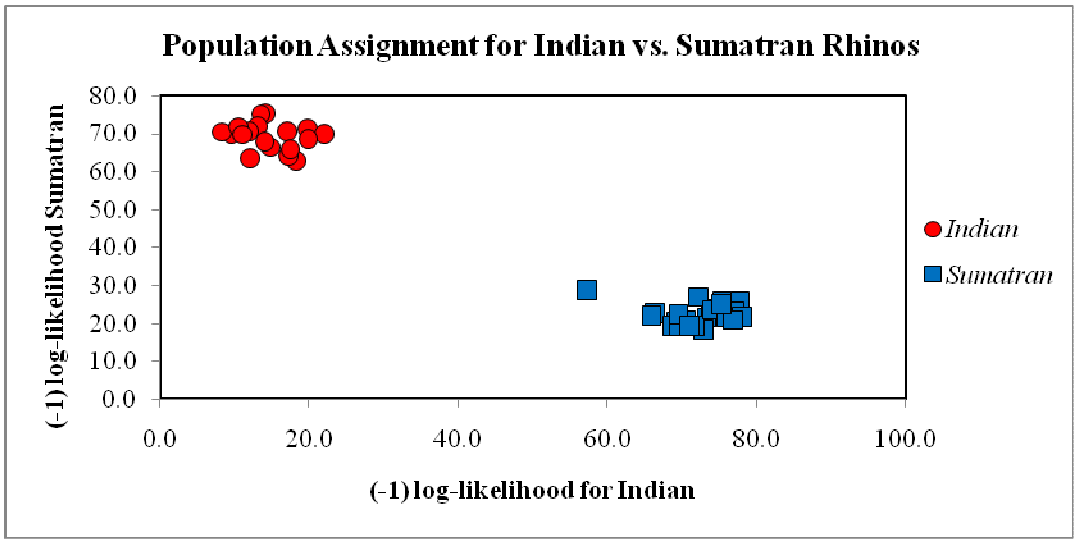

Population Assignment for Black bicornis vs. Sumatran Rhinos
(-1) log-likelihood Sumatran
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
(-1) log-likelihood for bicornis
bicornis
Sumatran


Population Assignment for Black minor vs. Sumatran Rhinos
(-1) log-likelihood Sumatran
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
(-1) log-likelihood for minor
minor
Sumatran


Population Assignment for Black *michaeli* vs. Sumatran Rhinos
(-1) log-likelihood Sumatran
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
(-1) log-likelihood for *michaeli*
michaeli
Sumatran


Population Assignment for Southern White vs. Sumatran Rhinos
(-1) log-likelihood Sumatran
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
(-1) log-likelihood for Southern white
Southern white
Sumatran


Population Assignment for Northern White vs. Sumatran Rhino
(-1) log-likelihood Sumatran
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
(-1) log-likelihood for Northern white
Northern white
Sumatran


Population Assignment for Indian vs. Sumatran Rhinos
(-1) log-likelihood Sumatran
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
(-1) log-likelihood for Indian
Indian
Sumatran