PHYLOGEOGRAPHY AND GENETIC DIVERSITY OF THE KENYAN BLACK RHINOCEROS (*Diceros bicornis michaeli* Zukowsky 1965)

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Phylogeography and Genetic Diversity of the Kenyan Black Rhinoceros (*Diceros bicornis michaeli* Zukowsky 1965)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

Dedicated to my family Nthambi, Mbula and Muya, my mum Anne, and all my siblings – the wind beneath my wings

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

A	Allele
AMOVA	Analysis of molecular variance
BLAST	Basic Local Alignment Search Tool
Bp	Nucleotide base pairs
D. b.	Diceros bicornis
d.f.	Degrees of freedom
D-loop	Mitochondrion hypevariable region
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
ESU	Evolutionarily significant unit
FCA	Factorial correspondence analysis
Fst	Wright's fixation index
h	Haplotype diversity
He	Expected heterozygosity under HWE
Heq	Expected heterozygosity under mutation-drift equilibrium
HKY85	Hasegawa-Kishino-Yano (1985) model of nucleotide substitution
Ho	Observed heterozygosity
HWE	Hardy-Weinberg equilibrium
IAM	Infinite allele model
IBD	Isolation by distance

IRF	International rhino foundation
IUCN	International union for the conservation of nature
Kya	Thousand years
MNA	Mean number of allele
mtDNA	Mitochondrial DNA
Mya	Million years
Ne	Effective population size
NJ	Neighbor-joining
nM	Number of migrations per generation
PCR	Polymerase chain reaction
RI	Harpending raggedness index
rRNA	Ribosomal ribonucleic acid
SMM	Stepwise mutation model
SSD	Sum of squared deviations
TMRCA	Time to the most recent common ancestor
tRNA	transfer RNA
µM	Micromolar
π	Nucleotide diversity
τ	Tau
ɵ	Theta
ΦCT	Fst analogue as defined by Weir and Cockerham (1984)

ABSTRACT

The Kenyan black rhinoceros population declined by over 98% in less than thirty years due to habitat destruction and extreme poaching in the 1970s; to a mere 381 animals in 1987 that were distributed in few isolated areas. Various government bodies that have managed wildlife in Kenya adopted the sanctuary approach to manage the remaining black rhinoceros subpopulations. Initially, this approach focused on creating four high security black rhinoceros nucleus breeding sanctuaries that begun taking threatened black rhinoceros in the early1980s. The approached proved very successful in rehabilitating black rhinoceros populations and new sanctuaries were seeded in Kenya. By 2008 the sanctuaries had increased to 14 and where holding over 650 animals.

However the translocations were not based on any empirical genetic information and thus, posed the risk of introducing outbreeding depression and breakdown of locally adapted genotypes in the black rhinoceros subpopulations. Kenya Wildlife Service (KWS) has also partitioned the metapopulation into lowland and highland subpopulations that are managed separately with strong emphasis in avoiding translocation of black rhinoceros between them. The genetic effect of this management strategy is not known. This study focused on generated information on the genetic status in the extant Kenyan black rhinoceros subpopulations. The information will be used by KWS in the formulation of a genetically viable management strategy for the Kenyan black rhinoceros subpopulations.

Twelve subpopulations were sampled for this study. General standard molecular methods were employed. Genetic information was obtained from 408bp mitochondrial D-loop sequence from 170 individuals and 145 individuals were genotyped at nine autosomal loci. Both model based and standard methods were used to examine the data.

Both mtDNA and microsatellite (nDNA) markers detected moderate genetic diversity in the Kenyan black rhinoceros metapopulation (h =0.78±0.027, n = 170; Ho =0.70±0.087, n=145) that is consistent with previous studies on *Diceros bicornis michaeli.* However, mtDNA and nDNA diversity varied between subpopulations; while Masai Mara had the highest mtDNA diversity, the least nDNA diversity, Lewa WC had exactly the opposite. The lack of genetic diversity detected by microsatellite data in Masai Mara unlike that detected by mtDNA illustrates the stochastic nature of the correlation between nDNA and mtDNA diversity in subdivided small populations

Findings from this study suggest that Masai Mara is fairly distinct subpopulation, with the highest inbreeding and isolation level. They also suggest that there is no distinct lowland - highland subpopulation grouping and that there is no historical gene flow barrier. The highest component of genetic diversity is still partitioned among individuals, hence to preserve genetic variability in the various subpopulations it will be important to conserve as many individuals as possible and in the event of translocation; evaluate keenly the genetic orientation of both the donor and recipient subpopulations.

CHAPTER ONE

1.1 GENERAL INTRODUCTION

1.1.1 General background on phylogeography

The term phylogeography was invented by Avise in 1987 (Avise *et al.*, 1987) from phylogeny (evolutionary relationships) and geography (spatial distribution). It is the study of principles and processes governing the geographical distribution of genealogical lineages by comparing the evolutionary relationship of genetic lineages especially of closely related species' (Avise, 2000) with their geographical location. The major goal of phylogeography is to understand which factors have more influence on the current geographical dispersion of genes, populations and species (Freeland, 2005).

Phylogenetic techniques have previously been used to study the population structure of cryptic as well as straightforward studied organisms (Allendorf and Luikart, 2007) to delineate evolutionary significant units for conservation purposes. Roca *et al.,* (2001) used these techniques to demonstrate that the African elephant (*Loxodonta africana*) is comprised of the savanna and forest elephants that have distinct evolutionary relationship. Johnson (2008) using the same techniques worked on forest elephants (*Loxodonta africana cyclotis*) in central parts of Africa and demonstrated that they are evolutionary distinct from the savanna elephants (*Loxodonta africana africana*). Similar

techniques have been used by Zhao *et al.,* (2008) to examine the complex population genetic and demographic history of the Salangid (*Neosalanx taihuensis).*

This study surveyed the phylogeography of the black rhinoceros subpopulations in Kenya in order to ascertain their evolutionary genealogical lineages in relationship to their current geographical locations. The overall goal was to determine whether the subpopulations have undergone sufficient evolutionary isolation to warrant delineation into distinct conservation management units.

1.1.2 Evolution of the black rhinoceros

There are five species of rhinoceros (**Plate 1.1**) surviving in the world today. They belong to the family rhinocerotidae of the order Perissodactyla (Tougard *et al.*, 2001). The term Perissodactyla is derived from the Greek words; *perissos*, meaning odd numbers, and *daktulos*, meaning a finger or toe. Therefore, all members of this order are odd-toed ungulates (ungulates meaning hoofed-animals), with the axis of symmetry of the foot passing through the central toe, a characteristic also known as mesaxonic (Lacombat, 2005). Other extant members in the Perissodactyla order are the equidae and tapiridae families (**Figure 1.1**). The black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros species are native to Africa, whereas the Indian (*Rhinoceros unicornis*), Sumatran (*Diceros sumatrensis*) and the Javan (*Rhinoceros sondaicus*) rhinoceros are indigenous to Asia. The white, black and Java rhinoceros have two horns, while the Indian and Sumatran rhinoceros have only one horn.

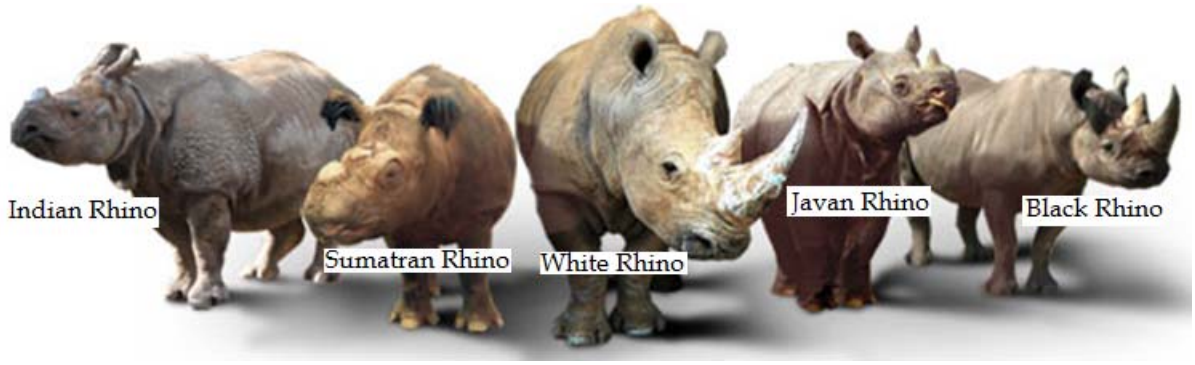


Plate 1.1: The five surviving species of odd-toed ungulates in the family Rhinoceroscerotidae. The Indian and Javan rhinoceros have one horn and skin folds around the shoulders and the rear side. The upper legs and shoulders of the Indian rhinoceros are covered in wart-like bumps. All other rhinoceros species have smooth skins and two horns. The Sumatran rhinoceros is the smallest and more hairy and archaic than the others. Source RCC (2008)

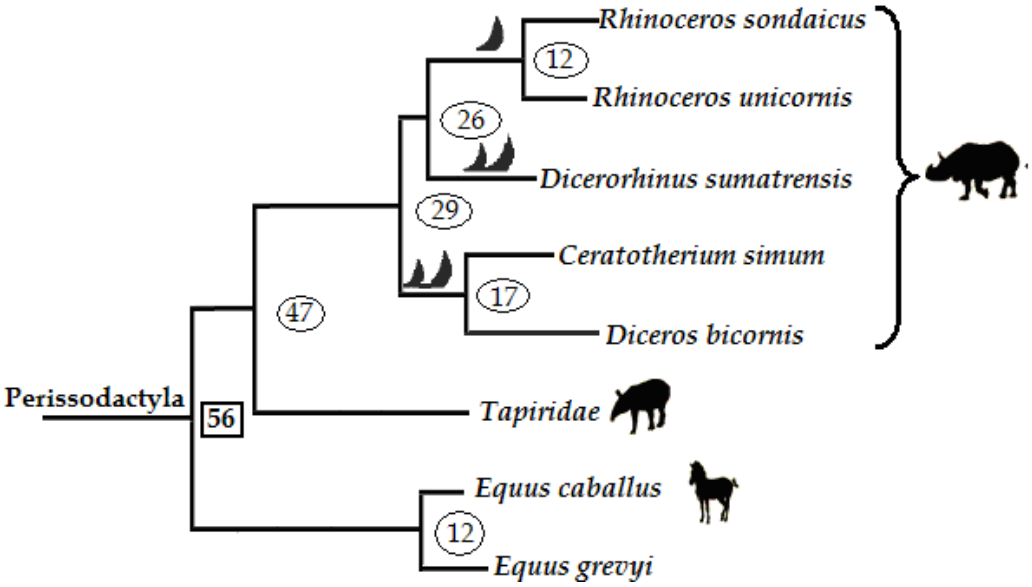


Figure 1.1: Evolution of modern rhinoceros. The encircled numbers represent divergence dates estimated for the different Perissodactyla splits, calibrated on the equid–ceratomorph divergence time at 56 Million Years Ago. The number of horns of each rhinoceroscerotids clade is shown (Tougard *et al.*, 2001).

Perissodactyls evolved in North America, where a fossil of the oldest known rhinoceros-like mammal; the *Hyrachyus eximus* (**Figure 1.2**), dating back to the Early Eocene Epoch[1] (Eldredge, 2005) was discovered (Cerdeño, 1995, 1999; Antoine, 2003; Orlando *et al.*, 2003). This animal was much smaller than the modern day rhinoceros and was morphologically more similar to the early tapirs and horses and did not have a horn (Lacombat, 2005). Horns only became a defining characteristic in the evolutionary history of rhinoceros in the late Eocene, with the appearance of the Rhinocerotidae family (Lacombat, 2005). Currently, horns are the major feature of rhinoceros; and they are used in the field for the identification of individuals among other characteristic, and in taxonomy (Orlando *et al.*, 2003).

[1] Epochs are defined moments in time that describe the beginning of a distinctive historical period or era or the characteristics of the distinctive historical period. The Age of Mammals epochs is called the Cenozoic Era, and include the following known epochs:--

Epoch	**Began**
Paleocene	~65 million years ago
Eocene	~58 million years ago
Oligocene	~37 million years ago
Miocene	~23 million years ago
Pliocene	~ 5 million years ago
Pleistocene	~ 2 million years ago
Holocene or Recent	~ 100 thousand years ago

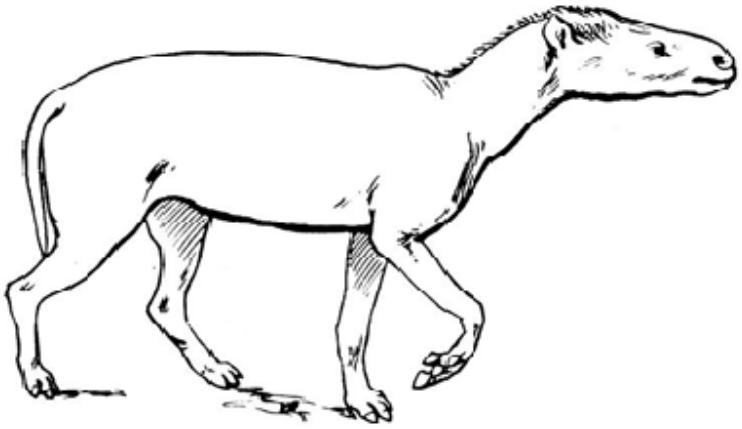


Figure 1: *Hrachyus eximius*; A species in the extinct *Hrachyus* genus of Perissodactyla mammals that lived in Eocene Europe (around 50 Million Years - Mya). The 1.50 m long animal is thought to be the ancestor of modern Perissodactyls (source: http://www.ivorybill.com/cb/extm/Hyrachyus-eximius.htm).

More distinct forms of rhinoceros evolved in the late Eocene all over Europe and Asia through a series of complex evolutionary pathways within the Perissodactyla order that saw several families become extinct during the fifth major extinction event (Frankham *et al.*, 2002; Eldredge, 2005) that occurred in the late Pliocene and early Eocene epochs, and leaving only three families that extant today (McKenna and Susan, 1997; Cerdeño, 1999; Tougard *et al.*, 2001).

The earliest members of the Rhinocerotidae were a diverse group of land mammals including 41 genera and 142 species. At one time, they were much more diverse and abundant than today, ranging from Europe, Asia, North America, and Africa (Cerdeño, 1999). A wave of extinctions in the middle Oligocene period (Frankham *et al.*, 2002; Eldredge, 2005) wiped out most of the smaller species. Several independent lineages

survived the fifth major extinction phase (Lacombat, 2005). The *Menoceras* lineages were pig-sized rhinoceros that occurred in Europe and North America up to the early Miocene period, which ended about 5.3 Million Years (Mya) (Lacombat, 2005). The males had two horns growing side-by-side, while the females were hornless (Lacombat, 2005). *Teleoceras* was a local lineage of *Menoceras* in North America that went extinct later in the Pliocene era around 5 Mya. Teleoceras were grazers and had shorter legs than the modern rhinoceros and a barrel chest, making their built more like that of hippopotamus (Hanson, 1989). Like the hippo, they were semi-aquatic (Hanson, 1989). Teleoceras had a single small nasal horn (Prothero, 2005).

The woolly rhinoceros (*Coelodonta antiquitatis*, **Plate 1.2**) is perhaps the latest member of the rhinocerotidae to go extinct (Orlando *et al.*, 2003). Evolutionarily, it is more closely related to the Sumatran Rhinoceros (Orlando *et al.*, 2003). The woolly rhinoceros appeared around one Mya in China and 600 Thousand years (Kya) in Europe and have survived until as recently as 10 Kya. *Coelodonta* was a large genus, with morphological adaptations to live in both cold and dry climates. Its most distinctive adaptation was the thick coat of long brown hair and a body septum that separated the nasal bone in two parts to warm the air easily (Osborn, 1915; Orlando *et al.*, 2003). Their fossils were discovered throughout Europe and Asia, although apparently they did not manage to extend their distribution into North America or to Ireland (Hanson, 1989; Orlando *et al.*, 2003).

Rhinoceroses are considered a good example of a major animal group that has outlived its evolutionary development peak and is an outmoded evolutionary group which is probably living its eventual extinction phase in geological times (Hooyer, 1976). Climate change and increase in human population are the major factors that could have fuelled the extinction at different rates (Eldredge, 2005).

Plate 1.2: The Woolly rhinoceros: Appeared around one Mya in China and may have survived until as recently as 10,000 years ago (Osborn, 1915; Orlando *et al.*, 2003)

1.1.3 Distribution and conservation status of modern rhinoceroses with emphasis on the black rhinoceros

Rhinocerotidae appeared in Asia during the Oligocene period around 37 Mya (Cerdeño, 1999; Antoine, 2003). The genus Paraceratheriumin the subfamily *Indricotheres* was

the most famous of rhinocerotidae in Asia as they are believed to be the largest terrestrial mammals that ever existed (Cerdeño, 1999; Antoine, 2003). These browsing rhinoceros were hornless and are thought to have been almost six meters high and nine meters long (Antoine, 2003). Their weight would have been close to 20 tones (Cerdeño, 1999; Antoine, 2003). Their upper teeth were tusk-shaped, while the lower teeth pointed forward (Lacombat, 2005). Fossils records indicate that Asia was the final departure point for the dispersal of modern rhinoceros during the Miocene to late Pleistocene periods (Hanson, 1989; Cerdeño, 1999; Antoine, 2003). All the European rhinoceroses were derived from the Asian ones (Cerdeño, 1999; Antoine, 2003).

The Indian and Javan rhinoceros are more closely related to each other than to any other rhinoceros species, and are thought to have evolved into separate lineages, around 4-2 Mya from a common one horned Asian rhinoceros species (Tougard *et al.*, 2001). Both species belong to the same genus - *Rhinoceros* - have a single horn, and have thick skin folded around the rear areas, making them appear armored, unlike other rhinoceros species that have smooth skin (Cerdeño, 1999; Lacombat, 2005). The Sumatran rhinoceros; also called the "hairy rhino" because of its hairy body; is the smallest among the exact rhinoceros species. Its skin does not appear armored. The Sumatran rhinoceros is more closely related to the now extinct woolly rhinoceros (*Coelodonta antiquitatis*) than to the other modern rhinoceros species (Orlando *et al.*, 2003; IRF, 2008). The Sumatran rhinoceros are more ancient than the Javan and Indian rhinoceros, with the first emerging more than 20 - 14 Mya. Because of its similarity with other

extinct rhinoceros species, is frequently referred to as a living fossil.

The lineage of rhinoceros in Africa is traced back to the Asiatic two-horned rhinoceros, approximately 14 Mya, at the end of the Miocene (Hooyer, 1976). There are two rhinoceros species in Africa. The black rhinoceros is considered to be more primitive and the older of the two species to emerge from the Asian rhinoceros. The white rhinoceros diverged from within the black rhinoceros between 2 and 5 Mya (Hooyer, 1976; Lacombat, 2005). Some taxonomists doubt whether they should remain separated into two genera because they are so closely related and can still mate and successfully produce viable offspring (Robinson *et al.*, 2005). Large free ranging populations of black rhinoceros roamed over vast areas of Africa in the 18th Century (**Figure 1.3**). The difference between the current distribution and the historical distribution of black rhinoceros in Africa is striking. Many countries like Burkina Faso, Ivory Coast, Ghana, Togo, Benin, Nigeria, Chad, Central African Republic, Sudan and Mozambique have lost their rhino populations altogether due to poaching and habitat loss (IUCN, 2008).

There are four recognized black rhinoceros subspecies living in different parts of Africa (IUCN 2008); a fifth subspecies (*Diceros bicornis bruceii*) may still survive in Ethiopia but according to data presented at the IUCN-SSC-AfRSG meeting held in Kenya in June 2004 there could be four individuals of this subspecies in Ethiopia and its population trends were unknown (AfRSG, 2004). Although the ranges of the four recognized subspecies overlap in some areas, the different subspecies exhibit ecological partitioning

in exploiting different habitats (**Figure 1.3**). For example, the South-western black rhinoceros (*Diceros bicornis bicornis*) is better adapted in the desert and dry savannah areas of Namibia and south-western South Africa and the scattered open woodlands of southern Angola, western Botswana and western South Africa (Emslie and Brooks, 1999). The South-central black rhinoceros subspecies (*Diceros bicornis minor*) is the most numerous and occupy the wetter areas of central Tanzania south through Zambia, Zimbabwe and Mozambique to northern and eastern South Africa (Emslie and Brooks, 1999). East African black rhinoceros subspecies (*Diceros bicornis michaeli*) is primarily in northern Tanzania and Kenya (Hall-Martin, 1984; Emslie and Brooks, 1999; Okita-Ouma *et al.*, 2007). The West African black rhinoceros subspecies (*Diceros bicornis longipes*) was tentatively declared extinct in 2006 (IUCN, 2006; Times online, 2006) after researchers failed to find any animal in its last known habitat in Northern Cameroon. Surveys conducted in *Diceros bicornis longipes* range areas in 1996–97 indicated that at least 10 rhinos remained, with a possible eight others unconfirmed (Emslie and Brooks, 1999)

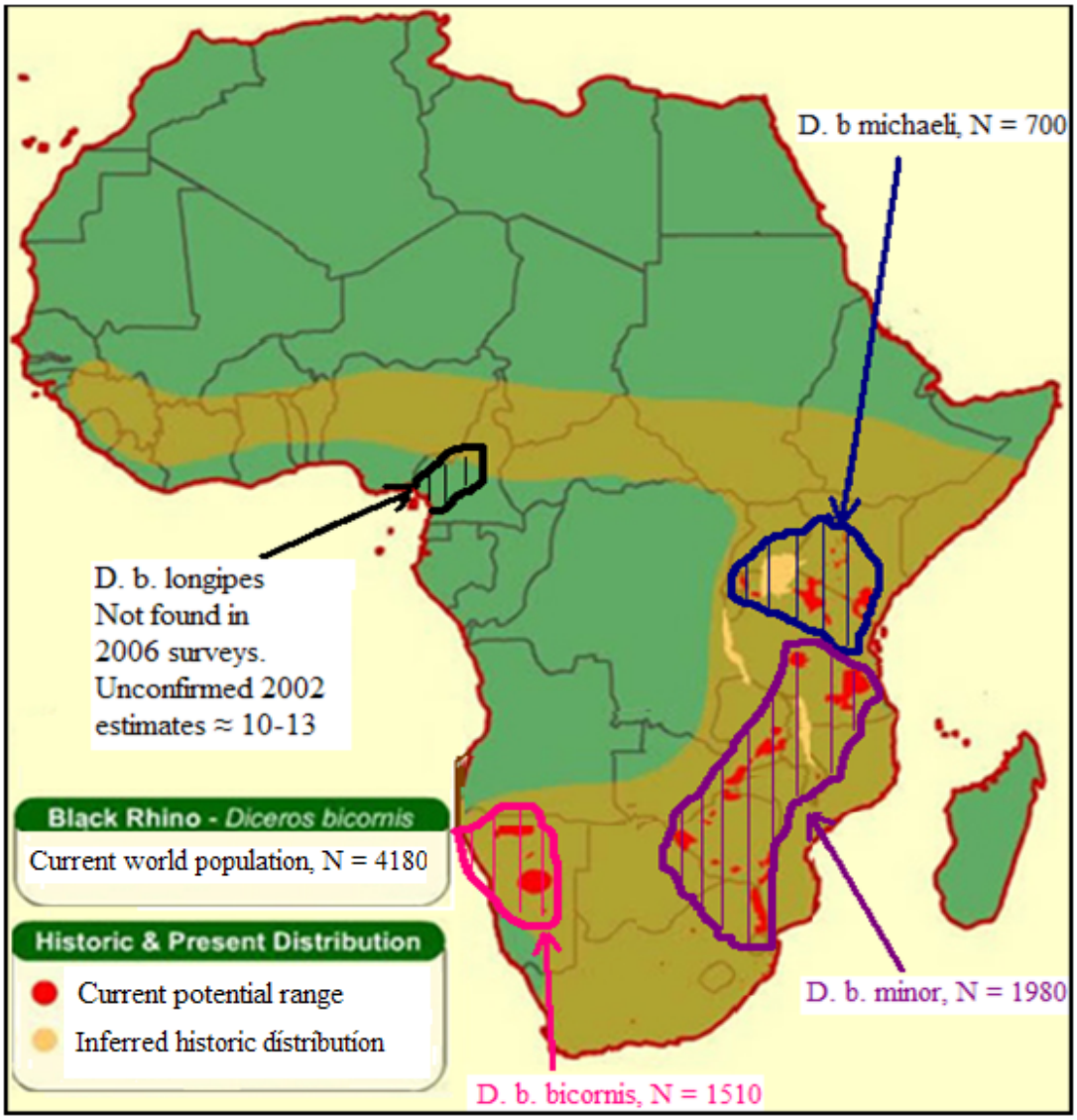


Figure 1.3: Current and inferred historical distributions of black rhinoceros subspecies populations in Africa. Map is modified from International Rhino Foundation report (IRF, 2008). The current census population size numbers (N) indicated after each subspecies names are adopted from International Union Conservation for Nature report (IUCN SSC AfRSG, 2008)

1.1.4 Threats to the survival of black rhinoceros in Africa

It is not known why rhinoceros became extinct in North America even though much of their evolution occurred there, however, it is thought that perhaps a wave of extinctions that swept across the earth in the late Pliocene (Eldredge, 2005) may have been the cause. The woolly rhinoceros perhaps was hunted to extinction by early human in Europe and Asia (Antoine, 2003). Black rhinoceros ranged in most parts of sub-Saharan Africa in the 18th Century (**Figure 1.3**). In Kenya, there are folklores about rhinoceros in almost all indigenous communities with local places and people being named after rhinoceros, an indication that the species was once quite common. Reports from early foreign hunters in Africa (Neumann, 1898; Patterson, 1909; Lloyd-Jones and Brevet-Major, 1925; Barclay, 1932) and Kenya in particular (Lloyd-Jones and Brevet-Major, 1925; Hunter, 1952) indicated that rhinoceros were numerous, since there was always a possibility of an attack by black rhinoceros hiding in almost every bush. Currently, rhinoceros in Africa and Asia are on the verge of extinction mainly due to heavy poaching for their horn and loss of their habitat.

The horn of the rhinoceros is used to make ornamental handles for daggers (Jambiyas) in Yemen, and traditional medicine in the Far East (Martin and Vigne, 2003). In 1970s, OPEC pushed up oil prices in Saudi Arabia, increasing demand for Yemeni workers. The Yemen workers remitted huge amounts of money back to Yemen, and some of this money was spent on buying expensive Jambiyas (Leader-Williams, 1992; Martin and Vigne, 2003). Consequently, poaching for rhinoceros horn in Africa and Asia surged,

resulting in a major crash in rhinoceros populations (Martin and Vigne, 2003). Wealthy Yemenis have sustained demand for Jambiyas and some are known to buy it for speculative purposes that if fresh supply of rhinoceros horns is completely wiped out then they stand to make huge profits from their stocks. In Eastern and Central Africa, poaching of both black and southern white saw resurgence in Kenya from 2001 and has virtually or actually exterminated the Northern White Rhino in the Democratic Republic of Congo (Martin and Vigne, 2003; Dean and Foose, 2006).

Contrary to the common believe that rhinoceros horn is mainly used in traditional Chinese medicine (TCM) as an aphrodisiac, research has shown that it is used widely to cure almost everything in addition to impotence and sexual inadequacy (Dinerstein, 2003; Martin and Vigne, 2003; Dean and Foose, 2006). From prevention and cure of devil possession, hallucinations, bewitching, nightmare and evil spirits, to medical conditions such as snake poisoning, typhoid, headache, feverish colds, rectal bleeding, heavy smallpox, etc. Users also believe that continuous consumption and/or administration of rhinoceros products lightens the body and makes one very robust (Dinerstein, 2003). Rhinoceros horn is much used as an aphrodisiac in traditional Asian medicine in Taiwan, Singapore, Japan, South Korea, Malaysia, Brunei, Macau, and Thailand (Spinage, 1962; Guggisberg, 1966; Leakey, 1969).

The major Asian importers of African rhinoceros horn were China, Hong Kong and Taiwan Nationals (Martin and Vigne, 2003). There is no scientific evidence that

rhinoceros horn medicines works; Europeans who have tried them never felt any change (Hunter, 1952), but traditional use continues to drive demand on which poachers thrive (Guggisberg, 1966; Martin and Vigne, 2003).

Land clearance for human settlement, agriculture, logging either authorized or illegal is also another key reason for the rapid decline of rhinoceros populations in Africa and Asia (Foose, 1992; Dean and Foose, 2006; IRF, 2008). For example, in Kenya between 1948 and 1957, a government-sponsored settlement scheme was implemented in the Makueni District, near the Chyulu Hills (Guggisberg, 1966). But the settlement was constrained by harsh climatic conditions, tsetse fly and human-wildlife conflict - mainly livestock depredation by wildlife. To ensure that the program succeeded the colonial Government had to implement a land clearance scheme. Bush was cleared for tsetse fly control, and a Game Warden named J.A. Hunter was brought in from Ngorongoro, Tanzania to control wildlife. Hunter boasts that he shot some 1,000 Black Rhino that were causing problems in the Chyullu Hills area (Hunter, 1952; Dean and Foose, 2006). Poaching was also rampant in this area from early 1970s to late 1980s. Today, only about 15 black rhinoceros survive in the Chyulu Hills National Park (Okita-Ouma *et al.*, 2007).

In areas where political conflicts are flaring and normal law and order has broken down, it is almost impossible to protect rhinoceros and other endangered species from poachers, some of whom are militias looking for resources to fund insurgences (Dean

and Foose, 2006). Particular examples of places where political conflict has been matched by a rise in poaching include the Democratic Republic of Congo, Zimbabwe and Nepal (Dean and Foose, 2006). Northern White Rhinos have been eradicated from the Garamba National Park in the Democratic Republic of the Congo (DRC) because of illegal hunting linked to the proliferation of arms and ammunition and displaced persons from the civil war in southern Sudan. This situation worsened during the last six or seven years since civil wars broke out in DRC (Dean and Foose, 2006).

The change of land policy in Zimbabwe in 2000 resulted in a breakdown of law and order and many areas where rhinoceros were living in privately owned conservancies were invaded by people with no understanding or interest in conservation, resulting in increased poaching and habitat loss (Dean and Foose, 2006). Maoist insurgency in Nepal has led to increased violence throughout the Country. Nepal's military had to reduce the number of soldiers assigned to protect the parks so as to deal with insurgence leading to an upsurge in poaching (Dean and Foose, 2006).

1.1.5 Black rhinoceros conservation efforts in Kenya

Early in the 1900s, pioneer naturalists in Europe and America began to notice the imminent demise of wildlife in Africa (Spinage, 1962; Leakey, 1969; Akama, 1998). Pristine natural lands in most colonized territories were rapidly declining due to increases in native people populations and the influx of more settlers demanding prime land for agriculture, industrialization and uncontrolled hunting practices (Ogutu, 1993).

In 1903, British conservationists formed the Society for the Preservation of the Fauna of the Empire (Akama, 1998). This society was instrumental in organizing conservation awareness campaigns throughout Europe and North America. Their goal was to sensitize the public in general and the British Government in particular, on the social and ecological value of nature conservation. Conservationists in Europe also put pressure on governments such as Britain, France, Germany and Italy, which had colonies in Africa and other parts of the Third World, to initiate policies and programs of nature protection (Akama, 1998) and control poaching. The society urged the British Government to establish adequate nature reserves before the country was completely settled by farmers and ranchers and the opportunity for doing so be lost forever (Leakey, 1969; Akama, 1998). The outbreak of Second World War, however, reduced the drive to create nature reserves in Kenya.

In 1939, the British government, as a result of pressure from British conservationists, appointed a game committee to study and make recommendations regarding setting up game parks in Kenya (Nash, 1982; Akama, 1998). Accordingly, the game committee made certain recommendations which were approved by the colonial legislature in 1945 (Akama, 1998). The recommendations of the game committee led to the creation of the pioneer national parks in Kenya which included Nairobi in 1946, Amboseli in 1947,

Tsavo in 1948 and Mt. Kenya in 1949 (Akama, 1998) .

After the creation of National Parks and other private game reserves in Kenya, conservation efforts for endangered species such as the black rhinoceros focused on the translocation of isolated individuals from areas that had been opened up for agriculture and/or settlement (**Figure 1.4, Table 1.1**). In 1961-1962, seven black rhinoceros (*Diceros bicornis michaeli*) were translocated from the Kibwezi area in Kenya to Addo Elephant National Park in South Africa to establish the first population in the Park (Hall-Martin, 1984). This was about 15 years after J. A. Hunter killed 1000 rhinoceroses from this area. In the following years, between 1963 -1968, more animals were translocated from Kibwezi and Makueni area (Darajani and Kiboko), Kitengela Area, Kapiti Plans and Nyeri Forest to Nairobi National Park to re-establish the first breeding nucleus of black rhinoceros in Kenya. More animals were moved in subsequent years and by 1999, a total of 35 animals had been brought to Nairobi National Park from different regions of the country. Later translocations of rhinoceros from Nairobi National Park to other areas were meant to either start new sanctuaries or restock areas deprived by poaching. By 2008 years, 73 animals were moved out of the park to all reserves in the country except Masai Mara Game Reserve and Chyullu Hills National Park (**Figure 1.4**). The biggest translocation of rhinoceros occurred in 1996, when 11 animals were moved to Tsavo East National Park.

Solio Game Reserve also played a major role in receiving animals from different parts of

the country similar to the Nairobi National Park. Most translocations into Solio took place in the 1970s. This was the second breeding nucleus. Animals come from Kiboko, Darajani, Tsavo East National Park, Lamuria, Embu, Kibwezi, Isiolo and Rimuruti. The animals were translocated to other areas after attaining a viable population. To date 89 animals have been moved out of Solio Game Ranch, with the largest single movement of 28 animals in 2007 to Ol Pejeta Game Ranch. Lake Nakuru National Park has donated the third highest number of individuals for restocking rhinoceros sanctuaries in Kenya; with 20 animals having been moved out since 1988 (Okita-Ouma, 2004).

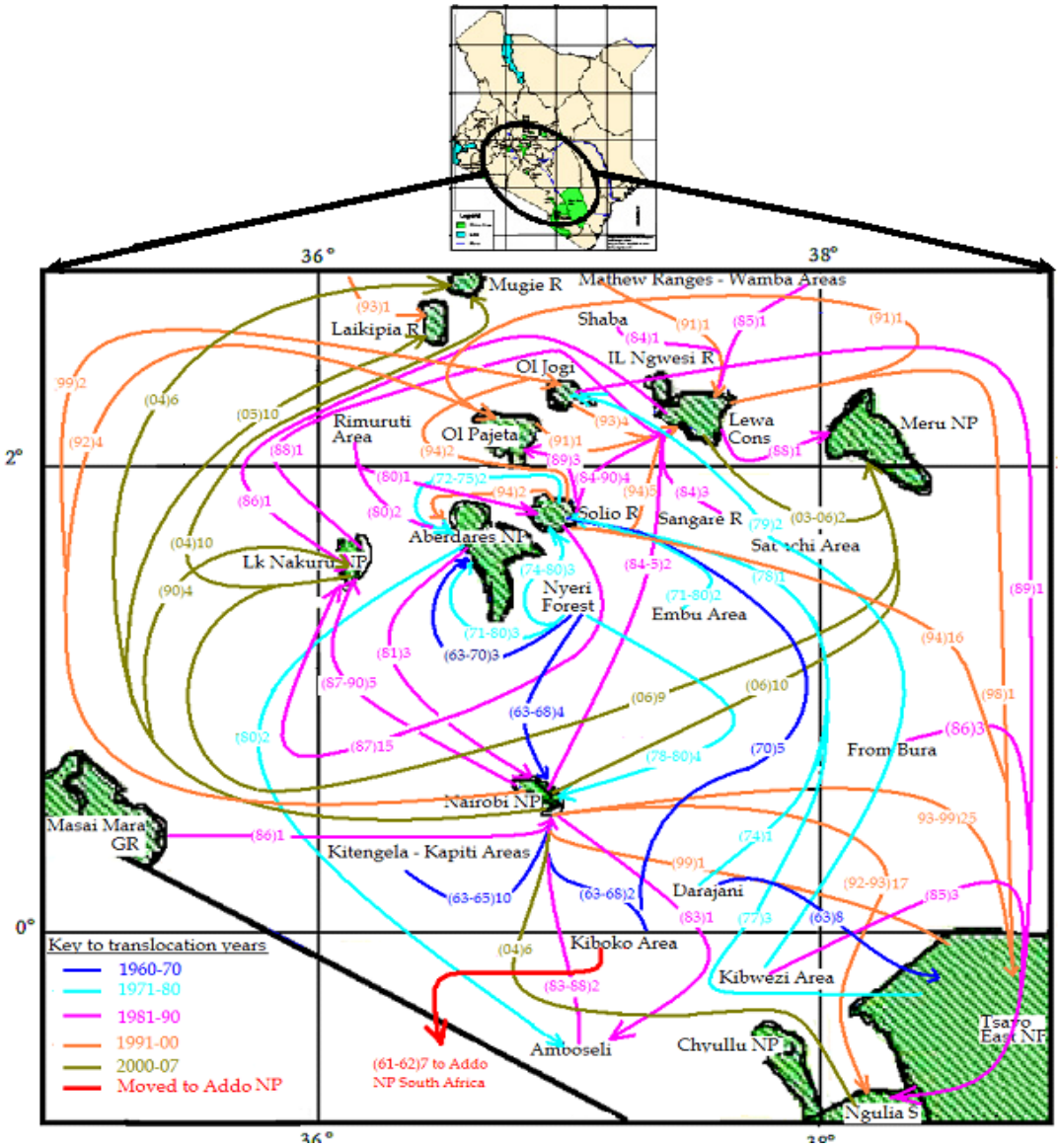


Figure 1.4: Diagrammatic representations of the locations of black rhinoceros conservation areas in Kenya showing the major black rhinoceros translocations routes between 1960 and 2007. Digits in brackets are the year(s) when translocation occurred. Digits outside brackets are number of rhinoceros translocated. Between 1960 to early 1980's (blue and magenta lines), translocations were from unprotected areas to sanctuaries. Subsequent movements (1980's onwards) were for restocking specific areas, saving isolated individuals or to boost subpopulation size. Mara and Chyullu have remained relictual with no known immigration. Laikipia also remained relictual until 2005.

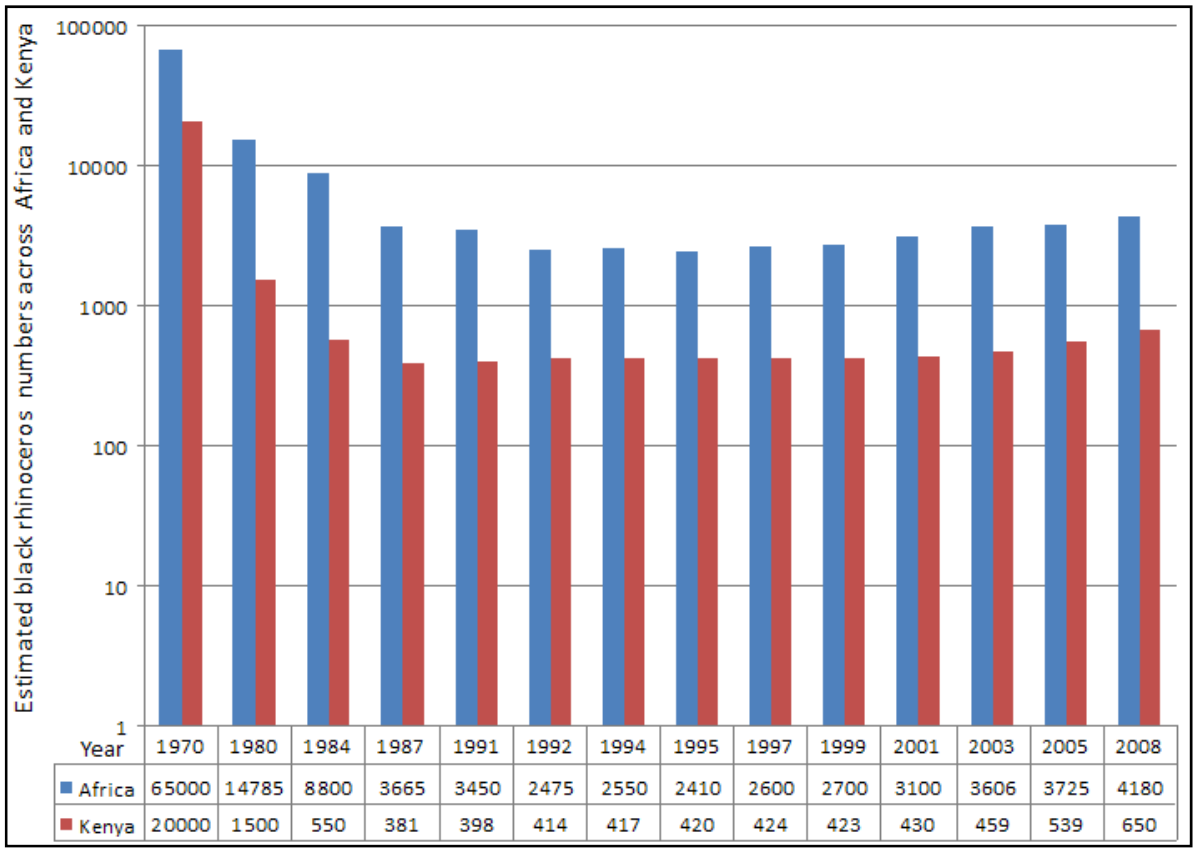
Table 1.1 Known translocation history of Kenyan black rhinoceros, 1961-2008. N = Current census population size, n = sample size for samples used in this study. Digits in brackets indicate the year(s) translocation was possibly carried out, while digits outside brackets indicate the number of rhinoceros that were translocated. NP = National Park, WC = Wildlife Conservancy, GR = Group Ranch,= National Reserve. Source - KWS rhino programme census data.

Subpopulation	N (2007)	n (sample size)	Lowest population size	Total number translocated in (61-08)	Origin of rhinoceros (Year - in two digits) and Number	Total number translocated out (61-08)	Recipient location (Number and Year)
Aberdare NP	30	9	37 (1989)	12	Solio (72-75)2 (94)2, Rimuruti (80)2, Nyeri Forest (63-80)6	5	Nairobi NP (81)3, Amboseli (80)2,
Chyulu NP	21	9	2 (1992)	0	0	0	0
Laikipia WC	12	9	10 (2002)	11	Other areas (93)1, Nairobi NP (05)10,	0	0
Lewa WC	55	33	12 (1991)	25	Ol Jogi (93)4, Ol Pejeta (91)1, Mathew Ranges (93)1, Solio (84)3 (90)1 (94)5, Lk Nakuru (88)1, Nairobi NP (84)1 (85)1, Wamba (85)1, Sabachi (85)1, Losai (90)1, Sangare Ranch (84)3, Shaba (84)1	7	Lk Nakuru (86)1, Tsavo East NP (98)1, Ol Pejeta (89)1 (91)1, Meru NP (88)1 (03)1, (06)1
Lk Nakuru NP	68	20	20 (1989)	21	Lewa (86)1, Nairobi NP (87)1 (90)4, Solio (87)15	20	Lewa (88)1, Mugie R (04)10, Meru NP (06)9
Masai Mara	33	30	24 (1990)	0	0	1	Nairobi NP (86)1
Nairobi NP	68	62	57 (1989)	35	Kitengela (63-68)5, Kapiti (63-	73	Amboseli (83)1,

Subpopulation	N (2007)	n (sample size)	Lowest population size	Total number translocated in (61-08)	Origin of rhinoceros (Year - in two digits) and Number	Total number translocated out (61-08)	Recipient location (Number and Year)
					68)5, Aberdares (81)3, Nyeri Forest (63-68)4 (78-80)4, Darajani (63-68)8, Kiboko (63-68)2, Masai Mara (86)1, Amboseli (83)1 (88)1, Tsavo East NP (99)1		Ol Pajeta (92)4, Lk Nakuru (87)1 (90)4 Lewa (84)1 (85)1, Ngulia (92)6 (93)3, Ol Jogi (99)2, Meru NP (06)10, Tsavo East NP (93)4 (96)11 (99)11, Tsavo West NP (90)1 (91)1 (92)6, Mugie NP (04)6
Ngulia Rhino Sanctuary	68	23	9 (1989)	20	Other Areas (93)3, Nairobi NP (90)1 (91)1 (92)6 (96)1, Ol Jogi (89)1, Bura (86)3, Kibwezi (85)3, Tsavo West NP (89)1,	25	Nairobi NP (04)6
Ol Jogi Ranch	27	15	10 (1989)	12	Solio (89)2 (94)2 (07)2, Kibwezi (79)2, Ol Pajeta (07)2, Ol Jogi (99)2	13	Lewa (93)4, Ol Pajeta (07)4, Tsavo East (97)1 (99)4
Ol Pejeta Ranch	77	37	4 (1989)	48	Nairobi NP (92)4, Solio (89)3 (93)8 (07)28, Lewa (89)1 (91)1, Ol Jogi (07)4	3	Lewa (91)1, Ol Jogi (07)2

Subpopulation	N (2007)	n (sample size)	Lowest population size	Total number translocated in (61-08)	Origin of rhinoceros (Year - in two digits) and Number	Total number translocated out (61-08)	Recipient location (Number and Year)
Solio Ranch	69	28	49 (2002)	26	Kiboko (70)5 (74)1, Darajani (74)1, Tsavo East NP (71-77)3, Isiolo (72)1, Rimuruti (80)1, Kibwezi (78)1, Embu (71,80)2, Lamuria (75,79)9, Nyeri Forest (74)1 (80)2	89	Ol Pejeta (89)3 (93)8 (07)28, Lk Nakuru (87)15, Lewa (84)3 (90)1 (94)5, Aberdares (94)2, Ol Jogi (89)2 (94)2, Tsavo East NP (94)16 Mugie (04)4
Tsavo East NP	50	20	2 (1989)	59	Darajani (63)8, Solio (94)16, Nairobi NP (93)4 (96)11 (98)1 (99)11, Ngulia (96)1, Ol Jogi (96)1 (97)1 (99)4, Lewa (98)1	4	Solio (71, 77)3, Nairobi NP (99)1
Mugie Ranch	25	0		20	Solio (04)4, Nairobi NP (04)6, Lk Nakuru (04)10	0	
Meru NP	17	0		27	Mt Kenya (81)6, Lewa (88)1 (03)1 (06)1, Nairobi NP (06)10, Lk Nakuru NP (06)9	0	
Addo NP (RSA)					Kibwezi (61-62)7	0	

Nairobi National Park, Solio Game Ranch and Lake Nakuru National Park are the nucleus populations accredited with the successful rehabilitation of the black rhinoceros in Kenya from about 280 animals in the late 1970's to the current size of over 600 animals (**Figure 1.5**). Demographically, these three populations have influenced significantly the vital change rates in recipient sanctuaries. Genetically, they are the recent contact points where all rhinoceros genotypes in Kenya got mixed.



Year	1970	1980	1984	1987	1991	1992	1994	1995	1997	1999	2001	2003	2005	2008
Africa	65000	14785	8800	3665	3450	2475	2550	2410	2600	2700	3100	3606	3725	4180
Kenya	20000	1500	550	381	398	414	417	420	424	423	430	459	539	650

Figure 1.5: Black rhinoceros population size trend across Africa and in Kenya from 1970-2008 in a $\log_{10}$ scale, showing the sharp decline and slow recovery. Data source: Okita-Ouma *et al.*, (2007) and IUCN (2008).

1.2 GENERAL BACKGROUND ON GENETIC DIVERSITY

Genetics is the science of understanding heritable variations in living organisms. Not all heritable variations in a population are expressed phenotypically. Hence, it is impossible to visually discriminate between individual's variations, thus the need to examine individuals at the molecular level in order to infer their finer heritable variation. Deoxyribonucleic acid (DNA) is the main hereditary molecule found in the chromosomes of all eukaryotic cells. Some DNA is also found in the mitochondria (Avise, 1994). The hereditary materials are given different terms at different organizational levels; thus the gene (**Figure 1.6**) is the basic unit of inheritance and corresponds to a union of genomic sequences encoding a coherent set of potentially overlapping functional products (Gerstein *et al.*, 2007). A locus is a known segment of DNA, and could even be an entire gene or in the case of microsatellites simply a defined segment of DNA that may have no functional products. Alleles are different forms of the same locus or gene that differ in DNA base sequences, e.g. A_1, A_2, A_3, etc being different alleles found in the same locus (Frankham *et al.*, 2002). Genotypes are the combination of alleles present at a locus in an individual e.g. A_1A_1, A_1A_2, A_2A_2 etc. Genotypes are homozygote (A_1A_1, A_2A_2) or heterozygotes (A_1A_2). Sometimes, genotypes could be compound including two or more loci, e.g. $A_1A_1B_1B_1$, $A_2A_2B_1B_2$,.

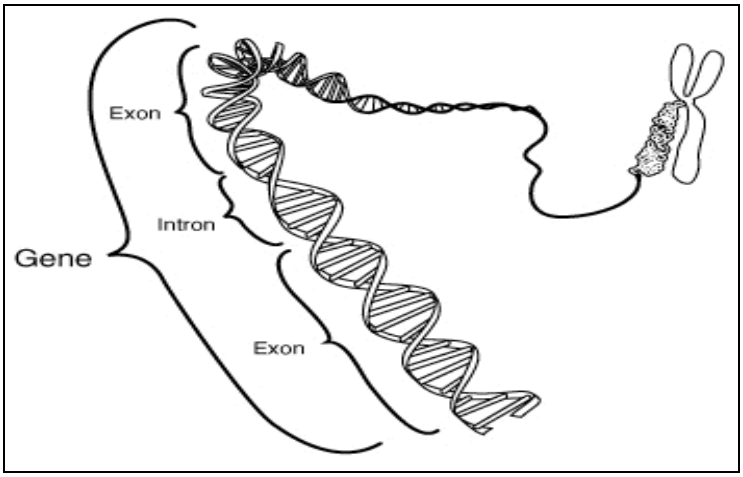


Figure 1.6: A simple structure of the gene. (Source: National Human Genomic Research Institute; www.genome.gov//Pages/Hyperion/DIR/VIP/ Glossary/Illustration/gene)

Genetic diversity is the extent of heritable variation in a population, or species, or across a group of species (Frankham *et al.*, 2002). It is gives populations the ability to evolve in response to ever changing environmental conditions (Lacy, 1997).

1.2.1 Choice of genetic markers for genetic diversity studies

In the absence of direct DNA sequences linking genotype to phenotype in natural populations, genetic markers are used to investigate populations characteristics at genomic level; either to measure genetic variation or to identify genetic variants linked to specific characters (such as disease or some other phenotype characteristics). A genetic marker is a molecular character which may or may not have been characterized at the sequence level (e.g. mitochondrion DNA), but which tell us something about the underlying genetic structure of the individual, population or species being studied (Freeland, 2005). There are a large variety of molecular markers available that have become available in the last forty years and these enable

studies of population and evolutionary genetics in a wide range of organisms, including conservation genetic studies of endangered species (Frankham *et al.*, 2002). Examples of currently popular molecular markers include microsatellites and mitochondrial DNA sequence.

Microsatellites are short tandem repeats (STRs) or simple sequence repeats (SSRs) that are highly polymorphic nuclear DNA markers which are abundant in the eukaryotic genome (Blouin *et al.*, 1996; Dakin and Avise, 2004). Through microsatellite markers studies, scientists have been able to determine various underlying population characteristics including population structure and differentiation (Pritchard *et al.*, 2000; Balloux and Lugon-Moulin, 2002; Laroche and Durand, 2004; Harley *et al.*, 2005), individual identities (Blouin *et al.*, 1996; Reed *et al.*, 1997), estimate relatedness (Goossens *et al.*, 2005; Robinson *et al.*, 2005), pedigrees (Paetkau *et al.*, 1997; Orlando *et al.*, 2003), estimate census (Goossens *et al.*, 2006) and effective population sizes (Nunney and Campbell, 1993). Recent genetic studies in rhinoceros have isolated and characterized microsatellite markers for *Diceros bicornis* (Brown and Houlden, 1999; Cunningham *et al.*, 1999; Nielsen *et al.*, 2007) and for related rhinoceros species (Florescu *et al.*, 2003; Zschokke *et al.*, 2003; Scott *et al.*, 2004).

Mitochondrial DNA is a circular molecule containing 37 genes, all of which are essential for normal mitochondrial function (**Figure 1.7**). Thirteen of these genes are involved in oxidative phosphorylation. The remaining genes are transfer RNA (tRNA) and ribosomal RNA (rRNAs) genes. The control region (including the D-

loop or hypevariable region) is a non coding region including the origin of replication and is therefore the most variable region within the mammalian mtDNA genome, characterized by rapid change in sequence and length (Saccone *et al*. 1991).

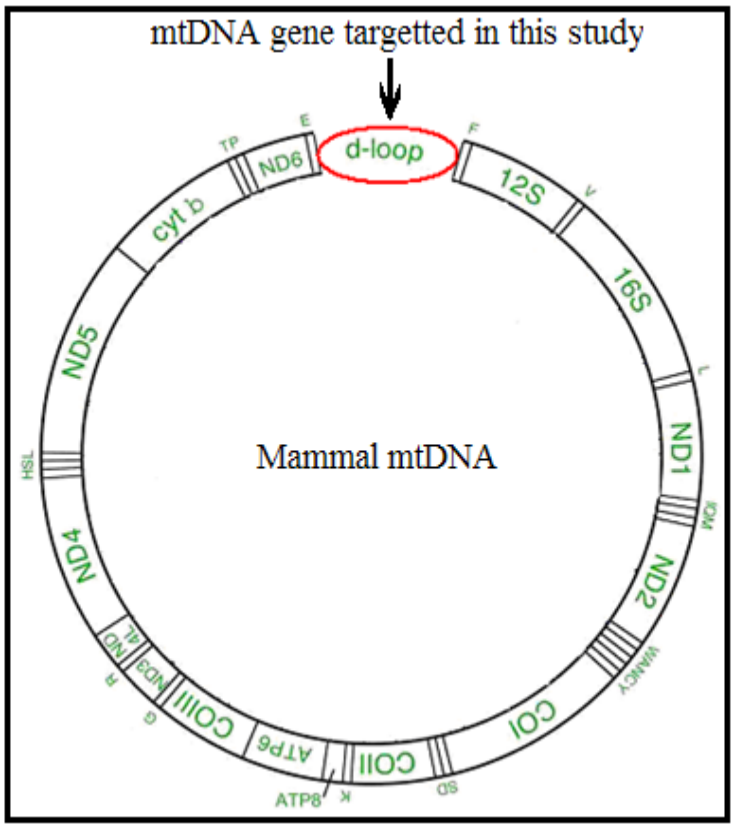


Figure 1.7: Mitochondria DNA showing the D-loop gene targeted in this study. Source: Adapted from Saccone *et al.*, (1991)

Mitochondrial markers were first introduced to population genetics in the 1970's, and prompted a revolutionary shift towards historical, phylogenetic perspectives on intra-specific population structure (Nei, 1987; Slatkin, 1987). Because of the predominantly maternal, non-recombining mode of mtDNA inheritance and rapid evolutionary rate, the molecule often provides haplotypes that can be ordered phylogenetically within a species, yielding intra-specific phylogenies interpretable as a matriarchal component of the organism's pedigree, and which can be augmented by

analysis of phylogenetic networks which can be used to indicate reticulate evolution (Beebee and Rowe, 2007). This region has been particularly applied to studies of genetic variability (Nunney and Campbell 1993), phylogeography (including the black rhinoceros, Brown and Houlden 2000 (O'Ryan *et al.*, 1994; Morales *et al.*, 1997), and assigning evolutionary significant and management units in wildlife management (Moritz 1994).

It is essential that methods for DNA analysis are sensitive and specific enough to be able to detect target DNA in different types of non-invasively collected or archaic samples (e.g. hair, faeces, skin, muscle, bone or horn). Molecular scatology has become the method of choice for most field studies (Kohn and Wayne, 1997).

However, applications have been constrained due to the intensive laboratory approach required to ensure correct genotyping of faecal DNA (Taberlet *et al.*, 1999). Recent research has focused on improving the reliability of results generated from DNA analysis when the source of material yields only tiny amounts of DNA (Reed *et al.*, 1997; Flagstad *et al.*, 1999; Gerloff *et al.*, 1999; Morin *et al.*, 2001). Field samples are subjected to a variety of conditions, which degrade the already small amount of DNA that may be present. Currently, field collection techniques have focused on preserving the sample and reducing the action of degrading enzymes before the sample can reach the laboratory (Wasser *et al.*, 1997; Frantzen *et al.*, 1998).

Since the polymerase chain reaction (PCR) is so powerful that it can amplify target

DNA millions of times, it is essential to avoid degraded or contaminated templates (Taberlet *et al.*, 1996; Goossens *et al.*, 2000). Recent experimental procedures have been developed to reduce genotyping errors (Taberlet *et al.*, 1996; Goossens *et al.*, 2000), in order to avoid the errors of analysis due to the inclusion of nuclear inserts of mtDNA (Sorenson and Fleischer, 1996; Greenwood and Pääbo, 1999), and to identify possible sources of error (Bradley and Vigilant, 2002). Using dung from black rhinoceros as a source of DNA should provide sufficient material for comparative analysis, since fresh rhinoceros dung piles are relatively easy to locate. It is feasible to generate reliable results from rhinoceros dung and this has been demonstrated by (Garnier *et al.*, 2001).

Hence any genetic analysis of the populations studied must include quantitative measures of variability of nuclear as well as mitochondrial DNA in order to interpret historic events such as potential hybridization, population founder events and dispersal patterns. The present study adopts this approach, in investigating mitochondrial variation in the control region, as well as using neutral nuclear polymorphic markers (microsatellites).

1.2.2 Quantifying genetic diversity within a population

One of the simplest measures of genetic diversity is the estimate of allelic diversity (often designated A), which is the average number of alleles per locus (Falconer and Mackay, 1996; Frankham *et al.*, 2002; Freeland, 2005). This measure is sensitive to sample size, and therefore its accuracy is compromised by the number of individuals

screened unless it is bootstrapped or jackknifed. Proportion of polymorphic (P) sites in a population can also give an indication of genetic diversity levels in a population. This measure is of some use in studies based on relatively invariant loci such as allozymes (Falconer and Mackay, 1996). It is also simple and sensitive to sample size. It cannot be used with microsatellites because the basic tenets for choosing a microsatellite is polymorphism at that particular site, hence P= 1 for this marker.

Genotype frequencies are used in various ways to estimate more robust measures of genetic diversity. The simply done by dividing the number of individuals that are heterozygotes at a particular locus by the total number of individuals sampled (Falconer and Mackay, 1996; Frankham *et al.*, 2002; Freeland, 2005). This ratio is called observed heterozygosity (H_o). Gene diversity (h) is another quantity used to estimate genetic diversity (Nei, 1973). This method is much less sensitive to sampling effects when compared to other methods. It is calculated as:

$$h = 1 - \sum_{i=1}^{m} x_i^2$$

Where x_i is the frequency of allele i and m is the number of alleles that have been found at that locus. For any given locus, h represents the probability that two alleles randomly chosen from a population will be different form one another (Nei, 1973; Freeland, 2005). In a randomly mating population, h is equivalent to the expected heterozygosity (He), and represents the frequency of heterozygotes that would be expected if a population was in Hardy-Weinberg Equilibrium (HWE), and for this reason h is represented as He. Most calculations of He will be based on multiple loci, in which H_e is calculated for each locus and then averaged over all loci to

present a single estimate of diversity for each population (Nei, 1973; Freeland, 2005).

The HWE principle (**Figure 1.8**) propose that allele and genotype frequencies will remain at an equilibrium over time so long as there is random mating (panmixia) in a infinitely large population with no disturbing forces such as mutations, migrations, selection, genetic drift, linkage disequilibrium or chance (Raymond and Rousset, 1997; Freeland, 2005). Hence, the HWE provides a null hypothesis that makes it possible to detect if a population has non random mating, migration or selection. Non-random mating could be based on ancestry (inbreeding and crossbreeding) or could be genotype at particular loci (assortive and dissortive mating). This principle is the basis of conservation genetics applied to wildlife populations. Obviously, populations are always being affected by one or more of these disturbing forces, but HWE genotype frequencies provide a benchmark to gauge genetic diversity in a population (Mills, 2007). If the population is out of HWE, efforts are made to establish why; thereby taking the first step towards exposing mechanisms acting on a population's genetic composition (Mills, 2007).

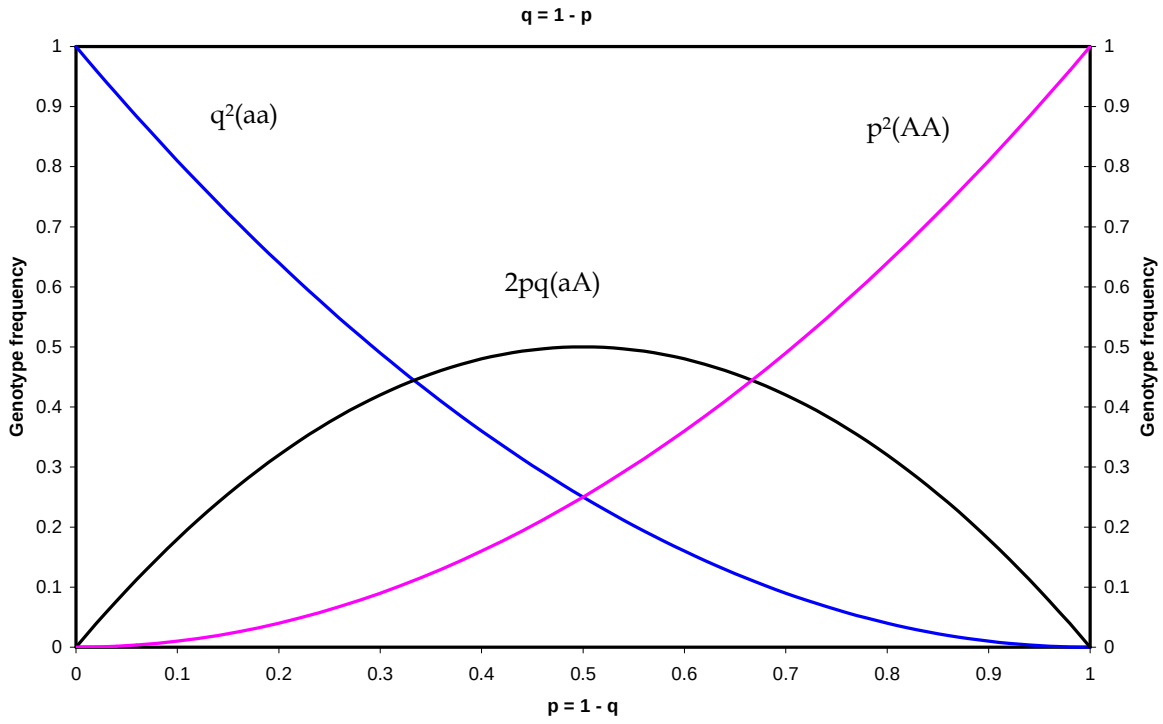


Figure 1.8: The combination of homozygote and heterozygote frequencies that can be found in populations that is in HWE. The frequency of heterozygotes is at maximum when p=q=0.5. When the allele frequencies are between 1/3 and 2/3, the genotype with the highest frequency will be the heterozygote. Modified from Freeland (2005)

Human error as a result of improper sampling constitutes the first source of genotyping errors that could result in genotypic or allelic frequency deviation from HWE (Falconer and Mackay, 1996; Freeland, 2005; Mills, 2007). Inadequate sampling will lead to flawed estimates of allele frequencies making HWE conclusions unreliable. Presence of null alleles is another reason why reason why observed heterozygosity may be lower than expected heterozygosity (Goossens *et al.*, 2000; Dakin and Avise, 2004; Chikhi and Bruford, 2005). This happens mostly where only one allele of a heterozygote is amplified during polymerase chain reaction (PCR) and hence genotyped erroneously as a homozygote (Pompanon *et al.*,

2005). This is known as allelic drop out (Dakin and Avise, 2004; Wehausen *et al.*, 2004) and can be corrected by adopting a multi-samples, multi-extracts approach for microsatellite analysis (Goossens *et al.*, 2000). If observed heterozygosity is significantly less than expected heterozygosity, then there is the possibility that the samples are from two or more randomly mating populations that have different allele frequencies, and the proportions of homozygotes is higher in the aggregate sample mean than it would be if the populations were analysed separately. This is known as the Wahlund effect (Frankham *et al.*, 2002; Freeland, 2005).

If neither null alleles nor Wahlund effect were responsible for an observed heterozygosity deficit, then assortative mating is probably taking place. It is important to eliminate deviations from HWE that are associated with assortive genotypes (linkage disequilibrium) in order to remain with only ancestry associated deviations (Nei, 1973; Falconer and Mackay, 1996; Frankham *et al.*, 2002; Freeland, 2005; Mills, 2007). Linkage disequilibrium can occur for a number of reasons, the most common being the proximity of two loci in a chromosome. Therefore, in the analysis of multiple loci, it is always prudent to test for linkage disequilibrium before ruling out the possibility that there are fewer independent loci for genetic analysis than expected (Falconer and Mackay, 1996).

1.2.3 Causes of low genetic diversity in small populations

Generally, larger populations are likely to harbour more individuals with variable alleles (Nei, 1973; Falconer and Mackay, 1996; Frankham *et al.*, 2002; Freeland,

2005; Mills, 2007). When they are broken down into smaller units by e.g. habitat fragmentation, catastrophe, poaching, pollution or climatic change etc., the new subpopulation will only contain a subset of the alleles and hence have a lower genetic diversity compared to larger original population.

Individuals in small populations are more likely to breed with their close relatives because they have no alternative choice for mates. This increases the chance of rare and injurious recessive alleles coming together and confer homozygous disadvantage to the inbred offspring. Inbred offspring have low total fitness and tend to die young further affecting the demography of the small population and thus making it smaller. Certainly, the inbred offspring will take away with them some rare alleles and erode further the extant weak genetic diversity in the subpopulation (Mills and Tallmon, 1999). Studies have reported greatly elevated rates of extinction in inbred populations of laboratory and domestic animals and plants (Frankham and Ralls, 1998; Frankham *et al.*, 2002). Their sizes reduce further as they continue to inbreed (Frankham *et al.*, 2002). Therefore, there is a feedback between reduced population size, loss of genetic diversity and inbreeding, popularly referred to as the extinction vortex (**Figure 1.9**).

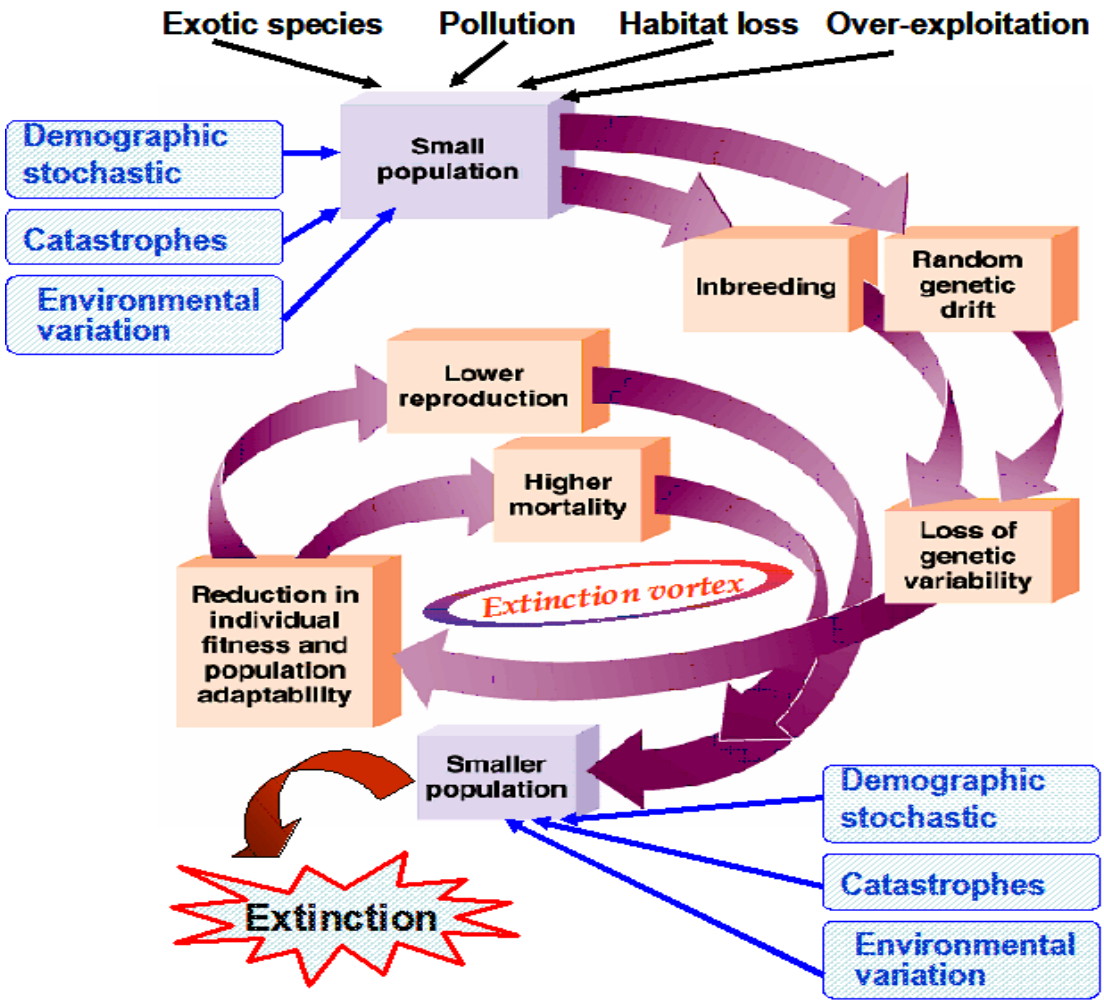


Figure 1.9: Model of the extinction vortex; demonstrating the possible links between human impacts, inbreeding, loss of genetic diversity and demographic instability in a downward spiral towards extinction. Redrawn from Frankham *et al.*, (2002).

Small populations of a classically k-selected species like that of a large mammal are susceptible to stochastic demographic threats such as biased sex ratio, slow life history and fluctuation in individual reproduction (Garnier *et al.*, 2001). Further, random environmental factors such as disease epidemics and natural catastrophes may result in further population contraction (Gilpin and Soulé, 1986; Foose and Seal, 1991; Foose, 1992).

In the wild, inbreeding and loss of genetic diversity has been shown to be the major cause of extinction of seven butterfly populations in Finland in 1996 (Saccheri *et al.*, 1998). After accounting for the effects of demographic and environmental variables in all the 42 butterfly populations that were under study, extinction rates were higher in populations that had lower heterozygosity; an indication of inbreeding (Saccheri *et al.*, 1998). The majority of extinctions of plants and animals have occurred in island species, and more endemic species become extinct compared non-endemic species (Myers, 1979; Newman, 1995) because gene flow into island populations is limited. Circumstantial evidence points to inbreeding and loss of genetic diversity as contributing to the extinction proneness of island populations of many species. The greater extinction likelihood in endemic than non-endemic island species is predicted more by compromised genetic diversity than by demographic and ecological considerations (Myers, 1979; Newman, 1995; Frankham and Ralls, 1998).

Genetic drift is a process that causes a population's allele frequency to change from one generation to the next simply as by chance (Freeland, 2005). This is more likely to happen where reproduction success within a population is variable, with some individuals producing more offspring than others. As a result, not all alleles will be reproduced at the same extent, and therefore allele frequency will fluctuate from one generation to the other in a non-adaptive random manner (Freeland, 2005). Therefore evolutionary changes that occur in populations that experience genetic drift are non-adaptive. Drift effects are more profound in small populations where in the absence of selection, drift will drive each allele to either fixation or extinction

within a relatively short period of time, and therefore the overall effect will be to decrease genetic diversity (Myers, 1979; Newman, 1995; Moehlman *et al.*, 1996; Frankham *et al.*, 2002; Freeland, 2005; Allendorf and Luikart, 2007). Genetic drift is therefore influenced more by the effective size of a population rather than the census size of a population.

A small and isolated large mammal population may still retain relatively high levels of genetic diversity for a long period of time, particularly if it is a relic of a once large outbred population that has undergone recent decline or if genetic drift is being opposed by balancing selection or by selection against inbred individuals (Coltman *et al.*, 1999; Chikhi and Bruford, 2005; Goossens *et al.*, 2006). Conservation of small populations of large wildlife species such as the black rhinoceros should take these potentially serious genetic threats into account (Amos and Hoelzel, 1992).

One way of reducing the effects of genetic drift in small populations is by maximizing their effective population size (Frankham and Ralls, 1998), through managing them as metapopulations. This can be achieved partly by translocating individuals between the few surviving populations of an endangered species. However, it may not always be advisable to translocate individuals between genetically distinct sub-populations because this could lead to the break-up of allelic combinations that have been combined through local adaptation in the remaining sub-populations (Allendorf and Leary, 1986). The inadvertent mixing of genetic material from locally adapted populations may lead to outbreeding depression (Templeton, 1986; Saccheri *et al.*, 1999), which counters the aims of any

conservation plan (Swart and Ferguson, 1997). The cautious management of endangered species therefore requires thorough knowledge of the genetic structure of the component populations.

1.2.4 Demographic and genetic considerations in the long term survival of small and isolated populations

Population size is a key factor in defining the demographic characteristics of any population. The World Conservation Union (IUCN) uses population size as a key attribute in designating the conservation status of any species. A population with less than 50 mature adults is considered critically endangered with extinction (Freeland, 2005). Vital processes that influence population size include birth, immigration, death and emigration rates. However, the magnitudes of these rates themselves are dependent on the initial size of the population (Mills, 2007). Natural populations of most species are structured into separate local random mating units (demes) and these units gives rise to primary genetic variations within and/or between a local population (Allendorf and Luikart, 2007). Understanding population genetic structure is therefore essential for identifying population conservation units (Moritz, 1994).

1.2.5 Previous studies on the black rhinoceros genetic diversity

Investigations on the genetic variation in the extant black rhinoceros populations in Southern Africa in late 1980s were conflicting, initially suggesting that *D. bicornis* was lacking in genetic variation (Osterhoff and Keep, 1970; Merenlender *et al.*,

1989; Ashley *et al.*, 1990). Furthermore, Ashley *et al.* (1990) did not detect significant regional differentiation in mtDNA. In contrast, Swart (1994) showed that the southern African *Diceros bicornis bicornis* retained a degree of genetic variation resembling that of large outbred population, although he did not perform a geographic analysis of genetic variation. Swart and Ferguson (1997) who studied the Etosha population in Namibia, performed a geographic analysis on the species and confirmed previous taxonomic hypotheses that southern African *Diceros bicornis* comprised two subspecies; *Diceros bicornis bicornis* in the west and *Diceros bicornis minor* in eastern southern Africa. Although the two taxa did not differ discretely, there appeared to be an east-west cline in genetic variation, suggesting that in order to maintain the genetic structure of the species in southern Africa, individuals from the eastern side of the subcontinent should not be translocated to the western side and *vice versa*.

Morales and Melnick (1994) examined the phylogeny of the living rhinoceros through molecular systematics using blood samples from five black rhinoceros and found no genetic variation within the southern Africa (South Africa and Zimbabwe black rhinoceros) populations. However this could be due to the limited power of restriction enzymes to detect intraspecific variation in mtDNA (12 and 16s ribosomal RNA) because of the less variable nature of ribosomal genes (Brown and Houlden, 2000).

Studies of the control region of the mtDNA genome have been shown to give contradicting results on genetic variation in black rhinoceros at subspecies level.

Ashley *et al.,* (1990), O'Ryan and Harley, (1993) and O'Ryan *et al*., (1994) found little or no genetic differentiation between the subspecies, while Swart and Ferguson (1997) revealed a pattern of differentiation among three *Diceros bicornis minor* populations and *Diceros bicornis bicornis* (from Etosha). Brown and Houlden (2000) detected high levels of variation (2.6%) in mtDNA control region between *Diceros bicornis minor* and *Diceros bicornis michaeli* subspecies. The differences in results here stem from the use of different mtDNA markers. The best resolution was obtained using control region sequences.

Using microsatellite data from nine loci and 121 black rhinoceros individuals, Harley *et al.,* (2005) demonistrated that *Diceros bicornis michaeli* retained the most genetic diversity (heterozygosity = 0.675) compared with *Diceros bicornis minor* (heterozygosity = 0.459) and *Diceros bicornis bicornis* (heterozygosity = 0.505). Harley *et al.,* (2005) therefore managed to show that there is detectable genetic variation in the extant rhinoceros population that is sufficient to infer the impact of known bottlenecks in these populations. The sample size in this study was about 50% of the census population size of 582 individual in the 12 subpopulations selected for this study. It is therefore expected that such a sample size will yield sufficient information that will be used to detect finer genetic variation between the Kenyan black rhinoceros subpopulations. This will improve the information on the genetic structure of the extant eastern black rhinoceros (*Diceros bicornis michaeli*) subspecies which is scanty.

1.3 PROBLEM STATEMENT

Kenyan black rhinoceros have been translocated across the country since 1960s. These translocations were in most cases necessary to save isolated individuals that were in most cases stranded in areas opened up for agriculture and/or settlement. There were no genetic considerations given to these translocations prior to their implementation. Further, there are no comparative genetic studies that have being carried out in the 14 black rhinoceros subpopulations in Kenya to examine if these translocations had any effect on the subpopulations' genetic diversity. Even though there are no obvious phenotypic indicators of reduced fitness in any of the 14 subpopulations that may suggest reduced genetic diversity, this does not imply that genetic diversity in these subpopulations has not been affected, and that the translocations carried out to date have not resulted in maladaptive gene combinations that could potentially lead to outbreeding depression. Hence, lack of this knowledge may jeopardize the Kenya Wildlife Service's (KWS) goal of attaining a minimum population growth rate of 5% per year and reaching the expected total of 650 rhinoceros by 2010 and 1000 rhinoceros by 2020 by managing the sub-populations as metapopulations through regular translocations (Okita-Ouma *et al.*, 2007). So far, no empirical studies have been done to assess whether the Kenyan black rhinoceros subpopulations are genetically distinct and whether they should be treated as separate evolutionary significant units, or whether there exists any signature of genetic admixture and or loss of diversity.

1.4 JUSTIFICATION OF THE STUDY

A great deal of valuable research on the black rhinoceros in Kenya has been conducted already, and is currently being applied in the management of this species. However, this has mainly concentrated on ecology (Goddard, 1967, 1970; Oloo *et al.*, 1994; Mukewa, 1995; Muya and Oguge, 2000; Patton and Jones, 2007), behaviour (Goddard, 1966; Morinte and Keter, 2000), breeding performance (Wanjohi, 1987; Brett, 1998; Okita-Ouma, 2004), diseases (Obanda *et al.*, 2008), management (Brett, 1993; Okita-Ouma *et al.*, 2007) and security (Leader-Williams, 1992; Martin and Vigne, 2003), while relatively little has been done on their genetics (Swart *et al.*, 1994; Brown and Houlden, 2000; Scott, 2008). Yet, genetic diversity especially of small population and its distribution within and between populations is as important as any other factor in assessing the overall conservation status of a species (Frankham, 1995; Freeland, 2005; Bergl *et al.*, 2008). It is therefore relevant to study the genetic diversity in black rhinoceros populations in Kenya in order to gain an understanding of their genetic status. This information will then be used by the Kenya Wildlife Service to develop long-term conservation strategies to manage this critically endangered species, taking into account their genetic differences and/or similarities.

1.5 RESEARCH QUESTIONS

The following research questions were examined in this study:

1. What are the current levels of genetic diversity in the Kenyan black rhinoceros subpopulations?

2. How long have the Kenyan black rhinoceros subpopulations been isolated from each other?
3. What is the pattern and extent of genetic variation within these populations?
4. What are the genetic and demographic responses of the black rhinoceros populations to habitat fragmentation and anthropogenic pressure (poaching) by?
5. How related are the animals within and among subpopulations?
6. How can genetic data be integrated into management of the black rhinoceros populations?
7. What is the impact of translocations?

1.6 RESEARCH HYPOTHESIS

This study tested the following hypotheses about Kenyan black rhinoceros population genetics:

1. That genetic differentiation among black rhinoceros populations in Kenya is proportional to their known demographic history and genetic exchange i.e. that those populations that have remained small and isolated have lower levels at genetic diversity and high inbreeding coefficients, but that larger populations and those that have received recent translocations have higher diversity and lower inbreeding coefficient.

2. That those populations known to have received immigrants from diverse geographic regions show the highest genetic diversity and signature of

admixture (i.e. Linkage disequilibrium and deviation from Hardy-Weinberg Equilibrium due to Wahlund effects)

3. That mitochondrial and nuclear marker recapitulates the same genetic scenario since they undergo similar evolutionary and selection pressure

1.5 STUDY OBJECTIVES

General objective

The overall goal of this study was to assess and determine the genetic diversity within and among black rhinoceros subpopulations in Kenya and generate information for use in the formulation of metapopulation management strategies of black rhinoceros in Kenya

Specific objectives

The specific objectives of the study were to:--

1. Determine the current level of genetic variations in the Kenyan black rhinoceros subpopulations.
2. Determine the extent at which the present genetic variation is partitioned in Kenyan black rhinoceros subpopulations.
3. Determine whether mitochondrial and nuclear markers recapitulate the same genetic scenario.

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CHAPTER TWO

2.1 GENERAL METHODOLOGIES

2.1.1 Study site

295 samples were collected from out of the 14 isolated black rhinoceros subpopulations in Kenya (**Table 2.1** and **Figure 2.1**). Two subpopulation; Meru National Park and Mugie Ranch; were not sampled because they were seeded recently. For example, Mugie subpopulation was seeded by animals from Solio Ranch, Nairobi National Park and Lake Nakuru National Park in 2004, while all the animals taken to Meru National Park before 2006 were poached and the current population was seeded from Nairobi National Park, Lewa Wildlife Conservancy and Lake Nakuru National Park in 2006. So sampling the source subpopulation captured the genetic diversity in the seeded subpopulations.

The samples included tissues, serum and dung. The 12 subpopulations that were sampled are completely separated from each other by human settlement. Even the ones that are within other conservation areas are completely ring fenced by an electric fence (e.g. Ngulia and Tsavo West National Park) at the time of sampling.

Table 2.1: Location, acreage in square kilometers, sample size and amplification information of the 12 sampled Kenyan black rhinoceros subpopulations.

Location	Area ($Km^{2)*}$	N	n	Type	Dn	+Ve n	n mtDNA	n msat
Aberdares NP	100	30	9	Dung	18	5	5	5
Chyulu NP	471	21	9	Dung	18	4	3	6
Laikipia WC	397	12	9	Dung	18	3	3	3
Lewa WC	247	55	33	Dung	66	27	23	28
Lake Nakuru NP	144	68	20	Tissue	20	15	13	14
Masai Mara GR	1510	33	30	Tissue	30	14	10	16
Ngulia RS	90	68	23	Dung	46	30	15	14
Nairobi NP	117	68	62	Tissue	62	51	28	35
Ol Jogi RH	249	27	15	Dung	30	12	10	10
Ol Pajeta RH	300	77	37	Tissue	37	33	30	30
Solio RH	69	69	28	Tissue	28	26	25	26
Tsavo East NP	13,747	50	20	Dung	40	5	5	5
Total		578	295		413	225	170	192

GR = Game Reserve, NP = National Park, RH = Ranch, WC = Wildlife Conservancy, N = Census population size, n = Sample size, Dn = Working sample size after extracting dung samples twice +Ve n = Number of samples that shown presence of DNA in Agarose gel test., n mtDNA = Number of samples that amplified with mitochondria DNA markers, n msat = Number of samples that amplified with microsatellite markers., *The acreages indicated refer to the area used for black rhinoceros conservation.

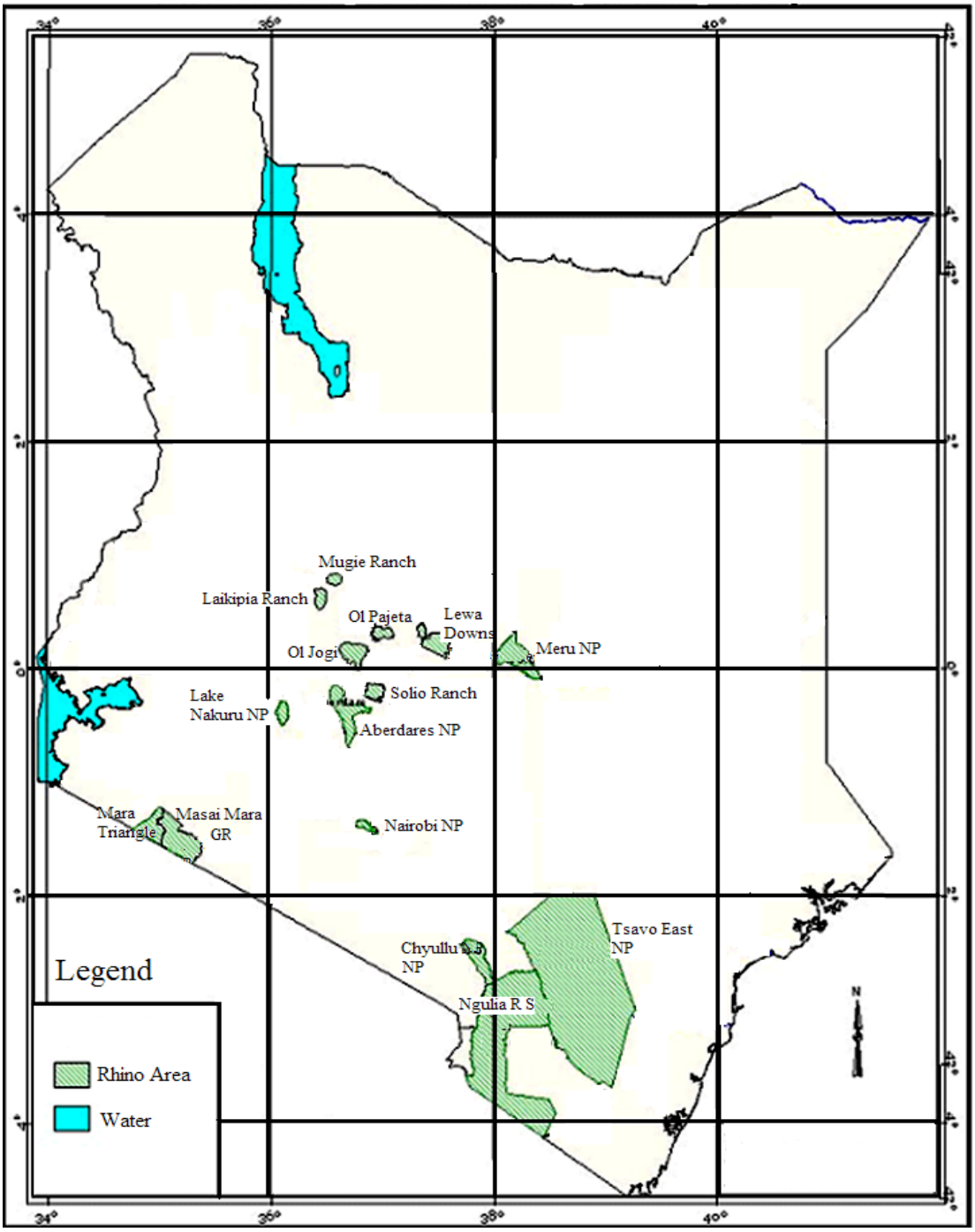

Figure 2.1: Locations of 14 black rhinoceros subpopulations in Kenya. Only 12 subpopulations Laikipia WC were sampled for this study. Mugie Ranch and Meru National Park subpopulations were not sampled. Map Source: KWS GIS Department.

2.1.2 Sampling plan

The bottleneck model developed by Frankel and Soulé (1981) suggest that of estimating heterozygosity retention in a population to be approximately equal to 1 – 1/(2N); where N is the population size after the bottleneck. This model predicts that 10 individuals are able to retain 95% of the genetic variation of the original population after a bottleneck. Since all the subpopulations in Kenya have undergone bottleneck, the target minimum sample size for this study was at 10% of the census subpopulation size. It was envisaged that this sample size target will capture sufficient genetic variation in each subpopulation to draw reliable inferences. In some subpopulation the samples sizes were more than 10%, but some did not amplify after priming. The metapopulation sample size was almost 50% of the census population size and therefore adequate.

Sampling was carried out in collaboration with Kenya Wildlife Service (KWS). Staff from KWS Veterinary Department regularly collects biopsy from various wildlife carcasses in Kenya that they come across during their work in the field. During black rhinoceros ear notching or translocation programmes, veterinary staff also collects whole blood in serum tubes and a small piece of ear lobe tissue

The samples are usually stored at -20^{o}C in 70% ethanol or at room temperature in 25% DMSO at the KWS Head Offices Veterinary Laboratory. As a consequence, the KWS veterinary laboratory has a large collection of tissue and serum samples of different animals that are catalogued and stored. From their large collection of biopsies, 177 samples of black rhinoceros tissues, serum and whole blood were sampled for this study. Some serum and tissue samples may have degenerate chemically even though

physically they were looking good and hence yielded very low DNA (**Table 2.1**). The degeneration may have been caused by the frequent thawing and freezing of the samples due to frequent power blackouts experienced in Kenya.

Dung sampling in Kenya was facilitated by the KWS rhinoceros Management Programme. The programme has KWS staff located in every conservation area that has rhinoceros. Their main responsibility is surveillance and monitoring of rhinoceros (**Plate 2.1, F**). Over time, these staff have established the identity of each rhinoceros its ranging patterns and its core territory. Therefore, it was possible to collect dung samples from different rhinoceros in each sanctuary with minimum error of double sampling. Black rhinoceros dung is unique in structure and smell from the dung of other browsers and is hence easily identified. Black rhinoceros and elephant dung can be confused in few instances especially when a rhinoceros drops a small bolus of dung outside its regular midden especially when scared or in flight.

Plate 1: Pictorial illustrations of black rhinoceros dung sampling activities in Kenya. A – Wandera, Lindsey (partly hidden) and Corynne spotting black rhinoceros in Ol Jogi, B – black rhinoceros in Lewa, C - Muya breaking a sampling stick, D – Muya using a sampling stick to fill a sample bottle with black rhinoceros dung, E – Lindsey training field staff on dung collection techniques, F – rhinoceros surveillance team at Laikipia Ranch

Sanctuary management were informed at least one month's prior to any sampling visit, giving them ample time to intensify their monitoring efforts and increase their certainty on the location of all the rhinoceros in their area. This made it possible for maximum sampling to be carried out with reasonable effort. Fresh dung samples were targeted, emphasizing the exterior portion of the bolus containing a mucous lining using non-reusable pieces of stick (**Plate 2.1**). Rhinoceros have a unique usage of their toilets which they also use to mark their territory and hardly any two rhinoceros share a toilet. Immature rhinoceros that are still under the care of their mothers are except, but the young animals tend to drop their faeces at the peripheral of the toilet. Despite planning, weather changes or variation in rhinoceros movement sometimes made sampling activity difficult. In this case, unit team leaders were trained on sampling techniques (**Plate 2.1**) and informed on the optimum number of samples required from their area of jurisdiction and given sampling material to continue sampling. Each conservation area had a different target based on the population size.

DNA was extracted from 295 samples from Kenya; 118 extracts from dung and 177 extracts from serum and/or tissue. The both the tissue and dung samples were collected in duplicate, but only the dung samples were extracted in duplicate as a measure for improving genotyping accuracy as stipulated in various protocols (Navidi *et al.*, 1992; Taberlet *et al.*, 1996; Goossens *et al.*, 2000; Valière *et al.*, 2007), so cumulative samples used were 413 (**Table 2.1**). The duplicate tissue sample was necessary just in case the other sample did not yield good DNA. The samples were fairly well distributed across the 12 initial black rhinoceros sub-populations.

2.1.3 Reliability of noninvasive genetic sampling

Noninvasive genetic sampling refers to the remote gathering of target DNA from material outside the skin (hair or feathers), or material sloughed, shed or passed outside the body (faeces, urine, regurgitated pellets, **Plate 2.1**), without having to capture or disturb the animal. Non-invasive sampling is thus viewed as a gentler, noninjurious approach to studying animal populations, and hence, more ethically and scientifically acceptable because it has less negative effects on research subjects (Taberlet *et al.*, 1999).

Debate stills ranges on about the accuracy of genotypes generated from samples collected non-invasively, especially where the samples are likely to have low quantities of target DNA (e.g. hair) and are laden with genetic material of other non-target species (Taberlet *et al.*, 1999; Miquel *et al.*, 2006; Valière *et al.*, 2007). Several studies have, however, concurred that hair or dung sampled noninvasively is a sufficient sources of target DNA (Bayes *et al.*, 2000; Fernando *et al.*, 2003; Broquet and Petit, 2004; Maudet *et al.*, 2004; Roon *et al.*, 2005; Okello *et al.*, 2008). Broquet *et al.,* (2007) demonstrated clearly that genotyping success did not differ between hair and faecal extracted DNA, and success in faeces-based analyses was not consistently influenced by the diet of focal species. They concluded that factors other than the source of DNA must be contributing to the variability they witnessed in their review. Dung is now generally accepted as a source of DNA, and appropriate measures have been designed to limit genotyping errors due to low DNA quantity (Fernando *et al.*, 2003; Dakin and Avise, 2004; Hoffman and Amos, 2005; Broquet *et al.*, 2007).

Pompanon *et al.,* (2005) reviewed in depth the potential causes of genotyping errors, consequences and solutions. They grouped causes into four broad categories: -- 1.Variation in DNA sequence, 2. Low quality or quantity of the DNA, 3. Biochemical artefacts and 4. Human factors.

2.1.3.1 Variation in DNA sequence

Errors linked to the DNA sequence are generated by mutations in the flanking sequence of the template DNA involved in the marker-detection process (Callen *et al.*, 1993; Paetkau and Strobeck, 1995). These errors result in production of null allele genotypes. Primer mutation also causes production of null alleles; either by failing to amplify one allele – usually the larger allele (Oosterhout *et al.*, 2004) or generate a different sized allele – a false allele – a condition called size homoplasy[2]. Usually, only substitutions close to the 3′ end of the primer or insertions or deletions cause problems. Either way, the resulting genotype will be different from the actual target species genotype (Navidi *et al.*, 1992; Taberlet *et al.*, 1996; Gagneux *et al.*, 1997; Goossens *et al.*, 2000; Broquet *et al.*, 2007).

2.1.3.2 Low quantity or quality of DNA

Low DNA quantity and/or quality is also likely to increase genotyping errors (Callen *et al.*, 1993; Taberlet *et al.*, 1996; Flagstad *et al.*, 1999; Maudet *et al.*, 2004; Broquet *et al.*,

[2] A homoplasy is a character that is shared by multiple species due to some cause other than common ancestry. West-Eberhard, M. 2003. *Developmental Plasticity and Evolution*. Oxford Univ. Press., p. 353–376.

2007). Extreme dilution of DNA extract or degradation of the target DNA molecules in the extract will result in low concentration of good quality DNA molecules (Fernando *et al.*, 2003; Pompanon *et al.*, 2005). This increases the probability of contaminant molecules getting amplified (Taberlet *et al.*, 1996) resulting in the production of null alleles genotypes and stutter bands from unused primer (Oosterhout *et al.*, 2004).

2.1.3.3 Biochemical artifacts

Sometimes, during the elongation phase of a PCR, Taq polymerase has a tendency of adding non-template nucleotide (usually an adenine) to the 3′ end of the newly synthesized strand (Magnuson *et al.*, 1996; Brownstein *et al.*, 2006). This '+A artefact' is common, and creates an artefactual band or peak on the readout gel or trace (Pompanon *et al.,* 2005). The relative proportions of the true fragment and the +A artefactual fragment are very sensitive to the sequence of the 5′ end of the primer used in the genotyping assay and the PCR conditions used, especially long elongation times that promote the +A artefact. This biochemical artefact produces a null allele causing genotyping error (Magnuson *et al.*, 1996; Pompanon *et al.*, 2005; Brownstein *et al.*, 2006).

2.1.3.4 Human error

Human factors constitute the highest source of error in genotyping studies. The error include allelic scoring error that make 80.0% of the total genotyping errors while data input and allelic dropout constitute 10.7%, and 6.7% of the total genotyping errors

respectively (Hoffman and Amos, 2005; Pompanon *et al.*, 2005). The remaining 2.7% probably resulted from sample mix-up, pipetting error or contamination. This means that human factors were responsible for about 93% of the genotyping errors. Among the various causes of error, allele calling might be the easiest to correct as it comes at the end of genotyping processes when all the data is collated together. However, the risk of human scoring error strongly depends on the quality of the data. By erroneously increasing the number of genotypes that are observed in a population sample, genotyping errors can strongly compromise the results of any study based on multilocus genotypes (Pompanon *et al.*, 2005).

2.1.4 Ways of minimizing genotyping errors

There is no universally accepted instructions that can be adopted to eradicate all genotyping errors in a study because sources of errors vary from one laboratory to another (Pompanon *et al.*, 2005). The optimal strategy therefore is determined by several factors; including the biological question, tolerable error rate, sampling possibilities, available equipment and technical skills, financial support and time constraints.

In general, since samples of poor quality and limited technical skills increase genotyping error rate (Paetkau, 2003), sample quality and available technical skills determine the number of genotyping replicate experiments that are necessary to attain a specific scientific goal,. In all the scenarios, a pilot study helps in assessing theoretical error rate that is compatible with the data analysis, and also in estimating the real error

rate on the basis of the analysis of a subset of the samples (Pompanon *et al.*, 2005; Valière *et al.*, 2007). Even after a successful pilot study, quality controls should be carried out in real time in each step and each batch of experiments, so that as many types of error as possible are detected in good time for correction. Replicate genotyping is a good procedure for detecting stochastic allelic dropout, but will not detect highly reproducible errors such as null alleles. These require a Hardy-Weinberg test or inheritance studies that will need results from the pilot study (Pompanon *et al.*, 2005; Valière *et al.*, 2007).

In this study, samples were collected in duplicate. Only dung samples were extracted in duplicate. All extracts were amplified three times. Hence each dung samples was amplified a minimum of six times as recommended by various authors (Goossens *et al.*, 2000; Pompanon *et al.*, 2005; Okello *et al.*, 2008). A randomly selected 10% of the samples were used to determine genotyping error rate per loci (**Figure 2.2**). Finally micro-checker software (Oosterhout *et al.*, 2004) was used to cross check the levels of genotyping error rate.

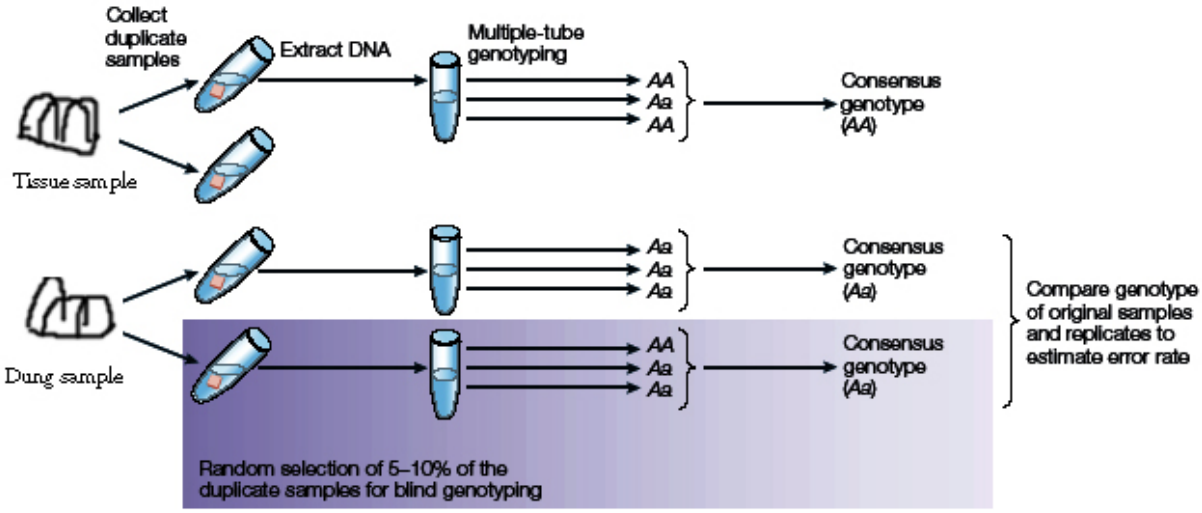

Figure 2.2: Multiple-tube genotyping approach was used to generate consensus genotypes. Dung samples were expected to have a higher genotyping error rate than tissue samples and the multiple-tube approach was complimented by multiple-sample extraction for dung samples. For tissue samples, 2 positive PCRs were used to decide a consensus genotype. In dung samples the criteria were 5 positive PCRs. In both cases 10% blind replicates were used to determine genotyping error. Diagram adapted from Pompanon *et al.*, (2005).

2.2 MOLECULAR TECHINIQUES

2.2.1 DNA extraction

Total genomic DNA was extracted from both dung and tissue samples using respective standard procedures. DNeasy® Tissue Kit was used for tissue samples while QIAmp® DNA Stool Mini Kit (QIAGEN® Germany) was used for dung following the manufactures instructions. Different kits were used because dung samples contain numerous contaminants that have to be removed before extraction; hence kits for extracting DNA from dung contain reagents that are not required in the process of extracting DNA from tissue sample. All extractions were done in a biological safety

hood category II at Cardiff University. Each extraction was preceded by a decontaminated process where the bench was sterilized with bleach (10%) and equipments (including pipettes, racks, tubes, tips etc) were exposed to UV-light inside the UV hood for >30 minutes.

Tissue samples were extracted first and then followed by dung samples, since tissue samples took shorter time to work on. Each extraction included a negative control; a blank sample with the entire kit reagent minus the tissue or dung sample. Care was taken to avoid cross-contamination between different samples.

2.2.2 Mitochondrial DNA amplification and sequencing

Polymorphism in the D-loop (**Appendix 1, Page 174**) of the black rhinoceros mitochondria DNA has previously been used successively to study the evolutionary relationships in this species (Morales *et al.*, 1997; Brown and Houlden, 2000; Fernando *et al.*, 2006). In this study, the same loci has been targeted and amplified using primers mt15996L (5′-TCCACCATCAGCACCCAAAGC-3′), located in the tRNAPro gene flanking the control region, and mt16502H (5′-TTTGATGGCCCTGAAGTAAGAACCA-3′), located in the central conserved domain of the control region (Brown and Houlden, 1999).

PCR reactions were performed in a final volume of 20 µl containing 1 µl of DNA extract, and 19 µl of reaction mix that contained 10 µl of master mix from QIAGEN multiplex kit, 2 µl of primer mix (to make 0.2 µM primer mix concentration from

stocks of 100 µM concentration of both forward and reverse primers), 2 µl of Q solution and 5 µl of water. Amplifications were carried out in a Perkin Elmer 9700 programmable DNA thermocycler as follows: activation step for 15 minutes at 95^{o}C followed by 35 cycles of 94^{o}C denaturation for 30 seconds, primer annealing at 58^{o}C for 90 seconds and 60 seconds of primer extension at 72^{o}C, and a final extension phase at 72^{o}C for 10 minutes. 3 µl PCR products were electrophorised on a 1.5 % agarose gel stained with 1.5 µl ethidium bromide (concentration 10mg/ml) and label with a 100 bp ladder. The gels were visualized on a UV transilluminator and photographs taken (**Plate 2.2**). Both extraction and amplification reaction blanks were also visualized in the transilluminator as a real time measure for potential contamination.

5 µl PCR products were purified using Exonuclease (Exo I) -Shrimp Alkaline Phosphate (SAP) protocol (http://www.nucleics.com/DNA_sequencing_support/exonucleaseI-SAP-PCR-protocol.html). The ration of Exo I to SAP was 0.1:1. SAP manufacture's concentration is always 1unit/ µl while that of Exo I vary between 10units/ µl and 20units/ µl. Therefore, if concentration of Exonuclease I 10units/ µl, then equal volumes of Exo I and are used. If the concentration of Exo I is 20units/ µl then the volume of Exo I used is half that of SAP. The Exonuclease I removes leftover primers, while the Shrimp Alkaline Phosphatase removes unused dNTPs. This was done in the PCR products in their original tubes thus minimizing the potential to contaminate PCR products. The PCR cycle include an incubation period of 60 minutes at 37^{o}C followed by a single cycle of 15 minutes at 80^{o}C, then a cooling phase at 4^{o}C to infinite.

1 µl of the PCR product was sequenced using 0.5 µl Big Dye Ver 1, and 2.5 µl Better Buffer in 2 µl of water and 1.5 µl of each primer (at 1.6 µM). The sequenced products were electrophoresed at Cardiff University using Applied Biosystems Model 3100 capillary sequencers. Later, large volume of samples were sequenced and electrophorised commercial at Macrogen Inc, South Korea.

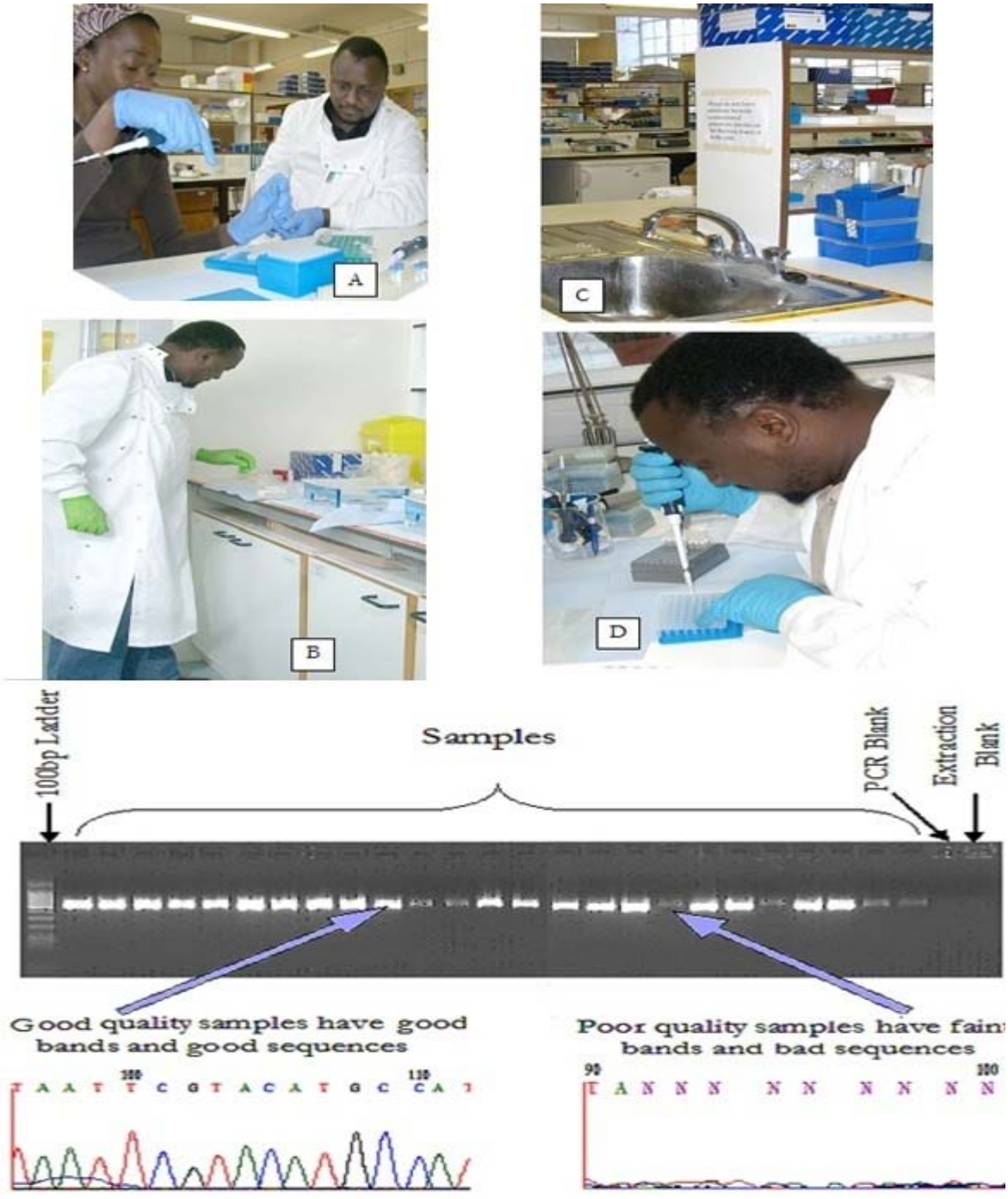

Plate 2: A pictorial depiction of laboratory work: - A - Mireille Johnson and Shadrack Muya choosing samples for pilot study; B – Shadrack Muya preparing samples to run in agarose gel electrophoresis; C – The molecular ecology laboratory at Cardiff University; D – Shadrack Muya loading a 96 well plate with samples; E - Agarose gel electrophoresis photograph and Capillary electrophoregram.

2.2.3 Microsatellite Amplification

12 microsatellite loci (**Table 2.2**) previously characterized for the black rhinoceros by Brown and Houlden (1999) and Cunningham *et al*. (1999) were used in this study. Each microsatellite locus was tested alone first and optimized as a single-plex. The singleplexes where then multiplexed based on their annealing temperature and amplified product size (**Table 2.2**). The screening process was carried out with a PCR reaction conducted in a 10 µl volume containing 5 µl of QIAGEN Multiplex PCR Master Mix (from QIAGEN® Multiplex PCR Kit), 1 µl of the 10X primer mix (0.2 µM of each primer, forward and reverse), 2 µl of DNA, 1 µl of 0.5X Q-Solution (provided in the kit) and 1 µl of water. The amplification profile consisted of an activation step at 95˚C for 15 minutes, followed by 35 cycles of 94˚C denaturation for 30 seconds; 90 seconds of multiplex primer annealing from 49˚C to 64˚C and 10 minutes of primer extension at 72˚C. Negative blanks of extraction and PCR reaction products were included in each batch of amplifications. The PCR products were electrophoresd in 1.5% agarose gel stained with 1.5 µl ethidium bromide (10 mg/ml concentration) and label with a 100 bp ladder, visualised on a UV transilluminator and photographed.

2.2.4 Genotyping Criteria

Tissue samples were genotyped three times to generate a consensus genotype for each locus. An individual was typed as heterozygous if both alleles appeared in 2 positive PCRs within the three replicates, and a homozygote was typed if it appeared in all the three replicates. Inconsistent genotypes were repeated ones and they remained inconsistent, they were eliminated (Goossens *et al.*, 2000).

Table 2.1 Primers used in the microsatellite analysis of Kenyan black rhinoceros populations. Sample size (n) = 192, Census population size (N) ≈≥ 600. Primers published by Brown and Houlden (1999) and Cunningham *et al.*, (1999).

Locus	Dye	Primer pair sequence 5'-3'	Repeat size	Size (bp)	Ta (^{o}C)	Multiplex
BR4 F	FAM (Blue)	CCC CTA AAT TCT AGG AAC AC	(CA)19	124 - 146	49	Multi C at 49^{0}C
BR4 R		CCA AAG ACC ACC AGT AAT TC			49	
BR6 F	HEX (Green)	TCA TTT CTT TGT TCC CCA TAG CAC	(CA)15	126 - 158	51	
BR6 R		AGC AAT ATC CAC GAT ATG TGA AGG			51	
DB23 F	HEX (Green)	CCT CAG CAA TAA GGG GAG GAT TAG C	(CA)12	179 - 185	55	Multi D at 55^{0}C
DB23 R		GTT GAT TCT CTG CCC CTG AGT TTG GG			55	
DB66 F	FAM (Blue)	CCA GGT GAA GGG TCT TAT TAT TAG C	(CA)7TA(CA)16	187 - 205	58	
DB66 R		GGA TTG GCA TGG ATG TTA CC			58	
BR17 F	FAM (Blue)	ACT AGC CCT CCT TTC ATC AG	(AT)5(GT)58	123 - 135	60	Multi B at 60^{0}C
BR17 R		GCA TAT TGT AAG TGC CCC AG			60	
DB1 F	HEX (Green)	AGA TAA TAA TAG GAC CCT GCT CCC	(CA) 14	121 - 127	60	
DB1 R		GAG GGT TTA TTG TGA ATG AGG C			60	
DB4 F	NED (black)	CCT AAG CCC CCT TTA CCT TG	(CA) 15	185 - 204	60	
DB4 R		GAC CAA TAA ACT CTT AGC AAA ATG G			60	
DB5 F	HEX (Green)	GAC CCC CAT GTT CAC TGC	(CA) 13	185 - 204	60	
DB5 R		AGG TCC ATC CAT TTT GTC CC			60	
DB30 F	HEX (Green)	GCG ACT ATG ACA TAC AAC TAT CTA C	(CA)21	201 - 205	64	Multi A at 64^{0}C
DB30 R		GGT CAA GGA TTA TTC TGA CTA GC			64	
DB44 F	FAM (Blue)	GGT GGA ATG TCA AGT AGC GG	(CA)4G(CA)16	170 - 184	64	
DB44 R		CTT GTT GCC CCA TCC CTG			64	
DB49 F	HEX (Green)	GTC AGG CAT TGG CAG GAA G	(CA)14	152 - 162	64	

DB49 R		CAG GGT AAG TGG GGG TGC			64
DB52 F	NED (black)	CAT GTG AAA TGG ACC GTC AGG	(ca)21	210 - 220	64
DB52 R		ATT TCT GGG AAG GGG CAG G			64

Dung samples were genotyped six times to generate a consensus genotype for each locus. An individual was typed as heterozygous if both alleles appeared in 4 positive PCRs within the six replicates, and a homozygote was typed if it appeared at least five times. Ambiguous genotypes were repeated one more time or eliminated (Goossens *et al*. 2000). A random replicate of 10% of the samples was genotyped to estimate error rate (Pompanon *et al.*, 2005)

2.3 DATA ANALYSIS

2.3.1 Mitochondrial DNA analysis

Sequences were assembled, aligned and edited with SEQUENCHER Ver. 3.0. Consensus sequences were trimmed into 520 bp sequences saved in NEXUS (*.nex) format for downward analyses.

Genetic diversity within populations was determined using haplotypes diversity (probability that two randomly chosen haplotypes in a sample are different) and nucleotide diversity (probability that two randomly chosen homologous nucleotides are different) estimates at two scales: (i) total sample, (ii) between/within populations and the various methods as detailed in the following subsections.

2.3.2 Phylogenetic relationships

Median joining network (MJN - which basically are the hamming distance or the sum of differences between two sequence types) derived by NETWORK V4.1.1.1 (Bandelt *et al.*, 1995) were used to define black rhinoceros phylogenetic relationships in this study. MJN also allow multifurcations and overlaying of sequences on geographic location to visualize phylogenetic information topologically, while Maximum likelihood (ML) or maximum parsimony (MP) phylogenies are more effective in portraying sequence intraspecific relationships among populations. Both ML and MP are cladistic methods and are based on the assumption that a set of sequences evolved from a common ancestor by a process of mutation and selection without mixing (Kitching, 1998). These methods work best with a known out group so that comparisons can be made between a finite number of alternate trees rather than calculating all possible trees for a given set of sequences (DeSalle *et al.*, 2002).

Maximum likelihood estimates predicts ancestral sequences at branch points in the tree (nodes) and can provide information about the timing of the acquiring of a mutation (DeSalle *et al.*, 2002). ML was carried out to test the strength of the black rhinoceros phylogeny, and determine the substitution model (or model of evolution) that best fitted the data according to a hierarchical likelihood ratio test. This was done using TREEFINDER (Jobb, 2008). Node support was tested using 1000 bootstrap replicates and dendrogram was constructed using ML based on the coalescent process for a neutral infinite-sites model

assuming a large constant population size. A bootstrap consensus tree was compared to the original tree from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed and the percentage of replicate trees in which associated taxa clustered together is shown next to the branches.

Sequences of other African black rhinoceros subspecies in published in the GenBank/EMBL were used to show the magnitude of variation in the Kenyan black rhinoceros subpopulation. Similarly GenBank/EMBL sequences for white rhinoceros were used as outer group.

2.3.3 Analysis of population demography

Past demographic information of black rhinoceros in Kenya were tested using ARLEQUIN Ver. 3.1.1 (Excoffier *et al.*, 2005). Pairwise haplotype mismatch distribution in the total sample and for the haplogroups identified by the MJN analysis were compared taking note of the distribution of the observed pairwise nucleotide site differences with the expected distribution in an expanding population. In a single origin, demographically expanding population, mismatches should follow a unimodal Poisson distribution, whereas in populations at demographic equilibrium or with sub-groups or genetic substructure, the distribution is expected to be multimodal (Rogers and Harpending, 1992).

Mismatch distribution also allowed to estimation whether the population has experienced

any the demographic expansion event. The possibility of a demographic expansion were further tested using Tajima's D and Fu's FS tests of neutrality to examine whether all mutations are selectively neutral. A negative value of Tajima's D statistic reflects a relative excess of low-frequency polymorphisms, and Fu's Fs, which is a powerful test for rejecting the hypothesis of neutrality of mutations, tends to be negative when there is an excess of recent mutations. Both tests were therefore used to detect the signal of a demographic expansion, where low frequency mutations are expected, especially in K-selected animals.

Analysis of molecular variance (AMOVA) between groups was performed using ARLEQUIN Ver. 3.1.1 (Excoffier *et al.*, 2005).

2.3.4 Criteria of microsatellite DNA analysis

It is usually advisable to apply several analysis methods on the same data while measuring genetic diversity using microsatellite markers (Slatkin, 1987; Goldstein, 1997; Paetkau *et al.*, 1997). If the results from different analysis methods of which each is based on different hypotheses of mutation or different algorithms are in agreement, then, there is a greater confidence that interpretations inferred from the data are correct (Okello *et al.*, 2008). This approach was followed in this study.

2.3.5 Genetic diversity, Hardy-Weinberg equilibrium and linkage disequilibrium

The software GENETIX 4.05 (Belkhir *et al.*, 2004) and GENEPOP (Bandelt *et al.*, 1995) were used in this study to perform all standard population genetic analyses: mean number of alleles per locus (*A*), allele frequencies differentiation between populations at each locus, gene diversity (*He*) and observed heterozygosity (*Ho*). Heterozygote deficiency was tested, as compared to Hardy-Weinberg equilibrium for each locus. Generally the MNA is highly dependent on the sample size. This is because; the presence of unique alleles in populations that occur in low frequencies whose frequency tends to increase with increase in population size. Also the number of observed alleles tends to increase with increases in population size (Nei, 1987). Therefore, the comparison of the MNA between samples of different sizes may not be meaningful unless sample sizes are more or less the same (Nei, 1987; Hart and Clark, 1989). However, MNA is a good indicator of genetic diversity especially where samples are genotyped in replicate.

Fisher's exact test (F_{IS}) was performed using GENEPOP Ver. 3.1b and GENETIX 4.05 to estimate the observed (Ho) and expected (He) heterozygosities and to test the genotypic distribution for conformance to Hardy-Weinberg Equilibrium (Rice, 1989). Significant positive values of F_{IS} indicate heterozygote deficiency, suggesting that the samples could be from different distinct populations (Wahlund's effect) (Frankham *et al.*, 2002; Freeland, 2005; Allendorf and Luikart, 2007) or there is significant inbreeding or linkage disequilibrium. Results that have a significantly negative F_{IS} value indicate

heterozygote excess. Fisher's exact tests were also used to determine linkage disequilibrium (Raymond and Rousset 1997). *P*-Values from multiple tests were assessed for significance using sequential Bonferroni correction for type II error (Rice 1989).

2.3.6 Genetic relationships between subpopulations

Genetic relationships between subpopulations were measured using their genetic distances (Cavalli-Sforza *et al.*, 1994) in order to estimate the level of genetic divergence between them (Avise, 1994). Thus when the genetic distance is large the genetic similarity is low and the time they diverged from each other greater while when the genetic distance is small the genetic similarity is high and the time they diverged from each other is smaller (Avise, 1994).

Brown and Houlden (2000) estimated a nucleotide substitution rate (μ) of 0.02 substitutions/site/Mya in the black rhinoceros mitochondrial control region. This substitution rate is consistent with rates of about 0.02 substitutions/site/Mya reported for most large mammals (Slade *et al.*, 1994; Wooding and Ward, 1997; Oakenfull *et al.*, 2000; Moodley and Harley, 2005).

One of the common measures of genetic distance in use today is Nei's *standard genetic distance (D_S)* (Nei, 1972). The value of D_S is proportional to the evolutionary time when

the effects of mutations and genetic drift set in the population. However Nei *et al*., (1983) noted that the modified Cavalli-Sforza and Edwards genetic distance measure D_A is more efficient in determining the true topology of an evolutionary tree being constructed using allele frequency data, especially if the populations are closely related (Cavalli-Sforza *et al*. 1994). D_A has also been reported to increase more slowly with time and to maintain a linear relationship for longer periods of time (Nei *et al*. 1983). In this study, both D_S and D_A distances were estimated using the program POPGENE (Yeh *et al.*, 2000).

2.3.7 Population genetic structure and admixture signature analysis

Genetic structuring or differentiation within a population reflects the number of alleles exchanged between populations that influence the genetic composition of individuals within these populations (Balloux and Lugon-Moulin 2002). Gene flow between populations determines the effects of selection and genetic drift, generation of new polymorphisms and effective local population size (Balloux and Lugon-Moulin 2002).

In this study, Nei's coefficient of gene variation (G_{ST}) and Wright's inbreeding coefficient (F_{ST}) were used to describe the population genetic differentiation measured using GENEPOP (Raymond and Rousset 1997). In practical terms, G and F statistics describe whether the majority of genetic variation in a population is distributed among or within subpopulations. In species with low G_{ST} (approaching 0), the majority of variation is found within subpopulations; individuals within subpopulations are likely to be

genetically different, but each subpopulation contains the same complement of alleles in similar frequencies Where G_{ST} is high (approaching 1), individuals within a subpopulation are relatively similar but the subpopulations are significantly different. Most species fall somewhere in between these extremes (Nei, 1975; Wright, 1978).

The genetic structure of the black rhinoceros population was investigated using a clustering method based on Bayesian model in STRUCTURE Ver. 2.2 (Pritchard *et al.*, 2000). The number of populations (K) is treated as an unknown parameter processed by the Markov Chain Monte Carlo (MCMC) computations. Several runs for each K, from K = 1 to 6, were performed in order to verify the consistency of the results. The mean posterior probability, which is the mean value of the log likelihood of the data at each step of the MCMC, was calculated for each K over its runs and was also used to identify the true number of populations K using the maximum value of the mean likelihood.

CHAPTER THREE

3.0 PHYLOGEOGRAPHY OF THE KENYAN BLACK RHINOCEROS

ABSTRACT

Kenya lost over 98% of its black rhinoceros (*Diceros bicornis michaeli*) between the 1960s and 1990s, leaving a mere 400 animals by 1993 isolated in small populations. The population is currently on a recovery path and currently stands at over 600 animals found in small isolated subpopulations, each of less than 100 animals. Differential evolutionary selection pressures are expected to apply in such isolated subpopulation, and may drive them into separate ecological evolutionary units. This study examined the Kenyan black rhinoceros mtDNA control region genetic diversity and its spatial structuring in Kenyan subpopulations. Different hypothesized subpopulation structuring scenarios were examined; including the lowland and montane conservation units designated by Kenya Wildlife Service (KWS).

Genetic information was obtained from 408bp mitochondrial control region sequence from 170 individuals. Both model based and standard methods were used to examine the data. The sample comprised 16 maternal lineages, moderate haplotype diversity (0.73±0.137) and low nucleotide diversity (0.007±0.003). The geographic and altitudinal distribution of haplotypes was not phylogeographically structured. This level of genetic diversity and structuring in the Kenyan black rhinoceros is consistent with

their demographic population history of a recent drastic population bottleneck and slow recovery.

Findings of this study imply that substantial levels of genetic diversity still exist within the Kenyan black rhinoceros gene pool. The hypothesis of lowland and montane population units is not supported from a genetic perspective. Management strategies that involve translocation among populations at a rate of at least one breeding migration per generation are therefore advocated in order to control any further loss in genetic diversity due to drift and/or inbreeding.

3.1 INTRODUCTION

The current distribution of the black rhinoceros (*Diceros bicornis*) is limited to Africa, south of the Sahara. Their evolutionary lineage is traced back to a common ancestor with the Asiatic two-horned rhinoceroses, approximately 14 Mya, at the end of the Miocene (Hooyer, 1976). The African black and white rhinoceroses share a more recent common ancestor between 2 and 5 Mya (Hooyer, 1976; Lacombat, 2005). *D. bicornis* has four recognized extant subspecies. *Diceros bicornis bicornis* is distributed in the south-western areas of Namibia, South Africa, southern Angola and western Botswana. *Diceros bicornis minor* is the most numerous and occupies the wetter areas south of central Tanzania through to Zambia, Zimbabwe and Mozambique to northern and

eastern South Africa. *Diceros bicornis michaeli* is primarily found in northern Tanzania and Kenya. A West African subspecies (*Diceros bicornis longipes*) has been tentatively declared extinct (IUCN, 2006; Times online, 2006). A putative fifth subspecies (*D. b. bruceii*) may still survive in Ethiopia but according to African Rhino Specialist Group report (2004) its population trends are not clear, and hence it is impossible to declare whether this species is already extinct. A sixth subspecies (*Diceros bicornis somaliensis*) that ranged in Somalia and, according to museum catalogues, is now extinct.

Diceros bicornis minor is the most immediate southern neighbour of *Diceros bicornis michaeli* and historically their ecological ranges may have overlapped when the distribution black rhinoceros in Africa was continuous (Cooke, 1972, and Figure 3 chapter 1), but Brown and Houlden (2000) showed that the extant *Diceros bicornis minor* and *Diceros bicornis michaeli* are reciprocally monophyletic with respect to their mitochondrial DNA, separated by 2.6% nucleotide divergence. They are thus likely to have separated around 0.93 - 1.3 Mya. The two subspecies appear to have accumulated sufficient genetic divergence and deserve to be management as separate evolutionary units (Moritz, 1994).

In the early 1900s, black rhinoceros were widely distributed in Kenya (**Figure 1.3, Page 11**). This is confirmed by the fact that there are oral traditions about rhinoceros in

almost all indigenous Kenyan communities and local names for places and people named after rhinoceros in many regions of Kenya. Reports from early foreign hunters in Africa (Neumann, 1898; Lloyd-Jones and Brevet-Major, 1925; Barclay, 1932) and Kenya in particular (Patterson, 1909; Hunter, 1952) indicated that rhinoceros were numerous in Africa. Analogous to most large mammals, the recent history of black rhinoceros in Kenya and elsewhere has been characterised by population fragmentation, primarily as a result of European colonization. Heavy poaching of black rhinoceros for their horns and loss of their habitat to agriculture and settlement further reduced their distribution in Kenya to isolated individuals and/or small populations scattered across their former range. However, British conservationists had already realized the imminent demise of wildlife in Africa in the early 1900s. They therefore pressurized the colonial government to set aside land for wildlife conservation in Kenya (Spinage, 1962; Leakey, 1969; Akama, 1998). In 1946, the conservationists' efforts bore fruit with the gazetting of the first national park in Kenya - the Nairobi National Park. The park, along with the private Solio Game Ranch, was later designated a breeding nucleus rhinoceros sanctuary and received several marooned black rhinoceros between the 1960s and 1980s from areas that had been opened up for agriculture and/or settlement (**Figure 1.4, Page 19**).

More sanctuaries – both public and private – were created in Kenya between the 1970s and 1990s (KWS, 2003). These sanctuaries offered security to rhinoceros that had been threatened by habitat loss and poaching. However, many of these subpopulations are

small (less than 100 total individuals, **Table 2.1, Page 46**) and genetic drift may thus become a major force in shaping their destinies. Inbreeding, coupled by extreme drift may eventually lead to a reduction in genetic diversity and total fitness (e.g. Saccheri *et al.*, 1998; Saccheri *et al.*, 1999), making the small populations face increased extinction risk. This kind of extinction vortex that is fuelled by an interactive negative feedback between reduction in population growth and inbreeding (Frankham *et al.*, 2002) is a clear possibility in the small black rhinoceros subpopulations of Kenya. The Masai Mara and Chyullu populations have remained relic and have no records of any immigrants (**Table 1.1 20**). The Chyullu population was discovered recently while fear for security levels in the unfenced Masai Mara discouraged translocation of rhinoceros to Masai Mara.

However, it is possible that the black rhinoceros in Kenya have not yet reached a critical stage in its genetic bottleneck because of its long generation time (≈ 7-10 yrs), as a classical *K*-selected species, compared to the recent nature of the species' population decline that took place in the later 60's and early 70's where Kenya lost over 98% of its black rhinoceros (from 20,000 animals in 1960s to less than 400 animals in 1990s) (Okita-Ouma *et al.*, 2007). Although some unique haplotypes and alleles are likely to have been lost as a result of the decline, if the hypothesized historical long-term population stability and gene-flow implies that, substantial genetic diversity could still remain in the remnant populations.

A basic understanding of Kenyan black rhinoceros genetics is also of importance in determining units for conservation and management strategies. Various wildlife management regimes that have protected wildlife in Kenya have effected numerous translocations of black rhinoceros between locations, but none of these translocation has been guided by genetics and to a larger extent, they have been based on other practical reasons, such as security of isolated animals or removal of problematic individuals or to increase the population size of a particular sanctuary. Currently, other than the slow recovery rate in some populations epitomized by the Aberdares National Park (Okita-Ouma *et al.*, 2007) no obvious phenotypic characteristic has been found to suggest that the Kenyan black rhinoceros subpopulations are experiencing inbreeding-related loss of fitness. However, drift-inbreeding forces may only manifest deleteriously in the long term (Frankham and Ralls, 1998; Frankham *et al.*, 2002; Freeland, 2005).

Current Kenya Wildlife Service (KWS) black rhino conservation policy includes the assumption that the Kenyan black rhinoceros exists in two main groupings referred to as the lowland and highland populations (Okita-Ouma *et al.*, 2007). The highland population designation was reviewed recently and renamed the montane forest population. It mainly encompasses the Aberdares National Park – Salient black rhino populations in Kenya (Okita-Ouma *et al.*, 2007), with all other subpopulations forming the lowland group. One major difference in these two main populations' environment

other than relief is the presence of Tsetse flies in the lowlands. Therefore, in the event of black rhinoceros translocation, efforts are made not to translocate black rhinoceros between these two major groups (i.e. lowland to montane forests and vice verse). KWS envisages that by doing so, they will minimize the chances of introducing locally adapted animals into environmental conditions that are different to their source environment. This approach implies that some populations are destined to remain small, and recently KWS has admitted that it has been difficult to build the montane forest population in Aberdares NP-Salient area to more than 20 animals while the total population in the park fluctuate around 30 animals. Hence this small population currently has no chance of obtaining fresh genetic input and continues to be prone to the extinction, that vortexed by the negative feedback associated with small isolated population, and could jeopardize the KWS goal of attaining a minimum population growth rate of 5% per year and reaching a confirmed total of 650 rhinos by 2010 and 1000 rhinos by 2020.

This study examines the spatial genetic structuring and diversity of mitochondrial DNA in the Kenyan black rhinoceros. The grouping of Kenyan black rhinoceros populations into lowland and montane units by KWS was also examined using both standard and model based approaches to determine whether there is any significant haplotype diversity within groups and phylogenetic structure to render the units genetically distinguishable. Finally in the light of genetic evidence, appropriate conservation of

strategies for the Kenyan black rhinoceros is discussed.

3.1.1 RESEARCH HYPOTHESIS

This study tested the following hypotheses about Kenyan black rhinoceros population genetics:

1. That Kenyan black rhinoceros subpopulations are not genetically differentiated
2. That subpopulation's genetic diversity is not in tandem with their known demographic history

3.1.2 STUDY OBJECTIVES

General objective

The overall goal of this study was to assess and determine the genetic diversity within and among black rhinoceros subpopulations in Kenya and generate information for use in the formulation of metapopulation management strategies of black rhinoceros in Kenya

Specific objectives

The specific objectives of the study were to:--

1. Determine the current level of genetic variations in Kenyan black rhinoceros subpopulations.

2. Determine the extent at which the present genetic variation is partitioned in Kenyan black rhinoceros subpopulations.

3.2 MATERIALS AND METHODS

3.2.1 Sampling

Tissue and dung samples were collected from 12 out of the 14 current locations in Kenya between 2005 and 2007 (**Table 2.1, Page 46 and Figure 2.1, Page 47**) as follows:-- Aberdares NP, (= 9), Chyulu NP, (= 9), Laikipia WC, (= 9), Lewa WC, (= 33), Lake Nakuru NP, (= 20), Masai Mara GR, (= 30), Ngulia RS, (= 23), Nairobi NP, (= 62), Ol Jogi RH, (= 15), Ol Pajeta RH, (= 37), Solio RH, (= 28), Tsavo East NP, (= 20). Sample collection techniques varied between tissue/serum and dung sample materials. Tissue and serum samples for this study were obtained from sample stocks kept by the KWS Veterinary Department which collects blood and tissue samples routinely during its work. This accounted for samples from five subpopulations (**Table 2.1 Page 46**). The samples are stored in 70% ethanol at -20^{o}C or at room temperature in 25% DMSO at the KWS Head Offices Veterinary Laboratory. Seven subpopulations lacked inadequate tissue/serum samples and hence, fresh dung samples were collected following published methods (Johnson, 2008) and were stored in 70% ethanol.

3.2.2 DNA Markers

This study is based on mitochondrial DNA (mtDNA) because these markers are non-recombining (haploid), rapidly evolving molecules that are predominantly maternally inherited, accumulates mutations more quickly than nuclear genes and are well suited to phylogeographic analysis (Avise, 1994). MtDNA produces haplotypes that can be ordered phylogenetically within a species, yielding intra-specific phylogenies interpretable as a matrilineal component of the organism's population history. The analysis of mtDNA phylogenetic networks can also indicate reticulate evolution (Beebee and Rowe, 2007). This marker has been used successfully to study genetic variability (Nunney and Campbell, 1993), phylogeography (O'Ryan *et al.*, 1994; Morales *et al.*, 1997), including the phylogeography of black rhinoceros (Brown and Houlden, 2000) and to assign evolutionary significant and management units in wildlife management (Moritz, 1994).

3.2.3 Molecular methods

Total genomic DNA was extracted from both tissue and dung samples using standard procedures. The Qiagen DNeasy® Tissue Kit (Qiagen, Hilden, Germany) was used to isolate DNA from blood and tissue samples while QIAmp® DNA Stool Mini Kit was used to isolate DNA from dung samples. For both methods, the manufacturer's instructions (QIAGEN® Germany) were followed.

PCR reactions were performed in a final volume of 20 µl containing 1 µl of DNA extract, and 19 µl of reaction mix that contained 10 µl of master mix from QIAGEN multiplex kit, the primers mt15996L and mt16502H, which yield a PCR product of 520 base pairs (Brown and Houlden, 2000) were used to a final concentration of 0.2 µM, 2 µl of Q solution (Qiagen Hilden, Germany) and 5 µl of water were also added. Amplifications were carried out in a Perkin Elmer 9700 thermocycler as follows: activation step for 15 minutes at 95˚C followed by 35 cycles of 94^{o}C denaturation for 30 seconds, primer annealing at 58˚C for 90 seconds and 60 seconds of primer extension at 72^{o}C, and a final extension phase at 72 ˚C for 10 minutes. PCR products were electrophorised on a 1.5 % agarose gel. A 520bp fragment was sequenced using the primers mt15996L (Brown and Houlden, 1999), located in the tRNA^{Pro} gene flanking the control region, and mt16502H (Brown and Houlden, 1999), located in the central conserved domain of the control region. The PCR products were purified using the Qiagen PCR purification kit and subsequently sequenced in forward and reverse directions commercially at Macrogen Inc, Korea.

Sequence chromatograms were checked by eye, reading errors were corrected and sequences were aligned on SEQUENCHER Ver. 3.1.1 software (Gene Codes Corporation, 1988). The control region fragments were authenticated by BLAST search. DAMBE (Xia and Xie, 2001) was used to identify haplotypes from the aligned sequences.

3.2.4 Analysis of genetic diversity and differentiation

Genetic diversity of control region was estimated by determining haplotype diversity (the probability that two haplotypes randomly chosen from the population will be different from one another; *h*) and nucleotide diversity (the probability that two randomly chosen homologous nucleotides are different; π). The analysis was executed using ARLEQUIN Ver. 3.1.1 (Excoffier *et al.*, 2005) and DnaSP Ver. 4.0 (Rozas *et al.*, 2003).

Hierarchical genetic structuring of control region sequences in the Kenyan black rhinoceros population was inferred using analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.1.1 based on F-statistics (FST also called fixation index, Wright, 1951) and variance measured by Φ_{CT}. Statistical significance was estimated using 1000 permutations. Five grouping scenarios were explored. Scenario one involved grouping Masai Mara subpopulation verses all other 11 subpopulations. This scenario was considered on the basis that Masai Mara subpopulation is a relict population that has never received any immigrants under the KWS translocation programme. Scenario two adopted the KWS approach of montane forest populations (Aberdares) versus all other 11 subpopulations referred to as the lowland population. Scenario three is based on the hypothesis that before the drastic population decline the Kenyan black rhinoceros was a single panmictic population and the relict populations – Mara and Chyullu can be hypothesized to have retained the genetic signature of the pre-bottleneck population, while all other populations

have undergone mixing from the numerous translocations (KWS, 2003). Both scenarios four and five are based on geographic proximity and historical demographic information of each subpopulation in order to test for fragmentation pattern of genetic structure. In scenario four Masai Mara and Lake Nakuru subpopulations were grouped together based on their geographic proximity and similar reasons guided the grouping of the Aberdares, Lewa, Ol Jogi, Laikipia and Solio populations together. Scenario five considers the relic populations of Masai Mara, Chyullu and Laikipia as individual groups that have not undergone any recent mixing and hence isolated by distance. The grouping scenario which maximized the among group variance (measured in Φ_{CT}) was assume to be the most plausible (Moodley and Harley, 2005).

A Median Joining Network (MJN) developed using NETWORK 4.1.1.1 (Bandelt *et al.*, 1999) to construct the most parsimonious network phylogeny linking all haplotypes in the Kenyan black rhinoceros subpopulations. Branch lengths were scaled according to the number of mutations separating linked haplotypes.

A haplotype neighbour joining (NJ) phylogenetic tree was estimated using MEGA4 (Tamura *et al.*, 2007) and the topology was confirmed using the maximum likelihood (ML) coalescence method using substitution model HKY[(Optimum),(Empirical)], where the 'Optimum', stands for maximum likelihood optimization of the substitution rate parameters [(TC:0.4444729, TA:0.027763549, TG:0.027763549, CA:0.027763549, CG:0.027763549,

AG:0.4444729), and the 'Empirical' stands for frequency parameter list for empirical estimation values (T:0.3178088, C:0.24501758, A:0.29873175, G:0.13844186)]. Node support was tested using 1000 bootstrap replicates and a consensus tree was constructed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were not shown. Sequences for *Diceros bicornis bicornis* and *Diceros bicornis minor* were used in this study to show the magnitude of the differences within *Diceros bicornis michaeli*. The *D. b bicornis* where sequenced from dung samples collected from Palmwag, Namibia by Michael WB, Dr Paul O'Donoghue and staff from Save the Rhino Trust in Namibia, while *Diceros bicornis minor* sequences were obtained from GenBank/EMBL (accession numbers AF187825-AF187827). White rhinoceros (*Ceratotherium simum simum*) sequences were also obtained from GenBank (accession number AF187839) and used as outgroup.

3.2.5 Analysis of population demography

Past demographic information of black rhinoceros in Kenya was examined by mismatch distribution analysis of the number of nucleotide differences between pairs of mitochondrial haplotypes, implemented in ARLEQUIN 3.1.1 (Excoffier *et al.*, 2005). Parameters expected under the sudden expansion model (Theta and Tau) were calculated for the entire population data set and a goodness of fit of the sum of squared deviations (SSD) and the Harpending raggedness index (RI) between the observed and expected mismatch distributions were computed.

Calculations for the divergence times for the Kenyan black rhinoceros maternal lineages were based on the HKY model of nucleotide substitution: $\mu = \pi/2T$ where μ is the general mutation rate of animals, π is the nucleotide diversity and T is the divergence time. The μ for black rhinoceros have been estimated at ≥ 0.02 per Mya, based on the 7 Mya divergence between white and black (Cooke, 1972; Brown and Houlden, 2000), thereby allowing for the inference of intraspecific coalescence times.

3.3 RESULTS

3.3.1 Genetic diversity

3.3.1.1 Sequences and haplotype analysis

DNA sequences were trimmed and analysed providing 408 bp for 170 Kenyan black rhinoceros. The sequences included polymorphic sites at positions 51, 74, 75, 79, 83, 85, 166, 195, 197, 232, 233, 247, 261, 284, 376, 385 and 404 of which 16 were transitions and there were no insertions or deletions. The sequences also revealed 16 distinct haplotypes in the Kenyan black rhinoceros (GenBank/EMBL accession numbers FJ227483-FJ227498, **Appendix 2, Page 176**). Haplotypes H01 to H05 and H13 were confined to one population each. H01 was present only in Ol Pajeta Ranch, H02 was limited to Masai Mara, H03 was limited to Ngulia Rhino sanctuary H04 and H05 were found in Tsavo East National Park only, while H13 was limited to Laikipia Wildlife Conservancy subpopulation (**Table 3.1**). 43% of the samples shared the H16 haplotype, which was present in 11 subpopulations in Kenya (**Figure 3.1**), and was only absent in the Laikipia Wildlife Conservancy.

Table 3.1: Geographic distribution of Kenyan black rhinoceros control region haplotypes

Haplotype	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA	Total
H01										1			1
H02						1							1
H03							1						1
H04												1	1
H05												1	1
H06					1			1		1			3
H07								2	1		1		4
H08						1		1		4	1		7
H09						3		1					4
H10				3				4	2	2			11
H11				2	4	1		4	1	2	10		24
H12		2				1	1			4			8
H13			2										2
H14	4		1	1	1	1	2	1	1	2		1	15
H15					3	2	3	4		2			14
H16	3	1		15	4	1	7	10	5	12	13	2	73
Total	7	3	3	21	13	11	14	28	10	30	25	5	170

The final row summarises the total number of haplotypes in each population, while the final column summarises the total number of individual black rhinoceros sharing a haplotype. The colours used in each haplotype are made to enhance the visualization of each haplotype in Figure 3.1. ABE, Aberdares National Park; CHY, Chyullu National Park; LAK, Laikipia Wildlife Conservancy; LEW, Lewa Wildlife Conservancy; LKN, Lake Nakuru National Park; MAR, Masai Mara Game Reserve; NGU, Ngulia Rhino Sanctuary; NNP, Nairobi National Park; OLJ, Ol Jogi Ranch; OLP, Ol Pajeta Ranch; SOL, Solio Ranch; TSA, Tsavo National Park.

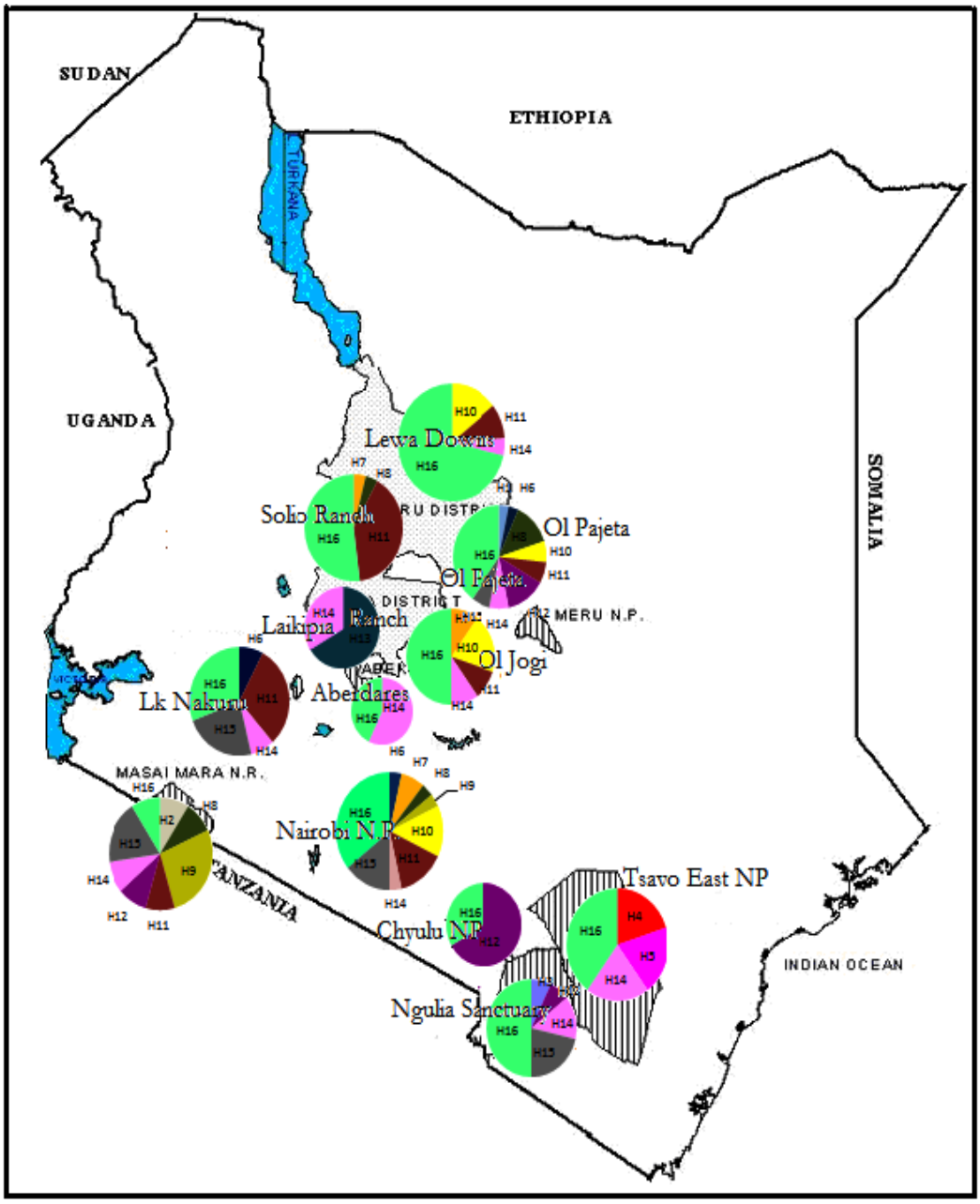

Figure 3.1: Geographic distributions of black rhinoceros control region haplotypes in Kenya. The haplotypes are represented by different colours and sample sizes defined in Table 3.1. The size of the circles represents the number of individuals sampled from the subpopulation.

3.3.1.2 Haplotype and nucleotide diversity

The average haplotype diversity in the entire Kenyan black rhinoceros metapopulation was moderate (0.73 ± 0.137, n=170), but the values varied considerably when each subpopulation was considered alone (**Table 3.2**). The Masai Mara Game Reserve subpopulation had highest haplotype diversity (0.93 ± 0.07, n=11), while Lewa Wildlife Conservancy had the lowest (0.48 ± 0.12, n=21). The average nucleotide diversity was low (0.0072 ± 0.003 n=170) but the values also varied considerably when each subpopulation was considered alone. Lake Nakuru National Park subpopulation had the highest nucleotide diversity (0.012 ± 0.006, n=13) while Aberdares National Park had the lowest (0.0014 ± 0.0014, n=7).

Table 3.2: Mitochondrial genetic variation in Kenyan black rhinoceros based on 408 base pair control region sequences

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA	Mean	SD	Total
n	7	3	3	21	13	11	14	28	10	30	25	5	14.167	9.63	170
A	2	2	2	4	5	8	5	9	5	9	4	4	4.917	2.54	16
h	0.57	0.67	0.67	0.48	0.81	0.93	0.73	0.83	0.76	0.81	0.59	0.9	0.73±0.137	0.14	
π	0.002	0.007	0.005	0.005	0.012	0.011	0.006	0.009	0.006	0.009	0.009	0.008	0.007±0.003	0.002	

n = Sample size; A = Number of haplotypes in each population; *h* = Haplotypes diversity; π = Nucleotide diversity. ABE = Aberdares National Park; CHY = Chyullu National Park; LAK = Laikipia Wildlife Conservancy; LEW = Lewa Wildlife Conservancy; LKN = Lake Nakuru National Park; MAR = Masai Mara Game Reserve; NGU = Ngulia Rhino Sanctuary; NNP = Nairobi National Park; OLJ = Ol Jogi Ranch; OLP = Ol Pajeta Ranch; SOL = Solio Ranch; TSA = Tsavo National Park.

3.3.2 Phylogeography

3.3.2.1 Individual-based

The phylogeny of *Diceros bicornis michaeli* was inferred from geographic distribution of haplotypes, (**Figure 3.1**), a median joining network (MJN) multifurcating (**Figure 3.2**) and a bifurcating maximum likelihood phylogenetic tree (**Figure 3.3**). From the geographic distribution of haplotypes, it is apparent that control region sequences are not strongly structured and the demographic relationship between the Kenyan black rhinoceros subpopulations is complex.

In the median joining tree, haplotypes were divided into three main groups joined together by two median vectors; mv1 and mv2. Haplotype H10 is a single group that shares mv2 with H11 and H06. Haplotype H07 and H14 link mv1 with H16. The 16 haplotypes were related to each other by a varying degree of mutations, but not more than three substitutions between adjacent haplotypes in the network, with a 99% sequence similarity index.

Phylogenetic relationships between rhinoceroses based on maximum likelihood analysis using white rhinoceros as outgroup showed strong bootstrap support for three maternal lineages (**Figure 3.3**) within the black rhinoceros where each subspecies; i.e. *Diceros bicornis bicornis*, *Diceros bicornis minor*, and *Diceros bicornis michaeli* form a monophyletic group (**Figure 3.3**). Three haplotypes (H01, H09 and H12) in the Kenyan black rhinoceros population grouped together but had a mixed geographic distribution.

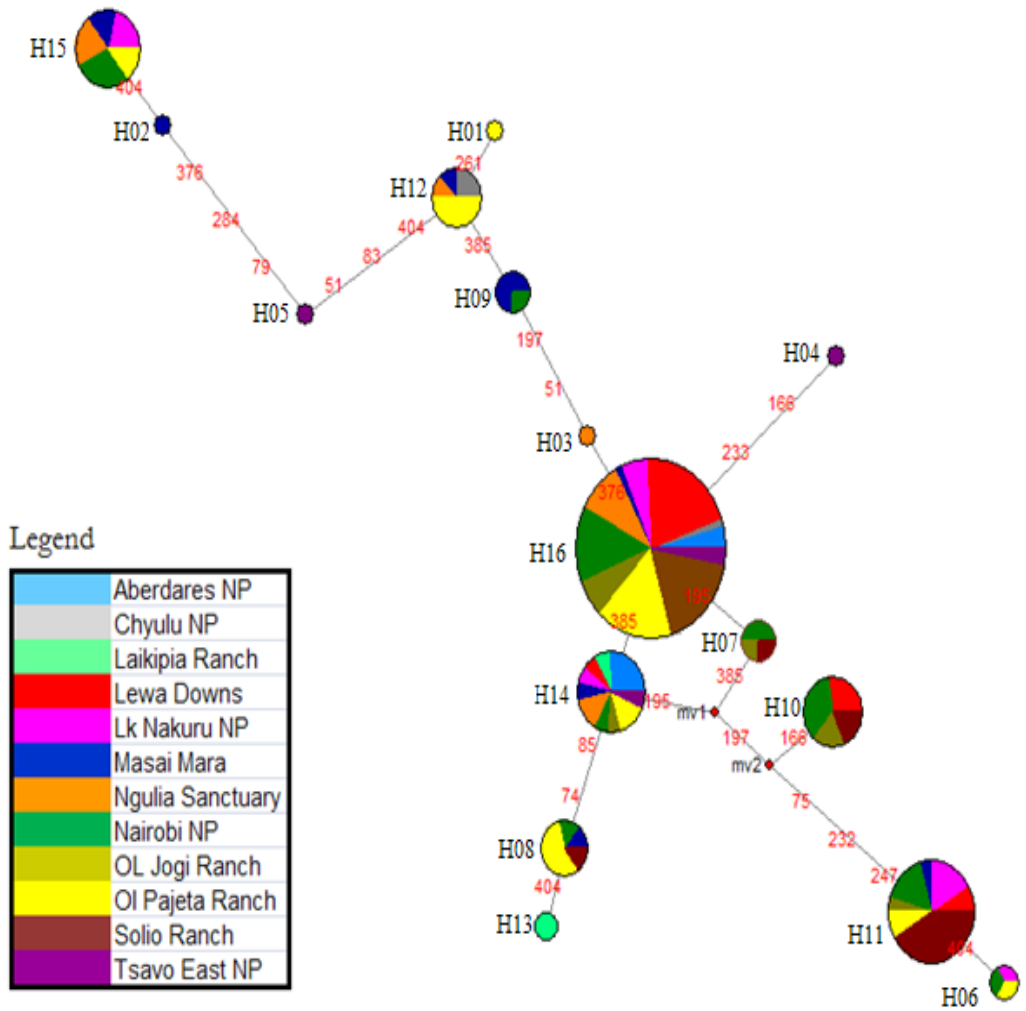

Figure 3.2: Median joining networks (MJN) of Kenyan black rhinoceros sequences. Each circle represents a haplotype and its size is proportional to the haplotype frequency in different subpopulation. Small red squares are median vectors of unsampled or extinct ancestral sequences. Red numbers indicate the nucleotide position at which variation occurred, and number of links between haplotypes indicates the number of mutations that haplotypes have undergone from one another. Each colour represents the subpopulation where a haplotype was sampled.

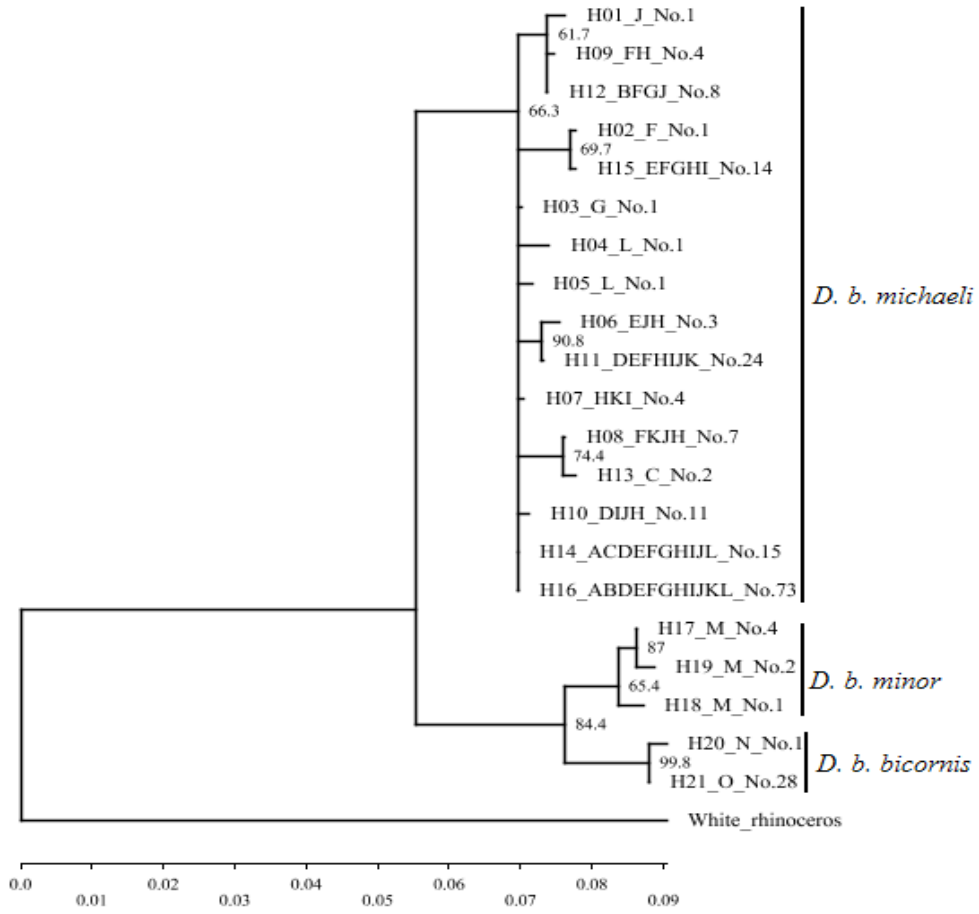

Figure 3.3: Maximum likelihood (ML) phylogenetic tree of the black rhinoceros control region haplotypes H01 to H21, with white rhinoceros (*Ceratotherium simum simum*) as outgroup, *D. b bicornis* and *Diceros bicornis minor* show the magnitude of the differences within *Diceros bicornis michaeli*. Alphabets letters A to O indicate the population location as follows:-- A = Aberdares National Park, B = Chyullu National Park, C = Laikipia Wildlife Conservancy, D = Lewa Wildlife Conservancy, E = Lake Nakuru National Park, F = Masai Mara Game Reserve, G = Ngulia Rhino Sanctuary, H = Nairobi National Park, I = Ol Jogi Ranch, J = Ol Pajeta Ranch, K = Solio Ranch, L = Tsavo National Park, M = Chete, Zimbabwe, N = Damaraland, Namibia, O = Kunene region, Namibia. Figures after No. indicate the number of individuals sharing a haplotype. Statistical bootstrap values for the nodes in the tree was obtained based on 1000 bootstrap replications in the computer program Treefinder version of October 2008 (Jobb, 2008). Only supports of above 50% are indicated.

3.3.2.2 Frequency-based

Generally the fixation index (Φ_{CT}) for the Kenyan black rhinoceros was low (**Table 3.3**) implying low levels of population structure. AMOVA supported neither a two grouped partitioning of the Kenyan black rhinoceros population with Masai Mara or the Aberdares against all other subpopulations grouped together (P = 0.268±0.0185 and 0.501±0.0153 respectively), even though Masai Mara accounted for 5.6% of the total variation in the population, while the montane forest – lowland grouping hypothesis is unsupported and hence remain unresolved. The hypothesis of regional substructuring was not statistically supported (P = 0.0674±0.0073, **scenario three Table 3.3**). There was a strong support (P = 0.0058±0.0026) for the Masai Mara, Chyullu and Laikipia (remained relictual until 2005) based grouping (**scenario four**, **Table 3.3**) suggesting that these relictual isolated subpopulations have retained a genetic status substantially different at control region with respect to all other subpopulations grouped together and historical isolation hypothesis for the relict subpopulations with respect to all other subpopulations mixed together is strongly support

Table 3.3: Analysis of molecular variance (AMOVA) among Kenyan black rhinoceros subpopulations based on mitochondrial haplotypes showing statistical support for various grouping scenarios

Grouping scenario	Hypothesis	Φ_{CT}	P-Values	% of variation
Two groups (1-MAR, 2-all other 11 Pops)	That only Masai Mara is different	0.0546	0.268±0.0185	5.46
Two groups (1-ABE, 2-all other 11 Pops)	Lowland –Highland structuring	-0.0053	0.501±0.0153	-0.53
Three groups (1-LKN/MAR, 2-ABE/LEW/OLJ/LAK/SOL, 3- all other five Pops)	Regional substructuring	0.0247	0.0674±0.0073	2.47
Four groups (1-MAR, 2-CHY, 3-LAK, 4- all other nine subpopulations)	Relictual subpopulations are different (historical isolation)	0.1168	0.0058±0.0026	11.68

The fixation index Φ_{CT} measures the proportion of genetic variation occurring among groups. The maximum value of fixation index (Φ_{CT}) is one. The % variation is the amount of diversity in the population associated to the partitioned group. ABE = Aberdares National Park; CHY = Chyullu National Park; LAK = Laikipia Wildlife Conservancy; LEW = Lewa Wildlife Conservancy; LKN = Lake Nakuru National Park; MAR = Masai Mara Game Reserve; NGU = Ngulia Rhino Sanctuary; NNP = Nairobi National Park; OLJ = Ol Jogi Ranch; OLP = Ol Pajeta Ranch; SOL = Solio Ranch; TSA = Tsavo National Park

3.3.3 Divergence time

The coalescence time of the Kenyan black rhinoceros mitochondrial lineages was calculated at 0.18 Mya (95% CI: 10 – 225 Kya). This coalescence time was calculated using the equation $\mu = \pi/2T$, where μ is the nucleotide substitution rate and π is the average nucleotide diversity in the Kenyan black rhinoceros. Brown and Houlden (2000) had earlier estimated that the black rhinoceros nucleotide substitution rate was around 0.02 substitutions/site/Mya, while this study estimated that the nucleotide diversity in the Kenyan black rhinoceros is around 0.0072±0.003. Substituting these values for μ and π in the equation $\mu = \pi/2T$ it was possible to estimate the time the Kenyan black rhinoceros control region haplotypes diverged from their most recent common ancestor. The μ value used in this study is consistent with other mutation rate values reported in other studies on large mammals (Slade *et al.*, 1994; Wooding and Ward, 1997; Oakenfull *et al.*, 2000; Moodley and Harley, 2005).

3.3.4 Population demography

The expected mismatch for the Kenyan black rhinoceros control region data set was described by parameters estimated from the sudden expansion model ($\Theta_0 = 0.002$, $\Theta_1 = 6.404$, $\tau = 5.812$). The observed and expected mismatch distributions were not significantly different (P(SSD)>0.05, P(RI)>0.05) (**Figure 3.4**). In mismatch distribution analysis, a Gaussian shaped unimodal distribution would suggest population that has undergone a period of rapid expansion in the past.

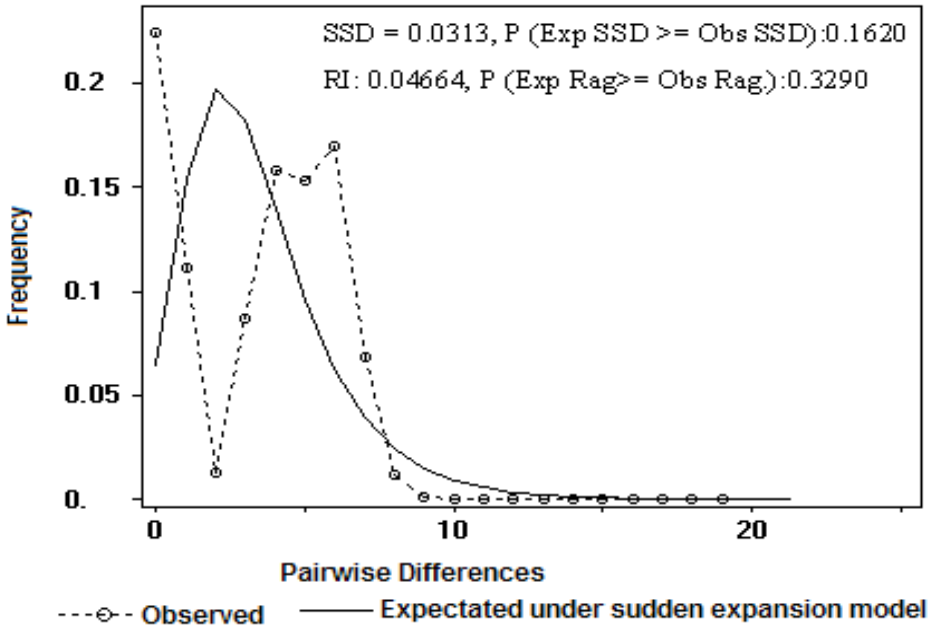

Figure 3.4: Pairwise differences frequencies mismatch distribution of the Kenyan black rhinoceros. SSD is squared deviations, Exp is Expected, Obs is observed and RI is Harpending's the raggedness index

3.4 DISCUSSION

3.4.1 Genetic variation

This study presents the first extensive analysis of the mitochondrial control region genetic structuring and variation in the Kenyan black rhinoceros population in relation to their historical demography. A total of 16 maternal lineages were established in this population signifying that the Kenyan black rhinoceros is not genetically depauperate at the control region, as had earlier been suggested for the black rhinoceros (Ashley *et al.*, 1990; O'Ryan and Harley, 1993; O'Ryan *et al.*, 1994) and in spite of the recent drastic bottleneck experienced by this population substantial genetic variation has been

conserved. Other studies have also reported that black rhinoceros have moderate haplotype diverse despite the recent drastic bottleneck throughout their range (Brown and Houlden, 2000; Tougard *et al.*, 2001; Goossens *et al.*, 2005; Scott, 2008). Eight Zimbabwean black rhinoceros (*Diceros bicornis minor*) studied by Brown and Houlden in 2000 had five haplotypes. Average haplotype diversity in this study was moderate (0.73±0.137) and the finding is consistent with that of *D. b minor* (h = 0.86, n = 8, Brown and Houlden, 2000). Persistence of haplotypes at low frequency in some localities further suggests that the effect of the recent population crash on haplotype diversity is low (**Table 2.2**).

Kenyan black rhinoceros mtDNA has lower average nucleotide diversity than many African mammals examined to date. For example, the endangered mountain zebra (*Equus zebra*) has much higher haplotype (0.918±0.016) and nucleotide (0.01521±0.001) diversity (Moodley and Harley, 2005) than the Kenyan black rhinoceros. Western lowland gorillas have a nucleotide diversity of 0.062 (Clifford *et al.*, 2004). The common warthog and Savannah elephants in Kenya have nucleotide diversity of 0.015, and 0.0168 respectively (Muwanika *et al.*, 2003; Okello *et al.*, 2008). The low nucleotide diversity therefore shows, more clearly than haplotype diversity, the serious impact of recent population reductions on the genetic diversity of the Kenyan black rhinoceros. Masai Mara and Lake Nakuru populations have the highest nucleotide diversity in Kenya (0.012 and 0.011 respectively). Perhaps, the high nucleotide diversity in these two populations is a suggestion of prehistoric shared genetic diversity

that formed part of a genetic continuum in this subregion, since due to their close geographical proximity; they may have exchanged more genetic material at point in time among themselves than with other subpopulations.

3.4.2 Population structuring

Haplotype sharing among populations was high, with haplotypes H14 and H16 being shared among 10 populations out of the total 12 sampled populations implying some historical genetic exchanges must have occurred. Based on demographic historical information, it was expected that drift-inbreeding mediated population structuring would be evident in at least three relic populations – Masai Mara, Chyullu and Laikipia (remained relictual until 2005) – that have no demographic history of immigration. This expectation was well supported by information generated in AMOVA. While frequency based AMOVA suggested a structure based on geographical proximity, especially for the relictual subpopulations, geographical mapping of the haplotypes did not portray any regional pattern of haplotype distribution at the level of the individual haplotype, neither using a median-joining network (**Figure 3.2**), nor a maximum likelihood phylogeny (**Figure 3.3**) inferred any phylogeographical structuring. However, the maximum likelihood analysis clearly shows that the three black rhinoceros subspecies are monophyletic. Three haplotypes (H01, H09 and H12) are grouped together, even though the haplotypes are located in areas separated by long distances. Perhaps, this could be due to the numerous translocations that have characterized rhinoceros management in Kenya; haplotypes that have evolved closely together getting separated.

Low nucleotide diversity (**Table 3.2**) indicates that haplotypes are closely related. This is reflected in a relatively recent divergence time of between 10,000 and 225,000 years for the coalescence of Kenyan black rhinoceros mitochondrial lineages. The mismatch distribution (**Figure 3.4**) of the Kenyan black rhinoceros data does not support the hypothesis of a recent population expansion, and it is likely that the Kenyan black rhinoceros population was relatively abundant over time. The significantly ragged mismatch distribution does hint at a recent demographic fluctuation, possibly due to the population reductions of the 1970s – 1990s and this is further supported utilization of median joining vectors in creating the median joining network (**Figure 3.2**) as it implies either that some haplotypes were lost in the population bottleneck or that they were not sampled.

3.4.3 Conservation implications

This study shows that the Kenyan black rhinoceros has lost some genetic diversity through the recent drastic bottleneck. However, evidence of drift-inbreeding mediated population structuring was not observed in the mitochondrial control region data used in this study. Perhaps, population translocations and a slow generation time may have helped curb the action of genetic drift. Compared to other African mammals that obtain conservation support as genetically viable population such as elephants, warthog or cheetahs (O'Brien *et al.*, 1983; Muwanika *et al.*, 2003; Okello *et al.*, 2008), the Kenyan black rhinoceros retains genetic diversity at the mtDNA control region and is not

showing signs of drift-inbreeding related fitness loss. This diversity will be further improved by continuing the strategy of metapopulation management. Studies have shown that one migration per generation is needed to purge genetic paucity caused by drift-inbreeding (Frankham *et al.*, 2002; Freeland, 2005).

There is also no evidence of population structuring in Kenyan black rhinoceros. Therefore, the partitioning of Kenyan black rhinoceros populations into lowland and montane forest populations by KWS lacks a genetic basis. There could be other valid reasons as to why KWS may partition the populations this way, but conservation managers should be aware of the genetic danger of inbreeding and drift associated with slow recovery of populations seeded with less than 10 individuals (Saccheri *et al.*, 1998; Miller and Waits, 2003; Bergl *et al.*, 2008). Moreover, the management policy of keeping highland-lowland populations separate may have exacerbated the reduction of genetic diversity in the Aberdares-Salient population, and it now has significantly lower nucleotide diversity than any other population in Kenya. This study advocates translocations from lowland population into the Aberdares-Salient population as soon as possible. This study also recommends metapopulation management approach for the Kenyan black rhinoceros that involves at least one translocation per generation.

CHAPTER FOUR

4.0 NUCLEAR GENETIC DIVERSITY AND ITS STRUCTURE IN THE KENYAN BLACK RHINOCEROS SUBPOPULATIONS

ABSTRACT

Habitat destruction and extreme hunting reduced the Kenyan black rhinoceros population by over 98% in thirty years to a mere 400 in 1993 in few isolated sanctuaries. Stringent conservation efforts have seen the number of sanctuaries increase to 14 by 2007. Translocations carry the risk of breaking down local adaptations due to outbreeding depression. Again Kenya Wildlife Service has delineated the metapopulation into lowland and montane subpopulation and avoids translocations between the subpopulation. This could enhance inbreeding driven genetic drift. The objective of this study was to determine the current level of genetic variations in the Kenyan black rhinoceros subpopulations and the extent at which the present genetic variation is partitioned within the subpopulations.

Twelve subpopulations were sampled for this study. Standard molecular methods were employed to extract DNA from both dung and tissue samples. A total of 145 individuals were genotyped at nine autosomal loci. Fifty-two different alleles were observed in the whole sample. Number of alleles per locus ranged from 4-12 with 8.69 as the mean, while mean number of alleles (MNA) per the nine loci per subpopulation ranged from 4.6 - 6.7.

Genetic diversity varied considerably between the subpopulations. Masai Mara has the least genetic diversity (Ho = 0.48) while Lake Nakuru and Tsavo East NP had the highest (0.8). The Kenyan black rhinoceros metapopulation has substantial genetic diversity that is at Hardy-Weinberg Equilibrium (HWE). The least diverse subpopulations had the lowest mean number of alleles, highest inbreeding coefficients and most severe demographic decline. Translocations were identified as the key basis for maintenance of genetic diversity in the metapopulation. In future, managed translocations should take account of the genetic differentiation between subpopulations detected in this study. The long term effective population size (Ne) of Kenya black rhinoceros metapopulation is 1376 individuals, and since the current population is far below the Ne, attaining the recommended Ne is urgently required.

Findings from this study call for further study on all *D. b michaeli* subpopulations including those in Tanzania to refine the understanding of their demographic and evolutionary status.

4.1 INTRODUCTION

4.1.1 Historical demographic dynamics and genetic structure

The temporal and spatial demographic history of any species impacts strongly on its long term structure, effective size, genetic diversity and differentiation (Bergl *et al.*, 2008). Processes such as genetic divergence, geographical and reproductive isolation, could be affected by any factor that varies the demographic trajectory of any insular subpopulation significantly in time and space (Whittaker *et al.*, 2008). Additional factors such as habitat loss and/or fragmentation can lead to local extirpations resulting in a greater loss of subpopulation size. Genetic structuring among subpopulation reflects the number of migrants exchanged between subpopulations and is expected to closely reflect the demographic history of a subpopulation. In contrast larger populations with a history of gene flow are expected to show less genetic structure with limited private polymorphisms and increased local effective subpopulation size (Balloux and Lugon-Moulin, 2002). In small and fragmented populations, genetic diversity may be reduced owing to increased levels of drift and inbreeding. This reduced diversity is often associated with decreased fitness and a higher threat of extinction (Bergl *et al.*, 2008).

Knowledge of the past demographic history of many animal species is often incomplete or totally lacking since human populations lacked the capacity and/or interest to accurately document this information. The San people of Southern Africa documented many animals; including rhinoceros, on cave rock paintings at the Lapala Wilderness

area and Goudriver recording their life and times long before civilizations even existed. However, these paintings are of little help in reconstructing prehistoric demographic histories since at best they can only suggest presence of certain species, but not subpopulation dynamics. Data from hunting expeditions or other statistics may provide some information on the recent demographic history of game species. However, even in these cases the statistics are often deficient and may only reflect the number of killed animals, which is not always correlated with subpopulation size (Aspi *et al.*, 2006). Currently, several coalescent-based modeling methods are available (Beaumont, 2004) that have the ability to infer demographic history of any contemporary populations from patterns of multilocus variation. Bayesian based approaches (Dawson and Belkhir, 2001) provide powerful means for testing various subpopulation historical characteristics; from the effect of alternative histories of drift and gene flow on within-subpopulation microsatellite allele frequencies (Moodley and Harley, 2005) to the determination of subpopulation structure using diploid allele frequencies (Ciofi *et al.*, 1999; Pritchard *et al.*, 2000; Dawson and Belkhir, 2001). These methods, which take advantage of the complete genetic dataset, have the advantage over standard summary conventional statistics and the patchy data obtained from adventure hunting histories in that they are flexible and can evaluate complex statistical models (Moodley and Harley, 2005)

The demographic history of black rhinoceros in Africa must be characterized by multiple evolutionary processes; including colonizations, migrations, subpopulation

expansions, mutation, genetic drift and selection. The results of these multiple evolutionary process is the formation of seven currently recognized black rhinoceros subspecies out of which only four are currently extant in Africa (IRF, 2008). Inferring the time when relevant evolutionary events occurred or the magnitude of their interactions from molecular data is not easy. Using Bayesian coalescent-based methods, it is now possible to estimate when and how these processes took place.

Currently only five rhinoceros species are extant; three species (*Rhinoceros unicornis*, *Diceros sumatrensis* and *Rhinoceros sondaicus*) are endemic to Asia, while the other two (*Ceratotherium simum* and *Diceros bicornis*) are endemic to Africa. *Diceros bicornis bicornis* is distributed in the south-western areas of Namibia, South Africa, southern Angola and western Botswana. *Diceros bicornis minor* is the most numerous and occupies the wetter areas south of central Tanzania through Zambia, Zimbabwe and Mozambique to northern and eastern South Africa. *Diceros bicornis michaeli* is found in northern Tanzania and the whole of Kenya; the Kenyan populations of which will hereafter be referred to as the Kenyan black rhinoceros.

4.1.2 Demographic history of the Kenyan black rhinoceros

Historically *Diceros bicornis michaeli* was distributed from southern Sudan, Ethiopia, and Somalia through Kenya into northern-central Tanzania. Currently, its stronghold is only Kenya, where 85% of the total subpopulation is located. Small numbers are also found in Rwanda and Tanzania (around 125 animals with Tanzania hosting 98% of

these). A free-ranging subpopulation of this subspecies was established outside its range in 1961-1962, when seven animals were translocated from Kibwezi area in Kenya to Addo Elephant National Park in South Africa to establish the first subpopulation in the Park (Hall-Martin, 1984).

There is overwhelming evidence that historically, black rhinoceros were abundant in Kenya (Neumann, 1898; Patterson, 1909; Lloyd-Jones and Brevet-Major, 1925; Barclay, 1932; Hunter, 1952; Brett, 1993) and were widely distributed across all habitats except the cold mountain tops and the coastal region. This implies that there was a high probability of uninterrupted movement of reproducing individuals across different locations ensuring continuous gene flow across the entire gene pool. By 1970 the estimated subpopulation of black rhinoceros in Kenya was approximately 20,000 individuals (**Figure 1.5, Page 23**). The subpopulation declined further and by 1990 it was less than 400 individuals (Brett, 1993; Gakahu, 1993; Emslie and Brooks, 1999; Okita-Ouma *et al.*, 2007), separated into 13 subpopulation each of less than 50 animals; a 98% decline in 30 years. The individuals that were lost comprised a large proportion of the genetic variation and hence the resulting subpopulation must have been less genetically diverse compared to the historical subpopulation. Gene flow was also interrupted by human disturbance activities such as poaching and habitat fragmentation.

Various governments that governed Kenya since the 20th Century realized the need to have increased efforts to halt the demise of this rhinoceros subpopulation to extinction.

Initial efforts were geared towards the creation of national parks and translocation of marooned individuals in threatened habitats to these national parks. Poaching still continued in the national parks and since the 1990s, the focus of black rhino conservation in Kenya has shifted to the development of highly-protected, small-scale, fenced rhino sanctuaries providing intensive protection for the species both in government and private wildlife areas. This was coupled with a matrix of translocations of animals from one location to another over time (**Figure 1.4, Page 19**). The impacts of these demographic changes on the structure of genetic diversity of the subspecies have not been known until now.

A number of studies have used microsatellite markers to examine aspects of rhinoceros genetics (Brown and Houlden, 1999; Florescu *et al.*, 2003; Zschokke *et al.*, 2003; Scott, 2008). Most of the studies were engaged in optimization of black rhinoceros microsatellites (Brown and Houlden, 1999; Cunningham *et al.*, 1999; Nielsen *et al.*, 2007). Harley *et al.,* (2005) used microsatellite markers to examine genetic variation and population structure in the black rhinoceros.

The objective of this study was to comprehensively investigate the genetic diversity and structure of *Diceros bicornis michaeli* within and among 12 Kenyan populations using 12 polymorphic microsatellites. The results were used to understand the genetic diversity and its partitioning in the Kenyan black rhinoceros subpopulations.

4.2 MATERIALS AND METHODS

4.2.1 Sampling

Twelve black rhinoceros subpopulations were sampled in Kenya. Sampling was carried out in collaboration with KWS Staff from the Veterinary Department and rhinoceros Monitoring Programme. The KWS staff collected most of the blood and tissue samples. The samples were stored in 70% ethanol at -20^{o}C or at room temperature in 25% DMSO at the KWS Head Offices Veterinary Laboratory. The tissue and/or blood samples were supplemented with dung samples in those subpopulations where they were not adequate. All dung samples were collected and stored them in 70% ethanol (**Table 2.1, Page 46**).

4.2.2 Laboratory procedures

Total genomic DNA (gDNA) was extracted from both dung and tissue samples using standard procedures as elaborated in Chapter 2. The DNeasy® Tissue Kit was used to isolate DNA from tissue samples while the QIAmp® DNA Stool Mini Kit was used to isolate DNA from dung samples. In both extractions the manufacturer's instructions (QIAGEN® Germany) were followed. DNA was amplified using polymerase chain reaction (PCR) and genotyped with a multiplex panel of 12 polymorphic microsatellite loci:- BR17; BR4; BR6; DB1; DB23; DB30; DB4; DB44; DB49; DB5; DB52; DB66 (Brown and Houlden, 1999; Cunningham *et al.*, 1999) that were constitution into four multiplex called A, B, C and D (**Table 2.2, Page 63**).

Each microsatellite locus was tested alone first and optimized as a single-plex. Single-plex is a PCR involving only one primer. The single-plexes where then multiplexed based on their annealing temperature and amplified product size (**Table 2.2, Page 63**). The screening process was carried out with a PCR reaction conducted in a 10 µl volume containing 5 µl of QIAGEN Multiplex PCR Master Mix (from QIAGEN® Multiplex PCR Kit), 1 µl of the 10X primer mix (0.2 µM of each primer, forward and reverse), 2 µl of DNA, 1 µl of 0.5X Q-Solution (provided in the kit) and 1 µl of water. The amplification profile consisted of an initial denaturation step at 95˚C for 15 minutes, followed by 35 cycles of 94˚C denaturation for 30 seconds; 90 seconds of multiplex primer annealing from 49˚C to 64˚C and 10 minutes of primer extension at 72˚C. Negative blanks of extraction and PCR reaction products were included in each batch of amplifications as negative control. The PCR products were electrophoresd in 1.5% agarose gel stained with 1.5 µl ethidium bromide and visualised using UV light in transilluminator and photographed. All PCR microsatellite fragment products were analysed through capillary electrophoresis on an ABI 377 sequencer (Perkin-Elmer Applied Biosystems). Only the dung samples were extracted twice. Both the duplicate dung sample and the single tissue sample were amplified three times (sequentially) in order to minimize genotyping errors.

4.3 DATA ANALYSIS

4.3.1 Analysis procedure and processing softwares

The ABI electropherogram files received from Macrogen International in Korea and Cardiff University Central Biotechnology Services were scored using Peak Scanner™ Software Ver. 1.0 (Perkin-Elmer Applied Biosystems). Single peaks were recorded as homozygous, while double peaks were recorded as heterozygous. The scored microsatellite fragments were larger than 100bp and hence were scored as 3-digit alleles and recorded per locus across the subpopulation in Microsoft©excel spreadsheet. Allele scores were independently cross checked by Prof Michael Bruford and Dr Benoit Goossens and a consensus scored was derived.

Most genetic analysis softwares are able to read or import data saved in GENEPOP (Raymond and Rousset, 1997) data input format. So the 3-Digit alleles excel spreadsheet data was formatted in text file format for GENEPOP. This software has the ability to convert 3-Digit alleles data file to a 2-Digit allele format and gives a copy of the new 2-Digit allele data for use with other softwares such as POPGENE Ver 1.32 (Yeh *et al.*, 2000) and TFPGA (Miller, 1997). POPGENE software estimates genetic diversity descriptive statistics and establishes standardized genetic distance matrices (Nei, 1972) and matrices of genetic distances corrected for small samples (Nei, 1978). Testing of different grouping scenarios was done with analysis of molecular variance (AMOVA) implemented by ARLEQUIN Ver. 3.1.1 software (Excoffier *et al.*, 2005).

The program STRUCTURE ver 2.0 (Pritchard *et al.*, 2000) was used to infer the number of populations (delta K) without prior information of the sampling locations using mean posterior probabilities.

The program GENETIX 405 (Belkhir *et al.*, 2004) generates several genetic diversity descriptive statistics including Factorial Correspondence Analysis (FCA) that shows the relationship among multilocus genotypes of individual black rhinoceros in different subpopulations in two or three dimensional space. This analysis is unique to this software. In addition this software exports data files to ARLEQUIN VER. 3.1.1. This software has many useful analyses including AMOVA, yet it is not easy to format its data input files, so GENETIX plays this critical function.

4.3.2 Locus inspection for genotyping errors

The program micro-checker Ver. 2.2.3 (van Oosterhout *et al.*, 2004) was used to identify possible null alleles, large allele dropout, scoring errors due to stutter peaks, and possible typographic errors in each locus; thus ensuring statistically acceptable inferences (van Oosterhout *et al.*, 2004). Each sample was replicated three times. Since dung samples were already duplicated, then, it implies that each dung sample was replicated six times.

4.3.3 Linkage disequilibrium analysis

Genotypic linkage disequilibrium (LD) was estimated using the correlation coefficient implemented in POPGENE software. LD analysis was necessary to determine whether there were any loci that were inherited together before proceeding with the analysis since independent assortment is a key assumption in genetic diversity modeling. A permutation approach was applied to determine the significant level of any LD at 95% confidence level. The program GENEPOP (online edition) was used to prepare data for further analysis by other softwares.

4.3.4 Subpopulation genetic diversity, inbreeding and past demographic history

4.3.4.1 Genetic diversity and inbreeding analysis

The program POPGENE Ver 1.32 (Yeh *et al.*, 2000) was used to calculate the number of alleles (na) per loci and the minimum number of alleles (MNA) per subpopulation. GENETIX software was used to calculate Nei's unbiased estimate of expected heterozygosity (HE, Nei, 1973). The observed genotype frequencies were compared with those expected under Hardy-Weinberg equilibrium (HWE) per locus and across all subpopulations and their inbreeding coefficient (F_{IS}) calculated using 1000 permutations (Weir and Cockerham, 1984). This gave an indication of the likely levels of inbreeding and/or genetic drift per subpopulation.

4.3.4.2 Analysis of past demographic history

The Garza and Williamson (2001) method of detecting past demographic history dynamics was used in this study. In this method, the mean ratio (M-ratio) of the number of alleles in a locus to their size range in allele size is calculated. In essence, the M-ratio is a measure of the number of unoccupied potential allelic states or, the average size of gaps between the largest and the smallest allele present in the sample. Following a reduction in population size, genetic drift will tend to create larger gaps, which will cause the value of M to decrease (Garza and Williamson 2001). For calculation purposes, the mean ratio is summarized as M=k/r, where *k* is the number of alleles and *r* = $S_{max} - S_{min} + 1$, where S_{max} is the size of the largest allele, and S_{min} is the size of the smallest allele in the sample. The value of M ratio is smaller (usually less than 0.647, Garza and Williamson, 2001) in recently reduced populations than in equilibrium populations. This method was preferred in this study in comparison to many other available methods because it has ability to predict demographic history for each subpopulation and for the entire metapopulation thereby making it possible to guide policy on translocation of animals between subpopulations.

4.3.5 Subpopulation differentiation and structuring analysis

4.3.5.1 Subpopulation pairwise differences

Genetic differentiation and gene flow among populations were estimated using the Fst

analogue (theta θ_{CT} of Weir and Cockerham (1984), also implemented by GENETIX. Estimates of theta (θ) were used to obtain Fst values (Weir and Cockerham, 1984) for subpopulation pairs in this study. Theta values were preferred compared to Fst values generated by the Wright's (1951) original method because the theta values take into account the effects of uneven samples sizes and the number of sampled populations. The probability that estimated θ_{CT} values were greater than they would have occurred by chance was tested with 1000 permutations using the program ARLEQUIN VER. 3.1.1.

4.3.5.2 Analysis of molecular variance to test subpopulation relatedness

Genetic structure in the Kenyan black rhinoceros subpopulation was investigated by partitioning the total genetic variance into hierarchical components from intra-individual differences to inter-individual differences, and/or to inter-subpopulation differences in an analysis of molecular variance framework, implemented in ARLEQUIN VER. 3.1.1 (AMOVA, Excoffier *et al.*, 2005)). The subpopulations were defined according to their geographical locality (**Table 2.1, Page 46**). Four grouping scenarios were tested. Scenario one involved grouping Mara verses the other 11 subpopulations. This scenario was considered on the basis that Mara is a relict subpopulation that has never received any immigrants under the KWS translocation programme. Scenario two adopted the KWS approach of montane forest subpopulation (Aberdares) verses the other 11subpopulations referred to as the lowland subpopulation. Scenario three is based on the hypothesis that before the drastic subpopulation decline the Kenyan black rhinoceros

was a single panmictic subpopulation and the relict subpopulations – Masai Mara and Chyullu have retained a genetic signature of the pre-bottleneck panmictic subpopulation, while all other populations have undergone mixing from the numerous translocations. Scenarios four was based on based on geographic proximity and historical demographic information of each subpopulation in order to test isolation hypothesis. In this scenario, Masai Mara and Lake Nakuru subpopulations are grouped together based on their geographic proximity and for similar reasons Aberdares, Lewa, Ol Jogi, Laikipia and Solio subpopulations were grouped together. Grouping with the highest value for θ_{CT} that is significant is the most likely subpopulation structure (Moodley and Harley, 2005).

4.3.5.3 Subpopulation gene flow analysis

Gene flow between subpopulations was estimated by determining possible number of effective migrants (Nm) between the subpopulations using Wright's (1951) formula Nm = 0.25(1 - Fst)/Fst per generation.

4.3.5.4 Individual genetic structuring analysis

GENETIX was used to visually explore patterns of genetic differentiation between individual animals in all populations using Factorial Correspondence Analysis (FCA) based on allele frequencies (Belkhir *et al.*, 2004).

4.3.5.5 Bayesian based subpopulation structuring analysis

Subpopulation structure of black rhinoceros in Kenya was further analysed by Bayesian clustering implemented in the program STRUCTURE ver 2.2.3 (Pritchard *et al.*, 2000) The program uses a Markov Chain Monte Carlo (MCMC) approach to infer the number of populations (*K*) in a data set without prior information of the sampling locations. A model with population admixture in which the allele frequencies were correlated within populations was assumed (Falush *et al.*, 2003) because of the numerous translocation that have characteristic rhinoceros management in Kenya since 1961. Most prior parameters were set to their default values as recommended in the STRUCTURE 2.0 user's manual (Pritchard *et al.*, 2000) The length of the burn-in period and the number of MCMC iterations were set to 10000 and 100000 respectively. The range of possible numbers of partitions in the data (*K*) tested was 1 to 12, assuming at a most structured level, that each subpopulation is a subpopulation of its own. Ten runs for each value of *K* were performed, in order to verify that the estimates are consistent across runs. The mean posterior probability was calculated for each *K* over each set of runs, and the true *K* was determined as the maximal value of the estimated logarithm of mean posterior probability of the data *ln* Pr(X/K).

4.3.6 Long-term effective subpopulation size

The long-term effective subpopulation size (Ne) of Kenyan black rhinoceros populations were calculated using expected heterozygosity under mutation-drift equilibrium (Heq)

and the assumed microsatellite mutation rate (μ) (Ohta and Kimura, 1973; Nei, 1987). Demographic or temporally spaced genetic diversity data were not available to allow calculation of more precise Ne estimates (Nei *et al.*, 1975; Waples, 1989; Frankham, 1995; Bergl *et al.*, 2008). The Ne estimates reflect historical changes in subpopulation size (Avise, 2000) and were calculated according to both the IAM: $\mathrm{Ne} = \mathrm{Heq}/(4\mu(1 - \mathrm{Heq}))$ and the SMM: $\mathrm{Ne} = ((1/(1 - \mathrm{Heq}))\text{^}0.5 - 1)/8u$ where *u* is the mutation rate. Ne was estimated assuming mutation rates ranging from 10^{-3} to 10^{-4} for IAM and SMM respectively, which are average mutation rates for autosomal microsatellite loci under these mutation models (Miller and Waits, 2003; Bergl *et al.*, 2008).

4.4 RESULTS

4.4.1 Elimination of genotyping errors

MICRO-CHECKER analysis indicated that two loci (DB4 and DB52) had strong evidence of stuttering and large allele dropout and were consequently dropped from subsequent analysis. A third locus (DB49) had high numbers of missing values for several alleles making occurrence comparison inaccurate. Analysis of presence of null alleles indicated that null alleles were likely to be present in locus DB52 as was suggested by the general excess of homozygotes than expected under HWE for most allele size classes in this locus in all subpopulations. The binomial test could not be conducted for locus DB4 because more than 50% of the alleles at this locus were of one allele size class. The remaining nine loci that had no signs of null alleles were scored in more than 95% of samples. All remaining nine loci did not show any statistically significant linkage disequilibrium.

4.4.2 Genetic diversity, inbreeding and past demographic reductions

4.4.2.1 Allele polymorphism as a measure of genetic diversity

A total of 145 individuals from 12 populations were analysed for the nine loci (**Appendix 3, Page 174**) variations. Fifty-two different alleles were observed in the whole sample and the mean number of alleles per locus was 8.67 ± 3.02 and ranged

from 4 alleles in DB23 to 12 alleles in DB30, BR4 and DB5 (**Table 4.1**). Allele frequency distributions by locus and subpopulation are shown in **Appendix 3.** The frequencies of the alleles generally showed multimodal distributions. Locus BR4, DB5 and DB30 were the most polymorphic, while locus DB23 was the least polymorphic.

Table 4.1: Total number of alleles per locus (na) observed in the Kenyan black rhinoceros subpopulation. Locus BR4, DB5 and DB30 were the most polymorphic locus while DB23 was the least polymorphic

	DB44	DB30	BR17	DB1	DB5	BR4	BR6	DB66	DB23
	169	191	124	118	184	118	131	179	173
	173	195	126	122	186	122	139	191	179
	175	197	128	124	188	124	141	195	181
	177	199	130	126	190	126	143	197	185
	179	201	132	128	192	128	145	199	
	181	203	134	130	194	130	149	201	
	183	205	136		196	132		203	
	187	207			198	134		205	
	189	209			202	136		207	
		215			204	138		209	
		229			206	144			
		231			210	146			
na	9	12	7	6	12	12	6	10	4

4.4.2.2 Number of alleles (MNA) and observed heterozygosity (Ho) as a measure of genetic diversity

The mean number of alleles (MNA) for the nine loci examined in the Kenya black rhinoceros metapopulation was 5.5 ± 0.88 alleles and ranged from 4.6 for Masai Mara to 6.8 for Ngulia Rhino Sanctuary (**Table 4.2**) subpopulation. In addition, Masai Mara had the lowest Ho of 0.48 while both Tsavo East NP and Lake Nakuru NP had the highest Ho of 0.80. Only Masai Mara had a discernible heterozygote deficit of more than 0.1 in magnitude when Ho was subtracted from He, even though this difference was not significant at 95% (**Table 4.2**). The mean Ho and He for the metapopulation were very similar (0.70±0.087 and 0.69±0.034 respectively), suggesting that in overall the metapopulation is at Hardy Weinberg Equilibrium (HWE).

Table 4.2: Genetic diversity and bottleneck variations in the Kenyan black rhinoceros population as demonstrated by nine polymorphic loci.

Demography			Genetic status			Inbreeding (W&C)		Bottleneck evidence	
Subpopulation	**N**	**n**	**He**	**Ho**	**MNA**	**Fis**	**P(0.95)**	**M**	**Variance**
Masai Mara	68	12	0.62	0.48	4.6	0.273	0.083	0.224	0.007
Laikipia WC	12	5	0.68	0.67	4.7	0.112	0.562	0.240	0.011
Ol Jogi RH	27	8	0.67	0.64	5.0	0.097	0.470	0.253	0.012
Aberdares NP	30	6	0.66	0.68	4.8	0.085	0.480	0.243	0.009
Chyulu NP	21	6	0.70	0.72	5.2	0.081	0.650	0.245	0.008
Ol Pajeta RH	77	10	0.67	0.68	5.1	0.033	0.778	0.252	0.012
Tsavo East NP	50	5	0.73	0.80	5.0	0.010	0.705	0.269	0.008
Lewa WC	37	26	0.70	0.70	6.7	0.005	0.566	0.316	0.010
Nairobi NP	68	14	0.73	0.76	6.6	-0.005	0.654	0.309	0.009
Ngulia RS	68	17	0.71	0.74	6.8	-0.013	0.500	0.304	0.010
Solio RH	69	24	0.73	0.77	6.7	-0.031	0.492	0.299	0.014
Lake Nakuru NP	55	12	0.71	0.80	5.6	-0.094	0.270	0.268	0.010
Mean			**0.69± 0.034**	**0.70±0.087**	**5.5±0.088**	**0.0461±0.93**	**0.513±0.19**	**0.268±0.031**	**0.010±0.002**

Significance level was tested using 1000 bootstraps. N = census subpopulation, n = sample size, Ho = Observed heterozygosity, He = Nei's expected heterozygosity, MNA = Mean number of alleles per locus per subpopulation, W&C = Weir and Cockerham's (1984) method for estimating F-statistic, M is the mean ratio of alleles number per locus to their size range in the same locus per subpopulation. Variance is the interlocus discrepancy of M.

4.4.2.3 Inbreeding coefficients (Fis)

The mean probabilities that any two alleles are related by descent (F) varied from -0.094 in Lake Nakuru NP subpopulation to + 0.273 (p=0.083) in Masai Mara subpopulation. Four populations (Lake Nakuru, Nairobi, Solio and Ngulia) exhibited overall negative inbreeding coefficient (**Table 4.2**). All the other populations showed a positive inbreeding coefficient not significantly different from zero and the overall mean inbreeding coefficient was positive (0.046), although not significant at 95% bootstraps (1000 permutations).

4.4.2.4 Past demographic history

All subpopulations of the Kenyan black rhinoceros have shown dramatically low M-ratio values of the number of alleles in a locus relative to their size ranges (**Table 4.2**). Masai Mara had the lowest value (0.224), followed by Laikipia (0.24) and Aberdares (0.243). Lewa had the highest value (0.316). These low M values suggest that Masai Mara subpopulation has undergone the most severe bottleneck compared to Lewa subpopulation.

4.4.3 Subpopulation differentiation

4.4.3.1 Subpopulation pairwise differentiation

Negative pairwise Fst values (**Table 4.3**) imply that the true Fst values for those pairs of subpopulations are extremely small and are probably not significantly different from zero (Long, 1986; Foster *et al.*, 2006). In general, higher Fst values obtained in this study implied genetic differentiation among populations. The Masai Mara subpopulation has the highest pairwise Fst values, while Laikipia Wildlife Conservancy has the lowest. The Masai Mara subpopulation therefore appears to be more differentiated relative to the other populations (**Table 4.3**).

Table 4.3: Pairwise genetic differentiation (*Fst*; Weir & Cockerham, 1984) and their statistical significance level in brackets (ns = not significant, * = significant at p = 0.05, and ** = significant at p = 0.001) between Kenyan black rhinoceros subpopulations (above diagonal) and their standard distance (Nei 1972) below diagonal.

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA
ABE		0.010^{ns}	-0.020^{ns}	0.005^{ns}	0.014^{ns}	0.115*	0.018^{ns}	0.031*	0.019^{ns}	0.012^{ns}	-0.003^{ns}	-0.003^{ns}
CHY	0.261		-0.039^{ns}	0.038*	0.007^{ns}	0.151**	0.029^{ns}	0.006^{ns}	0.007^{ns}	-0.011^{ns}	0.012^{ns}	0.003^{ns}
LAK	0.203	0.164		0.005^{ns}	-0.002^{ns}	0.139*	0.018^{ns}	0.013^{ns}	-0.016^{ns}	-0.029^{ns}	-0.008^{ns}	-0.020^{ns}
LEW	0.141	0.250	0.168		0.036**	0.129**	0.050**	0.046**	0.065**	0.035*	0.012*	0.008^{ns}
LKN	0.194	0.190	0.183	0.184		0.144**	0.032*	0.004^{ns}	-0.005^{ns}	0.005^{ns}	0.010^{ns}	0.021*
MAR	0.503	0.729	0.684	0.466	0.600		0.154**	0.159**	0.140**	0.166**	0.121**	0.090*
NGU	0.209	0.269	0.251	0.229	0.210	0.681		0.033*	0.051**	0.064**	0.021*	-0.013
NNP	0.233	0.171	0.213	0.200	0.107	0.660	0.195		0.007^{ns}	0.028*	0.029*	0.013^{ns}
OLJ	0.243	0.221	0.186	0.291	0.118	0.577	0.292	0.137		0.012^{ns}	0.024*	0.047*
OLP	0.198	0.145	0.125	0.179	0.125	0.675	0.318	0.177	0.172		0.025*	0.033*
SOL	0.121	0.177	0.138	0.086	0.114	0.469	0.147	0.159	0.171	0.157		-0.011^{ns}
TSA	0.259	0.307	0.261	0.193	0.279	0.503	0.170	0.236	0.412	0.318	0.143	

ABE = Aberdares NP, CHY = Chyulu NP, LAK = Laikipia WC, LEW = Lewa WC, LKN = Lake Nakuru NP, MAR = Masai Mara, NNP = Nairobi NP, NGU = Ngulia RS, OLJ = Ol Jogi RH, OLP = Ol Pajeta RH, SOL = Solio RH, TSA = Tsavo East NP

4.4.3.2 Analysis of Molecular Variance

AMOVA results were specific to the different grouping scenarios tested. Grouping scenario one pitting Masai Mara subpopulation against a single pool of all other subpopulation was not statistically significant (P = 0.070 ± 0.010, **Table 4.4**), even though this grouping scenario had the highest fixation value (Φ_{CT} = 0.108, **Table 4.4**) and accounted for 10.82% of the total variation in the Kenyan black rhinoceros subpopulation. Scenario two grouping pitting the Aberdares subpopulation against a single pool of all other subpopulations as is currently defined by KWS in partitioning lowland and montane forest subpopulation was not statistically significant, either and the fixation index value (**Table 4.4**) was negative. Scenario three grouping was based on the hypothesis that before the drastic decline the Kenyan black rhinoceros population was panmictic and the two relict subpopulations – Masai Mara and Chyullu have retained a similar genetic signature of the pre-bottleneck subpopulation, while all other populations have undergone genetic mixing from the numerous translocations (scenario three, **Table 4.4**) is also statistically unsupported and therefore remain unresolved. The hypothesis of regional substructuring due to fragmentation and isolation also had no statistical support (P> 0.05, scenario four, **Table 4.4**) and remains unresolved. This grouping scenario accounts for only 0.77% of the variation in the total subpopulation.

Table 4.4: Analysis of molecular variance (AMOVA) among Kenyan black rhinoceros subpopulations based on nuclear microsatellite data showing statistical support for various grouping scenarios

Grouping scenario	Hypothesis	Φ_{CT}	P-Values	% of variation
Two groups (1-MAR, 2-all other 11 Pops)	That only Masai Mara is different	0.0546	0.070±0.007	10.82
Two groups (1-ABE, 2-all other 11 Pops)	Lowland –Highland structuring	-0.020	0.735±0.010	-1.98
Three groups (1-LKN/MAR, 2-ABE/LEW/OLJ/LAK/SOL, 3- all other five Pops)	Regional substructuring	0.008	0.136±0.008	0.77
Four groups (1-MAR, 2-CHY, 3-LAK, 4- all other nine subpopulations)	Relictual subpopulations are different (fragmentation and isolation)	0.055	0.0603±0.001	5.52

The fixation index Φ_{CT} measures the proportion of genetic variation occurring among groups. The maximum value of fixation index (Φ_{CT}) is one. The % variation is the amount of diversity in the subpopulation associated to the partitioned group. ABE, Aberdares National Park; CHY, Chyullu National Park; LAK, Laikipia Wildlife Conservancy; LEW, Lewa Wildlife Conservancy; LKN, Lake Nakuru National Park; MAR, Masai Mara National Reserve; NGU, Ngulia Rhino Sanctuary; NNP, Nairobi National Park; OLJ, Ol Jogi Ranch; OLP, Ol Pajeta Ranch; SOL, Solio Ranch; TSA, Tsavo National Park

4.4.3.3 Gene flow between subpopulations

Gene flow between the subpopulations was estimated by determining the number of breeding adults that are migrants per generation using F-statistics as stipulated by Wright's (1951) method. The values obtained for Nm show the approximate number of breeding adults that are migrants per generation, in a typical island model of subpopulation structure. All the populations shown that they had more than one breeding adult migrant per subpopulation (**Table 4.5**) and therefore have attained the basic requirements of maintaining genetic homogeneity (Frankham *et al.*, 2002; Freeland, 2005). The source of gene flow could be translocations or prehistoric panmictic mixing. Even the Masai Mara subpopulation, known to not have received recent translocations, showed an Nm value of more than one.

Table 4.5: Pairwise estimates of number of breeding adults that are migrants per generation (Nm) flow between Kenya black rhinoceros subpopulations

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA
ABE												
CHY	23.81											
LAK	***	***										
LEW	54.89	6.32	50.05									
LKN	17.82	35.23	***	6.79								
MAR	1.92	1.4	1.54	1.69	1.49							
NGU	13.96	8.39	13.5	4.75	7.6	1.37						
NNP	7.9	44.12	19.01	5.19	66.49	1.32	7.38					
OLJ	12.82	38.16	***	3.62	***	1.54	4.66	34.14				
OLP	20.26	***	***	6.91	54.43	1.26	3.63	8.75	20.21			
SOL	***	21.35	***	20.3	25.72	1.81	11.46	8.27	10.28	9.87		
TSA	***	96.96	***	30.74	11.42	2.52	***	18.47	5.06	7.22	***	

Cells with asterisk (***) indicates the Fst values between these subpopulations is less than or equal to zero hence Nm values are infinitely very large; that is the subpopulations are subset of each other. ABE = Aberdares NP, CHY = Chyulu NP, LAK = Laikipia WC, LEW = Lewa WC, LKN = Lake Nakuru NP, MAR = Masai Mara, NNP = Nairobi NP, NGU = Ngulia RS, OLJ = Ol Jogi RH, OLP = Ol Pajeta RH, SOL = Solio RH, TSA = Tsavo East NP.

4.4.3.4 Individual genetic structuring

Factorial Correspondence Analysis among multilocus genotypes of individual Kenyan black rhinoceros (**Figure 4.1**) indicated that part of the Masai Mara subpopulation is clustered alone while a second group clusters together with all the other subpopulations in a mixed population, again confirming the results already reported previously.

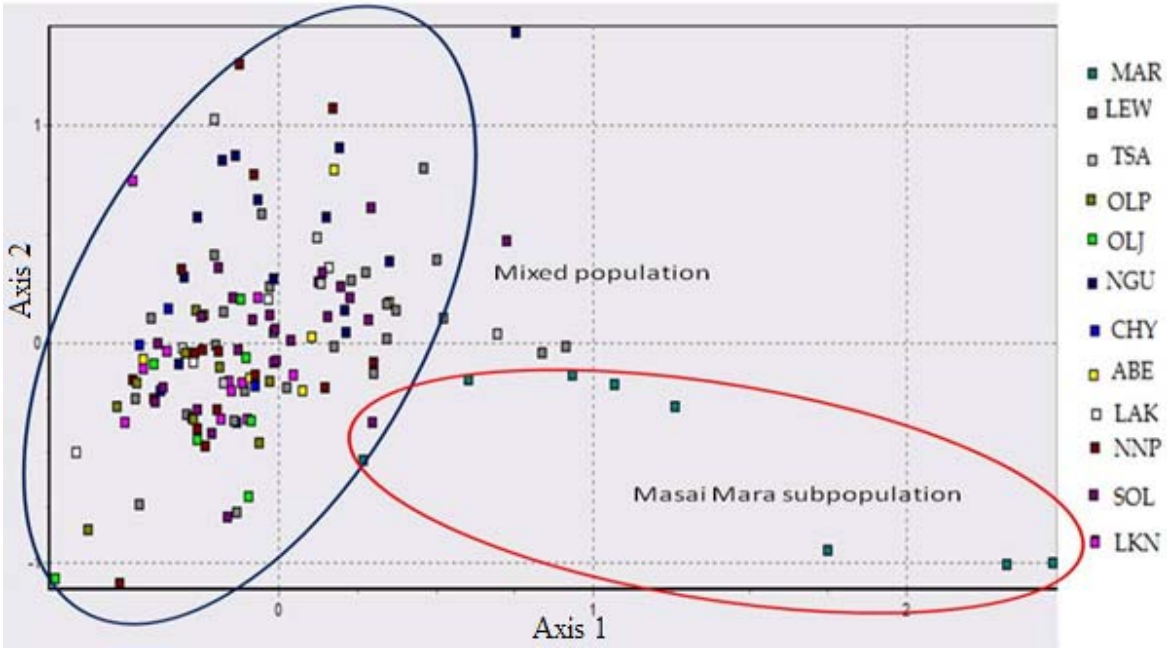

Figure 4.1: Factorial Correspondence Analysis showing two dimensional relationships among multilocus genotypes of individual Kenyan black rhinoceros. ABE = Aberdares NP, CHY = Chyulu NP, LAK = Laikipia WC, LEW = Lewa WC, LKN = Lake Nakuru NP, MAR = Masai Mara, NNP = Nairobi NP, NGU = Ngulia RS, OLJ = Ol Jogi RH, OLP = Ol Pajeta RH, SOL = Solio RH, TSA = Tsavo East NP

4.4.3.5 Bayesian subpopulation structuring

The estimated logarithm of probability of the data, *ln* Pr(X|K), was maximal at $K = 5$ (**Table 4.6**). STRUCTURE software minimal posterior probability revealed existence of five cryptic population structuring (**Table 4.6**), but visual inspection suggested that the best delta k is two closely followed by three, where Ngulia and Masai Mara standalone as populations while all the rest essentially group together (**Figure 4.2**). It is always advised in the STRUCTURE manual in cases where the K value isn't obvious from the delta K value to examine the plots inconjuction with prior biological knowledge to interpret K value.

Table 4.6: Inference for the number of populations (*K*), the minimal posterior probability of the number of populations was maximum with *K* = 5.

K	ln Pr(X|K)
1	-4219.5
2	-4105.5
3	-4028.6
4	-3983.6
5	**-3946.4**
6	-3987.7
7	-3995.1
8	-4114.1

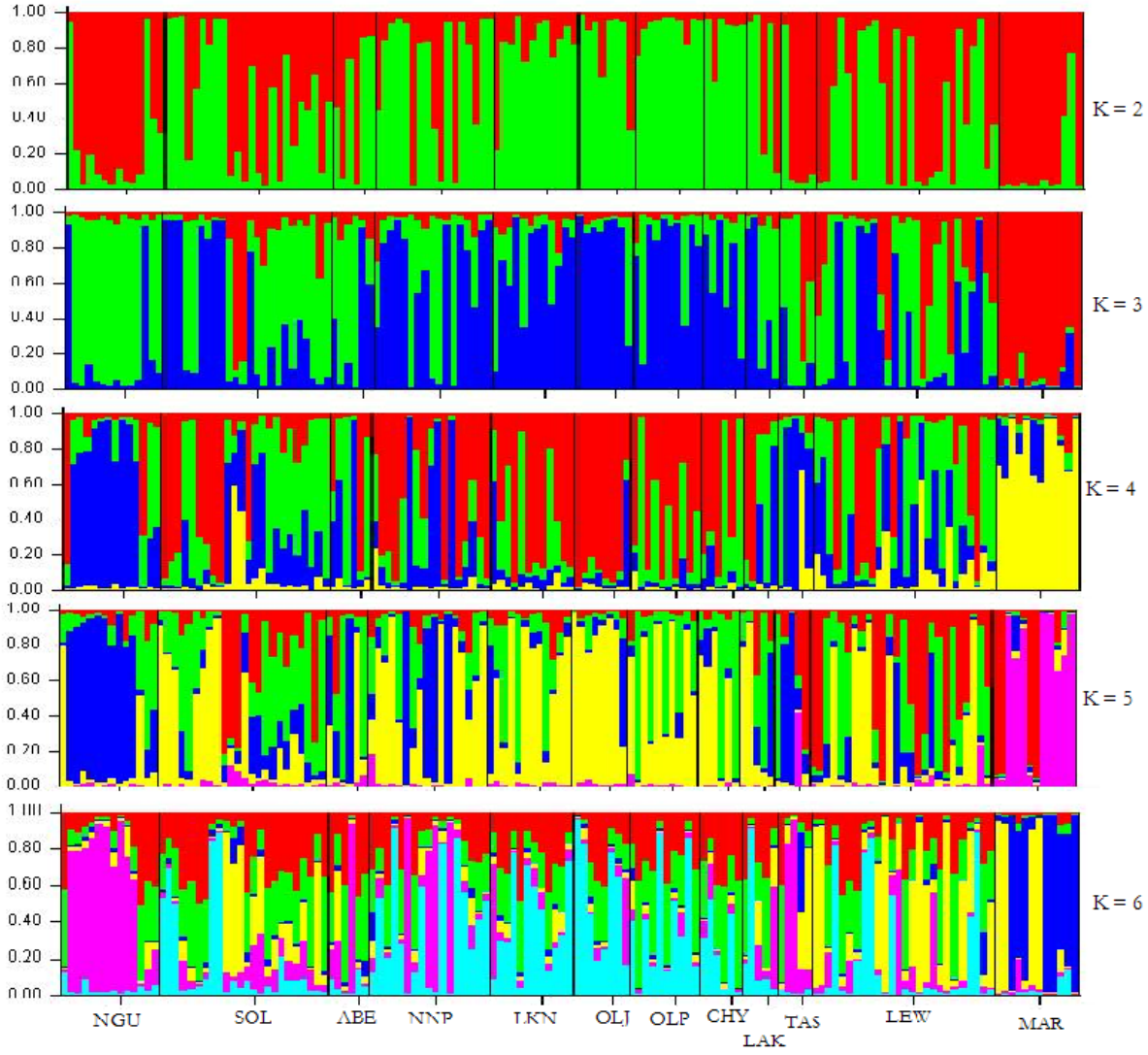

Figure 4.2: Clustering results (*K* = 2 - 6) for all sites, according to STRUCTURE analysis. Each individual is represented by a vertical coloured line partitioned in segments according to subpopulation. Sampled sites are separated by black vertical line and labeled below the figure. ABE = Aberdares NP, CHY = Chyulu NP, LAK = Laikipia WC, LEW = Lewa WC, LKN = Lake Nakuru NP, MAR = Masai Mara, NNP = Nairobi NP, NGU = Ngulia RS, OLJ = Ol Jogi RH, OLP = Ol Pajeta RH, SOL = Solio RH, TSA = Tsavo East NP subpopulation

4.4.4 Effective long term population size

The long-term effective population size (Ne) of Kenya black rhinoceros populations is 1376 as calculated by the bottleneck software, assuming step-wise mutation model (SMM) with a mutation substitution rates ranging from 10^{-4}. The current ratio of effective to census subpopulation size (Ne/N) is 2.29 for the SMM model.

4.5 DISCUSSION

4.5.1 Genotyping errors

This study used both tissue and dung samples therefore the tissue samples acted as the positive control for the dung samples. Genotyping consistency was achieved by adopting a multi-tube multi-sample approach. This method proved to be adequate for this study, thereby eliminating the necessity of adopting other genotyping accuracy improving methods such as Mendelian analysis (Okello *et al.*, 2005). Further improvement was achieved by checking the data using MICROCHEKER and hence the loci that remained after eliminating three problematic loci attained the basic statistical analysis requirements.

4.5.2 Genetic diversity

This study presents the first extensive analysis of genetic diversity in the Kenyan black rhinoceros metapopulation based on microsatellite markers. Despite the historically documented bottleneck in the Kenyan black rhinoceros (Gakahu, 1993), the results

showed moderate levels of genetic diversity for the metapopulation as a whole (**MNA = 5.5±0.088, Mean Ho = 0.70±0.087 and He = 0.69± 0.034**). Scott (2008) studied museum skins of different subspecies of black rhinoceros and found similar values of genetic diversity in a smaller sample of *Diceros bicornis michaeli* (**MNA = 5.3; Mean Ho = 0.57 and He = 0.64**) and also established that *D b michaeli* had the highest level of microsatellite genetic diversity among the extant rhinoceros subspecies. Harley *et al.,* (2005) also shown that *Diceros bicornis michaeli* was more genetically diverse (**Ho = 0.675**) compared with *Diceros bicornis minor* (0.459) and *Diceros bicornis bicornis* (0.505).

However in this study, not all subpopulations had high genetic diversity. Masai Mara subpopulation had the lowest mean number of alleles (MNA = 4.6), lowest observed heterozygosity (**Ho = 0.48**), lowest M value in the bottleneck tests (0.224) and highest inbreeding coefficient (Fis = 0.273) value (**Table 4.2**). A number of reasons could explain why Masai Mara subpopulation has such low genetic diversity compared to the other subpopulations. The MNA of Masai Mara is only two alleles fewer than the highest MNA observed in the Lewa Wildlife Conservancy subpopulation thus making it more difficult to explain the deficit in observed heterozygosity satisfactorily from MNA perspective. However, the Masai Mara subpopulation is entirely relictual and has no history of immigration. Due to this isolation, it is likely to have experienced stronger genetic drift compared to other subpopulations. The hypothesis of stronger drift in this subpopulation is more plausible given its small M value compared to other

subpopulations (**Table 4.2**). Prolonged isolation and demographic decline implies that over time, most of the animals in the subpopulation were closely related and any mating that took place was possibly between closely related individual. This kind of negative feedback involving demographic decline, isolation, and inbreeding enhances further the random forces of genetic drift thus creates perfect grounds for extinction vortex forces (Frankham and Ralls, 1998) to drive genetic diversity to this comparatively low value.

Another possible explanation as to why the observed heterozygosity in Masai Mara is so low compared to its expected heterozygosity is that perhaps this subpopulation is made up of more than one subpopulation (Wahlund effect). The Wahlund effect hypothesis is credible given that Masai Mara is unfenced and may be receiving or has historically received immigrants from the adjacent expansive Serengeti ecosystem in Tanzania. It is also possible that not all the animals in Mara-Serengeti ecosystem are *Diceros bicornis michaeli*. Possibly there could be some outbreeding of animals in this ecosystem with *Diceros bicornis minor* since historically, Tanzania forms the northern end of *Diceros bicornis minor* distribution thus making the Wahlund effect case more probable. Perhaps if the specific individuals that are from the same subpopulation are isolated, Ho values at HWE will be realized.

Lake Nakuru NP, Tsavo East NP, Solio RH, Ngulia RS and Nairobi NP subpopulations are the oldest sanctuaries created to receive isolated black rhinoceros from different locations in Kenya and have been successful nuclei units that have been used to restock

other areas. Thus they have enjoyed substantial genetic mixing and perhaps this is why they are the only subpopulations with negative inbreeding coefficient. Up to date, Lake Nakuru NP has donated 20 animals, Solio RH 89 animals, Ngulia RS 25, Nairobi NP 73 and Tsavo East NP 4. High heterozygosity values indicate low level of inbreeding, low or no selection pressure and large number of alleles that have been retained as the population has not undergone genetic drift and fixed or lost some alleles. These findings suggest that translocations had positive effects in the genetic diversity in the Kenyan black rhinoceros subpopulations.

4.5.3 Population structuring

Patterns of Kenya black rhinoceros subpopulation structuring were investigated by various methods employing both standard model based analysis for microsatellite markers. These included pairwise Fst, AMOVA, factorial correspondence analysis and Bayesian clustering analysis.

A total of 66 subpopulations pairs were analysed for genetic differentiation (**Table 4.3**). Thirty four pairwise comparisons had pairwise Fst values that were statistically significantly different from zero. The Masai Mara subpopulation had pairwise Fst values that statistically different from all other subpopulations, followed by Lewa Wildlife Conservancy subpopulation which was not significantly different from Aberdares NP, Laikipia Wildlife Conservancy and Tsavo East NP pairwise Fst values.

Some subpopulations had pairwise Fst values that were negative suggesting that these subpopulations had allele frequencies that were very similar; most probably due to continuous gene flow between them. Assessment of their demographic history made this view more credible. For example, the Aberdares subpopulation had negative pairwise Fst difference with Likipia WC, Solio RH and Tsavo East NP subpopulations (Fst = -0.020, -0.003, -0.003 respectively). Demographic data (**Figure 1.4, Page 19 and Table 1.1 Page 20**) indicates that between 1961 and 2008, at least 12 animals were translocated to the Aberdares from Solio, Rimuruti (Laikipia District) and Nyeri forest (Mt Kenya). In 1983, three animals where moved from Aberdares to Nairobi National Park, which also received several animals from the Tsavo region between 1961 and 2008. Solio also obtained animals from Rimuruti, Nyeri Forest and Tsavo region between 1961 and 2008. Laikipia Wildlife Conservancy had a small relict subpopulation of 11 (**Table 1.1 Page 20**) until 10 more animals were added in 2005 from Nairobi National Park. Therefore, animals in the Aberdares and Solio had a common origin in the Rimuruti and Nyeri forest in early 1970's. In addition, some animals were moved from Solio to Aberdares and the alleles of these animals may have originated in Tsavo. Animals in Laikipia have strong links with animals in Tsavo, Solio and the Aberdares; with Nairobi National Park as the central mixing ground. Another good example is the Lake Nakuru National Park subpopulation and Ol Jogi Ranch (Fst = -0.005, $P<0.05$). Records of translocation of black rhinoceros between these two locations indicate that these subpopulations have animals with origins at Solio Ranch between 1987 and 2007.

Pairwise Fst comparisons between subpopulations are indicative tools to guide future translocation efforts. This is because, if the objective of the specific translocation is to boost genetic diversity, then low Fst values are expected due to translocation of animals between subpopulations which will tend to be non- significantly different from one another, even if their overall pairwise Fst value is positive. In general, Fst values of 0-0.05 are considered as indicators of little differentiation; while values of 0.05-0.25 indicate moderate differentiation and values of >0.25 represent pronounced genetic differentiation, although this scales to population size (Freeland 2005). The significantly different Fst between Masai Mara and other subpopulations suggest impeded gene flow between Masai Mara and other subpopulations, which is consistent with translocation records.

Grouping scenarios were tested based on different hypothesizes. The hypothesis that Masai Mara was the only different subpopulation was rejected by AMOVA (**Table 4.4**) even though this grouping suggested that Masai Mara subpopulation accounted for more than 10% of the total genetic variation in the metapopulation. Similarly, the KWS hypothesis of lowland and montane forest grouping was rejected with a negative θ_{CT} suggesting possible significant gene flow between lowland subpopulations and montane forest subpopulation. Further, this kind of grouping contributed negatively in the overall genetic variation suggesting that it is invalid. Even demographic history (**Table 2.1**) indicates that some animals were moved from Solio, Laikipia and Mt Kenya forest in

various. Therefore, it must be based on other factors other than genetic considerations, but since it is one of the poor performing subpopulation in terms of population growth (Okita-Ouma *et al.*, 2007), there is a strong case to associate this poor performance with relatively high level of inbreeding coefficient observed in this subpopulation (**Table 4.2**) and recommend immediate genetic argumentation through immigration.

The third grouping hypothesis testing regional geographical proximity is also unsupported, and perhaps it cannot withstand historical demographic scrutiny due to the numerous translocations that have characterised the management of black rhinoceros in Kenya. The fourth grouping hypothesis implicating panmictic mixing of the prehistoric Kenyan black rhinoceros subpopulations expected to be portray a genetic signature in the three relictual subpopulations (Masai Mara , Chyullu NP and Laikipia WC) group against the other subpopulations is rejected (**Table 4.4**). However, this kind of grouping accounted for 5.52% of the total genetic variations in the metapopulation. .

Gene flow analysis based on the number of migrants per generation (**Table 4.5**) confirms that historically, the Kenyan black rhinoceros have exchanged breeding immigrants. However, the Masai Mara subpopulation had the least number of detectable migrant. Factorial correspondence analysis of individual animals in the metapopulation identified four individuals in Masai Mara that skewed to the extreme right. This occurrence supports further the possibility of existence of more than one subpopulation in Masai Mara and emphasis the need for further study in this subpopulation.

Posterior Bayesian clustering revealed that the most likely genetic structuring for Kenyan black rhinoceros subpopulation is 5 cryptic subpopulations, however visual examination of the K plots suggest a grouping of K = 3 as the most probable grouping. In this grouping, Masai Mara is one group and Ngulia is another while the rest is a mixed group.

4.5.4 Conservation Implications

This study reveals varied levels of genetic diversity in the Kenyan black rhinoceros subpopulations. It is low in Masai Mara, Laikipia WC and the Aberdares NP subpopulations, but high in Lake Nakuru NP and Tsavo East NP subpopulations and moderate in the rest of the subpopulations. Therefore there is need to focus on the genetic management of subpopulations that are low in genetic diversity. Of interest is that the same populations that have low genetic diversity have also undergone the severest demographic decline and have history of isolation. It is not documented why Masai Mara subpopulation has experienced sustained isolation. Perhaps its status as a National Reserve and the fact that it is not fenced and therefore with lingering security status might have contributed to its isolation. However, this isolation has contributed negatively to the genetic diversity of this subpopulation. The Aberdares NP subpopulation has been isolated intentionally and has been qualified as a montane forest subpopulation. Genetically this classification is unsubstantiated and should be re-examined from the management perspective. In the long run, continued isolation may

increase the probability of an extinction vortex in this subpopulation. The Laikipia WC subpopulation had been isolated for a long duration until it received some 10 immigrant from Nairobi NP in 2005. Perhaps with time, this introduction may improve genetic diversity in this subpopulation. This study recommends that animals should be translocated into Masai Mara and Aberdares NP.

Potential candidates for translocation to Masai Mara could be animals indigenous to Lewa WC or Lake Nakuru NP. These subpopulations are selected based on their significant different pairwise Fst values with Masai Mara and also on the fact that their current population size is large enough to afford to donate animals. The Aberdares NP would also benefit from translocation of some animals indigenous to Masai Mara and Nairobi NP, again based on pairwise Fst differences.

The Kenyan black rhinoceros metapopulation apparently lacks any significant ecological units that currently deserve conservation attention. Instead, its genetic diversity is approximately at equilibrium when considered as one group. Therefore, a continued programme of translocation should be conducted to enhance and retain the current genetic diversity experienced at the metapopulation level. Populations that have benefited with earlier translocation and have acted as breeding nucleus such as Lake Nakuru NP, Solio RH, Ngulia RS and Nairobi NP have shown negative inbreeding coefficients and genetic diversity that is at HWE; suggesting that translocation had a positive impact to their genetic diversity and should be encouraged.

The findings of this study call for further research on all *Diceros bicornis michaeli* subpopulations including those in Tanzania to refine the understanding of their evolutionary interactions.

CHAPTER FIVE

5.1 LEVELS OF GENETIC DIVERSITY AS REPORTED BY THE TWO MARKERS

5.1.1 Genetic variation in the Kenyan black rhinoceros based on the two markers

Both mtDNA and microsatellite (nDNA) markers detected moderate genetic diversity in the Kenyan black rhinoceros metapopulation (h =0.78±0.027, n = 170; Ho =0.70±0.087, n=145). The level of genetic diversity uncovered is consistent with previous studies on *Diceros bicornis michaeli* (Brown and Houlden, 2000; Tougard *et al.*, 2001; Scott, 2008). However, mtDNA and nDNA diversity varied between subpopulations. While Masai Mara had the highest genetic diversity for mtDNA (h = 0.93, π = 0.011, **Table 3.2, Page 92**) it had the lowest nDNA diversity (Ho = 0.48, MNA = 4.6, **Table 4.2, Page 124**). This subpopulation also had the highest inbreeding coefficient (+0.273) and the strongest evidence for a genetic bottleneck, as is illustrated in the population having the lowest M –ratio (M = 0.224, **Figure 5.1**). The lack of genetic diversity detected by microsatellite data in Masai Mara unlike that detected by mtDNA may be related to the stochastic effects of genetic drift after the severe bottleneck that Masai Mara seems to have undergone, compared to the other subpopulations and also the extremely shallow population history of divergence that approximate to within the past 10,000 years. Thus, mtDNA with its smaller effective population size, faster genetic drift and haploid maternal inheritance can actually provide a better view through this extremely short

timeframe (Nyakaana *et al.*, 2002; Hoarau *et al.*, 2004) but genetic drift may not always eliminate rare haplotypes and chance changes in allele frequencies can have the opposite effect to that predicted under neutral theory when a single locus is examined (e.g. Goodman *et al.*, 2001).

In contrast, the Lewa WC subpopulation showed exactly the opposite pattern of Masai Mara in that it had the lowest haplotype diversity, a high observed heterozygosity, the highest nucleotide diversity and the lowest bottleneck effect. This finding is consistent with the demographic history of this population in that its original stock of animals was rescued from northern parts of Kenya where gene flow was fairly high because of the low human density in this area thus rhinoceros movement was likely to have been high. However this result again illustrates the stochastic nature of the correlation between nDNA and mtDNA in subdivided small populations.

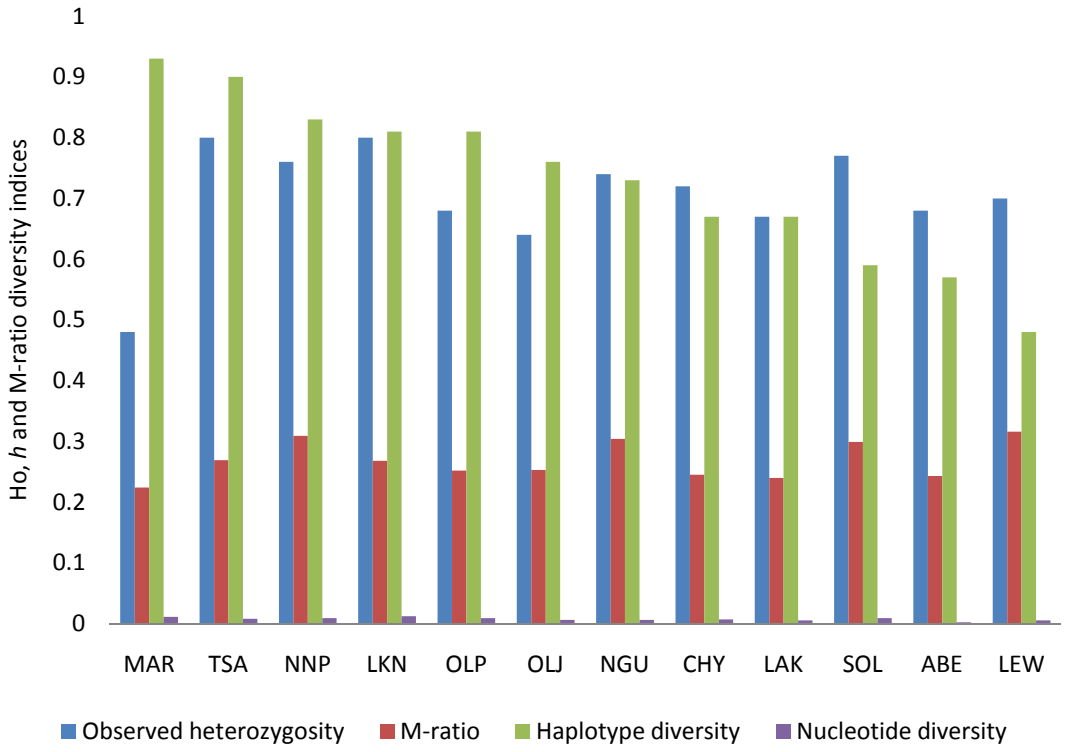

Figure 5.1: Mitochondrial and nuclear DNA genetic variation in the Kenyan black rhinoceros subpopulations. MAR = Masai Mara Game Reserve; TSA = Tsavo National Park NNP = Nairobi National Park; LKN = Lake Nakuru National Park; OLP = Ol Pajeta Ranch; OLJ = Ol Jogi Ranch; NGU = Ngulia Rhino Sanctuary; CHY = Chyullu National Park; LAK = Laikipia Wildlife Conservancy; SOL = Solio Ranch; ABE = Aberdares National Park; LEW = Lewa Wildlife Conservancy.

Between the two extremes the other subpopulations such as Nairobi NP, Ngulia RS, Lake Nakuru NP and Solio RH that have mitochondrial and nuclear diversity that are fairly similar and decrease together with decrease in bottleneck effects. All these subpopulations are similar in a number of interesting ways; for instance all the subpopulations are at HWE, have no evidence for inbreeding and low bottleneck effects. Similarly, all theses subpopulations have experienced substantial artificial gene flow

through translocations, since they are nuclei breeding sanctuaries in the rhinoceros population recovery strategy in Kenya (Okita-Ouma, 2004). It seems likely that the artificial gene flow into these populations is the reason why their mtDNA and nuclear DNA variations are similar (**Figure 5.1**) and their large population size is at HWE.

The Kenyan black rhinoceros is not as low in genetic diversity as some other celebrated species in Africa which have undergone demographic bottlenecks such as the cheetah (O'Brien *et al.*, 1983; Menotti-Raymond and O'Brien, 1993; Marker *et al.*, 2008), and has higher diversity compared to other black rhinoceros populations in Africa (Brown and Houlden, 2000; Scott, 2008). Findings from this study show varying levels of significant pairwise genetic differentiation between the subpopulations. Masai Mara is the only subpopulation that is significantly different from all other subpopulations, and perhaps this could be explained by several factors including genetic drift, as illustrated by a history of extreme demographic isolation from all other Kenyan black rhinoceros subpopulations. Additionally, it is possible that not all black rhinoceros in Masai Mara are *Diceros bicornis michaeli*; since the Masai Mara is unfenced there is a possibility of historical exchange of genetic material within the Mara – Serengeti ecosystem and perhaps some of the animals may be *Diceros bicornis minor* since Tanzania is the northern range for this subspecies (IRF, 2008).

5.1.2 Population structure of the Kenyan black rhinoceros based on the two markers

Findings from the mtDNA and nDNA AMOVA are congruent in that they fail to resolve three hypotheses i.e. that Masai Mara is substantially distinct subpopulation from the other Kenyan black rhinoceros subpopulations and hence it should be managed as a separate conservation unit. The lowland - highland hypothesis is also not resolved and completely lacks even historical demographic support. The hypothesis of regional substructuring based on geographical proximity is also not supported suggesting the absence of gene flow barriers that regionally structure the subpopulations or that those recent translocations have erased any signal of regional substructuring for nuclear DNA. Results between the markers concerning fragmentation and isolation for the relictual subpopulation were incongruent, where a significant relationship was found for the mitochondrial mtDNA (scenario four, **Table 3.3, Page 97**). This phenomenon has also been observed in African elephants (Okello *et al.,*., 2008) and has been dubbed 'cytonuclear genomic dissociation' by Roca *et al.,* (2007) and its thought to occur because of mitochondrial ploidy and inheritance modalities (Birky *et al.,*., 1989; Avise 1994; Hoarau *et al.,*., 2004; Roca *et al.,*., 2007). Thus this suggests that based on the mtDNA markers, the relictual subpopulations namely Masai Mara, Chyullu NP and Laikipia WC (that remained relict until 2005) are significantly isolated from each and this finding is supported by the demographic history of these subpopulations. This observation has a historical demographic support since Masai Mara and Chyullu subpopulations have no history of immigration.

CHAPTER SIX

6.1 GENERAL DISCUSSION

6.1.1 Introduction

Conservation research on the black rhinoceros in Kenya has, to date, focused on ecology (Goddard, 1967, 1970; Oloo *et al.*, 1994; Mukewa, 1995; Muya and Oguge, 2000; Patton and Jones, 2007) behaviour (Goddard, 1966; Morinte and Keter, 2000), breeding performance (Wanjohi, 1987; Brett, 1998; Okita-Ouma, 2004), disease (Obanda *et al.*, 2008), management (Brett, 1993; Okita-Ouma *et al.*, 2007) and security (Leader-Williams, 1992; Martin and Vigne, 2003), while relatively little research has been done on their genetics (Swart *et al.*, 1994; Brown and Houlden, 2000; Scott, 2008). Yet, genetic diversity, especially in small populations, and its distribution between populations can be as important as any other factor in assessing the overall conservation status of a species (Frankham, 1995; Freeland, 2005; Bergl *et al.*, 2008). There is no baseline data available for use in determining whether the genetic diversity currently present in the Kenyan black rhinoceros is declining or increasing.

This study presents the first extensive genetic analysis of the Kenyan black rhinoceros using both mitochondrial genes (mtDNA) and nuclear genes (nDNA). Direct comparison between mitochondrial and nuclear genetic variation was necessary because evolutionary and demographic processes (especially dispersal resulting in gene-flow,

mutation and drift) differentially affect each class of genes independently (Hoarau *et al.*, 2004; Hellberg, 2006; Shearer *et al.*, 2008) and the use of more than one marker made the study more informative. Previous studies have shown that effects of genetic drift are often larger at the mitochondrial genes compared to nuclear genomes. This is because nuclear genes have biparental, diploid inheritance, whereas mitochondrial genes are haploid, predominantly uniparentally (maternally) inherited (Avise, 1994), and hence the effective populations size for mitochondrial DNA is expected to be on average four times smaller than that of nuclear DNA and is thus subject to larger drift effect even where both DNA experience similar selection pressure (Birky *et al.*, 1989; Hoarau *et al.*, 2004). Also, mitochondrial DNA lacks the mutation repair capabilities ascribed to chromosomal DNA, hence mutations accumulate more quickly in mitochondrial genes (Birky *et al.*, 1989), and this makes them ideal candidates for analysis of genetic differentiation between populations (Hoarau *et al.*, 2004).

Analyses of geographical distribution of mitochondrial genes reveal female population history (Avise 1994) and thus they are the markers of choice for phylogeographic studies based on maternal lineages. On the other hand, nuclear genes have a higher level of polymorphism (Jarne and Lagoda, 1996) and thus higher ability to detect even subtle population differentiation (e.g. Shaw *et al.*, 1999) than mitochondrial genes, although in some cases they have been found to produce similar (e.g. Buonaccorsi *et al.*, 2001) or even lower resolution than mtDNA (e.g. Bérubé *et al.*, 1998). Therefore since this study used the two markers to determine the amount of available genetic diversity in the

Kenyan black rhinoceros and its mode of partitioning within and among subpopulations, the results are likely to be more accurate and the findings from this study could have substantial implications in the conservation of the Kenyan black rhinoceros populations in future.

6.1.4 Conclusion

From the finding of this study, a few highlights are clear on the genetic diversity still present in the Kenyan black rhinoceros subpopulations, the extent of its partitioning in the metapopulation and whether the two markers recapitulate the same scenario in measuring genetic diversity.

1. Despite the recent bottleneck, the Kenyan black rhinoceros is not as depauperate in genetic diversity as expected, and has higher diversity compared to other black rhinoceros population in Africa as detected by both mtDNA and nDNA markers.
2. However, the Kenyan black rhinoceros metapopulation has lower average haplotype diversity compared to most large African mammals.
3. Translocations have maintained gene flow that has probably helped in reducing the effects of genetic drift in Kenyan black rhinoceros subpopulations thus enabling them retain some moderate levels of genetic diversity.
4. Masai Mara has, however, experienced higher levels of genetic drift and/or inbreeding because it has been isolated for a long time from the other subpopulations and it has also undergone a more severe bottleneck. However, it does not represent a genetically distinct or a separate monophyletic

subpopulation and thus does not meet the definition of ecological significant unit (ESU) as classified by Moritz (1994).

5. The numerous translocations that have characterised the management of black rhinoceros in Kenya have masked any phylogeographic structuring in the subpopulations, thus both markers were incongruent in the analysis of genetic partitioning. While mtDNA markers detected significant separation between the relict subpopulations, nDNA markers did not.
6. The highland – lowland grouping is not genetically supported and even the demographic history of the animals that were translocated to constitute the Aberdares subpopulation (the main mountainous subpopulation, Okita-Ouma *et al.*, 2007) strongly suggest substantial recent genetic mixing.
7. To maintain the present moderate genetic diversity in the metapopulation, a careful translocation programme should be constituted and this program should consider the pairwise genetic differences between the subpopulations in order to avoid translocating animals between genetically similar subpopulations.

6.2 OVERALL CONSERVATION AND MANAGEMENT IMPLICATIONS

Survival of threatened species relies on a clear understanding of the factors causing the extinction threat and extensive adaptive management interventions that can reduce or eliminate the threat. The main threat to the survival of black rhinoceros in Kenya and elsewhere is hunting and loss of habitat (Moehlman *et al.*, 1996; Dean and Foose, 2006;

IUCN, 2008). Both threats are directly or indirectly linked to increase in human population (Laroche and Durand, 2004; Bergl *et al.*, 2008). The result of the two main threats on the Kenyan black rhinoceros population was drastic reduction into small isolated subpopulations; sometimes only individual animals remained isolated completely from any other population.

Findings from this study reveal that the highest component of genetic diversity is still partitioned among individuals, implying that individual animals in the total populations are responsible for driving the genetic diversity. Therefore, to preserve genetic variability in the various subpopulations it will be important to conserve as many individuals as possible and in the event of translocation; evaluate the genetic orientation of individual(s) identified for translocation, with respect to the recipient subpopulation, with the view of establishing the level of differentiation between the individual targeted for translocation and recipient population. This could be very expensive, so the most realistic approach is to consider the pairwise genetic differences to identify which populations are genetically differentiated and allow this to guide the most useful translocations.

Masai Mara is not a unique subpopulation and is in severe need of translocation to address the current low genetic diversity in the subpopulation. Continued isolation of the Masai Mara subpopulation may drive it into monomorphism and could trigger an extinction vortex (Frankham *et al.*, 2002) Similarly, the highland – lowland grouping is

negatively affecting the Aberdares NP subpopulation and should be revised. The breeding performance for this subpopulation is low (Okita-Ouma *et al.*, 2007), perhaps due to the low genetic diversity in this subpopulation.

Future studies will be necessary perhaps using two or more mitochondrial genes and perhaps sample the entire Masai Mara subpopulation and analyse further its phylogeography. The study should also be extended to other subpopulations and may examine more closely the paternity patterns within the specific populations. Therefore, further management recommendations should be informed by detailed genetic studies of each subpopulation.

REFERENCE

AfRSG ed. 2004. *IUCN-SSC-AfRSG meeting* Kilaguni, Tsavo West: African Rhino Specialized Group (AfRSG)

Akama, J. S. 1998. The evolution of wildlife conservation policies in Kenya. *Association of Third World Studies, Inc*, pp. 1-11.

Allendorf, F. W. and Leary, R. F. 1986. *Heterozygosity and fitness in natural populations of animals*. Sinauer Associates, Sunderland, Massachusetts.

Allendorf, F. W. and Luikart, G. 2007. *Conservation and the genetics of populations*. Blackwell Publishing.

Amos, B. and Hoelzel, A. R. 1992. Applications of molecular genetic techniques to the conservation of small populations. *Biological Conservation* 61, p. 133–144.

Antoine, P. O. 2003. Middle Miocene elasmotheriine Rhinocerotidae from China and Mongolia: taxonomic revision and phylogenetic relationships. *Zoologica Scripta* 32, p. 95–118.

Ashley, M. V., Melnick, D. J. and Western, D. 1990. Conservation genetics of the Black Rhinoceros (Diceros bicornis). I: Evidence from the mitochondrial DNA of three populations. *Conservation Biology* 4, p. 71–77.

Aspi, J., Roininen, E., Ruokonen, M., Kojola, I. and Vila, C. 2006. Genetic diversity, population structure, effective population size and demographic history of the Finnish wolf population. 10.1111/j.1365-294X.2006.02877.x. *Molecular Ecology* 15(6), pp. 1561-1576.

Avise, J. C. 1994. *Molecular markers, natural history and evolution*. New York: Chapman and Hall.

Avise, J. C. 2000. *Phylogeography: The History and Formation of Species*. Cambrigde, MA.: Harvard University Press.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E. and Lamb, T. 1987. Intraspecific Phylogeography: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. *Annual Review of Ecology and Systematics* 18, pp. 489 - 522.

Balloux, F. and Lugon-Moulin, N. 2002. The estimation of population differentiation

with microsatellite markers. *Molecular Ecology* 11, pp. 155-165.

Bandelt, H. J., Forster, P. and Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* 16, p. l37–148.

Bandelt, H. J., Forster, P., Sykes, B. C. and Richards, M. B. 1995. Mitochondrial portraits of human populations. *Genetics* 141, pp. 743-753.

Barclay, E. N. 1932. *Big Game Shooting Records; Together with Biographical Notes and Anecdotes on The Most Prominent Big Game Hunters of Ancient and Modern Times*. H.F. & G. Witherby.

Bayes, M. K., Smith, K. L., Alberts, S. C., Altmann, J. and Bruford, M. W. 2000. Testing the reliability of microsatellite typing from faecal DNA in the savannah baboon. *Conserv Genet.* 1, pp. 173-176.

Beaumont, M. A. 2004. Recent developments in genetic data analysis:
what can they tell us about human demographic history? *Heredity* 92, p. 365–379.

Beebee, T. and Rowe, G. 2007. *An Introduction to Molecular Ecology*. Oxford: Oxford Univeristy Press, p. 384.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. and Bonhomme, F. 2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations.Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France).

Bergl, R. A., Bradley, J. B., Nsubuga, A. and Vigilant, L. 2008. Effects of habitat fragmentation, population size and demographic history on genetic diversity: the cross river gorilla in a comparative context. *American Journal of Primatology* 70(9), pp. 848-859.

Bérubé, M., Aguilar, A., Dendanto, D., Larsen, F., Di Sciara, G. N., Sears, R., Sigurjonsson, J., Urban, R. J. and Palsboll, P. J. 1998. Population genetic structure of North Atlantic, Mediterranean Sea and Sea of Cortez fin whales, Balaenoptera physalus (Linnaeus 1758): analysis of mitochondrial and nuclear loci. *Mol. Ecol.* . 7(585–599).

Birky, C. W., Fuerst, P. and Maruyama, T. 1989. Organelle gene diversity under migration, mutation, and drift — equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics* 121(613–627).

Blouin, M. S., Parsons, M., Lacaille, V. and Lotz, S. 1996. Use of microsatellite loci

to classify individuals by relatedness. *Molecular Ecology* 5, p. 393–401.

Bradley, B. J. and Vigilant, L. 2002. False alleles derived from microbial DNA pose a potential source of error in microsatellite genotyping from faeces. *Molecular Ecology Notes* 2, pp. 602-605.

Brett, R. A. 1993. Conservation strategy and management plan for the black rhinoceros (Diceros bicornis) in Kenya: Rhino conservation programme. Sponsored by the Zoological society of London, p. 69.

Brett, R. A. 1998. Mortality factors and breeding performance of translocated black rhinos in Kenya: 1984-1995. *Pachyderm* 26, pp. 69-82.

Broquet, T., Ménard, N. and Petit, E. 2007. Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. *Conserv Genet* 8, pp. 249-260.

Broquet, T. and Petit, E. 2004. Quantifying genotyping errors in non-invasive population genetics. *Mol Ecol* 13, pp. 3601-3608.

Brown, S. M. and Houlden, B. A. 1999. Isolation and characterization of microsatellite markers in the black rhinoceros (*Diceros bicornis*). *Molecular Ecology* 8, pp. 1559-1561.

Brown, S. M. and Houlden, B. A. 2000. Conservation genetics of the black rhinoceros (Diceros bicornis). *Conservation Genetics.* 1, p. 365–370.

Brownstein, M. J., Carpten, J. D. and Smith, J. R. 2006. Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. *BioTechniques* 20, pp. 1004-1010.

Buonaccorsi, V. P., McDowell, J. R. and Graves, J. E. 2001. Reconciling patterns of inter-ocean molecular variance from four classes of
molecular markers in blue marlin (Makaira nigricans). *Mol. Ecol.* 10, p. 1179– 1196.

Callen, D. F., Thompson, A. D., Shen, Y., Phillips, H. A., Richards, R. I., Mulley, J. C. and Sutherland, G. R. 1993. Incidence and origin of "null" alleles in the (AC)n microsatellite markers. *AM J Hum Genet.* 52(5), pp. 922-927.

Cavalli-Sforza, L. L., Menozzi, P. and Piazza, A. 1994. *The history and geography of human genes*. Princeton, New Jersey: Princeton University Press.

Cerdeño, E. 1995. Cladistic analysis of the Family Rhinocerotidae (Perissodactyla). *Am.*

Mus. Novit. 3143, p. 1–25.

Cerdeño, E. 1999. Diversity and evolutionary trends of the family Rhinocerotidae (Perissodacyla). *Palaeogeogr. Palaeoclimatol. Palaeoecol.* 141, p. 13–34.

Chikhi, L. and Bruford, M. W. 2005. *Mammalian population genetics and genomics*. CAB International Publishing., pp. 539-584.

Ciofi, C., Beaumont, M. A., Swingland, I. R. and Bruford, M. W. 1999. Genetic divergence and units for conservation in the Komodo dragon Varanus komodoensis. *Proc. Roy. Soc. Lond. B* 266, p. 2269–2274.

Clifford, S., Anthony, N. M. and Bawe-Johnson, M. 2004. Mitochondrial DNA phylogeography of western lowland gorillas (Gorilla gorilla gorilla). *Mol. Ecol.* 13(1551-1565).

Coltman, D. W., Pilkington, J. G., Smith, J. A. and Pemberton, J. M. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* 53, pp. 1259-1267.

Cooke, H. B. S. 1972. The fossil mammal fauna of Africa. In: Keast, A. and Erk, F.C. eds. *Evolution, Mammals and Southern Continent*. State University of New York, p. 89–139Albany.

Cunningham, J., Harley, E. H. and O'Ryan, C. 1999. Isolation and characterisation of microsatellite loci in black rhinoceros (Diceros bicornis). *Electrophoresis* 20, pp. 1778-1780.

Dakin, E. E. and Avise, J. C. 2004. Microsatellite null alleles in parentage analysis. *Heredity* 93, p. 504 – 509.

Dawson, K. J. and Belkhir, K. 2001. A Bayesian approach to the identification of panmictic populations and the assignment of individuals. *Genet. Res.* 78, p. 59–77.

Dean, C. and Foose, T. J. 2006. The threat to rhino's survival. In: Blumer, E. *et al.,* eds. *The North American Save the Rhinos Campaing*. Florida: International Rhino Foundation.

DeSalle, R., Giribet , G. and Wheeler, W. 2002. *Molecular Systematics and Evolution: Theory and Practice*. Birkhäuser Basel.

Dinerstein, E. 2003. *The Return of the Unicorns : The Natural History and Conservation of the Greater One-Horned Rhinoceros*. Columbia Univ Press.

Eldredge, N. 2005. *The Sixth Extinction* American Institute of Biological Sciences. Available at: <URL: http://www.actionbioscience.org/newfrontiers/eldredge2.html> [Accessed: July 5 2008]

Emslie, R. and Brooks, M. 1999. African Rhino. Status Survey and Conservation Action Plan.IUCN/SSC African Rhino Specialist Group. IUCN: Gland, Switzerland and Cambridge, UK. p. ix + 92

Excoffier, L., Laval, L. and Schneider, S. 2005. Arlequin ver. 3.1.1: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1, pp. 47 - 50.

Falconer, C. S. and Mackay, T. F. C. 1996. *Introduction to Quantitative Genetics*. Harlow: Longman Group Ltd.

Falush, D., Stephens, M. and Pritchard, J. K. 2003. Inference of population structure from multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* 164, p. 1567–1587.

Fernando, P., Polet, G., Foead, N., Ng, L. S., Pastorini, J. and Melnick, D. J. 2006. Genetic diversity, phylogeny and conservation of the Javan rhinoceros (Rhinoceros sondaicus). *Conservation Genetics* 7(3), pp. 439-448.

Fernando, P., Vidya, T. N. C., Rajapakse, C., Dangolla, A. and Melnick, D. J. 2003. Reliable noninvasive genotyping: fantasy or reality? *Journal of Heredity.* 94, pp. 115-123.

Flagstad, O., Roed, K., Stacy, J. E. and Jakobsen, K. S. 1999. Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with a magnetic bead protocol. *Molecular Ecology* 8(879-883).

Florescu, A., Davila, J. A., Scott, C., Fernando, P., Kellner, K., Morales, J. C., Melnick, D., Boag, P. T. and Van Coeverden De Groot, P. 2003. Polymorphic microsatellites in white rhinoceros. *Molecular Ecology Notes* 3., pp. 344-345.

Foose, T. J. ed. 1992. *International Rhino Conference, 1991* San Diego, USA: International Rhino Foundation

Foose, T. J. and Seal, U. S. eds. 1991. *Metapopulation Analysis Workshop Briefing Book* Nairobi, Kenya: IUCN/SSC/CBSG/KWS

Foster, C., Aswath, K., Chanock, S., McKay, H. and Peters, U. 2006. Polymorphism analysis of six selenoprotein genes: support for a selective sweep at the glutathione peroxidase 1 locus (3p21) in Asian populations. *BMC Genetics* 7(1), p. 56.

Frankel, O. H. and Soulé, M. E. 1981. *Conservation and Evolution*. Cambridge: Cambridge University Press, UK, p. 299.

Frankham, H. and Ralls, K. 1998. Inbreeding leads to extinction. *Nature* 392, pp. 441-442.

Frankham, R. 1995. Effective population size/adult population size rations in wildlife: a review. *Genet Res* 66, p. 95–107.

Frankham, R., Balloua, J. D. and Briscoe, D. A. 2002. *Introduction to conservation genetics*. 1st ed. Cambridge University Press, pp. 72-174.

Frantzen, M. A. J., Silk, J. B., Ferguson, J. W. H., Wayne, R. K. and Kohn, M. H. 1998. Empirical evaluation of preservation methods for faecal DNA. *Molecular Ecology*. 7, pp. 1423-1428.

Freeland, J. R. 2005. *Molecular Ecology*. The Open University, Milton Keynes: John Wiley and Sons, LTD, p. Pp 388.

Gagneux, P., Boesch, C. and Woodruff, D. S. 1997. Microsatellite scoring errors associated with non-invasive genotyping based on nuclear DNA amplified from shed hair. *Mol Ecol* 6, pp. 861-868.

Gakahu, C. G. ed. 1993. *International conference on rhinoceros conservation and biology* . San Diego: Zoological Society of San Diego

Garnier, J. N., Bruford, M. W. and Goossens, B. 2001. Mating system and reproductive skew in black rhinoceros. *Molecular Ecology* 10, pp. 2031-2041.

Garza, J. C. and Williamson, E. G. 2001. Detection of reduction in population size using data from microsatellite loci. *Mol Ecol* 10, p. 305–318.

Gene Codes Corporation 1988. An international software firm specializing in bioinformatics software for DNA sequence analysis.Ann Arbor, Michigan:

Gerloff, U., Hartung, B., Fruth, B. and G., H. eds. 1999. *Royal Society of London*

Gerstein, M. B., Bruce, C., Rozowsky, J. S., Zheng, D. D. J., Korbel, J. O.,

Emanuelsson, O., Zhang, Z. D., Weissman, S. and Snyder, M. 2007. What is a gene, post-ENCODE? History and updated definition. *Genome Research* 17(6), p. 669–681.

Gilpin, M. E. and Soulé, M., E. 1986. Minimum viable populations: the process of species extinction. In: Soulé, M.E. ed. *Conservation Biology: The Science of Scarcity and Diversity*. Sinauer Associates, pp. 19-34Sunderland.

Goddard, J. 1966. Mating and courtship of the black rhinoceros (Diceros bicornis L.). *East African Wildlife Journal* 4, pp. 69-75.

Goddard, J. 1967. Home range, behaviour, and recruitment rates of two black rhinoceros populations. *East African Wildlife Journal* 5, pp. 133-150.

Goddard, J. 1970. Food preferences of black rhinoceros in the Tsavo National Park. *East African Wildlife Journal* 8, pp. 145-162.

Goldstein, D. B., and Pollock, D. D. 1997. Launching microsatellites: a review of mutation processes and methods of phylogenetic inference. *Journal of Heredity* 88, pp. 335 - 342.

Goodman, S. J., Tamate, H. B. and Wilson, R. e. a. 2001. Bottlenecks, drift and differentiation: the population structure and demographic history of sika deer (Cervus nippon) in the Japanese archipelago. *Molecular Ecology* 10, p. 357–1370.

Goossens, B., Chikhi, L., Ancrenaz, M., Lackman-Ancrenaz, I., Andau, P. and Bruford, M. W. 2006. Genetic Signature of Anthropogenic Population Collapse in Orang-utans. *PLoS Biol* 4(2), p. e25.

Goossens, B., Chikhi, L., Jalil, M. F., Ancrenaz, M., Lackman-Ancrenaz, I., Mohamed, M., Andau, P. and Bruford, M. W. 2005. Patterns of genetic diversity and migration in increasingly fragmented and declining orang-utan (Pongo pygmaeus) populations from Sabah, Malaysia. *Molecular Ecology* 14, pp. 441-456.

Goossens, B., Chikhi, L., Utami, S. S., de Ruiter, J. and Bruford, M. W. 2000. A multi-samples, multi-extracts approach for microsatellite analysis of faecal samples in an arboreal ape. *Conservation Genetics* 1, pp. 57-162.

Greenwood, A. D. and Pääbo, S. 1999. Nuclear insertion sequences of mitochondrial DNA predominate in hair but not in blood of elephants. *Molecular Ecology* 8, pp. 133-137.

Guggisberg, C. A. W. 1966. *S.O.S. rhino*. Nairobi: East African Publishing House, p. 174.

Hall-Martin, A. 1984. Kenya's black rhino in Addo, S Africa. *Newsletter of the African Elephant and Rhino Group* 3, pp. 11, fig. 11.

Hanson, C. B. 1989. *Teletaceras radinskyi, a new primitive rhinocerotid from the late Eocene Clarno Formation, Oregon.* New York: Oxford Univ. Press, p. 379–398.

Harley, E. H., Baumgarten, I., Cunningham, J. and O'Ryan, C. 2005. Genetic variation and population structure in remnant populations of black rhinoceros, Diceros bicornis, in Africa. *Molecular Ecology* 14, p. 2981–2990.

Hart, D. L. and Clark, A. G. 1989. *Darwinian evolution in the mendelian populations. Principles of Population Genetics*. Massachusetts: Sinauer Associates Inc, Sunderland.

Hellberg, M. E. 2006. No variation and low synonymous substitution rates in coral mtDNA despite high nuclear variation. *Bmc Evolutionary Biology.* 6.

Hoarau, G., Piquet, A. M.-T., van der Veer, H. W., Rijnsdorp, A. D., Stam, W. T. and Olsen, J. L. 2004. Population structure of plaice (*Pleuronectes platessa L.*) in northern Europe: a comparison of resolving power between microsatellites and mitochondrial DNA data. *Journal of Sea Research* 51,(3-4,), pp. 183-190.

Hoffman, J. I. and Amos, W. 2005. Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. *Mol. Ecol.* 14, pp. 599-612.

Hooyer, D. A. 1976. Phylogeny of the rhinocerotids of Africa. *Annals of the South African Museum* 71, pp. 167-168.

Hunter, J. A. 1952. *Hunter*. London: Hamish Hamilton London.

IRF. 2008. *Internatinal rhino foundation* IRF. Available at: <URL: http://www.rhinos-irf.org/woolly> [Accessed: July 5th 2008]

IUCN 2006. West African black rhino feared extinct. News release of 07 July 2006.The World Conservation Union (IUCN).

IUCN. 2008. *IUCN Red List of Threatened Species* Available at: <URL:

www.iucnredlist.org> [Accessed: 19 January 2009]

IUCN SSC AfRSG. 2008. *Diceros bicornis. In: 2008 IUCN Red List of Threatened Species* IUCN 2008. Available at: <URL: www.iucnredlist.org> [Accessed: 19 January 2009]

Jarne, P. and Lagoda, P. J. L. 1996. Microsatellites, from molecules to populations and back. *Trends Ecol. Evol.* 11, p. 424– 429.

Jobb, G. 2008. *TREEFINDER version of October 2008.* Distributed by the author. Available at: <URL: www.treefinder.de> [Accessed: 20th January 2009]

Johnson, M. B. 2008. *Genetic variation of the forest elephant (Loxodonta africana cyclotis) across central Africa.* PhD Thesis, Cardiff University.

Kitching, I. 1998. *Cladistics: The Theory and Practice of Parsimony Analysis*.

Kohn, M. and Wayne, R. K. 1997. Facts from faeces revisited. *Trends in Ecology and Evolution* 12, pp. 223-227.

KWS 2003. Conservation and management strategy for the black rhino (Diceros bicornis michaeli) in Kenya 2000-2005.Nairobi: Kenya Wildlife Service (KWS). Unpublished report.

Lacombat, F. ed. 2005. *Save the rhinos: EAZA Rhino Campaign 2005/6* London: European Association of Zoos and Aquaria

Lacy, R. C. 1997. Importance of genetic variation to the viability of mammalian populations. *Journal of Mammalogy* 78, p. 320–335.

Laroche, L. and Durand, J. D. 2004. Genetic structure of fragmented populations of a threatened endemic percid of the Rhône river: Zingel asper. *Heredity* 92, pp. 329-334.

Leader-Williams, N. 1992. *The World Trade in Rhino Horn*. Cambridge, UK: Traffic International.

Leakey, L. 1969. *Animals of East Africa*. New York: National Gerographic, p. 199.

Lloyd-Jones, L. and Brevet-Major, W. 1925. *"Havash"! Frontier Adventures in Kenya [Big Game Hunting with The King's African Rifles].* Arrowsmith.

Long, J. C. 1986. The allelic correlation structure of Gainj- and Kalam-speaking people. I. The estimation and interpretation of Wright's F-statistics. *Genetics* 112,

pp. 629-647.

Magnuson, V. L., Ally, D. S., Nylund, S. J., Karanjawala, Z. E., Rayman, J. B., Knapp, J. I., Lowe, A. L., Ghosh, S. and Collins, F. S. 1996. Substrate nucleotide-determined non-templates addition of adenine by Taq DNA polymerase: implications for PCR-based genotyping and cloning. *BioTechniques* 21(4), pp. 700-709.

Marker, L. L., Wilkerson, A. J. P., Sarno, R. J., Martenson, J., Breitenmoser-Wursten, C., O'Brien, S. J. and Johnson, W. E. 2008. Molecular Genetic Insights on Cheetah (*Acinonyx jubatus*) Ecology and Conservation in Namibia. *J. Hered* 99, pp. 2-13.

Martin, E. and Vigne, L. 2003. Trade in rhino horn from eastern Africa to Yemen. *Pachyderm* 34, p. 75–87.

Maudet, C., Luikart, G., Durray, D., Von Hardensberg, A. and Taberlet, P. 2004. Low genotyping error rates in wild ungulate faeces sampled in winter. *Mol Ecol Notes* 4, pp. 772-775.

McKenna, M. C. and Susan, B. K. 1997. *Classification of Mammals Above the Species Level.* New York: Columbia University Press, p. 631.

Menotti-Raymond, M. and O'Brien, S. J. 1993. Dating the genetic bottleneck of the African cheetah. *PNAS* 90(8), pp. 3172-3176.

Merenlender, A. M., Woodruff, D. S., Ryder, O. A., Kock, R. and Váhala, J. 1989. Allozyme variation and differentiation in African and Indian rhinoceroses. *Journal of Heredity* 80, p. 377–382.

Miller, C. R. and Waits, L. P. 2003. The history of effective population size and genetic diversity in the Yellowstone grizzly (Ursus arctos): Implications for conservation
10.1073/pnas.0735531100. *Proceedings of the National Academy of Sciences of the United States of America* 100(7), pp. 4334-4339.

Miller, M. P. 1997. *Tools for population genetic analyses (TFPGA): A Windows program for the analysis of allozyme and molecular population genetic data; 1997* Available at: <URL: http://herb.bio.nau.edul~miller/tfpga.htm> [Accessed: 10th Nov 2008]

Mills, L., S 2007. *Conservation of wildlife populations*. Oxford: Blackwell Publishing Ltd, p. 407.

Mills, L. S. and Tallmon, D. A. 1999. The role of genetics in understanding forest fragmentation. In: Rochelle, J.A. *et al.,* eds. *Forest Fragmentation: Wildlife and Management Implications*. Brill Academic Publishing, pp. 171 - 184Leiden.

Miquel, C., Bellemain, E., Poillot, C., Bessiere, J., Durand, A. and Taberlet, P. 2006. Quality indexes to assess the reliability of genotypes in studies using noninvasive sampling and multiple-tube approach. *Molecular Ecology Notes* 6(4), pp. 985-988.

Moehlman, P. D., Amato, T. G. and Victor, R. 1996. Genetic and demographic threats to the black rhinoceros population in the Ngorongoro Crater. *Conservation Biology* 10, pp. 1107-1114.

Moodley, Y. and Harley, E. H. 2005. Population structuring in mountain zebras (Equus zebra): The molecular consequences of divergent demographic histories. DOI 10.1007/s10592-005-9083-8. *Conservation Genetics*, pp. 1-16.

Morales, C. J., Andau, P. M., Supriatna, J., Zainuddin, Z. Z. and J., M. D. 1997. Mitochondrial DNA variability and conservation genetics of the Sumatran rhinoceros. *Conservation Biology* 11, pp. 539-543.

Morales, C. J. and Melnick, D. J. 1994. Molecular systematics of the living rhinoceros. *Molecular Phylogenetics and Evolution* 3, pp. 128-134.

Morin, P. A., Chambers, K. E., Boesch, C. and Vigilant, L. 2001. Quantitative polymerase chain reaction analysis of DNA from noninvasine samples for accurate microsatellite genotyping of wild chimpanzees (Pan troglodytes verus). *Molecular Ecology* 10, pp. 1835-1844.

Morinte, J. and Keter, P. 2000. Black rhino kills male lion. *CCA Ecological Journal* 2, p. 37.

Moritz, C. 1994. Defining evolutionary significant units for conservation. *Trends in Ecology and Evolution* 9, pp. 373-375.

Mukewa, E. N. 1995. *Habitat use by white rhinos (Ceratotherium simum) in Lake Nakuru National Park, Kenya.* Moi University.

Muwanika, V., Siegismund, H. R. and Okello, J. B. A. 2003. A recent bottleneck in the warthog and elephant populations of Queen Elizabeth National Park, revealed by a comparative study of four mammalian species in Uganda national parks. *Animal Conservation* 6(237-245).

Muya, S. M. and Oguge, N. O. 2000. Effects of browse availability and quality on black rhino (Diceros bicornis michaeli Groves 1967) diet in Nairobi National Park, Kenya. *African Journal of Ecology.* 38, pp. 62-71.

Myers, N. 1979. *The sinking ark: A new look at the problem of disappearing species*. Netherlands: Pergamon Press.

Nash, R. 1982. *Wilderness and the American Mind*. New York: Yale University Press.

Navidi, W., Arnheim, N. T. and Waterman, M. S. 1992. A Multiple-Tubes Approach for Accurate Genotyping of Very Small DNA Samples by Using PCR: Statistical Considerations. *American Journal of Human Genetics* 50, pp. 347-359.

Nei, M. 1972. Genetic distance between populations. *The American Naturalist* 106, pp. 283-292.

Nei, M. ed. 1973. *Proceedings of National Academy of Sciences Conference* USA: .

Nei, M. 1975. Molecular population genetics and evolution. In: Neuberger, A. and Tatum, E. eds. *Frontiers of Biology*. Vol. 40. Amsterdam: North-Holland.

Nei, M. 1987. Genetic distance and molecular phylogeny. In: Ryman, N., and Utter, F ed. *Population Genetics and Fishery Management*. University of Washington Press, pp. 193-223Seattle, USA.

Nei, M., Marayuma, T. and Chakraborty, R. 1975. The bottleneck effect and genetic variability in populations. *Evolution* 29, p. 1–10.

Nei, M., Tajima, F. and Tateno, Y. 1983. Accuracy of estimated phylogenetic trees from molecular data. II.Gene frequency data. *Journal of Molecular Evolution* 19, pp. 153-170.

Neumann, A. H. 1898. *Elephant-Hunting in East Equatorial Africa*. California: London & Encino.

Newman, D. K. 1995. *Importance of genetics on the survival of small populations: genetic drift, inbreeding, and migration. PhD thesis.* University of Montana.

Nielsen, L., Meehan-Meola, D., Kilbourn, A. and Alcivar-Warren, A. 2007. Characterization of microsatellite loci in the black rhinoceros (Diceros bicornis) and white rhinoceros (Ceratotherium simum): their use for cross-species amplification and differentiation between the two species. *Conservation Genetics*.

Nunney, L. and Campbell, K. A. 1993. Assessing minimum viable population sizes: demography meets population genetics. *Trends in Ecology and Evolution* 8, pp. 234-239.

Nyakaana, S., Arctander, P. and Siegismund, H. 2002. Population structure of the African savannah elephant inferred from mitochondrial control region sequences and nuclear microsatellite loci. *Heredity* 89, pp. 90-98.

O'Ryan, C., Flamand, J. R. B. and Harley, E. H. 1994. Mitochondrial DNA variation in black rhinoceros (Diceros bicornis): conservation management implications. *Conservation Biology* 8, p. 495–500.

O'Ryan, C. and Harley, E. H. 1993. Comparisons of mitochondrial DNA in black and white rhinoceroses. *Journal of Mammalogy* 74, p. 343–346.

Oakenfull, E., Lim, H. and Ryder, O. 2000. A survey of equid mitochondrial DNA: Implications for the evolution, genetic diversity and conservation of Equus. *Conserv. Genet.,* 1,, p. 341– 355.

Obanda, V., Gakuya, F., Lekolool, I., Chege, S., Okita, B. and Manyibe, T. 2008. Ciliated intestinal protozoa of black (Diceros bicornis michaeli) and white rhinoceroses (Ceratotherium simum simum) in Kenya. *African Journal of Ecology* 46(2), pp. 144-148.

O'Brien, S. J., D.E., W., Goldman, D., Merril, C. R. and Bush, M. 1983. The Cheetah is depauperate in genetic variation. *J. Science* 221(4609), pp. 459 - 462.

Ogutu, Z. A. 1993. Responding to population pressure in the rural Kenya. *Geography Journal* 4, pp. 409-419.

Ohta, T. and Kimura, M. 1973. A model of mutation appropriate to estimate the number of electrophoretically detectable alleles
in a finite population. . *Genet Res* 22, p. 201–204.

Okello, J. B. A., Masembe, C., Rasmussen, H. B., Wittemyer, G., Omondi, P., Kahindi, O., Muwanika, V. B., Arctander, P., Douglas-Hamilton, I., Nyakaana, S. and Siegismund, H. R. 2008. *Population Genetic Structure of Savannah Elephants in Kenya: Conservation and Management Implications* Journal of Heredity. Available at: <URL: [Accessed: 20th July 2008]

Okello, J. B. A., Wittemyer, G., Rasmussen, H. B., Douglas-Hamilton, I., Nyakaana,

S., Arctander, P. and Siegismund, H. R. 2005. Noninvasive Genotyping and Mendelian Analysis of Microsatellites in African Savannah Elephants 10.1093/jhered/esi117. *J Hered* 96(6), pp. 679-687.

Okita-Ouma, B. 2004. *Population performance of Black Rhinoceros (Diceros bicornis michaeli) in six Kenyan rhino sanctuaries.* University of Kent.

Okita-Ouma, B., Amin, B. and Kock, R. 2007. Conservation and management strategy for the black rhino (Diceros bicornis michaeli) and management guidelines for the white rhino (Ceratotherium simum simum) in Kenya (2007-2011).Nairobi: KWS. p. 157

Oloo, T. W., Brett, R. and Young, T. P. 1994. Seasonal variation in the feeding ecology of black rhinoceros (Diceros bicornis L.) in Laikipia Kenya. *African Journal of Ecology* 32, pp. 142-157.

Oosterhout, C. V., Hutchinson, W. F. and Wills, D. P. M. 2004. Micro - checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4, pp. 535-538.

Orlando, L., Leonard, J. A., Thenot, A., Laudet, V., Guerin, C. and Hänni, C. 2003. Ancient DNA analysis reveals woolly rhino evolutionary relationships. *Molecular Phylogenetics and Evolution* 28, p. 485–499.

Osborn, H. F. 1915. *Men of the Old Stone Age*. New York: Charles Scribner's Sons, p. 545.

Osterhoff, D. R. and Keep, M. E. 1970. Natural variation in the blood proteins of white and black rhinos. *Lammergeyer* 11, pp. 50-53.

Paetkau, D. 2003. An empirical exploration of data quality in DNA based population inventories. *Mol. Ecol.* 12, pp. 1375-1387.

Paetkau, D. and Strobeck, C. 1995. The molecular basis and evolutionary history of a microsatellite null allele in bears. *Mol. Ecol.* 4, pp. 519-520.

Paetkau, D., Waits, L. P., Clarkson, P. L., Craighead, L. and Strobeck, C. 1997. An empirical evaluation of genetic distance statistics using microsatellite data from bear (Ursidae) populations. *Genetics* 147, pp. 1943-1957.

Patterson, J. H. 1909. *In The Grip of The Nyika; Further Adventures in British East Africa.* Macmillan and Co., Ltd.

Patton, F. and Jones, F. 2007. Determining minimum population size and

demographics of black rhinos in the salient of Aberdare National Parl, Kenya. *Pachyderm* 43, pp. 63-72.

Pompanon, F., Bonin, A., Bellemain, E. and Taberlet, P. 2005. *Genotyping errors: causes, consequences and solutions* Nature Publishing Group. Available at: <URL: www.nature.com/reviews/genetics> [Accessed: July 8th 2008]

Pritchard, J., Stephens, M. and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155, pp. 945-959.

Prothero, D. R. 2005. *The Evolution of North American Rhinoceroses*. Cambridge: Cambridge University Press, p. 218.

Raymond, M. and Rousset, F. 1997. GENEPOP, Version 3.1b: an updated version of GENEPOP (Version 1.2), Population genetics software for exact tests and ecumenism. *Journal of Heredity* 86, p. 248–249.

Reed, J. Z., Tollit, D. J., Thompson, P. M. and Amos, W. 1997. Molecular scatology: the use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. *Molecular Ecology* 6, pp. 225-234.

Rice, W. R. 1989. Analyzing tables of statistical tests. *Evolution* 43, p. 223–225.

Robinson, T. J., Trifonov, V., Espie, I. and E.H., H. 2005. Interspecific hybridization in rhinoceroses: Confirmation of a Black × White rhinoceros hybrid by karyotype, fluorescence in situ hybridization (FISH) and microsatellite analysis. *Conservation Genetics.* 6(1), pp. 141-145.

Roca, A. L., Georgiadis, N., Pecon-Slattery, J. and O'Brien, S. J. 2001. Genetic evidence for two species of elephant in Africa. *Science.* 293, pp. 1473-1477.

Rogers, A. R. and Harpending, H. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Mol. Biol. Evol.* 9, pp. 552-569.

Roon, D. A., Thomas, M. E., Kendall, K. C. and Waits, L. P. 2005. Evaluating mixed samples as a source of error in non-invasive genetic studies using microsatellites. *Mol Ecol* 14, pp. 195-201.

Rozas, J., Sánchez-Delbarrio, J. C., Messeguer, X. and Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19, pp. 2496-2497.

Saccheri, I. J., Kuussaari, M., Vickman, P., Fortelius, W. and Hanski, I. 1998. Inbreeding and extinction in a butterfly metapopulation. *Nature* 392, pp.

491-494.

Saccheri, I. J., Wilson, I. J., Nichols, R. A., Bruford, M. W. and Brakefield, P. M. 1999. Inbreeding of bottlenecked butterfly populations: estimation using the likelihood of changes in marker allele frequencies. *Genetics* 151, pp. 1053-1063.

Saccone, C., Pesole, G. and Sbisa, E. 1991. The main regulatory region of mammalian mitochondrial DNA: structure-function model and evolutionary pattern. *Journal of Molecular Evolution* (33).

Scott, C. A. 2008. *Microsatellite variability in four contemporary rhinoceros species: implications for conservation* [Unpublsihed MSc Thesis]. Queen's University Kingston. Available at: <URL: http://www.rhinoresourcecenter.com/ref_files/1218825705.pdf> [Accessed: 13th October 2008]

Scott, K. D., Lange, L., Scott, L. J. and Graham, G. C. 2004. Isolation and characterization of microsatellite loci from Helicoverpa armigera Hubner (Lepidoptera: Noctuidae). *Molecular Ecology Notes* 4(204-205).

Shaw, P. W., Pierce, G. J. and Boyle, P. R. 1999. Subtle population structuring within a highly vagile marine invertebrate, the veined
squid Loligo forbesi, demonstrated with microsatellite DNA markers. *Mol. Ecol.* 8, p. 407– 417.

Shearer, T. L., Van Oppen, M. J. H., Romano, S. L. and Worheide, G. 2008. Slow mitochondrial DNA sequence evolution in the Anthozoa (*Cnidaria*). *Molecular Ecology* 11, p. 2475–2487.

Slade, R. W., Moritz, C. and Heideman, A. 1994. Multiple nuclear-gene phylogenies: Application to pinnipeds and comparison with a mitochondrial DNA gene phylogeny. *Mol. Biol. Evol.,* 11, p. 341–356.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. *Science* 236, pp. 787-792.

Sorenson, M. D. and Fleischer, R. C. eds. 1996. *Multiple independent transpositions of mitochondrial DNA control sequences to the nucleus, Proceedings of the National Academy of Sciences* USA:

Spinage, C. A. 1962. *Animals of East Africa*. London: Collins.

Swart, M. K. J. and Ferguson, J. W. H. 1997. Conservation implications of genetic

differentiation in Southern African Populations of Black Rhinoceros (Diceros bicornis). *Conservation Biology* 11, pp. 79-83.

Swart, M. K. J., Ferguson, J. W. H., du Toit, R. and Flamand, J. R. B. 1994. Substantial genetic variation in southern African black rhinoceros. *Journal of Heredity* 85, p. 261–266.

Taberlet, P., Griffin, S., Goossens, B., Questiau, S., Manceau, V., Escaravage, N., Waits, L. P. and Bouvet, J. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* 24, pp. 3189-3194.

Taberlet, P., Waits, L. P. and Luikart, G. 1999. Noninvasive genetic sampling: look before you leap. *Trends in Ecology and Evolution* 14, pp. 323-327.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, pp. 1596-1599.

Templeton, A. R. 1986. Coadaptation and outbreeding depression. In: Soulé, M.E. ed. *Conservation Biology: The Science of Scarcity and Diversity*. Sinauer Associates, p. 105–116Sunderland, MA.

Times online 2006. West African black rhino 'is extinct'. *The Times Online, July 2006.* July 7.

Tougard, C., Delefosse, T., Hanni , C. and Montgelard, C. 2001. Phylogenetic Relationships of the Five Extant Rhinoceros Species (Rhinocerotidae, Perissodactyla) Based on mitochondrial Cytochrome b and 12S rRNA Genes. *Molecular Phylogenetics and Evolution* 19 April(1 April), p. 34–44.

Valière, N., Bonenfant, C., To?go, C., Luikart, G., Gaillard, J. and Klein, F. 2007. Importance of a pilot study for non-invasive genetic sampling: genotyping errors and population size estimation in red deer. *Conservation genetics* 8, pp. 69-78.

van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. and Shipley, P. 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4, p. 535–538.

Wanjohi, E. W. 1987. *Non-invasive monitoring of reproductive status of the black rhinoceros (Diceros bicornis) in captivity and in the wild.* University of London.

Waples, R. S. 1989. A generalized approach for estimating effective population size

from temporal changes in allele frequency. *Genetics* 121, p. 379–391.

Wasser, S. K., Houston, C. S., Koehler, G. M., Cadd, G. G. and Fain, S. R. 1997. Techniques for application of faecal DNA methods to field studies of Ursids. *Molecular Ecology* 6, pp. 1091-1097.

Wehausen, J. D., Ramey, R. R. and Epps, C. W. 2004. Experiments in DNA extraction and PCR amplification from bighorn sheep feces: the importance of DNA extraction method. *Journal of Heredity* 95, pp. 503-509.

Weir, B. and Cockerham, C. 1984. Estimating F-statistic for the analysis of population structure. *Evolution* 38, pp. 1358 - 1370.

West-Eberhard, M. 2003. *Developmental Plasticity and Evolution*. Oxford Univ. Press., p. 353–376.

Whittaker, R. J., Triantis, K. A. and Ladle, R. J. 2008. A general dynamic theory of oceanic island biogeography. *Journal of Biogeography* 35(6), pp. 977-994.

Wooding, S. and Ward, R. 1997. Phylogeography and pleistocene evolution in the North American black bear. *Mol. Biol. Evol.,* 14, p. 1096–1105.

Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics* 15, pp. 323-354.

Wright, S. 1978. *Variability within and among natural populations*. Chicago: University of Chicago Press.

Xia, X. and Xie, Z. 2001. DAMBE: Data analysis in molecular biology and evolution. *Journal of Heredity* 92, pp. 371-373.

Yeh, F., Yang, R. and Boyle, T. 2000. *POPGENE version 1.32* Department of Renewable Resources at the University of Alberta, Canada. Available at: <URL: http://www.ualberta.ca/~fyeh/info.htm> [Accessed: 17th July 2008]

Zhao, L., Zhang, J., Liu, Z., Funk, S., Wei, F., Xu, M. and Li, M. 2008. Complex population genetic and demographic history of the Salangid, (*Neosalanx taihuensis)*, based on cytochrome b sequences. *BMC Evolutionary Biology* 8(1), p. 201.

Zschokke, S., Gautschi, B. and Baur, B. 2003. Polymorphic microsatellite loci in the endangered Indian rhinoceros, Rhinoceros unicornis. *Molecular Ecology Notes* 3, pp. 233-235.

APPENDICES

Appendix 1 A summary of the studies that have used rhinoceros mtDNA marker

Author	Target mtDNA Gene	Primer Sequence	Primer ID	Results
(Morales and Melnick, 1994)	1.6 kb segment of rRNA(12S), Valine tRNA and 16S)	5'-TGGGATTAGTACCCCACTAT-3'	LGL284	Significant - supported the traditional subdivision based on horns
		5'-TGATTATGCTACCTTTGCAC[A/G]GT-3'	LGL384	
(Brown and Houlden, 2000)	450 bp 5' end of the control region (d-loop)	5'-TCCACCATCAGCACCCAAAGC-3'	mt15996L	Significant - detected divergence at 0.93-1.3my
		5'-TTTGATGGCCCTGAAGTAAGAACCA-3'	Mt16502H	
(Morales *et al.*, 1997)	Control region D-Loop	5'-TACACTGGTCTTGTAAACC-3'	L15926	Significant - delineated ESUs
		5'-AAGGCTAGGACCAAACCT-3'	H00651	
(Tougard *et al.*, 2001)	12S rRNA Gene	5'-AAAGCAAGGCACTGAAAATGCCTAGA-3'	R1	Significant
		5'-TCTTCTGGGTGTAGGCCAGATGCTTT-3'	S2	
	Cytb	5'-ACCAATGACATGAAAAATCATCGTT-	L7	

Author	Target mtDNA Gene	Primer Sequence	Primer ID	Results
		3'		
		5'-TCTCCATTTCTGGTTTACAAGAC-3'	H6	
(Fernando *et al.*, 2006)	12S rRNA	5'-GCCYAGATGAGMCYACCARCT-3'	RH-12S-F	Significant delineated Javan rhinoceros into 2 ESU
		5'-TACRCTTACCTTGTTACGACT-3'	RH-12S-R	
	D-Loop	5'-CATCAACACCCAAAGCTGAAA-3'	RH-D-F1	
		5'-ATGGGCCCGGAGCGAGAACGA-3'	RH-D-R1	

Appendix 2 The 16 distinct haplotypes in the Kenyan black rhinoceros.

>Haplotype01

CAAAGTACCCCCATCATGTAACATACCAGTATTGACGTCACTTTTCTTACCCGCTAT
GTAATTCGTACATGCCATGTTGTAAGTACATCCCGGGTATGTATATCGTGCATTAAA
TTGTTTGCCCCATGCATATAAGCATATGTACTACATCATTAATGTTATACGACATAT
CAAGTTATTAATCGAGTATAGATTTTTAACATAGTGCATGGATATTTATATCCTAAA
GGTTACTGTTGATTTTACATAATACATATTGTTATTGATCGTACATACCCCATCCAAG
TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
ATGCCGCGGGAAATCATCAATCCTTCCACTCAATGCCCTTGTTCTCGCTCCGGGCCC
ATAACA

>Haplotype02

CAAAGTACCCCCATCATGTAACATACCAGTATTGACGTCACTTTTCTTGCCCGCTAT
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TTGTTTGCCCCATGCATATAAGCATATGTACTACATCATTAATGTTATACGACATAT
CAAGTTATTAATCGAGTATAGATTTTTAACATAGTGCATGGATATTTATATCCTAAA
GGTTACTGTTGATTTTACATAATACATATTATTATTGATCGTACATACCCCATTCAAG
TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
ATGCCGCGGGAAATCATCAATCCTTCCACTTAATGCCCTTGTTCTCGCTCCGGGCCC
AAAACA

>Haplotype03

CAAAGTACCCCCATCATGTAACATACCAGTATTGACGTCACTTTTCTTGCCCGCTAT
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CAAGTTATTAATCGAGTATAGATCTTTAACATAGTGCATGGATATTTATATCCTAAA
GGTTACTGTTGATTTTACATAATACATATTATTATTGATCGTACATACCCCATCCAAG
TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
ATGCCGCGGGAAATCATCAATCCTTCCACTCAATGCCCTCGTTCTCGCTCCGGGCCC
ATAACA

>Haplotype04

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AAGTTATTAATCGAGTATAGATCTTTAACATAGTGCATGGATATTTATATCCTAAAG
GCTACTGTTGATTTTACATAATACATATTATTATTGATCGTACATACCCCATCCAAGT
CAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACCA
TGCCGCGGGAAATCATCAATCCTTCCACTTAATGCCCTCGTTCTCGCTCCGGGCCCA
AAACA

>Haplotype05
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CAAGTTATTAATCGAGTATAGATTTTTAACATAGTGCATGGATATTTATATCCTAAA
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TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
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AAAACA

>Haplotype06
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TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
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AAAACA

>Haplotype07
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TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
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ATAACA

>Haplotype08
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TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
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ATAACA

>Haplotype09
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CAAGTTATTAATCGAGTATAGATTTTTAACATAGTGCATGGATATTTATATCCTAAA
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TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
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ATAACA

>Haplotype10
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AAGTTATTAATCGAGTATAGGTTTTTAACATAGTGCATGGATATTTATATCCTAAAG

GTTACTGTTGATTTTACATAATACATATTATTATTGATCGTACATACCCCATCCAAGT
CAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACCA
TGCCGCGGGAAATCATCAATCCTTCCACTTAATGCCCTTGTTCTCGCTCCGGGCCCA
TAACA

>Haplotype11
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TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
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ATAACA

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CAAGTTATTAATCGAGTATAGATTTTTAACATAGTGCATGGATATTTATATCCTAAA
GGTTACTGTTGATTTTACATAATACATATTATTATTGATCGTACATACCCCATCCAAG
TCAAATCATTTCTAGTCAACACGCATATCACCACCAATATTCCGGCGCTTAATCACC
ATGCCGCGGGAAATCATCAATCCTTCCACTCAATGCCCTTGTTCTCGCTCCGGGCCC
ATAACA

>Haplotype13
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CAAGTTATTAATCGAGTATAGATCTTTAACATAGTGCATGGATATTTATATCCTAAA
GGTTACTGTTGATTTTACATAATACATATTATTATTGATCGTACATACCCCATCCAAG
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AAAACA

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CAAGTTATTAATCGAGTATAGATCTTTAACATAGTGCATGGATATTTATATCCTAAA
GGTTACTGTTGATTTTACATAATACATATTATTATTGATCGTACATACCCCATCCAAG
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ATAACA

>Haplotype15
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ATAACA

Appendix 3 Kenya black rhinoceros Msat data for Genetic Diversity Analysis

Title line:"Kenya black rhinoceros Msat data for Genetic Diversity Analysis"

DB44

DB30

BR17

DB1

DB5

BR4

BR6

DB66

DB23

Pop

ABE001 169183 191195 126134 118130 184206 128128 143143 195207 179179

ABE002 177183 195199 126130 122130 186204 130132 141141 191201 179179

ABE003 181181 195197 124130 130130 198204 126128 141141 191195 179179

ABE006 173177 197197 130130 122128 186204 130132 141141 199207 179179

ABE007 169181 195205 126130 122124 186204 128128 139143 201207 173181

ABE008 000000 195197 126130 130130 198198 126128 139145 000000 179179

Pop

CHY001 169183 203203 126134 122122 186198 122130 141145 195205 179179

CHY002 177183 203207 124126 122128 198206 136144 143143 201207 181181

CHY003 169169 195209 124134 124130 186196 122128 139139 195205 179179

CHY004 183189 195201 124130 122124 198204 128130 141145 191195 179179

CHY005 181181 201207 126130 122122 196202 126128 141141 191205 179181

CHY006 181181 199203 124126 122126 198198 128130 141143 195203 179179

Pop

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LAK002 181181 203203 124124 122130 186202 128130 139141 205205 179179

LAK003 169181 195207 126126 128130 188198 122136 139145 191199 173179

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LAK005 181183 195197 130134 126130 186204 126128 143143 199199 179179

Pop

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LEW007 169181 195205 126130 122124 186204 128128 139143 201207 179181
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Pop
OLP008 169169 195195 126130 122130 186202 000000 000000 000000 179181

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SOL019 169181 195203 126126 124124 184188 128130 141141 191205 179179
SOL020 181183 195203 126126 122124 184188 128136 141143 191201 179179
SOL021 181183 205209 126126 122124 188198 134136 141143 199201 179179
SOL022 000000 195197 126130 122124 186204 126128 141141 000000 179181
SOL024 181181 197203 124126 122130 198204 130132 141141 191207 179179

SOL025 169169 195199 126130 124130 198204 134136 141143 191191 179179

SOL026 000000 199199 126130 122130 184198 128130 131143 195203 179181

Pop

TSA001 183189 195201 124130 122124 198204 128130 141145 191195 179179

TSA002 177183 197203 130132 122130 186188 130132 141143 191199 173179

TSA003 169169 197203 126126 122126 188190 130136 141141 199199 181181

TSA004 181181 195201 126130 130130 184198 126132 139143 191199 173185

TSA005 169183 195197 126130 122124 190204 122134 139143 191207 181181

Appendix 4 Allele frequency distribution by size, locus and subpopulation

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA	Totals
DB44													
(N)	5	6	5	20	11	11	14	15	8	10	22	5	132
169	0.2	0.25	0.2	0.075	0.2727	0.1818	0.25	0.4667	0.3125	0.2	0.2045	0.3	0.2428
173	0.1	0	0	0	0.0455	0.0455	0.0714	0	0	0	0	0	0.0219
175	0	0	0	0.075	0	0	0	0.0667	0.0625	0	0	0	0.017
177	0.2	0.0833	0	0.05	0.0455	0.4545	0.1786	0.0667	0.0625	0	0.0455	0.1	0.1072
179	0	0	0	0.075	0.0455	0	0	0	0	0	0.0455	0	0.0138
181	0.3	0.3333	0.6	0.4	0.2727	0.2273	0.3571	0.0667	0.375	0.3	0.4773	0.2	0.3258
183	0.2	0.25	0.2	0.325	0.2727	0.0909	0.1429	0.3	0.1875	0.5	0.2273	0.3	0.2497
187	0	0	0	0	0.0455	0	0	0	0	0	0	0	0.0038
189	0	0.0833	0	0	0	0	0	0.0333	0	0	0	0.1	0.0181
DB30													
(N)	6	6	5	24	11	11	14	16	8	10	24	5	140
191	0.0833	0	0	0	0	0	0	0	0	0	0	0	0.0069
195	0.4167	0.1667	0.2	0.375	0.2727	0.4091	0.0357	0.25	0.25	0.15	0.3333	0.3	0.2633
197	0.3333	0	0.2	0.3125	0.1818	0	0.3571	0.125	0	0.2	0.1875	0.3	0.1831
199	0.0833	0.0833	0	0.0625	0	0	0	0.0625	0	0.05	0.0625	0	0.0337
201	0	0.1667	0	0.1042	0.2273	0.3182	0.0714	0.1875	0.25	0.25	0.1458	0.2	0.1601
203	0	0.3333	0.4	0.125	0.1364	0.0455	0.25	0.25	0.1875	0.2	0.125	0.2	0.1877
205	0.0833	0	0	0.0208	0	0.0455	0.0714	0.0313	0.125	0.1	0.0833	0	0.0467

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA	Totals
207	0	0.1667	0.2	0	0.1364	0.0909	0.0714	0.0625	0.1875	0.05	0.0208	0	0.0822
209	0	0.0833	0	0	0.0455	0	0.1071	0.0313	0	0	0.0417	0	0.0257
215	0	0	0	0	0	0	0.0357	0	0	0	0	0	0.003
229	0	0	0	0	0	0.0455	0	0	0	0	0	0	0.0038
231	0	0	0	0	0	0.0455	0	0	0	0	0	0	0.0038
BR17													
(N)	6	6	5	26	12	9	14	17	8	10	24	5	142
124	0.0833	0.3333	0.3	0.1731	0.125	0.0556	0	0.1471	0.125	0.35	0.1667	0.1	0.1633
126	0.3333	0.3333	0.4	0.4038	0.4167	0.2222	0.4643	0.5588	0.5625	0.35	0.4583	0.4	0.4086
128	0	0	0	0	0	0	0	0	0	0.05	0	0	0.0042
130	0.5	0.1667	0.1	0.3654	0.1667	0.6667	0.25	0.0882	0.0625	0.2	0.2083	0.4	0.2645
132	0	0	0.1	0.0385	0.25	0.0556	0.1429	0.1176	0.125	0.05	0.125	0.1	0.092
134	0.0833	0.1667	0.1	0.0192	0.0417	0	0.1071	0.0882	0.125	0	0.0208	0	0.0627
136	0	0	0	0	0	0	0.0357	0	0	0	0.0208	0	0.0047
DB1													
(N)	6	6	5	26	12	10	14	17	8	10	24	5	143
118	0.0833	0	0	0	0	0	0	0	0	0	0	0	0.0069
122	0.25	0.5833	0.3	0.2115	0.2917	0.35	0.4286	0.4412	0.375	0.2	0.2708	0.4	0.3418
124	0.0833	0.1667	0	0.25	0.2083	0	0.0714	0.2059	0.0625	0.15	0.2708	0.2	0.1391
126	0	0.0833	0.1	0.0769	0.125	0.15	0.1786	0.1471	0.125	0.05	0.0417	0.1	0.0981
128	0.0833	0.0833	0.1	0.0192	0	0	0.1071	0	0	0.15	0	0	0.0453

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA	Totals
130	0.5	0.0833	0.5	0.4423	0.375	0.5	0.2143	0.2059	0.4375	0.45	0.4167	0.3	0.3687
DB5													
(N)	6	6	5	25	11	12	14	17	8	10	24	5	143
184	0.0833	0	0	0.16	0.0455	0.4583	0.1786	0.1471	0.0625	0	0.1667	0.1	0.1168
186	0.25	0.1667	0.3	0.18	0.2727	0.1667	0.1071	0.1471	0.25	0.2	0.1042	0.1	0.187
188	0	0	0.1	0.08	0.1818	0	0.1786	0.1176	0.0625	0.1	0.1042	0.2	0.0937
190	0	0	0	0.02	0	0	0.0357	0.0588	0.0625	0	0.0417	0.2	0.0349
192	0	0	0	0	0	0.0833	0	0	0	0	0	0	0.0069
194	0	0	0	0.04	0	0	0	0	0	0	0	0	0.0033
196	0	0.1667	0	0.02	0	0	0.0357	0	0	0	0.0208	0	0.0203
198	0.25	0.4167	0.3	0.16	0.1364	0.1667	0.0714	0.0588	0.0625	0.5	0.1875	0.2	0.2092
202	0	0.0833	0.1	0.1	0.0455	0	0	0.1765	0.25	0.1	0.0833	0	0.0782
204	0.3333	0.0833	0.2	0.24	0.2727	0.125	0.25	0.2059	0.125	0.1	0.2292	0.2	0.197
206	0.0833	0.0833	0	0	0.0455	0	0.0714	0.0882	0.125	0	0.0625	0	0.0466
210	0	0	0	0	0	0	0.0714	0	0	0	0	0	0.006
BR4													
(N)	6	6	5	25	11	11	14	16	8	9	24	5	140
118	0	0	0	0.02	0	0	0.0357	0	0	0	0.0208	0	0.0064
122	0	0.1667	0.1	0.12	0	0.1818	0.1071	0.0313	0.125	0.2222	0.0625	0.1	0.1014
124	0	0	0	0	0.0455	0.0909	0	0.0313	0	0	0.0208	0	0.0157
126	0.1667	0.0833	0.1	0.18	0.0909	0.5455	0.1429	0.0313	0.0625	0.0556	0.1875	0.1	0.1455

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA	Totals
128	0.5	0.3333	0.4	0.16	0.1364	0	0.1786	0.2188	0.4375	0.2778	0.2083	0.1	0.2459
130	0.1667	0.25	0.3	0.08	0.2727	0	0.2143	0.1875	0.0625	0.3333	0.25	0.3	0.2014
132	0.1667	0	0	0.08	0	0	0.1429	0.1875	0.0625	0	0.0417	0.2	0.0734
134	0	0	0	0.12	0.1818	0.1818	0.0357	0.125	0	0	0.0417	0.1	0.0655
136	0	0.0833	0.1	0.2	0.0455	0	0.1071	0.0625	0	0	0.1458	0.1	0.0704
138	0	0	0	0	0.1364	0	0	0.0313	0	0	0.0208	0	0.0157
144	0	0.0833	0	0.02	0.0909	0	0	0.0313	0.25	0.1111	0	0	0.0489
146	0	0	0	0.02	0	0	0.0357	0.0625	0	0	0	0	0.0099
BR6													
(N)	6	6	5	26	11	11	14	17	8	9	24	5	142
131	0	0	0	0	0	0	0	0	0	0	0.0208	0	0.0017
139	0.1667	0.1667	0.3	0.0577	0.3636	0.3182	0.0714	0.1765	0.5	0.3333	0.1875	0.2	0.2368
141	0.5	0.4167	0.3	0.2885	0.3182	0.0909	0.5	0.4412	0.3125	0.3889	0.4375	0.4	0.3662
143	0.25	0.25	0.3	0.5962	0.2273	0.2727	0.3571	0.3235	0.1875	0.2778	0.2708	0.3	0.3011
145	0.0833	0.1667	0.1	0.0577	0.0909	0	0.0714	0.0588	0	0	0.0833	0.1	0.0677
149	0	0	0	0	0	0.3182	0	0	0	0	0	0	0.0265
DB66													
(N)	5	6	5	25	11	12	14	15	8	9	23	5	
179	0	0	0	0	0	0	0.0357	0	0	0	0	0	0.003
191	0.2	0.1667	0.2	0.34	0	0.3333	0.0714	0	0	0.0556	0.2826	0.4	0.1708
195	0.2	0.3333	0.1	0.12	0.1818	0	0.1071	0.1	0	0.1111	0.1304	0.1	0.1237

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA	Totals
197	0	0	0	0.02	0	0	0.0357	0.0333	0	0	0	0	0.0074
199	0.1	0	0.3	0.12	0	0	0.2143	0.1667	0	0.0556	0.0652	0.4	0.1185
201	0.2	0.0833	0.1	0.08	0.1364	0.5833	0	0.2	0.375	0.1667	0.2174	0	0.1785
203	0	0.0833	0.1	0.08	0	0	0.0357	0.0333	0	0.1111	0.0217	0	0.0388
205	0	0.25	0.2	0.16	0.3636	0.0833	0	0.3	0.3125	0.3333	0.0652	0	0.1723
207	0.3	0.0833	0	0.08	0.3182	0	0.5	0.1333	0.3125	0.1667	0.2174	0.1	0.1843
209	0	0	0	0	0	0	0	0.0333	0	0	0	0	0.0028
DB23													
(N)	6	6	5	26	12	12	14	17	8	10	24	5	145
173	0.0833	0	0.2	0.0962	0	0	0.1429	0.1176	0.0625	0.1	0.0833	0.2	0.0905
179	0.8333	0.75	0.7	0.75	0.7917	0.25	0.5	0.7647	0.75	0.85	0.6042	0.3	0.6537
181	0.0833	0.25	0.1	0.1154	0.2083	0.0833	0.3214	0.0882	0.1875	0.05	0.3125	0.4	0.1833
185	0	0	0	0.0385	0	0.6667	0.0357	0.0294	0	0	0	0.1	0.0725