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Phylogeography of the eastern black rhinoceros (*Diceros bicornis michaeli*) in Kenya

Shadrack M Muya*

Faculty of Science, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

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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. The aim of this study was to examine the spatial genetic structuring and diversity of mitochondrial DNA in the Kenyan black rhinoceros. This study was motivated by the fact that currently, Kenya Wildlife Service (KWS) black rhino conservation postulates that the Kenyan black rhinoceros exists in two main subpopulation referred to as the lowland and montane populations based on exposure to tsetse fly infestation; present in lowlands but absent in montane ecosystem. 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. 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.

***Corresponding Author:** Dr. Shadrack Muya ✉ smuya@jkuat.ac.ke

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 continuou, 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. 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 (Barclay, 1932; Lloyd-Jones & Brevet-Major, 1925; Neumann, 1898) and Kenya in particular (Hunter, 1952; Patterson, 1909) 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 (Akama, 1998; Leakey, 1969; Spinage, 1962). 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.

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) 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, Kuussaari, Vickman, Fortelius, & Hanski, 1998; Saccheri, Wilson, Nichols, Bruford, & Brakefield, 1999), making the small populations face increased extinction risk. This kind of extinction vortex that is fueled by an interactive negative feedback between reduction in population growth and inbreeding (R. Frankham, Balloua, & Briscoe, 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. 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, Amin, & Kock, 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 (H. Frankham & Ralls, 1998; R. Frankham *et al.*, 2002; Freeland, 2005).

The aim of this study was to examine the spatial genetic structuring and diversity of mitochondrial DNA in the Kenyan black rhinoceros. This study was motivated by the fact that currently, Kenya Wildlife Service (KWS) black rhino conservation policy postulates that the Kenyan black rhinoceros exists in two main groupings referred to as the lowland and montane populations (Okita-Ouma *et al.*, 2007). The Montane population mainly encompasses the Aberdares National Park (Okita-Ouma *et al.*, 2007), with all other subpopulations forming the lowland group. The key major environmental difference between two main groupings is the presence of Tsetse flies in the lowlands. Thus, translocation of black rhinoceros translocation between lowland and montane ecosystems is not allowed. 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 thus examined the grouping of Kenyan black rhinoceros populations into lowland and montane units by KWS 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. The findings are used to advise appropriate conservation of strategies for the Kenyan black rhinoceros is discussed.

Materials and methods

Sampling

Tissue and dung samples were collected from 12 out of the 14 current locations in Kenya between 2005

and 2007 as follows:-- Aberdares NP, (n = 9), Chyulu NP, (n = 9), Laikipia WC, (n = 9), Lewa WC, (n = 33), Lake Nakuru NP, (n = 20), Masai Mara GR, (n = 30), Ngulia RS, (n = 23), Nairobi NP, (n = 62), Ol Jogi RH, (n = 15), Ol Pajeta RH, (n = 37), Solio RH, (n = 28), Tsavo East NP, (n = 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. The samples are stored in 70% ethanol at -20°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.

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 & Rowe, 2007). This marker has been used successfully to study genetic variability (Nunney & Campbell, 1993), phylogeography (Morales, Andau, Supriatna, Zainuddin, & J., 1997; O'Ryan, Flamand, & Harley, 1994), including the phylogeography of black rhinoceros (Brown & Houlden, 2000) and to assign evolutionary significant and management units in wildlife management (Moritz, 1994).

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 20µl containing 1µl of DNA extract, and 19µl of reaction mix that contained 10µl of master mix from QIAGEN multiplex kit, the primers mt15996L and mt16502H, which yield a PCR product of 520 base pairs (Brown & Houlden, 2000) were used to a final concentration of 0.2 µM, 2µl of Q solution (Qiagen Hilden, Germany) and 5µl 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°C denaturation for 30 seconds, primer annealing at 58˚C for 90 seconds and 60 seconds of primer extension at 72°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 & Houlden, 1999), located in the $tRNA^{Pro}$ gene flanking the control region, and mt16502H (Brown & 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. 1.1 software (Gene Codes Corporation, 1988). The control region fragments were authenticated by BLAST search. DAMBE (Xia & Xie, 2001) was used to identify haplotypes from the aligned sequences.

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. 1.1 (Excoffier, Laval, & Schneider, 2005) and DnaSP Ver. 4.0 (Rozas, Sánchez-Delbarrio, Messeguer, & Rozas, 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 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 & Harley, 2005).

A Median Joining Network (MJN) developed using NETWORK 4.1.1.1 (Bandelt, Forster, & Röhl, 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, Dudley, Nei, & Kumar, 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.

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 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 $\geq$ 0.02 per Mya, based on the 7 Mya divergence between white and black (Brown & Houlden, 2000; Cooke, 1972), thereby allowing for the inference of intraspecific coalescence times.

Results

Genetic diversity

Sequences and haplotype analysis

DNA sequences were trimmed and analysed providing 408bp 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). 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 1). 43% of the samples shared the H16 haplotype, which was present in 11 subpopulations in Kenya (Fig. 1), and was only absent in the Laikipia Wildlife Conservancy.

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 Fig. 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.

Table 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

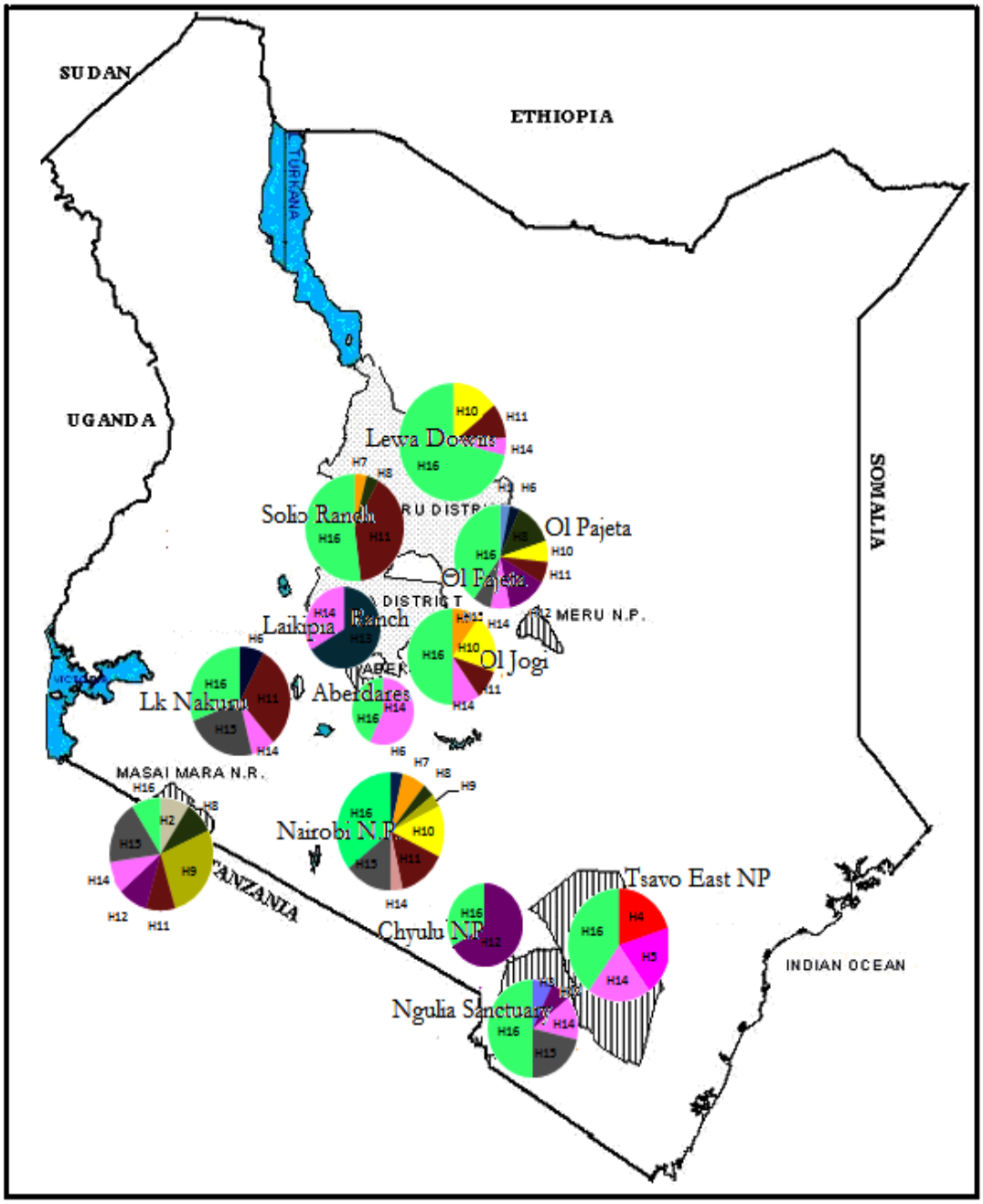

Fig. 1. Geographic distributions of black rhinoceros control region haplotypes in Kenya. The haplotypes are represented by different colours and sample sizes defined in Table 1. The size of the circles represents the number of individuals sampled from the subpopulation.

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 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 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.

Phylogeography

Individual-based

The phylogeny of *Diceros bicornis michaeli* was inferred from geographic distribution of haplotypes, (Fig. 1), a median joining network (MJN) multifurcating (Fig. 2) and a bifurcating maximum likelihood phylogenetic tree (Fig. 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 (Fig. 3) within the black rhinoceros where each subspecies; i.e. *Diceros bicornis bicornis*, *Diceros bicornis minor*, and *Diceros bicornis michaeli* form a monophyletic group (Fig. 3). Three haplotypes (H01, H09 and H12) in the Kenyan black rhinoceros population grouped together but had a mixed geographic distribution.

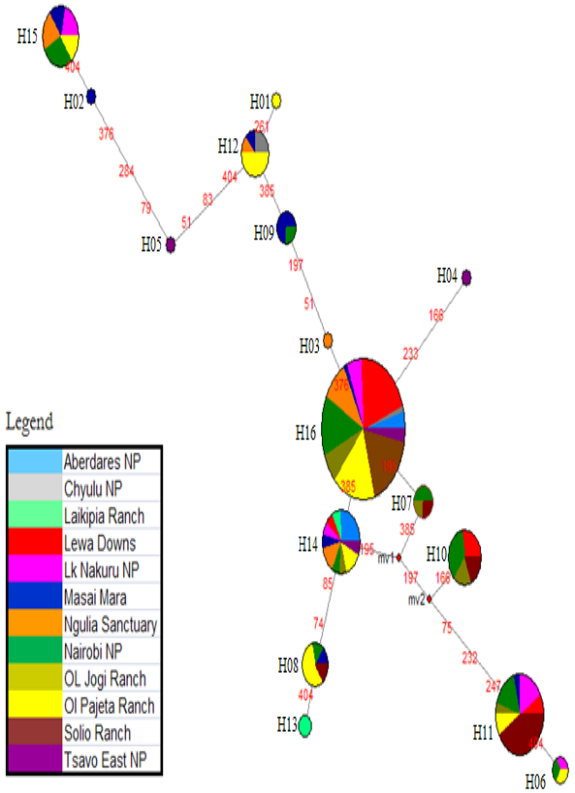

Fig. 2. Median joining networks (MJN) of Kenyan black rhinoceros sequences. Each circle represents a haplotype and its size is proportional to the haplotype frequency in different subpopulation. Small red squares are median vectors of unsampled or extinct ancestral sequences. Red numbers indicate the 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.

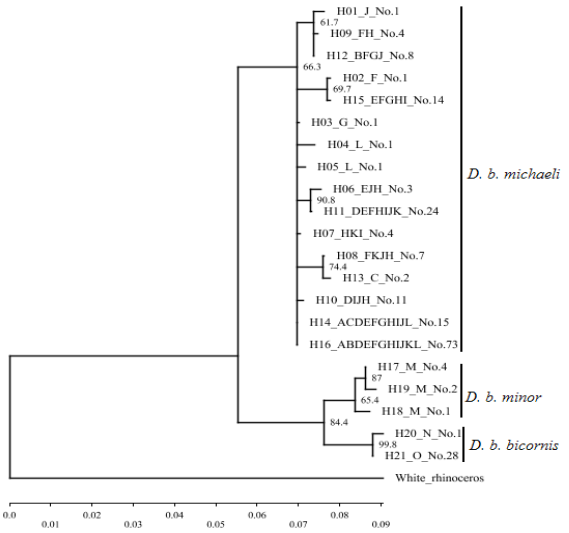

Fig. 3. Maximum likelihood (ML) phylogenetic tree of the black rhinoceros control region haplotypes H01 to H21, with white rhinoceros (*Ceratotherium simum simum*) as outgroup, *D. b bicornis* and *Diceros bicornis minor* show the magnitude of the differences within *Diceros bicornis michaeli*. Alphabets 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. Fig.s 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.

Frequency-based

Generally the fixation index (Φ_{CT}) for the Kenyan black rhinoceros was low (Table 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). 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) 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. 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

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±0.00 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 (Moodley & Harley, 2005; Oakenfull, Lim, & Ryder, 2000; Slade, Moritz, & Heideman, 1994; Wooding & Ward, 1997).

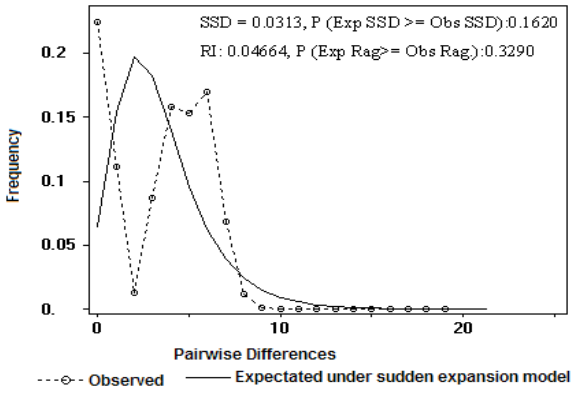

Fig. 4. Pairwise differences frequencies mismatch distribution of the Kenyan black rhinoceros. SSD is squared deviations, Exp is Expected, Obs is observed and RI is Harpending's the raggedness index.

Population demography

The expected mismatch for the Kenyan black rhinoceros control region data set was described by parameters estimated from the sudden expansion model (Θ_0 = 0.002, Θ_1 = 6.404, τ = 5.812). The observed and expected mismatch distributions were not significantly different (P(SSD)>0.05, P(RI)>0.05) (Fig. 4). In mismatch distribution analysis, a Gaussian shaped unimodal distribution would suggest population that has undergone a period of rapid expansion in the past.

Discussion

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, Melnick, & Western, 1990; O'Ryan *et al.*, 1994; O'Ryan & Harley, 1993) 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 & Houlden, 2000; Goossens *et al.*, 2005; Scott, 2008; Tougard, Delefosse, Hanni , & Montgelard, 2001). 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 & 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.

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 & Harley, 2005) than the Kenyan black rhinoceros.

Western lowland gorillas have a nucleotide diversity of 0.062 (Clifford, Anthony, & Bawe-Johnson, 2004). The common warthog and Savannah elephants in Kenya have nucleotide diversity of 0.015, and 0.0168 respectively (Muwanika, Siegismund, & Okello, 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.

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 (Fig. 2), nor a maximum likelihood phylogeny (Fig. 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 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 (Fig. 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 (Fig. 2) as it implies either that some haplotypes were lost in the population bottleneck or that they were not sampled.

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 (Muwanika *et al.*, 2003; O'Brien, D.E., Goldman, Merril, & Bush, 1983; 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 (R. 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 (Bergl, Bradley, Nsubuga, & Vigilant, 2008; Miller & Waits, 2003; Saccheri *et al.*, 1998). 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.

Reference

AfRSG. 2004. Meta-population planning for D. b. michaeli. Paper presented at the IUCN-SSC-AfRSG meeting, Kilaguni, Tsavo West.

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

Ashley MV, Melnick DJ, Western D. 1990. Conservation genetics of the Black Rhinoceros (Diceros bicornis). I: Evidence from the mitochondrial DNA of three populations. Conservation Biology **4,** 71-77.

Avise JC. 1994. Molecular markers, natural history and evolution. New York: Chapman and Hall.

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

Barclay EN. 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.

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

Bergl RA, Bradley JB, Nsubuga A, 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),** 848-859.

Brown SM, Houlden BA. 1999. Isolation and characterization of microsatellite markers in the black rhinoceros (*Diceros bicornis*). Molecular Ecology **8,** 1559-1561.

Brown SM, Houlden BA. 2000. Conservation genetics of the black rhinoceros (Diceros bicornis). Conservation Genetics **1,** 365-370.

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

Cooke HBS. 1972. The fossil mammal fauna of Africa. In A. Keast & F. C. Erk (Eds.), Evolution, Mammals and Southern Continent (pp. 89–139). Albany: State University of New York.

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

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

Frankham R, Balloua JD, Briscoe DA. 2002. Introduction to conservation genetics (1st ed.): Cambridge University Press.

Freeland JR. 2005. Molecular Ecology. The Open University, Milton Keynes: John Wiley and Sons, LTD.

Gene Codes Corporation. 1988. An international software firm specializing in bioinformatics software for DNA sequence analysis. (Version Ver 3.1.1). Ann Arbor, Michigan. Retrieved from http://www. genecodes.com

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

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

Hunter JA. 1952. Hunter. London: Hamish Hamilton London.

IUCN. 2006. West African black rhino feared extinct. News release of 07 July 2006. Retrieved from

Jobb G. 2008. TREEFINDER version of October 2008. Retrieved from www.treefinder.de

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

KWS. 2003. Conservation and management strategy for the black rhino (Diceros bicornis michaeli) in Kenya 2000-2005. Retrieved from Nairobi:

Lacombat F. 2005. The evolution of the rhinoceros. Paper presented at the Save the rhinos: EAZA Rhino Campaign 2005/6, London.

Leakey L. 1969. Animals of East Africa. New York: National Gerographic.

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

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

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

Morales CJ, Andau PM, Supriatna J, Zainuddin ZZJMD. 1997. Mitochondrial DNA variability and conservation genetics of the Sumatran rhinoceros. Conservation Biology **11,** 539-543.

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

Muwanika V, Siegismund HR, Okello JBA. 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).

Neumann AH. 1898. Elephant-Hunting in East Equatorial Africa. California: London & Encino.

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

O'Brien SJDEW, Goldman D, Merril CR, Bush M. 1983. The Cheetah is depauperate in genetic variation. J. Science **221(4609),** 459 - 462.

O'Ryan C, Flamand JRB, Harley EH. 1994. Mitochondrial DNA variation in black rhinoceros (Diceros bicornis): conservation management implications. Conservation Biology **8,** 495-500.

O'Ryan C, Harley EH. 1993. Comparisons of mitochondrial DNA in black and white rhinoceroses. Journal of Mammalogy **74,** 343-346.

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

Okello JBA, Masembe C, Rasmussen HB, Wittemyer G, Omondi P, Kahindi O, . . . Siegismund HR. 2008. Population Genetic Structure of Savannah Elephants in Kenya: Conservation and Management Implications.

Okita-Ouma B, Amin B, 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). Retrieved from Nairobi:

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

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

Saccheri IJ, Kuussaari M, Vickman P, Fortelius W, Hanski I. 1998. Inbreeding and extinction in a butterfly metapopulation. Nature **392,** 491-494.

Saccheri IJ, Wilson IJ, Nichols RA, Bruford MW, Brakefield PM. 1999. Inbreeding of bottlenecked butterfly populations: estimation using the likelihood of changes in marker allele frequencies. Genetics **151,** 1053-1063.

Scott CA. 2008. Microsatellite variability in four contemporary rhinoceros species: implications for conservation. Department of Biology. Retrieved from http://www.rhinoresourcecenter.com/ref_files/1218825705.pdf

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

Spinage CA. 1962. Animals of East Africa. London: Collins.

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

Times online. 2006. West African black rhino 'is extinct'. The Times Online, July 2006. Retrieved from http://www.timesonline.co.uk/tol/news/world/article684547.ece

Tougard C, Delefosse T, Hanni C, 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),** 34-44.

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

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

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