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RESEARCH ARTICLE

Substantial molecular variation and low genetic structure in Kenya's black rhinoceros: implications for conservation

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Abstract Kenya's black rhinoceros population declined by more than 98% from 20,000 individuals in the 1970s to around 400 individuals in 1990 due to the effects of poaching, at which time the surviving individuals were isolated in a series of demographically inviable subpopulations. An initial management exercise translocated the survivors into four high security sanctuaries to control poaching and enhance breeding, and this measure successfully arrested the decline. Subsequently, new sanctuaries were established and the metapopulation size reached 650 animals by 2008. However, translocations and the current management strategy that partitions the metapopulation into 'montane' and 'lowland' rhinoceros may have substantial consequences at the population level and their impact on population genetic diversity has not been investigated. In this study, 12 of the 16 extant subpopulations were analysed using 408 bp of mitochondrial control region sequence ($n = 170$) and nine microsatellite loci ($n = 145$). Both markers detected moderate to high genetic diversity ($h = 0.78 \pm 0.027$, $n = 170$; $H_O = 0.70 \pm 0.087$, $n = 145$) consistent with previous studies on *Diceros bicornis michaeli*. However, mtDNA and nDNA diversity varied substantially between subpopulations. The results suggest that the Masai Mara is more differentiated, inbred and isolated than other subpopulations. It also suggests that there are neither distinct montane and lowland groups nor other detectable historical barriers to gene flow. Instead the large majority of genetic diversity was partitioned at the level of individuals; highlighting the need to conserve as many individuals as possible. Future translocations should consider the genetic profile of individuals and the demographic history of both the donor and recipient subpopulations.

Keywords Black rhinoceros · Conservation genetics · Microsatellites · Population fragmentation · Translocation

Introduction

At the beginning of the 20th Century, Kenya's black rhinoceros (*Diceros bicornis michaeli*) were so abundant (Neumann 1898; Patterson 1909; Lloyd-Jones and Brevet-Major 1925; Barclay 1932; Hunter 1952; Brett 1993) that they were viewed as agricultural pests that potentially impeded human land use. For example, in 1950, over 1000 animals were shot in the Kibwezi area (Hunter 1952) to facilitate human settlement. In the 1970s, the species numbered around 20,000 individuals (IUCN SSC AfRSG 2008) and still had a wide distribution in Kenya. However, the population declined catastrophically due to poaching and human settlement during the following 20 years, to less than 400 animals by 1990 (Brett 1993; Gakahu 1993; Emslie and Brooks 1999; Okita-Ouma et al. 2007). This population collapse resulted in small, isolated, demographically inviable populations scattered across

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fragmented regions in Kenya, with many facing local extinction. An ambitious translocation program (Table 1) for isolated rhinoceros populations was therefore initiated. The program initially focused on moving rhinoceros into four high security sanctuaries to enhance the breeding prospects of rescued individuals (Merz 1994). Gradually additional sanctuaries were established, being seeded from the initial four and consequently the population had grown to over 650 animals in 16 subpopulations by 2008 (Emslie et al. 2009). However, the genetic consequences of these translocations are not known and could have unforeseen consequences; including precipitating the breakdown of locally adapted genotypes (Frankham et al. 2002; Allendorf and Luikart 2007). In addition, the Kenya Wildlife Service (KWS) currently operates a management strategy that focuses on maintaining an isolated montane forest and the lowland subpopulations separately (Okita-Ouma et al. 2007). This approach may, on the one hand, lead to conservation of locally adapted genotypes within the two subpopulations but may alternatively further isolate an already genetically restricted population, exposing the subpopulation into further, genetically mediated risk. The impact of these management strategies on the population's genetic diversity has not been investigated (Table 2).

Earlier genetic studies of black rhinoceros have examined various aspects of *ex-situ* conservation using mitochondrial (mt; Ashley et al. 1990; Jama et al. 1993; Morales and Melnick 1994; O'Ryan et al. 1994) and nuclear DNA (Brown and Houlden 1999; Garnier et al. 2001; Zschokke et al. 2003; Nielsen et al. 2008; Scott 2008). For example, Swart et al. (1994), using mtDNA, demonstrated the persistence of genetic diversity in small populations of black rhinoceros that had undergone demographic contraction; highlighting the need to conserve even small populations. Behavioural observations in Lewa Down Wildlife Conservancy indicated that black rhinoceros males are polygynous, with a high variance in reproductive success, suggesting a potential benefit of moving males among populations to reduce mate competition and increase male effective population size (Merz 1994). This observation was subsequently confirmed by Garnier et al. (2001) using microsatellite markers in a population in Zimbabwe.

Within the context of previous studies, and the available management options for Kenya's remaining population, we examined both mitochondrial control region sequence variation and nine microsatellite loci to assess the genetic structure of the Kenya's black rhinoceros metapopulation and address the following questions:

i. Has genetic variation within the Kenya's black rhinoceros metapopulation been substantially affected by the recent demographic decline?
ii. To what extent are Kenyan black rhinoceros subpopulations genetically differentiated from each other and what are the effects of translocations and the separate management of lowland and highland populations?
iii. Do mitochondrial and nuclear markers recapitulate the same demographic history in these populations?
iv. In light of the genetic evidence, what are the appropriate conservation strategies for the management of Kenya's black rhinoceros metapopulation?

Materials and methods

Sampling

Sampling was carried out in 12 of Kenya's 16 current black rhinoceros subpopulations. Two subpopulations—Meru National Park and Mugie Ranch were not sampled because they were established between 2004 and 2006 with animals from Solio Ranch, Nairobi National Park, Lake Nakuru National Park and the Lewa Down Wildlife Conservancy. Hence sampling the source subpopulations was expected to capture the genetic composition of these subpopulations. The fourth unsampled subpopulation, Masai Mara Triangle, is not distinct from Masai Mara National Reserve because the animals occupy the same unfenced ecosystem. The Aberdares National Park is the only montane park in Kenya inhabited by black rhinoceros and is managed separately by KWS with translocation not allowed in or out this subpopulation. Only fresh dung samples were obtained in this subpopulation, since tissue or blood samples were not available. All other 15 subpopulations are considered as lowland subpopulations and translocations are allowed. A total of 295 samples were analysed. They included tissue (86), serum (54), whole blood (37) and dung (118) collected from known different individuals. Serum, whole blood and tissue samples were preserved in either 70% ethanol at -20°C or in 25% dimethylsulphide (DMSO) saturated with sodium chloride at room temperature. Dung samples were preserved in 90% ethanol.

Molecular methods

A DNeasy® Tissue Kit was used to extract DNA from tissue and serum while a QIAmp® DNA Stool Mini Kit was used to isolate DNA from dung samples. In both cases the manufacturer's instructions (QIAGEN® Hilden, Germany) were followed. The extracts were dissolved in 150 µl of elution buffer and stored at −20°C. Extractions blanks and positive controls were used to monitor contamination during extraction and amplification.

Table 1 Known translocation history of Kenyan black rhinoceros, 1961–2008

Subpopulation	*N* (2007)	LPN (year)	IN (61–08)	Donor location (Year) and number	OUT (61–08)	Recipient location (Year) and number
Aberdare NP	30	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	2 (1992)	0	0	0	0
Laikipia WC	12	10 (2002)	11	Other areas (93) 1, Nairobi NP (05) 10,	0	0
Lewa WC	55	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 (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	24 (1990)	0	0	1	Nairobi NP (86) 1
Nairobi NP	68	57 (1989)	35	Kitengela (63–68) 5, Kapiti (63–68) 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	73	Amboseli (83) 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	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,	6	Nairobi NP (04) 6
Ol Jogi Ranch	27	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	4 (1989)	49	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
Solio Ranch	69	49 (2002)	27	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	2 (1989)	58	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		20	Solio (04) 4, Nairobi NP (04) 6, Lk Nakuru (04) 10	0	
Meru NP	17		28	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	

N Current census population size, *LPN* lowest population size; digits in brackets indicate the year(s) translocation was possibly carried out, *IN* total number of animals translocated in while, *OUT* total number of animals translocate out, *NP* national park, *WC* wildlife conservancy, *NR* national reserve. Digits outside *brackets* indicate the number of rhinoceros that were translocated. *Source*: KWS rhino programme census data

Table 2 Location, area in square kilometers, and population size and sampling information of the 12 sampled Kenyan black rhinoceros subpopulations

Location	Area (km^2)*	*N*	*n*	nDNA +ve Samples	mtDNA +ve Samples	Sample composition
Aberdares NP	100	30	9	6	7	Dung
Chyulu NP	471	21	9	6	3	Dung
Laikipia WC	397	12	9	5	3	Dung
Lewa WC	247	55	33	12	13	Dung
Lake Nakuru NP	144	37	28	26	21	Tissue and serum
Masai Mara GR	1510	68	30	12	11	Tissue and whole blood
Ngulia RS	90	68	30	14	28	Dung
Nairobi NP	117	68	47	17	14	Tissue, whole blood and serum
Ol Jogi RH	249	27	15	8	10	Dung
Ol Pajeta RH	300	77	37	10	30	Tissue, whole blood and serum
Solio RH	69	69	28	24	25	Tissue, whole blood and serum
Tsavo East NP	13,747	50	20	5	5	Dung
Total		582	295	145	170	

GR Game reserve, *NP* National park, *RH* Ranch, *WC* Wildlife conservancy, *N* Census population size, *n* Sample size, *nDNA +ve samples* number of sample that produced incontestable microsatellite genotypes, *mtDNA +ve samples* number of samples that produced good sequence chromatographs. * The area indicated refers to the area used for black rhinoceros conservation as some of these Parks are larger than the areas indicated in this table and have areas that are designated for as rhinoceros sanctuaries (Okita-Ouma et al. 2007)

MtDNA amplification PCR reactions were performed in 20 µl containing 1 µl of DNA extract, 10 µl of master mix from the QIAGEN multiplex PCR kit (Qiagen Hilden, Germany), a final concentration of 0.2 µM of primers mt15996L and mt16502H (Brown and Houlden 2000), 2 µl of Q solution (Qiagen) and 5 µl of water. Amplifications were carried out in a Perkin Elmer 9700 thermocycler at 95°C for 15 min followed by 35 cycles of 30 s at 94°C, 90 s at 58°C, 72°C for 60 s, and a final extension of 72°C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel to check amplification. A 520 bp fragment was sequenced using the PCR primers, which anneal to sites located in the $tRNA^{Pro}$ gene flanking the control region, and the central conserved domain of the control region. Products were purified using a QIAGEN PCR purification kit and sequenced in forward and reverse directions by Macrogen Inc, Korea. Chromatograms were checked by eye, corrected and sequences were aligned using SEQUENCHER v3.1.1 software (Gene Codes Corporation 1988) and BLAST aligned (Altschul et al. 1990) to confirm that they were authentic *D. b. michaeli* sequences.

Nine polymorphic microsatellite markers—DB44, DB30, BR17, DB1, DB5, BR4, BR6, DB66, and DB23 (Brown and Houlden 1999; Cunningham et al. 1999) were amplified using a modified multiple-tubes PCR approach (three repetitions for each tissue/serum derived DNA sample and six repetitions for each dung-derived DNA sample (regardless of whether the consensus genotype was a homozygote of heterozygote) respectively (Taberlet et al. 1996). Amplifications were carried out in 10 µl containing 5 µl of QIAGEN Multiplex PCR Master Mix, 0.2 µM of each primer, forward and reverse, 2 µl of DNA, 1 µl of 0.5X Q-Solution and 1 µl of water. The nine primers were assorted into four multiplexes using three dyes (HEX, FAM and NED) based on their annealing temperature similarity and product size range. The multiplex conditions were optimized at Cardiff University, and the final PCR products were analysed at Macrogen Inc, Korea. Electropherogram files were scored using Peak Scanner Ver. 1.0 (Applied Biosystems 2010). Allele scores were independently cross-checked and a consensus score derived. MICROCHECKER 2.2.3 (van Oosterhout et al. 2004) was used to identify null alleles, scoring errors and allelic dropout for each locus.

Statistical analysis

GENETIX 4.05 was used to calculate the mean number of alleles (MNA) per subpopulation and Nei's unbiased estimate of expected heterozygosity (H_E, Nei 1973). Observed and expected genotype frequencies were compared per locus and across all subpopulations and their inbreeding coefficient (F_{IS}) calculated using 1000 permutations (Weir and Cockerham 1984). The program POPGENE (Yeh et al. 2000) was used to estimate genotypic linkage disequilibrium (LD). DnaSP Ver. 4.0 (Rozas et al. 2003) was used to estimate mitochondrial haplotype (h) and nucleotide (π) diversity.

A parsimony network linking all haplotypes was developed using NETWORK 4.1.1.1 (Bandelt et al. 1999).

Branch lengths were scaled according to the number of mutations separating linked haplotypes. Pairwise genetic differentiation among subpopulations was estimated using F_{ST} (Weir and Cockerham 1984) and Nei's standard genetic distances, and was implemented in ARLEQUIN 3.1.1. Hierarchical genetic structure was inferred using analysis of molecular variance (AMOVA; Excoffier et al. 2005) for both haplotype and genotype data. Four hypothetical grouping structures were explored based on alternative putative demographic histories. The first considered Masai Mara subpopulation as isolated from the subpopulations that have historical records of exchange. The second hypothesis was based on the assumption of a unique montane forest subpopulation (Aberdares) versus the other subpopulations (referred to as lowland subpopulation by KWS). The third hypothesis was based on an assumption of a historically panmictic population in which subpopulation genetic similarity is assumed to correlate with geographic proximity. In this scenario, the Masai Mara subpopulations formed one group in western Kenya. Aberdares, Lewa Down, Ol Jogi, Ol Pajeta, Laikipia, Lake Nakuru and Solio subpopulations forming another group in the Kenyan highlands, while the Nairobi, Tsavo East, Ngulia and Chyulu subpoulations formed a third group. The fourth hypothetical structure was based on the assumption that the three relictual subpopulations—Masai Mara, Chyulu and Laikipia—retained a genetic signature of their pre-bottleneck population structure. The hypothesis with the highest significant value for θ_{CT} (the among population molecular variance component) was inferred as the most likely structure (Weir and Cockerham 1984).

Genetic structure was further investigated in two ways; first, through Bayesian clustering of genotypes, and second, through testing of the relative likelihood of a drift versus gene flow genealogical model. The Bayesian clustering method was implemented using STRUCTURE 2.2 (Pritchard et al. 2000). The number of clusters (K) tested ranged between one and 12; by assuming that at a most structured level, each subpopulation would cluster as its own group. Ten runs for each value of K were performed, in order to verify that the results were consistent across runs. The mean posterior probability (Pr(X|K)) was calculated for each value of K over each set of runs, and the most likely K value was determined as the maximum value of the logarithm (ln) of mean posterior probability of the data. The maximal ln Pr(X|K) was at $K = 5$ (data not shown). The clustering was further visually inspected through incremental plotting of $K = 2$–12 for all sites, but did not change for values of $K > 5$ (data not shown) and therefore only plots for $K = 2$–5 were retained for visual analysis. Further analysis was carried out to establish the mostly likely value of K, using an *ad hoc* statistic, delta K, which is the mean of the absolute value of the difference between successive values of $L'(K)$, i.e., $|L''(K)| = |L'(K+1) - L'(K)|$ divided by the product of standard deviation and $L(K)$. This corresponds to the second order rate of change of $L(K)$ with respect to K (Evanno et al. 2005). The most likely K was assumed to be the K value at the largest peak of delta K distribution. A population admixture model (Falush et al. 2003) was assumed in view of the fact that numerous translocations have taken place in Kenya's black rhinoceros metapopulation.

Hierarchical Bayesian-based models were implemented using the 2MOD software to assess the relative likelihoods for a pure drift versus a gene-flow/drift equilibrium demographic model of population history, following the method described in Ciofi et al. (1999). The probability density distributions were visualized using the BOA (Bayesian Output Analysis) programme (Brian 2007) in R version 2.8.1 (R Development Core Team 2006) to examine the most likely historical parameter values.

Results

Genetic diversity

170 mitochondrial DNA sequences of 408 bp in length were obtained after editing, containing 16 polymorphic sites that were all transitions. Sixteen haplotypes were found (GenBank/EMBL accession numbers FJ227483-FJ227498), six of which (H1–H5 and H13) were found in only one of the subpopulations (H01 was found only in Ol Pajeta, H02 and H03 only in Mara, H04 and H05 only in Tsavo east while H13 was found only in Laikipia). 43% of samples shared haplotype H16, which was present in 11 subpopulations and was only absent from the Laikipia sample (Fig. 1). Mean haplotype diversity (h) was relatively high ($h = 0.73 \pm 0.14$) but values varied considerably between subpopulations. The Masai Mara subpopulation had the highest haplotype diversity ($h = 0.93 \pm 0.07$, $n = 11$), in spite of its historical isolation (Table 1), while Lewa Down had the lowest ($h = 0.48 \pm 0.12$, $n = 21$). Average nucleotide diversity was low ($\pi = 0.0072 \pm 0.003$, $n = 170$) but once again values varied considerably between subpopulations. One hundred and forty-five individuals from all 12 populations amplified reliably for nine polymorphic loci. The mean number of alleles per locus was 8.67 ± 3.02 and ranged from four alleles in DB23 to 12 alleles in DB30, BR4 and DB5 (data not shown). Masai Mara had both the lowest expected and observed heterozygosity ($H_E = 0.62$ and $H_O = 0.48$). Nairobi NP, Solio GR and Tsavo East NP shared a similar expected heterozygosity value ($H_E = 0.73$), while Lake Nakuru NP and Tsavo East NP had similar observed heterozygosity values ($H_O = 0.8$) that

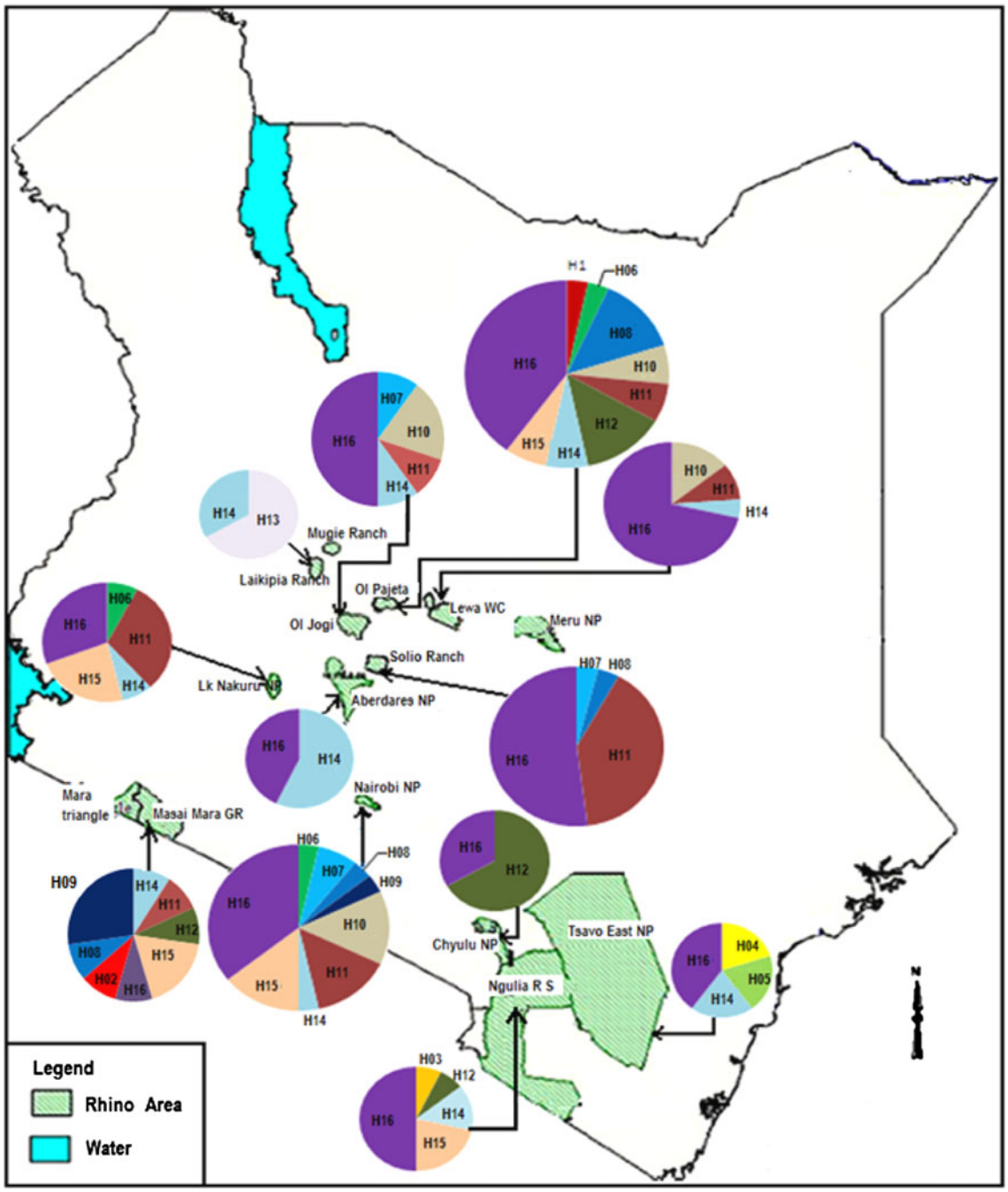


Fig. 1 Location of the 12 Kenyan black rhinoceros subpopulations and the geographic distributions of control region haplotypes. The haplotypes are represented by different numbers and the pie sizes are in proportion to their frequency

were the highest in the metapopulation (Table 3). The overall metapopulation mean H_E and H_O values were 0.70 ± 0.087 and 0.69 ± 0.034 respectively. F_{IS} varied from -0.079 in Lake Nakuru NP to +0.269 in Masai Mara (Table 3). Lake Nakuru, Nairobi, Solio and Ngulia subpopulations had negative F_{IS} values, while all other subpopulations showed positive F_{IS} values with Masai Mara GR having the highest, followed by Laikipia WC and Ol Jogi RH respectively (Table 3).

Population structure

The genetic structure of Kenya's *D. b. michaeli* was inferred from geographic mapping of haplotypes (Fig. 1), a

Table 3 Genetic variation in Kenyan black rhinoceros based on nine polymorphic loci and 408 base pair control region sequences

Subpopulation	Nuclear DNA diversity				Mitochondrial DNA diversity		
	H_E	H_0	MNA	F_{IS} (W&C)	A	*h*	π
Masai Mara GR	0.62	0.48	4.6	0.269	8	0.93	0.011
Laikipia WC	0.68	0.67	4.7	0.127	2	0.67	0.005
OL Jogi GR	0.67	0.64	5	0.111	5	0.76	0.006
Aberdares NP	0.66	0.68	4.8	0.058	2	0.57	0.002
Chyulu NP	0.7	0.72	5.2	0.053	2	0.67	0.007
Ol Pajeta	0.67	0.68	5.1	0.035	9	0.81	0.009
Lewa WC	0.7	0.7	6.7	0.017	4	0.48	0.005
Tsavo East NP	0.73	0.8	5	0.014	4	0.9	0.008
Ngulia Sanctuary	0.71	0.74	6.8	−0.008	5	0.73	0.006
Nairobi NP	0.73	0.76	6.6	−0.011	9	0.83	0.009
Solio GR	0.73	0.77	6.7	−0.035	4	0.59	0.009
Lake Nauru NP	0.71	0.8	5.6	−0.079	5	0.81	0.012
Mean ± SD	0.69 ± 0.03	0.7 ± 0.09	5.57 ± 0.88	0.046 ± 0.091	4.92 ± 2.54	0.73 ± 0.14	0.007 ± 0.003

He Nei's expected heterozygosity, *Ho* observed heterozygosity, *MNA* mean number of alleles per locus per subpopulation, F_{IS} (*W&C*) inbreeding fixation index Weir and Cockerham's (1984) method for estimating the mean probabilities that any two alleles are related by descent, *A* number of haplotypes in each population, *h* haplotypes diversity, π nucleotide diversity, *SD* standard deviation

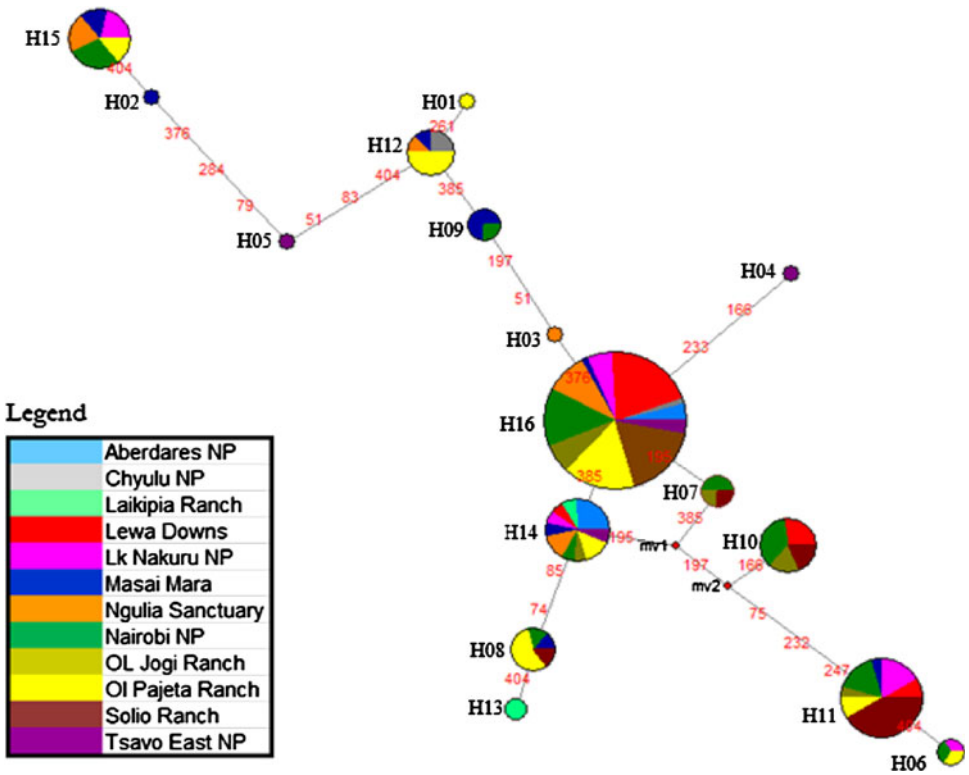


Fig. 2 Median joining networks (MJN) of Kenyan black rhinoceros sequences. Each circle represents a haplotype and its size is proportional to the haplotype frequency in different subpopulation. *Small squares* are median vectors of unsampled or extinct ancestral sequences. The numbers indicate the nucleotide position at which variation occurred, and number of links between haplotypes indicates the number of mutations that haplotypes have undergone from one another. Each pie represents the subpopulation where a haplotype was sampled

median joining network (Fig. 2), analysis of molecular variance and structure cluster plots. Haplotype distribution did not illustrate any strong geographical patterns but rather a complex distribution among subpopulations (Fig. 1). While the median joining network showed a similarly complex pattern, it indicated relatively few substitutions between adjacent haplotypes in the network, never exceeding three base pairs, and with a 99% sequence similarity index (Fig. 2). Several pairs of subpopulations had negative pairwise F_{ST} values (Table 4) implying that those pairs of subpopulations are genetically indistinguishable (Long 1986; Foster et al. 2006). The Aberdares, Chyulu and Laikipia populations had non-significant F_{ST} values for all pairwise comparisons except with Masai Mara; while in contrast the

Table 4 loci pairwise genetic differentiation (F_{ST}; Weir and Cockerham 1984) and their statistical significance level in brackets (*ns* not significant, * $P = 0.05$, and ** $P = 0.001$) between subpopulations (above diagonal) and their standard distance (Nei 1973) below diagonal

	ABE	CHY	LAK	LEW	LKN	MAR	NGU	NNP	OLJ	OLP	SOL	TSA
ABE	0	0.027^{ns}	-0.013^{ns}	0.008^{ns}	0.023^{ns}	0.151*	0.029^{ns}	0.032*	0.036^{ns}	0.016^{ns}	-0.003^{ns}	0.011^{ns}
CHY	0.261	0	-0.03^{ns}	0.047**	0.013^{ns}	0.173**	0.038**	0.004^{ns}	0.023^{ns}	-0.008^{ns}	0.016^{ns}	0.017^{ns}
LAK	0.203	0.164	0	0.01^{ns}	-0.004^{ns}	0.153**	0.022^{ns}	-0.006^{ns}	-0.002^{ns}	-0.034^{ns}	-0.004^{ns}	-0.018^{ns}
LEW	0.141	0.25	0.168	0	0.038**	0.127**	0.053**	0.033**	0.075**	0.037**	0.013**	0.011^{ns}
LKN	0.194	0.19	0.183	0.184	0	0.156**	0.034**	0^{ns}	-0.001^{ns}	0.006^{ns}	0.006^{ns}	0.029*
MAR	0.503	0.729	0.684	0.466	0.6	0	0.167**	0.158**	0.155**	0.17**	0.111**	0.11*
NGU	0.209	0.269	0.251	0.229	0.21	0.681	0	0.025*	0.063**	0.064**	0.023*	-0.012^{ns}
NNP	0.233	0.171	0.213	0.2	0.107	0.66	0.195	0	0.006^{ns}	0.024**	0.014*	0.019^{ns}
OLJ	0.243	0.221	0.186	0.291	0.118	0.577	0.292	0.137	0	0.016^{ns}	0.029*	0.065*
OLP	0.198	0.145	0.125	0.179	0.125	0.675	0.318	0.177	0.172	0	0.02^{ns}	0.043**
SOL	0.121	0.177	0.138	0.086	0.114	0.469	0.147	0.159	0.171	0.157	0	-0.016^{ns}
TSA	0.259	0.307	0.261	0.193	0.279	0.503	0.17	0.236	0.412	0.318	0.143	0

Note that MAR is significantly differentiated from other subpopulations

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

Masai Mara subpopulation had significant pairwise F_{ST} values for all comparisons (Table 4). The Masai Mara subpopulation also possessed the largest pairwise Nei's distance values, with a mean of 0.595 ± 0.097. The mean Nei's distance value for all other populations is 0.235 ± 0.031 (calculated from Nei's distance values in Table 4).

Generally the mtDNA and nDNA ϕ_{CT} values were low (mean $\phi_{CT} = 0.076 \pm 0.048$ and 0.049 ± 0.041 for mtDNA and nDNA respectively) irrespective of the evolutionary hypothesis examined. The AMOVA results do not statistically support the hypothesis that the Masai Mara subpopulation is genetically distinct with respect to all other subpopulations ($P = 0.235 \pm 0.012$ and 0.084 ± 0.009 respectively for mtDNA and nDNA Table 5), although it accounted for 5.46 and 10.82% of the variance in the metapopulation (mtDNA and nDNA, respectively). However, all pairwise F_{ST} values were statistically significant (Table 4), implying that the Masai Mara subpopulation is demographically distinct to other subpopulations. The montane forest–lowland evolutionary hypothesis was statistically unsupported for both mtDNA and microsatellites, having a negative ϕ_{CT} value for both mtDNA and nDNA. The hypothesis of panmictic subpopulations due to geographic proximity was not statistically supported by mtDNA markers ($P = 0.380 \pm 0.0137$), but was supported by nDNA markers ($P = 0.012 \pm 0.003$), and accounted for 4% of the variation in the metapopulation (Table 5). The relictual hypothesis was significantly supported by mtDNA ($P = 0.002 \pm 0.014$), accounted for 11.68% of the genetic variation in the metapopulation and was close being significant with microsatellites ($P = 0.055 \pm 0.006$). In all evolutionary hypotheses examined, genetic partitions at the population and individual levels were statistically significant. Differences at the individual level accounted for over 80% of all the genetic variation in the metapopulation (Table 5).

The estimated natural logarithm of mean posterior probability of the data (ln Pr(X|K)), was maximal at $K = 5$ (data not shown) suggesting the existence of five subpopulations, however visual inspection of the STRUCTURE plots suggested $K = 3$ was more plausible, identifying Masai Mara, Ngulia and the remaining populations as genetically distinct clusters. The delta K distribution also had its highest value at $K = 3$ (data not shown), further corroborating the STRUCTURE plot (Fig. 3). The relative likelihood of gene flow—drift equilibrium model versus drift model, estimated using the Markov Chain Monte Carlo procedure described in Ciofi et al. (1999) revealed that for all Kenya's black rhinoceros metapopulations the gene flow model is more likely than the drift model with a posterior probability of $P = 0.952$ (data not shown) (Fig. 4).

Discussion

Genetic diversity

This study represents the first extensive in situ investigation on the genetic status of the Kenya's black rhinoceros, using both mtDNA and nDNA markers. The first finding of this study is that Kenya's black rhinoceros retains relatively high levels of genetic diversity (for example mean $H_O = 0.73 \pm 0.03$) and has high diversity compared to other black rhinoceros populations in Africa recorded to date (Harley et al. 2005 [$H_O = 0.436$ for *D. b. minor* and $H_O = 0.523$ for *D. b. bicornis*], and Scott 2008 [mean

Table 5 Analysis of molecular variance (AMOVA) among subpopulations based on mitochondrial control region (CR) and microsatellite loci data showing statistical support for various grouping scenarios

Demographic hypothesis	Analysis grouping scenario	Partition level	Mitochondrial CR			Partition level	Microsatellite Loci		
			ϕ_{CT}	*P*-values ± SD	% variation		ϕ_{CT}	*P*-values	% variation
1. Masai Mara is different	Two groups: (MAR verses all others 11 subpops)	Groups	0.0546	0.235 ± 0.011	5.46	Groups	0.108	0.084 ± 0.009	10.82
		Populations	0.1250	0.001 ± 0.001	7.05	Populations	0.023	0.000 ± 0.000	2.09
		Individuals	0.0745	0.000 ± 0.000	87.49	Individuals	0.130	0.000 ± 0.000	87.08
2. Montane—Lowland structuring	Two groups: (ABE verses all others 11 subpops)	Groups	−0.005	0.523 ± 0.013	-0.53	Groups	−0.02	0.721 ± 0.012	-1.98
		Populations	0.0773	0.000 ± 0.000	8.27	Populations	0.044	0.000 ± 0.000	4.58
		Individuals	0.0823	0.000 ± 0.000	92.26	Individuals	0.026	0.000 ± 0.000	97.40
3. Panmictic subpopulations due to geographic proximity	Three groups: (MAR = Grp 1, NNP/TSA/NGU/CHY = Grp 2, others = Grp3)	Groups	0.0005	0.380 ± 0.0137	0.05	Groups	0.040	0.012 ± 0.003	4.05
		Populations	0.0820	0.000 ± 0.000	8.15	Populations	0.020	0.000 ± 0.000	1.91
		Individuals	0.0816	0.000 ± 0.000	91.80	Individuals	0.060	0.000 ± 0.000	94.04
4. Relictual subpopulations	Four groups: (MAR = Grp1, CHY = grp2, LAK = Grp3, others = Grp 4)	Groups	0.117	0.002 ± 0.0014	11.68	Groups	0.055	0.0557 ± 0.006	5.52
		Populations	0.167	0.002 ± 0.0014	4.97	Populations	0.025	0.000 ± 0.000	2.41
		Individuals	0.056	0.000 ± 0.000	83.34	Individuals	0.080	0.000 ± 0.000	92.07

ϕ_{CT} is fixation index. The % variation is the amount of diversity in the subpopulation associated to the partitioned group

Grp 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, *SD* standard deviation

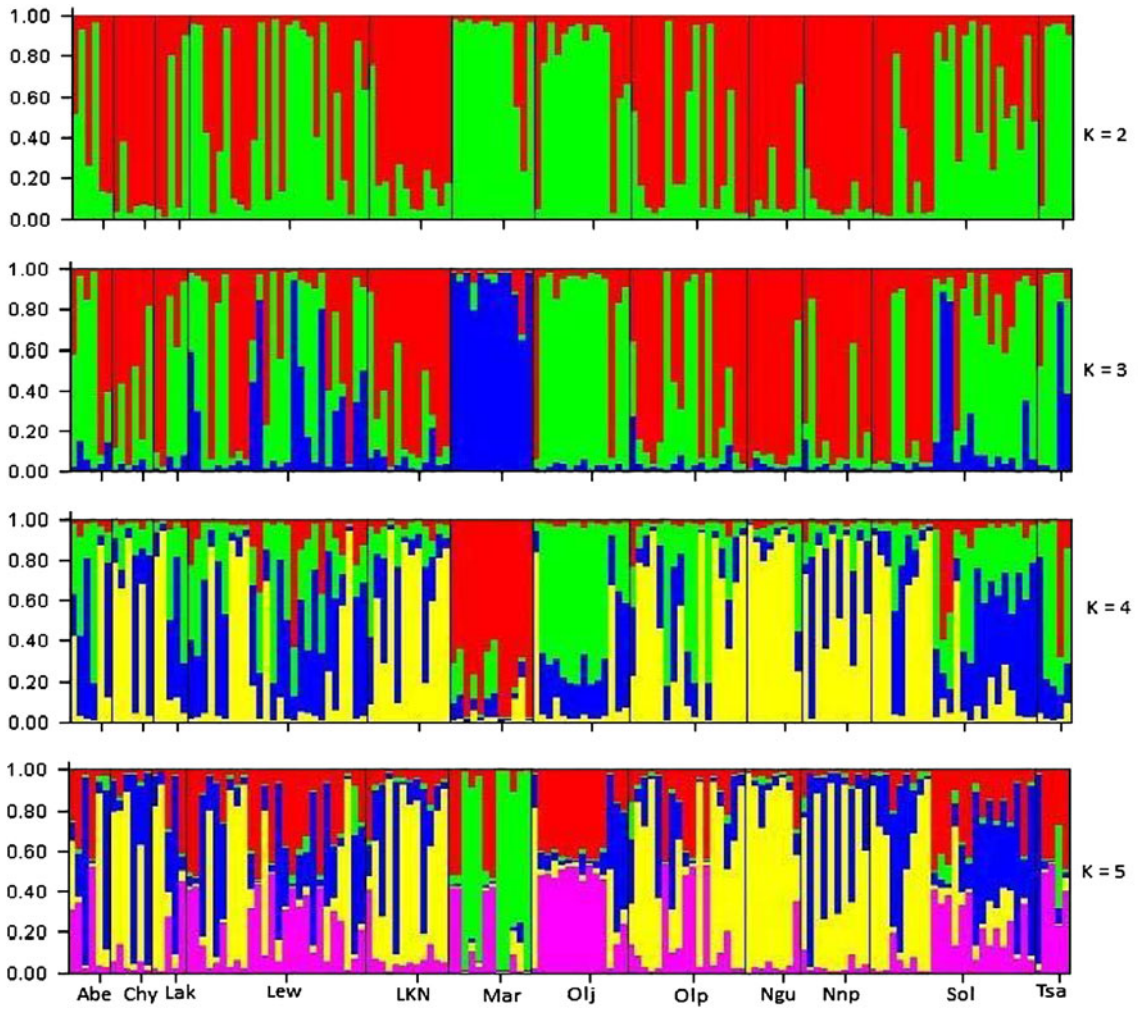
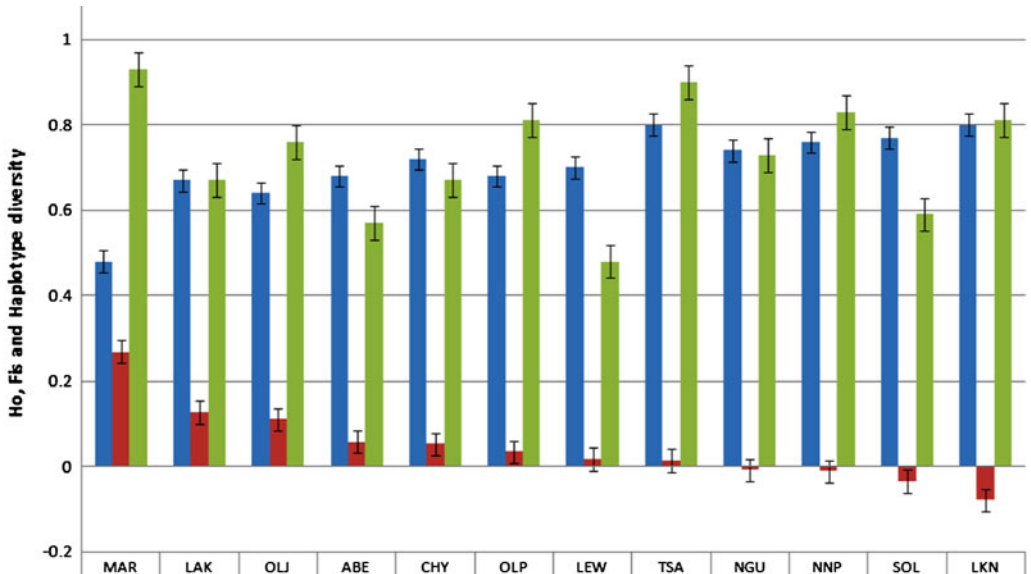


Fig. 3 Clustering results ($K = 2$–5) for all sites, according to STRUCTURE analysis. Sampled sites are separated by dark black vertical line and labeled below the figure. *ABE* Aberdares NP, *CHY* Chyulu NP, *LAK* Laikipia WC, *LEW* Lewa WC, *LKN* Lake Nakuru NP, *MAR* Masai Mara, *NNP* Nairobi NP, *NGU* Ngulia RS, *OLJ* Ol Jogi RH, *OLP* Ol Pajeta RH, *SOL* Solio RH, *TSA* Tsavo East NP subpopulation

$H_O = 0.477$ for *D. b. minor* and $H_O = 0.401$ for *D. b. bicornis*]). However, findings from this study show widely varying levels of genetic diversity and differentiation within and among different subpopulations, which are likely to have a variety of explanations.

The congruence of genetic diversity patterns revealed by mtDNA and microsatellites was not consistent across subpopulations. For example, Masai Mara had the lowest observed heterozygosity ($H_O = 0.48$) and highest haplotype diversity ($h = 0.93$) respectively (Table 3). This may be explained on the basis of population history and differential stochastic effects of genetic drift on a specific marker class (Hoarau et al. 2004). Masai Mara has undergone an extended and severe bottleneck compared to the other subpopulations, and mtDNA, with its smaller effective population size, faster genetic drift and haploid maternal inheritance is expected to reflect population contraction more rapidly than nuclear DNA (Nyakaana et al. 2002; Hoarau et al. 2004). However, genetic drift may not always eliminate rare alleles 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 Tsavo East National Park subpopulation had both high values for haplotype diversity and observed heterozygosity and extremely low F_{IS} values (Table 3). This finding is consistent with the population history of this population in that its original stock of animals was rescued from several parts of Kenya (Table 1) thus resulting in increased admixture. However this result again may illustrate the stochastic nature of the correlation between nDNA and mtDNA in subdivided small populations.

Between the two extremes, other subpopulations such as Nairobi NP, Ngulia RS, Lake Nakuru NP and Solio RH have similar mitochondrial and nuclear diversity and inbreeding coefficients (Table 3). All these subpopulations



	MAR	LAK	OLJ	ABE	CHY	OLP	LEW	TSA	NGU	NNP	SOL	LKN
Heterozygosity (H0)	0.48	0.67	0.64	0.68	0.72	0.68	0.7	0.8	0.74	0.76	0.77	0.8
Inbreeding (FIS)	0.269	0.127	0.111	0.058	0.053	0.035	0.017	0.014	-0.008	-0.011	-0.035	-0.079
Haplotype diveristy (h)	0.93	0.67	0.76	0.57	0.67	0.81	0.48	0.9	0.73	0.83	0.59	0.81

Fig. 4 Nuclear and Mitochondrial DNA genetic diversity in 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

are similar in a number of interesting ways; for instance all the subpopulations are at HWE and have experienced substantial gene flow through translocations, since they represent breeding nuclei for the rhinoceros population recovery strategy in Kenya (Okita-Ouma 2004) and have been receiving individuals from different locations, breeding from them and releasing them into other areas, thus functioning as sources of genetically admixed individuals. We hypothesise that gene flow into these populations has already contributed towards the homogenisation of their mtDNA and nuclear allele frequencies (Table 3).

Population structure

Masai Mara was the only subpopulation with pairwise F_{ST} values statistically distinct from all other subpopulations and possessed the largest pairwise Nei's distance values (Table 4), although the AMOVA analysis was inconclusive (Table 5). This result may be explained by several factors including genetic drift, as illustrated by a history of demographic isolation from all other subpopulations (Table 1). Additionally, it is possible that not all black rhinoceros in the Masai Mara are *D. b. michaeli*; since the Masai Mara is unfenced and has a potential for genetic interaction with other black rhinoceros in the larger Mara–Serengeti ecosystem and it is possible that some of the animals may be *D. b. minor* since Tanzania is the northern range for this subspecies (IRF—Internatinal Rhino Foundation 2008).

AMOVA results on population genetic structuring generated by the two markers are congruent in the sense that they are inconclusive on the hypothesis that the Masai Mara subpopulation is genetically distinct from other subpopulations, and do not support the existence of a montane–lowland genetic discontinuity. The latter, habitat-based division, currently used as a management tool by KWS, also lacks a historical basis, since there is evidence of previous translocations in and out of this subpopulation to other areas in Kenya prior to current management policy (Table 1).

The same analysis did, however, capture different levels of genetic diversity in the third and fourth evolutionary hypothesis of Kenya's black rhinoceros. While the mtDNA marker did not support the hypothesis of panmictic subpopulation due to geographical proximity, the nDNA marker statistically supported this hypothesis (Table 5), even though, as is often the case for microsatellite data, the large majority of the variance is found within populations. The significance of the among population variance component may be due to the fact that microsatellite markers are known to be more sensitive indicators of local genetic structure than mitochondrial DNA (e.g. Sunnucks 2000). These findings may suggest that local translocations have obscured the genetic signature of demographic differentiation that might

otherwise have been more obvious, or that natural gene flow is ongoing. In contrast, mtDNA results supported the hypothesis of distinct relictual subpopulations (Masai Mara, Chyulu NP and Laikipia WC, that remained genetically intact until 2005) while nDNA markers did not ($P = 0.056$). Weaker nuclear structure has also been observed in African elephants (Okello et al. 2008) and has been attributed to the difference in effective size for nuclear and mitochondrial DNA (Birky et al. 1989; Avise 1994; Hoarau et al. 2004; Roca et al. 2007). This observation suggests that mtDNA may be detecting a pre-management genetic signal and that the relictual subpopulations were historically isolated from each other. This finding is supported by the known population history of these subpopulations since the Masai Mara and Chyulu subpopulations have no recorded history of immigration (Table 1) and hence gene flow between these subpopulations is likely to be historically very low. The relative merits of these hypotheses must be placed in the context, however, of the fact that between 83 and 92% (mtDNA) and 87 and 97% (microsatellite DNA) of the variance is partitioned within populations and thus variation among individuals must be considered the most biologically significant variance component within this dataset.

Findings from the Bayesian clustering analysis suggest low genetic structuring in the metapopulation with three evident subpopulations: Masai Mara, Ngulia and the remainder (Fig. 3, K = 3). While it is known that Masai Mara has no historical record of immigrations (Table 1), Ngulia has experienced some level of genetic management and hence this result is more difficult to explain. One possible explanation is that some unusual demographic process has occurred in this population compared to the others, which has altered allele frequencies sufficiently to be detected using STRUCTURE (Evanno et al. 2005). For example, it is possible that translocated individuals have either not bred or, conversely, have been extremely successful. Both explanations might result, through drift, in a signature being created that is detected by the algorithm. This requires further study.

Conclusions, conservation and management implications

It is evident that despite the recent bottleneck, Kenya's black rhinoceros is not as depauperate in genetic diversity as might have been expected from experience with other animals that have undergone a drastic demographic decline. Kenya's black rhinoceros population has high genetic diversity (Table 3) compared to other black rhinoceros population in Africa as reported by Harley et al. (2005) and Scott (2008). Translocations have maintained gene flow that has helped to reduce the effects of genetic drift in most of the subpopulations thus enabling them retain genetic diversity, although Masai Mara has experienced higher levels of genetic drift and inbreeding because it has been isolated for long from the other subpopulations and has undergone the most severe bottleneck. Despite this, the subpopulation does not represent an evolutionary significant unit (ESU) as classified by Moritz (1994), although its allele frequencies are the most distinct and thus it could potentially be classified as a separate management unit (MU).

The montane–lowland management hypothesis is not genetically supported, and the demographic history of the Aberdares subpopulation suggests substantial genetic mixing prior to the recent management policy (Table 1). Therefore it is unsurprising that these populations are undifferentiated and we conclude that the current management plan to keep these populations separate is unnecessary and recommend that individuals can be moved, if deemed demographically appropriate, between lowland and montane habitats.

We suggest that to maintain the present genetic diversity in the metapopulation, future translocations should consider the patterns and profile of genetic differentiation among the donor subpopulations to maximize the genetic impact of translocations in the recipient populations, with the aim to maintain heterozygosity at as high a level as possible.

The findings from this study reveal that the highest component of genetic diversity in Kenyan black rhinos is still partitioned among individuals (Table 5), implying that individuals in the total population maintain the genetic diversity. Therefore, to preserve genetic variability in the various subpopulations it is important to conserve as many individuals as possible and in the event of translocation; evaluate the genetic profile of the individual(s) identified, with respect to the recipient subpopulation. If this proves to be too expensive an approach, a cheaper alternative is to consider pairwise genetic differentiation among subpopulations (Table 4) and to incorporate the results of the Bayesian clustering to identify which populations are genetically differentiated and allow this to guide the most useful translocations.

Although Masai Mara is genetically the most distinct of all subpopulations; it remains genetically the least diverse and although the fitness-related effects of drift or inbreeding are undocumented in this population, additional genetic material could soon be required to address the low diversity in the subpopulation. The continued demographic isolation of the Masai Mara subpopulation (if treated as a separate management unit) could result in further losses of genetic variation which could affect its long term viability (Frankham et al. 2002). Similarly, the montane–lowland separate management practice by KWS may negatively

affect the genetically isolated Aberdares NP subpopulation and we recommend this be revised. In other cases, the cumulative effects of recent and ongoing translocations render population-specific recommendations superfluous, and we would recommend periodic translocations to minimize reductions in male effective population size and maximize the maintenance of genetic variation within these sanctuaries.

The survival of threatened species relies on a clear understanding of the factors causing the extinction threat and adaptive management interventions that can reduce or eliminate that threat. Although judicious genetic management of the remaining Kenyan black rhinoceros population is of great importance, it is important to point out that the main threats to the survival of black rhinoceros in Kenya and elsewhere are currently a resurgence in poaching and loss of habitat (Moehlman et al. 1996; Dean and Foose 2006; IUCN 2008). Both threats are directly or indirectly linked to increases in human populations (Laroche and Durand 2004; Bergl et al. 2008) in Africa and elsewhere and must be a primary focus of conservation activities for the foreseeable future.

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