Genetic Structure of Hartebeest Populations Straddling a Transition Zone between Morphotypes

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ABSTRACT

Variation in mitochondrial and microsatellite DNA was used to define the genetic structure of hartebeest populations straddling a transition zone between two morphotypes (*Alcelaphus buselaphus cokei* and *A. b. lelwel*) in Kenya. Previous analyses of mtDNA from hartebeest populations across Africa supported the refugium hypothesis; that present day hartebeest morphotypes diverged in allopatry, due to climatic changes. In this analysis of morphologically distinct populations in close geographical proximity, majority of genetic variation was found within populations, with relatively little distinction, and varying levels of subdivision, among populations. Patterns of shared alleles, and reduced tendency for mtDNA haplotypes to cluster phylogenetically according to morphotype, depicted gene flow between populations. There was sharp disparity between apparently seamless genetic transition between populations and marked disjunction in gross morphology over short distances (<100km). Hartebeest in the transitional zone between *A. b. lelwel* and *A. b. cokei*, including populations in Ruma, Meru National Parks and Laikipia, are the only remaining examples, each genetically and morphologically different from the other, of what appears to be resumed contact between two lineages that diverged in allopatry. Our results underscore the importance of using genetic and morphological information to explicitly define evolutionary processes as targets for conservation in Africa.

KEY WORDS: African bovids, hartebeest, Kenya, conservation genetics, mtDNA, microsatellite, morphology

INTRODUCTION

Conservation in Africa has necessarily emphasized proximate threats to the persistence of large mammals, such as habitat loss and illegal hunting. Community-based conservation is increasingly promoted to provide essential space for wide-ranging wildlife species in human-occupied areas that cannot be formally protected as national parks or reserves. The importance of 'ultimate' threats that are fundamentally biological, such as genetic integrity in small populations, have been stressed in the literature (e.g. Greig 1979; Amos and Balmford, 2001), but under-attended *in situ*. This is partly because ultimate threats are too often assumed to

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become relevant only after proximate threats have been overcome, and partly from lack of funds or capacity. The ultimate threats merit more attention because the survival of large mammals in relatively small and decreasingly natural areas that have become 'islands in a sea of humanity' will entail more than maintaining species lists, or patterns of relative abundance. To the extent possible, fragmented landscapes must be actively managed as functionally intact ecological communities, aiming to perpetuate natural processes that have been operating over vast ecological space and long evolutionary time (Young and Clarke, 2000). These include relatively complex processes, such as gene flow and natural selection that often do not translate in simple ways into appropriate conservation action. This study was intended to advance the application of these principles to the conservation of hartebeest (*Alcelaphus bubelaphus*) in Kenya.

Modern hartebeest lineages appeared at least 740,000 years ago (Vrba, 1995a), and diverged during bouts of radiation that coincided with rapid climate change (Arctander *et al.*, 1999; Flagstad *et al.*, 2001; Pitra *et al.*, 1998; Matthee and Robinson 1999). Seven extant 'subspecies', described according to variation in horn shape and size, coat colour and body size (Sidney, 1965), are considered either as variants within a single species (*Alcelaphus buselaphus*; Kingdon, 1982) or as two species, comprising a southern lineage (*A. lichtensteini,* including *lichtensteini* and *caama*; Cillie, 1987) , and a northern lineage (*A. buselaphus,* including *major*, *lelwel*, *tora*, *swaynei* and *cokei*) where several of the subspecies appears to be of relatively recent origin (< 200,000 year bp; Flagstad *et al.*, 2001). Phylogenetic analysis of mitochondrial DNA sequences supported the latter classification (Arctander *et al.*, 1999, Flagstad *et al.*, 2001) in that the northern and southern clades are reciprocally monophyletic. Within each of these clades, lineages tend to cluster according to morphotype, but not exclusively, such that groups defined by morphotypes are polyphyletic.

Hartebeest were among the most widely distributed antelopes in Africa, but have declined rapidly in number and distribution, and seem especially vulnerable to local extinction. For example, *A. b. cokei* became locally extinct in Kenya's Amboseli National Park and Mwea National Reserve within the last decade. Two subspecies with severely restricted ranges in northern east Africa are critically endangered (*A. b. tora* and *A. b. swaynei*). A third, *A. b. lelwel*, is declining across range states that include Central African Republic, southern Sudan, northern D.R. Congo, northern Uganda, and central Kenya. All but the latter have severely limited conservation means or potential.

Central Kenya marks the transition between *A. b. lelwel*, at the south-eastern extremity of its range, and *A. b. cokei,* which is patchily distributed across southern Kenya and adjoining regions of northern Tanzania (Fig. 1). Hartebeest were once abundant across this zone but have been extirpated by settlement over large areas. Hartebeest with *A. b. lelwel* morphology (Fig. 2) remain at only two locations in Kenya. Ruma National Park near Lake Victoria holds a declining population of a few hundred. Ruma NP is small (140 km^2) and surrounded by such dense humanity, and may not endure as a protected area. The other population is in Laikipia District, north-west of Mount Kenya, numbering less than 1000. While nowhere formally protected, many of the properties that comprise the hartebeest range in Laikipia are currently well-protected in the functional sense.

Resolving the systematics of the Laikipia hartebeest is important because they warrant exceptional conservation status, for two reasons. First, as a 'flagship' species for a spectacular and biologically diverse area that hosts more wildlife than all of Kenya's protected areas except the Masai Mara Reserve, but lacks formal protection status. Second, given the precarious status of *A. b. lelwel* elsewhere, the Laikipia population has potential to be one of the most secure populations of this morphotype remaining in Africa. The Meru NP population has dwindled to less than 30 and may be supplemented by translocation in the near future. Genetic data that might assist in the decision to supplement this population, and select the appropriate source population, were lacking.

To address these systematic and conservation genetic issues, we used mitochondrial DNA sequences and a suite of nuclear (microsatellite) markers to describe the structure of hartebeest populations straddling the transition zone between *A. b. lelwel* and *A. b. cokei* in Kenya, against the wider context of population genetic variation in hartebeest across eastern Africa.

MATERIALS AND METHODS

Sample collection:

Samples were collected as skin using the biopsy dart method (Karesh *et al.*, 1989), as fragments cut from salted skins, or as fresh dung. DNA was extracted from skin using the standard proteinase-K-phenol-

chloroform method (Sambrook *et al.,* 1989), or from dung using the *Dynabeads DNA Direct Kit* (Dynal AS, Oslo; Flagstad *et al.,* 1999). One pellet from each individual was placed in a plastic bag, 400 µL phosphate-buffered saline (PBS, pH 7.4) added, and the surface of the pellet gently massaged to wash off epithelial cells. The supernatant was transferred to a 1.5 ml eppendorf tube and extracted using the magnetic beads procedure described by Rudi *et al.* (1997). DNA extracts were eluted in 40 µL of Tris-EDTA buffer (TE) for 5 minutes at 65°C and diluted 10 times in distilled water prior to amplification.

Building on the results of Arctander *et al.* (1999) and Flagstad *et al.* (2001) who analysed D-loop sequences from 134 individuals representing 7 locations in Africa, we added 75 homologous hartebeest mitochondrial DNA sequences from 4 new locations in Kenya (Laikipia, Ruma NP, Naivasha, and Meru NP; the latter were collected too late to feature in the mitochondrial analysis) and 2 new locations in Tanzania (Mara-Serengeti NP and Ngorongoro Crater; Figure 1, Table 1).

Fig. 1. Distribution of hartebeest in Africa (after East 1998), with different symbols denoting morphotypes. Symbols are filled grey if mtDNA sequences from that location were used in this analysis, and black where populations were added by this study (see legend below).
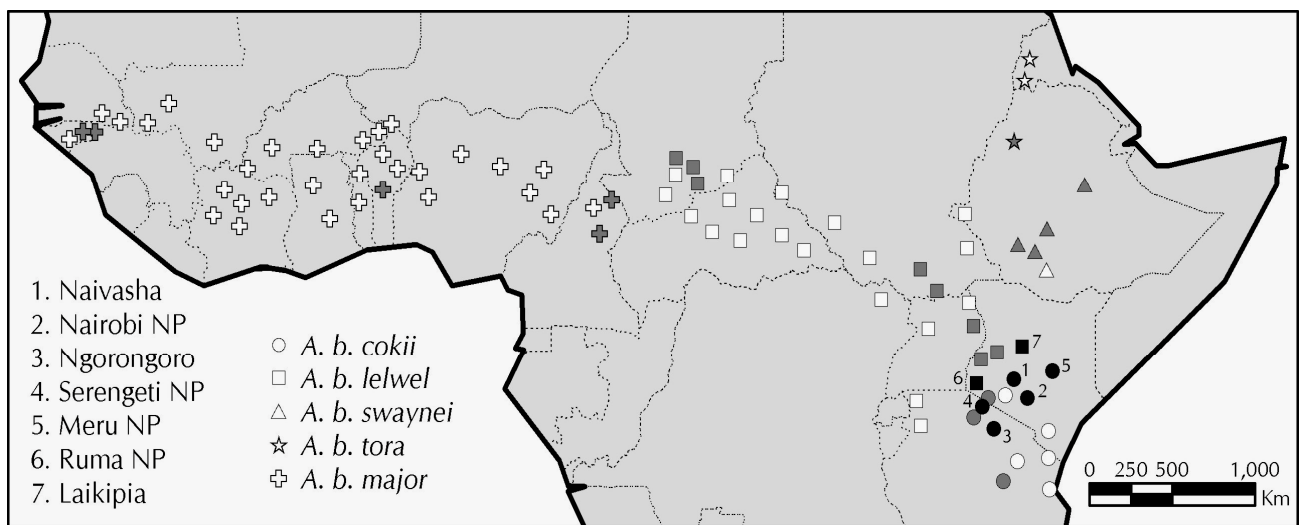
Table 1. Summary of provenance and number of samples used in this analysis. Numbers in bold denote new samples added by this study to samples from which results have been published previously (Arctander *et al.,* 1999; Flagstad *et al.,* 2001).

Morphotype	Country	Locality	Sample Type	DNA Analysis	
				mtDNA	**microsatellite**
cokei	Kenya	Naivasha	Skin	16	14
cokei	Kenya	Nairobi NP	Skin	6+8	11
cokei	Tanzania	Ngorongoro Crater	Skin	11	13
cokei	Tanzania	Mara-Serengeti NP	Skin	15+14	17
cokei	Kenya	Meru NP	Skin	-	5
lelwel	Kenya	Ruma NP	Skin / Dung	2 / 6	2 / 6
lelwel	Kenya	Laikipia	Skin / Dung	6 / 12	4 / 12
lelwel	Uganda + Congo+ Sudan			11	
lelwel	Chad			5	
swaynei	Ethiopia + Somalia			49	
tora				5	
major				11	
buselaphus				1	
lichtensteinii	Tanzania	Outgroup		2	
caama	South Africa	Outgroup		1	
		TOTAL		181	84

mtDNA PCR amplification and sequencing:

A 475 bp region of the mitochondrial D-loop adjacent to the tRNA[pro] gene was targeted for PCR and sequencing (Hultmann *et al.,* 1989) using the primers applied by Arctander *et al.,* 1999 and Flagstad *et al.,* 2001. The primers employed were 5'-AATAGCCCCACTATCAGCACCC-3' (L15394; specific to hartebeest) paired with 5'-TATGGCCCTGAAGTAAGAACCAG-3' (H15947; "mammalian" primer targeting CSB-D; Southern *et al.,* 1988). Primers are light- and heavy-strands corresponding to the 5' primer end in *Homo sapiens* mitochondrial genome as given in data base entry HSMITG, accession number X93334.

DNA samples were extracted and amplified at Mpala Research Centre in a 50 µl reaction volume containing ~10 ng of target DNA, 0.5 µl (10 µM) of each primer, 200 µmol dNTPs, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3) and 1 U of Taq polymerase. A Hybaid OMN-E thermalcycler was used with 35 cycles, consisting of a 30-second denaturation at 94°C, a 30-second annealing at 60°C and a 45-second extension phase at 72°C. A 4-minute pre-denaturing step at 94°C and a 7-minute post-cycle extension temperature at 72°C were employed.

Two different amplification approaches were applied for the dung extracts. The first approach was identical to the amplification of skin tissue extracts except for the use of 45 cycles instead of 35. A second approach was employed when PCR products were insufficient for sequencing. Thirty cycles of amplification under the same conditions as for the skin tissue extracts were followed by a second step with 5 µl of the amplified product subjected to 40 cycles. The second step was run with the same temperature profiles as above. The PCR conditions, however, were changed to 50 mM KCl, 10 mM Tris-HCl, 2.85 mM $MgCl_2$, 12.5 pmol of each primer, 400 µM of each nucleotide, and 1 U of Taq polymerase. All PCR products were run on a 1% agarose gel and viewed using ethidium bromide staining and ultraviolet transillumination.

Enzymatic clean-up of PCR Products:

PCR products were prepared for sequencing by removing excess primers and dNTPs with Exonuclease I (*Exo I*) and shrimp alkaline phosphatase (SAP; Amersham Pharmacia Biotech, Greece). Five µl of the enzyme mix (SAP 0.74 µl, Exo I 0.38 µl and dH_2O, 3.88 µl) were added to 20 µl of PCR product, spun down and incubated through successive steps of 37°C for 30 minutes and 80°C for 15 minutes, for digestion of excess primers and dNTPs, and to permanently inactivate enzymes, respectively. Enzyme-purified PCR products were sequenced using the BigDye Terminator system (ABI 3700, Applied Biosystems; Sanger, 1977).

Microsatellite DNA

Samples used for microsatellite DNA analysis:

A total of 84 samples obtained from 5 localities in Kenya (Ruma NP, Laikipia, Nairobi NP, Naivasha and Meru NP) and 2 localities in Tanzania (Ngorongoro and Mara-Serengeti NP) were used in this analysis (Table 1, Figure 1).

PCR amplification of microsatellite loci:

A total of 7 pairs of fluorescent-labeled primers were used to genotype hartebeest DNA samples on an ABI automated sequencer (Table 2; sequences obtained from O. Flagstad). A Perkin Elmer 9700 thermalcycler was used with a 10 µl reaction containing 1 µl of template DNA, 50 mM KCl, 10 mM Tris-HCl, 1.35 mM of $MgCl_2$, 200 µM of each nucleotide, 5 picomoles of each primer and 0.5 U of Taq polymerase. Hot start was initiated through a 5 minute denaturing step at 94°C. This was followed by 35 cycles of 95°C for 30 seconds, annealing temperature ranging from 50-57°C for 30 seconds and elongation at 72°C for 45 seconds (Flagstad *et al.,* 1999). The cycles were followed by a final elongation temperature of 72°C for 10 minutes.

For electrophoresis, the PCR products from each DNA were pooled and run together with an internal size standard (TAMRA 350, Applied Biosystems). The software GENESCAN and GENOTYPER (Applied Biosystems) were used to automate measurement of allele length and to quantify peak heights in samples containing multiple alleles per locus. Data from samples that amplified poorly for each particular locus were discarded.

Table 2.   Microsatellite primer sequences and sources.

Locus	Annealing Temp. (C)	Allele size range (bp)	F-Forward primer R-Reverse primer	Reference
ETH225	52	136-138	F-GATCACCTTGCCACTATTTCCT	**Barendse *et al.*, 1994**
			R-ACATGACAGCCAGCTGCTACT	
FCB48	57	146-164	F-GACTCTAGAGGATCGCAAAGAACCAG	**Buchanan *et al.*, 1994**
			R-GAGTTAGTACAAGGATGACAAGAGGCAC	
FCB304	57	129-161	F-CCCTAGGAGCTTTCAATAAAGAATCGG	**Buchanan & Crawford 1993**
			R-CGCTGCTGTCAACTGGGTCAGGG	
MAF209	57	121-145	F-TCATGCACTTAAGTATGTAGGATGCTG	**Buchanan & Crawford 1992**
			R-GATCACAAAAAGTTGGATACAACCGTGG	
MCM38	50	128-150	F-TGGTGAATGGTGCTCTTCATACCAG	**Hulme *et al.*, 1994**
			R-CAGCCAGCAGCCTCTAAAGGAC	
MCM58	52	164-178	F-CTGGGTCTGTATAAGCACGTCTCC	**Hulme *et al.*, 1994**
			R-CAGAACAATAAACGCTAAACCAGAGC	
SMHCC	57	177-213	F-ATCTGGTGGGCTACAGTCCATG	**Groth & Wetherall 1994**
			R-GCAATGCTTTCTAAATTCTGAGGAA	

Data Analysis - Mitochondrial DNA

Phylogeny reconstruction and choice of DNA substitution model:

Since previous studies (Arctander *et al.* 1999, Flagstad *et al.,* 2001) showed the 'southern' hartebeest (*lichtensteinii* and *caama*) form a monophyletic clade, one *caama* sequence and two *lichtensteini* sequences were used as outgroups in this phylogenetic analysis. Sequencher 3.1 (Gene Codes Corporation, Ann Arbor MI) was used to examine sequence quality, and the nucleotide sequences were compared to registered sequences in GenBank and confirmed as a fragment of the hartebeest mitochondrial DNA. Mitochondrial DNA D-loop sequences were aligned using CLUSTAL X (Thomson *et al.,* 1997) and Se-Al (Rambaut, 2002) excluding 11 base pairs of uncertain alignment. Final analyses were based on a 489 base pair alignment of mitochondrial D-loop.

To select a model of DNA substitution that best fits the data, a maximum likelihood ratio test implemented in the program Modeltest ver 3.06 (Posada & Crandall, 1998) was used. The model selected by hierarchical likelihood ratio tests (K81 uf+I+G) was implemented in PAUP*4.0b10 using the Modeltest generated likelihood settings: base frequencies, A = 0.3517, C = 0.2540, G = 0.1262, T = 0.2681; number of substitution types = 6; substitution rate matrix = (1.00 67.17 3.40 67.17); shape parameter of the gamma distribution = 0.41; and assumed proportion of the invariable sites = 0.40. These settings were used for Neighbor Joining (NJ) and for Maximum Likelihood (ML) analyses. For Maximum Likelihood analysis, a starting tree obtained by random addition underwent TBR branch swapping. Bootstrap resampling support was based on 100 (ML), 500 (MP) or 1, 000 (NJ) replicates, with TBR (MP) or NNI (ML) branch swapping of starting trees obtained by NJ (for ML bootstrap) or simple stepwise addition.

Population genetic structure was analysed using ARLEQUIN software version 2.001 (Schneider *et al.,* 2000), with Tamura Nei distances (Gamma correction, A = 0.01; allowing for unequal mutation rates among sites and considering the degree of divergence between different haplotypes), and 0.05 as the allowed level of missing data. Analysis of Molecular Variance (AMOVA; Excoffier *et al.,* 1992) was used to examine the extent of differentiation among 'subspecies' and sampling locations. The significance of Φ-statistics was tested using 1,000 replications. The exact test of population differentiation used 100,000 steps in Markov chain and 3,000 dememorization steps. Haplotype diversity within populations (H) was estimated using the equation of Nei and Tajima (1981). Nucleotide diversity (π, the average number of differences between two DNA sequences at each nucleotide site) was estimated according to Nei & Li (1979).

Mismatch frequency distributions were used to draw inferences about historical demography of hartebeest populations. Mismatch distribution used 100 bootstrap replicates, while neutrality tests used 16,000 simulated samples. Departures between observed and expected distributions under the expansion hypothesis (Rogers and Harpending, 1992) were tested using the chi-square test of goodness of fit in the program ARLEQUIN.

Microsatellite DNA analysis

Genetic variation:

Genetic variability in hartebeest from 8 sampling locations was determined by examining the mean number of alleles per locus, allele frequencies at each locus, observed heterozygosity (*Ho*), and Nei's unbiased expected heterozygosity (H_E; Nei 1987, Minch *et al.,* 1996).

Heterozygosity:

An unbiased estimate of gene diversity or expected heterozygosity (H_E) was derived for each locus per population combination as previously described (Nei, 1987).

Hardy-Weinberg Equilibrium (HWE):

The test for deviations from HWE was performed as previously described (Raymond and Rousset, 1995). For all comparisons in this analyses, the Markov chain was set to 100 batches, 1,000 iterations and 1,000 dememorizations.
Genotypic linkage disequilibrium was also examined for all pairwise combinations of loci in each population, as previously described (Raymond and Rousset, 1995). The null hypothesis (H_o) tested was: genotypes at one locus are independent from genotypes at a second locus. The algorithm used is based on analysis of contingency tables, and each contingency table is analyzed using the Markov chain method in a similar manner to the test for HWE expectations described above (Raymond and Rousset, 1995).

Population tree:

Genetic similarity (*p*) was estimated using the proportion of shared alleles, which has been shown to be appropriate for closely related populations (Bowock *et al.,* 1994). The distance measure *1-p* was used to reconstruct a population tree (Felsenstein, 1993). Confidence estimates for the UPGMA tree topologies were based on 1,000 bootstraps.

Structure:

In order to identify subgroups that have distinctive allele frequencies a model-based clustering algorithm was applied as previously described in *Structure* analysis (Pritchard *et al.,* 2000). *Structure* places individuals into K clusters, where K is chosen in advance but can be varied across independent runs of the algorithm. *Structure* was run for hartebeest for which all 7 microsatellites amplified successfully, using values of K between 2 and 5, without using prior population information, and the run was performed using two million steps following a burning of 200,000. For each value of K, four parameter combinations were used (admixture and correlated; no admixture and correlated; admixture and not correlated; no admixture and not correlated), with 3 structure runs for each combination of K and parameters.

Genetix:

Genetic differentiation was studied by performing correspondence analysis on the matrix of microsatellites for samples amplifying at least 6 of 7 loci using *Genetix* 4.05 software as described previously (Belkhir *et al.,* 2002; Bierne *et al.,* 2003) and with populations partitioned into locales and subspecies.

Fig. 2. Frontal and lateral views of male horn morphology in Nairobi NP (A; *A. b. cokei*), Meru NP (B; resembles *A. b. cokei*), Solio Ranch, Laikipia District (C; greater resemblance to *A. b. lelwel*), and Ruma NP (D; resembles *A. b. lelwel*). (Photos by N. Georgiadis.)
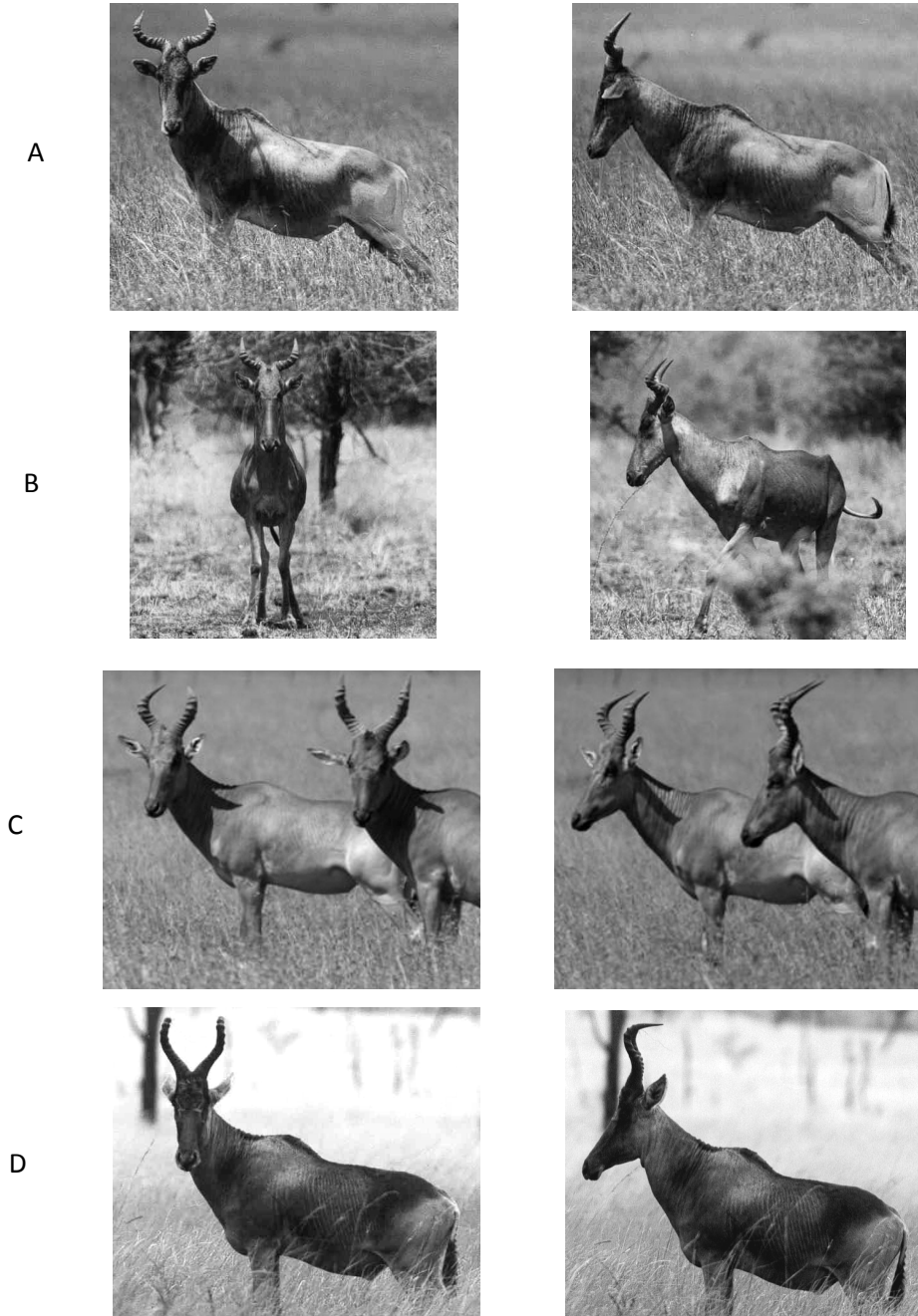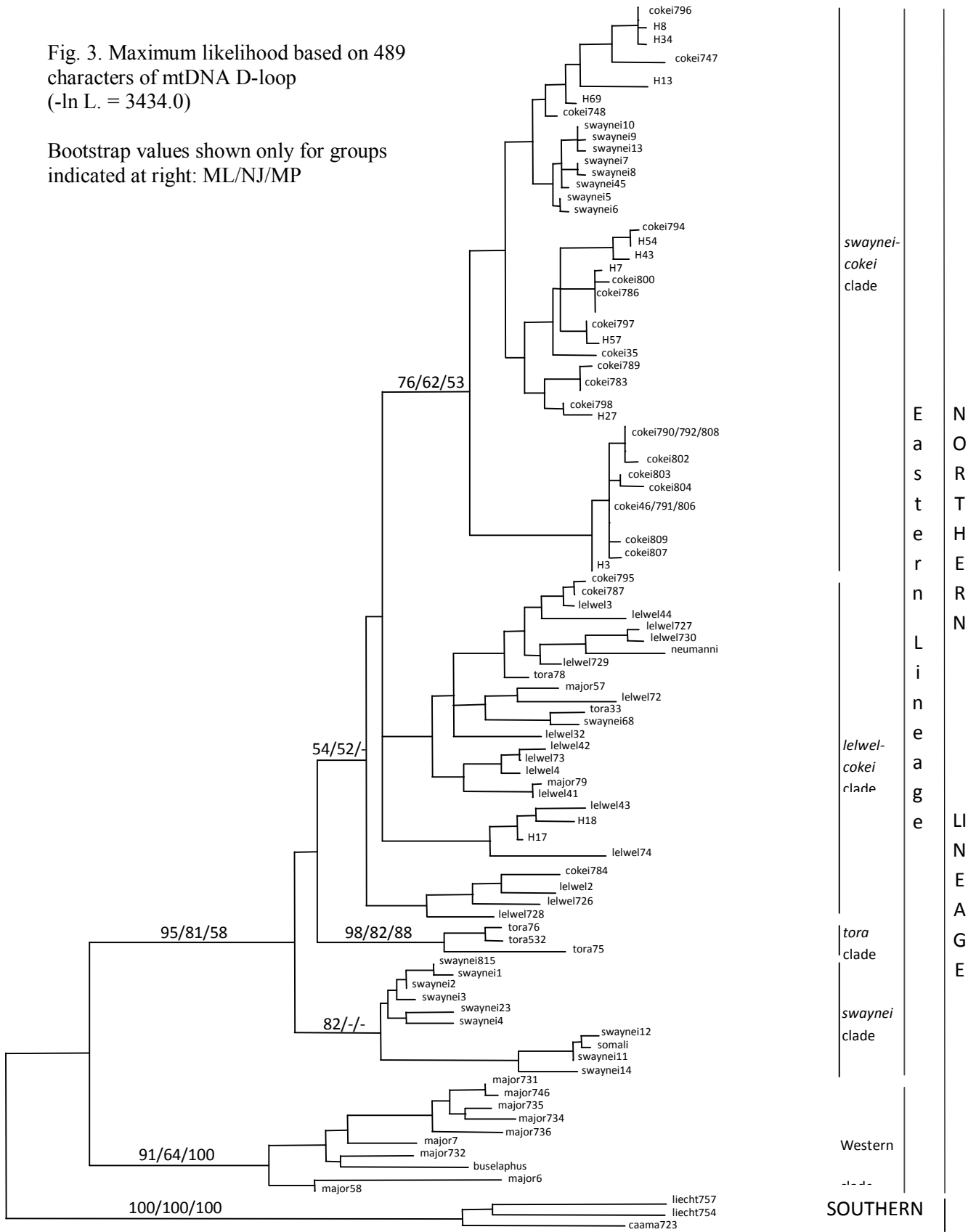
Fig. 3. Maximum likelihood based on 489 characters of mtDNA D-loop (-ln L. = 3434.0)

Bootstrap values shown only for groups indicated at right: ML/NJ/MP

0.01 substitutions/site

Fig. 4. Plots of genetic distance versus geographical distance (km) for pairs of populations based on mtDNA data (A, using standard genetic distance of Nei, 1987) and microsatellite DNA data (B, using *1-b*; Bowock et al. 1994). Symbols distinguish pairs in which both populations had *cokei* morphology (triangles), both populations had *lelwel* morphology (circles), and one population had *cokei* morphology, the other *lelwel* (squares). Values for all remaining pairs are symbolized by crosses. Filled symbols in A distinguish pairs involving the Ruma NP population. Filled symbols in B distinguish pairs involving the Meru NP population.
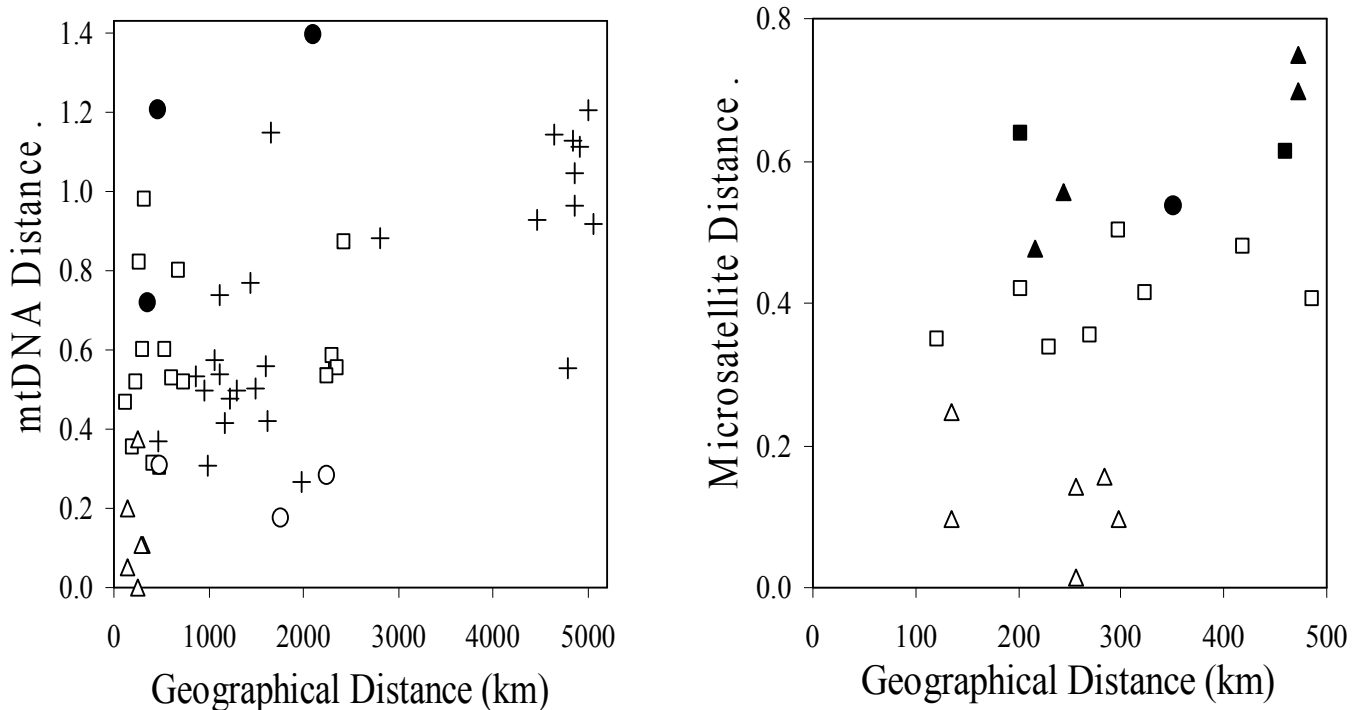
RESULTS

Mitochondrial DNA

Phylogenetic relationship:

Phylogenetic analysis was based on a 489 bp alignment of 89 unique mitochondrial D-loop sequences, combining 12 unique sequences added from this study in Kenya and Tanzania with 77 previously published sequences (Arctander *et al.,* 1999; Flagstad *et al.,* 2001). All available sequences were included in the phylogenetic analysis to enhance accuracy (Pollock *et al.,* 2002).

All new sequences generated in this study clustered within the 'Eastern Lineage' of Flagstad *et al.* (2001), and bootstrap support was enhanced (Fig. 3). All haplotypes from individuals with *A. b. cokei* morphology clustered within the '*swaynei-cokei* clade', with one exception. This was cokei784, which fell within the '*lelwel* group' (Flagstad *et al.,* 2001) of the '*lelwel-cokei* clade' (Figure 3), and was shown by this study to be distributed widely across the *A. b. cokei* range, but was not found in populations having *lelwel* morphology.

Two closely related haplotypes with high frequencies in Laikipia (H17 and H18) clustered with a haplotype from the Uganda/Kenya border (lelwel43) to form a clade distinctive from both the '*lelwel* group' (Flagstad *et al.,* 2001) and the '*swaynei-cokei* clade' (Figure 3). The remaining haplotypes from Laikipia fell within the '*swaynei-cokei* clade', often on the same branch as haplotypes from populations having *cokei* morphology. The sole haplotype from Ruma NP (H13), another population with *lelwel* morphology, also fell within the '*swaynei-cokei* clade', forming a relatively long branch that lacked representation in any other population. These results confirm and compound the mitochondrial polyphyly of hartebeest subspecies comprising the 'Eastern Lineage'.

Genetic variation:

A slightly different set of sequences was used in the population genetic than the phylogenetic analysis because several published hartebeest sequences were of uncertain provenance, and several others differed by indels that were not used as characters in the phylogenetic analysis. Among 75 samples added by this study from 6 locations, a total of 23 unique haplotypes was found, 16 not encountered in previous studies. Seven of the 16 new haplotypes were found only in one population (Laikipia), and the remaining new haplotypes did not feature in more than 2 populations. Added to those previously published, the total was 82 haplotypes among 178 samples from 11 locations (Table 3).

The observed transition/transversion ratio was 8.00, and among-site variation was moderate, with α = 0.01. Compared to results from previous studies (Wilhelmus *et al.,* 1999; Flagstad *et al.,* 2001; Arctander *et al.,* 1999; Flagstad *et al.,* 2000), moderate to high genetic, haplotype, and nucleotide diversity was recorded in all populations except Ruma NP (Table 4).

Population Genetic Subdivision:

A hierarchical analysis of molecular variance (AMOVA) revealed significant Φ statistics, with the highest proportion of genetic variation observed within populations (50.55%; d.f. = 166; Φ_{ST} = 0.497). A substantial proportion of the variance was distributed among morphotypes (36.76%; d.f. = 5; Φ_{CT} = 0.366), leaving the lowest proportion distributed among populations within morphotypes (12.70%; d.f. = 6; Φ_{SC} = 0.207; *P < 0.001* in all cases).

Estimates of genetic subdivision (F_{st}) revealed significant structure between all but 4 population pairs, including some with the same morphotype (Table 5). The only exceptions were between Mara-Serengeti NP and two other populations with *A. b. cokei* morphology, Ngorongoro and Naivasha. The Uganda and Chad populations (both have *A. b. lelwel* morphotype) were also insignificantly subdivided, as were the *A. b. tora* and Chad populations. This was the only instance of lack of subdivision between morphotypes, probably due to low samples sizes.

Geographical proximity was weakly related to mtDNA distance between populations. To detect possible isolation-by-distance, the genetic distance between each pair of populations was plotted against the geographic distance separating them (Fig. 4). Geographical proximity was only weakly related to mtDNA distance between populations. A positive but scattered trend was observed overall that rose steeply and then leveled off at geographic distances greater than about 500 km. In general, genetic distances between populations having similar morphology (*cokei-cokei* and *lelwel-lelwel* pairs) were low, relative to genetic distances between populations of different morphology. This was true irrespective of geographic distance, suggesting genetic disjunction between *lelwel* and *cokei* morphotypes. However, genetic distances between Ruma NP and other *lelwel* populations were among the highest, probably as a result of the monormorphic status of the Ruma NP samples.

Table 3. Frequency of mitochondrial haplotypes (with all 'duplicate' labels) within each population.

Haplotype	Naivasha	Nairobi NP	Ngoro-ngoro	Mara-Serengeti NP	Ruma NP	Laikipia	Uganda Congo Sudan	Chad	Total
cokei796/H79/H8	1	0	0	1	0	1	0	0	**3**
cokei783/H2	0	0	6	5	0	1	0	0	**12**
H34	0	1	0	0	0	0	0	0	**1**
cokei747/H51/H20	1	0	0	0	0	1	0	0	**2**
H13/H32/H25/H14/H33/H37/H81/H82	0	0	0	0	8	0	0	0	**8**
H69/cokei748	0	0	0	0	0	1	0	0	**1**
cokei794/H61	0	0	0	2	0	0	0	0	**2**
H54	0	0	0	1	0	0	0	0	**1**
H43	0	1	0	0	0	0	0	0	**1**
H7	1	0	0	0	0	0	0	0	**1**
cokei800	0	0	0	1	0	0	0	0	**1**
cokei786/H5/H47/H11/H19/H41/H56/H6/H68/H74/H10/H29/H44	5	1	1	4	0	2	0	0	**13**
cokei797/H58/H63	1	0	0	2	0	0	0	0	**3**
H57/H36/H53	2	1	0	0	0	0	0	0	**3**
cokei789783/H2/H21/H23/H26/H28/H30/H4/H55/H65/H62/H64	0	0	0	1	0	0	0	0	**1**
cokei798	0	0	0	1	0	0	0	0	**1**
H27	0	0	1	0	0	0	0	0	**1**
cokei792/H12/cokii808/H39/H46/H66	1	2	0	4	0	0	0	0	**7**
cokei802/792/790	0	0	0	1	0	0	0	0	**1**
cokei803	0	1	0	0	0	0	0	0	**1**
cokei804	0	1	0	0	0	0	0	0	**1**
cokei791/H9/H35/H40/H42/H50/H52/H70/cokei46/cokei806	3	4	0	1	0	1	0	0	**9**
cokei809	0	1	0	0	0	0	0	0	**1**
cokei807	0	1	0	0	0	0	0	0	**1**
H3	0	0	0	1	0	0	0	0	**1**
cokei795	0	0	0	1	0	0	0	0	**1**
cokei787	0	0	0	1	0	0	0	0	**1**
lelwel3	0	0	0	0	0	0	1	0	**1**
lelwel44	0	0	0	0	0	0	1	0	**1**
lelwel727	0	0	0	0	0	0	1	0	**1**
lelwel730	0	0	0	0	0	0	1	0	**1**
lelwel729	0	0	0	0	0	0	1	0	**1**
lelwel72	0	0	0	0	0	0	0	1	**1**
lelwel32	0	0	0	0	0	0	1	0	**1**
lelwel42	0	0	0	0	0	0	0	1	**1**
lelwel73	0	0	0	0	0	0	0	1	**1**
lelwel4	0	0	0	0	0	0	0	1	**1**
lelwel41	0	0	0	0	0	0	1	0	**1**
lelwel43	0	0	0	0	0	0	1	0	**1**
H18/H76/H72/H75/H77/H78	0	0	0	0	0	4	0	0	**4**
H17/H73	0	0	0	0	0	7	0	0	**7**
lelwel74	0	0	0	0	0	0	0	1	**1**
cokei784/H1/H67/H22/H24/H31	1	0	3	2	0	0	0	0	**6**
lelwel2	0	0	0	0	0	0	1	0	**1**
lelwel726	0	0	0	0	0	0	1	0	**1**
lelwel728	0	0	0	0	0	0	1	0	**1**
Total	**16**	**14**	**11**	**29**	**8**	**18**	**11**	**5**	**112**

Table 4. Summary of mitochondrial variation within each population.

Population	No. Samples	Polymorphic sites	No. of transitions	No. of transversions	Indels	Mean no. of pairwise differences	Haplotype diversity (H)	Nucleotide diversity (π)
Naivasha	16	55	52	3	1	22.66 ± 10.54	0.85	**0.050 ± 0.02**
Nairobi NP	14	32	23	5	4	11.21 ± 5.42	0.92	**0.033 ± 0.01**
Ngorongoro	11	47	38	3	9	25.26 ± 12.03	0.70	**0.055 ± 0.03**
Mara-Serengeti NP	29	61	43	5	19	19.53 ± 8.90	1.00	**0.057 ± 0.02**
Ruma NP	8	0	0	0	0	0.00 ± 0.00	0.00	**0.00 ± 0.00**
Laikipia	18	54	52	2	1	26.67 ± 12.26	0.83	**0.063 ± 0.03**
Uganda+Congo+Sudan	11	42	38	2	3	20.375 ± 9.765	1.00	**0.063 ± 0.03**
Chad	5	45	38	6	1	26.769 ± 14.214	1.00	**0.058 ± 0.03**
A.b. swaynei	49	76	66	2	9	31.77 ± 14.10	0.87	**0.068 ± 0.03**
A.b. tora	5	55	50	5	2	42.00 ± 22.11	1.00	**0.090 ± 0.05**
A.b. major	**11**	**73**	**60**	**6**	**10**	**45.38 ± 21.35**	**0.97**	**0.129 ± 0.06**

Table 5. Estimates of F_{ST}, an index of genetic subdivision between pairs of populations, based on variation in mitochondrial (below diagonal) and microsatellite (above diagonal) DNA. Asterisk denotes significance at $p \leq 0.05$.

Population	Naivasha	Nairobi NP	Ngoro-ngoro	Mara / Seren-geti NP	Meru NP	Ruma NP	Laikipia	Uganda / Sudan / Congo	Chad	*A.b swaynei*	*A.b. tora*
Naivasha		0.024	0.016	0.016	0.095*	0.099*	0.071*	-	-	-	-
Nairobi NP	0.180*		0.018	0.083	0.166*	0.172*	0.139*	-	-	-	-
Ngorongoro	0.102*	0.313*		0.041	0.147*	0.091*	0.089*	-	-	-	-
Mara-Serengeti NP	-0.014	0.104*	0.050		0.204*	0.139*	0.112*	-	-	-	-
Meru NP	-	-	-	-		0.185*	0.161*	-	-	-	-
Ruma NP	0.403*	0.624*	0.560*	0.371*	-		0.182*	-	-	-	-
Laikipia	0.298*	0.452*	0.261*	0.268*	-	0.511*		-	-	-	-
Uganda / Sudan / Congo	0.435*	0.528*	0.395*	0.401*	-	0.660*	0.242*		-	-	-
Chad	0.425*	0.585*	0.400*	0.394*	-	0.798*	0.233*	0.086		-	-
A.b. swaynei	0.391*	0.417*	0.393*	0.379*	-	0.522*	0.438*	0.409*	0.409*		-
A.b. tora	0.427*	0.537*	0.342*	0.394*	-	0.683*	0.340*	0.252*	0.194	0.308*	
A.b. major	**0.649***	**0.700***	**0.618***	**0.681***	**-**	**0.677***	**0.672***	**0.610***	**0.554***	**0.601***	**0.426***

Mismatch Frequency Distributions: Distributions of the number of nucleotide differences between each pair of haplotypes were examined within the 6 populations for which samples were added in this study (Ruma NP was omitted because there was no nucleotide variation). All populations exhibited multimodality (not shown), and Chi-square tests invariably indicated a significant departure between observed frequencies and unimodality expected under the population expansion hypothesis (Nee *et al.*, 1996; *P < 0.0001* in all cases). Although multimodal patterns are consistent with long-term population stability (Rogers and Harpending, 1992), in this case the incidence of > 22 pairwise differences between haplotypes within a population was more likely due to the presence of haplotypes representing both the *swaynei-cokei* and *lelwel* clades of the mtDNA phylogeny. Both were present in all populations except Nairobi NP.

Microsatellite DNA

Allelic variation and linkage: Among 84 hartebeest samples from 7 populations a total of 55 alleles were recorded at 7 microsatellite loci (Table 6). No significant differences were observed in allele frequency distribution at each of the seven microsatellite loci using Fisher's exact test (*P < 0.05*; data not shown). Exact tests for genotypic linkage disequilibrium between pairs of microsatellite loci across all populations revealed no evidence for linkage (*P > 0.05* in all cases; data not shown). Allelic variation among populations was also

not significantly different. Observed heterozygosity was relatively low in Meru NP and Laikipia, and high in Ruma NP (Table 6), but there was no significant difference between observed and expected heterozygosities in all the populations tested (Table 6).

There were significant departures from Hardy-Weinberg Equilibrium in Nairobi NP, Ngorongoro and Mara-Serengeti NP, all due to apparent heterozygote deficiency. In tests on individual loci only FCB304, which had the most alleles, was not in HWE (P < 0.001), also showing heterozygote deficiency in four populations (Nairobi NP, Naivasha, Ngorongoro and Mara-Serengeti NP).

Population Genetic Subdivision:

Pairwise analysis of population subdivision revealed generally low F_{ST} values (Table 5). None of the estimates involving both populations with *A. b. cokei* morphology was significantly greater than zero. By contrast, all other population pairs were significantly subdivided, including those in which both populations had *A. b. lelwel* morphology. Private alleles were found in Naivasha (2), Meru NP (3), and Ruma NP (1).

Table 6. Summary of microsatellite variation within each population.

Locus		Naivasha	Nairobi NP	Ngorongoro	Mara-Serengeti	Meru NP	Ruma NP	Laikipia	All populations
ETH225	No. of individuals	14	11	13	11	5	8	17	
	No. of alleles	3	3	4	4	2	2	2	
	H_O	0.71	0.88	0.45	0.90	0.20	1.00	0.45	**0.56**
	H_E	0.83	0.77	0.73	0.86	0.51	0.66	0.4	**0.65**
FCB48	No. of individuals	14	11	13	11	5	8	16	
	No. of alleles	4	2	2	3	2	3	3	
	H_O	0.53	0.11	0.55	0.35	0.25	0.50	0.60	**0.41**
	H_E	0.49	0.11	0.50	0.36	0.53	0.42	0.68	**0.44**
FCB304	No. of individuals	14	11	13	11	5	8	17	
	No. of alleles	9	7	8	6	2	5	4	
	H_O	0.67	0.25	0.50	0.25	0.80	1.00	0.50	**0.56**
	H_E	0.89	0.86	0.88	0.84	0.53	0.82	0.77	**0.79**
MAF209	No. of individuals	14	11	13	11	5	8	16	
	No. of alleles	6	2	5	4	2	5	5	
	H_O	0.63	0.50	0.37	0.30	0.00*	0.42	0.80	**0.43**
	H_E	0.77	0.40	0.80	0.50	0.53	0.50	0.84	**0.62**
MCM38	No. of individuals	14	11	13	11	5	8	16	
	No. of alleles	6	5	6	6	2	4	4	
	H_O	0.83	0.63	1	0.91	0.2	0.87	0.69	**0.8**
	H_E	0.78	0.74	0.83	0.68	0.2	0.65	0.62	**0.73**
MCM58	No. of individuals	14	11	13	16	5	8	17	
	No. of alleles	5	5	4	4	3	3	2	
	H_O	0.75	0.60	0.25	0.15	1.00	1.00	0.00*	**0.53**
	H_E	0.76	0.78	0.82	0.78	0.71	0.67	0.53	**0.72**
SMHCC	No. of individuals	14	11	13	11	5	8	17	
	No. of alleles	8	6	6	7	3	3	5	
	H_O	0.71	0.88	0.45	0.75	0.80	1.00	0.45	**0.72**
	H_E	0.83	0.77	0.73	0.79	0.73	0.66	0.40	**0.70**
Mean	No. of alleles	5.86 (2.12)	4.29 (1.98)	5.14 (1.95)	4.86 (1.46)	2.29 (0.49)	3.57 (1.13)	3.57 (1.27)	
	H_O	0.668 (0.05)	0.507 (0.06)	0.507 (0.06)	0.611 (0.05)	0.378 (0.08)	0.743 (0.06)	0.488 (0.07)	
	H_E	0.714 (0.05)	0.604 (0.10)	0.745 (0.04)	0.672 (0.06)	0.511 (0.05)	0.586 (0.06)	0.618 (0.06)	
	F_{IS}	**0.064**	**0.160**	**0.319**	**0.090**	**0.260**	**-0.267**	**0.210**	

Population tree:

When individuals for which allelic information was available for all 7 loci were used to derive a distance-based UPGMA tree, populations with *A. b. cokei* morphology clustered together with strong bootstrap support. However, clustering did not accord with the degree of their geographic separation (Fig. 5).
Basal to this *A. b. cokei* group were populations with *A. b. lelwel* morphology at Laikipia and Ruma NP, but only the branch to the former was well supported. Despite its *A. b. cokei* morphology, the position of the Meru NP population was not well supported. When all individuals were included for which allelic information was available for at least 6 loci, a tree with similar topology was obtained but with lower bootstrap support (Fig. 5).
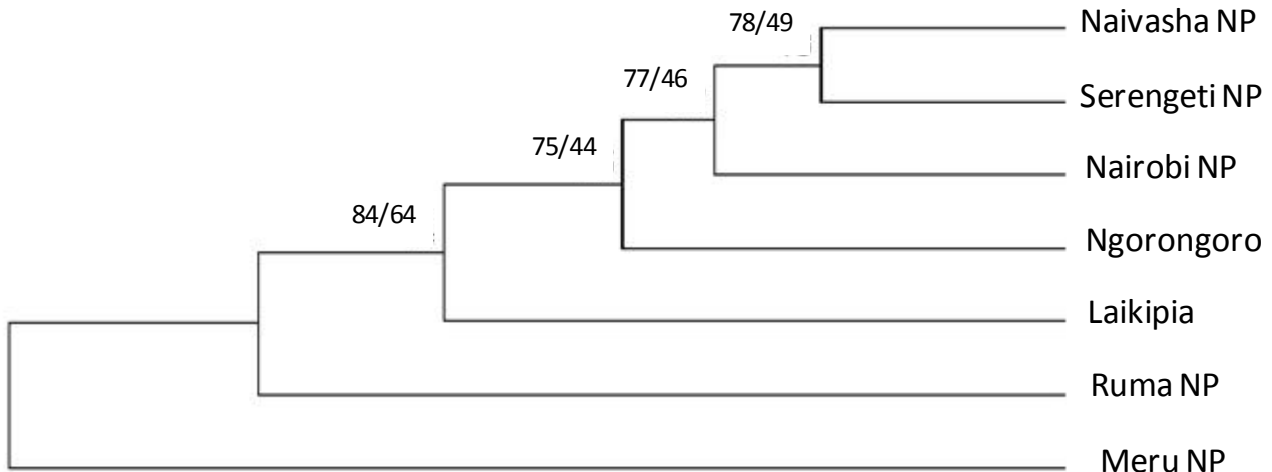
Fig. 5. An UPGMA population tree derived from genetic distances based on microsatellite data. Bootstrap values are indicated for trees based on individuals in which data from 7 microsatellite loci, and at least 6 microsatellite loci, were available.

Structure and Genetix analyses:

In all algorithmic analyses carried out, estimated membership in different clusters was similar across individuals and populations. Thus *Structure* did not reveal any genetically distinctive populations. Further analysis using *Genetix* with populations grouped by locales and subspecies revealed genetic distinctiveness for Ruma and for Meru.

DISCUSSION

A survey of rapidly evolving genetic markers revealed relatively little distinction and varying levels of subdivision among hartebeest populations in Kenya that straddle the transition zone between two morphotypes. The analysis enhanced results of previous studies focusing solely on mtDNA, showing the majority of genetic variation to exist within populations, and less variation partitioned between morphotypes. However, our analysis of populations in such close geographical proximity reduced the tendency for mtDNA haplotypes to cluster phylogenetically according to morphotype. Populations with *lelwel* morphology shared divergent mitochondrial haplotypes with populations that are morphologically *cokei* in ways that suggest both retention of shared ancestral polymorphism, and more recent gene flow. Shared ancestral polymorphism is consistent with the observed pattern of a haplotype in the *lelwel* clade (cokei784) being absent from populations with *lelwel* morphology, but widespread among *cokei* populations. Conversely, gene flow was invoked by the pattern of more derived haplotypes belonging to the cokei clade being present not only in populations with *cokei* morphology but also in populations with *lelwel* morphology. For example, cokei796, cokei747, cokei786, cokei783, and cokei46 were widespread in Serengeti, Nairobi, Naivasha and Ngorongoro, and also present in Laikipia. The reciprocal pattern was not observed, that is, haplotypes belonging to the lelwel clade were not found in both *cokei* and *lelwel* populations.

Microsatellite distinctions between populations and morphotypes were more subtle than mitochondrial distinctions. Alleles showed little tendency to be abundant within one morphotype and absent from the other. While lower indices of population subdivision were observed for biparentally inherited microsatellites than for maternally inherited mitochondrial markers, the haploid, uniparental mode of inheritance of mtDNA would have contributed to this effect (Avise, 1994), as would greater philopatry among females than males. Interpretation of these patterns as to the degree of hybridization between *lelwel* and *cokei* is limited by lack of microsatellite information from populations further from the contact zone, in the 'core' of the *lelwel* range.

These patterns are consistent with the refugium hypothesis of Arctander *et al.* (1999) and Flagstad *et al.* (2001), which holds that present day hartebeest morphotypes originally diverged in isolation during recent bouts of climatic changes. However, our results further suggest that *cokei* and *lelwel* later resumed contact, and that maternal gene flow was predominantly from populations with *cokei* morphology to those with *lelwel* morphology, particularly in Laikipia and Ruma. Such a biased pattern would result, for example, if in fights between males, the more robust horns of *A. b. lelwel*, with the long pedicel providing superior leverage (J. Kingdon, pers. comm.), conferred an advantage over males with *cokei* horn morphology.

Our genetic results also agree with the assignment by Stuart and Stuart (1963) and East (1998) of all hartebeest in the transitional zone as hybrid between *A. b. lelwel* and *A. b. cokei*. This includes the Laikipia, Ruma NP, and Meru NP populations. Although limited variation in horn shape and size was evident within populations, our field observations of gross morphology showed the hartebeest at Ruma NP and Laikipia to resemble *A. b. lelwel* in horn shape, and the Meru NP hartebeest to resemble *A. b. cokei* (a quantitative analysis of skull morphology is in progress). Thus, there was a sharp disparity between the subtle, apparently seamless genetic transition between morphotypes and the marked disjunction in gross morphology over short distances. For example, about 100 km separates southern Laikipia from Naivasha, eastern Laikipia from Meru NP, and Ruma NP from the northern Masai Mara, yet morphological distinctions and affinities to their respective 'parental' phenotypes were readily apparent within each of these pairs. These patterns differed fundamentally from those in wildebeest, also an Alcelaphine, in which mitochondrial differences were marked between morphologically distinct populations in close geographic proximity (Templeton and Georgiadis, 1995; Arctander *et al.,* 1999).

Earlier observations, documented when hartebeest distribution was less fragmented than today, support the notion that the transition zone between morphotypes was remarkably narrow. For example, 'Laikipia hartebeest' were consistently distinguished from 'hybrids' in early game reports of the British administration in Kenya (1912, 1914). G. H. Goldfinch (1914) noted that 'Laikipia hartebeest appeared to share the same ground, but not the same herds, with hybrids near Lake Olbolossat' [in south-west Laikipia] on a seasonal basis. He also noted 'there appears to be a [hybrid] form…near Meru'. And the last remaining individual of a population considered to be hybrid between *lelwel* and *cokei*, that went extinct in the Nakuru area of the Rift Valley in 1967, had the appearance of a more equal mix between the two (Gosling 1969). Some individuals on Solio Ranch in southern Laikipia have a similar appearance (Fig. 2C).

Consistent with their emergence in the last 140,000 years (Flagstad *et al.,* 2001), subtle genetic differences between populations suggest that gross morphological distinctions between *lelwel* and *cokei* evolved recently, and entail relatively few loci. Sexual selection and hybrid inferiority may have played a role in maintaining morphotypic distinctions, despite historical gene flow.

Conservation implications

In this case, gross morphology might superficially appear to provide a superior basis than genetics for management action intended to conserve evolutionary diversity in hartebeest. An interpretation of the genetic data alone might have concluded that a subset of the populations in this study is isolated by distance, but not distinct from each other in fundamental or qualitative ways. In the case of the Ruma NP population, analysis based solely on mtDNA would have been further misleading in two senses. First, in implying phylogenetic affinity to the cokei clade rather than the lelwel clade. And second, in implying that genetic variation in the population was low. Low mtDNA variation at Ruma NP suggests the population has bottlenecked in the past, but given that it had the highest microsatellite heterozygosity, nuclear variation appeared unaffected (in all other populations, levels of nuclear and mitochondrial variation were moderate to high, compared to other

African bovid species; Alpers *et al.,* 2004, and references therein). The very high observed heterozygosity in Ruma may also be indicative of ongoing hybridization in this population. Even though firm conclusions cannot be drawn from this population due to low sample size (sample size were half that of Laikipia population even though the number of alleles were similar between Ruma and Laikipia populations).

But gross morphology could not – as genetic data can – illuminate the processes that underlie the observed morphological patterns. In this case, both morphological and genetic data were required to define the targets of conservation. Hartebeest populations in Laikipia, Ruma NP and Meru NP are important in that they provide the only remaining examples, each different from the other, of what appears to be resumed contact between two morphotypes that diverged in allopatry, a process that has been ongoing across a vast area for thousands of generations.

Yet all these populations are threatened. Dwindling numbers in Meru NP prompted conservation managers to propose supplementing the population from elsewhere. Contrary to East (1998), in which the Meru NP population is incorrectly grouped with Laikipa and Ruma NP on morphological grounds, similarities in gross morphology would suggest a more suitable source population to be Naivasha or Nairobi NP. While this would be unlikely to result in reproductive incompatibilities, our microsatellite results suggest the Meru NP hartebeest to be genetically subdivided from the 'core' *cokei* populations, and the only remaining hybrid population with *cokei* morphology. A preferable strategy, aiming to conserve the products of hybridization, would be to breed up the remaining individuals at Meru in a large area surrounded by a predator-proof fence. The Laikipia population is also declining and is nowhere formally protected. However, the property holding the highest densities (Solio Ranch in the extreme south) is unlikely to remain intact and hartebeest within this Ranch should be moved to a more permanently secure location. Similarly, suitable refuges for the Ruma NP hartebeest should be identified should this protected area not survive pressures from surrounding humanity.

Conservation protocols and practices are ill-defined for hybrids between species (IUCN 2004) morphotypes. By emphasizing recognizable species, this inevitably relegates hybrids and variants to a lesser status, forcing much of the attention on the important but narrow question of whether or not products of inter-breeding might be viable. In practice, conservation managers do well to evaluate reproductive compatibility before merging two populations. Too rarely considered are the implications of disrupting more subtle evolutionary processes. Often there is no option (particularly in captivity), but in many African countries, including Kenya, where there has been little mixing of populations by translocation, opportunities to conserve ongoing evolutionary processes persist, and should be pursued. If these more subtle but valuable evolutionary processes are not defined as soon as possible and explicitly integrated into national conservation strategies, they will not be conserved. Defining evolutionary processes is possible through partnerships between national and international agencies, and with funding from zoos and non-government conservation organizations (NGO's).

Acknowledgements

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