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Population genetics of the roan antelope (*Hippotragus equinus*) with suggestions for conservation

D. L. ALPERS,* B. J. VAN VUUREN,* P. ARCTANDER† and T. J. ROBINSON*
**Department of Zoology, University of Stellenbosch, Private Bag XI, Matieland 7602, South Africa, †Department of Evolutionary Biology, University of Copenhagen, Copenhagen, Denmark*

Abstract

The roan antelope (*Hippotragus equinus*) is the second largest African antelope, distributed throughout the continent in sub-Saharan savannah habitat. Mitochondrial DNA (mtDNA) control region sequencing (401 bp, *n* = 137) and microsatellite genotyping (eight loci, *n* = 137) were used to quantify the genetic variability within and among 18 populations of this species. The within-population diversity was low to moderate with an average mtDNA nucleotide diversity of 1.9% and average expected heterozygosity with the microsatellites of 46%, but significant differences were found among populations with both the mtDNA and microsatellite data. Different levels of genetic resolution were found using the two marker sets, but both lent strong support for the separation of West African populations (samples from Benin, Senegal and Ghana) from the remainder of the populations studied across the African continent. Mismatch distribution analyses revealed possible past refugia for roan in the west and east of Africa. The West African populations could be recognized together as an evolutionarily significant unit (ESU), referable to the subspecies *H. e. koba*. Samples from the rest of the continent constituted a geographically more diverse assemblage with genetic associations not strictly corresponding to the other recognized subspecies.

Keywords: conservation genetics, control region, ESU, microsatellites, nested clade analysis, roan antelope

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Introduction

The roan antelope (*Hippotragus equinus*) is the second largest African antelope, exceeded in size only by the eland (*Taurotragus oryx*). The species is fairly widely distributed throughout the continent in sub-Saharan savannah habitat, but at a finer scale shows a patchy and discontinuous distribution (Kingdon 1982; Skinner & Smithers 1990; East 1999). In some areas of Africa, large local populations of roan antelope still exist, but their numbers have decreased dramatically over the past few decades due, primarily, to the pressures of commercial hunting, epizootic diseases (De Vos 1992; Ebedes 1992; Meltzer 1992) and habitat disturbances (Kingdon 1982; Skinner & Smithers 1990; Meltzer 1992; Happold 1995). It is a popular game ranch species and there is substantial commercial movement of animals around the continent. The species is currently listed as threatened and its continued survival is dependent on active conservation measures (IUCN 1996).

Correspondence: T.J. Robinson. E-mail: tjr@sun.ac.za.

The absence of detailed population genetic information has led conservation agencies to employ management programmes based on the only evidence available: morphological subspecies descriptions. Ansell (1971), using morphological rather than genetic evidence, recognized six *H. equinus* subspecies (*equinus*, *cottoni*, *langheldi*, *bakeri*, *charicus* and *koba*), but the validity and geographical limits of these geographical races have not been strictly defined (Roberts 1951; Ansell 1971; Kingdon 1982; Magin & Kock 1997). Conservation policies based on poorly defined subspecific categories, which may not reflect the underlying genetic diversity, are at best inefficient and at worst might reduce the long-term evolutionary potential of the species. This study aimed to quantify roan antelope genetic variation and from this directly impact conservation management.

In the broadest sense, conservation of the genetic diversity and integrity of a species or subspecies relies on identifying the critical genetic units and then managing these units in a co-ordinated manner (Lesica & Allendorf 1995; Triggs *et al*. 1989; McCauley 1991). These critical units are often referred to as evolutionarily significant units (ESUs) (Ryder

1986) and designation requires significant differentiation with both mitochondrial and nuclear DNA (Moritz 1994). Matthee & Robinson (1999) made a preliminary investigation of roan antelope mitochondrial DNA (mtDNA) variation by sequencing the control region from 13 samples from four of the six subspecies recognized by Ansell (1971). They found that all four of these subspecies had distinct mtDNA evolutionary lineages indicating historical isolation and possibly representing four different ESUs. This current study extends this earlier work by including one more subspecies (*H. e. charicus*), using larger sample sizes and including microsatellite markers. Our aim was to define genetic units of conservation for the roan antelope over the majority of its range.

We also investigated whether the roan antelope showed similar phylogeographical patterns to other large African savannah bovids. Environmental variation during the Pleistocene was characterized by cold and arid glacial periods oscillating with warmer and wetter interglacial periods, with associated expansion and contraction of savannah habitats (deMenocal 1995). Repeated isolation of populations into one or more favourable refugia in West, East and South Africa during periods of global warming with range expansion events during more arid periods has been postulated as the explanation for phylogeographical patterns in hartebeest (*Alcelaphus buselaphus*; Arctander *et al*. 1999; Flagstad *et al*. 2001), topi (*Damaliscus lunatus*), wildebeest (*Connochaetes taurinus*; Arctander *et al*. 1999) and buffalo (Van Hooft *et al*. 2002).

Materials and methods

Samples, DNA extraction and polymerase chain reactions

The new tissue samples used in this project were skin samples obtained opportunistically from 18 localities throughout Africa (Fig. 1) and stored at room temperature in 20%DMSO/saturated NaCl. Thirteen samples were previously extracted

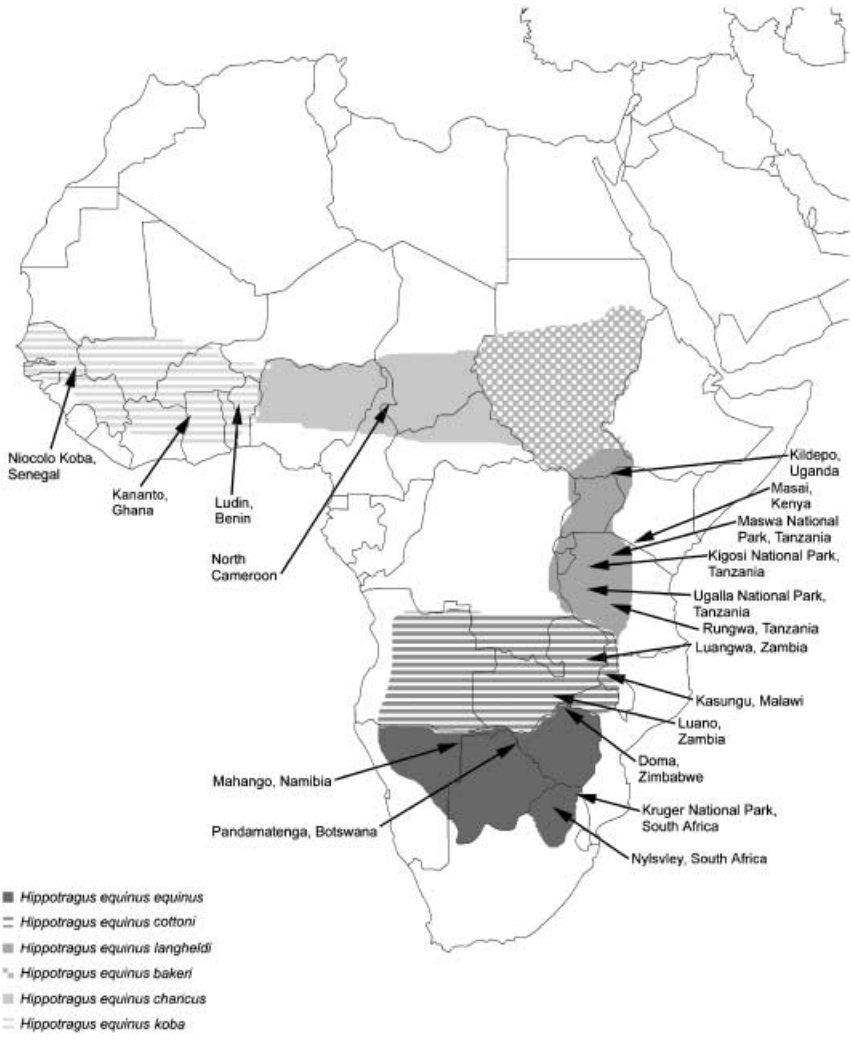

Fig. 1 Map of Africa showing the sampling localities for the roan antelope (*H. equinus*) specimens used in this study. The shaded areas indicate subspecies boundaries, after Ansell (1971).

by Matthee & Robinson (1999). All samples and are currently held at either the Department of Zoology, University of Stellenbosch, South Africa (n = 82), or the Department of Evolutionary Biology, University of Copenhagen, Copenhagen, Denmark (n = 55). DNA was extracted using standard phenol/chloroform procedures (Sambrook *et al*. 1989). Polymerase chain reactions (PCR) were carried out for control region sequence analyses and for microsatellite genotyping.

Using primers (N777 and DLH1) and protocols described in Matthee & Robinson (1999), 420 bp of the mtDNA control region were amplified for 137 samples. There were 5 indels, totalling 19 bases, which were excluded for the phylogenetic analyses, leaving 401 bp for these analyses. Our samples included all but one (*Hippotragus equinus bakeri*) of the six recognized subspecies (Ansell 1971). PCR products for sequencing were cleaned using the Qiagen PCR purification kit (cat# 28106). Both strands of all PCR products were cycle sequenced using BigDye dye termination chemistry from Perkin–Elmer and all unincorporated dye label was removed by sephadex columns before the samples were run on an ABI Prism377 sequencer (Applied Biosystems). Sequences were checked using Sequence Navigator (ABI, version 1.01) and aligned by eye.

The microsatellite analyses involved samples from 137 samples from 13 populations. Four of the six recognized subspecies were included, excluding *H. e. charicus* and *H. e. bakeri*. Eight dinucleotide microsatellite loci were amplified (*BM757*, *BM804*, *BMC3224*, *BMS4008*, *OARFCB48*, *IDVGA-68*, *TAMLS113.3*, *OARFCB304*). The primers were originally designed from cattle or sheep (MARC cattle map; Bishop *et al*. 1994; Mezzelani *et al*. 1995; Kappes *et al*. 1997) and all have been mapped to different chromosomes in cattle (Table 1). PCR conditions varied among the loci (Table 1). Approximately 50 ng of template DNA was used per reaction. For each locus the forward primer was fluorescently labelled with TET, 6-FAM or HEX (PE-Biosystems) for electrophoresis on an ABI 377 DNA sequencer. Commercially prepared size standard markers (Genescan TAMRA) were run with every sample. Data were collected, analysed and genotyped using ABI commercial software, GENESCAN version 2.1 and GENOTYPER version 2.0 (Applied Biosystems).

Data analyses

Control region analyses. Phylogenetic relationships among the representative mtDNA haplotypes were estimated using maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) methods carried out by PAUP* version 4.0b8 (Swofford 1998). The robustness of these analyses was assessed using bootstrap replications, 1000 replications for the MP and NJ and 100 replications for ML. The settings for the nucleotide substitution model used in the ML and NJ analysis were selected by hierarchical likelihood ratio testing using MODELTEST 3.06 (Posada & Crandall 1998, 2001) and the model selected was the Hasegawa *et al*. (1985) (HKY85) model with invariable sites and rate heterogeneity (HKY + I + Γ). The same likelihood settings were used in a Bayesian phylogeny analysis using the program MR BAYES (Huelsenbeck & Ronquist 2002), which assesses robustness from a posteriori probabilities. This Bayesian analysis constructed a 50% majority-rule tree from 30 001 trees inferred after the likelihood of the a posteriori distribution had stabilized (from three consecutive runs of 1×10^6 generations after 100 000 initial 'burnin'

Table 1 PCR conditions for eight microsatellite loci with roan antelope

Locus	Direction	Sequence (5′- to 3′)	Annealing temp. (°C)	No of PCR cycles	$MgCl_2$ conc. (mM)	Chromosome no. in cattle*
BM757	forward	TGGAAACAATGTAAACCTGGG	55	35	2.5	9
	reverse	TTGAGCCACCAAGGAACC				
BM804	forward	CCAGCATCAACTGTCAGAGC	60	30	2.5	26
	reverse	GGCAGATTCTTTGCCTTCTG				
BMC3224	forward	CCATCACTGCTATTCTACCTCC	55	30	2.5	29
	reverse	CACAGCCAATTTCTGATTTCAG				
BMS4008	forward	CGGCCCTAAGTGATATGTTG	58	35	2.5	1
	reverse	GAAGAGTGTGAGGGAAAGACTG				
OARFCB48	forward	GACTCTAGAGGATCGCAAAGAACCAG	55	35	2.5	17
	reverse	GAGTTAGTACAAGGATGACAAGAGGCAC				
IDVGA68	forward	TCAGAGGGGCAGACAGTGAT	58	30	2.0	16
	reverse	GAGGGACTTGGGAGGGAAAC				
TAMLS113.3	forward	TTACTGCTGAGCCACCGG	60	30	1.5	23
	reverse	GATGGGGGTCACAAACTGAC				
OARFCB304	forward	CCCTAGGAGCTTTCAATAAAGAATCGG	58	30	2.0	22
	reverse	CGCTGCTGTCAACTGGGTCAGGG				

*See MARC cattle map http://sol.marc.usda.gov/genome/cattle/cattle.html

generations). As mammal mtDNA control region sequences are characterized by a bias towards transitions over transversions (Brown *et al*. 1982), parsimony analysis was performed using differential weighting of transversions (TV) and transitions (TI) (empirically determined). The TI : TV ratio was calculated using MEGA 2.1 (Kumar *et al*. 2001). Parsimony searches were heuristic with bootstrapping carried out using the stepwise addition and the fast heuristic approach in PAUP* 4.0b8 (Swofford 1998). Published sable antelope (*Hippotragus niger*) sequences were chosen as outgroup sequences for the phylogenetic analyses (GenBank Accession nos. AF364773 and AF364774; Pitra *et al*. 2002).

Population differentiation was initially examined via the estimation of haplotype and nucleotide diversities, and pairwise F_{ST} values using Kimura 2-parameter (K2P) distances with gamma correction (Kimura 1980) as calculated by MODELTEST, using the program ARLEQUIN 2.000 (Schneider *et al*. 2000). An analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992), which utilizes both sequence divergence and frequencies, was also carried out with the mtDNA sequence data using ARLEQUIN 2.000 (Schneider *et al*. 2000). The distance used for the AMOVA was the K2P distance (gamma corrected as above) and two structures were tested. The first allocated the 18 populations into five subspecies groups: *H. equinus equinus, H. e. cottoni, H. e. langheldi, H. e. charicus* and *H. e. koba*. The populations were also separated into two major groups based on the results of the phylogeographical analyses; one group from West Africa (as represented by populations from Benin, Ghana and Senegal) and the other group represented by all the remaining populations (Botswana, Cameroon, Kenya, Kruger National Park, Malawi, Namibia, Nylsvley, Tanzania Kigosi, Tanzania Maswa, Tanzania Rungwa, Tanzania Ugalla, Uganda, Zimbabwe, Zambia Luano and Zambia Luangwa).

The mtDNA sequence data were used for a nested clade analysis (NCA) to test for statistically significant phylogeographical associations, based on phylogenetic relationships among haplotypes and the frequency of haplotypes in each population. Haplotype networks with 95% parsimoniously plausible connections were constructed using the program TCS 1.13 (Clement *et al*. 2000) with the statistical approach developed by Templeton *et al*. (1992). Gaps in the sequences were treated as missing data and three loops due to homoplastic ambiguities in the networks were resolved following the criteria suggested by Crandall & Templeton (1993): that rare haplotypes are more likely to be at a tip, common haplotypes interior within the network, and that haplotypes represented by single specimens are more likely found associated with haplotypes from the same population than from different populations (Crandall & Templeton 1993; Mardulyn 2001). These networks were then converted into a nested clade design using standard nesting rules (Templeton *et al*. 1987; Templeton & Sing 1993). Nested contingency analysis and nested geographical distance analysis were implemented with GEODIS 2.0 (Posada *et al*. 2000) using 1000 permutations. Geographical distances between populations were user-defined by measuring off a map along probable colonization routes, rather than accepting the default 'as-the-crow-flies' distances calculated by GEODIS from latitude and longitude coordinates. The results of the nested geographical analysis were interpreted for patterns of population structure and historical events using the latest version of Templeton *et al*.'s (1995) interpretative key (available at http://zoology.byu.edu/crandall_lab/dposada/documents/NCA-key(24Oct01).pdf). Only clades with either geographical or genetic variation were tested with the NCA.

A mismatch analysis was performed using ARLEQUIN 2.000 (Schneider *et al*. 2000) to test for evidence of population expansion in the clades identified by the TCS analysis. ARLEQUIN uses a parametric bootstrap approach (1000 simulations) to compute 95% confidence intervals for τ, the time since the expansion in units of mutational time ($\tau = 2\mu t$, where μ is the mutational rate and t is the generation time in years), θ_0, the initial population size ($\theta_0 = 2\mu N0$) and θ_1, the final population size ($\theta_1 = 2\mu N1$). ARLEQUIN also computes the raggedness statistic (Harpending 1994) and the sum of squared deviations (SSD), which test the fit of the observed data to the expected population growth model (Schneider & Excoffier 1999). To estimate t, we assumed a mutation rate of 13%/Myr (similar to hartebeest, Flagstad *et al*. 2001; African buffalo, van Hooft *et al*. 2002), and a generation time of 3 years (J. Krick personal communication).

Microsatellite analyses. Within population genetic diversity was measured by allelic diversity (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) and these were calculated by BIOSYSL (Swofford & Selander 1981). Each locus in every population was tested for deviations from Hardy–Weinberg equilibrium (HWE) proportions using permutation tests by ARLEQUIN 2.000 (Schneider *et al*. 2000).

Population differentiation was investigated using F_{ST} analyses. The global F_{ST} value (and its 95% confidence interval estimated by 15 000 bootstrap replications) was calculated using FSTAT 2.9.1 (Goudet 2000). Pairwise F_{ST} values were calculated using ARLEQUIN 2.000 with significance determined via permutation tests (3024 permutations). These pairwise F_{ST} values and the same user-defined matrix of distances used for the GEODIS analysis were used to test for isolation-by-distance. The F_{ST} values were first linearized using Slatkin's (1995) transformation, $F_{ST}/(1 - F_{ST})$, and the geographical distances were transformed using the natural log function. The correlation between these two matrices of transformed distances was calculated using a 10 000-permutation Mantel test (Mantel 1967), performed using ARLEQUIN 2.000 (Schneider *et al*. 2000). Relationships among the 13 populations were summarized by three underlying dimensions using factorial correspondence analysis

(FCA) of microsatellite genotypes implemented with GENETIX 4.04 (Belkhir *et al*. 2002). Genetic population structure was also analysed using AMOVA (Michalakis & Excoffier 1996) by partitioning the genetic diversity into two structures. The first structure allocated the 13 populations into four subspecies groups: *H. e. equinus*, *H. e. cottoni*, *H. e. langheldi* and *H. e. koba*. The second structure divided the populations into two groups, according to the same criteria as were used for the mtDNA data, with the West African populations as one group and the other populations as the other. The AMOVA was carried out using ARLEQUIN 2.000 (Schneider *et al*. 2000), with pairwise F_{ST} as the distance measure. The genetic relationships among populations were also assessed using D_A distances (Nei *et al*. 1983) computed for all pairwise comparisons and clustered using the NJ algorithm with the program DISPAN (Ota 1993). Robustness of the tree topology was tested via 10 000 bootstrap replications.

Table-wide rejection levels for multiple statistical tests were calculated using sequential Bonferroni correction throughout the analyses (Rice 1989).

Results

Control region sequence characteristics and phylogenetics

Figure 2 lists the 52 unique haplotypes obtained from the mtDNA control region sequences (the sequences were submitted to GenBank, Accession nos AF049373–AF049375, AF049378, AF068839, AF068840, AY179373–AY179429, AY575568–AY575605). The empirical TI : TV ratio was calculated as 12 : 1. Of the 401 bases used for the MP analyses, 143 were parsimony-informative. The parsimony analysis resulted in 120 equally parsimonious trees of 933 steps (consistency index [CI] = 0.7771). The localities of shared identical haplotypes are shown in Fig. 2. The minimum sequence divergence was 0.25% (one substitution) between samples from within Tanzania (haplotype 19, from Maswa and Kigosi, with haplotypes 18 and 28, both from Maswa). The maximum divergence among *Hippotragus equinus* haplotypes was 27.53% between haplotype 46 from Senegal and haplotype 12 from Botswana. The *H. equinus* ingroup clade had strong support (bootstrap values of 100% from NJ, MP and ML as well as a 100% credibility value from the Bayesian analyses, Fig. 2). There was also strong support for a separation between sequences from West Africa (Ghana, Benin and Senegal) and those from the rest of Africa (Fig. 2). Support for basal relationships among most of the other African populations was weak. Although numerous terminal clades had strong support (Fig. 2), there were no other strong geographical associations or clades represented by the recognized subspecies other than the split from West Africa (subspecies *H. e. koba*), even when only the eastern populations were analysed. It is important to note that although the haplotype representing *H. e. charicus* from Cameroon did not cluster with the West African lineage neither was it strongly associated with any other lineage.

Population structure based on control region sequences

Nucleotide diversity for all 18 populations ranged from 0 to 4.9% (highest for Luangwa, North Zambia; mean 1.6%). Nucleotide diversity within populations with sample sizes ≥ 10 ranged from 0.9 to 3.3% (mean 1.9%) (Table 2). There

Table 2 Frequencies of 52 different roan mitochondrial DNA haplotypes from 18 populations

Population	Latitude and longitude	Sample size	Number of haplotypes	Haplotype diversity	Nucleotide diversity
Namibia	19° S, 21° E	13	8	0.923	0.033
Kruger National Park, South Africa	23° S, 33° E	4	2	0.500	0.005
Nylsvley, South Africa	25° S, 29° E	5	2	0.400	0.001
Botswana	19° S, 26° E	8	2	0.536	0.023
Zimbabwe	16° S, 30° E	5	1	0.000	0.000
Luano, South Zambia	15° S, 29° E	5	3	0.800	0.036
Luangwa, North Zambia	12° S, 32° E	3	3	1.000	0.049
Malawi	11° S, 34° E	23	3	0.170	0.014
Rungwa, Tanzania	7° S, 34° E	9	4	0.833	0.015
Ugalla, Tanzania	6° S, 32° E	8	7	0.964	0.026
Kigosi, Tanzania	4° S, 32° E	4	4	1.000	0.038
Maswa, Tanzania	3° S, 34° E	10	5	0.756	0.009
Kenya	2° S, 36° E	1	1	1.000	0.000
Uganda	4° N, 34° E	1	1	1.000	0.000
Cameroon	10° N, 15° E	2	1	0.000	0.000
Benin	10° N, 2° E	2	2	1.000	0.002
Ghana	9° N, 2° W	10	4	0.800	0.026
Senegal	14° N, 15° W	24	8	0.862	0.015

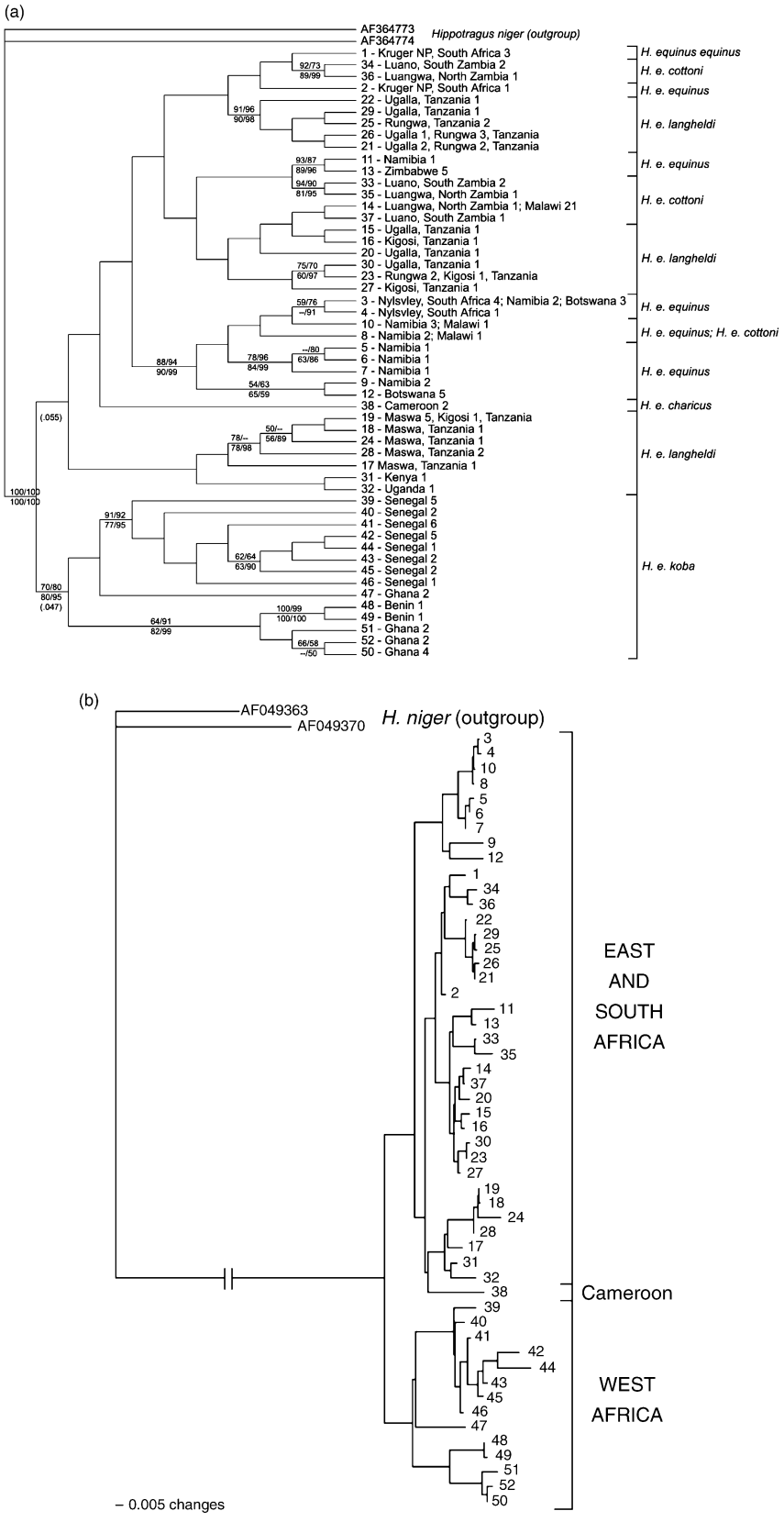

Fig. 2 Phylogenetic relationships among 52 unique *H. equinus* control region haplotypes. The trees shown are a cladogram (a) and a phylogram (b), both based on neighbour-joining (NJ) trees generated by PAUP* based on HKY85 distances, with an assumed proportion of invariant sites and a gamma rate for variant sites calculated by Modeltest (Posada & Crandall 1998). In the main picture the haplotype numbers are listed with the population locations they were detected in and their frequency in each of those population locations. Bootstrap values above the lines are maximum parsimony/ neighbour-joining (both from 1000 replications); below the lines maximum likelihood (100 replications)/ Bayesian credibilities (as percentages, based on 30 001 trees). Nucleotide diversity figures for the West and East Africa clades shown in brackets.

was significant population differentiation for over a third of the population pairs as 37.3% of the pairwise F_{ST} values remained significant at the $P < 0.05$ level after Bonferroni correction. Most of the pairwise values that were non-significant were between populations represented by small sample sizes. All of the populations with 10 or more samples had significant pairwise differences. With the AMOVA all the tests of differentiation at the different hierarchical levels were significant in both analyses. The AMOVA using the subspecies groups showed marginally more variance, 40.73%, among the subspecies groups ($\Phi_{CT} = 0.41$, $P < 0.0001$), than variance among populations within the subspecies groups, 39.62% ($\Phi_{SC} = 0.67$, $P < 0.0001$). Variance within populations was 19.65% ($\Phi_{ST} = 0.80$, $P < 0.0001$). In contrast, a clear majority of the variance explained by the second AMOVA was due to differences between the two geographical regions, West Africa vs. the other populations combined (48.70%; $\Phi_{CT} = 0.49$; $P = 0.004$). The smallest partition of variance was found within populations (15.20%, $\Phi_{ST} = 0.85$, $P < 0.0001$) and differences among populations within the two main regional groups accounted for 36.10% of the variance ($\Phi_{SC} = 0.70$, $P < 0.0001$).

Nested clade analyses

Haplotype networks of up to eight mutational steps could be constructed in a parsimonious fashion with a probability ≥ 0.95. Figure 3 shows four disjoint networks, Senegal (clade 2-13), Ghana (clade 2-12), Benin (clade 1-30) and East and South Africa (clade 4-1), and two single haplotypes

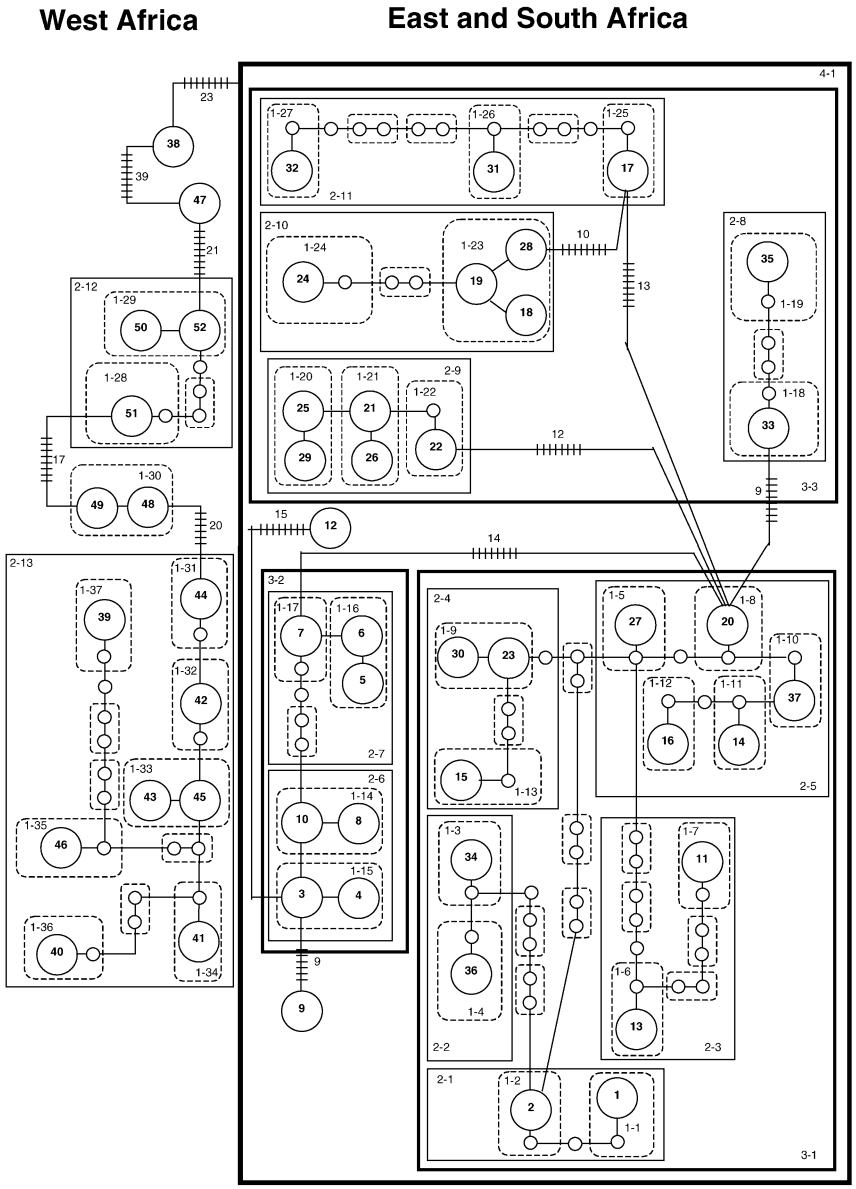

Fig. 3 The nesting design inferred from the cladogram estimation of the 52 mtDNA haplotypes detected for *H. equinus*. Each line in the network represents one mutational change. Small empty circles represent the inferred, nondetected interior haplotypes. The number inside each circle identifies the detected haplotypes, listed in Fig. 2. Smaller dashed-lined rectangles indicate the haplotypes grouped together into 1-step clades and the thin-lined rectangles indicate the nesting 2-step clades, while larger thick-lined rectangles indicate the 3-step clades and the largest, thickest-lined rectangle indicates the single 4-step clade. The closest possible connection between clade 4-1 and haplotype 38 (Cameroon) is through haplotype 32 (Uganda), although the number of steps, 23, is outside the 95% confidence range (max = 8).

Table 3 Inference chain based on results of geographical dispersion analysis. Only those clades that resulted in a rejection of the null hypothesis of panmixia are included in this table

Clade	Chain of inference	Inference
2-5	1-2-3-4 NO	Restricted gene flow with isolation by distance
3-1	1-2-3-5-6-13 YES	Long distance colonization
3-3	1-2-11-17-4 NO	Restricted gene flow with isolation by distance
4-1	1-2-3-4 NO	Restricted gene flow with isolation by distance
Total Cladogram	1-2-3-5-6-7 YES	Restricted gene flow/dispersal but with some long distance dispersal

Table 4 Results of the mismatch analyses

Clade	τ	Obs mean	θ_0	θ_1	Ragged.	$P_{(HARP)}$	$P_{(SSD)}$	$t_{exp.}$ (kyr)
3-1	14.750 (6.62–26.50)	8.015	0.0 (0.0–7.5)	9.9 (4.1–39.3)	0.115	0.015	0.061	190–763
3-2	0.442 (0.00–3.26)	2.526	1.4 (0.0–5.0)	696.4 (7.1–6561.4)	0.058	0.717	0.233	0–94
3-3	28.014 (18.50–35.74)	17.360	0.0 (0.0–10.8)	46.8 (25.1–734.3)	0.068	0.004	< 0.001	532–1028
2-12	12.020 (6.31–25.00)	11.286	7.3 (0.0–9.6)	7.3 (1.0–71.9)	0.449	0.042	0.017	182–719
2-13	5.010 (1.50–21.72)	5.736	3.6 (0.0–14.6)	13.1 (6.4–1597.2)	0.088	0.081	0.082	43–625

Clades are those used for the NCA (see Fig. 3). The expansion parameter (τ), the mismatch observed mean (Obs. mean), the mutation parameter before (θ_0) and following (θ_1) expansion, and the raggedness index (Harpending 1994) (Ragged.) were calculated for all major groups separately and for the groups of haplotypes according to the clades identified by the TCS analysis. Values in brackets refer to 95% confidence intervals. $P_{(HARP)}$ is the probability of getting a higher value of the raggedness index (Harpending 1994) by chance. $P_{(SSD)}$ (sum of squared deviations) is the probability of observing a less good fit between the model and the observed distribution by chance. The time, in thousands of years, since the most important demographic expansion ($t_{exp.}$) in each group was determined on the basis of the equation $\tau = 2\mu t$, assuming a mutation rate of 13%/Myr and an average generation time t of 3 years. The range of times is based on the lower and upper limits of the 95% confidence interval for τ.

from Ghana (haplotype 47) and Cameroon (haplotype 38). These networks are connected by nine or more mutational steps, outside the confidence limits for parsimony, so the precise connections between these networks are unknown from these data. Four clades contained significant D_n, D_c or I–T distances, which resulted in a rejection of the null hypothesis of panmixia: the 4-step East and South African clade (4-1), two of the three 3-step clades (3-1 and 3-3), and one of the 2-step clades (2-5) nested within clade 4-1 (detailed results available from the authors). The chains of inference from these results are listed in Table 3. Restricted gene flow with isolation-by-distance was inferred from haplotypes in clade 2-5 (geographically from North and South Zambia, Kigosi and Ugalla in Tanzania, and Malawi), clade 3-3 (Zambia, Tanzania, Kenya, and Uganda) and clade 4-1 (eastern and southern Africa). Long-distance colonization was inferred from haplotypes in clade 3-1 (Kruger National Park, South Africa; Zimbabwe; Zambia; Namibia; and Kigosi, Ugalla and Rungwa, Tanzania). Using the most probable connections among clades (Fig. 3) to identify internal and tip clades within the total cladogram resulted in an inference for the total network of restricted gene flow with some long-distance dispersal. Although the region between Cameroon and Uganda, occupied by the subspecies *H.e. bakeri*, was not sampled in this study, the NCA did not reveal any problems with sampling adequacy.

The mismatch distributions differed significantly among many of the clades (Table 4). All the clades had significantly different τ ($p < 0.05$) except for the two West African clades 2-12 and 2-13 and also between clade 3-1 and the same two West African clades. The largest τ was observed in the northeast clade, 3-3 ($\tau = 28.014$; see Table 4 for more details), suggesting that this is the oldest lineage, while the lowest τ, and therefore youngest lineage, was observed in the southern African clade, 3-2 ($\tau = 0.442$). The low τ for the Senegal clade 2-13 ($\tau = 5.010$) also suggests a recent expansion. A model of sudden population expansion was a significantly bad fit to the observed data for every clade except the two with evidence of recent expansions (southern Africa 3-2, $P_{(HARP)} = 0.717$, $P_{(SSD)} = 0.233$; Senegal 2–13, $P_{(HARP)} = 0.088$, $P_{(SSD)} = 0.082$). The other clades were signifcantly ragged ($P_{(HARP)} < 0.05$).

Microsatellite analyses

For the eight microsatellite loci the allelic diversity ranged from 1.7 to 5.9 (mean, 3.4) for the 13 populations and expected heterozygosity ranged from 0.24 to 0.70 (mean, 0.46) (Table 5). The West African populations were more

Table 5 Genetic variability at eight microsatellite loci in 13 roan antelope populations. Calculated using BIOSYSL with standard errors in parentheses

Country	Location/ Popn	Mean sample size per locus	Mean no. of alleles per locus	Percentage of loci polymorphic*	Mean heterozygosity	
					Observed	Expected†
Namibia	Mahango	10.6	3.8	75.0	0.425	0.503
		(0.7)	(0.7)		(0.141)	(0.122)
South Africa	Kruger Natl Park	3.1	2.1	62.5	0.250	0.342
		(0.2)	(0.5)		(0.122)	(0.120)
South Africa	Nylsvley	11.9	2.9	75.0	0.230	0.378
		(0.8)	(0.4)		(0.085)	(0.092)
Botswana	Pandamatenga	9	3	75.0	0.417	0.463
		(0)	(0.6)		(0.109)	(0.121)
Zimbabwe	Doma	5.9	1.8	50.0	0.308	0.243
		(0.1)	(0.3)		(0.129)	(0.099)
Zambia	Luano (S)	4.8	2.4	62.5	0.563	0.422
		(0.2)	(0.5)		(0.169)	(0.127)
Zambia	Luangwa (N)	2.4	1.7	42.9	0.262	0.286
		(0.2)	(0.4)		(0.145)	(0.149)
Malawi	Kasungu	15	2.9	75.0	0.375	0.360
		(0)	(0.6)		(0.115)	(0.108)
Tanzania	Ugalla	9.3	4.1	100.0	0.469	0.555
		(0.5)	(0.7)		(0.103)	(0.092)
Tanzania	Kigosi	8.6	4	100.0	0.604	0.584
		(0.7)	(0.7)		(0.130)	(0.108)
Tanzania	Maswa	13.9	4	100.0	0.417	0.485
		(1.0)	(0.8)		(0.107)	(0.101)
Ghana	Kananto	13.4	5.9	100.0	0.540	0.696
		(0.7)	(0.7)		(0.068)	(0.047)
Senegal	Niocolo Koba Natl Park	15.5	5.1	87.5	0.558	0.627
		(0.3)	(0.9)		(0.105)	(0.096)

*A locus is considered polymorphic if more than one allele was detected.
†Unbiased estimate (see Nei 1978).

variable than the East and South African populations, in particular Ghana was significantly more variable than all of the other populations except for the other western population of Senegal (Table 5). Of the 104 locus per population HWE tests only three remained significant ($p < 0.05$) after Bonferroni correction: *IDVGA68* in Nysvley and Namibia, and *BMS4008* in Ghana. No population or locus consistently deviated from HWE.

Significant population differentiation was observed. There was a moderate to high level of subdivision with a global F_{ST} value of 0.171 (95% confidence interval 0.141–0.201). This value could in fact indicate an even greater level of subdivision as Hedrick (1999) argues that although F_{ST} theoretically varies between 0 and 1, this is often not the case as $F_{ST} < 1 - H_E$ and therefore with these data the maximum F_{ST} would be ~0.54. Of the pairwise F_{ST} values, 33.3% remained significant after Bonferroni correction and most of these were pairs including either Senegal or Ghana, the only two West African populations in the microsatellite analyses. In fact, Senegal and Ghana were significantly different from all other populations, including each other. Among the other, non-West African populations the only significant pairwise differences between populations with sample sizes ≥ 10 were between Nylsvley, South Africa and Malawi and between Nylsvley and Maswa, Tanzania. A Mantel test of isolation-by-distance revealed an insignificant correlation between the transformed geographical and genetic distances. Figure 4 shows the three-dimensional diagram based on the FCA. The factor that explained most of the variance, axis 1 (27.39%), clearly differentiated the two West African populations (*H. e. koba*) from all the other populations, which clustered together in the same section of the diagram. The two West African populations were also different from each other along axis 2. Figure 4 also shows that among the other subspecies, the Tanzanian populations (*H. e. langheldi*) were the most diverse. When the West African populations were excluded from the FCA this greater variation within the Tanzanian populations was even more apparent (figure not shown). With the AMOVA all the tests of differentiation at the different hierarchical levels were significant in both analyses. In the first AMOVA, using four subspecies as groups, the majority of the

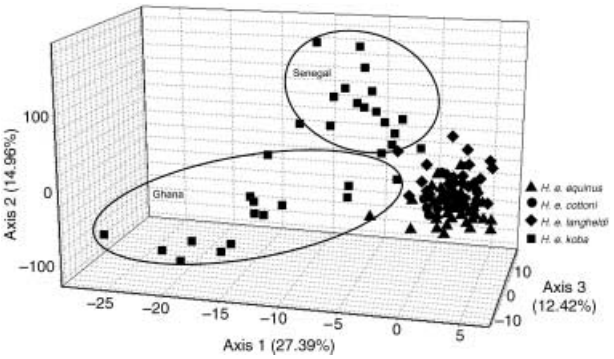

Fig. 4 Three-dimensional diagram showing the relationships among 13 roan populations based on factorial correspondence analysis (FCA) of microsatellite genotypes. Only the first three dimensions are shown, with percentage of variance explained in parentheses.

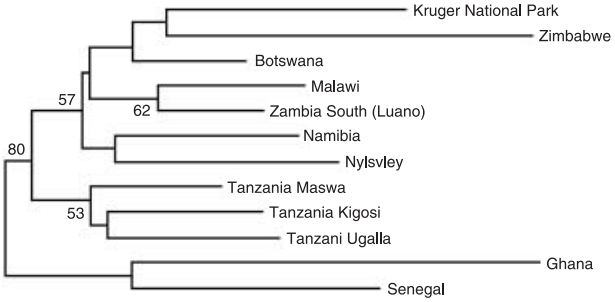

Fig. 5 Neighbour-joining (NJ) tree based on D_A genetic distances (Nei *et al.* 1983) among twelve roan populations calculated from eight microsatellite loci. Luangwa, North Zambia was excluded because of missing data for one locus. Numbers are bootstrap values from 10 000 replications.

variance, 76.80%, was within populations ($\Phi_{ST} = 0.23$, $p < 0.0001$), with slightly more variance between groups (12.78%, $\Phi_{CT} = 0.13$, $p < 0.0001$) than among populations within groups (10.42%, $\Phi_{SC} = 0.12$, $p < 0.0001$). Similarly, in the second AMOVA using two geographical groups, the majority of the variance (66.73%, $\Phi_{ST} = 0.33$, $p < 0.0001$) was within populations, with the smallest partition of variance being found among populations within groups (6.99%, $\Phi_{SC} = 0.09$, $p < 0.0001$). However, differences between the two groups accounted for 26.28% of the variance ($\Phi_{CT} = 0.26$, $p = 0.0127$). These genetic relationships among the roan populations were clearly represented by the NJ tree from genetic distances (Fig. 5). Although some structure was apparent among all the populations, most of it was only weakly supported and only one node had a bootstrap value greater than 70%: the West African populations (Senegal and Ghana) were separated from the rest of the populations by a bootstrap of 80%.

Discussion

Genetic diversity and phylogeography

The mtDNA sequence data showed comparable levels of nucleotide diversity to that found in other antelope species (0.42–5.72% in kob and 1.7% in puku populations, Birungi & Arctander 2000; 0.5–3.9% for wildebeest, 1.3–2.5% for topi and 1.5–5.4% for hartebeest, Arctander *et al*. 1999; 0.75–3.66% in impala, Nersting & Arctander 2001; 2.9–4.2% in Swayne's hartebeest, Flagstad *et al*. 2000). However, the average within-population microsatellite diversity was low relative to other species. In other studies in which microsatellite loci cloned from cattle or sheep were applied to other large ungulate species, such as Swayne's hartebeest (Flagstad *et al*. 2000) and buffalo (Van Hooft *et al*. 2000), the resultant diversity levels were higher than that found on average in the roan ($H_E = 0.70$, $A = 6.86$; $H_E = 0.76$, $A = 7.1$, respectively). Comparable levels of microsatellite variation were found, however, in some populations such as those from West Africa. The West African population of Ghana was significantly more variable than all of the other populations except for the other western population of Senegal. Although the small sample sizes for this roan study could have contributed to the lower recorded diversity levels and therefore cause the true diversity levels to be underestimated, this effect would be greater on allelic diversity than on expected heterozygosity (Nei *et al*. 1975). The expected heterozygosity levels found may reflect a genuine lower genetic diversity in roan populations than for several other studied antelope species (Arctander *et al*. 1999) as previous work on the genetic diversity of roan antelope populations based on allozymes (Grobler & Van der Bank 1993; Grobler & Nel 1996) also found lower levels of genetic diversity in the South African roan populations relative to the sable antelope (*Hippotragus niger*) and the gemsbok (*Oryx gazella*).

The relatively low to moderate variation within at least the nonwestern populations and the high level of population subdivision and significant pairwise differentiation detected by the mtDNA analyses across the species' range could indicate that the species as a whole may have undergone population crashes in the distant past. This is a scenario suggested by Grobler & Van der Bank (1993) and consistent with the theory put forward by Arctander *et al*. (1999) working on mtDNA variation in three other savannah antelope species. Arctander *et al*. (1999) studied the hartebeest, topi and wildebeest and suggested that these three species became extinct with the exception of a few refugia that subsequently underwent expansion periodically during the Pleistocene. None of the roan populations (or loci) in this study showed significant overall deviations from HWE, consistent with ancient bottlenecks allowing for the subsequent re-establishment of equilibrium. The

greater microsatellite diversity levels found in the western populations compared with the other populations, clearly shown by the FCA, may indicate a western refugia during times of range reduction. The mtDNA data, however, did not show greater diversity in the west, but similar levels of variability across the continent. The reason for this discrepancy between the microsatellite and mtDNA data is not entirely clear. Our sampling may not have been representative as in some cases there may have been a sampling bias towards related individuals within a maternal lineage if all individuals in a population sample were obtained from the same maternal group or herd (Skinner & Smithers 1990). Alternatively, the effects of lineage sorting and the smaller effective population size for mtDNA compared with autosomal markers may have meant that during population contraction to small isolated refugia in the west more mtDNA diversity was lost while microsatellite diversity was retained. Similar retention of microsatellite variation in the east and south may have been obscured by a greater movement and mixing of animals, particularly via male dispersal (Skinner & Smithers 1990) during range expansion. This scenario is consistent with the lack of significant population differences with microsatellites among the eastern and southern populations while the western populations are both significantly different to each other as well as to all of the rest.

In fact, with all of the analyses for both the mtDNA and microsatellite data there was significant structure at the hierarchical level that separated West African populations from the roan antelope populations elsewhere in Africa. Although a two-group population structure, consisting of a West African group and a group containing all other populations, explained most of the variation between groups in AMOVA analyses using both mtDNA and microsatellite data, the position of the *H. e. charicus* haplotype from Cameroon should not be considered as part of an eastern and southern clade. It is definitely not a western haplotype, but the *charicus* haplotype was much more distant from the haplotypes in the eastern clades we identified in the NCA than these haplotypes were from each other. However, we do not have any support from the analyses to define a central clade, to which haplotypes from *H. e. charicus*, and possibly, *H. e. bakeri*, could belong.

Genetic population structure at hierarchical levels below the east/west split, such as among western or eastern and southern populations, was discernable and provided a phylogeographical picture consistent with the general patterns of Pleistocene fluctuations in climate resulting in refugia with alternate expansion and contraction of populations similar to patterns found with other savannah bovids. Flagstad *et al*. (2001) emphasized an East African refugium for hartebeest, whereas Arctander *et al*. (1999) identified possible refugia in the east and west for hartebeest and topi and in the south for wildebeest. There are also indications that the eastern refuge probably comprised a mosaic of several refugia (Livingstone 1982; Arctander *et al*. 1999). The roan antelope mtDNA data are concordant suggesting refugia in the west and east of the species' range but, as suggested by the microsatellite analysis, with more recent gene flow superimposed upon this pattern at least in the east. There was some disagreement in the data as to whether the west or the east had provided the oldest refugia from which colonization took place. Although the microsatellite data may indicate a more diverse and, therefore possibly older, western refuge this was not found with the mtDNA data as when the West and East African populations were pooled into two regions the levels of mtDNA nucleotide diversity within these regions were both moderate and similar. The mismatch distribution analysis provided evidence for both a western and eastern refuge, but with the eastern refugium being older (532 000–1 028 000 years BP). Even though the exact dates should be treated with discretion, as estimates of divergence time based on control region calibration is questionable (Arctander *et al*. 1999 and references therein), the mismatch analysis revealed significantly different relative ages among clades. The largest τ was observed in the northeast clade, suggesting that this is the oldest lineage and the site of the refugia from which later expansions took place, however, there is also an indication of a western refugium in Ghana from which western population expansion may have taken place. The ragged mismatch distributions for most of the regions analysed are indicative of a deep history of multiple, overlaid expansions (Rogers & Harpending 1992), but there was also evidence of significantly rapid expansion more recently at the southern and western extremes of the population range. The NCA also revealed a highly fragmented phylogeographical structure with the very large distances between sequences and clades and the overall cladogram consistent with an inference of restricted gene flow with some long distance dispersal. Significant patterns of restricted gene flow and isolation-by-distance were apparent throughout East and South Africa and long-distance colonization was inferred from the clade containing haplotypes from Tanzania, Zambia, Zimbabwe, northern South Africa and Namibia. In contrast, no isolation-by-distance was discerned with the microsatellite data, perhaps due to homoplasy in the microsatellite data, but this might also reflect male-biased dispersal. The lack of significant geographical associations with microsatellite genetic structure among the eastern populations would also be consistent with the invasion of ancient eastern refugia by the descendants of animals who had previously migrated south, similar to what Pitra *et al*. (2002) postulated had occurred in sable antelope (*H. niger*). In summary, it appears that a once large panmictic population has undergone successive contractions and expansions out of ancient refugia in both West and East Africa

with the earliest expansion event probably originating from East Africa.

The genetic structuring apparent among the roan populations showed some geographical consistency, but interestingly did not concur strongly with the previously recognized subspecies boundaries. Previous mtDNA work carried out by Matthee & Robinson (1999) found a generally concordant pattern between the genetics and the recognized subspecies, but in our study, of the five roan subspecies included, only *H. e. koba*, the West African subspecies, held up strongly against the genetic data. With the other four, *equinus*, *cottoni*, *langheldi* and *charicus*, whose distributions range from Cameroon to South Africa, there were no strong phylogenetic associations corroborating the subspecies designations. It is important to note that some geographical regions were present in the mtDNA analyses, but were not represented in the microsatellite analyses. In fact, the subspecies *H. e. charicus* was not included in the microsatellite analyses and in the mtDNA analyses only represented by one haplotype. However, little support was found for a population structure based on the recognized subspecies in AMOVA using either mtDNA or microsatellite data; more variance was found within subspecies groups than between them. Pitra *et al*. (2002) analysing mtDNA in a related species, sable antelope (*H. niger*), found similar integration between eastern and southern subspecies previously identified from morphological characters.

Evolutionarily significant units (ESUs) and conservation strategies

Both the mtDNA and microsatellite variation revealed significant and historical separation between populations in West Africa and the rest of the continent. The monophyly of the western populations allows the designation of at least one ESU within the roan antelope species to the West of Africa and corresponding to the subspecies *H. e. koba*. Prudent conservation practice would maintain the genetic integrity of this east/west separation and prevent homogenization of the species. It would therefore be recommended to conservation managers of roan antelope populations that at the very least translocations between these broadly designated areas be avoided. As the data were either missing or limited for the investigation of the position of *H. e. charicus* and *H. e. bakeri* it would also be advised that the precautionary principle be applied and the translocation of animals from the geographical areas associated with these subspecies be avoided. Movement of animals around the remaining regions of the roan antelope's range would be considered less of a conservation concern. This is particularly the case as, for example in southern Africa, the situation with popular game species such as roan is often far from a natural state and taking a strict conservationist view is no longer practical. What is required is a pragmatic workable management plan that incorporates science, game ranch economics and social history. Different countries throughout Africa, however, will have different management situations and these countries may have other more serious threats to wildlife conservation to contend with than game ranch translocations, such as habitat destruction, uncontrolled poaching, excess hunting and civil unrest.

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This research was conducted while Deryn Alpers was a postdoctoral fellow in Terry Robinson's laboratory. Deryn's research covers the conservation and phylogenetics of a range of vertebrates. She is currently a research associate in the Department of Veterinary Biology at Murdoch University, Western Australia. Peter Arctander heads an evolutionary biology department with a research focus on population evolution and phylogeny, as well as the evolutionary studies of molecular processes (http://www.zi.ku.dk/evolbiology). Terence Robinson and Bettine Jansen van Vuuren's research includes projects in conservation genetics, molecular and conventional cytogenetics, and phylogenetics (http://www.sun.ac.za/zoology/).
