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An exploratory analysis of geographic genetic variation in southern African nyala (*Tragelaphus angasii*)

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Abstract

We report patterns of genetic variation based on microsatellite, allozyme and mitochondrial control region markers in nyala from geographic locations sampled in South Africa, Mozambique, Malawi and Zimbabwe. Highly significant differences were observed among allele frequencies at three microsatellite loci between populations from KwaZulu-Natal, Limpopo and Malawi, with the Malawi and KwaZulu-Natal groupings showing the highest differentiation ($R_{ST} = 0.377$). Allozyme frequencies showed minor, non-statistically significant regional differences among the South African populations, with maximum F_{ST} values of 0.048–0.067. Mitochondrial DNA analyses indicated a unique haplotype in each location sampled. Since none of these indices of population differentiation showed significant correlation to absolute geographic distance, we conclude that geographic variation in this species is probably a function of a distribution pattern stemming from habitat specificity. It is suggested that translocations among geographically distant regional populations be discouraged at present, pending a more elaborate investigation. Transfer of native individuals among local populations may, however, be required for minimizing the likelihood of inbreeding depression developing in small captive populations.

Key words: *Tragelaphus angasii*, evolutionary significant unit

Introduction

The nyala (*Tragelaphus angasii*) is a medium-sized African antelope with marked sexual dimorphism. The species is distributed from Malawi through Zimbabwe and Mozambique into Swaziland and South Africa (including Limpopo, Mpumalanga, and KwaZulu-Natal provinces). Nyala are associated with thickets and riverine vegetation and are seldom found more than a few 100 meters from cover (Skinner and Smithers 1990) which results in a patchy distribution throughout the range.

The translocation of conspecific game animals between farms and regions is common in the southern African game industry. Nyala are popular with tourists and sport hunters,

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providing economic benefits arising from ecotourism and hunting which, in turn, fuels the demand for permits to translocate and introduce nyala into public reserves and private game farms. This has led to nyala being translocated into Limpopo Province, where they are locally rare, from KwaZulu-Natal Province and Malawi, where their numbers are relatively high. South African conservation authorities have expressed concern that ill-advised mixing among populations could result in the swamping of distinct lineages. Conservation of genetic diversity below the species level has gained considerable prominence in recent years, with the goal of conserving not only the current spatial distribution of genetic variants, but also the processes that sustain current and future diversity (Bowen 1999; Moritz 2002). Phylogeographic information is lacking for most African mammals, including many of significant economic value to game ranching. It is not known whether possible geographic genetic differences in southern African antelope result from vicariance or have adaptive significance. Clearly therefore, there is a need for empirical studies that examine the spatial distribution of genetic variation and so inform legislation that regulates translocations of important game species.

Furthermore, commercial game ranching, the confinement of nyala on small nature reserves, and translocations of nyala have led to a fragmentation and redistribution of *T. angasii* populations. This practice has lead to an increase in the number of reproductively isolated groups which could potentially lead to inbreeding depression.

The aims of this study were: (1) to determine whether the genetic variation present in nyala is geographically structured, and (2) to determine levels of genetic diversity in geographically isolated nyala populations.

Material and methods

Populations sampled

Samples were obtained from localities in South Africa, Zimbabwe, Mozambique and Malawi (Fig. 1). Specimens included are as follows:

Limpopo Province, South Africa: 12 naturally occurring nyala specimens (i.e. the populations

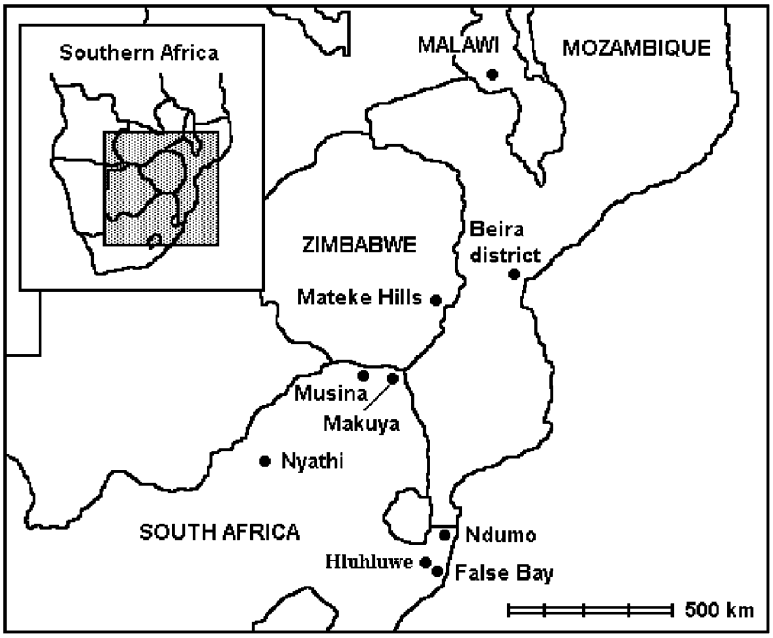

Fig. 1. Distribution of the locations sampled to assess geographical genetic variation in nyala.

have never been subjected to translocation) from the Musina and Makuya Nature Reserves, both controlled by Limpopo Province Environmental Affairs.
KwaZulu-Natal, South Africa: 12 nyala were collected in the naturally occurring populations of False Bay, Hluhluwe and Ndumo Nature Reserves. Three additional samples were also collected from the Nyathi Game Lodge (although the lodge is situated in Limpopo Province, these animals were imported from KwaZulu-Natal).
Malawi: 10 nyala from Malawi were sampled when a group of animals was translocated from Malawi into Limpopo Province. The precise origin of the Malawian specimens is not known.
Zimbabwe: A single specimen (skull) was included from the Mateke Hills, Zimbabwe.
Mozambique: One specimen was sampled in the Beira district, Mozambique.

Genetic and statistical analysis

The microsatellite study screened 12 animals from the Limpopo Province, 12 from the KwaZulu-Natal reserves and nine from Malawi. DNA was extracted using a commercial purification kit (Roche Diagnostics). Microsatellite polymorphism was screened at five loci – Bmc, Eth225 and Maf46 from Bishop et al. (1994); Bms1234 and Oarfc304 from Kappes et al. (1997). The markers were amplified in 7 µl PCR reaction volumes, with the forward primers labeled with fluorescent dyes. The reaction mixture consisted of 25–50 ng DNA, 4 pmol of each primer, 0.5 U DNA polymerase, 1 × buffer, 0.25 mM dNTP mixture, and 1.5 mM $MgCl_2$. Genotyping was done using an ABI 377 automated DNA sequencer and the GENESCAN and GENOTYPER software (Applied Biosystems). Statistical analysis of data started with screening for linkage disequilibrium (Weir 1979) using POPGENE (Yeh et al. 1999). We performed an Analysis of Molecular Variation (AMOVA), as described by Michalakis and Excoffier (1996) and implemented in ARLEQUIN (Schneider et al. 2000), to evaluate the relative components of variance within and between geographic locations. Population differentiation was also estimated using the stepwise mutation model-based *R*ST (Slatkin 1995; using *R*ST Calc, Goodman 1997). We also calculated the significance of correlations between the coefficients of population differentiation and absolute geographic distance using a Mantel test (from GENEPOP, Raymond and Rousset 1995). An assignment test was used to quantify the frequency at which individual nyala composite genotypes could be classified correctly to their respective geographical groups (using GeneClass, Cornuet et al. 1999). Genetic polymorphism in individual populations and for data pooled across the species was quantified as expected heterozygosity (H_e) and the number of alleles per locus (A), using POPGENE.
The allozyme study screened all 12 specimens representative of the Limpopo Province, as well as 12 nyala from the False Bay, Hluhluwe and Ndumo Nature Reserves in KwaZulu-Natal. Liver, muscle, kidney and blood samples were frozen in liquid nitrogen until analysis. Malawi animals could not be included in a comprehensive allozyme analysis, since only blood samples were available. We used starch gels and enzyme staining methods as described in Grobler et al. (1999). The nomenclature suggested by Van der Bank (2002) was used to designate loci and alleles. POPGENE was used to calculate allele frequencies, significance of allele frequency differences among populations, average expected heterozygosity (Nei 1975) and F_{ST} (Wright 1965). We again calculated the significance of correlations between the coefficients of population differentiation and absolute geographic distance using a Mantel test.
The mitochondrial DNA control region was sequenced for 19 nyala specimens (10 from Malawi, one from Mozambique, six from Limpopo Province [Nyathi = 3, Musina = 3], one from KwaZulu-Natal [Hluhluwe Game Ranch], and one from Zimbabwe). Genomic DNA was extracted using a standard phenol/chloroform/isoamyl alcohol protocol. Species-specific nyala primers (Nyala-L 5′-TATTTAAAC-TATTCCCTG-3′; Nyala-H 5′- TGGCTTATATG-CATGGGG-3′) were used in combination with H16498 (Shields and Kocher 1991) for amplification and sequencing of the mitochondrial DNA control region. Amplicons were extracted using the Nucleotrap Extraction Kit for Nucleic Acids (Macherey–Nagel). Manual sequencing was done with the Sequenase Kit v. 2.0 (United States Biochemical Corp.), with [α-^{32}P]dATP as the labeling agent. Single-stranded template was obtained by treatment of the purified product with dynabeads–streptavidin (Dynal AS). All DNA sequences generated were deposited in Genbank (accession numbers AY530163–AY530181). A minimum spanning network was constructed using TCS (Clement et al. 2000). The spatial distribution of mitochondrial variation was assessed using reeds AMOVA with sequence variation partitioned into within and among-population components. Phi-statistics were calculated using the TrN model (Tamura and Nei 1993), and significance values under the null hypothesis of panmixia were obtained with 1000 permutational randomizations.

Results

Microsatellite variability

Of the five microsatellite loci screened, three (Bmc3224, Bms1237, and Oarfc304) displayed allelic variation with the Eth225 and Maf46 loci fixed for single alleles (Table 1). Testing for linkage disequilibrium showed no significant ($P<0.05$) evidence of linkage between any of the microsatellite loci investigated. All populations exhibited unique alleles at the Bms1237 and Oarfc304 loci, while the Malawi population also had a unique allele at Bmc3224. Chi-square values indicated highly significant ($P<0.001$) differences among allele frequencies of the respective groups. Using an assignment test, with animals grouped as Malawi, Limpopo and KwaZulu-Natal populations in the data file, all genotypes were classified to their correct origins. Results from R_{ST} loosely correlate with geographic distribution (Table 2). The Malawi and KwaZulu-Natal groupings (geographically the most distant) showed the most differentiation ($R_{ST} = 0.377$), with slightly lower values between the Limpopo and KwaZulu-Natal groups ($R_{ST} = 0.254$). The groups from Malawi and Limpopo showed closest identity, with $R_{ST} = 0.062$.

Table 2. Population differentiation (R_{ST} values) from microsatellite data, among three regional groups of nyala

	Limpopo	KwaZulu-Natal	Malawi
Limpopo	—	—	—
KZN	R_{ST}: 0.254 ($P = 0.01$)	—	—
Malawi	R_{ST}: 0.062 ($P = 0.05$)	R_{ST}: 0.377 ($P = 0.01$)	—

Table 1. Alleles observed, allele frequencies, and coefficients of genetic diversity at five microsatellite loci in three populations of nyala. The statistical significance of allele frequency differences among populations is indicated below the locus names.

	Population			
Locus	Allele (bp)	Malawi ($n = 9$)	Limpopo ($n = 12$)	KZN ($n = 12$)
BMC3224	179	1.0	1.0	0.667
($P = 0.001$)	199	—	—	0.333
BMS1237	205	0.167	—	—
($P = 0.001$)	207	0.222	0.167	—
	209	0.611	0.125	0.042
	211	—	0.500	0.042
	213	—	—	0.167
	217	—	0.167	0.250
	219	—	0.042	—
	221	—	—	0.500
ETH225	152	1.0	1.0	1.0
MAF46	78	1.0	1.0	1.0
OARFC304	131	—	—	0.08
($P = 0.001$)	133	0.389	0.333	0.167
	135	0.333	0.333	0.167
	139	—	—	0.125
	141	—	—	0.167
	147	—	0.292	—
	149	0.278	—	—
	153	—	—	0.292
	155	—	0.042	—
Expected heterozygosity (H_e)		0.242	0.274	0.382
Average number of alleles (A)		1.8	2.4	3.0

Nevertheless, results of the Mantel test showed no significant correlation between absolute geographic distance and R_{ST} values ($P = 0.52$). Results from AMOVA show that only 12.8% of variation is found among geographic locations sampled, with 87.2% within locations. The KwaZulu-Natal population showed the highest levels of polymorphism as indicated by $H_e = 0.382$, with an average of three alleles per locus (Table 1). This compares to lower values of $H_e = 0.242$–0.274 and $A = 1.8-2.4$ in the other two populations.

Allozyme variability

Thirty-six allozyme loci were scored in nyala from Limpopo and KwaZulu-Natal provinces. Loci resolved (with EC numbers) were: Adh-1 and -2 (1.1.1.1), Ak-1 and -2 (2.7.4.3), Ck-1 and -2 (2.7.3.2), Gapdh1 and -2 (1.2.1.12), G3pdh-1 and -2 (1.1.1.8), Gpi-1 and -2 (5.3.1.9), Hk (2.7.1.1), Idh-1 and -2 (1.1.1.14), Idh (1.1.1.42), Ldh-1 and -2 (1.1.1.27), Mdh-1 and -2 (1.1.1.37), Mdhp-1 and -2 (1.1.1.40), Mpi (5.3.1.8), Pnp (2.4.2.1), Pep1-5 (3.4.-), Pgm-1 and -2 (5.4.2.2), Pgdh (1.1.1.44), Prt-1, -2, and -3, Sod-1 and -2 (1.15.1.1).

Polymorphism was found at the Ldh-1, Mdh-1 and Pep-2 loci (Table 3). Comparison of allele frequencies showed minor regional differences. At the Ldh-1 locus, nyala from Limpopo Province were fixed for a single allele, with a second allele observed in four heterozygous individuals in the KwaZulu-Natal populations. For Mdh-1, KwaZulu-Natal nyala were fixed for one allele, but a second allele was observed in one animal each from Musina and Makuya. Pep-2 showed allele frequency differences between populations. Two-way contingency chi-square analyses showed that none of the allele frequency differences at Ldh-1, Mdh-1 or Pep-2 were statistically significant ($P = 0.073-0.184$).

The F_{ST} value between nyala from Limpopo Province and KwaZulu-Natal was 0.06. To provide a measure of geographic scale, F_{ST} values were recalculated after subdividing the Limpopo sample into distinct Musina and Makuya groups (Table 4). Individual population sizes for three KwaZulu-Natal reserves were, however, considered too small for significance and could not be analysed independently. The F_{ST} values between the Limpopo populations and KwaZulu-Natal nyala were comparable at 0.048 and 0.067. In contrast, F_{ST} (0.016) was much smaller between the two Limpopo populations. The

Table 4. Pairwise comparison of F_{ST} values between Limpopo and KwaZulu-Natal populations, from allozyme results

	Musina	Makuya
Makuya	F_{ST}=0.016	—
Kwazulu-Natal	F_{ST}=0.048	F_{ST}=0.067

Table 3. Indices of allozyme genetic diversity and divergence within and among nyala populations. The statistical significance of allele frequency differences is indicated below the locus names

	Population		
Locus	Allele	KZN	Limpopo
Ldh-1	A	0.923	1.000
($P = 0.184$)	B	0.077	—
Mdh-1	A	1.000	0.909
($P = 0.073$)	B	—	0.091
Pep-2	A	0.955	0.773
($P = 0.079$)	B	0.045	0.227
Expected heterozygosity (H)		0.007	0.015
Proportion of polymorphic loci (P) (%)		5.6	5.6
Overall F_{ST}			0.06

Mantel test showed no significant correlation between absolute geographic distance and F_{ST} values, with $P = 0.5$. Average heterozygosity values suggested more diversity in the Limpopo Province compared to KwaZulu-Natal, with H values of 0.015 and 0.007, respectively, but the proportion of polymorphic loci was the same (5.6%) in both regions.

Mitochondrial control region sequence variation

Our aligned mitochondrial control region data set comprised 519 bp of which 13 characters were variable and 10 parsimony informative among nyala. All populations surveyed exhibited unique control region haplotypes. Three Nyathi specimens shared a haplotype with those from Hluhluwe which is not surprising since they were originally derived from the KwaZulu-Natal population. Sequence divergence estimates were low and ranged between 0.6% (Hluhluwe, including Nyathi vs. Zimbabwe; Malawi vs. Mozambique) and 2.1% (Mozambique vs. Zimbabwe). Phylogenetic analyses also further indicated a close association between Malawi and Mozambique populations (sequence divergence = 0.59%), and between the populations sampled in KwaZulu-Natal, Zimbabwe and the Northern Province (sequence divergence range between 0.59% and 0.98%). The Malawi–Mozambique specimens clustered with high bootstrap support (>85%) while only moderate support was found for the Zimbabwe–Musina–Hluhluwe populations (<70%). The minimum spanning network (Fig. 2) also showed these two groupings, separated by seven mutational steps. The haplotypes observed in the Malawi and Mozambique populations were separated by 3 mutational steps. Three or four steps separated populations within the KwaZulu-Natal–Musina–Zimbabwe group. Interestingly, haplotypes found in the Musina and Zimbabwe populations, the geographically closest localities, were separated by 5 steps. AMOVA analyses grouping the Malawi–Mozambique and KwaZulu-Natal–Northern Province–Zimbabwe populations indicated that 54% of the variation was among groups, with 46% between populations within groups. Since each population was characterized by a single maternal haplotype, no variation was documented within populations. An exact test of population differentiation showed no statistically significant

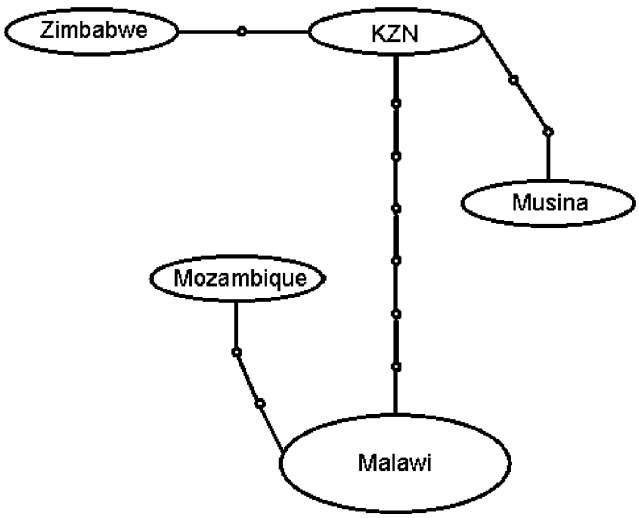

Fig. 2. Minimum spanning network showing the relationships among haplotypes detected from the mitochondrial DNA control region, for nyala from different geographic locations sampled.

differentiation among populations, although this may have been a result of small sample sizes. A Mantel test plotting *p*-distance against geographic distance resulted in an insignificant ($P = 0.23$) correlation.

Discussion

Moritz (2002) suggested criteria for the recognition of evolutionary significant units (ESUs) that require significant divergence of allele frequencies at nuclear loci and reciprocal monophyly for mitochondrial loci. The microsatellite (nuclear) markers screened during the current study showed significant divergence between regional populations at three out of five polymorphic loci. It is also notable that all nyala screened with microsatellites grouped to their correct geographical location in the assignment test. The allozyme results suggested some unique alleles in specific populations, but allele frequency differences were not significant. We note that Moritz (2002) cautions that the criterion of "significant divergence" imposes an arbitrary threshold on a continuum of divergence. It would thus be informative to screen for the possibly unique alleles observed at Ldh-1 and Mdh-1 using larger sample sizes. Our mtDNA analyses provided additional support for the recognition of ESUs, with all regional populations surveyed exhibiting unique control region haplotypes, although this support is limited considering the small sample sizes used for mtDNA analysis. The conservative deduction from combined nuclear and mtDNA results is that the existence of two potential ESUs in nyala, based on a Malawi group and a southern population, should be investigated by further sampling.

Molecular data alone may be insufficient for classification of regional populations as ESUs. Waples (1995) suggested that the concept of evolutionary significance might be too subjective to apply in practice and states that the key test question could be whether if the population became extinct, this would represent a significant loss to the ecological–genetic diversity of the species. Additionally, Moritz (2002) holds that molecular evidence for recognizing ESUs should be correlated with ecological and historical factors such as a history of fragmented isolation within species. Other factors which may warrant giving ESU status to populations include life-history traits or morphological traits that are unusual for the species, or existing in an unusual habitat relative to other populations of the same species. Current life-history and genetic data on nyala is too sparse to test these requirements. Differentiation among nyala populations is probably a reflection of a patchy distribution caused by the specific habitat preference of the species with a secondary relationship associated with geographic distance. Localized nyala populations of small effective size have presumably been subjected to the effects of random genetic drift in their historic past leading to differentiation over time. Current management practices, notably the enclosure of small nyala populations on game farms and small reserves, have presumably further reduced the frequency of genetically effective migration to below historic levels.

The challenge for conservation planners is to formulate a conservation strategy for nyala that will ensure that the processes that sustain current and future diversity are protected. The data at hand are indicative of some regional-level geographic genetic variation. Differentiation needs to be more rigorously assessed using larger sample sizes and more microsatellite loci. Given the limited data currently available it is suggested that translocations be discouraged at present.

Results from the current preliminary investigation does not suggest loss of genetic diversity at local level, but these results are mostly from relatively large populations from nature reserves and the situation may well be different on smaller enclosed farms. Until more definitive data are at hand, transfers of native animals among populations at a local scale may prove useful for reducing the likelihood of inbreeding depression developing in genetically isolated herds. Judicious translocations at local level should not affect the overall objective of conserving genetic differentiation between regional populations.

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Zusammenfassung

Genetische Variation bei Nyala (*Tragelaphus angasii*) im südafrikanischen Verbreitungsraum

Unsere Studie befaßt sich mit Mustern der genetischen Variation in Nyala-Antilopen. Beprobt wurden die geographischen Standorte Südafrika, Mozambique, Malawi und Zimbabwe. Zwischen den untersuchten Populationen von Kwa Zulu Natal, Limpopo und Malawi wiesen die Allelfrequenzen in drei Mikrosatellitenloci höchst signifikante Unterschiede auf, wobei Malawi und Kwa Zulu Natal die höchste Differentiation ($R_{ST} = 0.377$) hervorbrachte. Allozymfrequenzen zeigten kleinere, statistisch nicht signifikante regionale Unterschiede unter den südafrikanischen Populationen mit maximalen F_{ST}-Werten von 0.048–0.067. Mitochondriale DNA-Analysen deuten in jeder Population auf einen einzigartigen Haplotyp hin. Da keine dieser Anzeichen für Populationsdifferenziation einen signifikanten Zusammenhang zur absoluten geographischen Distanz unterstützen, schlußfolgern wir, daß geographische Variation in dieser Spezies wahrscheinlich eine Funktion von Verteilungsmustern als Folge von Habitatspezifität darstellt. Von der Einführung habitatfremder Tiere ist bis zu einer detaillierteren Untersuchung abzuraten. Der Transfer einheimischer Individuen unter lokalen Populationen ist dagegen zu empfehlen, um mögliche Inzuchtdepressionen, für welche kleine in Gefangenschaft lebende Populationen anfällig sind, zu minimieren.

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