Interspecific hybridization between greater kudu and nyala

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Abstract Hybridization of wildlife species, even in the absence of introgression, is of concern due to wasted reproductive effort and a reduction in productivity. In this study we detail an accidental mating between a female nyala (*Tragelaphus angasii*) and a male greater kudu (*T. strepsiceros*). The hybrid was phenotypically nyala and was identified as such based on mitochondrial DNA. Further genetic analysis based on nine microsatellite markers, chromosome number and chromosome morphology however, confirmed its status as an F1 hybrid. Results obtained from a reproductive potential assessment indicated that this animal does not have the potential to breed successfully and can be considered as sterile.

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Introduction

The nyala (*Tragelaphus angasii*) is a medium sized antelope distributed in the south-eastern parts of Africa, from Malawi through Zimbabwe and Mozambique into Swaziland and eastern South Africa (Skinner and Chimimba 2005). They have been introduced into areas outside of their historic range and now occur in Botswana, Namibia and the north-western parts of South Africa (Skinner and Chimimba 2005). The greater kudu (*T. strepsiceros*) i s a large antelope widespread throughout southern and eastern Africa and, although locally extinct in some areas due to hunting, the species' retains much of its former range (Skinner and Chimimba 2005). Within Tragelaphini there have been previous reports of hybridization, for example between eland (*Taurotragus oryx*) and greater kudu, as well as between eland and sitatunga (*Tragelaphus spekeii*) (Gray 1971). Hybridization has also been reported in a number of other ungulate taxa in South Africa such as the blue (*Connochaetes taurinus*) and black wildebeest (*C. gnu*) (Grobler et al. 2011). Anthropogenic hybridization occurs due to human interference and may lead to breeding of previously reproductively isolated taxa. Hybridization without introgression (Allendorf et al. 2001) is the mating between different species where the hybrid offspring is sterile. Sterilility can occur due to genetic incompatibility that can include either (or both) genic and chromosomal differences. The former can disrupt the cascade of events leading to the development of testis and other reproductive related occurrences (see for example Waters and Robinson

2008). Differences in the chromosomal complement of the parental species may lead to gametic imbalance due to malsegregation at meiosis. Hybridization can be problematic due to wasted reproductive effort (Allendorf et al. 2001). An example is the Cape mountain zebra (*Equus zebra zebra*) which has been reported to hybridize with feral donkeys *(Equus asinus*) resulting in sterile offspring. In this case, even without introgression, hybridization has threatened the Cape mountain zebra population due to reduced productivity (Brooke et al. 1986).

Hybridization resulting in sterile offspring has been reported in red hartebeest and blesbok as well as in eland and greater kudu (Robinson et al. 1991; Grobler and van der Bank 1995; Rhymer and Simberloff 1996). Chromo-some numbers vary widely within the Bovidae with diploid numbers (2n) ranging from 30 and 60 mostly due to Robertsonian (centric) fusions (Robinson and Ropiquet 2011). Within the *Tragelaphini* diploid numbers (2n) varies from a low of 30 in the greater kudu to 56 in the nyala. A characteristic of all tragelaphines is the presence of a Y-autosome translocation (Y;13) which results in males having one chromosome less than females (Rubes et al. 2008). The sitatunga (*T. spekei*) is an exception where both sexes are $2n = 30$. The interspecific differences in chromosomal number among the *Tragelaphini* allows for the simple detection of F1 hybrids via cytogenetics.

This paper highlights the value of using different approaches to study mammalian hybridization. Using phenotypic characteristics, conventional cytogenetics, microsatellite markers, mitochondrial DNA sequencing and a clinical reproductive assessment of the hybrid's fertility, we verified anecdotal reports of putative hybridization between a male greater kudu and a female nyala. In so doing we add to the growing literature on interspecific hybridization among African antelope.

Materials and methods

Immobilisation and sample collection

All procedures were conducted within the guidelines of the South African Veterinary Council (SAVC). The male putative hybrid was immobilised on a game farm in the North West Province of South Africa, via remote injection dart (Dan-Inject®). The shoulder height was determined in lateral recumbency and taken from the bottom of the hoof to the dorsal aspect of the scapula with the leg fully extended. The horns were measured using the Rowland Ward method for spiral-horned antelope (Ward 1903). Whole blood was collected and skin biopsies were taken and placed immediately into cell culture medium.

Microsatellite DNA analysis

DNA was extracted from the hybrid in addition to 11 nyala and 8 greater kudu reference samples using the ZR Genomic DNA™ Tissue MiniPrep (Zymo Research Corporation) following the extraction protocol. Nine cross-species autosomal microsatellites markers were used to genotype all individuals. Amplification was carried out using standard methods. Samples were run on an ABI 3130 sequencer and were genotyped using GeneMapper® v. 4.0 (Applied Biosystems, Inc.). Mean number of alleles per locus (A), observed heterozygosities (Ho) and expected heterozygosities (He) were used to estimate the level of genetic diversity (GenAlEx; Peakall and Smouse 2006). F_{ST}-based hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) was calculated using Arlequin 3.1 (Excoffier et al. 2005) in order to determine the level of genetic differentiation between species. As part of confirming the animal's hybrid status, the individual assignment of pure control samples and the putative hybrid was inferred by a Bayesian clustering analysis using STRUCTURE version 2.3.3 (Prichard et al. 2000). An a priori value of $K = 2$ accounted for the two parental species when we used the genetic admixture analysis and correlated allele frequencies model of the programme STRUCTURE. We assessed the average proportion of membership (Q_I) of the putative hybrid to the inferred clusters.

Mitochondrial DNA sequencing

All samples were identified by means of cytochrome b (Cytb) amplification and sequence analyses. Primers L14724 and H16498 were used to target a 427 bp region of the gene following standard protocols (Hsieh et al. 2001). Resulting sequence chromatograms were viewed and edited in the Chromas program embedded in MEGA5 (Tamura et al. 2011) prior to performing a BLAST nucleotide search (www.ncbi.nm.nih.gov/blast). Maximum likelihood analyses were inferred in MEGA5 while the best fit model of sequence evolution was selected under the Akaike Information Criterion (AIC) in jModeltest (Posada 2008). Nodal support for the Likelihood (ML) tree was assessed through 10,000 non-parametric bootstrap replications.

Cytogenetic analysis

Fibroblast cultures from the skin biopsies of the putative hybrid were established using conventional tissue culture techniques. Cells were grown at 37 °C in DMEM medium enriched with 15 % bovine fetal serum. Colcemid (0.01 μg/ml) was added ~60 min before harvest. Hypotonic treatment (0.075 M KCl) and fixation (3:1; methanol:

acetic acid) followed standard protocols. Air-dried metaphase preparations were made and stained with Giemsa.

Reproductive evaluation, semen collection and radioimmunoassay

The internal and external reproductive organs of the bull was examined by means of ultrasonography (Mindray Medical International Ltd.). The external organs were examined and then palpated to establish the presence of any abnormalities. Individual testicular length and circumference were measured with a calliper. Semen was collected by means of electro-ejaculation using a portable battery operated El Torro electro-stimulator (Electronic Research Group) following convensional techniques (Crosier et al. 2006). The sample was evaluated microscopically at a 200× magnification at 37 °C immediately after collection. Two sets of eosin/nigrosin smears were made. One set was made immediately (t0) and set two (t24) was made 24 h after ejaculation. These smears were examined under oil at 1000× magnification with phase contrast. For smears made at t24 the collected ejaculate was kept at 5 °C overnight in an upright position to allow the formation of sediment. After centrifugation at 300*g* for 15 min, aliquots were collected from the bottom of the tubes and a smear was made and evaluated microscopically. Whole blood samples were centrifuged (300×*g* for 15 min) and recovered serum stored at -20 °C until hormone analysis. Testosterone concentrations were determined by radioimmunoassay using a Coat-a-Count commercial kit for total testosterone (Diagnostic Products Corporation®) as previously described (Newell-Fugate et al. 2012).

Results

Phenotype

The hybrid, shown in Fig. 1c, was estimated to be 7 years of age. It was of intermediate size with a shoulder height of 120 cm and the shoulders and neck were less muscled than those of a greater kudu bull. General body colour was a fawn-grey, lighter than that of a typical nyala bull. Eleven, evenly spaced, faint white vertical stripes were visible on its flanks with only two faint white spots on the caudoventral thorax and lateral thighs. The animal had a distinct white chevron between the eyes, white lips and white spots on the cheeks, typically seen in both greater kudu and nyala bulls. The horns resemble those of a nyala bull with a fairly straight spiral and a less prominent keel than the wide corkscrew spiral and strong keel seen in greater kudu bulls. The horns were fairly smooth with whitish tips and had a length of 88 cm (34.6 inches), which exceeds the current record of 84 cm (33 inches) recorded for a nyala bull (Ward 1903).

Genetic analysis and assignment testing

A total of 35 alleles were detected with 15 specific to nyala, 15 to greater kudu; five were shared. Analysis by AMOVA (44 %) and pairwise F_{ST} ($F_{ST} = 0.498$, $P = 0.010$) indicated a high proportion of genetic differentiation among populations confirming two distinct species. This was futher suported by phylogenetic analysis. Distinct clades for greater kudu and nyala that includes the tested hybrid sample (consistent with the nyala as the female parent) was also recovered with 98 % bootstrap support (Fig. 2). Expected heterozygosity within pure control populations was 0.395 (nyala) and 0.431 (greater kudu). Observed heterozygosity was 0.528 (greater kudu) and 0.535 (nyala) respectively. The average number of alleles was similar (2.222) in both populations. Posterior probabilities (*Ln*) using bayesian admixture analysis indicated two distinct clusters (Fig. 3). The average proportion of membership for both pure populations was $Q_I > 0.995$. The criterion of $q_I > 0.90$ suggested by Barilani et al. (2007) can be used to identify individuals as either pure or hybrid. This criterion was considered adequate for this study given that the animal could clearly be identified as a hybrid ($q_I = 0.4$).

Cytogenetic analysis

Meiosis in greater kudu males (31,X,t(Y;13)) would yield 15,t(Y;13) gametes and in female nyala (56,XX), gametes with 28,X. Chromosomes of 50 metaphases were counted to establish the chromosome number of the putative F1 as $2n = 43$ (Supplementary 1). This is consistent with its status as an F1 hybrid. This is further underscored by the morphology of the hybrid's chromosomes. The male parent, a greater kudu ($2n = 31$), has a chromosomal complement that comprises a single pair of acrocentric autosomes, one unpaired acrocentric autosome (Y2), 13 pairs of bi-armed autosomes, the submetacentric t(Y;13) fusion chromosome (Y1), and an acrocentric X (i.e., 31,X,t(Y;13). The female parent, a nyala ($2n = 56$), has 26 autosomal pairs that are acrocentric in morphology, one pair that is submetacentric in morphology and two acrocentric X chromosomes (O'Brien et al. 2006) (i.e., 56,XX). Close inspection of the hybrid's chromosomes shows 28 acrocentrics (one inherited from the male kudu and 27 from the female nyala), 15 biarmed chromosomes (14 inherited from the male greater kudu and one from the female nyala gamete) in the $2n = 43$ complement.

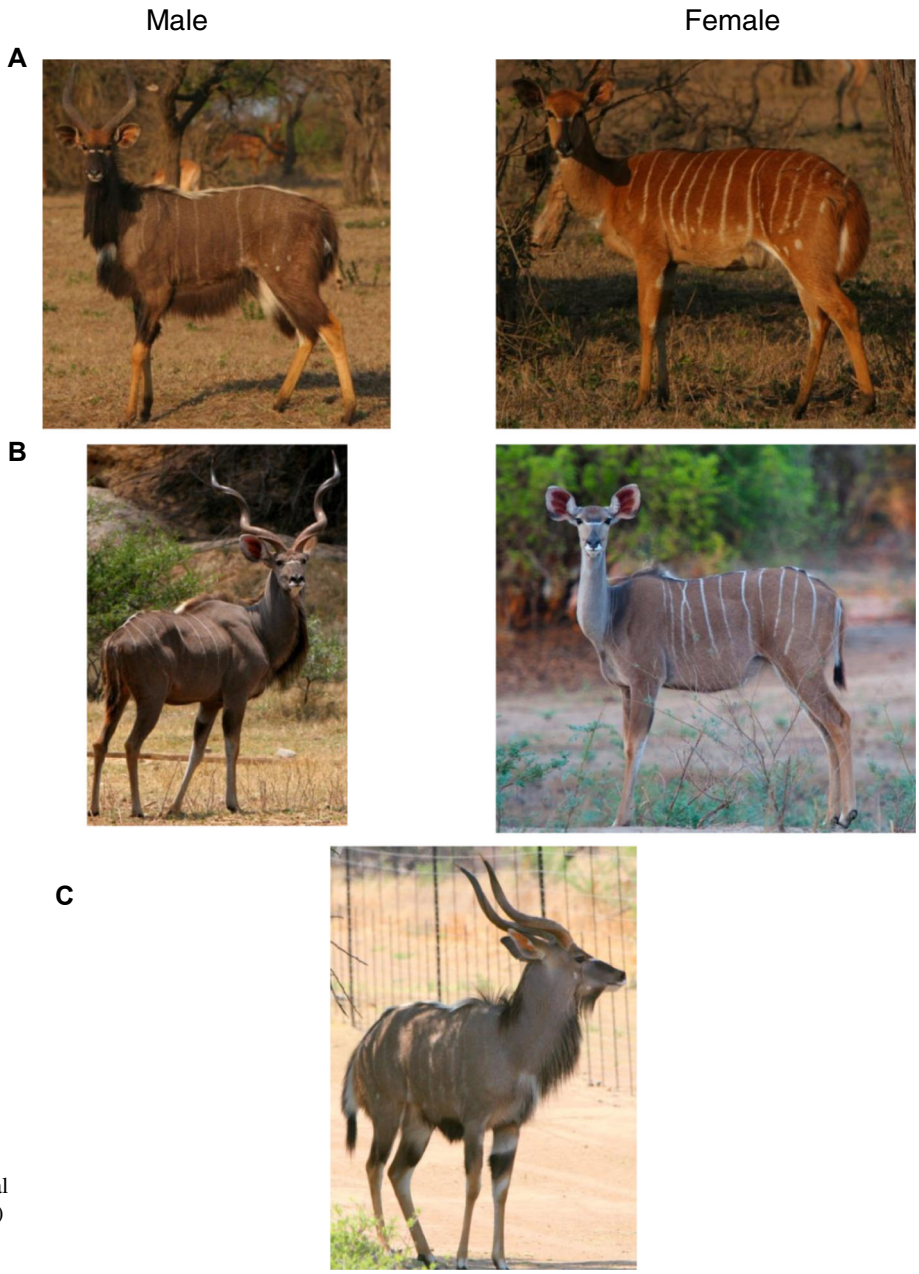

Fig. 1 Representative phenotypes of typical male and female nyala (**a**), greater kudu (**b**) and **c** the nyala × greater kudu hybrid

Reproductive analysis

The ultrasound evaluation of the internal reproductive organs and structure displayed no visual abnormalities that could interfere with the mobility of the testes within the scrotum. Both testes were symmetrical, the left testis length measured 5.8 cm with the right testis length at 6 cm. The collected ejaculate was clear in colour, the volume was 1.5 ml with pH 7.5. Evaluation of the eosin/nigrosin smears prepared at t0 had only a few epithelial cells recorded. However, smears prepared at t24 revealed the presence of additional epithelial debris and a single abnormal sperm cell as illustrated in Fig. 4. Ejaculate collected was considered azoospermic. The blood testosterone concentration measured 15.79 nmol/l and is considered high.

Discussion

This is the first study to describe the application of genetic and reproductive analysis tools to identify a hybrid animal resulting from a mating of a male greater kudu and a female nyala. Bayesian clustering, analysis of chromosome number and morphology confirmed hybridization. Although interspecific hybridization events are generally

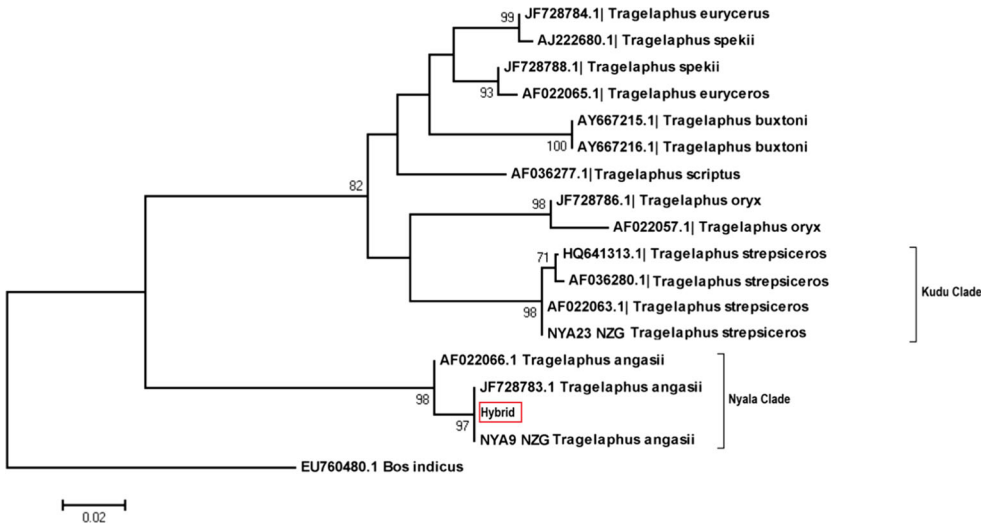

Fig. 2 Maximum likelihood tree of generated greater kudu, nyala and kudu-nyala hybrid *Cytb* gene fragments in combination with reference samples acquired from Genbank. All reference samples are prefixed with relevant Genbank accession numbers, while samples generated in this study are indicated with NYA. The hybrid sample is indicated in red

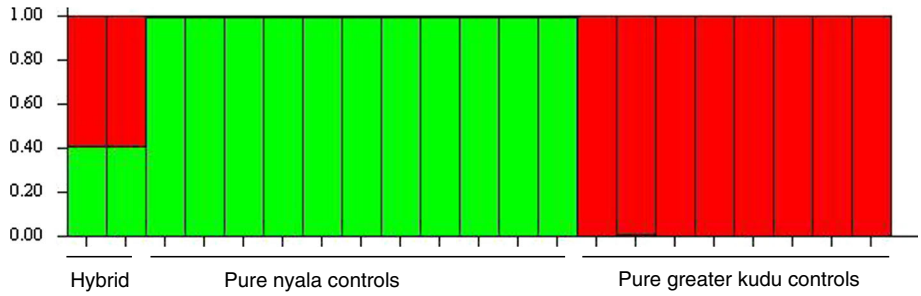

Fig. 3 STRUCTURE analysis (performed with K = 2) of microsatellite genotypes of pure nyala, pure greater kudu and hybrid animal (in duplicate). Each individual is represented by a *single vertical line*, with lengths proportional to the estimated membership in each cluster

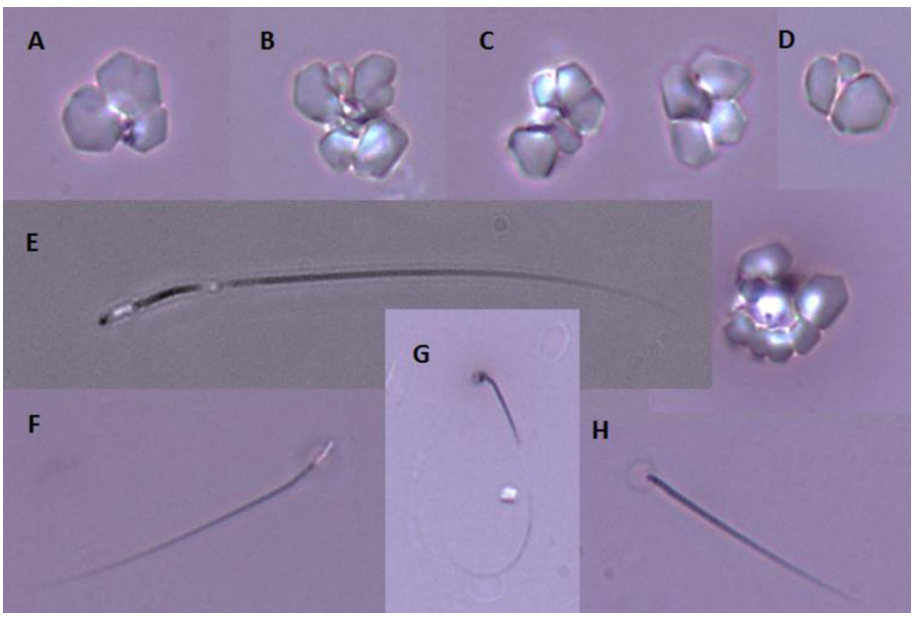

Fig. 4 Images recorded during evaluation of eosin/nigrosin smears (t24) taken at 1000× magnification. **a**–**d** series of epithelia cells possibly originating from the epididymal epithelial cells and squamous epithelial cells possibly derived from desquamation of the preputial epithelium cell. **e**, **f** Abnormal spermatozoa showing head, tail and abnormal mid-piece (spermatogenic defect). **g**, **h** Loose tail of spermatozoa

considered rare, crosses have been recorded between *T. scriptus* × *T. oryx* (Gray 1971) while other instances of interspecific hybridization among *Tragelaphini* (held under captive conditions) have been recorded between *T. spekei* × *T.* (*Boocerus) euryceros* and *T. spekei* × *T. imberbis* (Gray 1971). Hybrid offspring were recorded from all three

crosses (a fertile female hybrid in the case of *T. spe-kei* × *T. euryceros* (Gray 1971)). Hybridisation is often the result of situations where access to conspecifics is denied (Robinson et al. 2005), or where species that are found in separate, non-overlapping geographic areas (allopatry) are brought into artificial contact. Hybridization in the present study may have been due to the occurrence of both species in very low numbers on the game ranch, resulting in lim-ited access to conspecifics.

Based on the reproductive potential assessment of this individual we consider it to be sterile. The immature size of the scrotum, testes and penis was immediately evident. The size of the testes and penis resembled those of a juvenile and not a 7 year old bull. Reduction in testes and penis size has similarly been reported in hybrid male Arabian oryx (*Oryx leucoryx*; Eljarah et al. 2012). The authors indicated that the average testis length in the latter measured 5.9 cm, nearly half of the average measured for bulls under the age of 2 years. The scrotum circumference measured 14 cm in the hybrid, significantly less that the average scrotum circumference of 24 cm (Schoeman et al. 1987). The blood serum testosterone level in the hybrid animal identified in this study was high and is consistent with reports that the hybrid had been observed mounting cows on the farm. Studies conducted on the mating performance in Dorper rams indicated that there was a statistical non-significant correlation between the testis size and concentration level of plasma testosterone (Schoeman et al. 1987). The high concentration plasma testosterone levels recorded during examination of the hybrid could have been due to numerous contributing factors. The hybrid was examined during the start of the breeding season. In addition, the male hybrid in this case was the only bull with a harem of females, increasing the number of mating opportunities.

Although observations of sterility are consistent with the meiotic impairment anticipated from the chromosomal differences between the parental karyotypes, a single spermatozoan was nonetheless observed in our microscopic screening of the ejaculate (Fig. 4e, f). However, although some meiotic activity appears to occur in the greater kudu x nyala hybrid, the spermatozoan identified from t24 eosin/nigrosin is considered grossly abnormal (defect with a spermatogenic origin). The presence of abnormal spermatozoa has been previously reported in the ejaculate of a hybrid hinny (*Equus caballus* × *E. asinus*). The authors however indicated that spermatogenesis was disrupted at pachytene (Chandley et al. 1974). However, further analysis on other sterile hybrids within *Tragelaphus* would be required to confirm the occurrence of abnormal spermatozoon.

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Supplementary Material

Supplementary 1: Karyotype of the nyala x greater kudu hybrid.