

Genetic diversity in natural range remnants of the critically endangered hirola antelope

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The hirola antelope (*Beatragus hunteri*) is considered to be the most endangered antelope in the world. In the *ex situ* translocated population at Tsavo East National Park, calf mortality and the critically low population numbers might suggest low genetic diversity and inbreeding depression. Consequently, a genetic study of the wild population is pivotal to gain an understanding of diversity and differentiation within its range before designing future translocation plans to increase the genetic diversity of the *ex situ* population. For that purpose, we assessed 55 individuals collected across five localities in eastern Kenya, covering its entire natural range. We used the complete mitochondrial DNA control region and microsatellite genotyping to estimate genetic diversity and differentiation across its range. Nuclear genetic diversity was moderate in comparison to other endangered African antelopes, with no signals of inbreeding. However, the mitochondrial data showed low nucleotide diversity, few haplotypes and low haplotypic differentiation. Overall, the inferred low degree of genetic differentiation and population structure suggests a single population of hirola across the natural range. An overall stable population size was inferred over the recent history of the species, although signals of a recent genetic bottleneck were found. Our results show hope for ongoing conservation management programmes and that there is a future for the hirola in Kenya.

ADDITIONAL KEYWORDS: *Beatragus hunteri* – conservation genetics – Kenya – sanctuary – translocation.

INTRODUCTION

Conservation concerns typically arise for small or much reduced populations with low reproductive rates, which are vulnerable to extinction before they can adapt to new environmental challenges (Lynch & Lande, 1992). Often such populations have suffered from human activities forcing the species to suboptimal habitats at

the edges of their range (Channell & Lomolino, 2000). Such conservation concern for a threatened species may require urgent measures, and this urgency often does not permit an initial assessment of all-important factors for such actions. The assessment of the genetic diversity of such populations has often been ignored as a first step in conservation actions, despite its relevance in influencing and enhancing individual fitness and, ultimately, population persistence (Hedrick & Fredrickson, 2010; Weeks *et al.*, 2011; Batson *et al.*, 2015; Vaz Pinto *et al.*, 2015; Jansen van Vuuren *et al.*, 2017).

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Genetic evaluation of threatened populations is now considered key in recovery programmes that are being carried out through translocations (Pimm *et al.*, 2006; Hedrick, 2014; Batson *et al.*, 2015) by augmentation, introduction or re-introduction of individuals (IUCN, 1987; Weeks *et al.*, 2011; Kelly & Phillips, 2016).

The hirola antelope, *Beatragus hunteri* (Sclater, 1889), is ranked 69th in the Evolutionarily Distinct and Globally Endangered (EDGE) species list (www.edgeofexistence.org; accessed September 2019). It is endemic to north-east Kenya and, historically, also occurred in south-west Somalia (IUCN SSC Antelope Specialist Group, 2017). Karyotypic and phylogenetic analyses (Kumamoto *et al.*, 1996; Pitra *et al.*, 1997; Steiner *et al.*, 2014) support the placement of the hirola into its own genus. Its population decreased from ~14 000 in the 1970s to < 250 individuals today. It is listed as Critically Endangered under category A2 and C2, because the hirola has shown a continuing decline of > 80% over the past 16 years and, based on direct observation, a decline in area of occupancy and habitat quality, and levels of exploitation are continuing (IUCN SSC Antelope Specialist Group, 2017). If this species were to go extinct, it would be the first mammal genus to have disappeared since the extinction of the Tasmanian tiger, *Thylacinus cynocephalus* (Harris, 1808).

The main reason for this steep decline in numbers and range of the hirola was an outbreak of rinderpest virus (*Morbillivirus*) in the 1980s that led to mass

mortality of ruminants in eastern Kenya (Kock *et al.*, 1999). Poaching, predation, competition with livestock, habitat loss and degradation may also have contributed to this recent decline (Butynski, 2000; Andanje, 2002; Ali *et al.*, 2014a). The causes of non-recovery of hirola populations after the eradication of the rinderpest virus are yet to be understood fully, although habitat loss owing to tree encroachment has been pointed out as the main factor (Ali *et al.*, 2017).

In 1963, the first conservation efforts consisted of the translocation of wild animals from the Garissa district to the Tsavo East National Park (Fig. 1). This translocation to a new environment, although proximal to the original range of the species, might require genetic adaptation to this new ecosystem. An additional translocation was carried out in 1996, with the intention of boosting the genetic composition of the Tsavo population to help the persistence of the only *ex situ* population of hirola (Andanje, 2002). Surveys in 1995 and 2000 estimated the population to be 76 and 77 animals, respectively (Andanje, 1997, 2002; Butynski, 2000). The last aerial survey at Tsavo, carried out in 2011, suggests a smaller population of 67 hirola in nine herds (Probert *et al.*, 2014). As in other unsuccessful translocations (Pérez *et al.*, 2012), calf and juvenile mortality remained high in this population (Andanje & Ottichilo, 1999; Probert, 2011), suggesting signs of inbreeding (Berger, 1990; Butynski, 2000).

In 2012, a predator-proof fenced sanctuary was created in Ishaqbini Community Conservancy,

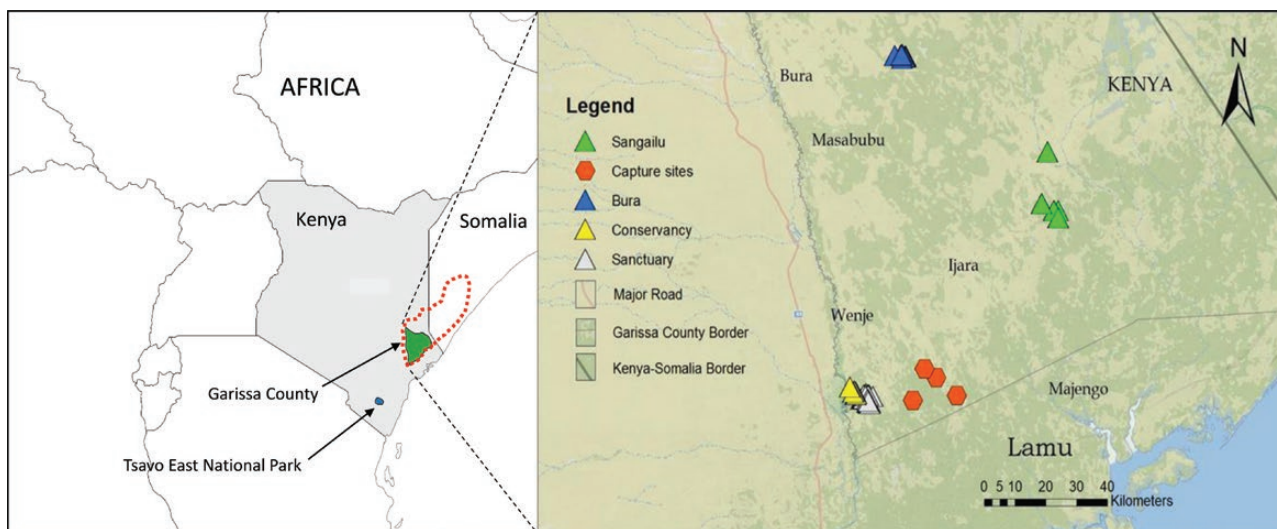


Figure 1. Study area and sampling points. Left image is East Africa and Kenya, showing the *ex situ* Tsavo East National Park hirola translocated population (in blue) and the Garissa conservancy (in green), representing the total distribution of the species. The red dotted area represents the formed species distribution. The right map is the Garissa County, from where all five conservancy localities were sampled. Abbreviations: BURA, Bura East conservancy; CONS, Conservancy (Ishaqbini Community Conservancy); SANG, Sangailu (Sangailu community conservation area); SANT, Sanctuary (2012 predator-proof fenced sanctuary); TRAN, capture sites (translocated; original capture sites for the 2012 Sanctuary).

POPULATION DIFFERENTIATION AND STRUCTURE

Analysis of molecular variance (AMOVA) and the pairwise fixation index (F_{ST}) were quantified in ARLEQUIN on both the mitochondrial and microsatellite datasets. In addition, for the mitochondrial dataset, a median-joining haplotype network (Bandelt *et al.*, 1999) was constructed using POPART v.1.7 (Leigh & Bryant, 2015) and included the museum and GenBank sequences. For the microsatellite dataset, a factorial correspondence analysis was performed in GENETIX v.4.05 (Belkhir *et al.*, 2004), together with a Bayesian analysis in STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000). A total of five independent simulations were run under models of admixture and correlated allele frequencies, starting with a burn-in of 500 000 iterations, followed by 1 000 000 Markov chain Monte Carlo (MCMC) runs, with values of clusters (K) set from one to ten. The results were processed using STRUCTURE HARVESTER v.0.6.94 (Earl & von Holdt, 2012; Supporting Information, Supplementary Material and Methods).

DEMOGRAPHIC CHANGES

Mitochondrial and microsatellite datasets (the five localities together) were used to infer demographic changes of the hirola across time. In the case of the mitochondrial dataset ($N = 52$), the neutrality tests Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) were calculated in ARLEQUIN v.3.5. Effective population size changes through time were inferred according to a Bayesian skyline plot constructed in BEAST v.1.8.2 (Drummond & Rambaut, 2007) and the nucleotide substitution model HKY as inferred in jModelTest2 v.2.1.4 (Posada, 2008; Supporting Information, Supplementary Material and Methods). For the microsatellite dataset ($N = 54$), BOTTLENECK v.1.2.02 (Cornuet & Luikart, 1996) was run to infer recent population bottlenecks. One thousand simulations were

performed using the two-phase mutation model, with a 70% stepwise mutation model and a 30% infinite allele model (Di Rienzo *et al.*, 1994). Wilcoxon signed-rank tests (one-tailed, for heterozygote excess) were adopted to test the significance of the analysis (Luikart *et al.*, 1998). In order to time calibrate the population tree, we fixed a strict clock and the mutation rate in the *CR* gene fragment to 6.5×10^{-8} substitutions per site/year, as used for the same locus in the roan antelope (Alpers *et al.*, 2004) and similar to the hartebeest (*Alcelaphus buselaphus* Pallas, 1766; Flagstad *et al.*, 2000) and the African buffalo *Syncerus caffer* Sparrman, 1779 (Van Hooft *et al.*, 2002). We selected a diffuse gamma distribution (shape = 1, scale = 1×10^{-8}). We ran two independent MCMC chains, each with 20 million states and sampling every 2000th state. Independent runs were evaluated for convergence and mixing by observing and comparing traces of each statistic and parameter in TRACER v.1.6 (Rambaut & Drummond, 2007; <http://beast.bio.ed.ac.uk/tracer>). We considered effective sampling size (ESS) values > 200 to be good indicators of parameter mixing. The first 10% of each run was discarded as burn-in, and samples were merged using LogCombiner v.1.8.2 (Drummond *et al.*, 2012).

RESULTS

MICROSATELLITE DATASET

Thirty-four of the 70 individual faecal samples obtained resulted in duplicate genotypes and were excluded from population genetic analyses. The final dataset consisted of 55 unique genotypes obtained from faecal samples ($N = 36$), from blood ($N = 18$) and from the museum tissue sample ($N = 1$). The number of individuals identified per sampling locality varied between three in SANG and 21 in SANT (Table 1).

The panel of 14 polymorphic microsatellites showed a low probability of finding two identical genotypes

Table 1. Genetic diversity parameters estimated for each sampling locality and for the overall dataset for both the 14 autosomal loci and the mitochondrial DNA control region (934 bp)

Population	Microsatellite markers							Mitochondrial DNA control region				
	N	N_a	H_o	H_e	F_{is}	AR	PA	N	N_h	H_d	S	π
TRAN	18	3.0	0.531	0.546	0.014	2.36	2	18	4	0.585	5	0.0015
SANT	21	2.79	0.531	0.529	-0.004	2.25	3	19	3	0.368	2	0.0005
CONS	8	2.71	0.545	0.558	0.025	2.34	1	8	3	0.607	4	0.0020
BURA	4	2.36	0.429	0.526	0.209	2.25	0	4	2	0.500	1	0.0005
SANG	3	2.36	0.524	0.533	0.022	2.36	1	3	3	1.000	6	0.0042
Overall	54	3.286	0.512	0.551	0.022	2.35	–	52	5	0.583	6	0.0014

Abbreviations: AR, allelic richness; BURA, Bura East; CONS, Ishaqbini Community Conservancy; F_{is} , inbreeding coefficient; H_d , haplotype diversity; H_e , expected heterozygosity; H_o , observed heterozygosity; N , sample size; N_a , mean number of alleles; N_h , number of haplotypes; π , nucleotide diversity; PA, number of private alleles; S , polymorphic sites; SANG, Sangailu; SANT, predator-proof fenced sanctuary; TRANS, individuals translocated in 2012.

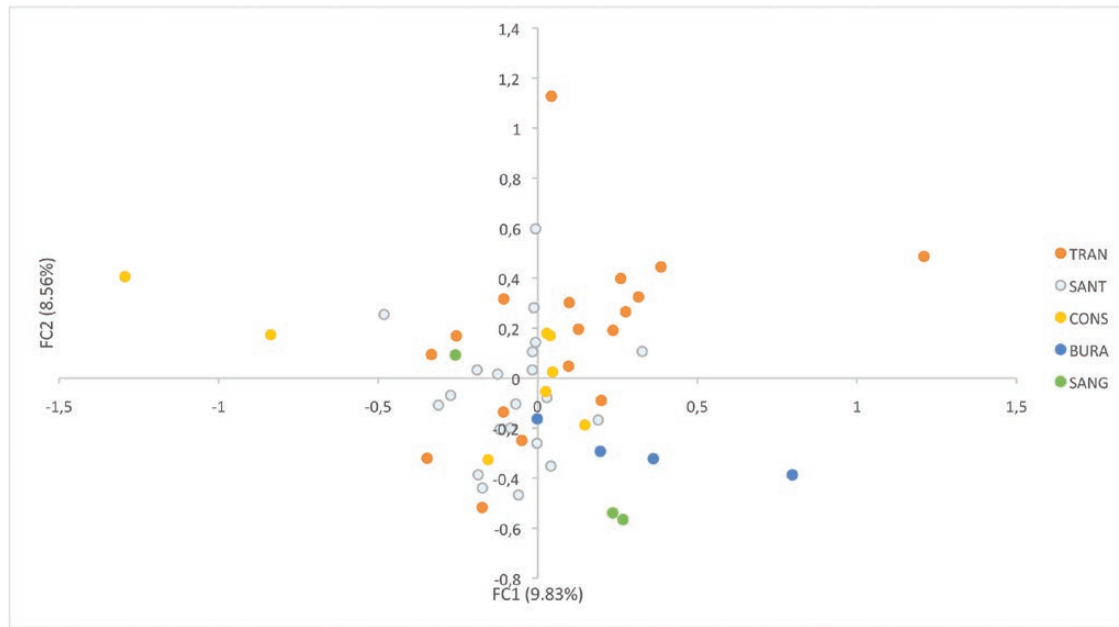


Figure 3. Factorial correspondence analysis performed in GENETIX using the 14 microsatellite markers. Abbreviations: BURA, Bura; CONS, Conservancy; SANG, Sangailu; SANT, Sanctuary; TRAN, translocated (original capture sites for the 2012 Sanctuary).

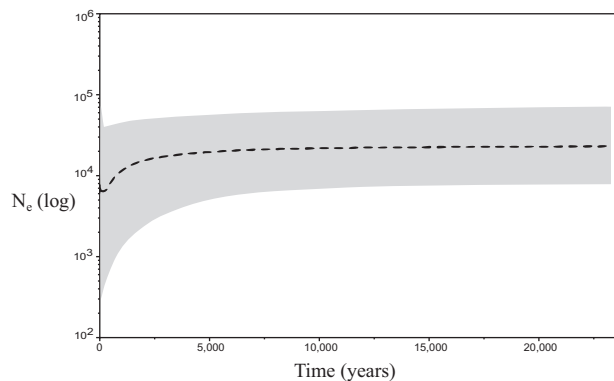


Figure 4. Mitochondrial control region Bayesian skyline plot. The y -axis indicates population size (N_e) and the x -axis represents time (in years) from present to past. The continuous line represents the median estimate, and the grey area shows the 95% confidence interval.

inbreeding depression. Here, we follow the assignment of a single wild hirola population according to the population definition based on ecological, evolutionary and statistical paradigms (see the review by Waples & Gaggiotti, 2006). The overall genetic diversity estimates in the wild range are moderate for nuclear loci and low for mitochondrial loci. Although signals of a recent genetic bottleneck are observed, no evidence of inbreeding is detected, and overall genetic diversity parameters are similar throughout the population, which might

indicate that all sampled areas could potentially be suitable for translocation purposes. Homogeneous genetic diversity throughout the reduced natural range of the hirola population is likely to indicate bottleneck effects increased through genetic drift. The lack of weak population differentiation suggests connectivity of herds throughout the species range, at least until recent times, and that ecological and ethological factors have been key to the survival of this species through herd mixing, which hints at the idea that such factors are likely to be absent for population growth in Tsavo. Population admixture maintains genetic variation and prevents genetic depression (Franklin, 1980). However, it is also a reason for failed translocations, resulting in outbreeding depression and genetic introgression from captive stocks (Storfer, 1999; see review by Bubac *et al.*, 2019). Such negative effects have been attributed to different selection pressures of populations causing population declines mostly by decreased fitness in suboptimal habitat conditions (Stearns & Sage, 1980; Dhondt *et al.*, 1990; King & Lawson, 1995; Storfer & Sih, 1998). The weak signs of a population structure throughout the natural range of the hirola indicate that such detrimental effects are unlikely in the remnant population range, but might be a concern in Tsavo National Park owing to limited habitat suitability. These findings are relevant for future and ongoing conservation management programmes, and a genetic characterization of this population would be essential to evaluate possible future translocations.

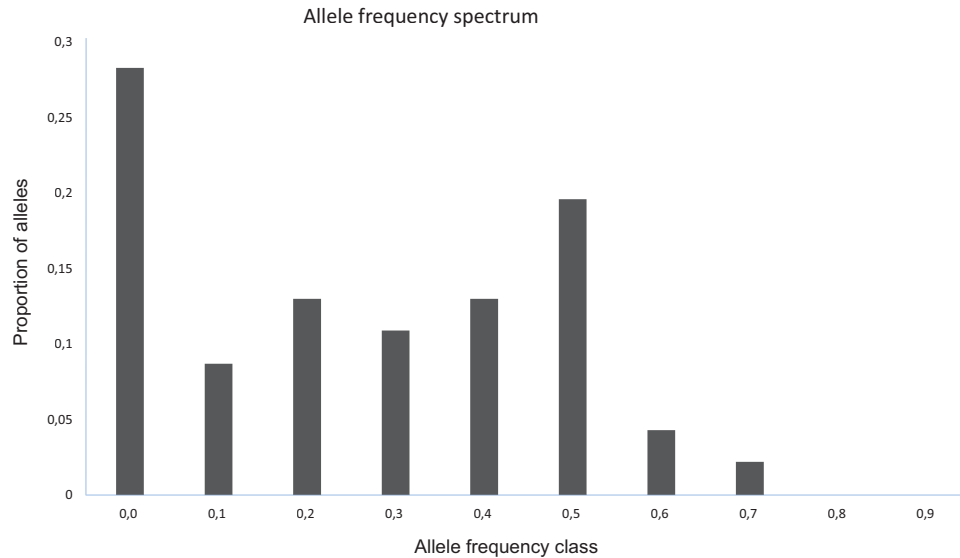


Figure 5. Plotting of allele frequency spectrum for the 14 microsatellite markers. Numbers along the x-axis represent classes of frequency of alleles (e.g. 0.0 represents alleles with frequency < 0.1). The observed ragged pattern suggests the occurrence of a recent bottleneck.

The overall mitochondrial genetic diversity is remarkably low when compared with populations of other endangered antelopes, such as the roan antelope [*Hippotragus equinus* (É. Geoffroy, 1803); Alpers *et al.*, 2004], dorcas gazelles [*Gazella dorcas* (Linnaeus, 1758); Godinho *et al.*, 2012], scimitar-horned oryx [*Oryx dammah* (Cretzschmar, 1827); Iyengar *et al.*, 2007], saiga [*Saiga tatarica* (Linnaeus, 1766); Campos *et al.*, 2010], mountain gazelle [*Gazella gazella* (Pallas, 1766)] and acacia gazelle [*Gazella arabica acaciae* (Mendelsohn, Groves & Shalmon, 1997); Hadas *et al.*, 2015]. Limited mitochondrial variation and weak population structure might reflect a historical pattern, as a consequence of one or more episodes of severe population declines in the distant past. This scenario would fit with the contraction of the species into refugia throughout the Pleistocene, similar to what has been described for other African bovids, such as the roan antelope, the hartebeest, the topi [*Damaliscus lunatus* (Burchell, 1824)] and the wildebeest [*Connochaetes taurinus* (Burchell, 1823)] (Arctander *et al.*, 1999; Alpers *et al.*, 2004).

Nevertheless, demographic analyses suggest a stable population size within the recent history, with only a non-significant population decrease in recent times, mostly throughout the Holocene.

Nuclear genetic diversity is moderate, within the range reported for other endangered antelopes that have experienced severe population declines in the past few decades, such as some populations of the roan antelope and captive/semicaptive populations of dorcas gazelles (Alpers *et al.*, 2004; Godinho *et al.*,

2012) and the South African oribi antelope (*Ourebia ourebi ourebi* Zimmermann, 1782; Jansen van Vuuren *et al.*, 2017). Interestingly, other species that have also suffered population declines, such as Swayne's hartebeest [*Alcelaphus busephalus swaynei* (Sclater, 1892)], which is an endangered subspecies from the same family as the hirola (Alcelaphinae), have higher genetic diversity than hirola (Flagstad *et al.*, 2000). In contrast, the genetic diversity of the hirola is higher than, for instance, for populations considered also to have sustained population bottlenecks, the Angolan giant sable (*Hippotragus niger varianti* Thomas, 1916; Vaz Pinto *et al.*, 2015) and the Parque Lecocq Zoo population of addax [*Addax nasomaculatus* (Blainville, 1816); Armstrong *et al.*, 2011]. Such a wide range of genetic diversity values observed across several antelope populations might reflect different levels of diversity before their recent population demographic changes or different magnitudes in the genetic bottlenecks experienced. Additionally, the different diversity values exhibited by several threatened antelope populations might also be a consequence of the use of different microsatellite panels (Queirós *et al.*, 2015). Interestingly, the museum sample, dating to 1937, did not present any different alleles from the contemporary samples, probably exhibiting the most common alleles present throughout the population before its decline and those that remained in the population thereafter. Additional samples from specimens acquired before the population bottleneck would clarify this issue and would also allow direct quantification of the diversity crash, but such museum

samples are extremely rare. The presence of private alleles in all localities except Bura suggests some degree of genetic differentiation among the localities, but is not sufficient to define a pattern of population structure.

ECOLOGICAL INSIGHTS

Since the 1980s, the hirola has declined dramatically in numbers, and its distribution has been reduced and fragmented (Butynski, 2000; Andanje, 2002; Ali *et al.*, 2014b, 2017; Ali, 2016), which is likely to restrict gene flow. However, our data suggest admixture between localities. This pattern could be explained by behavioural features of hirola, because individuals cover long distances to find forage in the dry season (Butynski, 2000) and have a tendency to change groups or form new ones (Andanje, 2002), contrasting with philopatric habits in other species that present a population structure (Simonsen *et al.*, 1998; Fernando *et al.*, 2000). In addition, no differentiation was expected between the sanctuary and the conservancy individuals owing to the small geographical distance between the capture sites used for the translocation into the sanctuary. Furthermore, the fenced sanctuary is too recent (5 years between translocation and sample collection) for individuals there to have differentiated genetically from the wild herds.

Despite the small numbers of hirola remaining in the wild, the estimated levels of genetic diversity were moderate, with no signs of inbreeding. The sanctuary provides clear evidence that this species would probably be able to increase in numbers in a predation-free area with suitable habitat, with no signs of inbreeding depression, which is consistent with the moderate estimates of genetic diversity obtained in the present study. Overgrazing by livestock, megafaunal extirpation and fire suppression are believed to be factors causing tree encroachment and subsequent lack of forage in the habitat of hirolas (Riginos, 2009; Goheen *et al.*, 2013; Daskin *et al.*, 2016; Ali *et al.*, 2017). Ali *et al.* (2017) found that tree encroachment was the main factor affecting habitat availability and that habitat suitable for hirola had decreased by 75% between 1984 and 2012. Despite studies predicting that tree cover increases predation rates on hirola, an assessment of the causes of mortality failed to support such conclusions (Ali *et al.*, 2017).

The factors suppressing the population growth of hirola in the wild (i.e. illness, predation, demographics, natural range) remain uncertain. Nevertheless, demographics and habitat range suitability might pose a constraint to hirola population growth. As an example, coastal topi [*Damaliscus lunatus topi* (Blaine, 1914)] suffered a severe population crash caused by

the rinderpest virus (*Morbillivirus*) but recovered fully, aided by their larger population size and more extensive natural range. In contrast to hirola, topi also extended their range in the dry season into the moist coastal forests of eastern Kenya, diminishing the lack of forage caused by the increase in tree cover (Butynski, 2000; Ali, 2018). Predation is also considered as one of the main factors suppressing population growth of hirola, but only when combined with other factors (Ali, 2016; Ali *et al.*, 2018). In fact, predators of hirola have not increased in abundance (Ali, 2016; Ali *et al.*, 2018). This implies that, as in other species, it is probable for hirola to persist with one of these factors but not in the presence of multiple stressors (Godinho *et al.*, 2012; Ali, 2016).

CONSERVATION IMPLICATIONS

Despite documented cases of a rapid increase in population numbers of the species through genetic capture at the predator-free sanctuaries (Weeks *et al.*, 2011), as may seem the case at the hirola sanctuary, its fenced perimeter might also constrain movements to more suitable habitats. For example, a severe nationwide drought reportedly killed 23 animals in 2017 (Cherver, 2018) as a consequence of limited habitat suitability within the sanctuary.

Conservation measures should aim to improve rangeland quality and maintain existing protected areas for hirola recovery. Nevertheless, the lack of human-mediated gene flow between managed populations is likely to decrease levels of genetic diversity and lead to inbreeding depression (Buk *et al.*, 2018; Serrouya *et al.*, 2019). It is therefore pivotal to secure larger habitat connectivity and range-wide conservation efforts. In addition, evidence of a genetic bottleneck suggests that this species has a higher chance of losing diversity over time and, as such, a lower chance of adaptation to changing environments. It is thus advisable to maintain gene flow among herds through habitat connectivity by reducing tree encroachment. In this sense, the current lapsset infrastructural project (<http://www.lapsset.go.ke>), set to run through the middle of the geographical range of the hirola, is likely to interrupt the connectivity among herds.

One of the future objectives of the sanctuary is to reintroduce individuals into the wild once the population numbers increase (King *et al.*, 2014). However, reintroductions through genetic restoration or genetic adaptation (Weeks *et al.*, 2011) should also be aimed at increasing genetic diversity in the sanctuary and at Tsavo East National Park and not only at increasing the number of individuals in the wild. The low number of founders in these two areas and the possibility of outbreeding depression through admixture of different gene pools should be evaluated carefully owing to higher

risk of genetic erosion at the sanctuary and at Tsavo. Although genetic parameters are similar between the sanctuary and the wild populations, they remain unknown at the Tsavo East National Park population. Therefore, it is pivotal to ensure long-term genetic monitoring programmes in this species, particularly in fenced populations, in order to avoid the loss of genetic diversity and to support management decisions. Additionally, future studies should take advantage of technological advances in conservation genetics, such as the implementation of genetic tagging, to understand spatial variation in population density, temporal variation in population growth, population connectivity and human–wildlife interactions (Lamb *et al.*, 2019).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1.1. Microsatellite markers tested for the 18 hirola blood samples. Details of the multiplex reactions are provided. Abbreviation: NA, no amplification of markers. *Markers were discarded owing to linkage disequilibrium and departures from Hardy–Weinberg equilibrium. **Markers were discarded owing to low polymorphism information content.

Table S1.2. Polymerase chain reaction conditions used for testing 18 hirola blood samples for a set of 72 different microsatellites developed for different species. Conditions are given for the 12 multiplex reactions conducted.

Table S1.3. Microsatellite markers amplified for all samples (blood, faeces and museum samples). The table provides information about the fluorescent dye used, the volume used in the multiplex and the source. This panel of markers was used for the further population genetics analyses.

Table S1.4. Summary diversity statistics for the 14 autosomal microsatellites amplified for all sampled used in this study: F_{is} , inbreeding coefficient; H_e , expected heterozygosity; H_o , observed heterozygosity; N , sample size; N_a , total number of alleles; and allele dropout rate.

Table S1.5. Results of the hierarchical analysis of molecular variance conducted for the mitochondrial DNA control region sequences. The P -value is determined through the frequency of more extreme variance components obtained randomly after 10 000 permutations. Abbreviations: BURA, Bura; CONS, Conservancy; SANG, Sangailu; SANT, Sanctuary; TRAN, translocated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table S1.6. Values of pairwise fixation index (F_{ST}) between populations using the microsatellite dataset at the top right and F_{ST} values between populations obtained using the mitochondrial DNA sequences at the bottom left. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table S1.7. Results of hierarchical analysis of molecular variance. The P -value is determined through the frequency of more extreme variance components than obtained randomly after 10 000 permutations. Abbreviations: BURA, Bura; CONS, Conservancy; SANG, Sangailu; SANT, Sanctuary; TRLC, translocated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table S1.8. Results of neutrality tests: Tajima's D and Fu's F_s . N is the sample size. All samples were non-significant ($P > 0.05$). Abbreviations: BURA, Bura; CON, Conservancy; SAN, Sanctuary; SANG, Sang; TRLC, translocated.

Figure S1.1. Inference of the most probable number of clusters (K) using the mean of estimated natural logarithm probability of data, obtained in STRUCTURE HARVESTER. $K = 1$ was chosen as the best solution.

Figure S1.2. Bar plot from STRUCTURE for $K = 2$ and $K = 3$.