

Conservation genetics and demography of the hirola antelope relict: an entire mammal genus on the brink of extinction

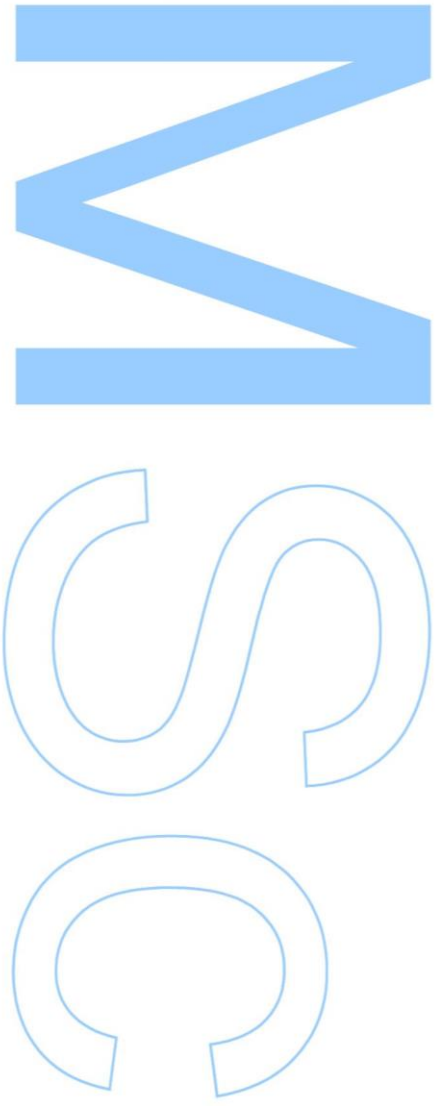
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2018

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____



Acknowledgements

I would first like to thank my supervisors Michael Jowers, João Queirós and Raquel Godinho for letting me participate in such a unique project, for their precious help, for always having an eye for detail and for the motivation they provided.

Thanks to Samer Angelone, Dr. Abdullah H. Ali (Director of the Hirola Conservation Programme), Mathew Mutinda (KWS- Field Veterinary Officer), Dr. Francis Gakuya (KWS Head of Veterinary Services), Moses Otiende (KWS), Isaac Lekolool (KWS) and everybody at the Kenya Wildlife Service for their collaboration, providing us with samples and information about hirola. Thanks to Daniel Klingberg Johansson of University of Copenhagen Zoological Museum for providing us with a museum sample.

Thanks to Paulo Célio for his support of the project, to José Carlos Brito for his help with the maps and to Rita Rocha for her advice on the analyses.Thanks to Susana Lopes, Diana Castro, Patrícia Ribeiro, Sofia Mourão, and everyone at CTM and CIBIO-InBIO, without whom this project would not have been possible.

A big thank you, Rute, for always being there for me with love and support, and sometimes a much needed cup of coffee.

My heartfelt thanks to my mother, for always having the patience and time to help me.

My sincere thanks to all the friends that walked this path with me for all their support

This work was supported by Norte Portugal Regional Operational Programme (NORTE2020), under the PORTUGAL 2020 Partership Agreement, through the European Regional Development Fund (ERDF)(NORTE-01-0145-FEDER-000007 to Nuno Ferrand).

KENYA WILDLIFE SERVICE

HIROLA CONSERVATION PROGRAM

CIBIO Research Center in Biodiversity and Genetic Resources

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Resumo

O hirola (*Beatragus Hunteri*) é considerado o antílope mais ameaçado do mundo. A sua extinção constituiria o primeiro desaparecimento de um género de mamíferos desde o tigre-da-tasmânia (*Thylacinus cynocephalus)* em 1936. Devido à perda de habitat e epidemias de peste bovina, entre outros fatores, os hirola sofreram um grave declínio no fim do século XX. A translocação recente para um santuário livre de predadores em 2012 criou alguma esperança para a conservação desta espécie. No entanto, o número de indivíduos existente sugere que a sua diversidade genética é reduzida devido a um possível efeito de *bottleneck.* Logo, um estudo genético dos hirola é necessário para futuras decisões relativas à conservação da espécie e para avaliar a existência de subestruturação genética entre grupos de vários locais para futuros programas de translocação e reintrodução. Neste estudo, foi obtida informação genética de 54 indivíduos (de uma população com menos de 500 indivíduos), através da recolha de fezes e amostras de sangue em vários locais na distribuição natural da espécie. Foi ainda obtida informação genética de uma amostra de tecido existente no museu Zoológico de Copenhaga. Um conjunto de 14 microssatélites autossómicos e a região de controlo mitocondrial completa foram utilizados para avaliar a diversidade e a subestruturação genética desta espécie, assim como a influência da história demográfica recente nos padrões genéticos.

A diversidade genética detetada (He = 0.551) foi moderada, contrastando com o número reduzido de indivíduos e recente declínio da espécie. No entanto, as sequências mitocondriais analisadas demonstraram um número diminuto de haplótipos, assim como uma diversidade nucleotídica e diferenciação haplotípica muito reduzidas. No santuário, os níveis de diversidade demonstraram ser semelhantes aos dos restantes locais. O nível reduzido de diferenciação sugere uma alta dispersão dos hirola pelo território natural, pelo menos até recentemente. Apesar de terem sido detectados sinais de um efeito de bottleneck, não foram encontradas evidências de consanguinidade populacional.

Devido ao número reduzido de indivíduos restantes e evidências que indicam que a queda populacional desta espécie criou um efeito de *bottleneck* genético*,* é aconselhável desenvolver estratégias para evitar erosão genética de modo a ser possível recuperar o número de hirolas para níveis historicamente registados.

Palavras-chave: Hirola, Antílope, Espécie ameaçada, Genética populacional, Genética da conservação, Diversidade genética, Estruturação populacional, Efeito de *Bottleneck*, Isolamento por distância.

Abstract

The hirola (*Beatragus hunteri*) is considered the most endangered antelope in the world. Its extinction would constitute the first disappearance of a mammalian genus since the Tasmanian tiger (*Thylacinus cynocephalus*) in 1936. Tree encroachment and a rinderpest outbreak that occurred in the 1980s, among other factors, have caused a severe decline in this species in the late 20th century. Recent translocation of wild herds to a new predator-proof sanctuary in 2012 has brought some hope to the conservation of this species. Nevertheless, the critically low population numbers of this species suggest that its genetic richness is low as a consequence of a possible bottleneck effect. Therefore, a genetic study of the hirola was in need for future conservation decisions on the species and to assess possible substructure in different locations for future translocation programmes. In the present study, genetic data from 54 individuals (from less than 500 remaining) was obtained from faeces and blood samples collected across several localities in the natural distribution range of the species in Kenya. Additionally, one museum sample was obtained from the Zoological Museum of Copenhagen. A set of 14 microsatellite loci and the complete mitochondrial control region were used to estimate genetic diversity and population structure, as well as to infer genetic imprint of recent demographic history of the species.

Patterns of nuclear genetic diversity were moderate (He=0.551), contrasting with the low numbers and recent decline of this species. However, the mitochondrial sequences obtained showed low nucleotide diversity, few haplotypes and low haplotypic differentiation. In the sanctuary, the levels of nuclear and mitochondrial diversity are similar to those in the wild. The low degree of differentiation inferred together with no evidence of population structure suggests dispersal of hirola across the natural distribution range, at least until recent times. Although signals of a genetic bottleneck were found, no inbreeding was detected.

Due to the species' low population numbers and evidence of a genetic bottleneck caused by the recent crash, it is advisable to develop strategies to avoid genetic erosion in order to recover the number of hirolas to historical levels.

Keywords: Hirola, Antelope, Endangered species, Population genetics, Conservation genetics, Genetic diversity, Population structure, Bottleneck, Isolation-by-distance.

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List of Abbreviations

AMOVA – Hierarchical analysis of molecular significance

AR – Allelic richness

BSP – Bayesian Skyline Plot

CON – Conservancy

CR1 – first fragment of the control region amplified

CR2 – second fragment of the control region amplified

dNTPs – deoxyribonucleotide triphosphates

ECM – Equal-capture model

ESS – Effective sample size

FC – Factorial component

FCA – Factorial correspondence analysis

FIS – Inbreeding coefficient

FST – Fixation index

HD – Haplotype diversity

HE – Expected heterozygosity

HKY – Hasegawa, Kishino and Yano

HO – Observed heterozygosity

HWE – Hardy-Weinberg equilibrium

ISAG – International Society for Animal Genetics

IUCN – International Union for Conservation of Nature

K – Number of genetic clusters

LD – Linkage disequilibrium

LRT – Likelihood ratio test

MCMC – Monte Carlo Markov chain

mtDNA – Mitochondrial DNA

NA – Number of alleles

NGO – Non- governmental organizations

NH – Number of haplotypes

NJ – Neighbor-joining

NRT – Northern Rangelands Trust

PA – Private alleles

PART – Partitioning

PCR – Polymerase chain reaction

PIC – Polymorphism information content

pID – Probability of identity

pIDsib – Probability of identity assuming siblings

QG – Queller's and Goodnight

RFLP- Restriction fragment length polymorphism

RTF - Hirola Task Force

S – Number of polymorphic sites

SAN – Sanctuary

SANG – Sangailu

TIRM – Two-innate-rates model

TRLC - Translocated

UTM – Universal Transverse Mercator

π – Nucleotide diversity

1. Introduction

1.1. The 6th extinction

The world is losing species at an alarming rate: up to 100 times faster than the natural "background" rate (Ceballos et al., 2015). Some scholars are even calling it an anthropogenic sixth mass extinction (Ceballos et al., 2015). This loss of biodiversity is one of the most critical environmental problems today and it is estimated that 25% of all mammals are threatened (IUCN, 2014) due to deterministic (habitat loss, over exploitation, introduced species and pollution) and stochastic (demographic, environmental, genetic and catastrophic) factors (Shaffer, 1981). Even in species that are not currently endangered, the loss of populations is frequent and widespread, at a much higher rate than natural species-level extinctions, having profound negative impacts on ecosystem diversity (Hughes et al., 1997; Ceballos & Ehrlich, 2002).

1.2. Hirola, the rarest antelope



Fig. 1- Hirola (*Beatragus hunteri*) Credit: Kenneth Coe, member of the Advisory Council of the Nature Conservancy's Africa Program (image displayed on the cover)

The hirola (Fig. 1), or Hunter's antelope (*Beatragus hunter;* Sclater, 1889), also known as Hunter's hartebeest, is the rarest antelope in the world and is endemic to north-east Kenya and south-west Somalia (where it may already be extinct; see Fig. 4; IUCN, 2017).

Its population decreased from around 14,000 in the 1970s to less than 500 individuals today, although some estimates are even lower, and the hirola is now considered critically endangered by the IUCN Red List of Threatened Species (IUCN, 2017).

1.2.1.Lessons from the past

The loss of the hirola can be compared with that of the Tasmanian tiger (*Thylacinus cynocephalus*) in 1936. This marsupial carnivore, the largest one in modern times, was unique as it was the last species of its family (Thylacinidae; Prowse et al., 2013). When attacks on sheeps in Tasmania started to be credited to this species, bounties on the Tasmanian tiger led to its intensive hunting; this, combined with disease, introduction of dogs and human intrusion in its habitat, led to the decline and eventual extinction of this iconic species (Prowse et al., 2013). Hirola's extinction would be the first of an entire mammal genus since the extinction of the Tasmanian Tiger and, thus, the first in contemporary human history.

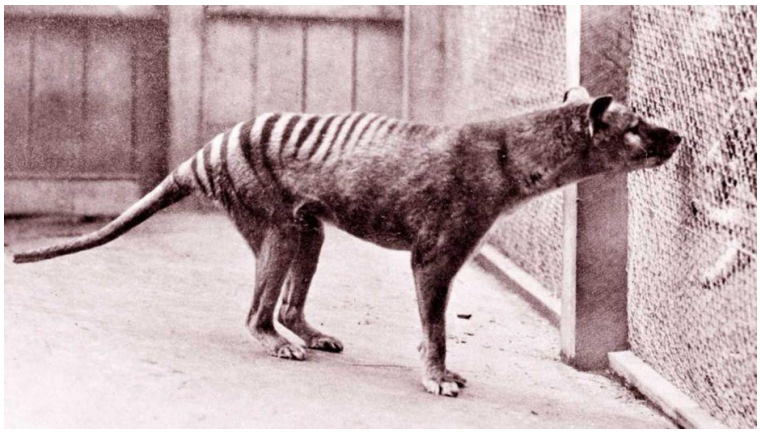


Fig. 2- Tasmanian Tiger at Hobart Zoo in 1933. Credit: National Archives of Australia

1.2.2.Taxonomy and etymology

The species was named and described by P. L. Sclater in 1889 as Hunter's antelope in honor of H.C.V. Hunter, who collected the type specimen in 1887, in the eastern bank of the Tana River; the common name, hirola, is believed to be derived from the Somali name for this animal, "Aroli" (Andanje & Ottichilo, 1999; Butynski, 2000). Hirola belongs to the subfamily Alcelaphinae of the family Bovidae; its taxonomy has been controversial and its morphology has led to this species being attributed to the genus *Damaliscus* (as

D. hunteri) or even a subspecies of the topi (as *Damaliscus lunatus hunteri)* before being placed in its own genus as *Beatragus hunter* (Andanje, 2002). Karyotypic and mithochondrial DNA analyses (Kumamoto et al., 1996; Pitra et al., 1997; Steiner et al., 2014) support the separation of hirola into its own genus (Fig.3). Male hirola exhibit the behaviour known as flehmen (a form of urine-testing to determine sexual receptivity), which is present in all bovids with the exception of *Damaliscus* and *Alcelaphus*; it has been argued that this is a strong argument in favour of the separation of hirola from *Damaliscus* or Alcelaphus (Estes, 1999). There are also fossil records that support this separation as these suggest that hirola is the only extant member of an older lineage than both Damaliscine and Alcelaphine antelopes (Kingdon, 1982).

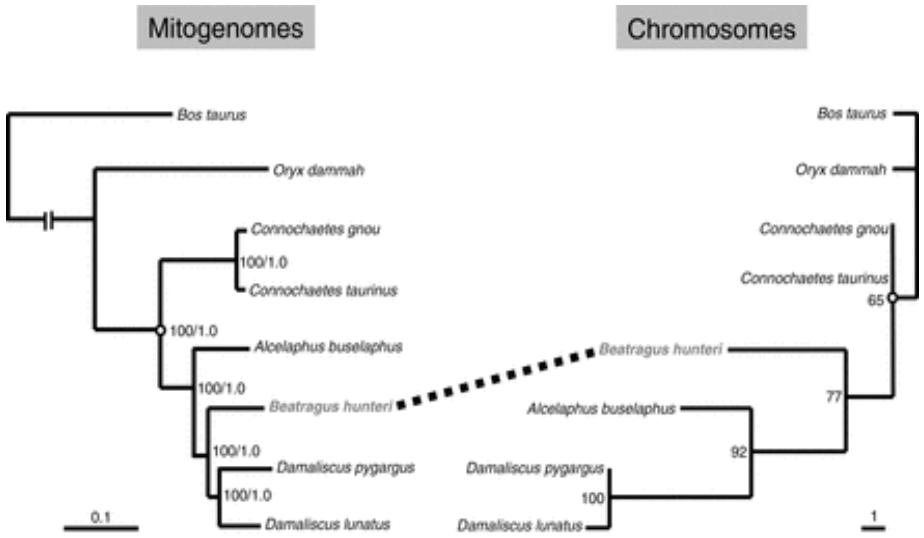


Fig. 3 - Comparison of phylogenetic trees obtained from the molecular analysis using complete mitochondrial genomes versus chromosomal characters. The Bayesian inference (left) showed maximum likelihood bootstrap values and posterior probabilities for each node, whereas the maximum parsimony tree (right) showed just maximum parsimony bootstrap values. The dotted line indicates the phylogenetic position of Beatragus hunteri (in bold) in both topologies. Image and caption credit: Steiner et al. (2014)

1.2.3.Species description

Hirola are sometimes described as being similar to species of *Damaliscus* and *Alcelaphus* but present differences in horn and body shape and coloration (Butynski, 2000). This medium-sized antelope possesses white markings around the eyes resembling spectacles, with an inverted white chevron between the eyes, which has earned this species the nickname of “the four-eyed antelope” (Andanje, 2002; Bain, 2010). It presents a tawny or yellow-brown coloration (Butynski, 2000). Hirolas weigh between 80 and 120 kilograms and stand 1 to 1.25 meters tall at their shoulder. They present curved lyrate-shape horns, rising from the brow, coming to long, slim, sharp

points (Andanje, 2002). Males and females look similar although males are slightly larger with thicker horns and darker coats (Kingdon, 1982; Butynski, 2000). There is no documented evidence for morphologic variation across this species' range.

1.2.4. Habitat

Hirola are adapted to arid environments, with an annual precipitation of 300-600 mm^2, and open to lightly bushed grasslands and wooded savannahs with scattered trees and small shrubs (Bunderson, 1981). They are more dispersed during the wet season than in the dry season due to the scarcity of pasture in the latter. Hirola have been described as a grazer species (Kingdon, 1982) but browsing has been observed during the dry season (Bunderson, 1981). They prefer short, green grasses (*Panicum infestum, Digitaria rivae, Latipes senegalensis* and *Cenchrus ciliaris*) but occasionally feed on forbs (*Portulaca oleraceae, Tephrosia subtriglora* and *Commelina erecta*) (Andanje & Ottichilo, 1999).

1.2.5.Social organization

Males of this species form bachelor herds, with up to 38 individuals, and females with offspring form groups that range from 5 to 40 animals and are often accompanied by a mature male. Hirola are seasonal breeders as the mating season peaks in March at the start of the main rainy season and most calves are born at the start of the short wet season (late September, October and November), after a gestation period of around 7.5 months (Andanje, 2002). When sub-adult males leave nursery herds, at around six months of age, they may join a bachelor group of older males, a group of Grant's gazelles (*Nanger granti*) or other dispersing female hirola or just stay alone (Andanje & Ottichilo, 1999). When old enough (32 months), they can form their own family herds or take the place of a male in control of a family herd (Andanje, 2002). They reach adult size at three years of age (Butynski, 2013). It is known that mature males can occupy and actively defend territories of up to 7 km^2, which they mark with their preorbital scent gland, if these present good quality pasture (Andanje, 2002; Butynski, 2013). Females leave their family groups when around nine months of age and frequently join an adult male, a group of Grant's gazelles or young males in mixed-sex yearling herds or just remain alone (Andanje, 2002). They mature at 1.5–2 years and first give birth at 2–3 years (based on records from captive animals; Butynski, 2013). Generation lenght of this species is

mentioned in the IUCN Red List as 5.4 years (IUCN, 2017) but recent estimates (Cooke et al.,) are of 6.3 years.

1.2.6. Interspecific interactions

Hirola are frequently in the company of other species, such as the above mentioned Grant's gazelle, the Grant's zebra (*Equus quagga boehmi*) (Andanje & Ottichilo, 1999), the fringe-eared oryx (*Oryx callotis*) (Lee et al., 2013) and the coastal topi (*Damaliscus lunatus topi)* (Kingdon, 1982), possibly to reduce need for vigilance and predation risk (Andanje, 2002). Hirola avoids association with kongoni (Coke's hartebeest - *Alcelaphus cokii*), even though they occur in Tsavo National park (see section 1.2.9.1) alongside hirola and use similar resources, which might be indicative of competition (Andanje, 2002). Hirola also suffer from competition for good quality pasture with livestock, which has been pointed out as a factor impeding their population recovery (Andanje, 2002; Ali et al., 2017, 2018). This species' main predators in the Garissa district are lions (*Panthera leo*) and African wild dogs (*Lycaon pictus*) (Andanje, 2002), although they can also be preyed on by cheetah (*Acinonyx jubatus*) and hyenas (*Crocuta crocuta*). Also, smaller predators like serval cat (*Felis serval*), caracal (*Felis caracal*) and black-backed Jackal (*Canis mesomelas*) can hunt young hirola (Kingdon, 1982; Andanje, 2002).

1.2.7. Distribution

Hirola are believed to be the relict population of a lineage (Kingdon, 1982) that, based on fossil evidence, originated 3.1 million years ago and was once widespread in Eastern Africa and probably also in South Africa (Kingdon, 1982). *Beatragus antiqus,* hirola's likely ancestor, was larger and had a broader skull and more upright horn cores and fossils of this extinct species were found in Tanzania, Ethiopia and Kenya, dating back to the Early Pleistocene. Fossils of *B. hunteri* were discovered in Tanzania, Ethiopia, Djibouti and possibly South Africa. Records of the extant species indicate that their original range lies somewhere to the south of Garissa town in Kenya, 30-50 km inland, from and parallel to the Indian Ocean, east of the Tana River, to the north of Kismayu on the west of the Juba River in Somalia (Butynski, 2000; Andanje, 2002). Bunderson (1976) estimated that hirola occupied 12,000 km^2 in Kenya and 2,000 - 3,000 km^2 in Somalia based on information from aerial surveys, giving a total range of 14,000-15,000

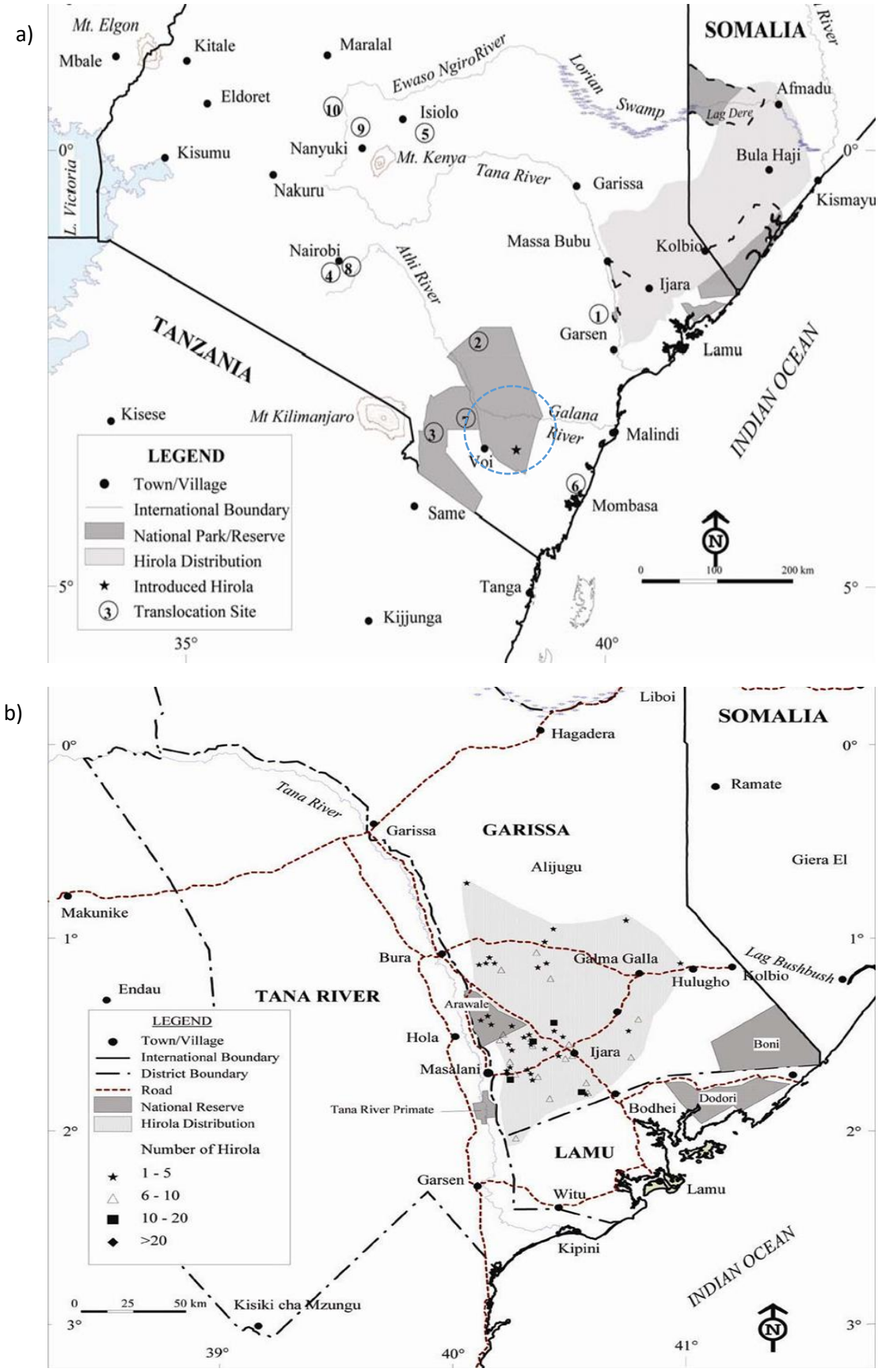


Fig. 4- a) Historical natural distribution range of hirola. Blue line and the number 2 (in the original map) indicate the location of Tsavo East National Park where there is currently an ex situ population of hirola (see section 1.2.9.1). Source: Hirola Evaluation Report (Butynski, 2000) b) Current natural distribution range (light grey) of hirola. Source: Hirola Evaluation Report (Butynski, 2000).

km^2, but, by 1996, their distribution in Kenya had been limited to only 42% of its original size (see Fig. 4 and Fig. 5; Butynski, 2000). Hirola is currently distributed in Kenya between Ijara, Bura and Galmagalla, over an area of around 1,200 km (Butynski, 2000). The current status on distribution range in Somalia is unknown but the former range has been affected by civil and military conflicts and it has been reported that the Somali population is probably extinct (Andanje & Ottichilo, 1999; IUCN, 2000).

1.2.8. Decline

The Kenyan population suffered a rapid decline in its numbers from around 14,000 in 1976 to less than 500 in 1995, estimated through aerial surveys (Bunderson, 1976; Ottichilo et al., 1995). The main reason for this was an outbreak of rinderpest (*Morbillivirus*) in the 1980s that led to mass mortality of hirola and other ruminants in Eastern Kenya although other factors like drought, poaching, predation, competition with livestock, habitat loss and degradation have also contributed to this recent decline (Butynski, 2000; Andanje, 2002; Ali et al., 2014b). Using the estimates of generation time of 6.3 for this species present in Cooke et al. (2018), the sudden decline occurred around 5 or 6 generations ago. In the Arawale National Reserve, population of hirola declined dramatically due to neglect of the reserve, which permitted the presence of poachers, livestock grazing and semi-permanent settlements (Magin, 1996; Butynski, 2000). Questions remained on why the eradication of rinderpest did not prompt the recovery of hirola in the subsequent years. Habitat loss due to tree encroachment has been pointed out as the main factor that is impeding recovery of the hirola (Ali et al., 2017).

1.2.9.Conservation efforts

1.2.9.1. Tsavo population

In 1963, a group of 30 individuals were translocated from its wild range, in Garissa district, to the Kenya's Tsavo East National Park, which covers an area of 13,000 km^2 and is situated in south-east Kenya (see Fig.4), but some perished soon after release and only less than 20 individuals remained (Andanje & Ottichilo, 1999; IUCN, 2000).

In 1996, C. Magin developed a hirola recovery plan with two main objectives: improved protection and management in the natural range and effective conservation of translocated populations in Kenya (Magin, 1996). The Hirola Task Force (RTF) was formed, composed of government organizations, NGOs and private individuals who

shared the common goal of conserving hirola in Kenya (Andanje, 2002). A study by Andanje and Ottichilo (1999) estimated a total of 76 hirola by 1995 in Tsavo. To apply Magin's recovery plan, the RTF undertook a translocation of hirola (n = 35) in 1996 from the natural distribution range to boost the population in Tsavo East National Park (Butynski, 2000). This additional translocation was intended to enable a closer scientific study of the species and to boost the genetic composition of the Tsavo population in order to help ensure the persistence of the only ex-situ population of hirola (Andanje, 2002). However, by December 2000, the population had decreased to 77 individuals (Andanje, 2002). Analysis of the growth rate and age structure of this population indicated high calf and juvenile mortality and a low recruitment rate (Andanje & Ottichilo, 1999), which is usually due to inbreeding or decline in genetic variability (Berger, 1990). The population in Tsavo East National Park is more vulnerable to adverse genetic factors due to the low number of founder individuals than the population in the natural range (Butynski, 2000). The Tsavo population mixes and is not as territorial as wild groups (Ali, 2013; Ali et al., 2014a, 2014b), which might suggest that there is higher genetic differentiation between wild groups than in the Tsavo's population. Genetic differentiation between and within populations is unknown.

1.2.9.2. Ishaqbini conservancy

In the year of 2005, four local communities, in collaboration with the Northern Rangelands Trust (NRT), proposed the establishment of the Ishaqbini Hirola Conservancy. This community-based conservation area, situated in the Ijara district in Garissa County, Kenya, covers around 215 km^2 along the eastern bank of the Tana River. Within this conservancy, there is a predator-proof fenced sanctuary that covers 25 km^2, which was established with 48 hirola in August 2012 (Ali, 2016). By the end of April 2016, the population had doubled to around 100 individuals in the sanctuary with a higher proportion of calves than in the rest of the conservancy (King et al., 2016). Herds inside the sanctuary are larger and have more calves and sub-adults than herds outside the sanctuary, suggesting less calf mortality (King et al., 2016). In the sanctuary, from its establishment in August 2012 to April 2016, only two calves died, immediately after birth, representing a 3% mortality, which contrasts with a 44% calf mortality in the conservancy (Ali et al., 2017). The calf mortality in the conservancy is similar to calf mortality in the wild (41.2%-69.8%; Andanje, 2002).

1.2.9.3. Other conservancies

There is also been an effort to increase the protected area in the hirola's native range. Three new conservancies have been established: Bura East Conservancy (5,195 km^2), Sangailu Conservancy (800 km^2) and Gababa Conservancy (330 km^2) (Ali, 2016). Recently, Bura East Conservancy received its registration certificate (Robb-McCord, 2017).

1.3. Genetic diversity and conservation

Biodiversity refers to the variety present in all organisms and to the interactions among them, including those they form with their abiotic environment (DeLong 1996). It is comprised of three levels which are all important to conserve. The species level is formed by the differences between species and the genetic level represents the genetic variability between conspecific individuals. At a higher level of organization, there is the ecosystem level which denotes diversity between ecosystems, and englobes habitat variation, biological communities and ecological processes (Frankham, 1995a).

The total number of genetic differences in a species or a population forms the genetic diversity. Since it was discovered, through the first population genetic studies, that species usually present a great number of polymorphisms (Harris, 1966; Lewontin & Hubby, 1966), these differences started to be considered as a requirement for biological evolution. Data from early genetic studies using allozymes and subsequent data from DNA sequencing showed that genetic diversity differs to a great extent among different species (McVean et al., 2005; Begun et al., 2007; Lack, 2015; Ellegren & Galtier, 2016). Genetic diversity increases over time as mutations occur due to DNA replication errors or mutagen-induced DNA damage (Ellegren & Galtier, 2016). The rate at which these mutations appear is variable between distinct areas of the genome (Hodgkinson & Eyre-Walker, 2011) and is also different between different species (Lynch, 2010), which might be one of the factors responsible for the high level of variation in genetic diversity (Ellegren & Galtier, 2016). The rate of allelic loss and fixation (when only one of the alleles remains) is also a determinant of genetic diversity (Ellegren & Galtier, 2016). Loci with neutral alleles are largely influenced by genetic drift (Charlesworth, 2009).

Genetic diversity is important for species to deal with changes in their environment. The current climate change, for example, has put a great pressure on species to adapt and change their behaviour (e.g. migrations) and distribution (McCarthy, 2001). Genetic diversity plays a great part in this as it determines the ability for a species to survive in a

new habitat by increasing the chance that some individuals have genetic differences. This improves their chances of surviving in unexpected conditions and, as such, improve their fitness (Sober, 1994). Another way in which genetic diversity is important for the resilience of endangered species is that these generally have small populations (Baillie et al., 2004), in which inbreeding is inevitable (Frankham et al., 2002). This causes homozygosity, which increases the chances of offspring being affected by recessive or deleterious traits (inbreeding depression; Charlesworth & Willis, 2009). In conclusion, low levels of genetic diversity increase the extinction risk by limiting potential for adaptation and by the accumulation of deleterious alleles (Reed et al., 2002). Therefore, it is pivotal to preserve genetic diversity, which has led to the field of conservation genetics (Primmer, 2009), which applies principles of population genetics to preserve the genetic diversity of species and their populations (Wayne & Morin, 2004).

1.4. Non-invasive sampling

In studies of endangered or elusive species, or in any study where disturbing the individuals of interest would be limited by ethical and practical constraints, the use of non-invasive genetic sampling is essential (Taberlet et al., 1999). There are many difficulties in amplifying DNA from non-invasive samples due to the typically low quantity and quality of existent DNA in these samples as well as to the presence of PCR inhibitors (Waits & Paetkau, 2005). However, many techniques have been developed to counteract these problems and non-invasive sampling has been widely used to study natural populations (Beja-Pereira et al., 2009; Oliveira et al., 2010; Chaves et al., 2012; Ferreira et al., 2018). Faeces are among the most widely used non-invasive samples as it is easier to find in the wild and more informative, especially when it is possible to collect them just after seeing the animals defecating (allowing to collect fresh, less degraded faeces and providing information about the species and sex of the individual; Beja-Pereira et al., 2009).

1.5. Molecular tools to assess genetic diversity

Like many fields in biology, the field of conservation genetics had its progress catalysed by technological advances, namely improvements regarding the choice of molecular markers. Studies of genetic diversity were not possible until the use of allozyme electrophoresis. This method was used to estimate the heterozygosity of allozymes (allelic variants of enzymes), which was generally assumed to reflect overall genetic

variability and, as such, it was believed it should be considered in decisions about the management of population and species (Bader, 1998).

This field had another major breakthrough with the development of DNA-based markers, which allowed to assess DNA variation itself instead of relying on variations in the electrophoretic mobility of the proteins (Schlötterer, 2004). The discovery of restriction endonucleases led to the use of restriction fragment length polymorphisms (RFLP) (Botstein et al., 1980). This concept was later applied to minisatellites and microsatellite analyses, the latter having the advantage of being smaller than the former, making it easier to amplify with polymerase chain reaction (PCR) (Schlötterer, 2004). The invention of PCR allowed, for the first time, to amplify any genomic region without isolating large amounts of clean DNA (Schlötterer, 2004).

1.5.1.Microsatellites

Microsatellites are short sections of DNA where a simple motif, generally 1-6 bp long, is repeated up to about 100 times (Richard et al., 2008). This is due to DNA replication slippage and the mismatch repair system (Schlötterer, 2000). The number of repeats of the motif varies, leading to high polymorphism levels among individuals (Bruford & Wayne, 1993). Microsatellite markers are highly polymorphic, abundant and fairly evenly distributed across eukaryotic genomes. This, coupled with their simple amplification and genotyping, even from non-invasive sources of DNA, their co-dominant nature and their typically high levels of allelic diversity at different loci, led to microsatellite markers being considered as the best molecular tools in population genetics, social structure, mating success and population movement (Schlötterer, 2000; Sunnucks, 2000), at least until the development of next-generation sequencing, which refers to new methods that reduced the cost of sequencing and produce high quality, robust data, with low noise (Buermans & den Dunnen, 2014).

1.5.2.Mitochondrial markers

In eukaryote cells, there is a comparatively small portion of DNA present in the mitochondria, known as mitochondrial DNA (mtDNA), in the form of a double-stranded, covalently closed circular molecule, with 37 coding genes, essential for normal mitochondrial function, and a control region in animals (Avise et al., 1987; Moritz, Dowling, & Brown, 1987). MtDNA is often used in population genetics and evolutionary

studies due to its lack of recombination (useful to assess clear genealogies and ancestry data), ease to isolate and assay and high mutation rate compared to nuclear DNA (Avise et al., 1987; Harrison & Quinn, 1989). It is often used to amplify DNA from non-invasive samples as it is easier to obtain from samples with low DNA quantity and quality, due to the high number of mitochondrial copies per cell (Harrison & Quinn, 1989; Waits & Paetkau, 2005). Nevertheless, it is limited by the fact that it is solely maternally inherited and, thus, may induce bias due to the fact that it is not susceptible to recombination and only reflects the female portion of the history of the species which may be different from the history of the species as a whole. The control region presents a displacement loop structure in vertebrates (D-loop), which has a function in the replication process (Moritz et al., 1987). Due to the fact that it is the most variable region of mtDNA, it is useful to assess phylogenetic relationships and intra-populational genetic diversity (Birungi & Arctander, 2000).

1.6. The genetic consequences of bottlenecks

In Africa, many large mammals have experienced severe recent population declines due to anthropogenic pressures and climatic fluctuations (Hilborn et al., 2006; Stoner et al., 2007). These population declines may lead to a great loss of genetic diversity (Groombridge et al., 2000; Weber et al., 2000; Wisely et al., 2002; Bellinger et al., 2003). This occurs because a limited number of randomly selected individuals create a founding population, leading to genetic drift. This effect is known as genetic bottleneck. A bottleneck decreases the population's ability to adapt to and survive environmental changes, like climate change or a shift in available resources (Lande, 1988). Alternatively, if the survivors of the bottleneck are the individuals with the greatest genetic fitness, the frequency of the fitter genes within the gene pool is increased, while the pool itself is reduced. Nevertheless, the reduction in the number of individuals leads to inbreeding and, consequently, increases homozygosity, increasing the potential for inbreeding depression to occur. Smaller population size can also cause deleterious mutations to accumulate (Lynch et al., 1995). There are many species with once widespread distributions that now exist only in remnant, isolated populations in protected areas or patchy distributions, with disrupted gene flow. This population fragmentation further increases the loss of genetic diversity, constituting a serious challenge for long-term conservation (Young & Clarke, 2000; Hedrick, 2005). On the other hand, the levels of genetic diversity increase very slowly with time as random mutations accumulate or when gene flow with another population occurs. Thus, the extinction risk of bottlenecked

populations with low genetic diversity can be decreased through the introduction of individuals from other populations (Frankham, 2015; Weeks et al., 2017; Hasselgren et al., 2018)

A number of studies have shown low genetic diversity on recently bottlenecked populations of ungulates (e.g.: Armstrong et al., 2011; Godinho et al., 2012; Vaz Pinto et al., 2015). Although no genetic studies have assessed the variation in remaining hirola populations, it is suspected that remaining genetic variation is low due to the population crash and the high calf and juvenile mortality found in the Tsavo population and in the Ishaqbini population outside of the sanctuary (Andanje, 2002; Probert, 2011; Ali et al., 2014, 2018). However, in the Ishaqbini sanctuary population, calf and juvenile mortality is low and the population is increasing rapidly, showing no signs of inbreeding depression (Ali et al., 2016; King et al., 2016).

To estimate the loss of genetic diversity caused by a bottleneck, pre-bottleneck museum samples are often used as they allow a comparison between haplotypes found in current samples and haplotypes that were present before the bottlenecks (Culver et al., 2008).

1.7. Inbreeding depression

Inbreeding depression is the decrease in fitness that results from low genetic variability, as a consequence of either increased homozygosity for deleterious recessive mutations, increased homozygosity for alleles at loci in which heterozygosity is the favoured genotype (‘overdominance’) or a combination of both. Deleterious alleles will generally be present in populations at low frequencies (mutation–selection balance), whereas overdominant alleles at a locus are maintained at intermediate frequencies by balancing selection. (Charlesworth & Willis, 2009)

Inbreeding has been proven to have a negative effect on reproduction and survival, including sperm production, mating ability, female fecundity, juvenile survival, mothering ability, age at sexual maturity and adult survival (Hedrick, 1995; Frankham et al., 2002; Johnson et al., 2010; Casas-Marce et al., 2013). Deleterious effects of inbreeding have been widely reported for wildlife in natural habitats (Keller & Waller, 2002; Dunn et al., 2011; Walling et al., 2011). There is also evidence that inbreeding depression has more impact in populations under stressful environmental factors (Miller, 1994). Nevertheless, the severity of inbreeding depression is dependent on several factors, like genetic purging (Lacy & Ballou, 1998) and the natural level of inbreeding/outbreeding of a species (Husband & Schemske, 1996; Charlesworth & Willis, 2009). In some cases,

repeated bottlenecks can lead to genetic purging diminishing the negative effects of post-bottleneck inbreeding (Amos & Harwood, 1998; Frankham, 2005). Evidence has shown that, to a certain intensity, inbreeding depression can be beneficial to the general fitness of the populations due to the effects of genetic purging, which eliminates deleterious mutations and alleles that diminish fitness; however, the effect of genetic purging seems to be modest in small populations as small deleterious effects tend to become effectively neutral and drift to extinction and empirical evidence has found only moderate effects of purging (Frankham, 2005).

As it has been shown that inbreeding reduces reproduction and survival, it is only natural that it increases extinction risk, which was first demonstrated in laboratory populations of *Drosophila*, houseflies and mice (Frankham, 1995b; Bijlsma et al., 1999; Bijlsma et al., 2000; Reed & Bryant, 2000; Reed et al., 2002; Reed et al., 2003), even in experiments with rates of inbreeding within the range of many endangered species (Reed & Bryant, 2000; Reed et al., 2003). Although the effect of inbreeding is harder to understand and assess in wild populations, there are studies that clearly demonstrate its contribution to extinction risk (Newman & Pilson, 1997; Saccheri et al., 1998; O'Grady et al., 2006).

Computer projections performed with conservative levels of inbreeding depression and taking into account the effects of demographic and stochastic factors and of genetic purging showed a clear negative effect of inbreeding depression on population viability across a broad taxonomic range (Brook et al., 2002; Frankham, 2005). There is the possibility that small populations are driven to extinction due to stochastic factors before low genetic diversity has an impact on the population ("Lande scenario") (Lande, 1988). However, even though quick population decline can diminish the effects of inbreeding depression, most threatened populations are not driven to extinction before being affected by adverse genetic factors (Spielman et al., 2004).

There are small surviving populations whose fitness is apparently normal (Craig, 1994; Elgar & Clode, 2001), which has led to doubts about the negative role of inbreeding and loss of genetic diversity on population viability and extinction risk. For example, Chatham Island black robins (*Petroica traversi*), golden hamsters (*Mesocricetus auratus*) and Mauritius kestrels (*Falco punctatus*) all survived population bottlenecks in which only a single breeding pair remained (Groombridge et al., 2000; Frankham et al., 2002). Black-footed ferrets (*Mustela nigripes*) recovered from an extreme bottleneck that left them with only 18 individuals and now has more than a thousand individuals across several populations (Wisely et al., 2002), the northern elephant seal (*Mirounga angustirostris*)

rebounded from 20-100 individuals a century ago to 175,000 today (Weber et al., 2000) and the plains bison (*Bison bison bison*) were once only distributed in five small herds (Hedrick, 2009). Nevertheless, these observations are highly selective and ignore the vast majority of cases of small populations that did not avoid extinction (Frankham, 2005).

It is important to devise conservation plans to reduce inbreeding in populations of conservation interest due to its effects on fitness and on the extinction risk of small populations (Frankham, 2005; Keller et al., 2007). One way to reduce inbreeding is the crossing of unrelated populations (Spielman & Frankham, 1992; Falconer & Mackay, 1996). This method has been proven to be effective in the wild in deer mice (*Peromyscus maniculatus*; Schwartz & Mills, 2005), gray wolf (*Canis lupus*; Vilà et al., 2003), greater prairie chicken (*Tymphanuchus cupido pinnatus*; Westemeier et al., 1998) and adders (*Vipera berus*; Madsen et al., 2004).

1.8. Why genetics matter in translocations

Although translocations have been an important tool in species conservation (IUCN, 2013), they are often unsuccessful and expensive and, as such, there is growing interest in the factors that determine their success, namely genetic ones (Fischer & Lindenmayer, 2000). There are different types of translocation (IUCN, 2013):

- Population restoration denotes “conservation translocations to within indigenous range” and is comprised of:

  - Reinforcement (movement of individuals into a population of conspecifics);

  - re-introduction (movement of an organism into a part of its native/historical range from which it has disappeared).

- Conservation introduction indicates “the intentional movement and release of an organism outside its indigenous range” and comprises:

  -Assisted colonisation, which aims to avoid the extinction of populations of the focal species;

  -Ecological replacement, which has the objective of translocating an organism to perform a certain ecological function.

Although the genetic effects of a translocation are often overlooked, there has been increasing evidence that these are crucial for population establishment and persistence

(Weeks et al., 2011). For instance, reinforcement can be used to increase the population size of a threatened species in order to alleviate the risk of stochastic loss but it can also decrease the risk of low genetic variability and, consequently, the risk of inbreeding depression (Hedrick, 1995; Westemeier et al., 1998; Vilà et al., 2003; Hedrick & Fredrickson, 2010; Young & Pickup, 2010). Several translocations have been performed to alleviate genetic threats in endangered species or populations, like, for instance, in the famous case of the Florida Panther (*Puma concolor coryi*), which presented severe inbreeding depression (Fischer & Lindenmayer, 2000; Johnson et al., 2010; Sheean et al., 2012; Ottewell et al., 2014). On the other hand, ignoring genetic factors in conservation management might lead to adverse effects like, for example, the use of inappropriate recovery strategies or the use of populations not adapted to the environment in which they are introduced (Frankham, 2005).

It is also important to keep a level of gene flow to ensure long-term success for populations that cannot be increased above 1,000 individuals, which is considered the minimum threshold to maintain adaptive potential (Willi et al., 2006; Weeks et al., 2011). This is vital for many endangered species with small remaining populations (Weeks et al., 2011). Genetic studies are also necessary to understand differentiation between populations before designing translocation plans due to the risk of outbreeding depression (Weeks et al., 2011). Ideally, a framework should be developed which considers taxonomic status, existence of fixed chromosomal differences, historical gene flow, evolutionary relationships, environmental differences between populations and the number of generations in different environments is the only way to assess the risk of outbreeding depression (Frankham et al., 2011). To implement successful conservation strategies, the risk of outbreeding depression must be weighed against the effect of low genetic diversity on immediate risk of population decline/extinction in the absence of translocation (Edmands, 2006; Lopez et al., 2009; Frankham et al., 2011; Weeks et al., 2011).

1.9. Objectives

This study aims to provide a genetic assessment of this rare and endangered species, the hirola. This species requires active conservation efforts to ensure its persistence. However, the lack of a detailed study on the remaining genetic diversity and gene flow does not allow informed conservation decisions regarding its management. As such, the main objectives of this study are to:

1) estimate the levels of genetic diversity existent in wild herds in the natural distribution range and in the sanctuary population;
2) to understand the degree of differentiation between groups of individuals in different geographic localities and investigate possible population structure in the natural distribution range;
3) to infer demographic history and understand the severity of the recent population crash.

This information should provide a basis for the future of the hirola conservation programme and for further research on this iconic endangered species. The data on genetic diversity and differentiation should provide premises to establish a framework for future translocations. Additionally, this study should increase the scientific knowledge pertaining to conservation of endangered species in fenced sanctuaries. Finally, it will, hopefully, draw attention to a species in a great need for active conservation and further study.

2. Material and Methods

2.1. Study area and sampling

The Republic of Kenya is located in East Africa and is bordered to the north by Ethiopia and South Sudan, to the south and south-west by Tanzania, to the west by Uganda and to the east by Somalia and the Indic Ocean (Fig. 6a). The study area is focused in the eastern area of the Garissa County, which is situated in north-eastern Kenya and is bordered by Somalia (Fig. 6b).

Faecal samples were collected, in December 2017 and February 2018, from herds within the predator-free fenced sanctuary (1°52'24. 94"S 40°11'13. 55"E) and from wild herds outside the sanctuary at the Ishaqbini Community Conservancy (1°54'19. 56"S, 40°12'49. 89"E), and in Bura East (1º02’64’’S 40º30’83’’E) and Sangailu (1º40’20’’S 40º71’61’’E) administrative divisions (Figure 6b). A museum sample was retrieved from an individual hunted in the Bura area in 1937. Blood samples were obtained from individuals captured in 2012 near the Ishaqbini Community Conservancy (up to 30 km away from the sanctuary) and then translocated into the sanctuary (Figure 3b). Sampling was carried out by Dr. Abdullahi Ali (Founder of the Hirola Conservation Programme) and Matthew Mutinda (Field veterinary officer of Kenya Wildlife Service), together with local rangers. Eighteen hirola blood samples were retrieved from the group of 48 animals translocated into the sanctuary in August 2012, which were immobilized with a combination of 3 mg Etorphine hydrochloride (M99; a narcotic) and 30 mg Azaperone (Stresnil; a tranquilizer) with 6 mg Diprenorphine hydrochloride as a reversal. Faecal samples (n = 84) were collected from wild herds [Sangailu (n = 12) and Bura (n = 19) localities], from the sanctuary (n = 37) and from the conservancy (outside the sanctuary; n = 16). Hereafter, these groups are named as populations. Geographic coordinates were registered for most of the faecal samples (Missing: n = 5) and for the capture sites of the 2012 translocation. Blood samples were preserved in filter paper and faecal samples were preserved in plastic vials containing absolute ethanol. Samples were then sent to Research Centre in Biodiversity and Genetic Resources (CIBIO – InBIO Associate Laboratory).

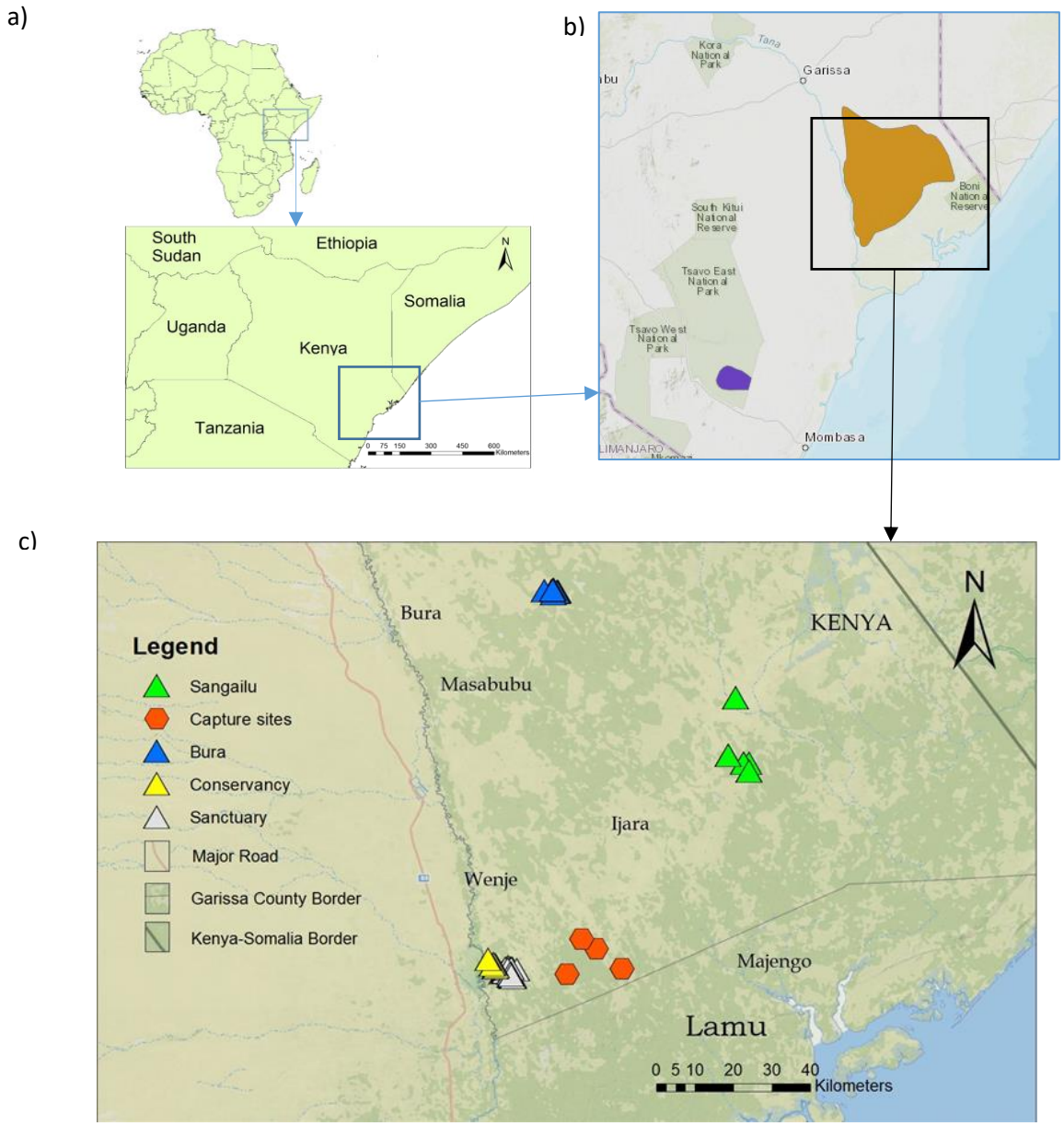


Fig. 5- Study area and sampling: (a) The location (marked with a square) of the sampling area in Kenya, in East Africa, and neighbouring countries; (b) Map showing the current distribution of hirola (source: IUCN, 2017): the Tsavo population (dark blue) and the natural range (yellow), where the sampling occurred; (c) Map showing the location on which sampling of hirola scats took place (Sanctuary, Conservancy, Bura, Sangailu) and of the capture sites used for the 2012 translocation (into the sanctuary).

2.2. DNA extraction and amplification

Small pieces of filter paper containing blood samples were cut and DNA was extracted using the EasySpin® Extraction Kit in columns for blood samples (QIAGEN, Germany), following manufacturer's instructions. The success of DNA extraction was assessed through gel electrophoresis using a solution of agar at 0.8% and included GelRed Nucleic Acid Stain. DNA extraction from non-invasive (faeces) and museum samples was

performed in a laboratory facilities with sterile conditions and positive air pressure to reduce possible contaminations. The E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek) was employed for faecal samples, following manufacturer's instructions. An ancient DNA extraction protocol was used for the museum sample (Dabney et al., 2013) . Polymerase Chain Reaction (PCR) amplifications of nuclear and mitochondrial markers were conducted in a T100TM BIO-RAD 96 Well Thermal Cycler and included a negative control in each reaction to assess possible contaminations. In the case of faecal samples a positive control (a sample from *Hippotragus niger variani*) was also included. The success of PCR amplifications was assessed through gel electrophoresis using a solution of agar at 2% and GelRed Nucleic Acid Stain.

2.3. Amplification of mitochondrial DNA control region

Amplification of the mitochondrial DNA control region was divided in two overlapping fragments (named CR1 and CR2) to facilitate amplification of DNA from non-invasive samples (Table 1). Hirola specific primers were designed based on the complete mitochondrial genome of hirola available in GenBank (accession number: NC_023542).

Table 1- Description of primers and PCR conditions used to amplify the CR1 and CR2 fragments of the mitochondrial DNA control region.

Primer	Primer sequence (5'-3')	PCR conditions		
CR1	F:aggaagaagctcatagccccac R:gcgagaagaggagtccctgcca	95ºC	15'	
		95ºC	30"	(45 cycles)
		48ºC	30"	
CR2	F:cgagcttaatcaccatgccgcgtg R:gtgccttgctttggttttaagc	72ºC	30"	
		60ºC	10'	
		10ºC	FOREVER	

Each 8 µl PCR contained approximately 3 µl of DNA, 5 µl Master Mix (QIAGEN), 3,2 µl H_20 and 0,4 µl of each primer (Forward and Reverse; diluted 1:10). PCR conditions were as follows: an initial denaturation step of 15 minutes at 95ºC; 45 cycles of denaturation at 95ºC for 30 seconds, annealing at 51ºC for 30 seconds and extension at 72ºC for 30 seconds; a final extension at 60ºC for 10 minutes. Following the PCR amplifications, an enzymatic clean-up using a mix of Exonuclease I and Thermosensitive Alkaline Phosphatase (both from Thermo Scientific) was performed in order to remove excess

primers and dNTPs. Conditions were as follows: 15 minutes at 37ºC and 15 minutes at 85ºC, following the manufacturer's instructions. Samples were sequenced bi-directionally using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's protocol, and run on a 3130xl Applied Biosystems® automated sequencer. Chromatographs were checked manually, assembled, aligned and edited using Sequencher® (Gene Codes).

2.4. Amplification of microsatellite markers from invasive samples

As no primers were available specifically for hirola, a set of 72 cross-specific microsatellite markers developed for the giant sable (*Hippotragus niger variani*) (n = 58; Vaz Pinto et al., 2015), domestic sheep (*Ovis aries*; n = 10; International Society for Animal Genetics - ISAG) and cattle (*Bos Taurus*; n = 4; ISAG) were tested for the 18 hirola blood samples. From the 72 markers tested, 44 were successfully amplified, although only 21 loci revealed to be polymorphic (see details in Table S1, Supplementary Material).

To overcome the poor quality of the amplifications, markers were amplified in a two-step PCR using a pre-amplification protocol and a M13 tailed primer (Piggott et al., 2004). Different mixes were used for pre and post PCRs. This was done for all multiplex PCRs with the exception of the cattle multiplex (see details in Table S2, Supplementary Material). For the pre-PCR, both the forward and reverse primers were used. For the post-PCR, only the reverse primer was used together with the M13-tail attached to the 5′ end. PCR amplifications were performed using approximately 3 µl DNA, 5 µl Master Mix (QIAGEN), 3,0 µl H_20 and 1 µl of primer mixes (Table S1). Genotyping of PCR products was conducted on a 3130xl Applied Biosystems® automated sequencer using Gene Scan™ 500 LIZ™ size standard (Thermo Fisher Scientific, United States of America). GeneMapper® v. 5.0 (Applied Biosystems™) was used to create bins, for each allele of each locus. Allele scoring was done through a semi-automated procedure, i.e. the automated allele calling done by the software was posteriorly checked through visual analysis in order to minimise scoring-related errors.

Departures from Hardy-Weinberg Equilibrium (HWE) were estimated for the 21 polymorphic markers using Genepop on the web v. 4.2 (Raymond & Rousset, 1995). Linkage disequilibrium (LD) between all pairs of loci was also computed in this software, with a dememorization number of 10,000, using 1,000 batches and 1,000 iterations per

batch. Significant departures from HWE or significant LD values were corrected applying a Bonferroni correction for tests with multiple comparisons (Dunn, 1958, 1961). Polymorphism information content (PIC) was calculated in CERVUS v3.0 (Kalinowski et al., 2007). Genetic diversity statistics per locus were calculated in GenAlEx v. 6.503 (Peakall & Smouse, 2012). These data were used for the selection of the most informative set of markers.

2.5. Amplification of microsatellite markers from non-invasive samples and museum sample

Based on the PIC and after testing for HWE and LD, 14 out of the 21 polymorphic microsatellites were selected for genotyping non-invasive samples: HPN93, HpN12, HpN2, HpN9, Hpn16, HPN10, HSC, ILST87, Inra06, Inra5, BM1824, HPN13, HPN21 and TGLA53 (see more details in Table 2). PCRs reactions were conducted as described previously for the invasive samples, with Pre-PCR and Post-PCR procedures. However, as it is difficult to obtain reliable genotypes from non-invasive samples, a *multiple-tubes* approach (Taberlet et al., 1996) was used: DNA obtained from each faecal sample was amplified four times and a consensus genotype for each sample per locus was defined after comparing the different replicates. This was performed in GIMLET v1.3.3 (Valière, 2002), together with quantification of allele dropouts and false alleles. Departures from Hardy-Weinberg Equilibrium (HWE) were estimated using Genepop on the web v. 4.2 (Raymond & Rousset, 1995). Linkage disequilibrium (LD) between all pairs of loci was also computed in this software, with a dememorization number of 10,000, using 1,000 batches and 1,000 iterations per batch. Significant departures from HWE or significant LD values were corrected applying Bonferroni correction for tests with multiple comparisons (Dunn, 1958, 1961).

Table 2- Microsatellite markers amplified for non-invasive samples and respective information regarding 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.

Multiplex	Locus	Species	Tail fluorescent dye	Volume in multiplex (ul/50ul)	Reference
M1	HPN93	Giant sable	6-FAM	0.4	Vaz Pinto et al., 2015
	HPN16	Giant sable	NED	0.8	Vaz Pinto et al., 2015
	HPN2	Giant sable	PET	2	Vaz Pinto et al., 2015
	HPN9	Giant sable	PET	0.7	Vaz Pinto et al., 2015
	HPN12	Giant sable	VIC	2	Vaz Pinto et al., 2015
M2	HSC	Sheep	VIC	0.5	Scott et al., 1991
	Inra5	Sheep	6-FAM	0.6	Vaiman et al., 1992
	Inra06	Sheep	PET	0.5	Vaiman et al., 1992
	ILST87	Sheep	NED	1.2	Kappes et al., 1997
	HPN10	Giant sable	PET	0.8	Vaz Pinto et al., 2015
M3	HPN13	Giant sable	PET	1.2	Vaz Pinto et al., 2015
	BM1824	Cattle	NED	0.6	Vaiman et al., 1992
	TGLA53	Cattle	6-FAM	0.7	Georges & Massey, 1992
	HPN21	Giant sable	VIC	2.7	Vaz Pinto et al., 2015

2.6. Data analysis

2.6.1. Probability of identity

To assess the reliability of the panel of microsatellites to identify unique genotypes, the probability of identity (pID; Paetkau & Strobeck, 1994) and the probability of identity assuming siblings (pIDsib; Waits, 2001) were calculated using GenAlEx v. 6.503 (Peakall & Smouse, 2012). Identical genotypes were determined using the Excel (Microsoft) macro IRMACRON (Amos et al., 2001).

2.6.2. Nuclear and mitochondrial genetic diversity

Genetic diversity was estimated for the five sampling areas: i) individuals captured in 2012 (TRLC); ii) sanctuary in 2018 (SAN); iii) conservancy (CON); iv) Bura (BURA); v) Sangailu (SANG), as well as for the overall dataset. To assess genetic diversity using the microsatellite dataset for each population and each locus, several descriptive diversity statistics were assessed in ARLEQUIN v 3.5 (Excoffier et al., 2005): number of

different alleles (NA), expected heterozygosity (HE), observed heterozygosity (HO) and inbreeding coefficient (FIS). The significance value for FIS was obtained through a randomisation test with 10,000 permutations and is equivalent to the frequency of permuted values that are larger than or equal to the original value. FSTAT v2.9.3. (Goudet, 1995) was used to infer allelic richness through a rarefaction analysis, based on a minimum sample size of three individuals, which is the smaller sample size (SANG) from the populations considered. As populations with a larger sample size are expected to have more alleles than populations with a smaller sample size, allelic richness is highly dependent on sample size. Thus, a rarefaction analysis was used, which aimed to overcome this problem and allow for true comparisons between populations with different sample sizes (Kalinowski, 2004).

Genetic variation in the mitochondrial sequences was estimated as the number of haplotypes, haplotype diversity (HD; Nei, 1987), number of polymorphic sites (S) and nucleotide diversity (π; Nei, 1987) using the software DnaSp v5.10 (Rozas, 2009).

2.6.3. Population differentiation and structure

Population differentiation and structure was assessed on microsatellite and mtDNA datasets. Firstly, AMOVAs (analysis of molecular variance), computed in ARLEQUIN (Excoffier et al., 2005), were performed at several levels: no groups assigned (equal separation between every group); grouping TRLC, SAN and CON; grouping TRLC and CON. The pairwise fixation index (FST) was estimated in ARLEQUIN using 50,000 permutations. Neighbor-joining (NJ; Saitou & Nei, 1987) trees were constructed based on pairwise FST values in MEGA 3.0 (Kumar et al., 2008).

For the microsatellite dataset, a factorial correspondence analysis was performed in GENETIX v.4.05 (Belkhir et al., 2004) to visualize structural patterns among populations. In addition, STRUCTURE v. 2.3.4 (Pritchard et al., 2000) was used to determine the existence of population substructure using microsatellite loci data. This software uses MCMC methods to place genotypes into groups whose members present similar patterns of variation and identifies populations that fit best for the detected patterns, under the assumption of HWE and LD. It assumes that there are different genetic clusters in the genotypes and, for each individual, proportions of its genome belonging to one or more genotypes are assigned to a cluster accordingly. K represents number of clusters. 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 MCMC

steps, with K values set from 1 to 10. The results were processed using STRUCTURE HARVESTER v. 0.6.94 (available at http://taylor0.biology.ucla.edu/struct_harvest/;Earl & vonHoldt, 2012) and the most probable number of K was estimated through the Evanno method (Evanno et al., 2005) and through the posterior probability of K (Pritchard et al., 2000).

GENELAND v 4.0.8 (Guillot et al., 2005) was used to infer population structure using the microsatellite dataset, based on the spatial distribution of the individuals. GENELAND is similar to STRUCTURE in the sense that it also implements a Bayesian assignment approach. However, by incorporating geographical information, it increases the power to correctly determine the underlying population structure, assuming populations occupy geographically delimited areas. UTM coordinates were used to run the spatial model. First, the uncorrelated allele frequency model was used to have an initial estimate of structure. The null allele model was not adopted. 10 independent runs were performed of K=1 to K=5. Each run consisted of 100,000 MCMC iterations with a thinning of 100 and a burn-in of 1,000. This was repeated under the correlated allele frequency model. The most likely number of clusters was determined as the modal K (from each independent run) with the highest posterior probability.

Isolation by distance (Wright, 1943) was tested through Mantel tests (Mantel, 1967) in GenALEx (Peakall & Smouse, 2012), using the pairwise FST values obtained for both the microsatellite and mitochondrial datasets genotype dataset and the logarithm of geographic distances calculated between populations, as described in Rousset (2000). Significant correlation was tested for the five possible pairs of geographic populations using 10,000 permutations. Besides the population-level analysis, an individual based approach was tested in Genepop on the Web (Raymond & Rousset, 1995), using the genotype data and geographic distances between individuals (the logarithm of geographic distances was not used in this approach as it would affect the analysis due to the presence of null and very low distances; Mantel, 1967).

Mean relatedness among individual samples within groups was estimated using the Queller and Goodnight (1989) regression-based as implemented in GenAlEx v. 6.503 (Peakall & Smouse, 2012).

PopArt v. 1.7 (Leigh & Bryant, 2015) was used to construct a median-joining mtDNA haplotype network (Bandelt et al., 1999), with a value of epsilon set to 1. Haplotypes were coloured according to the previously defined groups of samples. The museum sample and the sequence available in GenBank from Steiner et al. (2014) were included in this analysis.

2.6.4. Demographic history

The demographic history of hirola populations was inferred using both the nuclear and mtDNA datasets. Mitochondrial control region sequences were used to test for mutation-drift equilibrium on the whole dataset. Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) were estimated in ARLEQUIN v 3.5 (Excoffier et al., 2005). A positive significant D value indicates population decline and a negative significant D value suggests population expansion or selection (Rozas, 2009). A negative value of Fu's Fs indicates an excess of alleles, as is expected in the case of recent population expansion or genetic hitchhiking, and a positive value indicates a deficiency of alleles, expected after a recent population bottleneck or from overdominant selection (Fu, 1997). Fu and Li's D* (Fu and Li, 1993), Fu and Li's F* (Fu and Li, 1993), and Ramos-Onsins and Rozas' R_2 (Ramos-Onsins and Rozas, 2002) were estimated in DnaSP v. 5.10 (Rozas, 2009). The significance of Fu's F and Ramos-Onsins and Rozas' R_2 was tested using 1,000 coalescent simulations based on theta with a 95% confidence interval.

Mismatch distribution analyses were performed for the whole dataset and compared against a model of constant population size in DnaSP v 5.10 (Rozas, 2009). A mismatch distribution is expected to be ragged and erratic in populations that have been stable for a long time and smooth and unimodal in populations that have been growing for a long time or that experienced sudden growth (Harpending, 1994). To complement the mismatch distribution analysis, effective population size changes through time were estimated according to a Bayesian skyline plot (BSP) constructed in BEAST v1.8.2 (Drummond & Rambaut, 2007). jModelTest (Posada, 2008) suggested the Hasegawa, Kishino and Yano model (HKY) (Hasegawa et al., 1985) as the best fitting model. Two independent runs, with a length of chains defined to 20,000,000, were performed in BEAST and the runs were combined in LogCombiner v1.8.2 (Drummond & Rambaut, 2007) and analysed in Tracer v1.6 (Rambaut et al., 2018), with a 10% burn-in. Bayesian Skyline plots were produced using the combined trees file.

In the case of microsatellite markers, BOTTLENECK v. 1.2.02 (Cornuet & Luikart, 1996) was run for the whole dataset to infer recent population bottlenecks. This program is based on the assumption that, after a genetic bottleneck, the observed heterozygosity is higher than the expected heterozygosity at mutation drift-equilibrium because the number of different alleles at polymorphic loci has a faster reduction than the actual heterozygosity. Therefore, for n samples, it computes the distribution of the expected heterozygosity under mutation-drift equilibrium for each population sample and each

locus by simulating the coalescent process of n genes. One thousand simulations were performed under the stepwise mutation model (SMM; Kimura & Ohta, 1978) and the two-phase model (TPM; Di Rienzo et al., 1994), with 70% SMM and 30% IAM (infinite allele model; Kimura & Crow, 1964). Sign tests, standardized differences tests (Cornuet & Luikart, 1996) and Wilcoxon sign-rank tests (Luikart et al., 1998a) were adopted to test the significance of the distribution. The mode-shift indicator was used as a descriptor of the allele frequency distribution and, as such, to infer the existence of a bottleneck (Luikart et al., 1998b).

Besides the inferences made using DNA markers, the census population size of hirola was estimated using mark-recapture models implemented in the package Capwire (Pennell et al., 2013) in R v. 3.5.1 (R Foundation for Statistical Computing, 2018), using the information gathered from non-invasive genetic sampling (the translocated population was excluded). Two models were used: Equal-capture model (ECM) and Two-innate-rates model (TIRM). In the first model, all individuals are assumed to have an equal probability of being captured whereas the latter accounts for heterogeneity in the probability of capture. A likelihood ratio test (LRT) was performed to understand which is the most accurate model for this data. A partitioning algorithm (PART) was employed to remove individuals that were captured far too frequently, which may introduce a bias in the analysis. These analyses were performed for all the faecal samples as one population, for the sanctuary population and for the conservancy population. Bura and Sangailu were excluded due to the low sample size.

Additionally, maximum distance between duplicate samples was retrieved using ArcGIS v10.1 to assess possible patterns of movement of the individuals/herds.

3. Results

3.1. Microsatellite loci

3.1.1. Genotyping, quality control procedures, and identification of repeated genotypes

Twenty-one out of 44 markers that amplified for the blood samples were polymorphic (see Table S1, Supplementary Material). From these, four loci (HPN27, HPN29, HPN36 and SPS115) showed departures from Hardy-Weinberg (HW) equilibrium and three pairs of loci were in linkage disequilibrium (LD) (HPN27/HPN36, HPN29/HPN36 and HPN27/HPN29) after applying the Bonferroni correction for multiple comparisons. Hence, these four loci were discarded from further DNA amplifications from non-invasive samples. Other three loci were discarded based on small number of alleles and low polymorphism information content (HPN20, HPN45 and HPN116).

Eighty-four faecal samples were successfully amplified and genotyped for the 14 most informative microsatellite loci (see Table 2 in section 2.5). No samples were excluded due to low DNA quality. GIMLET estimated a very low frequency of genotyping errors (allelic dropout: 0.2%; false alleles: 0%) from the four replicates amplified for each faecal sample. No significant departures from HW equilibrium were found and no LD was found across the five populations after the Bonferroni correction for multiple comparisons (*p*-value = 0.00071 for HWE; *p*-value = 0.00055 for LD). However, two pairs of loci (HPN9/INRA06; INRA06/BM1824) were found to be in LD (p-value = 0.000216; p-value = 0.000212) in the Sanctuary population when analysed alone. Across all samples, the number of alleles per locus ranged from 2 to 6. Observed heterozygosity ranged from 0.379 (HPN9) to 0.841 (TGLA53), presenting an average of 0.512 (Table 3).

Table 3- Summary diversity statistics for the 14 autosomal microsatellite tested: n - sample size; NA - total number of alleles; HO - observed heterozygosity; HE - expected heterozygosity; FIS - inbreeding coefficient) and allele dropout rate.

Locus	n	NA	Allelic range	HO	HE	FIS	Dropout rate (%)
HPN93	54	2	224-226	0.519	0.536	-0.060	0
HPN12	54	4	147-155	0.565	0.630	0.020	0
HPN2	53	6	213-221	0.400	0.602	0.275	0
HPN9	54	3	168-174	0.379	0.507	0.190	0
HPN16	54	3	230-248	0.555	0.486	-0.233	0
HPN10	54	3	300-306	0.499	0.564	0.045	0
HSC	54	3	133-137	0.385	0.505	0.171	2.3
ILST87	54	3	141-143	0.442	0.514	0.071	1
INRA06	54	2	171-175	0.468	0.481	-0.052	0
INRA5	53	4	186-194	0.516	0.507	-0.115	0
BM1824	53	3	200-202	0.570	0.508	-0.226	0
HPN13	54	2	185-211	0.629	0.514	-0.337	0
HPN21	53	3	175-189	0.396	0.377	-0.136	0
TGLA53	54	5	229-241	0.841	0.805	-0.143	0
Overall	-	-	-	0.512 (±0.089)	0.538 (±0.127)	-0.038 (P=0.19)	-

The cumulative probability of identity (pID) and probability of siblings (pIDsibs) curves showed that the probability of finding two identical genotypes from different individuals (PI) is very low ($P < 10e^{-8}$), even between siblings (PIsibs) ($P < 10e^{-4}$).

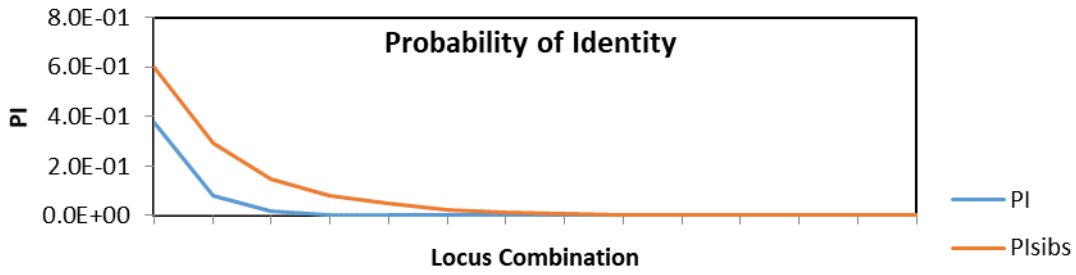


Fig. 6- Cumulative probability of identity (PI) and probability of identity between siblings (PIsibs).

Thirty-four samples resulted in duplicate genotypes and were excluded from population genetic analyses. The maximum number of duplicate samples of the same individual

was 8. Also, the maximum genetic distance measured between samples was 2350 meters (see Figs. S2 and S3 and Table S4). The final microsatellite dataset consisted of 36 unique genotypes obtained from faecal samples, 18 from blood and 1 genotype from the museum tissue sample (55 individuals in total).

3.1.2. Genetic Diversity

The levels of genetic diversity observed, using the microsatellite dataset, were similar between populations (Table 4). The overall observed heterozygosity was 0.512 (±0.038), whereas the expected heterozygosity was 0.551 (±0.089). The mean number of alleles (Na) per population ranged from 2.4 (Sangailu) to 3.0 (Translocated) and the allelic richness ranged from 2.25 (Sanctuary and Bura) to 2.36 (Translocated and Sangailu). The observed heterozygosity was lower than expected for Bura, higher than expected for Conservancy, Sanctuary and Sangailu populations and similar to expected in the Translocated population. Inbreeding coefficient was positive for all populations except Sanctuary and highest in the Bura population. In all populations, except Bura, private alleles (PA) were found, with the Sanctuary having the highest number of private alleles (PA = 3) (Table 4).

Table 4- Genetic diversity measures for each population and for the overall dataset for 14 autosomal loci: n - sample size; NA - number of alleles; H0 - observed heterozygosity; HE - expected heterozygosity; FIS - inbreeding coefficient; AR - allelic richness; PA - number of private alleles. TRLC = Translocated population; SAN = Sanctuary population; CON = conservancy population; BURA = Bura population; SANG = Sangailu population.

Pop	n	NA	HO	HE	FIS	AR	PA
TRLC	18	3.000 (±0.877)	0.531 (±0.124)	0.546 (±0.104)	0.014 (P= 0.430)	2.36	2
SAN	21	2.786 (±0.893)	0.531 (±0.141)	0.529 (±0.098)	-0.004 (P=0.541)	2.25	3
CON	8	2.714 (±0.914)	0.545 (±0.233)	0.558 (±0.099)	0.025 (P=0.430)	2.34	1
BURA	4	2.357 (±0.842)	0.429 (±0.267)	0.526 (±0.171)	0.209 (P=0.145)	2.25	0
SANG	3	2.357 (±0.842)	0.524 (±0.363)	0.533 (±0.163)	0.022 (P=0.529)	2.36	1
Overall	54	3.286 (±1.139)	0.512 (±0.038)	0.551 (±0.089)	0.022 (P=0.297)	2.35	-

3.1.3. Population differentiation and structure

The pairwise FST values ranged between -0.005 and 0.102, with an overall value of 0.028 (Fig. 8; and see Table S5, supplementary material). However, only a statistical significant FST value was found (*p*-value < 0.05), when comparing the translocated group and the Bura group.

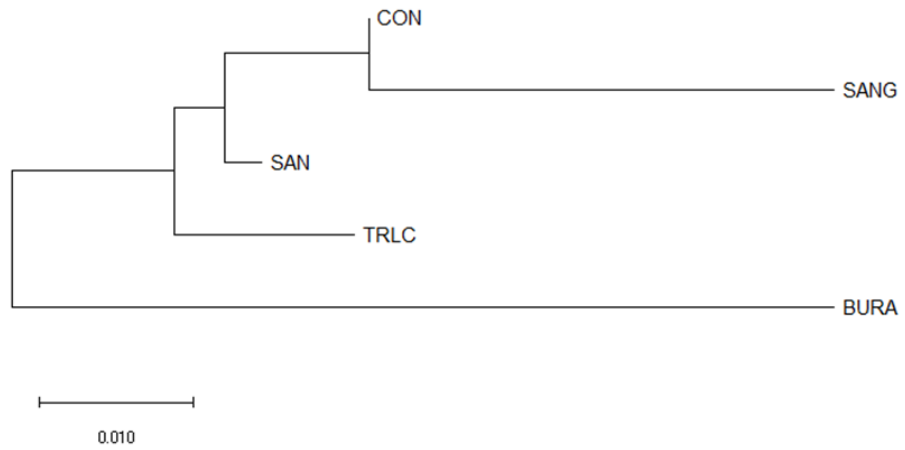


Fig. 7- Neighbor-Joining tree constructed through pairwise FST obtained for the five comparison populations using the 14 microsatellite markers. Only one FST value was found, between TRLC and BURA. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG = Sangailu.

The hierarchical AMOVA conducted using the microsatellite data revealed that the majority (>90%) of the variation was present within the populations and not among populations or groups, suggesting no clear genetic differentiation between populations (Table 5).

Table 5- Results of hierarchical AMOVA. The p-value is determined through the frequency of more extreme variance components than observed obtained randomly after 10,000 permutations. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG = Sangailu.

Group assignment	Source of variation	Sum of squares	Variance components	Percentage of variation
No groups	Among populations	17.257	0.133	4.19
	Within populations	149.095	3.043	95.81
TRLC + CON	Among groups	13.327	0.062	1.94
	Among populations within groups	3.929	0.08	2.51
	Within populations	149.095	3.043	95.55
SAN + CON + TRLC	Among groups	8.481	0.126	3.86
	Among populations within groups	8.775	0.092	2.81
	Within populations	149.095	3.043	93.33

No clustering pattern of the populations was observed from the factorial correspondence analysis (Fig. 4). The first factorial component (FC1) explains 9.83 % of variation and the second factorial component (FC2) explains 8.56% of the observed variation.

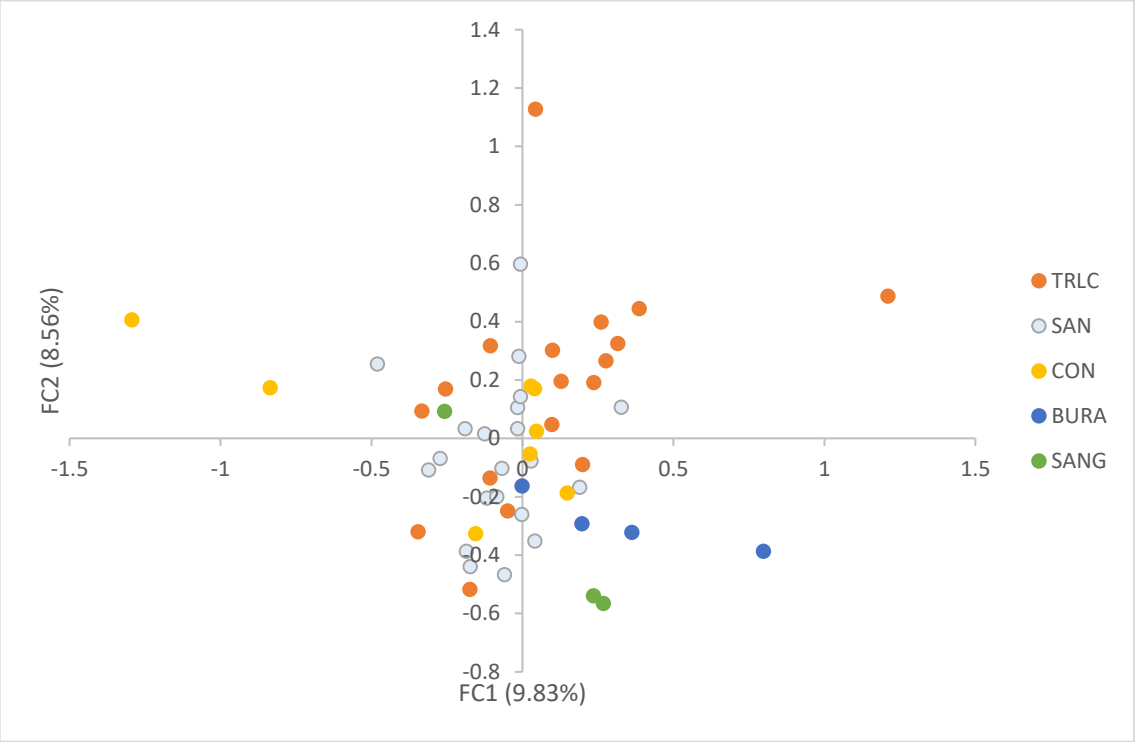


Fig. 8- Factorial correspondence analysis performed in GENETIX using the 14 microsatellite markers. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG = Sangailu.

The Bayesian clustering analysis carried out in STRUCTURE indicated K = 1 as the most probable number of clusters (Figs. 10 and 11).

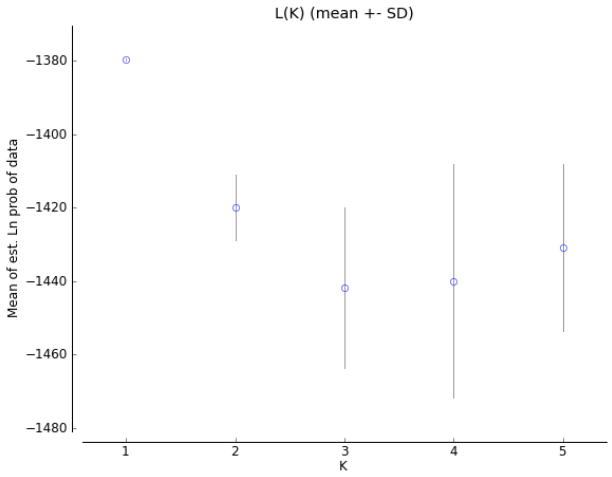


Fig. 9- Inference of the most probable number of clusters (K) using the mean of estimated Ln probability of data, obtained in STRUCTURE HARVESTER. K=1 was chosen as the best solution.

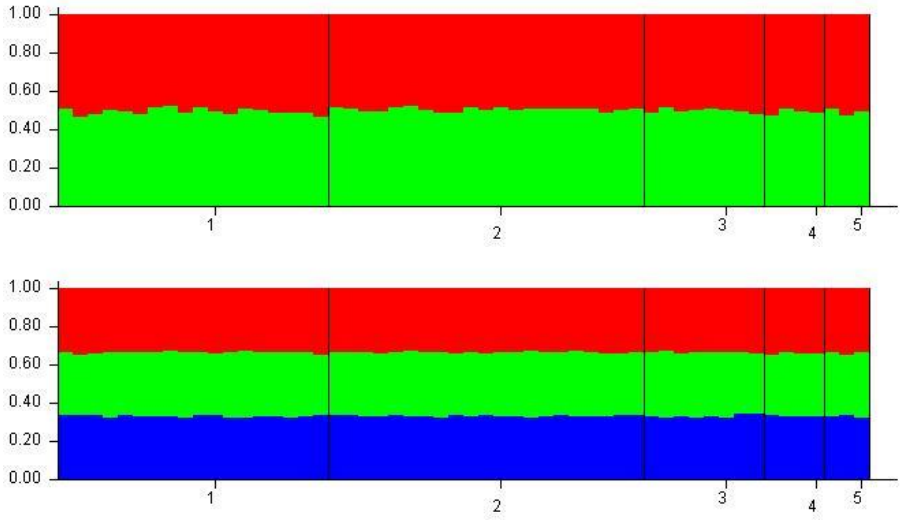


Fig. 10- Bar plot obtained in STRUCTURE for K=2 and K=3

The Bayesian approach incorporating spatial coordinates performed in GENELAND (under the uncorrelated model), did not indicate any clustering or geographical structuring patterns (see Fig S4, Supplementary Material). Under the correlated model, the clustering solution inferred by GENELAND was K = 4 (Fig. 12). However, these clusters do not correspond to a geographical pattern with the exception of the separation between Bura and the Translocated, Sanctuary and Conservancy populations, which corresponds to the furthest distance between populations. It is important to note that the independent runs performed showed inconsistent results (see Figs S5 and S6, Supplementary Material), which, combined with the lack of differentiation inferred under the uncorrelated model, suggests a weak signal of the data.

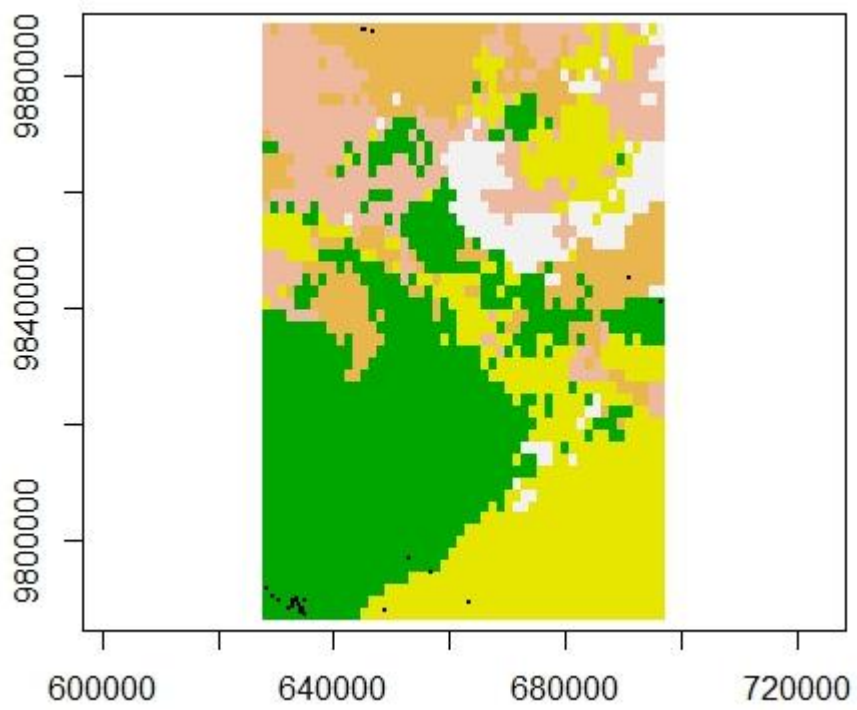


Fig. 11- Map of cluster membership for the run with highest average posterior probability (K=4). X and Y graph correspond to UTM coordinates.

The Mantel tests performed for all individuals and for the populations using the microsatellite data showed a significant pattern of isolation-by-distance (*p*-value < 0.05) (Fig. 13 and Fig. 14).

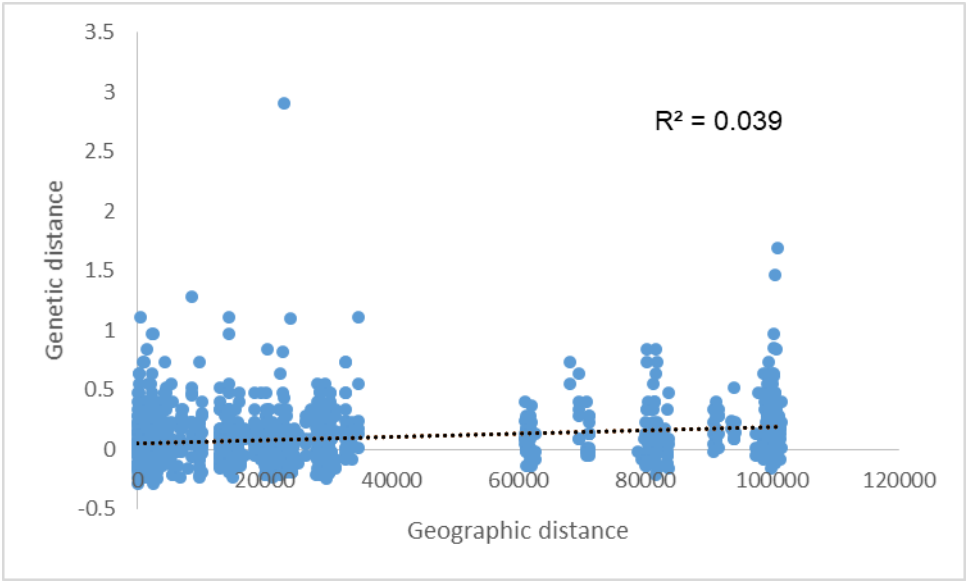


Fig. 12- Mantel test performed using microsatellite data to test the hypothesis of isolation by distance (R = Pearson correlation coefficient; P = P value). This graph shows the relationship between genetic distance and geographic distance.

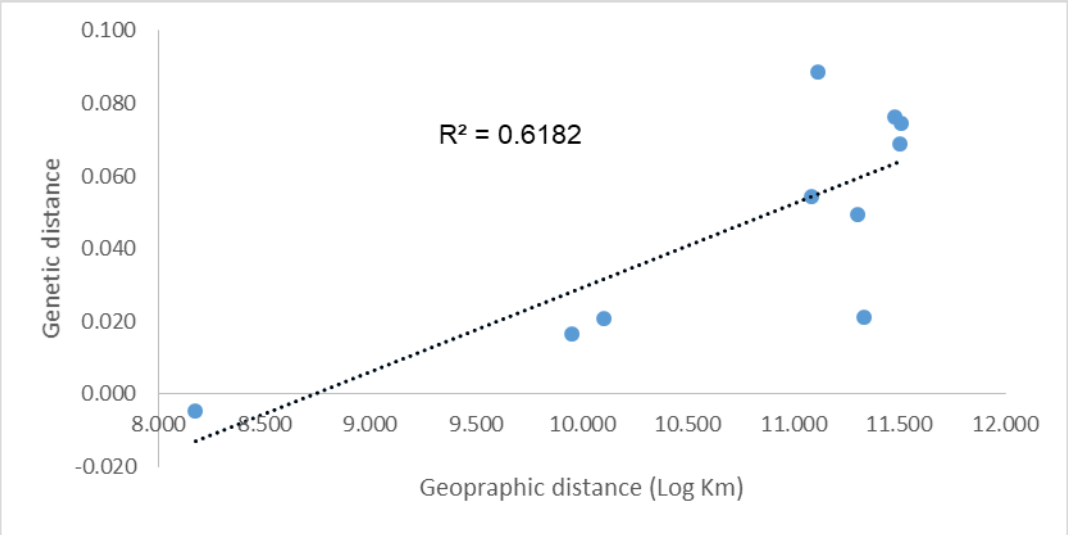


Fig. 13- Mantel test performed between populations, using microsatellite data to test the hypothesis of isolation by distance (R = Pearson correlation coefficient; P = P value). This graph shows the relationship between genetic distance and geographic distance.

Individual relatedness analysis (Table 6) showed mean values of Queller's and Goodnight (QG) estimator of relatedness that ranged from -0.4903 to -0.0001 across populations. Queller's and Goodnight estimator for the overall dataset was -0.0202.

Table 6 - Queller's and Goodnight (QG) estimator of individual relatedness for each population in averages. The minimum and maximum values are also displayed. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG= Sangailu.

Pop	Mean	Variance	Min	Max
TRLC	-0.0001	0.0489	-0.7449	0.677
SAN	-0.0416	0.0757	-0.8176	0.7774
CON	-0.1427	0.0894	-0.5842	0.7808
BURA	-0.2997	0.1331	-0.6911	0.4419
SANG	-0.4903	0.0582	-1.000	-0.1786
Overall	-0.0202	0.0462	-0.4966	0.4949

3.1.4. Demographic history

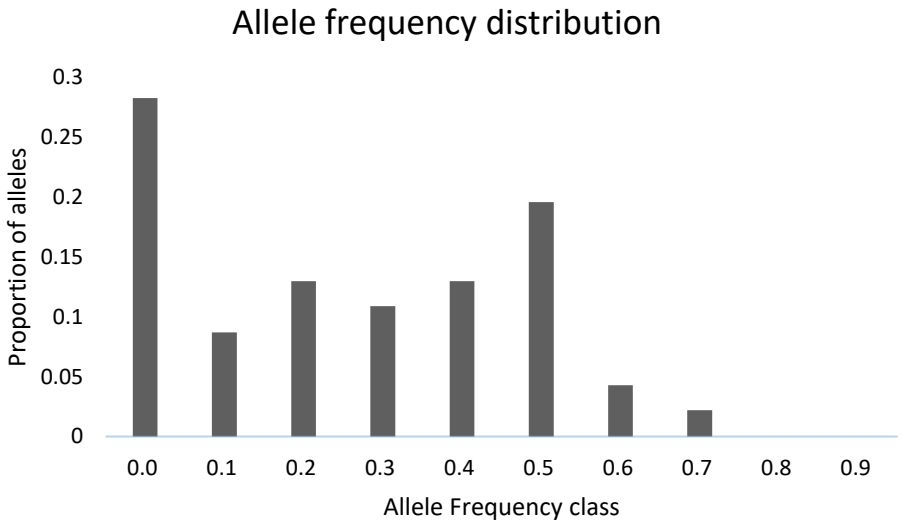


Fig. 14- Plotting of frequency distribution of allele classes for microsatellite markers. Figures along the x-axis represent classes of frequency of alleles (e.g. 0.0 represents alleles with frequency lower than 0.1) and figures along the y-axis represent the proportion of alleles in those classes. A non-shifted, or L-shaped, distribution was revealed as the low-frequency class (0.0) has more alleles than any of the other classes.

BOTTLENECK indicates no signals of recent population bottleneck when considering allele frequency, which showed a normal L-shaped distribution. In addition, the result of the sign test under the stepwise mutation model was not significant (p-value > 0.05). However, the standardized differences test and the Wilcoxon test (one-tail for heterozygote excess) showed a significant excess of heterozygotes under this model and, under the two-phase model, the sign test, the standardized differences test and the Wilcoxon test (one-tail for heterozygote excess) showed significant p-values < 0.05, indicating an excess of heterozygotes.

3.2. Mitochondrial DNA

3.2.1. Genetic diversity

The length of the mitochondrial control region was 934 base pairs long. A total of 87 samples were successfully amplified but, after, identity confirmation of individuals with the microsatellite data, 52 sequences (two samples that amplified for microsatellite loci did not amplify successfully for the mtDNA) remained, comprising 5 haplotypes and 6 polymorphic sites. The overall estimated haplotype diversity (HD) was 0.583 (± 0.067)

and the nucleotide diversity (π) was 0.0014 (± 0.0003) (Table 7). Haplotype diversity was lowest in the Sanctuary (0.368) and highest in Sangailu (1.000). Nucleotide diversity was very low, ranging from 0.0005 in Sanctuary and Bura to 0.0042 in Sangailu.

Table 7- Genetic diversity statistics from the mtDNA control region: n - number of sequences; NH - number of haplotypes; HD - haplotype diversity and its standard deviation; S - polymorphic sites; π - nucleotide diversity and its standard deviation. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG = Sangailu.

Population	n	NH	HD	S	π
TRLC	18	4	0.595 (±0.109)	5	0.0015 (±0.0004)
SAN	19	3	0.368 (±0.125)	2	0.0005 (±0.0002)
CON	8	3	0.607 (±0.164)	4	0.0020 (±0.0005)
BURA	4	2	0.500 (±0.265)	1	0.0005 (±0.0003)
SANG	3	3	1.000 (±0.272)	6	0.0042 (±0.0015)
Overall	52	5	0.583 (±0.067)	6	0.0014 (±0.0003)

3.2.2. Population differentiation and structure

The pairwise FST values ranged between -0.128 and 0.562 (Fig. 16). However, no significant FST values were found ($p > 0.05$) for any pairwise comparison of the populations (see Table S5, supplementary material).

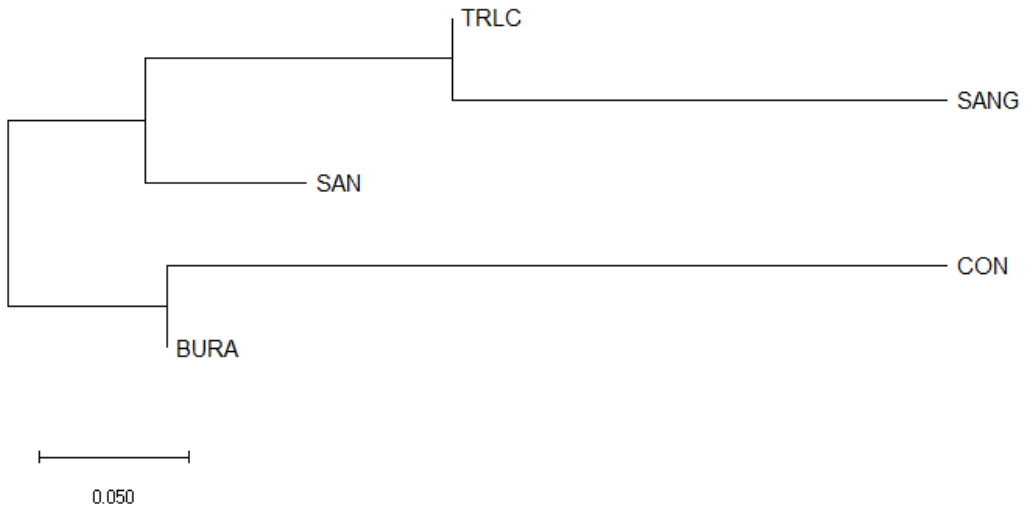


Fig. 15- Neighbor-Joining tree constructed through pairwise FST obtained for the five comparison populations using the mtDNA control region sequences. No FST values were considered statistically significant. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG = Sangailu.

The hierarchical AMOVA conducted using the mtDNA control region data under the assumption of no groups revealed that most of genetic variance (82.92%) was present within populations and only 17.08% was present between populations (Table 8). The analysis with the TRLC and CON grouped together showed similar variation within populations (81.33%). Analysis with the SAN, TRLC and CON as a group showed more variation among groups (20.6 %) and less variation within populations (71.63%). All three analyses were statistically significant ($p < 0.05$).

Table 8- Results of the hierarchical AMOVA conducted for the mtDNA control region sequences. The p-value is determined through the frequency of more extreme variance components obtained randomly after 10,000 permutations. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG = Sangailu.

Group assignment	Source of variation	Sum of squares	Variance components	Percentage of variation
No groups	Among populations	6.666	0.118	17.08
	Within populations	26.911	0.573	82.92
TRLC + CON	Among groups	5.645	0.091	12.92
	Among populations within groups	1.021	0.041	5.75
	Within populations	26.911	0.573	81.33
SAN + TRLC + CON	Among groups	3.760	0.165	20.6
	Among populations within groups	2.906	0.062	7.77
	Within populations	26.911	0.573	71.63

The median-joining network of the 54 mtDNA control region sequences, including the GenBank and the museum samples, showed 6 haplotypes, with the GenBank sequence constituting a unique haplotype (Fig. 17). The highest genetic divergence observed between haplotypes was of three mutations, between haplotypes 3 and 4. All the other haplotypes diverged only by a single mutation. The museum sample presented the most frequent haplotype.



Haplotype	SAN	TRLC	CON	BURA	SANG	MUSEUM	GENBANK
1	1	0	0	1	1	0	0
2	3	4	0	3	0	0	0
3	15	11	5	0	0	1	0
4	0	2	2	0	0	0	0
5	0	1	1	0	1	0	0
6	0	0	0	0	0	0	1

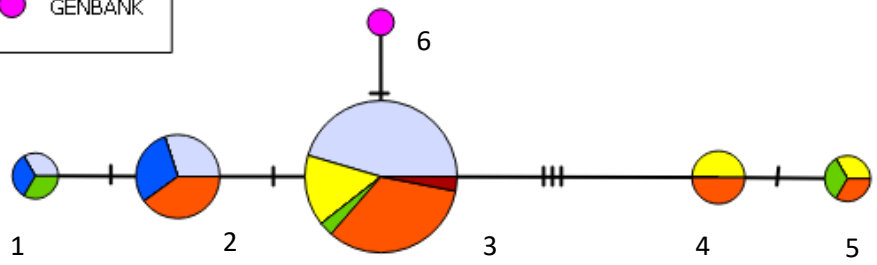


Fig. 16- Median-joining network based on mtDNA control region sequences. Populations are distinguished through different colours and node size is dependent on frequency of sequences. Each mutation is represented by a hatch mark. Table demonstrating the number of individual per haplotype and population. Note that Genbank and museum sequences were included in this analysis. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG= Sangailu.

The mitochondrial DNA based Mantel test (Fig. 17) did not show a significant correlation ($p > 0.05$) between genetic distance and geographic distance.

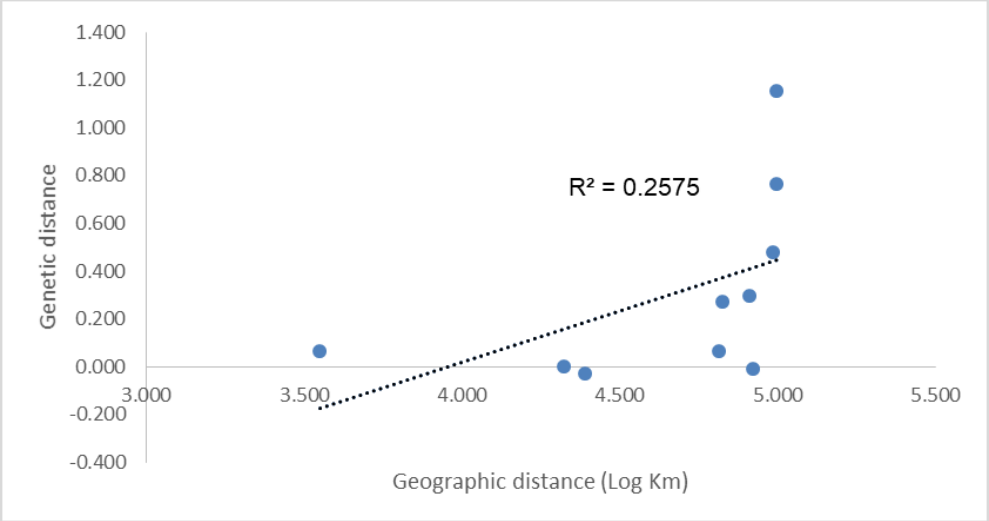


Fig. 17 - Mitochondrial DNA Mantel test of isolation-by-distance. This graph shows the relationship between genetic distance and geographic distance.

3.2.3. Demographic history

Fifty-two mtDNA control region sequences were used to analyse the demographic history through neutrality tests per each population and for the overall dataset. Some tests were not calculated for the Sangailu sequences because of the low sample size (n = 3). None of the neutrality tests performed were statistically significant (Table 9).

Table 9 - Results of neutrality tests: Tajima's D, Fu's F, Fu and Li's D and F, R2. n is the sample size, NH is the number of haplotypes and NS means non-significant result. TRLC = Translocated; SAN = Sanctuary; CON = Conservancy; BURA = Bura; SANG = Sangailu.

Population	n	Tajima's D	Fu's F	Fu and Li's D	Fu and Li's F	R_2
TRLC	18	-0.205	0.595	0.42	0.286	0.133
SAN	19	-0.485	-0.421	-0.574	-0.63	0.142
CON	8	0.889	1.412	0.568	0.706	0.2172
BURA	4	-0.612	0.172	-0.612	-0.479	0.433
SANG	3	-	-	-	-	0.2722
Overall	52	-0.021	0.715	1.172	0.931	0.1097

The overall mismatch distribution (Fig. 14) is slightly bimodal, suggesting population stasis. The raggedness index did not show a significant deviation from a model of population growth. The Effective sample size (ESS) values obtained in BEAST were significantly >200. The Bayesian skyline plot (Fig. 15) sho*w*ed a stable population size over time.

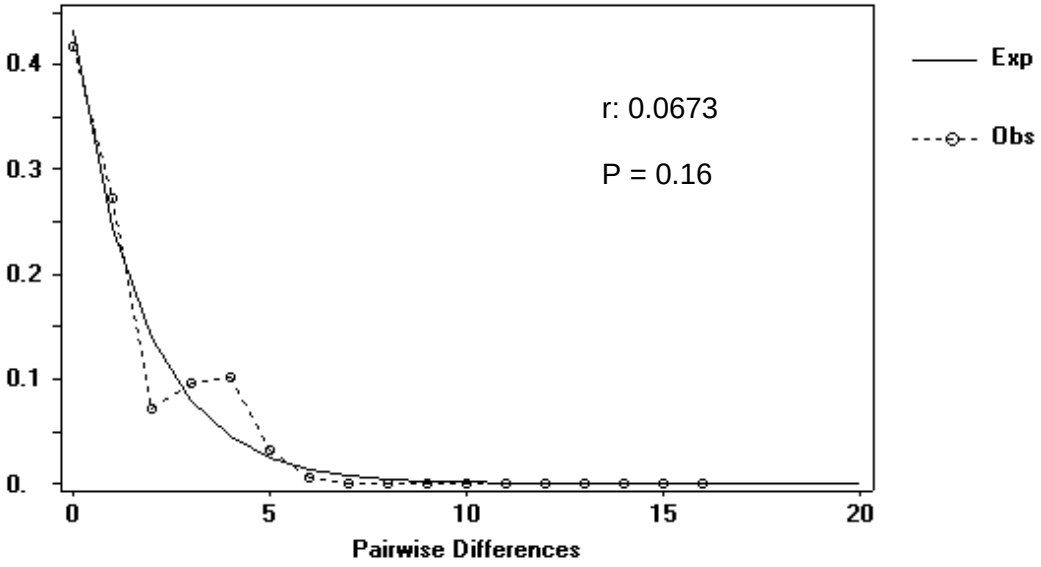


Fig. 18 - Mismatch distribution analysis with observed distribution, represented by a dotted line, and expected distribution, represented by a black line, under a model of constant population size.

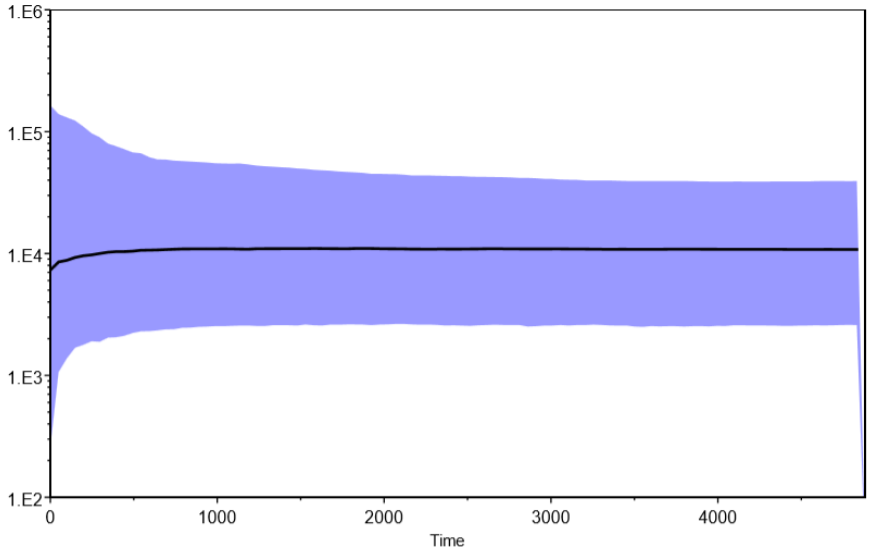


Fig. 19 - Bayesian skyline plots constructed in BEAST. The Y axis indicates population size and the X axis represents time (in years) from present to past. The solid line represents the median estimate and the blue area shows a 95% confidence interval.

3.2.4. Census population size estimation using non-invasive samples

Results from the package Capwire in R, which calculates population size through mark-recapture models, estimated a number of 21 animals in the sanctuary, 12 in the conservancy and 45 for all the populations under the equal-capture model (Table 10). For the two-innate-rates-model, for which a higher likelihood was obtained, the results estimated 42 individuals at the sanctuary, 13 at the conservancy and 65 for all the populations. By excluding individuals with a higher number of recaptures, the software estimated 61 individuals for the sanctuary and 69 for all the populations.

Table 10 - Census population size estimated using the mark-recapture models implemented in Capwire. The number of individuals estimated from equal-capture model (ECM) and two-innate-rates model (TIRM) are shown, together with the results excluding individuals with a higher number of recaptures (Partitioned) and confidence intervals for both models.

Population	Faecal samples (N)	Individuals (N)	ECM	TIRM	Conf. int. ECM (2.5 – 9.75)	Part.	Conf. int. TIRM (2.5 – 9.75)
Sanctuary	34	21	31	42	21 - 44	61	37 - 84
Conservancy	8	12	12	13	8 - 29	-	37 - 84
Total	70	36	45	65	37 - 52	69	53 - 93

4. Discussion

This is the first study to assess the genetic diversity, population structure and demographic history of the world's most endangered antelope, the hirola (*Beatragus hunteri)*. The overall genetic diversity estimates were moderate for the microsatellite loci and low for the mitochondrial data, although signals of a genetic bottleneck were observed. Lack of population structure suggests mixing of herds and gene flow throughout the species range. These results are relevant for future and ongoing conservation management.

4.1. Patterns of genetic diversity in Hirola

The overall genetic diversity estimates obtained from nuclear (microsatellites) and mitochondrial (control region) DNA content were contrasting. Firstly, nuclear genetic diversity was similar between all five hirola populations, with HO and HE ranging between 0.429 - 0.545 and 0.526 - 0.558, respectively. Despite the differences in sample size across sampling groups, the allelic richness obtained using a rarefaction analysis was similar among sampling groups (2.25 – 2.36), suggesting a non bias in genetic diversity in relation to population sampling (Kalinowski, 2004). The overall genetic diversity recovered from the microsatellite analyses (HO = 0.512; He = 0.551) was higher, for instance, than for populations considered to have sustained severe bottlenecks (see Table 11), like the Angolan population of the Giant Sable (*Hippotragus niger variani*; HE = 0.306; Vaz Pinto et al., 2015) and the Parque Lecocq Zoo population of Addax (Addax *nasomaculatus;* HE = 0.432; Armstrong et al., 2011). However, genotypic diversity was within the range of other endangered antelopes. For example, it is comparable to that of some populations of the roan antelope (*Hippotragus equinus*), a species that has experienced severe population declines in the past few decades (Alpers et al., 2004), and to captive/semi-captive populations of Dorcas gazelles (*Gazella dorcas)* that also showed signs of bottleneck (Godinho et al., 2012). Higher genetic variation has been shown for Swayne's hartebeest (*Alcelaphus busephalus swaynei*), an endangered subspecies from the same family as hirola (Alcelaphinae) that also suffered severe population declines (Flagstad et al., 2000). Hirola´s genetic diversity is somewhat unexpected for a species which recently suffered severe population declines with a low population size remaining, with an overall historical population size relatively low when

compared to other antelope species, confined to a small geographical range since before its decline (Butynski, 2000).

On the other hand, mitochondrial genetic diversity varied significantly among populations. The sanctuary population recovered low haplotype and nucleotide diversity as compared with all other populations, while the Sangailu population had the highest haplotype and nucleotide diversity. Curiously, the Sangailu population had a haplotype per individual (n = 3), which implies that the low sample size may not be representative of a possibly heterogenous population. The overall mitochondrial nucleotide diversity was remarkably low for this species ($\pi = 0.00141$), when compared with populations of other endangered antelopes (see Table 12): Roan antelope (*Hippotragus equinus;* Alpers et al., 2004); Dorcas gazelles (Godinho et al., 2012); scimitar-horned oryx (*Oryx dammah*; Iyengar et al., 2007), Prezwalsky's gazelles (*Procapra przewalskii*; Lei et al., 2003), *Saiga tatarica* (Campos et al., 2010), mountain (*gazelle gazelle*) and acacia gazelles (*Gazella arabica acacia*) (Hadas et al., 2015).

The contrast between the lower mitochondrial variation and nuclear analyses may reflect the effective population size of the strictly maternally inherited mitochondrial genome, which is much lower than that of the nuclear genome (Birky et al., 1983). Furthermore, it is possible that this lack of mitochondrial variation is an historical pattern, consequent of one or more episodes of severe declines in the distant past of this species. One possible explanation for the existence of severe declines in its past is 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 (*Alcelaphus busephalus*), *Damaliscus lunatus* and the wildebeest (*Connochaetes taurinus*) (Arctander et al., 1999; Alpers et al., 2004). However, no signs of population decline were found using the mitochondrial dataset.

Interestingly, the museum sample, dating to 1937, did not present different alleles from the other samples. Although one sample does not constitute a statistically significant number, it could be expected that an individual from before the population crash would present novel alleles not present in the rest of the post population crash dataset. This implies the observed pattern of genetic diversity in our dataset is a historical one. Nevertheless, this finding may be a reflection of the most common haplotypes present throughout the population before it declined and that remained fixed in the population thereafter.

4.2. Population structure

The results obtained from the Bayesian clustering analysis, the factorial correspondence analysis (FCA), AMOVAs and the median-joining network do not indicate any significant genetic substructure or population differentiation and, consequently, no geographical structure pattern. In contrast, a significant pattern of isolation-by-distance was evidenced from the microsatellite Mantel test.

Hirola population declines are thought to be a consequence of several biotic, abiotic and anthropogenic factors, such as disease, drought, predation, poaching, competition with livestock and habitat loss. As a result, hirola has declined dramatically and its natural range has been severely reduced, with most of its area being composed of sub-optimal habitat for the species (Butynski, 2000; Andanje, 2002; Ali et al., 2014a, 2017; Ali, 2016). Consequently, this likely led to the fragmentation of hirola's natural range, which, as a consequence, now concentrates in areas with suitable habitat, especially in protected areas where there have been efforts to reduce tree encroachment. In fact, an aerial survey from 2011 (King et al., 2011) showed a tendency for herds to concentrate in three locations (Fig S7, Supplementary Material). Although it is possible to detect genetic subdivision caused by fragmentation after a limited number of generations through standard population genetic markers (Epps et al., 2005; Proctor et al., 2005; Lesbarrères et al., 2006; Delaney et al., 2010), no clear pattern of differentiation was found between the populations sampled. This suggests that, before their decline, the hirola had a continuous distribution across their historical range and formed a relatively large, panmictic population with a high degree of connectivity and gene flow between localities, leading to a genetic homogenization effect. Genetic differentiation is likely to be diminished by the patterns of migration and aggregation of herds in the dry season, in which they search for water and better grazing areas and occasionally aggregate in small areas, and their tendency to change groups and form new ones (Andanje, 2002), contrasting with philopatric habits in other species that tend to lead to higher genetic subdivision (Simonsen et al., 1998; Fernando et al., 2000). The maximum distance obtained between duplicate samples (2350 meters) is consistent with the lack of movement of this species in the wet season, which lasts from November to May (Andanje, 2002; Mugalavai et al., 2008). In addition, no differentiation was expected between the sanctuary and the conservancy individuals due to the small geographical distance between the capture sites used for the translocation into the sanctuary and the Ishaqbini Conservancy. Additionally, the fenced sanctuary is too recent (5 ½ years since the translocation) to have differentiated genetically from the wild herds.

The mtDNA median-joining network recovered five haplotypes and little divergence between haplotypes, corroborating the lack of variation presented by the hierarchical AMOVA. This corroborates the lack of population structure found using the microsatellite dataset but may be due to high dispersal of the females. However, both females and males disperse from their natal groups (Andanje, 2002), suggesting there is not a bias caused by higher female dispersal. The mtDNA control region sequence obtained in GenBank, from another study (Steiner et al., 2014), recovered a different haplotype, not found in our study. However, the authors of that study emphasize the low quality of the control region obtained in that sample, suggesting this is likely due to a genotyping error, especially when considering this only potential mutation is the result of a transversion and not a transition.

4.3. Demographic history

The mark-recapture method to estimate the census population size of hirola (Miller et al., 2005) estimated much lower numbers of individuals than expected based on literature and reports from Kenya Wildlife Services rangers. For instance, the sanctuary population is known to have increased from 48 founder individuals to more than 100 (sightings reports from rangers in May 2017 suggested a total of 121 individuals) (Ali, 2016; “Hirola Conservation Programme Newsletter,” 2017b). Sightings reports from May 2017 suggest a total of around 40 animals in Bura and at least 20 in the Ishaqbini conservancy (“Hirola Conservation Programme Newsletter,” 2017b). A report from April 2017 indicates there were more than 40 animals in the Sangailu area (“Hirola Conservation Programme Newsletter,” 2017a). This would result in over more than 200 individuals in the sampled areas. However, our results estimated a maximum of around 90 individuals for the total of the sampled areas. This discrepancy is likely due to a limitation in the sampling area and on the dates of capture. The limitation on spatial and temporal span limits the sampling to faeces of the only herds present at that time and particular location. It would be interesting to expand the area of collection of scats and to perform this sampling on a more continuous basis to retrieve information on other herds and to provide a more accurate estimation of the demography of the remnant populations using genetic data.

Results from BOTTLENECK regarding allele frequencies showed that the mode of the distribution is in the low-frequency class, considered an L-shaped frequency distribution (recent bottlenecks typically cause a mode-shift in which the low-frequency class, i.e. < 0.1, has fewer alleles than one or more of the intermediate frequency classes, e.g. 0.1-0.2; Luikart et al., 1998a). However, visual interpretation of the graphical output shows a

high proportion of alleles in the intermediate classes and a distortion from a normal L-shaped distribution. This, combined with the results of the sign test, the standardized differences test and the Wilcoxon test under the two-phase model (TPM) and the results of the Wilcoxon test under the stepwise mutation model, which showed statistical significance for heterozygote excess (expected after a recent population bottleneck; Cornuet & Luikart, 1996), might suggest that a genetic bottleneck occurred, concordant with the population decline. However, the genotyping of more microsatellite loci would be advisable in order to have a more robust analysis when implementing this software (Cornuet & Luikart, 1996).

The neutrality tests (Tajima's D, Fu and Li's D* and F*, Fu's F and Ramos-Onsins and Rozas' R_2) did not significantly depart from neutrality. Populations that experienced a genetic bottleneck, like populations that have been stable for a long time, should present a ragged and multimodal distribution (Harpending et al., 1993; Weber et al., 2004; Johnson et al., 2007). Genetic evidence of population decline can be detected through a mismatch distribution analysis depending on the severity and duration of the decline (Harpending et al., 1998; Johnson et al., 2007). The raggedness index did not significantly depart from a model of population growth but the mismatch distribution presented a weak bimodal shape. In accordance with the bimodal shape of the mismatch distribution analysis and neutrality tests statistics, the Bayesian skyline plot, similarly, recovered no signs of population growth or decline but of stasis.

Due to their much higher mutation rate (Li et al., 2002), microsatellite markers are better suited than mitochondrial sequences to detect recent bottlenecks, which might explain the evidence found for a bottleneck using the microsatellite dataset but not using the mitochondrial sequences. Additionally, the lack of a bottleneck signal using the mitochondrial dataset might be related to the low polymorphism level in the mitochondrial sequences (Grant, 2015). Nevertheless, the absence of a mode-shift, which contrasts with the heterozygosity excess determined by the statistical tests, suggests that further investigation is needed to address this question.

4.4. Ecological factors and conservation implications

Despite the small numbers of hirola remaining in the wild, the estimated levels of genetic diversity were moderate with no signs of inbreeding contributing to population decline. Therefore, it is important to consider ecological factors to understand hirola's low numbers in the wild. The sanctuary is a clear evidence that hirola are 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 this study. Overgrazing by livestock, megafaunal extirpation and fire suppression are believed to be factors causing tree encroachment and subsequent lack of forage in hirola's range (Riginos, 2009; Goheen et al., 2013; Daskin et al., 2016; Ali et al., 2017). Even though studies have predicted that tree cover increases predation rates on hirola, an assessment of mortality causes failed to support such conclusions (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. Coastal topi (*Damaliscus lunatus topi*), which has the same habitat preferences as hirola, recovered in population numbers since the rinderpest (*Rinderpest morbillivirus*) outbreak, which would suggest the lack of suitable habitat is not what is suppressing the hirola's population growth (Butynski, 2000). However, topi, on the contrary to hirola, extends its 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, 2016). 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, hirola's predators have not increased in abundance and topi, to which predation is comparable, also suffered a decline similar to hirola in the 1980s and has since recovered in numbers (Ali, 2016; Ali et al., 2018). This indicates it is possible for hirola to persist under one of these factors but is unable to contend with a combination of both (Ali, 2016).

Conservation measures should focus on the improvement of rangeland quality and maintenance of existent 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 (Godinho et al., 2012; Buk et al., 2018). In addition, evidence of a genetic bottleneck suggests that this species has a higher chance of losing diversity over time and, as such, is at higher risk of extinction. As such, it is advisable to maintain gene flow between populations through translocation management. For instance, one of the future objectives of the sanctuary is to reintroduce individuals into the wild once the population numbers increase (King et al., 2016).

However, reintroductions should also be aimed at increasing genetic diversity in the sanctuary and not just at increasing the number of individuals in the wild. Due to the low number of founders (n = 48) and restricted gene flow in the sanctuary, this population is likely at high risk of genetic erosion (Butynski, 2000). Thus, it is also pivotal to ensure long-term monitoring programs of genetic diversity in this species, and particularly in fenced populations, in order to avoid the loss of genetic diversity and to support management decisions.

4.5. Limitations in this study and considerations for further research

The main problem associated with this study was the difficulty to collect samples and, consequently, the low sample size in Bura (n = 4) and Sangailu (n = 3) localities. This is due to the rareness of this species (<500 individuals remaining) and remoteness of the sampled area. Dissymmetry in sample sizes may affect the performance of STRUCTURE and a small sample size can lead to underestimation of the number of clusters (Evanno et al., 2005). Scattered sampling along the whole natural range of the hirola would be important to confirm the evidence of genetic isolation-by-distance. Increasing the sample size, especially in Bura and Sangailu, and retrieving further population samples would allow for more robust estimates of genetic diversity and provide more power to detect subtle patterns of differentiation and of demographic history (Grant, 2015). Increasing the number of microsatellite loci and genes analysed in future studies would improve the inference of genetic diversity and structure, as well as provide a more detailed demographic history (Avise, 2000; Heled & Drummond, 2008). On the other hand, future studies could benefit from advances in next-generation sequencing to provide more robust analyses and remove any possible bias introduced through the use of small numbers of neutral markers (Primmer, 2009; Allendorf et al., 2010). Even though non-invasive samples usually present low quality DNA, genomic analyses may be applicable with the use of special approaches to resolve issues that might arise from the use of this type of sampling, like, for example, the use of exon-capture (Russello et al., 2015). It is important to optimize these approaches as non-invasive sampling is frequently the only possible way to sample endangered and elusive species such as hirola (Beja-Pereira et al., 2009; Silva et al., 2015).

Additionally, more museum samples are needed in order to ascertain if the observed current genetic diversity is the consequence of a historical pattern to provide a better picture of the species' demographic history. However, all other attempts to obtain hirola

samples from other museums were unsuccessful. Furthermore, there are only circa 20 samples worldwide and the rarity of such specimens made invasive sampling of mounts and bones not possible. Another limitation in this study is the lack of samples from the hirola *ex-situ* population in the Tsavo National Park. It is important to assess genetic diversity in this population, as this may be the reason for the lack of increase of its size, and to infer any degree of differentiation from the natural population (Butynski, 2000). Samples from the Arawale National Reserve would also be important to assess the genetic diversity in this population, which was affected by the lack of maintenance and consequent degradation of this reserve (Magin, 1996; Butynski, 2000). Finally, non-invasive sampling on a larger spatial and temporal scale could be used to estimate movements in this species, especially their patterns of migration in the dry season to find good quality forage (Butynski, 2000).

5. Concluding remarks

This study constitutes the first genetic assessment of the most endangered antelope, the hirola. Altogether, this project provided novel information on the status of hirola through the use of genetic data. The main conclusions attained in this study were:

i. Nuclear genetic diversity is moderate when compared to other endangered antelopes, which points to a favourable future of this species, especially considering the recent success of the predator-proof sanctuary. This is contrasted by extremely low genetic diversity estimated using mtDNA sequences.
ii. There does not seem to be a restriction on gene flow in the natural range. However, a pattern of isolation by distance is apparent.
iii. Evidence of a genetic bottleneck was found using microsatellite loci but not mitochondrial data

The present study highlights the importance of addressing genetic diversity in endangered species to understand the possible factors of population decline and provide scientific knowledge for ongoing and future conservation management.

6. Glossary

Balancing selection - selection that maintains genetic variants in a population, for example heterozygote advantage.

Fitness - the reproductive success of an individual and its average contribution to the gene pool of the next generation.

Genetic diversity - the total number of genetic differences in a species or a population.

Genetic drift - change of allele frequency across generations due to random sampling of organisms.

Gene flow - transfer of genetic variation from one population to another.

Genetic hitchhiking – increase in frequency of an allele due to a selective sweep on another allele in the same DNA chain.

Genetic structure – any pattern in the genetic makeup of individuals within a population.

Genetic purging - reduction of the frequencies of deleterious mutations in inbred populations, thereby lowering their presence.

Inbreeding - the production of offspring from the mating or breeding of individuals or organisms that are closely related genetically.

Inbreeding depression - the reduction in biological fitness in a population as a result of inbreeding.

Microsatellites- short sections of DNA where a simple motif, generally 1-6 bp long, is repeated up to about 100 times.

Outbreeding - crosses between genetically distant individuals.

Outbreeding depression – loss of fitness resulting from outbreeding.

PCR inhibitors - factors which prevent the amplification of nucleic acids in the PCR.

Polymorphism - DNA difference between distinct individuals of a given species.

Restriction fragment length polymorphisms - different patterns of restriction fragments caused by single base substitutions.

Translocation - Human-mediated movement of living organisms from one area, with release in another (as defined by IUCN, 2013).

7. References

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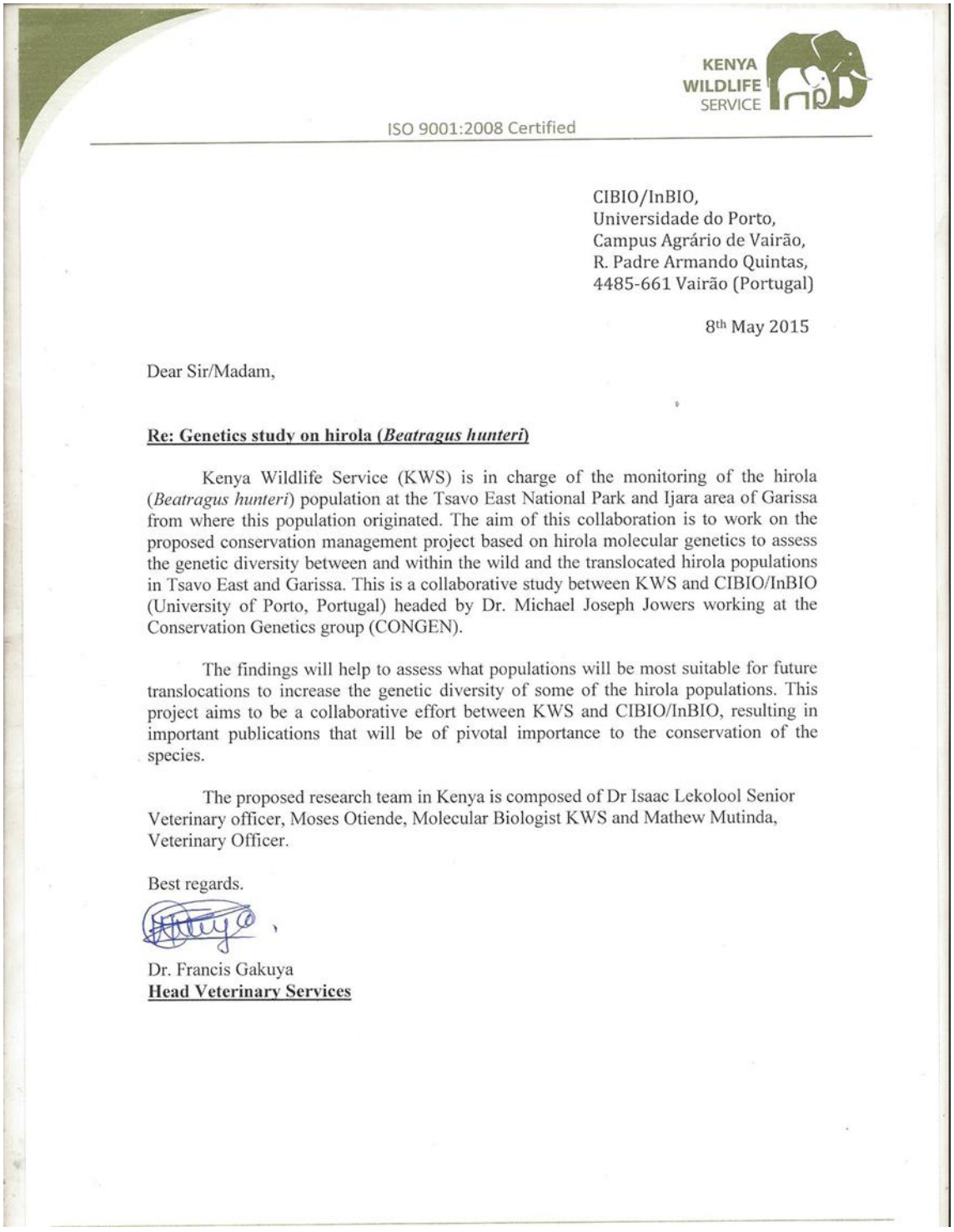
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8. Supplementary Material



KENYA WILDLIFE SERVICE

ISO 9001:2008 Certified

CIBIO/InBIO,
Universidade do Porto,
Campus Agrário de Vairão,
R. Padre Armando Quintas,
4485-661 Vairão (Portugal)

8th May 2015

Dear Sir/Madam,

Re: Genetics study on hirola (*Beatragus hunteri*)

Kenya Wildlife Service (KWS) is in charge of the monitoring of the hirola (*Beatragus hunteri*) population at the Tsavo East National Park and Ijara area of Garissa from where this population originated. The aim of this collaboration is to work on the proposed conservation management project based on hirola molecular genetics to assess the genetic diversity between and within the wild and the translocated hirola populations in Tsavo East and Garissa. This is a collaborative study between KWS and CIBIO/InBIO (University of Porto, Portugal) headed by Dr. Michael Joseph Jowers working at the Conservation Genetics group (CONGEN).

The findings will help to assess what populations will be most suitable for future translocations to increase the genetic diversity of some of the hirola populations. This project aims to be a collaborative effort between KWS and CIBIO/InBIO, resulting in important publications that will be of pivotal importance to the conservation of the species.

The proposed research team in Kenya is composed of Dr Isaac Lekolool Senior Veterinary officer, Moses Otiende, Molecular Biologist KWS and Mathew Mutinda, Veterinary Officer.

Best regards.

Dr. Francis Gakuya
Head Veterinary Services

Fig. S1- Permit letter by Dr. Francis Gakuya, Head of Veterinary Services of Kenya Wildlife Service (KWS).

Table S1 Microsatellite markers used and tested for hirola's invasive samples. Details of the multiplex reactions are provided. NA means no amplification of markers. Markers preceded by an asterisk (*) were discarded due to LD and departures from HWE. Markers preceded by two asterisks (**) were discarded due to low polymorphism information content.

Multiplex	Locus	Primer sequence 5'-3'	Tail fluorescent Dye	Volume in multiplex (ul/50ul)	Number of alleles	Allelic range	Polymorphism information content	Original reference
Cattle	TGLA53	F: GCTTTCAGAAATAGTTTGCATTCA R: ATCTTCACATGATATTACAGCAGA	6-FAM	1	4	181-189	0.676	Georges & Massey, 1992
	*SPS115	F: AAAGTGACACAACAGCTTCACCAG R: AACCGAGTGTCCTAGTTTGGCTGTG	6-FAM	0.4	5	269-271	0.477	Dongmei et al., 2010
	TGLA122	F: AATCACATGGCAAATAAGTACATAC R: CCCTCCTCCAGGTAAATCAGC	VIC	0.4	1	167	0	Georges & Massey, 1992
	BM1824	F: GAGCAAGGTGTTTTTCCAATC R: CATTCTCCAACTGCTTCCTTG	NED	0.6	4	209-211	0.438	Barendse et al., 1994
Giant Sable 1A	HPN17	F: TCTTACCCCCACTTCACTAA R:TATTCCTCCCTTTTCTCCTC	6-FAM	1	1	227	0	Vaz Pinto et al., 2015
	HPN2	F: CAATTCCCTGGGGATAAG R:CTGTCCAGACCACCAAATAC	PET	4	5	213-221	0.585	
	HPN21	F: TGCCAATTTAAAAATCTTAGC R: TGAACCAtGCTTAAAGATAACA	VIC	9.5	3	229-241	0.370	
	HPN9	F: TGTGAACAGCTGTGATGC R: TCTCCTGCCCTAGGATATT	PET	1.2	3	168-174	0.539	
Giant Sable 2A	HPN1	F:AAATGTTAAATCAGCCTTGC R:AGCAGTTCAAACTCCTTCAA	6-FAM	1.6	NA	NA	NA	Vaz Pinto et al., 2015
	HPN13	F: TATCCTTTCATCTCGGTGAC R:GAAGAATCCCATCAACAGAG	PET	2.4	2	200-202	0.369	
	HPN5	F: AGCATAGGTGCTGCTACAGT R:GGTGCAACTTCATCTAGACC	VIC	0.6	1	154	0	
	HPN7	F:TGCCAATTACACAGACAGAG R:TGCAGTAGTCTGTCGTTCAG	NED	2.4	NA	NA	NA	
Giant Sable 3A	HPN10	F: CTGTAGAATCCCAAGGACAG R: AATGTCCATCAAGGAATGAG	PET	1.6	3	186-194	0.550	Vaz Pinto et al., 2015
	HPN12	F: AAGACTTTGAGCTTCCATTG R: AATGGTTTTGTCCATCTCTG	VIC	1	4	230-248	0.443	
	HPN16	F: AGGGGCTGTTGTGCTTA R: CACTGGAGTTAGACCAATGG	NED	1.6	2	149-155	0.372	
	HPN8	F:TTTTGAGCAGCATACTCTGT R:ACTTTTGCTTACCAGCATTG	6-FAM	2	NA	NA	NA	
Giant Sable 4A	HPN11	F: AAGGAGGCAGGAAAGGAT R: GGCGGAGATATGTTCTTTG	6-FAM	2	NA	NA	NA	Vaz Pinto et al., 2015
	**HPN20	F: CTCTCAGAAAGACATAGAATCA R: CCTGCTATTAACCTCAGATG	VIC	1.8	2	178-180	0.364	
	HPN3	F: TCCCCTAATCAAAAGATAAAAA R:ACCGCCACCTAAGATCA	NED	1.4	NA	NA	NA	
	HPN6	F: ATTCAAGCCTTGGTCAGG R: ACAATGTTGTGTTAGTTTCAGGT	PET	1	1	142	0	
Giant Sable 1	HPN23	F: GGGCAAGTATGAAAGGCAT R: CACTCATCCTGGTACCGCTT	6-FAM	0.8	1	162	0	Vaz Pinto et al., 2015
	HPN25	F: GACACGACTGAGCGACTTCA R: TGCACCTCTTCAGTTTGGTT	6-FAM	0.6	1	109	0	
	HPN31	F: AAGGGAGGGGAGAGCTGATA R: CGCTTGTGTTCTTTCTACAGTGA	VIC	1	1	180	0	
	HPN38	F: TATGTATCCATCCACCCACC R:TCAGATGATAGAGATAGATGATAGGCA	PET	0.8	NA	NA	NA	
	HPN39	F: GTTTGCCCCTTTTGAATCCT R:AACTCTAGAACCCTCAAGGCG	NED	1.2	1	104	0	
	HPN41	F: TGGAGGATCTATTCCAGGGG R:CACTCCCACTCTAGTCACTCCC	NED	1.2	NA	NA	NA	
	**HPN45	F:AAGCAAGTAGAGTGCATTAAATAAAA R:GATTGTTGGCTGCTTTGCTA	6-FAM	2.2	2	207-210	0.171	
	HPN52	F: GCAGTCCACAGGATCACAAA R:ACGAACTTTCATTTGGCACC	PET	0.8	NA	NA	NA	
Giant Sable 2	HPN111	F: GTCGGACACAACTGAACCAC R:GCAGCTAGTTATTCTGAAATGGG	PET	1.2	NA	NA	NA	Vaz Pinto et al., 2015

	HPN24	F: CCTTTCAAGATGACTCCACATT R:GGCTGGATGAAGAGATGGAT	6-FAM	2.2	NA	NA	NA	
	HPN48	F: TTTTGTTCTAGATGTGTTGGATACTTG R:GCGTGTTACAGTCACAGCCTA	VIC	1.4	NA	NA	NA	
	HPN50	F: AGTCCATGGGGTCACAAAGA R:AGAACATTCGCTCGCAAACT	PET	1.4	NA	NA	NA	
	HPN60	F: GGGAGATTTTGAGAGGGGTT R:GAGGCTGCAGGATCATAGTG	6-FAM	0.8	NA	NA	NA	
	HPN68	F: TCCCAGTTCAGTCTCCACCT R:TGGATAAAACTTTTGACTAATAGAGCC	6-FAM	1	NA	NA	NA	
	HPN80	F: GGTTTGAAGGAAGCATGGTG R: GCTCTGCGACATACACATCC	NED	2	1	117	0	
	HPN81	F: GGAGAGGGCAATTGATGAAC R:TCCAACATTCAGTTTTAATGTCTAA	VIC	1.2	1	164	0	
	HPN91	F: CCTCCCTCCCTCTTCCCT R:AATTGGGATGCAAAGACGAG	NED	5	NA	NA	NA	
Giant Sable 3	HPN112	F: TACATGGAGTTGAAGGATATTATGTT R: ATCTGATCAGTTGGGAGGCA	6-FAM	0.8	1	110	0	Vaz Pinto et al., 2015
	HPN57	F: TCTTTACACACGTGGGAGCA R:CAGGATTCACAAGATAAAGAACCA	VIC	0.7	NA	NA	NA	
	HPN61	F: CAGATCAGATTCACCAGTATGGA R: AAGTGCTTTGGGAATCTTGG	VIC	0.8	1	173	0	
	HPN72	F: AGGTGTGCTTCCATATTTTTCTC R: TATGTATGTGCTTGTGGGCA	NED	1.2	1	116	0	
	HPN86	F: GCTTCTTAGAGGAGCCTGGA R:GCTAGGACATGGAAGCAACC	PET	1	NA	NA	NA	
	HPN89	F: ACATGAGAGGAGCTATGGAAGTT R:GGAGTCTAGTAACCAGAGGCCA	PET	2	NA	NA	NA	
	HPN93	F: AGGGAGAACAGATAAACATCCC R: GGACTAGGAAATAGGCAGTCCC	6-FAM	0.8	2	224-226	0.372	
Giant Sable 4	HPN106	F: AGGTACAGAGTTTTGTTGGGGA R:GAATACTGCCAACTGCCTCA	NED	4.5	1	264	0	Vaz Pinto et al., 2015
	HPN110	F: CGATACCTGGGTCAGGAAG R:GCTTAGTTTGTAGTCCCTTCTCTGCT	PET	2.4	NA	NA	NA	
	*HPN27	F: CCTCGGATAGGAGGAACAGG R: TGGAGAAACCATTTTCCCAG	6-FAM	0.8	2	120-122	0.171	
	HPN28	F: TTTCAGGTGAACACTGAAGGG R: CAGCCTGGATGAGAGGAGAG	PET	1	1	142	0	
	*HPN29	F: GGTCATAGGCCTTGGACTCA R: AGATGAGAAGATGGATGAGGG	NED	0.8	2	107-111	0.171	
	*HPN36	F: CCACTGTTACCTCCACCCAT R: TTTCATCAATCCATGCATCC	VIC	1.6	2	213-217	0.171	
	HPN46	F: CATAGGGACAATGCTGAAGAA R:CCATGGAGTCCCAAAGAGTC	6-FAM	2.6	NA	NA	NA	
	HPN64	F: GGGCTACACAAGTTCAGGGA R: AAGGAACTCAGGGAGCTTTC	NED	1.4	1	166	0	
	HPN92	F: TCATAGGGGAAGAGGAGAAGG R: TCTTGATCCCTGATGAGCAA	VIC	1.6	2	218-220	0.357	
Giant Sable 5	HPN101	F: TCATGCGTAGAGTTCTGGTGA R:GCAACCACTGATGTCAAAGAAG	VIC	1.4	1	319	0	Vaz Pinto et al., 2015
	HPN113	F: TGGGGCATTTATCTTGAGAAC R: GGTTGCAAAGAGTCGGACAT	PET	0.5	1	189	0	
	**HPN116	F: CAAGCATTGCCTCTGTCAAC R: TCCCACGAAGCACCTAGATT	VIC	2.05	2	230-236	0.050	
	HPN22	F: TTCATGAAGTTGGCTGAGCA R: TTGATGCCTGAATGGATGAC	PET	0.5	1	273	0	
	HPN37	F: TCCATCCACTTAGACACTCCC R: GTAGGTGAGGGATGGATGTG	PET	0.4	1	114	0	
	HPN47	F: CATCAAGTGCACCCTAACCC R: CGATGGCTGGGCCTTAAT	NED	0.35	1	152	0	
	HPN58	F: ATGTCTTCTTTGGCCTTCCC R:TGAATAGCATTTCTCAGGTATGTG	VIC	0.6	1	135	0	
	HPN75	F: TCAATCTCAGGACTTAGTTTGCAT R:CTTTAAAAGTACAAACACAAGACAATG	PET	1.7	NA	NA	NA	

	HPN79	F: AAGGAGTTGTAGGAGTTTTGTAGCTC R:GGAAGAAATAGGGCTTGGGA	VIC	2.05	NA	NA	NA	
Sheep 1A	Inra5	F:CAATCTGCATGAAGTATAAATAT R:CTTCAGGCATACCCTACACC	6-FAM	2.75	3	133-137	0.300	Vaiman et al., 1992
	CSRD247	F: GGACTTGCCAGACTCTGCAAT R: CACTGTGGTTTGCATTAGTCAGG	VIC	0.4	NA	NA	NA(	Davies et al., 1996
	OarFCB304	F: AAATGTGTTTAAGATTCCATACAGTG R: GGAAAACCCCCATATATACCTATA	NED	2	NA	NA	NA	Buchanan et al.,1993
	Inra06	F:AGGAATATCTGTATCAACCTCAGTC R:CTGAGCTGGGGTGGGAGCTATAAATA	PET	1.2	2	141-143	0.369	Vaiman et al., 1992
	D5S2	F: CCCACAGGTGCTGGCATGGCC R:CCATGGGATTTGCCCTGCTAGCT	PET	2.75	NA	NA	NA	FAO 2011
Sheep 2A	IlSTS087	F:AGCAGACATGATGACTCAGC R:CTGCCTCTTTTCTTGAGAGC	NED	0.8	NA	NA	NA	Kappes et al., 1997
	HSC	F:CTGCCAATGCAGAGACACAAGA R:GTCTGTCTCCTGTCTTGTCATC	VIC	1.2	3	300-306	0.503	Davies et al., 1996
	Inra63	F:ATTTGCACAAGCTAAATCTAACC R:AAACCACAGAAATGCTTGGAAG	6-FAM	0.35	1	185	0	Vaiman et al., 1994
	McM42	F: CATCTTTCAAAAGAACTCCGAAAGTG R: CTTGGAATCCTTCCTAACTTTCGG	VIC	0.7	NA	NA	NA	Hulme et al., 1994
	MAF65	F:AAAGGCCAGAGTATGCAATTAGGAG R:CCACTCCTCCTGAGAATATAACATG	VIC	0.6	NA	NA	NA	Buchanan et al., 1992

Table S2- Details of the multiplex PCR performed for amplification of invasive samples: Species, temperature of each step (temp), time of each step (' denotes minutes, '' denotes seconds) and number of repeats of each denaturation, annealing and extension cycle (Nº of cycles). Negative temperatures between brackets (e.g. -0.5ºC) signifies the decrease in annealing temperature in each cycle.

Multiplex	Pre-PCR conditions			Post-PCR conditions		
	Temp	Time	Nº of cycles	Temp	Time	Nº of cycles
Giant Sable 1, 2 and 4	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	60ºC (-0,5ºC)	90''	11X	60ºC	60''	11X
	72ºC	30''		72ºC	30''	
	95ºC	30''		95ºC	30''	
	55ºC	90''	9X	55ºC	45''	34X
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	
Giant Sable 3	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	64ºC (-0,5ºC)	90''	19X	64ºC (-0,5ºC)	60''	19X
	72ºC	30''		72ºC	30''	
	95ºC	30''		95ºC	30''	
	55ºC	90''	5X	55ºC	45''	26X
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	
Giant Sable 5	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	60ºC (-0,5ºC)	90''	11X	60ºC (-0,5ºC)	60''	11X
	72ºC	30''		72ºC	30''	
	95ºC	30''		95ºC	30''	
	55ºC	90''	9X	55ºC	45''	34X
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	
Sheep 1A and 1B	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	54ºC	90''	12x	54ºC	60''	37x
	72ºC	30''		72ºC	30''	

	95ºC	30''		95ºC	30''	
	53ºC	90''	8x	53ºC	45''	8x
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	
Giant Sable 1A, 2A and 3A	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	54ºC	90''	20x	54ºC	60''	45x
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	
Giant Sable 4A	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	56ºC (-0,5ºC)	90''	13X	56ºC (-0,5ºC)	60''	13X
	72ºC	30''		72ºC	30''	
	95ºC	30''		95ºC	30''	
	50ºC	90''	7X	50ºC	45''	32X
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	
Cattle	95ºC	15'				
	95ºC	45"				
	62ºC (-0.5ºC)	60''	7x			
	72ºC	45''				
	95ºC	45"				
	59ºC	60''	30x			
	72ºC	45''				
	95ºC	45"				
	53ºC	45"	8x			
	72ºC	45"				
	60ºC	30'				
	10ºC	FOREVER				

Table S3- Details of the Multiplex PCR for the amplification of the museum and non-invasive samples: Temperature of each step (Temp), time of each step (' denotes minutes, '' denotes seconds) and number of repeats of each denaturation, annealing and extension cycle (Nº of cycles). Negative temperatures between brackets (e.g. -0.5ºC) signifies the decrease in annealing temperature in each cycle.

Multiplex	Pre-PCR conditions			Post-PCR conditions		
	Temp (ºC)	Time	Nº of cycles	Temp (ºC)	Time	Nº of cycles
M1	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	60ºC (-0,5ºC)	90''	13X	60ºC	60''	13X
	72ºC	30''		72ºC	30''	
	95ºC	30''		95ºC	30''	
	54ºC	90''	7X	54ºC	45''	32X
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	
M2	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	54ºC	90''	20x	54ºC	60''	45x
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	
M3	95ºC	15'		95ºC	15'	
	95ºC	30''		95ºC	30''	
	60ºC (-0.5ºC)	90''	9x	60ºC (-0.5ºC)	60''	9x
	72ºC	30''		72ºC	30''	
	95ºC	30''		95ºC	30''	
	56ºC	90''	11x	56ºC	45''	36x
	72ºC	30''		72ºC	30''	
	60ºC	15'		60ºC	30'	
	10ºC	FOREVER		10ºC	FOREVER	

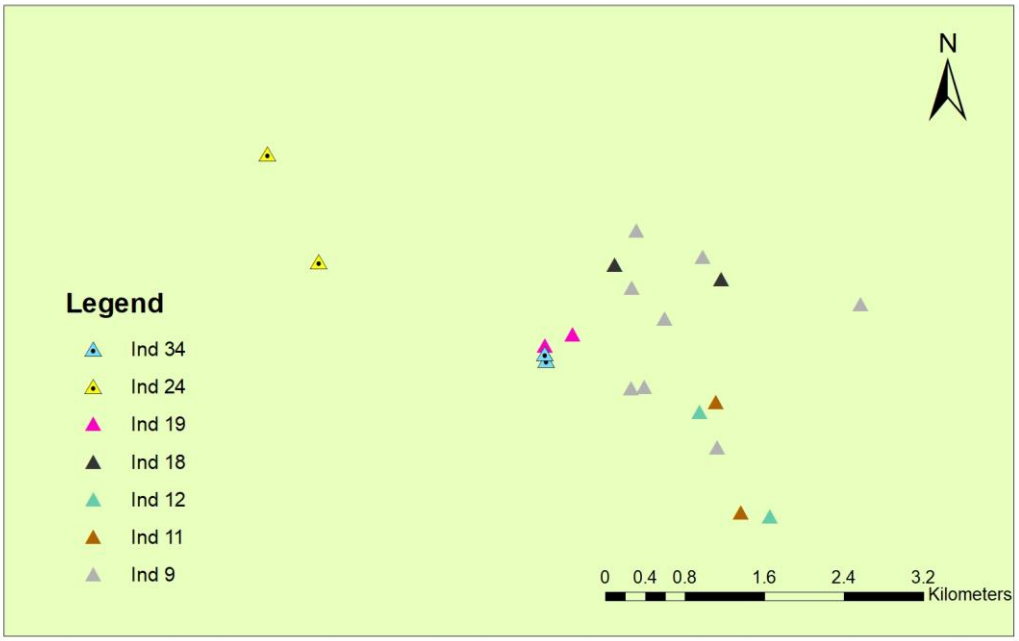


Fig. S2- Map of duplicate samples of individuals (Ind) in the sanctuary and in the conservancy (Ind 24). Same color represents the same individual.

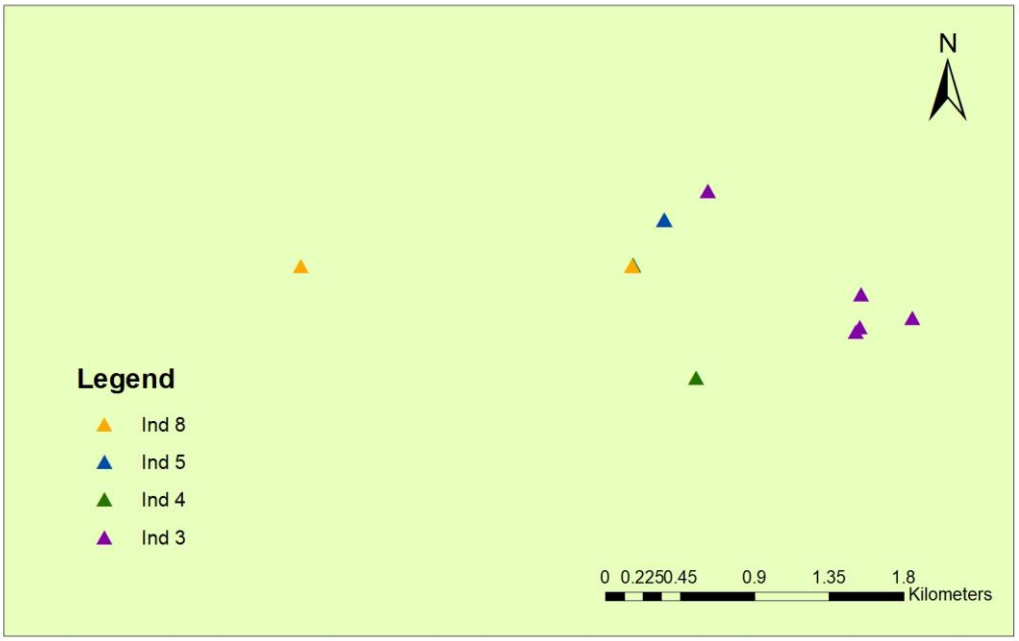


Fig. S3- Map of duplicate samples of individuals (Ind) in Bura East. Same color represents the same individual.

Table S4- Maximum distance between samples of the same individual.

Individual	Distance (m)
34	68
24	1203
19	301
18	1081
12	1268
11	1132
8	2023
5	3
4	780
3	1455

Table S5- Values of pairwise fixation index (FST) between populations using the microsatellite dataset on the top right and FST values between populations obtained using the mtDNA sequences on the bottom left. The single significant value is marked with an asterisk ($p > 0.05$).

Pop	**TRLC**	**SAN**	**CON**	**BURA**	**SANG**
TRLC		0.016	0.021	0.076*	0.055
SAN	0.031		-0.005	0.071	0.047
CON	0.315	0.459		0.072	0.024
BURA	-0.021	-0.128	0.152		0.102
SANG	0.044	0.325	0.562	0.320	

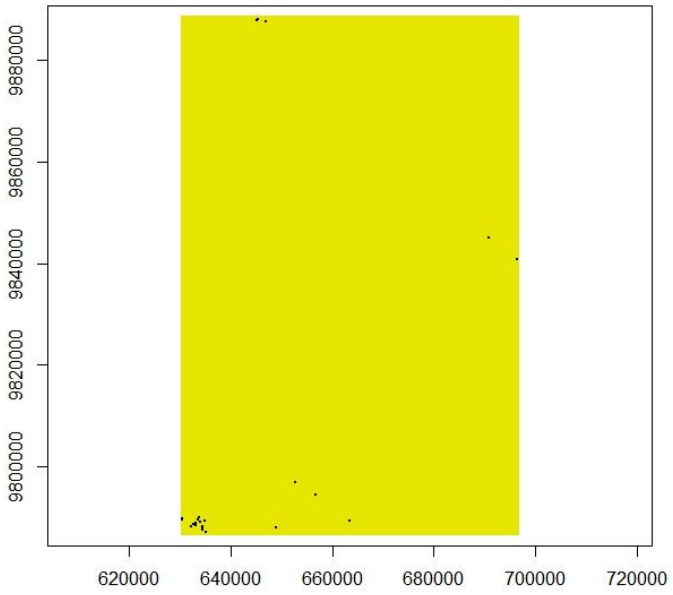


Fig. S7- Map of cluster membership obtained in GENELAND run with highest average posterior probability under the uncorrelated allele frequency model. X and Y graph correspond to UTM coordinates.

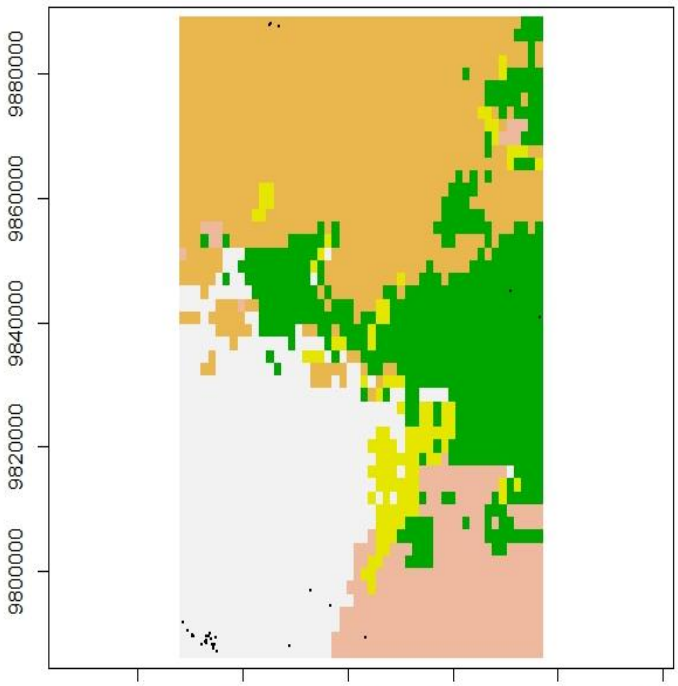


Fig. S4- Map of cluster membership obtained in GENELAND run with second highest average posterior probability under the correlated allele frequency model X and Y graph correspond to UTM coordinates.

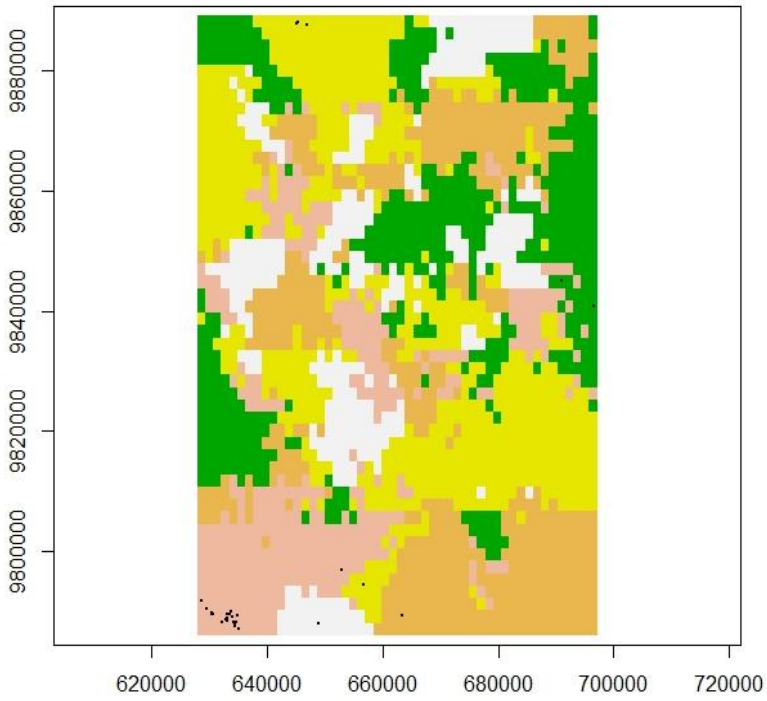


Fig. S10- Map of cluster membership obtained in GENELAND run with third highest average posterior probability under the correlated allele frequency model. X and Y graph correspond to UTM coordinates.

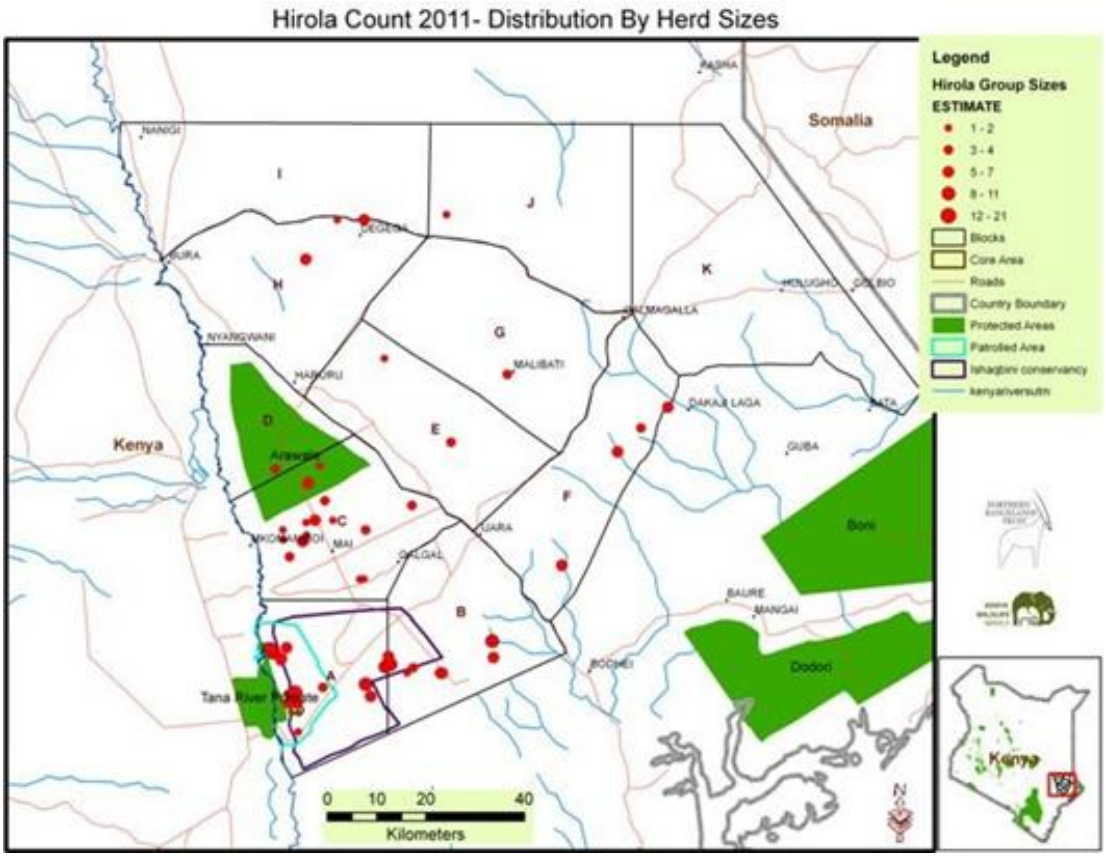


Fig. S13- Distribution of hirola observed during aerial survey performed in 2011. Credit: King et al. (2011)