

**Landscape genetics and behavioural ecology of mountain nyala
(*Tragelaphus buxtoni*) in the Southern highlands of Ethiopia**

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Ph.D. thesis

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*Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 1338*

ISSN 1501-7710

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Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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Acknowledgements

I would like to give my deepest appreciation and gratitude to my advisors Prof. Nils Chr. Stenseth, Dr. Leif Egil Loe, Dr. Eli Knispel Rueness and Prof Afework Bekele. This research wouldn't have been possible without their constructive comments and encouragement. I would like to express my heartfelt thanks to Nils Chr. inviting me to conduct a Ph.D. thesis at the Centre for Ecological and Evolutionary Synthesis (CEES), which has been a wonderful opportunity for my career, and for his marvelous encouragement throughout the work of this thesis. I am indebted to Leif Egil Loe for his unreserved suggestions, assistance and meticulous comments throughout the course of this thesis and the project. I also thank him and his wife for their hospitality and all kind of assistance during my stay in Oslo. I am very grateful to Eli for her guidance with the genetics laboratory, which was a new experience for me. I am thankful for Prof. Afework Bekele for his long standing support of this project.

I thank Dr. Stuart Williams for his great support in establishing this project. I thank all my field assistants: Shiferaw Tadesse, Awol Hulle, Usman Abdella, Kassem Keder, Ummer Garsso and Hana Adiss. They worked so hard through all of the harsh weather in the Bale mountains to make this project successful. I thank all those who helped with radio-telemetry and capture of mountain nyala in particular the veterinarian Dave Cooper. Thank you to Dr. Øystein Langangen, Dr. Dimitar Serbezov, and Dr. Paul Evangelista for their useful comments on the thesis. I am deeply indebted to the members of the Frankfurt Zoological society, in particular, Dr. Debra Randel, Alastair Nelson, Dr. Anouska Kinahan, Dr. Thadaigh Baggallay, and Dr. Karen Laurenson for their support in the field work during this project. I am also very thankful to Nanna Winger Steen and Emelita Rivera Nerli who were always keen to help in the laboratory. My sincere appreciation is also

extended to many friends including Addisu Mekonnen in spending many good days in the Bale Mountains.

This project would not be possible without all the funding organizations including Saint Louis Zoo, International Foundation for Science (IFS), Centre for Ecological and Evolutionary Synthesis (CEES), Sahal FARM-Africa SOS Sahel Ethiopia, Rufford Small Grants for Nature Conservation, Conservation des Espèces et des Populations, Riverbanks Zoo and Garden, and Frankfurt Zoological Society.

Finally, I would like to thank my mother Tenaye Mogess and my father Atickem Meshesha for their unwavering support and encouragement in all my pursuits. You have been always with me being the base of my whole carrier. I thank my wife Sebele Wengel Tadess and beloved son Esrom Anagaw.

List of papers

The thesis is based on the following four papers which are referred to by their Arabic numerals

Paper I. Atickem, A., Loe, L.E. , Langangen, Ø., Rueness, E.K., Bekele, A. and Stenseth, N.C. (2011) Estimating population size and habitat suitability for mountain nyala in areas with different protection status. *Animal conservation* 14: 409-418.

Paper II. Atickem, A., Rueness, E.K, Loe, L.E., Serbezov D., Bekele, A. and Stenseth, N.C. Population genetic structure and connectivity in the endangered Ethiopian mountain nyala (*Tragelaphus buxtoni*): recommending dispersal corridors for future conservation.

Submitted manuscript

Paper III. Atickem, A. and Loe, L.E. Livestock-wildlife conflicts in the Ethiopian highlands: assessing the dietary and spatial overlap between mountain nyala and cattle.

Submitted manuscript

Paper IV. Atickem A., Loe, L.E. and Stenseth, N.C. Sleeping with the enemy? Individual heterogeneity in use of human shields in mountain nyala. *Submitted manuscript*

Introduction

African antelope species have drastically declined during the last few decades. Out of the 91 species of antelopes existing in the world, most of which are native to Africa, 25 species are threatened with extinction (ASG, 2009). The steep decline is mainly associated with the impact of rapid human population growth (Homewood *et al.*, 2001). In sub-Saharan Africa countries that support more than 70 antelope species (East *et al.*, 1999), the human population has increased by 371% since 1950 (Bongaarts, 2009). According to East *et al.*, (1999), 50% of the antelope species will be threatened by extinction or be extinct by 2025 if the current trend is continued without intervention through immediate conservation action. With this thesis, I studied the behavioral ecology and population genetics of endangered mountain nyala (*Tragelaphus buxtoni*) to provide guidelines for the scientific conservation management plan of the species. The study is based on interdisciplinary scientific methods including high resolution satellite image analysis, habitat suitability modelling, non-invasive genetics tools and radio telemetry based behavioural ecology studies. These tools were utilized to develop an understanding of the conditions that could enable the endangered antelope to survive in the face of increasing human pressure.

In the past, establishing protected areas, mainly national parks, was believed to be the ultimate solution in sustainable conservation of wildlife species worldwide (Margules and Pressey, 2000). In sub-Saharan Africa countries, more than 3000 protected areas were established for wildlife conservation (Reid and Miller, 1989). Regardless of this however, wildlife species including antelopes continued to decline across the region including in the well established national parks of Kenya and Tanzania (Newmark, 2008; Western *et al.*, 2009). While a number of reasons are given to explain why the protected areas fail to achieve their goals, inefficient low of enforcement to stop illegal acts including poaching and livestock grazing (Leader-Williams and Milner-Gulland, 1993; Bonnington *et al.*, 2007; Mfunda and Røskaft, 2010; Gandiwa *et al.*,

2011) and escalating habitat loss and fragmentation (Caughley, 1994; Myers *et al.*, 2000; Fahrig, 2003; Johansson *et al.*, 2007) are believed to be the main reasons.

Delineation of wildlife areas and maintenance of network corridors between distant populations should ideally be based on solid knowledge of spatial ecology and landscape genetics of the species. This is essential to make the right management decisions and ultimately critical for successful long-term persistence of wildlife species. To obtain such information is thus a starting point for any conservation practice, and is what I intended to do in my thesis.

Once protected areas are established, it is critical to manage the area by keeping it safe from human influence including habitat loss and keep the network corridors to the surrounding landscape intact (Wikramanayake *et al.*, 2004; Rouget *et al.*, 2006). Habitat loss may remove resources necessary to support viable wildlife populations and disrupts natural functions of the ecosystem (Caughley, 1994; Fahrig, 2003). It also causes habitat fragmentation that may decrease the attractiveness of the remaining habitat and also cause loss of genetic variation by decreasing connectivity (Chetkiewicz *et al.*, 2006), in turn accelerating population declines. Landscape genetics, a research area that combines population genetics, landscape ecology, and spatial statistics (Manel *et al.*, 2003), has become an emerging interdisciplinary science for the conservation of wildlife species in a fragmented landscape (Storfer *et al.*, 2010). Landscape genetics determines how landscape structure affects dispersal/gene flow for a given species (Savage *et al.*, 2010). Advancements in non-invasive molecular techniques provide powerful tools to evaluate functional connectivity (Beja-Pereira *et al.*, 2009), while the technological innovations in Geographic Information System (GIS) and increasingly available satellite image enable more precise information of the vegetation types and the physical landscape structure, which are a key components in resource selection of wildlife species (Kliskey *et al.*, 1999). Conservation application of landscape genetics is mainly to use least-cost modelling to suggest

ecological networks that best connect wildlife habitats via corridors (Jongman and Pungetti, 2004; Li *et al.*, 2010; Richard and Armstrong, 2010).

The other important threat for the wildlife species in the Sub-saharan Africa protected areas is escalating conflict with the pastoral communities. Increasing human population, accompanied by intensive agriculture and infrastructure has caused the pastoral people to invade the last remaining wildlife habitats and increased the grazing land conflict in wildlife protected areas (Prins, 1992; Hilborn *et al.*, 2006). Livestock could affect wildlife species as a result of scramble competition for pastures (Madhusudan, 2004) and/or interference competition, such as preventing wildlife from accessing resources (Johanson, 1993). Several studies have reported declines in native wild ungulate populations following the introduction of livestock (Lamprey and Reid, 2004; Young *et al.*, 2005; Kittur *et al.*, 2010).

Human expansion towards protected areas also increases human-wildlife interactions, which in turn affect wildlife species through behaviorally-mediated indirect effects, depending on the nature of interaction. The level of the behavioral impacts of human on wildlife species depends on the amount of human-wildlife interaction in the past. For instance, hunted and non-hunted ungulate species respond to human presence differently. Human-induced disturbances generate stronger flight response in hunted population than in non-hunted populations (Stankowich and Blumstein, 2005; Stankowich, 2008). If not subjected to human hunting over an extended time, ungulates are reported to approach humans to reduce the risk of predation; a phenomenon called the human shield hypothesis (Berger, 2007). Elk (*Cervus elaphus*), for instance, use localities with high human activity, which is avoided by their principal predator, wolves (*Canis lupus*) (Hebblewhite *et al.*, 2005). The conservation value human shield may have for endangered species has recently been highlighted (Leighton *et al.*, 2010). However, the general conclusion of the contribution of human shield for conservation is difficult to determine as human presence is also associated with a number of other negative effects.

Study species

Mountain nyala (*Tragelaphus buxtoni*) is a sexually dimorphic spiral-horned antelope endemic to the south eastern highlands of Ethiopia (Figure 1, Kingdon, 1997; Evangelista *et al.*, 2007). Its population size is likely to have declined in the past few decades possibly as a consequence of human impact, direct persecution and habitat loss. The species is currently categorized as an endangered species (EN A1a, C1) by IUCN (Sillero-Zubiri, 2008). Mountain nyala has remained unstudied since its initial discovery in 1908, when it was categorized as a type of greater kudu (*Tragelaphus strepsiceros*; Lydekker, 1910a). The limited knowledge of the species has so far been provided mainly by the trophy hunters and *ad hoc* observations and few short term studies (Evangelista *et al.*, 2007). Mountain nyala is the most important trophy species in Ethiopia, generating 1.4 million US dollars per year (Lindsey *et al.*, 2007a, 2007b).



Figure 1a



Figure 1b

Figure 1. Mountain nyala (1a, male; 1b, female)

Study area

The study was primarily carried out in the Bale massif of Ethiopia elevated above 1800 m a.s.l. (14,775 km²; Figure 2), the lowest elevation range where the mountain nyala is found (Evangelista *et al.*, 2008). The range is part of Conservation International's Eastern Afromontane hotspot (Brooks *et al.*, 2004), providing key conservation areas for wide range of other endemic mammals of Ethiopia including Ethiopian wolf *Canis simensis* and Bale monkey *Chlorocebus djamdjamentis* (Yalden and Largen, 1992). The rapid human population growth (3.2% annually), which has been closely followed by extensive cultivation, overgrazing and consumption of fuelwood, put enormous pressure on the biodiversity of the area (Stephens *et al.*, 2001). Mountain nyala population are restricted to within protected areas of the Bale Mountains National Park (BMNP; Gaysay area, the BMNP headquarters, Web Valley, Senetti Plateau and Harena Forest) established in 1970 (Waltermire, 1975) and within controlled hunting areas (i.e. Hanto, Hora, Abasheba, Odobullu, Shedem-Berbere and Dodola; Figure 2). Law enforcement to prevent illegal human resource use including firewood collection and livestock grazing is implemented in part of the BMNP (Gaysay area and headquarters) and two hunting concessions (Abasheba and Odobullu). For the population genetic study, we also included the population in Kakka-Galama Mountains of Arsi (Kakka-Galama), a region that harbor a remnant mountain nyala population north of Bale mountains. The climate of the Bale and Arussi Mountains varies depending upon on altitudinal gradient. In general however, the area is subject to dry season from November to February and wet season from March to October (Hillman, 1986).

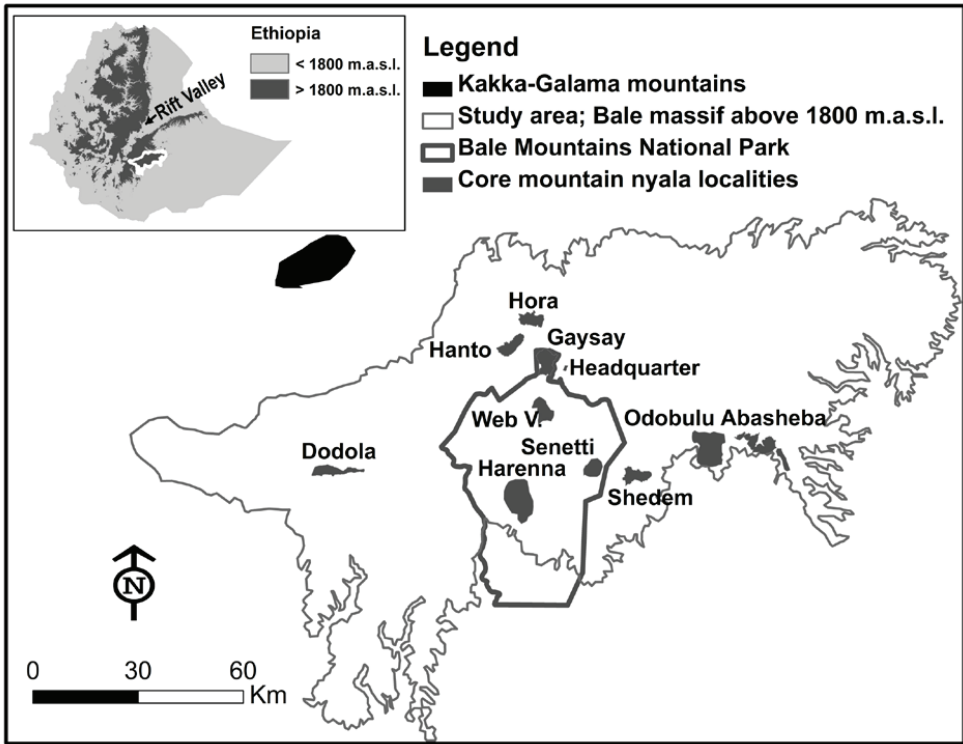


Figure 2. The study area in south-eastern highlands of Ethiopia

Objective of the study

This study aims to fill the major gaps in the conservation science of mountain nyala through integrating multiple research fields including ecology, genetic and behavioural aspects of the species. Specific objectives are as follows:

- 1) To determine the spatial distribution pattern and estimate population size of mountain nyala from faecal pellet counts and/or visual counts.
- 2) Build a resource selection function at the landscape level to quantify predictors of mountain nyala presence.

- 3) To identify appropriate genetic markers for studying the population structure of the mountain nyala and determine the level of genetic population differentiation among the 6 main populations.
- 4) To evaluate the effects of habitat fragmentation on gene flow and design dispersal corridors to facilitate dispersal among the populations.
- 5) To examine the potential for competition between livestock and mountain nyala based on diet overlap and area level abundance correlation across 7 study sites.
- 6) To examine the behavioural interactions of mountain nyala and local people in localities under different protection status and investigate if human shield behaviour was occurring.

Materials and methods

Immobilising mountain nyala

Seven adult female and seven adult male mountain nyala were immobilised by using a remote injection system by a CO₂ powered Dan-Inject JM Special gun (Dan-Inject, SA) in Gaysay area, primarily for fitting them with collars. The immobilising mixture used was Thiofentanil oxalate (A3080® 10mg/ml), Xylazine (Rompun® 2%) and Hyaluronidase (Hyaluronidase®). Reversal was achieved using Naltrexone (Trexanol® 50mg/ml) and Yohimbine (Yohimbine® 6.25mg/ml) or Atipamizole (Antisedan® 5mg/ml). Females were collared with Tellus GPS collars (Followit Holding AB, Sweden) and males were collared with VHF-collars (Telemetry Solutions, USA). The study on foraging behaviour and human shield (Paper III and IV) were based on these collared animals.

Habitat classes, slope and elevation

In all of the four papers of this thesis, we used habitat variables (forest, pasture, bush land, agriculture, human settlement and Erica forest/shrub) derived from 10 m SPOT images, classified by maximum likelihood classification algorithm in ERDAS Imagine (ERDAS, 1994; Dean and Smith, 2003). The degrees of the slope and elevation values were derived from 90 m resolution Digital Elevation Model (DEM) by using spatial analyst in ArcGIS 9.3.

Paper I - Estimating population size and habitat suitability for mountain nyala in areas with different protection status

The first paper was dedicated to answer the most basic conservation issue of mountain nyala: to obtain a population estimate and to develop a habitat suitability model for the species. Because much of the mountain nyala habitat consisted of dense forest too thick to allow good visibility, we used pellet count method for the population estimate, as follows:

$$Da = Ds/Pi * I$$

where Da= density of the animal, Ds= total number of faecal pellets encountered per area, Pi= mean time to decay of the pellets and I= defecation rate (Rivero *et al.*, 2004; Periago and Leynaud, 2009). Total number of faecal pellets encountered per area (Ds) was censused on randomly selected plots (4 x 5 m²) laid on line transects established at 200 m distance intervals in each locality (Krebs, 1989). A total of 1515 plots was censused within 352 km² of core mountain nyala areas. Pellet group degradation rate (Pi) was estimated by linear combination of average degradation rate of pellet groups (open area 33.5 days; n=31, SD=8.6 and area with cover, 44 days; n=29, SD=12) weighted with the proportion of open and closed habitat within each locality. The mountain nyala defecation rate (I) was estimated at 22.3 pellet groups per day per

animal from the population in Gaysay area with known density from six repeated total visual counts (range in estimated density: 23–25 individuals/km²).

The model of habitat selection was carried out by two steps. First, logistic regression was used to determine key environmental differences between known mountain nyala areas (n=10) and randomly selected areas (13; size of 5*6 km²) which did not harbor mountain nyala population. A total of 1515 plots in mountain nyala areas and 1553 plots in areas without mountain nyala were sampled randomly and checked for presence/absence of mountain nyala pellet. The most parsimonious model was selected based on the Akaike Information Critereon (AIC) value (Burnham and Anderson, 1998). The second modeling step investigated how the probability of detecting mountain nyala pellets varied within mountain nyala areas based on absence or presence of pellets groups within the random plots. Logistic mixed models (using function lmer in R library lme4: R Development Core Team, 2011) was implemented with the presence of pellets within the plots as a binomially distributed response variable, and mountain nyala area as a random intercept. The top competing models were included in model averaging and extrapolated on all pixels within the study area by entering the fixed effects parameter estimates from the average model into the Spatial Analyst raster calculator in ArcGIS 9.3.

Paper II - Population genetic structure and connectivity in the endangered Ethiopian mountain nyala (*Tragelaphus buxtoni*): recommending dispersal corridors for future conservation

The second paper focused on studies of genetic population structure of mountain nyala and combined this information with the pervious habitat suitability study to assess effects of habitat fragmentation on dispersal of the species and to suggest conservation corridors. Genomic DNA was extracted from 378 pellet samples by using Dynabeads[®] MyOne™ SILANE (Invitrogen Dynal AS, Oslo, Norway) and amplified by using 12 polymorphic microsatellite markers. The

markers were obtained from cross species amplification of thirty-five primer pairs, originally developed for cattle *Bos taurus* and sheep *Ovis aries*. Control regions of mitochondrial DNA (370bp) was amplified using the primers MT4 (Arnason *et al.*, 1993) and B16168H (Simonsen *et al.*, 1998).

Possible genotyping errors were tested by MICRO-CHECKER 2.2.3. (Van Oosterhout *et al.*, 2004). Genetic diversity of the microsatellite data was measured using FSTAT (Goudet, 2002) and Departure from HWE, and linkage disequilibrium were estimated using GENEPOP 4.0 (Rousset, 2008). We applied a Bayesian maximum likelihood approach to infer the number of populations, as suggested by the microsatellite data, using the software STRUCTURE 2.2.3 (Pritchard *et al.*, 2000). We estimated gene flow (based on microsatellite data) using a genetic assignment method implemented in BayesAss+ 1.3 (Wilson and Rannala, 2003).

Corridors between the mountain nyala populations were derived through applying least-cost path analysis (Coulon *et al.*, 2004), as implemented in the ArcToolbox in ArcGIS 9.3. The resistance value of any given pixel in the cost raster was calculated as 1 minus the habitat suitability value (Chetkiewicz *et al.*, 2006; Spear *et al.*, 2010). Genetic distance was correlated with three different measures for dispersal distance/resistance: 1) linear Euclidian distance between populations (km), 2) the least-cost path distance (km) and 3) ‘cumulative resistance’ (the sum of resistance values along the least-cost path) by using Mantel tests (Mantel, 1967).

Paper III - Livestock-wildlife conflicts in the Ethiopian highlands: assessing the dietary and spatial overlap between mountain nyala and cattle

The third paper focuses on the assessment of potential adverse effects of livestock on the mountain nyala through resource competition. With this study, we determine the potentials of diet overlap and abundance correlation between livestock and mountain nyala. Foraging behaviour of mountain nyala was studied from scan sampling (Parker *et al.*, 2003) with 10 minutes interval

recorded as grazing if the observed animal ingested grass and browsing if the animal was feeding on leaves. For observations of browsing, the plant species was recorded. Correlation in abundance and spatial overlap was estimated from faeces counts within randomly assigned plots (4*5 m²) along transect lines spaced out with 200 meter intervals (Krebs, 1989). The abundance correlation between livestock and mountain nyala was assessed by Pearson's product-moment correlation. We modeled the effect of cattle faeces presence on mountain nyala faeces presence with a generalized linear mixed model (function `glmmPQL` in library `MASS`) by using R version 2.13.1 (R Development Core Team, 2011). Effectiveness of the law of enforcement in livestock grazing restriction in Gaysay area was estimated based on the livestock pellet count in the area and number of livestock caught while grazing within the Gasay area. Data on the number of confiscated livestock was acquired from the BMNP office. The BMNP management impose penalty of US\$0.78 per confiscated cattle caught while grazing within the Gasay area

Paper IV- Sleeping with the enemy? Individual heterogeneity in use of human shields in mountain nyala

With the fourth paper, we examine the nature of human shield behaviour in the protected Gaysay area and (with less detailed data) in the trophy hunting concessions. The “human shield” hypothesis, where prey species use humans as shield from natural predation, was tested on mountain nyala subjected to predation from the spotted hyena (*Crocuta Crocuta*), one of the most important large nocturnal predator in Africa (Silvestre *et al.*, 2000; Breuer, 2005).

The definition of a human visit for a given night was defined for a given collared animal to be recorded within 50 meter from a house. The gradient in hyena abundance from the park area towards the settlements was estimated from faeces observations along transects lines. The relationship between hyena abundance and distance to human households were first explored with a Generalized Additive Model (GAM) (Wood, 2006). Daily variation in distance to the

closest house was analyzed with a mixed-GAM using the function `gamm` in the R package `mgcv` (Wood, 2006). To assess the difference in movement in nights with and without human visits we calculated the step lengths (in meters) and the relative angles (in radians) between movement steps (small angle = straight-lined movement) using the function `ltraj` in the R package `adehabitat` (Calenge, 2006). Probability of being active was analysed with a mixed-GAM from activity data recorded in GPS collared females (1 = active, 0 = passive; Godvik *et al.*, 2009). Because no animals were radio-collared in the hunting concessions, Abasheba and Odobullu, the difference in human visits of mountain nyala was assessed through faeces sampling along transect lines in and around human settlements. To substantiate the difference in how mountain nyala perceive humans between the protected Gaysay area and the hunting concessions we also investigated the difference in vigilance using the following standard metrics (Reimers *et al.*, 2009). Perception of the predation risk by humans was studied through a qualitative relationship of “flight initiation distance”, distance at which an animal begins to flee from an approaching predator (Griffin, 2007), “Start distances”, distance between predator and prey when approach begins (Blumstein, 2003), “Alert distance”, distance at which prey become alert standing with its head and neck upright and looking at the approacher (Blumstein *et al.*, 2005), “assessment time”, elapsed time between alert posture that permit measurement of the actual time spent attending to the approaching threat and flight (Stankowich and Coss, 2006) and “distance moved”, distance a prey animal flees from an approaching predator before stopping either; Taylor and Knight, 2003). This metrics were recorded in both hunted and non-hunted populations.

Results

Population estimate and habitat suitability

The total Mountain Nyala population in the Bale mountains was estimated at 3756 individuals (95% CI: 2506–7135). While BMNP contributed with only 31.9% of the mountain nyala population, the two hunting concessions in Besmena-Odo Bulu and Abasheba Demaro harbour 53.5% of the total population. Mountain nyala abundance is strongly affected by human influence and Mountain nyala was never found in areas with more than 50% human-influenced habitat. Due to data paucity in the interval 5-50% percent human influence (i.e., areas were either heavily influenced or quite pristine) we could not estimate a more accurate threshold for mountain nyala tolerance for humans. Forest habitat was found to be the most preferred habitat of mountain nyala. The total potential suitable habitat of mountain nyala across the Bale massif was estimated to 8333 km² where high probability class if limited to 3169 km². Law of enforcement in mountain nyala areas increase the probability for high mountain nyala abundance. The probability of detecting pellets in a plot was 3.7 times higher in the well-protected areas (Gaysay Valley, Besmena-Odo Bulu and Abeshaba Demaro) compared to unprotected or less protected areas.

Landscape genetics of mountain nyala

The average allelic richness in the mountain nyala populations was 5.39 ranging from 4.94 to 6.06. In general, we detected limited gene flow between the mountain nyala populations. With the exception of Gaysay-Headquarter, all of the population-pairs were significantly differentiated from pair-wise F_{ST} estimate. According to the STRUCTURE analysis, peripheral populations Kakka-Galama, Abasheba and Dodola form three distinctive genetic clusters while the individuals representing the populations of Gaysay, Headquarter and Odobullu were assigned the same cluster. All of the sampled populations, with the exception of Dodola, were suggested to be

partially inbred. Three mtDNA haplotypes were defined by two transitions. Haplotype H3 was not present in Gaysay, Headquarter and Kakka-Galama. The pair-wise genetic distance values observed between the mountain nyala populations generally showed a clear pattern of isolation by distance. After accounting for the geographic distance of the least-cost path there was no residual effect of “cumulative resistance” on the genetic distance indicating that the habitat resistance has little impact on the pattern of genetic differentiation observed among the populations. The “cumulative resistance” of corridors was less correlated with the genetic distance than the geographic and the least-cost distance. Our least-cost path model suggested nineteen dispersal corridors (along three main pathways) that could potentially interconnect the mountain nyala populations across the Bale Massif. Three of these could be important for connecting the most distant population-pairs, Gaysay-Dodola (62 km), Gaysay-Abaseheba (59 km) and Dodola-Abaseheba (120 km).

Livestock-wildlife conflicts in the Ethiopian highlands: assessing the dietary and spatial overlap between mountain nyala and cattle

In contrary to the allegation that mountain nyala is a browser antelope, this study revealed that mountain nyala is a mixed feeder where average yearly grazing proportion of female and male was 87 % and 48 % respectively. Hence, the potential of diet overlap with grazing cattle is much higher than what was expected previously. There is a considerable spatial overlap mountain nyala across the mountain nyala range and cattle dung was found in 22 % of plots used by mountain nyala. The abundance of mountain nyala and cattle showed a significant negative correlation where highest mountain nyala abundances were recorded in localities with livestock grazing restriction. In Gaysay, despite the livestock grazing prohibition, cattle dung was found in 32 % of mountain nyala plots. In addition 16,160 head of cattle were confiscated in this area for illegal grazing in the two-year study period.

Human shields in mountain nyala

Mountain nyala went on excursions close to people 14 % of nights (when hyena is expected to be active) and 0 % during day, partly supporting the human shield hypothesis as a facultative strategy. There was no difference between sexes, but large individual heterogeneity in frequency of nightly visits in females (0 - 70.6 %) and males (0 - 26.7 %). The probability to detect faeces of the spotted hyena increased with distance from houses supporting presence of hyena decrease with the proximity of human settlement. Individuals moved longer between relocations and more directional when heading for humans compared to other nights supporting the movement of mountain nyala towards human is pre-planned and directional. Excursions towards humans occurred throughout the year, including dry season where no barley crop is available suggesting that forage is no attractor in causing the visit. In contrary to the prediction that individuals were expected to rest at night close to human households (because limited food was available), individuals were more active when staying within 50 m from human houses compared to further away. Attraction to human households was only confirmed in the protected area, and not in the hunting concessions. Flight initiation distances were 7 times greater in the hunting concessions when compared to the Gaysay area. In Gaysay, the alert distance was 130 meter, the assessment time was 12 seconds and the distance moved was 140 m. In the hunting concessions, flight occurred instantly after the focal mountain nyala saw the observer (n = 37) and hence alert distance was almost identical with flight initiation distance.

Discussion and conclusions

The increase and expansion of human populations is increasingly threatening the wildlife species of sub-Saharan Africa countries. Several antelope species have already become endangered or gone to extinction (ASG, 2009) and imminent conservation action is needed to save the remaining antelope species (Fahrig, 2003; Newmark, 2008). The endangered Ethiopian endemic

antelope, mountain nyala, remains little known regardless of its discovery dating back to 1908 (Lydekker, 1910b). This thesis aims to fill some of the gaps needed to conduct a scientifically based conservation and management of mountain nyala. I do this by highlighting the ecological requirements that need to be met to facilitate the future persistence of the species in the face of increasing human pressure in the Bale massif of Ethiopia.

Uncertainty in population sizes and high quality habitat of endangered species may lead wildlife managers and policymakers to make wrong decision in prioritizing efforts of conservation (Ferrier S., 2002; Webbon *et al.*, 2004; Freckleton *et al.*, 2006; McKechnie *et al.*, 2007). Here, I provide new insight for conservation management plan for mountain nyala based on substantial field data. The BMNP which has previously been suggested to contain the largest mountain nyala population, and remains the primary focus for conservation and research during the last four decades (Hillman, 1986; Malcolm and Evangelista, 2004; Refera and Bekele, 2004), was found to harbour less than a third of the total mountain nyala population of the Bale mountains. Much of the park consists of inferior nyala habitat and we recommend including the forested areas east and west of the current boundaries into the national park which is home for over 63.5 % of the total population estimate (ca 3756 individuals). Because mountain nyala selected forested areas to a larger degree, the standardized faecal pellet count method used in this study for the first time may be further developed and used in the future monitoring of mountain nyala. This is particularly important to assure the currently on-going trophy hunting to be sustainable.

The spatial distribution of mountain nyala was strongly affected by human influence through agriculture and human settlements. Areas exceeding 50 % human influence were never inhabited by Nyala, and the threshold is likely lower than that. Ensuring efficient law of enforcement against human influence within the best suitable habitat designated in this study, a 3,169 km² forest area, should be a priority in the future conservation of mountain nyala. Brown

(1969) suggested the ericaceous belt provided the best potential habitat for the Mountain Nyala. In this study, this habitat was found to be inferior choice of mountain nyala both at Geographic scale habitat selection and habitat selection within home range scale (3rd order selection; Atickem *et al.*, in prep). This difference may be resulted from two possibilities. First Erica forests may has been converted to agricultural land since Browns survey (Evangelista *et al.*, 2008) and the remaining pockets are less selected than other forest types. Second, Brown survey was restricted to the high elevation range of the mountains with low forest cover. Exclusion of eastern escarpment of Bale mountains that harbour the currently largest mountain nyala population from his survey may have lead him to underestimate the use of forests by mountain nyala.

Based on genetic data we found limited gene flow among the nyala populations except the populations in adjacent locations of Gaysay and Headquarter. The level of genetic diversity was generally relatively low and heterozygote deficiency was detected in all populations except in Dodola. It is therefore important to maintain some gene flow among these populations to reduce possible negative effects of inbreeding. Many studies have demonstrated that some migration between populations effectively prevents diversity loss by genetic drift (Ockinger and Smith, 2008). Genetic differentiation among pairs of populations generally followed a pattern of isolation by distance and we were not able to detect habitat features that explained the limited gene flow significantly better than the mere geographic distance (i.e., isolation-by-distance). Hence, I conclude that the observed limited gene flow is due to intrinsic species specific dispersal propensities (Sutherland *et al.*, 2000) rather than habitat resistance. Short dispersal which is sufficient for avoiding resource competition is common in many mammal species (Sutherland *et al.*, 2000) but longer dispersal distances is necessary to adaptive inbreeding avoidance and genetic population differentiation (Long *et al.*, 2008). Illegal hunting may be a additional factor in constraining dispersal as hunting may have dramatic effects on the behaviour of large mammals (Berger 2004; Johnson *et al.*, 2005). Isolated subpopulations may be subject to

an increased risk of local extinction (Frankham, 2005) and keeping migration corridors designed in this study coupled with ‘stepping stones’ may facilitate dispersal of mountain nyala (Chetkiewicz *et al.*, 2006). Corridors and stepping stones increase meta-population persistence through demographic and genetic rescue in the fragmented landscape (Richards, 2000). This is in particular important in the future due to the current expansion of human settlement and agriculture in the area (Stephens *et al.*, 2001) which is likely to worsen the isolation between the populations.

In contrary to the allegation on browsing foraging behavior of mountain nyala (Yalden and Largen, 1992; Gagnon and Chew, 2000), my study suggests that mountain nyala in Gaysay are mixed feeders (Hofmann and Stewart, 1972), with females being closer to being pure grazers throughout the year. The spatial overlap in habitat use between cattle and mountain nyala is high and mountain nyala did not avoided patches also used by livestock. Due to a similar foraging behaviour and a relatively high spatial overlap in habitat use, the potential grazing competition between mountain nyala and cattle is considered to be high (Illius and Gordon, 1992; Shipley *et al.*, 1994; Sitters *et al.*, 2009; Murray and Illius, 2000; Wilmshurst *et al.*, 2000). However, further work on diet analysis and availability of grazing resources is required to determine the extent of competition. Across the seven investigated mountain nyala areas, mountain nyala abundance was negatively correlated with livestock abundance. This is in line with a large number of other studies having reported a negative correlation between wildlife and livestock density (Aagesen, 2000; de Leeuw *et al.*, 2001; Lamprey and Reid, 2004; Namgail *et al.*, 2007). In localities which are well guarded by scouts, both localities in the BMNP and trophy hunting concessions, the mountain nyala abundance was 3.7 times which may be due to prevention of illegal grazing and/or poaching. Hence, I conclude presence of livestock negatively affect the abundance of mountain nyala and more efficient spatial restriction of livestock grazing should be a prioritized task for mountain nyala conservation. We did not find a negative effect of trophy hunting of

males on total abundance; however the sustainability of trophy hunting remains unknown due to short history of hunting in the studied populations.

Finally, this study supports the view that the effect of human-wildlife interactions can be strongly scale-dependent. I demonstrated a strong negative effect of humans on the large scale distribution of mountain nyala (Paper I). However, positive small-scale effect of humans giving shield for humans against predator hyena was noticed in the Gaysay area where anti-poaching law of enforcement implemented over 4 decades (Paper IV). The observed human shield behaviour is however associated with high individual heterogeneity ranging from 0 to 70% nocturnal visits. The antipredator behavioral differences observed between individuals (Wilson *et al.*, 1994) could be resulted from different sources including genetic differences (Dingemanse *et al.*, 2002; Abjornsson *et al.*, 2004; Brown *et al.*, 2007; Bleakley and Brodie, 2009) or physical or social environment (McElreath and Strimling, 2006) including previous experience with predator encounters (Berger *et al.*, 2001). In addition, in all mountain nyala that used the human shield strategy, this behavior was facultative. They visited humans only in some nights with a median frequency of 14 % both in males and females. If individual predation risk is high only during some nights, and if this is predictable (ex if cued by visual contact or sound of hyenas), excursions during a few critical nights could result in a substantial increase in survival probability. The human shield behavior was observed only in the well protected areas of Gaysay area and not in the remote eastern escarpments of Bale mountains exposed to legal and illegal hunting for many years. This finding is supported by other studies. For instance elk use areas of low wolf densities close to humans in Banff National Park Canada (Hebblewhite *et al.*, 2005) while the same species avoid humans to a larger extent than wolves in Bialowieza forest in Poland (Theuerkauf and Rouys, 2008) and in Yellowstone (Proffitt *et al.*, 2009). Given that the predator are ubiquitous and ungulates are endangered, human shield may in some cases be an

advantage for the conservation of the rare species, if humans presence does not reduce other limiting resources.

With this ever first detailed study on mountain nyala, we presented important findings on spatial distribution and population size, ecology and level of connectivity between different mountain nyala populations. Further work is required to make this research complete including studies on diet, home range and dispersal behavior, reproductive system and juvenile survival rate of the species both in the human dominated landscape of Gaysay area and intact forest of the eastern Bale mountains.

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Population genetic structure and connectivity in the endangered Ethiopian mountain nyala (*Tragelaphus buxtoni*): recommending dispersal corridors for future conservation.

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Keywords: Mountain nyala, landscape genetics, isolation by distance, dispersal corridors, gene flow, least-cost path

Abstract

Habitat fragmentation is an increasing threat to wildlife species across the globe and it has been predicted that future biodiversity will decrease rapidly without the intervention of scientifically-based management. In this study we applied a landscape genetics approach in order to determine a network design that will maintain connectivity among populations of the endangered mountain nyala (*Tragelaphus buxtoni*) in the fragmented highlands of Ethiopia. DNA was obtained non-invasively from 328 individuals and genetic population structure and gene flow were estimated using 12 microsatellite markers. In addition, a 475-bp segment of the mitochondrial control region was sequenced for 132 individuals. Potential dispersal corridors were determined from least-cost path analysis based on a habitat suitability map. The genetic data suggested limited gene flow between the sampled mountain nyala populations of the Bale Massif and the Arsi Massif. The genetic differentiation observed among five sampling areas of the Bale Massif generally followed a pattern of isolation by distance. We detected no impact of habitat resistance on the gene flow. In the future, however, the current expanding human population in the highlands of Ethiopia may reduce the current mountain nyala habitat and further limit migration. Maintaining habitat connectivity and facilitating survival of stepping-stone populations will be important for the future conservation of the species. The approach used here may also potentially be important for the study and conservation of other wildlife species inhabiting areas of increasing human encroachment.

Introduction

Population genetic theory and empirical studies show that long-term viability of populations is positively related to genetic diversity (Reynolds et al. 1999). Over the last few decades, however, accelerating habitat fragmentation has reduced genetic variability in many wildlife species (Fahrig 2003). For endangered species habitat fragmentation lead to enhanced extinction risk as genetic drift in small populations may cause inbreeding effects such as reduced fitness and evolutionary potential (Reed and Frankham 2003).

Most countries have established protected areas as the primary conservation tool to protect wildlife from impacts of humans and their livestock (Joppa et al. 2008). Unfortunately, the importance of connectivity between the protected area and the remaining distribution range of the species is often given lower priority (Margules and Pressey 2000). Isolated subpopulations may be subject to increased risk of local extinction (Frankham 2001) when the probability of receiving migrants from neighboring populations is low. To mitigate this, it is crucial to maintain landscape connectivity by conserving habitat corridors or ‘stepping stones’ (Bennet 2003). This is the cornerstone in the concept of conservation networks (Westemeier et al. 1998; Wikramanayake et al. 2004; Chetkiewicz et al. 2006; Rouget et al. 2006).

Functional connectivity in fragmented populations has traditionally been studied by direct observation of dispersal patterns through telemetry (Maehra et al. 2002; Graves et al. 2007). For rare, endangered species living in remote and inaccessible areas it is, however, rarely feasible to measure dispersal directly at appropriate spatial scales. In addition long-distance dispersal may be rare events with a low probability of detection through a small subset of GPS-marked animals. Landscape genetics is a rapidly growing discipline applied to analyse how

environmental features influence populations in terms of gene flow and genetic population structure (Bellemain and Taberlet 2004). It has become an increasingly important tool in conservation biology over the last decade (Storfer et al. 2010) as it provides information for designing corridors among fragmented populations and hence ensures functional connectivity (Li et al. 2010). Technological innovations in spatial analyses such as Geographic Information Systems (GIS) have massively improved the availability of large-scale habitat data through satellite images and enabled conservation biologists to find potential corridors through least-cost modelling (Li et al. 2010; Pullinger and Johnson 2010). These kinds of models are based on the assumption that the probability of dispersal is higher through suitable than non-suitable habitats and over shorter geographic distances (Beier and Noss 1998).

There is a rapid population decline of large mammals within East African reserves and isolation has been suggested as a major cause (Soulé et al. 1979; Burkey 1995). The highland of Ethiopia has unique flora and fauna with many endemic species including the mountain nyala (*Tragelaphus buxtoni*), the Ethiopian wolf (*Canis simensis*) and the Bale monkey (*Chlorocebus djamdjamensis*). The area has been increasingly fragmented by rapid expansion of human settlements, agricultural land and livestock pasture over the last decades (Evangelista et al. 2007; Evangelista et al. 2008). The mountain nyala (*Tragelaphus buxtoni*), a large antelope of ecological and economical importance, inhabits the mountains of south-eastern Ethiopia (Hillman 1993; Kingdon 1997). It is listed as endangered by the IUCN Red List (Sillero-Zubiri 2008) as a result of its small population size (Atickem et al. 2011) and suspected steady population decline. Currently an attempt to protect the mountain nyala is done within small patches of the Bale Mountain National Park (BMNP) as

well as in five trophy-hunting concessions in the Bale Massif (Evangelista et al. 2007; Atickem et al. 2011), where the majority of the species population exists. In addition, some remnant populations subsist in the adjacent Arsi Mountains (Evangelista et al. 2007).

It has been established that the large-scale distribution of mountain nyala is negatively affected by human settlements and agriculture (Atickem et al. 2011). The genetic population structure and habitat connectivity of the species have, nonetheless, never been investigated and limited information is available to guide conservation. It is known that in related species such as bushbuck *Tragelaphus scriptus* (Apio et al. 2010) and sitatunga *Tragelaphus spekei gratus* (Magliocca et al. 2002) males are the dispersing sex that defend harems during the breeding season. Unpublished data (A. Atickem) support that also male mountain nyalas range more widely than females and that adult males have a roam-to-mate breeding strategy.

The human development has increased in the mountain nyala range over the last two decades (Stephens et al. 2001) and it is important to investigate if the habitat is still suitable for dispersal between the populations. In this study we assess genetic diversity and the levels of population differentiation and gene flow among mountain nyala from six sampling areas. We combine this information with a previously developed habitat suitability map (Atickem et al. 2011) in order to suggest least-cost dispersal corridors that might be used to ensure future functional connectivity within the mountain nyala meta-population. The establishment of such an ecological network may also have importance for the general biodiversity maintenance of the Bale Mountains. Furthermore, the approach presented here could be applicable for the conservation of other species inhabiting remote areas.

Materials and Methods

Study area and fecal pellet sampling

The study area comprises two highland areas in Ethiopia, the Bale Massif and the Arsi Mountains. The 14775 km² Bale Massif elevates over 1800 m.a.s.l. (Fig. 1; N 6° 40'; E 39° 40'; the lowest elevation range for mountain nyala) and is the last stronghold of the mountain nyala. An estimated total of 3750 individuals exist in isolated patches within a landscape modified by human settlement and cultivated land (Atickem et al. 2011). Faecal pellets (the DNA source) were collected from five sampling areas in the Bale Massif; Gaysay, Headquarter, Abasheba Demaro (Abasheba), Besmena-OdoBullu (Odobullu) and Dodola, and in the Kakka-Galama Mountains of Arsi (Kakka-Galama), see Fig. 1. The sampling took place during the dry season between December and March in 2008/2009, and the pellets were preserved in 96% Ethanol. The sampling localities in the Bale Massif together contain approximately 85% of the total population and the mountain nyalas here are protected either by the BMNP or hunting concessions (Atickem et al. 2011). Gaysay and Headquarter are surrounded by human settlement and the animals in these two areas are quite habituated to humans. Odobullu, Abasheba and Dodola are trophy-hunting concessions and the populations in these areas are illusive and avoid human contact (Atickem et al. unpublished). It has been suggested that the original Dodola population was intensively hunted about two decades ago and may have been re-established by migrants from the surrounding mountains (Paul Evangelista Pers. Comm). In addition, small mountain nyala populations exist in the Bale Massif, including in the Harena Forest, Hanto, Hora, Shedem Berbere, the Web Valley and the Senetti Plateau (Fig. 1). The dry season last from November to February while the wet season last from March to October (Hillman 1986). The temperature varies with altitude. The Bale-

and Arsi Mountains are separated by extensive human development including cities, agriculture areas and roads. As we lacked habitat suitability data for the Arsi Mountains, the Kakka-Galama population, was excluded from our landscape genetics analysis.

Cross-species amplification of Bovidea microsatellite markers for mountain nyala

Thirty-five primer pairs originally developed for cattle (*Bos taurus*) and sheep (*Ovis aries*) were tested for their applicability in the mountain nyala. Of these, 14 gave discrete amplification products with good reproducibility and 12 were polymorphic (Table S1).

DNA extraction amplification and error checking

Genomic DNA was extracted from 378 pellet samples preserved in 96% ethanol. A thin slice (≤ 0.01 g) was cut from the surface of each pellet, transferred to 300 μ l lysis buffer (500 mM Tris 10 mM NaCl 50 mM EDTA) and incubated for 30 min at 56°C. 100 μ l of the lysate was mixed with 90 μ l isopropanol for DNA precipitation and 20 μ l of Dynabeads® MyOne™ SILANE (Invitrogen Dynal AS Oslo Norway) for DNA binding. The DNA was washed in 70 % ethanol twice and finally eluted in 100 μ l mqH₂O preheated to 80°C. One negative control was included for each eight samples.

The amplification of the 12 microsatellite markers was carried out in three multiplex panels. For panel 1 (BRIBO, ETH 225, CRM 60, ETH 10 and BM 719) the 50 μ l reaction mix included 25 μ l of 2* QIAGEN Multiplex PCR Master Mix (QIAGEN GmbH Hamburg, Germany), 50-100 ng DNA template, 8 pmol of each primer, 0.05 mg BSA (New England Biolabs) and mqH₂O. For panel two (BM4028,

INTRA40, BM3205, BM4025) and three (TGLA227, BMC3224, MCM58) the 50 μ l reaction mix in addition contained 50 nmol $MgCl_2$ (QiaGen), see (Table S2). The PCR program was as follows: Initial denaturation at 95°C for 15 min followed by 12 cycles of denaturation at 94°C for 1 min annealing at 58°C and extension of 72°C for 1 min and then 30 cycles of touchdown with the annealing temperature being reduced by 0.5 C per cycle. The program was terminated by a 10 min final extension at 72°C.

Fluorescently-labelled PCR products were separated with an ABI3730 Genetic Analyzer (Applied Biosystems Foster City CA.). Allele sizes and genotypes were scored using GENEMAPPER 4.0 (Applied Biosystems). The genotyping was carried out via the multi-tube approach where genotypes were assigned after three consistent replicates for heterozygotes and five consistent replicates for homozygotes (Taberlet et al. 1996; Gagneux et al. 1997). For ten individuals the amplification products for two different DNA extracts (with three replicates each) were compared in order to verify the consistency of the results.

In order to assess the ability of the markers in measuring population genetic differentiations, the probability of identity (PI; Paetkau et al. 1995) was estimated using the software Identity 1.0 (Wagner and Sefc 1999). PI is the probability of finding two individuals with identical genotypes in a given population when using a certain set of markers. Identical genotypes were detected using SPAGeDI 1.2 in order to avoid that any individual was sampled more than once (Hardy and Vekemans 2002). Possible genotyping errors due to stuttering short allele dominance and null alleles were tested by MICRO-CHECKER 2.2.3. (van Oosterhout et al. 2004). Possible effects of null alleles on the estimates of genetic diversity and population differentiation were assessed using the software FreeNa (Chapuis and Estoup 2007). FreeNa applies an expectation maximization algorithm and creates a data set

corrected for null alleles that is used to calculate global and pair-wise F_{ST} -values for each locus and across all loci (Chapuis and Estoup 2007).

A fragment of the mitochondrial DNA (mtDNA) control region (CR) was amplified using the primers MT4 (Arnason et al. 1993) and B16168H (Simonsen et al. 1998). The mtDNA amplification was performed in 15 μ l reactions containing 2.5 μ l HotStar PCR buffer (QIAGEN GmbH Hamburg, Germany), 5nmol dNTP, 0.01mg BSA (New England Biolabs), 50nmol Mgcl₂, 1.25 units HotStar *Taq* polymerase, 8 pmol of each primer, 50-100 ng template DNA and mqH₂O. The PCR program for the CR consisted of initial denaturation at 95°C for 15 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min.

Genetic diversity, population differentiation and gene flow

Genetic diversity for a total of 328 individuals was measured as the number of alleles per locus (A), allelic richness (AR), observed heterozygosity (H_O) and expected heterozygosity (H_E) under Hardy–Weinberg expectations (HWE; Nei and Chesser 1983; Nei 1987) using FSTAT (Goudet 2002; n=328). Departure from HWE, measured as F_{IS} (Wright 1978) and linkage disequilibrium, were estimated using GENEPOP 4.0 (Rousset 2008).

Population differentiation of the microsatellite data was assessed through a pair-wise F_{ST} -test implemented in GENEPOP 4.0 (Rousset 2008) with 1000 permutations. The same software was used to estimate pair-wise genetic distances [$F_{ST}/(1 - F_{ST})$] (Rousset 1997). We estimated gene flow (based on microsatellite data) using a genetic assignment method implemented in BayesAss+ 1.3 with 3000000 iterations, 999999 burn-in and 2000 sampling frequency (Wilson and Rannala 2003).

We applied a Bayesian maximum likelihood approach to infer the number of

populations as suggested by the microsatellite data using the software STRUCTURE 2.2.3 (Pritchard et al. 2000). STRUCTURE assigns individuals to their most likely population of origin without prior information on the sampling locations (Pritchard et al. 2000). We assumed the admixture model with correlated allele frequencies, a specified burn-in of 500000 interactions and 1000000 Markov Chain Monte Carlo (MCMC) replicates after the burn-in period. In order to ensure that the posterior probabilities converge consistently the program was run 10 times for each number of populations (K) between 1 and 5. The significance of the results was assessed according to Evanno et al. (2005) through a plot of the mean likelihood values ($\ln P(X|K)$) for each K. The rate of change of the likelihood function with respect to K (ΔK) evaluated the individual membership coefficient (Q; Pritchard et al. 2007).

The level of differentiation between pairs of populations based on mtDNA haplotypes was measured as pair-wise Φ_{ST} -values using Arlequin 3.5.1.2 (Excoffier and Lischer 2010). The same software package was used to test if any of the Φ_{ST} -values were significantly higher than under the null hypothesis of no population structure (10000 permutations) and to calculate the overall Φ_{ST} .

Corridor analysis

Corridors between the mountain nyala populations were derived applying least-cost path analysis (Chetkiewicz et al. 2006; Li et al. 2010) as implemented in the ArcToolbox in ArcGIS 9.3 (ESRI, Redlands, NY, USA). The cost raster was based on a habitat suitability map developed from logistic mixed modelling (using function lmer in R library lme4). The model was based on presence/absence of mountain nyala pellet samples assessed on 1515 randomly assigned plots, altogether covering 352 km². The presence of pellets within the plots (1 for pellet found, 0 for no pellet found)

was modelled as a binomially distributed response variable, and locality a random intercept. Land cover types (forest, *Erica* dominated shrub, grassland, bushland, agriculture and human settlement), slope and elevation (Chetkiewicz and Boyce 2009) were included as fixed effects. The model was extrapolated onto all pixels within the study area by entering the fixed effects parameter estimates from the model into the Spatial Analyst raster calculator in ArcGIS 9.3. The resistance value of any given pixel in the cost raster was calculated as 1 minus the probability of mountain nyala presence (Chetkiewicz et al. 2006; Spear et al. 2010). The resistance values ranged from 0.80 to 0.98 (Fig. 2). Based on this cost raster map, we developed three different measures for dispersal distance/resistance: 1) linear Euclidian distance between populations in km, 2) the least-cost path distance (also in km) derived applying least-cost path analysis (Adriaensen et al. 2003) as implemented in the ArcToolbox in ArcGIS 9.3 (ESRI, Redlands, NY, USA) and 3) ‘cumulative resistance’ (the sum of resistance values along the least-cost path) and relate it to genetic distance. Additional information on the method and results for the nyala habitat suitability map are provided in Atickem et al. (2011).

Mantel tests (Mantel 1967) as implemented in the package VEGAN (Oksanen et al. 2005) in R (R Development Core Team 2011) were used to estimate correlations between the pair-wise genetic distance values [$F_{ST} / (1 - F_{ST})$] and each of three distance/resistance metrics. Partial Mantel tests (Smouse et al. 1986) with 1000 permutations were used to determine the relative importance of habitat resistance on the genetic distance without the influence of linear geographic distance (Cushman and Landguth 2010).

Results

Amplification success and reproducibility

Of a total of 378 DNA extracts, 328 resulted in reliable multilocus genotypes recognized as unique samples by the SPAGeDI analysis. Of these 277 were complete genotypes, while 22, 3 and 26 individuals lacked one, two and three loci respectively. For the ten individuals, for which we compared genotypes from two different DNA extracts, all of the replicates (three per extract) gave identical results for each individual. The overall probability of identity (PI) for the 12 microsatellite loci was 1.47×10^{-11} . We found no evidence for scoring errors due to stuttering or allelic dropout. Although the MICRO-CHECKER analysis (van Oosterhout et al. 2004) indicated that 26 out of the 72 sampling-locus combinations might contain null alleles these were not associated with any particular locus and all of the loci were used. The FreeNA software estimated null allele frequencies between 0.06 and 0.22. The overall F_{ST} -value calculated with null-allele adjustment, 0.0546 (0.0330-0.0622), was slightly higher than that calculated without, 0.0548 (0.0435-0.0643). Given the small difference we assume that our estimates of genetic diversity and population differentiation are not biased by the presence of null alleles.

Sequences of the mtDNA CR for 132 individuals resulted in three haplotypes defined by two transitions. The haplotypes (H1-H3) were assigned the following GenBank accession numbers: JQ423261, JQ423262 and JQ423263.

Genetic diversity and Hardy–Weinberg equilibrium

The various diversity indices measured for each of the mountain nyala populations based on microsatellite data are summarised in Table 1. The number of alleles per locus ranged from 2 to 13 (mean = 8) while the average allelic richness was 5.39

ranging from 4.94 to 6.06. Five private alleles were detected in Kakka-Galama, two in Abasheba, one each in Dodola and Odobullu, all of them at low frequencies. The mean observed heterozygosity (H_O) and mean expected heterozygosity (H_E) were 0.56 (0.54-0.60) and 0.66 (0.61-0.69), respectively. All of the sampled populations, with the exception of Dodola, were suggested to be partially inbred ($F_{IS} = 0.025$ -0.197; Table 1). For the total population 15 of 66 locus-pairs were in linkage disequilibrium (Appendix A1), while only one of the populations (Abasheba) contained individuals that had a substantial proportion of their loci-pairs in linkage disequilibrium (24%).

Population structure and gene flow

The pair-wise, Bonferroni corrected, F_{ST} -values based on microsatellite data are shown in Table 2. With the exception of two areas (the adjacent Gaysay and Headquarter) all of the population-pairs were significantly differentiated at the 0.05-level.

The STRUCTURE analysis on multilocus genotypes indicated that the most likely number of genetic clusters chosen with the ΔK method was four ($\Delta K = 108$ for $K = 4$; $\Delta K < 55$ for all other K -values, see Fig. S1). The individuals sampled in Kakka-Galama, Abasheba and Dodola formed three distinctive genetic clusters while the individuals representing the populations of Gaysay, Headquarter and Odobullu were assigned to the same group (Fig 3a).

The distribution of the three mtDNA haplotypes over populations is displayed as pie charts in Fig. 3b. Haplotype H3 was not present in Gaysay, Headquarter and Kakka-Galama. A pairwise Φ_{ST} -test showed that Abasheba and Kakka-Galama were significantly different from all of the other populations ($p < 0.05$). The highest Φ_{ST} -

value (0.66) was observed between Abasheba and Kakka-Galama. As for the genotype data no differentiation was observed between Gaysay and Headquarter (Table 2). The overall Φ_{ST} was 0.37.

In general, we detected limited gene flow between the mountain nyala populations with the exception of from Gaysay to Headquarter and Odobullu (Fig. 4). The pattern of gene flow among the study locations was highly asymmetric and the population in Gaysay was suggested to be the main source of migrants.

Isolation by distance

The pair-wise genetic distances (based on microsatellite data) observed between the mountain nyala populations generally showed isolation by distance ($r^2 = 0.38$, $p < 0.001$; Fig. 5). The correlation between genetic distance and the least-cost distance was only marginally higher than that for linear geographic distance ($r^2 = 0.41$ $p < 0.001$; Fig. 5), and the least-cost dispersal corridors tended to form nearly straight lines between the populations (Fig. 6). The “cumulative resistance” of corridors was less correlated with the genetic distance than the geographic and the least-cost distance ($r^2 = 0.28$ $p = 0.0034$; Fig. 5). There was no residual effect of “cumulative resistance” on the genetic distance (after accounting for the length of the least-cost path; $r^2 = 0.12$ $p = 0.986$) indicating that the habitat resistance has little impact on the pattern of genetic differentiation observed among the populations.

Dispersal corridors

Our least-cost path model suggested 19 dispersal corridors as the best candidates for interconnecting the main mountain nyala populations across the Bale Massif (Table 2; Fig. 6). Based on visual inspection on the map (Fig. 6), the majority of these corridors

merge close to each source population. The main stretch connecting each of the population pairs are covered by a cluster of only 1-3 closely spaced pathways (Fig. 6) that we term “main corridors”. The lengths of the three main connecting corridors are 62 km (Gaysay-Dodola), 59 km (Gaysay-Abaseheba) and 120 km (Dodola-Abaseheba). The smaller mountain nyala populations of the Harenna Forest, Shedom, Senatte Plateau and Shedem Berbere (Fig. 1) are all situated close to these three corridors.

Discussion

Landscape genetics has become a key tool for the conservation of wildlife species in the face of the increasing threat of habitat fragmentation (Segelbacher et al. 2010). In this study we have optimized genetic markers and measured genetic variability and gene flow within and among the main populations of the endangered mountain nyala. We further combine the results from the genetic and ecological data in order to suggest habitat corridors that can potentially be used to secure future functional connectivity among mountain nyala populations within the increasingly fragmented Ethiopian highlands.

Working with low-quality DNA sources, such as feces, many cautions must be made. Five replicates were run for homozygote genotypes to minimize the risk of measuring artificially high levels of heterozygote deficit. It has been demonstrated that the presence of null alleles may slightly elevate the observed genetic differentiation and reduce the proportion of correctly assigned individuals by Bayesian cluster analysis (Carlson 2008). The low null allele frequencies observed in our data are expected to be of minor effect.

The average allelic richness observed for the mountain nyala (5.3) was slightly

lower than what we observed (6.4) for the same markers when analyzing 16 individuals of Menelik's bushbuck (*Tragelaphus scriptus meneliki*), another antelope endemic to southern Ethiopia. Two of the markers (BMC3224 and MCM58), have also been tested for 15 individuals of each of five African antelopes by Eblate et al. (2011). The average number of alleles per locus for these species was 7.8 and 6.8 respectively, compared to 7 for both loci in the mountain nyala. Considering the much higher number of genotyped individuals, the allelic diversity observed in the mountain nyala can be regarded as relatively low. However, the demographic history of the mountain nyala, a species that was first described in 1910 (Lydekker 1910), is largely unknown and the low variability does not necessarily result from population decline. The low number of mtDNA CR haplotypes may indicate a long-term low female effective population size.

Moderate levels of inbreeding (F_{IS}) were detected in all but one of the studied mountain nyala populations (Table 1). This may be caused by limited gene flow between the mountain nyala populations and be strengthened by other factors reducing the effective population size. A skewed sex ratio is common in ungulates, including *Tragelaphus* species (Owen-Smith 1993), due to lower survival rates for males (Bonenfant et al. 2002). The male to female ratio of the mountain nyala has been measured to (1:2.2) in the Gaysay and Headquarter area, where hunting is prohibited. The difference is expected to be larger in the hunting concessions where trophy hunters selectively shoot males. Additionally, non-random mating with high reproductive success of a few dominant males is expected in the polygynous mountain nyala (Evangelista et al. 2007; Refera and Bekele 2004) and may further have contributed to the observed inbreeding. The F_{IS} -value observed in the Dodola population (Table 1) did not deviate from HWE expectations. It is possible that this

population was established by immigration from various sources in the surrounding mountains where the nyala populations have been displaced by human settlements during the last few decades (Paul Evangelista Pers. Comm.). A relatively high degree of linkage disequilibrium was observed in the Abasheba. That the outermost populations in a species' distributional range may be particularly inbred has been demonstrated (Ortego et al. 2011) and our gene flow analysis suggests that Abasheba receives relatively few migrants (Fig. 4). We found nine microsatellite alleles that were unique to one population, five of them in Kakka-Galama and two in Abasheba. One of the mtDNA haplotypes was absent in three localities (Gaysay, Headquarter and Kakka-Galama; Fig. 3b), but Abasheba was the only of the Bale Mountain populations that were significantly differentiated (Φ_{ST}) from the other populations (Table 2).

Based on microsatellite data all of the studied populations were genetically differentiated with the exception of Gaysay and Headquarter (Table 2). These two adjacent locations are separated by human settlements and based on focal observations it was believed that the animals populating them represented two separate groups. Our results suggest that genetically they can be regarded as one population with Gaysay being, according to the gene flow analysis, the source of the majority of migrants (Fig. 4). The STRUCTURE analysis grouped the Odobullu population and the Gaysay-Headquarter population as one cluster. A relatively high degree of admixture can be observed within this cluster (Fig 3a), and the gene flow estimates indicated that the immigration to Odobullu from Gaysay is considerable (Fig. 4). The Gaysay area has been protected from hunting since the establishment of the BMNP in 1970 (Waltermire 1975) and the animals are habituated with human activities. This may explain that they are more prone to dispersal than mountain

nyalas from other areas. In the absence of other evolutionary forces, it is predicted from theoretical models that the exchange of only a few individuals per generation is sufficient to prevent significant genetic differentiation among populations (Wright 1931; Mills and Allendorf 1996). However, the threshold of gene flow to avoid genetic population differentiation from empirical studies of natural population remains unclear (Schemske et al. 1994; Newman and Tallmon 2001).

The genetic differentiation among pairs of mountain nyala populations generally followed a pattern of isolation by distance (Jenkins et al. 2010). We were not able to detect habitat features that explained the observed pattern of genetic differentiation significantly better than the mere geographic distance. Hence, we conclude that currently the habitat resistance has little effect in lowering the degree of connectivity among the studied nyala populations. The close to linear least-cost paths corroborate that habitat resistance has had limited impact on gene flow. We suggest that the pattern of genetic differentiation observed among the mountain nyala populations is constrained by intrinsic species-specific dispersal propensities (Sutherland et al. 2000) to a larger extent than habitat characteristics. In many ungulates (Haanes et al. 2011), including Tragelaphine species (Magliocca et al. 2002; Apio et al. 2010), males are the main contributors to genetic change over long distances. Based on studies on a limited number (7 males and 7 females) of collared mountain nyalas followed over two years, all the females were stationary throughout the period, while two of the males dispersed (Atickem et al. unpublished data). Although dispersal rates and distances for the mountain nyala are unknown, we find it most likely that males are the main dispersers. Hunting by humans has been reported to severely affect dispersal rates in some mammal species (Berger 2004). Male mountain nyala are subjected to trophy hunting in five controlled hunting areas in the

Bale Massif. In addition, poaching has been reported (Evangelista et al. 2007), but it is yet unknown to what extent the risk of legal hunting or poaching affects the dispersal rates and distances in the mountain nyala.

Habitat fragmentation has increased across the mountain nyala range during the last two decades due to human settlements, agricultural expansion and the growing livestock population (Stephens et al. 2001). In order to ensure long-perspective conservation of wildlife species, it is important to maintain connectivity that ensures gene flow among populations in ecological networks (Murphy et al. 2010). Our finding of close to linear least-cost distances suggests that the habitats of the areas separating populations are currently not what limits dispersal. The conservation goal should thus be to keep the degree of connectivity at today's level. We suggest that three of the nineteen corridors predicted by the least-cost path analysis should be given particular attention as they link the most distant localities (Fig. 6). The small nyala populations of the Hareenna Forest, Shedom, Senatte Plateau, Web Valley and Shedem Berbere (Fig. 1) are all situated close to these dispersal corridors and may act as 'stepping stones' (Stephanie et al. 2011; Bennet 2003). The specific corridors suggested here might also help preserving other key species of the study area such as the endemic Bale monkey *Chlorocebus djamdjamensis* (Mekonnen et al. 2010; Fig. S2) and ensure maintenance of the ecosystem.

Acknowledgements

This research was funded by Centre for Ecological and Evolutionary Synthesis (CEES) at the University of Oslo. We are grateful to Saint Louis Zoo Conservation and Rufford Small Grants for Nature Conservation for their financial support to field-work and the Ethiopian Wildlife Conservation Authority for permitting this research.

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Figure legends

Fig. 1 The study area in the Bale Massif in the southern Ethiopian Highlands. The number of genotyped/sequenced samples from each of the areas; Abasheba (78/24), Kakka-Gallama (34/21), Dodola (63/30), Gaysay (70/19), Headquarter (37/17) and Odobullu (46/21).

Fig. 2 Cost-raster map for the mountain nyala habitat in the Bale Massif. The values equal the inverse of the probability of mountain nyala presence in each pixel.

Fig 3a. Bar-plot showing the probability for each individual (multilocus genotype) to assign to each of the four clusters suggested by the Bayesian analysis. Each horizontal bar denotes an individual, and the four colors illustrate the different inferred clusters. The individuals are ordered after population of origin as given to the left of the bar plot. Fig 3b. Pie-charts displaying the proportion of each mtDNA haplotype (H1 = black , H2 =grey , H3 = white) in each of the mountain nyala populations. The sizes of the charts are proportional to the number of sequenced individuals.

Fig. 4 Gene flow estimated between the different mountain nyala populations. The arrows show the direction, and the amount of gene flow is given as, m , i.e. the fraction of individuals that migrates per generation. Confidence intervals for each value of m : Kakka-Galama to Gaysay ($7.03 \times 10^{-7} - 0.014$), to Dodola ($5.35 \times 10^{-6} - 0.019$); Gaysay to Kakka-Galama ($1.89 \times 10^{-4} - 0.049$), to Dodola ($1.61 \times 10^{-5} - 0.047$), to Odobullu ($0.07 - 0.2$), and to Headquarter ($0.24 - 0.327$); Dodola to Kakka-Galama ($3.73 \times 10^{-6} - 0.019$), to Gaysay ($2.08 \times 10^{-5} - 0.034$) and to Odobullu ($2.02 \times 10^{-4} - 0.04$); Odobullu

to Dodola ($4.26 \times 10^{-6} - 0.018$) and to Abasheba ($0.7 \times 10^{-6} - 0.038$); Abasheba to Odobullu (0.021-0.123); Odobullu to Headquarter ($9.34 \times 10^{-6} - 0.028$); Headquarter to Odobullu ($1.03 \times 10^{-4} - 0.03$) and to Gaysay ($8.08 \times 10^{-7} - 0.01$).

Fig. 5 Correlation of genetic distance between mountain nyala populations in the Bale Massif with a) geographical linear distance (straight connecting line; km), b) least-cost path distance (km) and c) the “cumulative resistance” of corridors. The cumulative resistance is the cumulative sum of resistance values along the least-cost path.

Fig. 6 Predicted corridors between the mountain nyala populations in the Bale Massif, as derived from least-cost path analysis of habitat suitability maps. The three main corridors that we recommend are shown in black.

Table 1. Genetic diversity of the mountain nyala based measured from microsatellite data.

| Locality | n | AR | H _O | H _E | F _{IS} |
|--------------|----|------|----------------|----------------|-----------------|
| Abasheba | 78 | 5.11 | 0.55±0.05 | 0.67±0.02 | 0.176 |
| Dodola | 63 | 4.94 | 0.60±0.04 | 0.61±0.02 | 0.025 |
| Gaysay | 70 | 5.31 | 0.57±0.07 | 0.67±0.04 | 0.142 |
| Headquarter | 37 | 5.62 | 0.54±0.08 | 0.67±0.04 | 0.197 |
| Kakka-Galama | 34 | 6.06 | 0.56±0.05 | 0.69±0.03 | 0.187 |
| Odobullu | 46 | 5.31 | 0.55±0.07 | 0.65±0.04 | 0.161 |

n = number of samples; AR = allelic richness; H_O = observed heterozygosity = H_E, expected heterozygosity; F_{IS} = inbreeding index. Bold values of F_{IS} represent significant deviations at the 0.001-level.

Table 2. Paired values of geographic distance (GDS), ‘the least-cost path distance (LPD), “cumulative resistance” (CR), F_{ST} (significant differentiation, $P < 0.0006$, indicated by asterisk), Genetic distance (GD; $F_{ST}/(1- F_{ST})$) and ϕ_{ST} values between pairs of mountain nyala localities.

| Paired mountain nyala localities | GDS (Km). | LPD (Km). | CR | Φ_{ST} | F_{ST} | GD |
|----------------------------------|--------------|--------------|------|-------------|----------|--------|
| Abasheba - Dodola | 120 | 127, 131 | 2397 | 0.55487 | 0.0618* | 0.0682 |
| Abasheba - Gaysay | 59 | 62, 69 | 1323 | 0.64031 | 0.0314* | 0.0308 |
| Abasheba -Headquarter | 51 | 48, 56 | 1204 | 0.63317 | 0.0391* | 0.0306 |
| Abasheba - Odobullu | 12 | 14 | 40 | 0.35019 | 0.0339* | 0.0259 |
| Odobollu - Headquarter | 42 | 44, 46 | 1013 | 0.09231 | 0.0218* | 0.0126 |
| Dodola - Gaysay | 59 | 62, 77 | 1465 | 0.02056 | 0.0422* | 0.0451 |
| Dodola - Headquarter | 65 | 68, 71 | 1553 | 0.01100 | 0.0598* | 0.0598 |
| Dodola - Odobullu | 94 | 98, 104 | 846 | 0.02300 | 0.0642* | 0.0628 |
| Gaysay - Headquarter | 5 | 4, 6 | 56 | 0.00 | 0.0179 | 0.0113 |
| Gaysay - Odobullu | 43 | 47, 51 | 1023 | 0.09709 | 0.0221* | 0.0210 |
| Kakka-Galama - Odobullu | 112 | - | - | 0.22950 | 0.0785* | 0.0872 |
| Kakka-Galama - Headquarter | 71 | - | - | 0.49636 | 0.0744* | 0.0667 |
| Kakka-Galama - Gaysay | 61 | - | - | 0.54246 | 0.0686* | 0.0722 |
| Kakka-Galama - Dodola | 52 | - | - | 0.26199 | 0.0904* | 0.0872 |
| Kakka-Galama - Abasheba | 123 | - | - | 0.66187 | 0.0751* | 0.0766 |

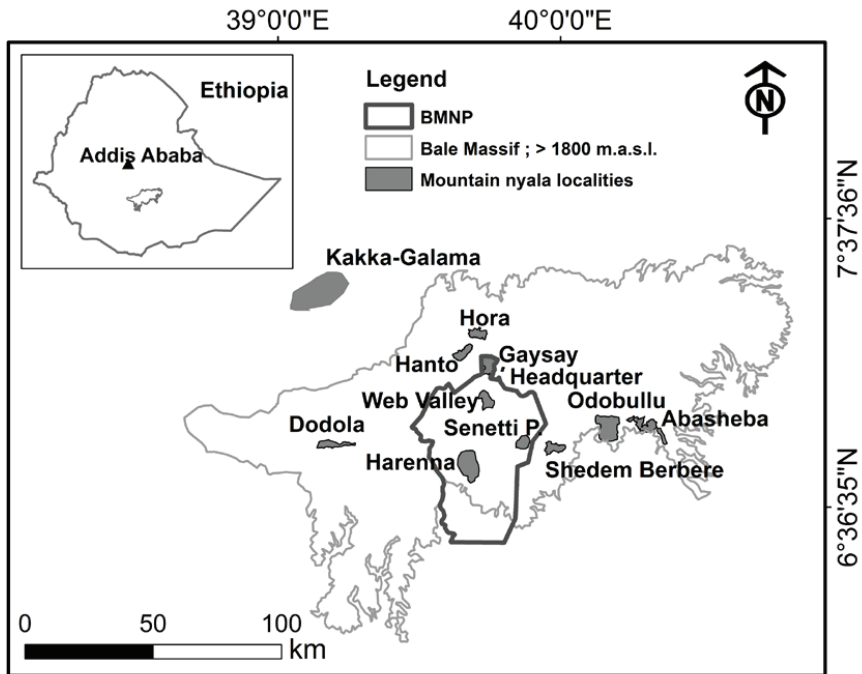


Fig. 1

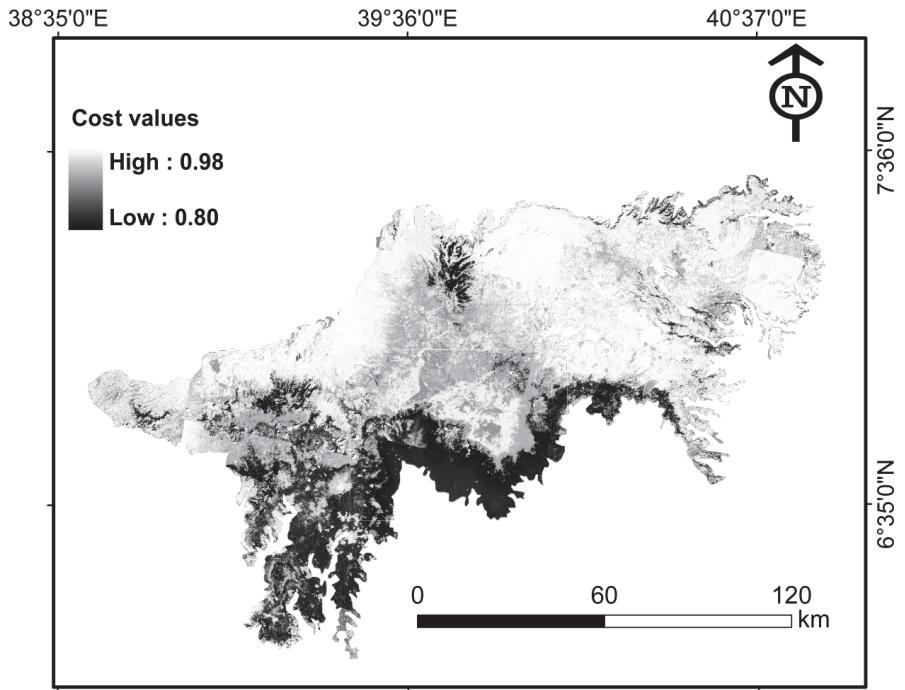


Fig. 2

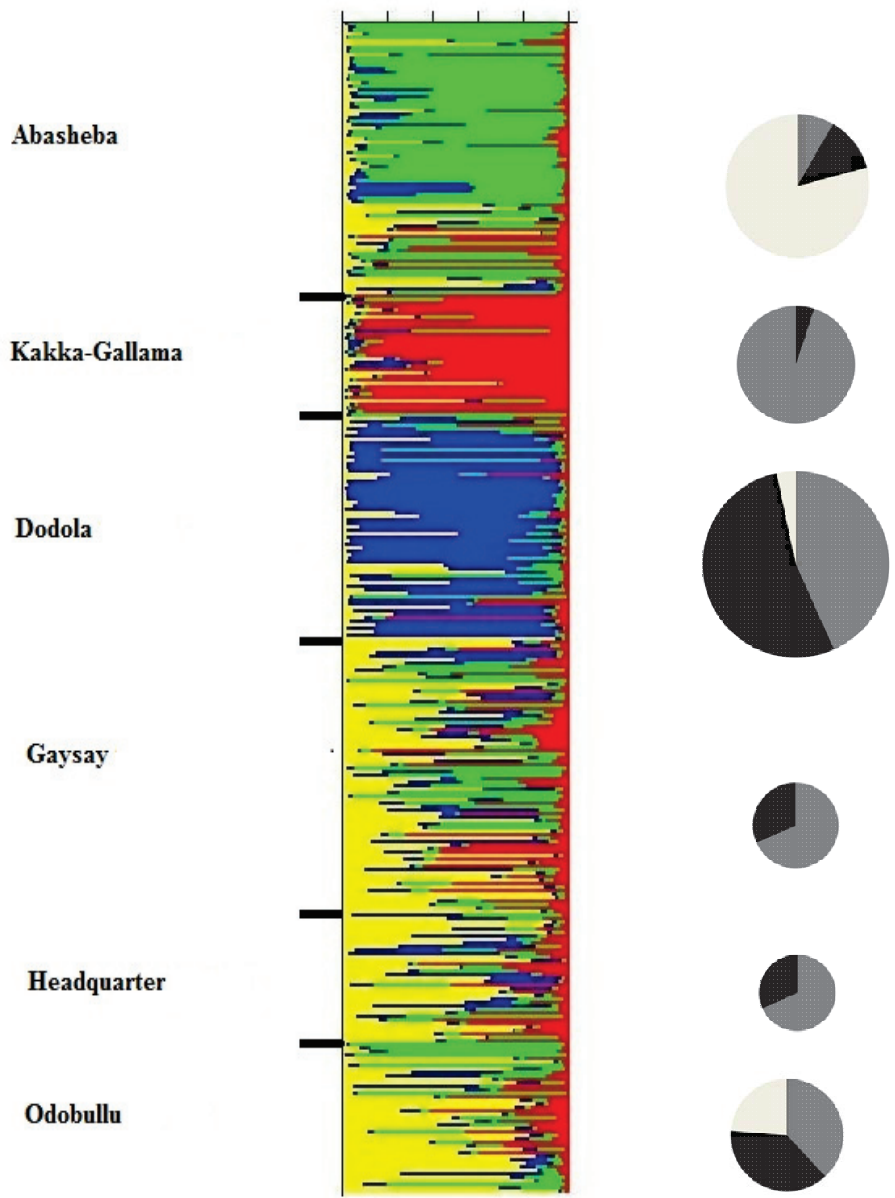


Fig 3a

Fig 3b

Fig 3.

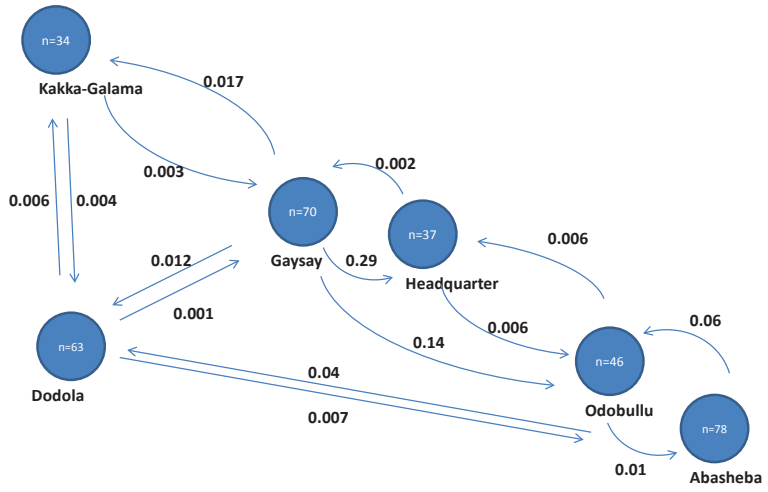


Fig. 4

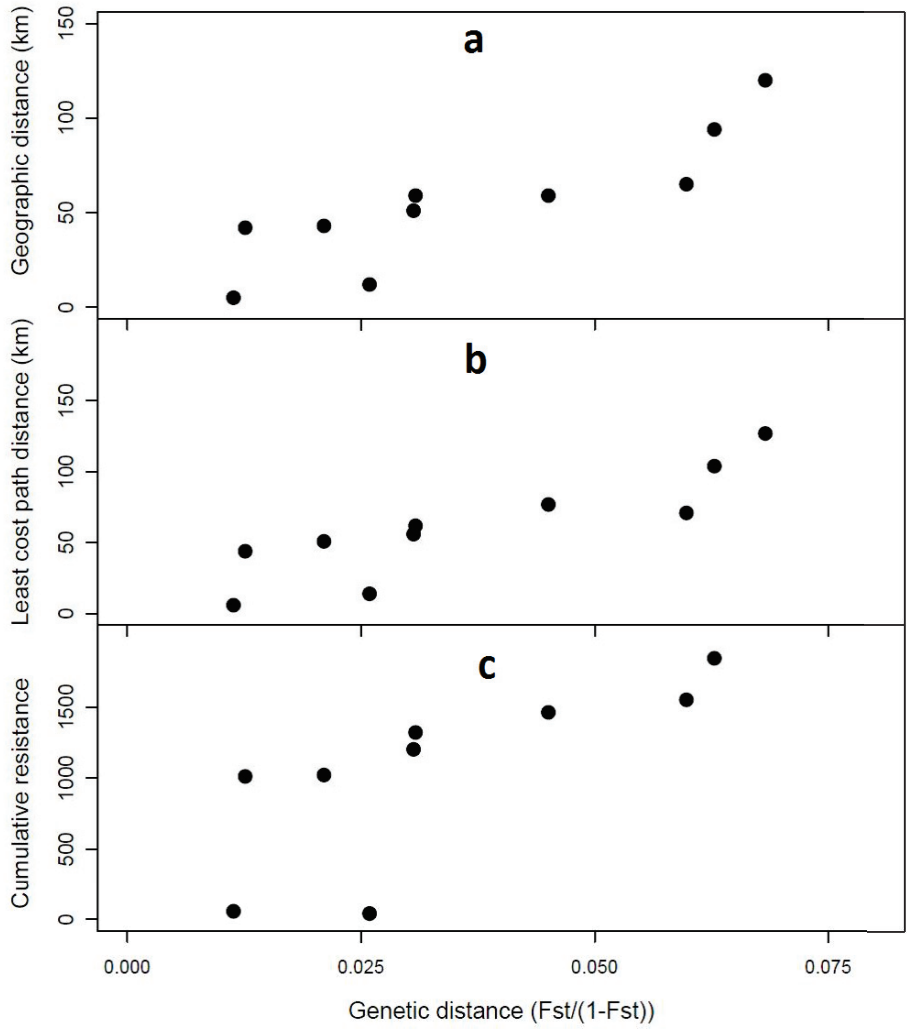


Fig. 5

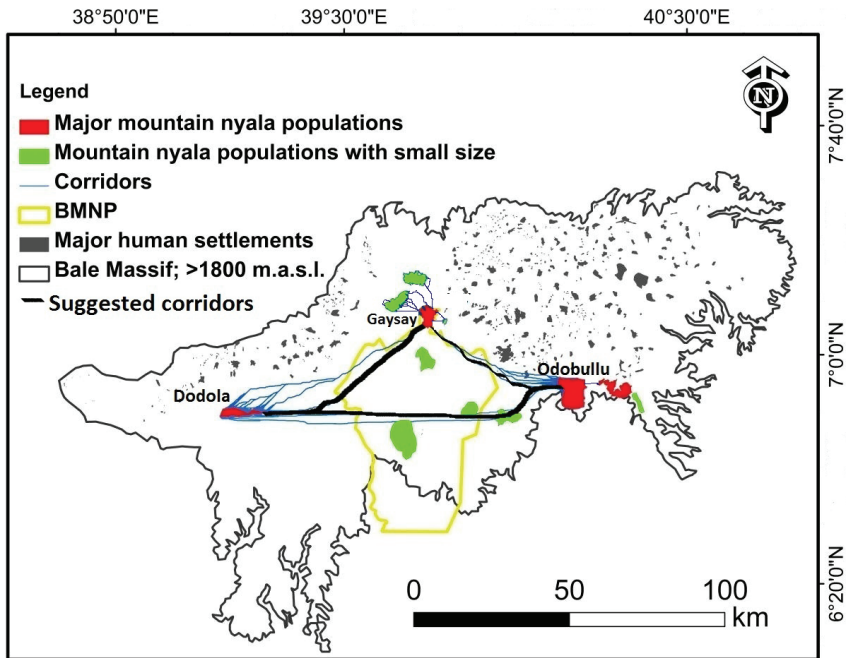


Fig. 6

Electronic supplementary Material

Table S1. Characteristics of the 35 microsatellite markers that were tested for the mountain nyala.

| Locus | Amplification | Size-range (bp) | Reference |
|----------|---------------|-----------------|-----------------------------|
| BM 3205 | + | 204-212 | Bishop et al. 1994 |
| BM4025 | + | 128-146 | Bishop et al. 1994 |
| BM4028 | + | 154-174 | Bishop et al. 1994 |
| BM719 | + | 139-161 | Bishop et al. 1994 |
| BMC3224 | + | 182-188 | Bishop et al. 1994 |
| BRIBBO | + | 157-252 | Bishop et al. 1994 |
| CSRM 60 | + | 160-188 | Moore et al. 1994 |
| ETH 10 | + | 211-215 | Toldo et al. 1993 |
| ETH 225 | + | 134-146 | Steffen et al. 1993 |
| INRA 40 | + | 205-230 | Vaiman et al. 1994 |
| MCM58 | + | 166-182 | Hulme et al. 1995 |
| TGLA227 | + | 64-115 | Georges and Massey 1992 |
| BM 1824 | + | 178 | Bishop et al. 1994 |
| BM2113 | + | 127 | Bishop et al. 1994 |
| BM 17132 | - | - | Stone et al. 1995 |
| BM 3517 | - | - | Bishop et al. 1994 |
| BM1225 | - | - | Bishop et al. 1994 |
| BM1237 | - | - | Bishop et al. 1994 |
| BM143 | - | - | Bishop et al. 1994 |
| BM1706 | - | - | Bishop et al. 1994 |
| BM1862 | - | - | Bishop et al. 1994 |
| CSSM 19 | - | - | Moore et al. 1994 |
| DIK 020 | - | - | Hirano et al. 1996 |
| ETH 3 | - | - | Toldo et al. 1993 |
| HEL 1 | - | - | Kaukinen and Varvio 1993 |
| HEL9 | - | - | Kaukinen and Varvio 1993 |
| ILST061 | - | - | Kemp et al. 1995 |
| ILST068 | - | - | Kemp et al. 1995 |

| | | | |
|----------|---|---|----------------------------|
| INRA 005 | - | - | Bishop et al. 1994 |
| INRA 032 | - | - | Vaiman et al. 1994 |
| INRA 037 | - | - | Vaiman et al. 1994 |
| INRA 063 | - | - | Vaiman et al. 1994 |
| TGLA 126 | - | - | Georges and Massey 1992 |
| TGLA 179 | - | - | Georges and Massey 1992 |
| TGLA 53 | - | - | Georges and Massey 1992 |

Table S2. The three multiplex panels used to amplify the 12 microsatellite markers used.

| Panel | Primers | The reaction mix (total volume 50 μ l) |
|-----------|---|---|
| Panel I | BRIBO ETH 225 CRM 60 ETH 10 and BM 719 | 25 μ l of 2* QIAGEN Multiplex PCR Master Mix, 50-100 ng DNA template, 8 pmol of each primer, 0.05 mg BSA (New England Biolabs) and mqH ₂ O |
| Panel II | BM4028 INTRA40 BM3205 BM4025 | 25 μ l of 2* QIAGEN Multiplex PCR Master Mix, 50-100 ng DNA template, 8 pmol of each primer), 0.05 mg BSA (New England Biolabs), 50 nmol MgCl ₂ and mqH ₂ O |
| Panel III | TGLA227 BMC3224 MCM58 | 25 μ l of 2* QIAGEN Multiplex PCR Master Mix, 50-100 ng DNA template, 8 pmol of each primer), 0.05 mg BSA (New England Biolabs), 50 nmol MgCl ₂ and mqH ₂ O |

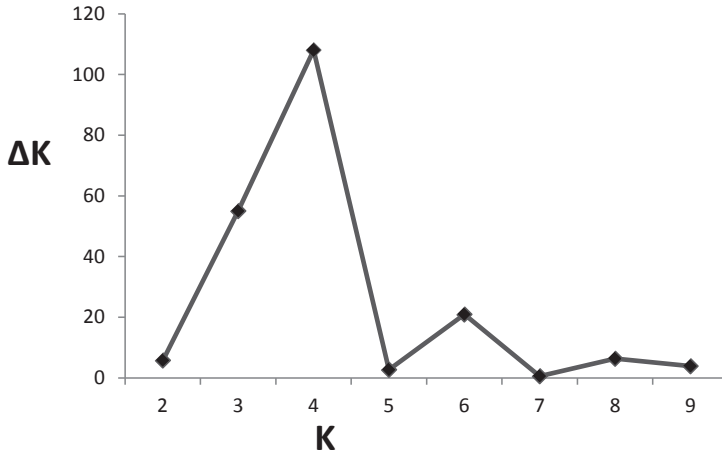


Fig S1. The magnitude of ΔK for each K value where the true K value represented by the uppermost level of the structure, four in this case.

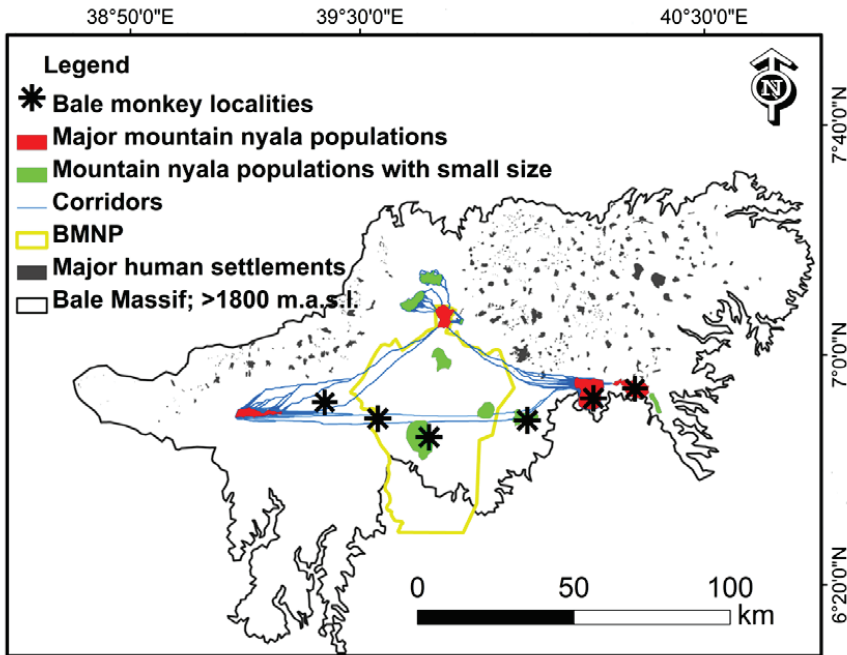


Fig. S2 The distribution pattern of Bale monkey along the least cost path corridors of mountain nyala.

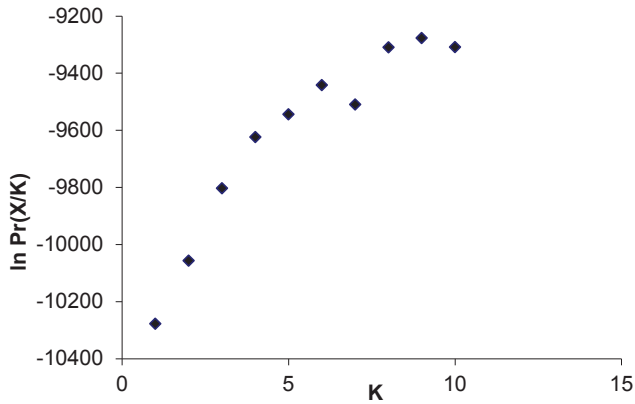


Fig S3. A plot of the log likelihood of the data ($\ln \Pr(X|K)$) values obtained from the STRUCTURE analysis for $K = 1-10$.

Appendix A1. Locus-pairs which were in linkage disequilibrium considering the whole population

BM4025 X BM4028, BM4025 X CSRM 60, BM4025 X INRA 40, BM4028 X BRIBBO, BM4028 X CSRM 60 60, BM4028 X INRA 40, BM719 X CSRM 60, BRIBBO X CSRM 60, BRIBBO X INRA 40, CSRM 60 X ETH 10, CSRM 60 X ETH 225, CSRM 60 X INRA 40, ETH 10 X ETH 225, ETH 10 X INRA 40, ETH 225 X INRA 40

