

The use of non-invasive molecular techniques to confirm the presence of mountain bongo *Tragelaphus eurycerus isaaci* populations in Kenya and preliminary inference of their mitochondrial genetic variation

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Abstract The mountain bongo antelope *Tragelaphus eurycerus isaaci* has rapidly declined in recent decades, due to a combination of hunting, habitat degradation and disease. Endemic to Kenya, mountain bongo populations have shrunk to approximately 100 individuals now mainly confined to the Aberdares mountain ranges. Indirect observation of bongo signs (e.g. tracks, dung) can be misleading, thus methods to ensure reliable species identification, such as DNA-based

techniques, are necessary to effectively study and monitor this species. We assessed bongo presence in four mountain habitats in Kenya (Mount Kenya National Park, Aberdare National Park, Eburu and Mau forests) and carried out a preliminary analysis of genetic variation by examining 466 bp of the first domain of the mtDNA control region using DNA extracted from faecal samples. Of the 201 dung samples collected in the field, 102 samples were molecularly identified as bongo, 97 as waterbuck, one as African buffalo and one as Aders' duiker. Overall species-identification accuracy by experienced trackers was 64%, with very high error of commission when identifying bongo sign (37%), and high error of omission for waterbuck sign (82%), suggesting that the two species' signs are easily confused. Despite high variation in the mtDNA control region in most antelope species, our results suggest low genetic variation in mountain bongo as only two haplotypes were detected in 102 samples analyzed. In contrast, the analysis of 63 waterbuck samples from the same sites revealed 21 haplotypes. Nevertheless, further examination using nuclear DNA markers (e.g. microsatellites) in a multi-locus approach is still required, especially because the use of mitochondrial DNA can result in population overestimation as distinct dung samples can potentially be originated from the same individual.

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Introduction

The mountain bongo *Tragelaphus eurycerus isaaci* is a critically endangered antelope sub-species (East 1999) now endemic to Kenyan montane forests. Bongo populations

have declined in the last decades and have been extinct in Uganda since 1913 (Bosley 2003). Currently, there are approximately 100 mountain bongos mostly restricted to the Aberdares Mountains in Kenya (Prettejohn 2004; Reillo 2002). The dramatic decline of these populations has been due to a combination of hunting, disease and habitat degradation (Kock et al. 1999; Estes et al. 2008).

Bongos are difficult to observe directly due to their cryptic and elusive behaviour. Assessing the presence and abundance of forest dwelling antelope species, such as bongo, often involves the analysis of indirect field signs such as faeces and tracks (Putman 1984; Bowkett et al. 2009). Methodologies based on faecal counts are commonly used, though there are many possible errors associated with these methods (Neff 1968) such as variable defecation rates, unknown decaying times and difficulty in identifying pellets of sympatric species (van Vliet et al. 2008a, b). Discriminant analysis of morphometric measurements of pellets from ten sympatric West African ungulates has demonstrated that only six species could be correctly identified in the field (Hibert et al. 2008). The reliability of pellet count methodologies can be improved by association with other techniques, such as direct observation, radio-tracking and molecular approaches. For example, a combination of pellet counts, camera trapping, direct counts along transect lines, and radio-tracking has been used to estimate the abundance of forest antelopes (Lunt et al. 2006; Rovero and Marshall 2004). In another study, molecular methods were employed to test the accuracy of identifying sympatric antelope species in the African forests using dung pellets appearance. It was demonstrated that the precision of species identification by experienced trackers using pellets is low, ranging from 58 to 76% (Bowkett et al. 2009).

Molecular techniques using non-invasive sampling (e.g. faeces, hair, etc.) have now been successfully applied to many studies of endangered mammals, such as population structure in brown bears (Perez et al. 2009), sex determination in wolves (Sastre et al. 2009), estimation of effective population sizes in giant pandas (Zhan et al. 2006) and dietary habits in carnivores (Farrell et al. 2000). This approach can be particularly useful in rare, endangered and secretive species like bongo. Recently, Ntie et al. (2010) used mitochondrial sequences to distinguish small faecal pellets from Central African ungulates in Gabon, of the genera *Cephalophus*, *Neotragus*, *Tragelaphus* and *Hymoschus*. Although confounded by a lack of phylogenetic resolution among several duiker (*Cephalophus*) species, the study provided evidence that sufficient resolution was available within the sequences used to provide a potentially valuable tool to identify the presence of many forest ungulates in cases where faecal morphology is extremely cryptic.

We used molecular methods to: (1) to establish a molecular protocol for identification of mountain bongo using dung pellets; (2) investigate the presence of the elusive mountain bongo in four montane forest habitats in Kenya: Mount Kenya National Park, Aberdare National Park, Eburu and Mau forests; (3) to validate field observations for input in habitat distribution models; and (4) to conduct a preliminary analysis of bongo genetic diversity using mitochondrial DNA control region. Understanding the current distribution of the few remaining bongo populations is crucial for an ongoing, multi-phase recovery effort (Reillo 2002).

Materials and methods

Sample collection and field identification

Dung samples were collected from the forests of Mt Kenya, Aberdares, Mau and Eburu, which were historically known as mountain bongo habitats prior to the population decrease in the 1950s (Estes et al. 2008; Kingdon 1982). These forests also support a diverse range of antelopes including waterbuck *Kobus ellipsiprymnus*, eland *Tragelaphus oryx*, several duiker species (including *C. monticola* and *C. harveyi*) and African buffalo *Syncerus caffer*. To date, the Aberdares Mountain is the only location where the occurrence of bongo has been confirmed by field observations conducted by the Bongo Surveillance Project (unpublished data). The other areas chosen for this study were selected either because they constitute suitable habitats for bongos or have historically contained bongo populations.

Bongo dung consists of a number of flattened pellets (roughly 2–3 cm diameter) clumped together into one to several large piles. Fresh dung piles were identified by their shiny surface and damp, greenish interior when crushed, and by using associated tracks and other signs (e.g. bite marks on known browse plants) when present to guide identification. Samples were collected during tracking expeditions conducted by the Bongo Surveillance Team (BST), which looked explicitly for bongo signs in areas where they were believed to most likely occur, and also during research conducted to collect distribution data for use in habitat modeling (Estes et al. 2008, 2010). Dung sample collection and species attributions were based on the judgment of the lead tracker(s), including several samples ascribed to waterbuck and other species, which produce dung that may look similar to bongo's. The sampling protocol specified the collection of one sample per dung pile. If there was any question as to whether separate piles were commingled, only one sample was collected. A total of 212 dung samples were collected from 115 sites within the four montane forest areas: Mt. Kenya National

Park and Reserve ($n = 35$, 25 sites), Aberdare Conservation Area ($n = 158$, 86 sites), Eburu ($n = 6$, 3 sites) and Mau ($n = 2$, 1 site) (Fig. 1). Of these, the trackers attributed 13 to waterbuck or other species, while the remainder was attributed to bongo. All dung samples collected were preserved in 95% ethanol at room temperature while in the field until DNA extraction.

We assessed the accuracy of trackers' species identifications using an error matrix, which contains three different accuracy assessment measures, expressed as rates. The first two are species-specific: the error of omission, which results when trackers overlook dung from a particular species at a particular site; and the error of commission, caused when trackers mistakenly attribute dung to that particular species when it was produced by a different species. The third measures the rate of tracker accuracy, both within species and overall. These metrics are calculated as follows:

$$\text{Error of Omission} = 100(N_{\text{omit}}/N_{\text{actual}})$$

$$\text{Error of Commission} = 100\left(N_{\text{commit}}/\sum N_{\text{identified}}\right)$$

$$\text{Identification accuracy} = (N_{\text{corr}}/(N_{\text{corr}} + N_{\text{omit}} + N_{\text{commit}}))$$

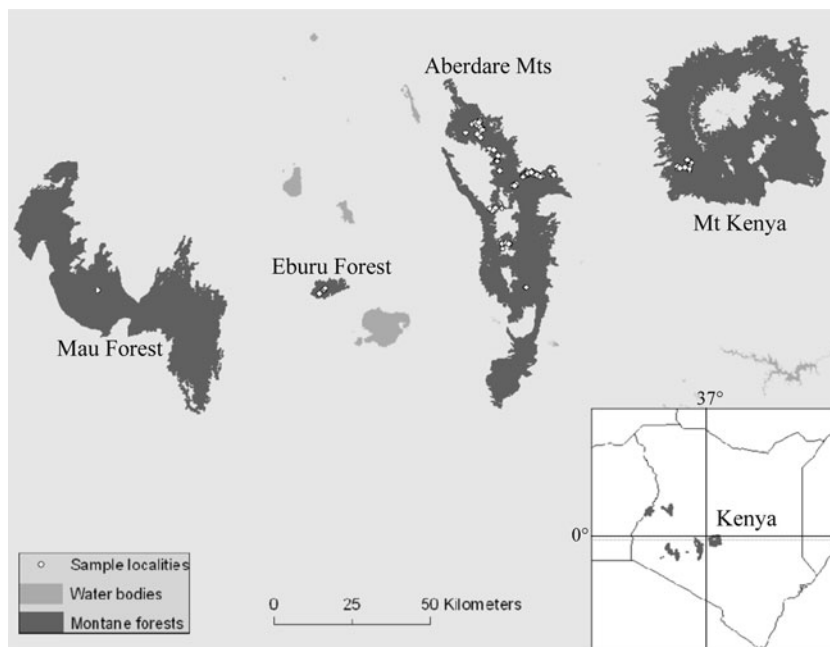
where N_{omit} is the number of sites containing genetically confirmed samples of species X that were overlooked by the trackers, N_{commit} is the number of sites where the trackers identified samples as belonging to species X when they were genetically confirmed as belonging to species Y (or Q or Z), N_{corr} is the number of sites correctly attributed to species X by the trackers, N_{actual} is the total number of sites that the genetic analysis attributed to species X, and

$N_{\text{identified}}$ is the total number of sites attributed to species X by the trackers. Note that we use site as the unit for determining accuracy, rather than sample, as we assumed that multiple samples collected at a given site belonged to a single species. This assumption was based on our initial hypothesis that bongo sign has a characteristic appearance that is not easily confused with other species, and that there is little habitat overlap between bongo and waterbuck (the species whose sign pose the greatest risk of misidentification), which would minimize the probability of collecting samples from more than one species at a single site. To account for the possibility that genetic analyses could show that certain sites contained more than one species (due to misidentifications by trackers), at each site we assigned each recorded species the proportion of the total number of samples that were genetically attributed to it (e.g. 0.5 bongo and 0.5 waterbuck at a two-sample site with 1 confirmed bongo sample and 1 confirmed waterbuck sample). We defined sites using the global positioning system (GPS) coordinates we recorded for each collected sample. Typically, if several dung piles were within 10–20 m of one another, a single GPS reading centered amongst them was collected and used to define the location of the site; however, the GPS readings for a few of the multi-sample sites collected by the BST may describe a larger site radius of 100–200 m.

Genetic identification

Total genomic DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN, Crawley, UK) according to manufacturer's instructions. At the time of the study, there

Fig. 1 Sample sites of bongo antelope within the four surveyed montane forests, shown in relation to the region (*inset*)



were no mountain bongo mtDNA control region sequences available on GenBank to allow us to correctly assign samples as belonging to mountain bongo. Therefore, firstly we used Cytochrome *b* gene sequences available on GenBank (accession numbers AF036276, and AF022065) to design two specific primers (Bongo Forward 5'-GCT ACGTACTACCATGAGGACAAATAT-3' and Bongo Reverse 5'-TGGTGTGTTGAGTGGGTTTG-3') to amplify a fragment of 406 bp of Cytochrome *b* gene.

An initial set of 13 DNA samples were sequenced using these primers and four of them were positively identified as bongo antelope mitochondrial DNA. Species identity was confirmed by using BLAST (Basic Local Alignment Search Tool; Altschul et al. 1997). These four samples identified as mountain bongo were then used as positive controls for amplification of the mtDNA control region. The evidence indicating that the sequences amplified in this study are authentic mitochondrial DNA are: (1) single PCR fragments on agarose gels; (2) clear sequences with no ambiguities; (3) absence of stop codons, deletions, insertions or frame-shifts. Furthermore, we could be confident about the authenticity of the bongo sequences used in GenBank for identification of field-generated data because one (accession number AF030264) originated from a cell-line immortalised by the Centre for Research on Endangered Species, Zoological Society of San Diego, and the two other available sequences were extremely similar to this exemplar.

A 466 bp fragment of the left domain of the mitochondrial DNA control region (D-loop), was amplified using general primers: MT4 (5'-CCTCCCTAAGACT CAAGGAAG-3' Arnason et al. 1993) and B16168H (5'-GG TTGCTGGTTTCACGCGGCATG-3' Simonsen et al. 1998). These primers have been reported to reliably amplify mitochondrial control region sequences in African bovids (Nersting and Arctander 2001; Simonsen et al. 1998; Birungi and Arctander 2000). Amplifications were carried out using the Qiagen Multiplex Kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. The PCR cycling profile was: an initial activation step at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 90 s and 72°C for 90 s and a final elongation step of 72°C for 10 min. The amplicons were electrophoresed in a 1.5% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotium) and visualized under ultra-violet light. PCR products were purified with Exonuclease *I* and Shrimp Alkaline Phosphatase (SAP) enzymes (USB Corp, USA) at 37°C for 60 min and inactivated at 80°C for 15 min. Purified PCR products were then sequenced using BigDye™ Terminator Sequencing kit (Version 3.1, ABI) and analysed on a semi-automated genetic analyzer (ABI 3130XL).

Chromatograms were checked and aligned using SEQUENCHER (Version, 3.02, Gene Codes Corporation).

The observed number of haplotypes (N_H), haplotype diversity (H_D) $\{h = (1 - \sum f_i^2) n / (n-1)\}$ (Nei 1987) and nucleotide diversity $\{\pi = \sum \pi_{ij} / n (n-1) / 2\}$ (Nei 1987) were calculated using DnaSP (version 4.1) software (Rozas et al. 2003). Species identity was confirmed by using BLAST scores (Altschul et al. 1997).

Results and discussion

Species identification

All dung samples collected could be successfully amplified and sequenced using the mtDNA control region primers MT4 and B16168H. Only five DNA samples had to be re-extracted and 20% of the PCRs had to be repeated to yield good quality products. Out of the 201 samples analyzed, 102 were identified as bongo, 97 as waterbuck, one as African buffalo and one as Aders' duiker *Cephalophus adersi* (Table 1). With sampling site as the classification unit, 64.12 sites contained correctly identified bongo dung and nine contained correctly identified waterbuck dung, while trackers incorrectly classified the dung of waterbuck dung or other species (Ader's duiker, buffalo) as bongo dung at 37.88 sites (Table 2). Overall tracker identification accuracy was 64%, but the high error of commission in identifying bongo (37%), and the correspondingly high error of omission for discerning waterbuck sign (82%), suggest that trackers have great difficulty in distinguishing bongo from waterbuck dung, despite experience and the presence of additional signs (tracks, feeding sign) aiding identification. The very low bongo error of omission (1.5%) might be an underestimate, since we collected all samples that trackers identified as belonging to bongo, while ignoring most samples identified as waterbuck or other species. A more balanced sampling design is thus needed to properly determine how easily bongo sign are overlooked. Despite this, these error rates are similar to those reported by Bowkett et al. (2009), using dung morphology to differentiate antelope species in the Udzungwa Mountains of Tanzania. Hibert et al. (2008) also experienced difficulty discriminating dung pellets of some sympatric antelope species from West African savannas; four out of ten species analyzed could not be distinguished by morphological measurements. Our results illustrate the importance of incorporating molecular methods into faecal-count studies, especially to aid species identification. Correct identification of cryptic and/or rare species is critical for estimating presence, abundance and geographical range. The use of anecdotal data is often unreliable and can result in a negative impact on conservation efforts (McKelvey et al. 2008). Here we show that, in the absence of molecular techniques, tracker identifications would have

Table 1 Molecular species identification in each of the four main sampling regions in Kenya

	Bongo	Waterbuck	African buffalo	Aders' duiker	Total per region
Mt Kenya	21	14	–	–	35
Aberdare	76	81	1	–	158
Eburu	4	1	–	1	6
Mau	1	1	–	–	2
Total per species	102	97	1	1	201

Table 2 Molecular species identifications compared to tracker identifications, presented as an error matrix, with sampling site as the unit of comparison, rather than individual sample

Tracker identifications	mtDNA identification				Accuracy measures		
	Bongo	Waterbuck	Other	Total	Omissions	Commissions	Tracker accuracy
Bongo	64.12	0.00	1.00	65.12	1.54	37.13	62.26
Waterbuck	37.13	9.00	3.00	49.13	81.68	0.00	18.32
Other	0.75	0.00	0.00	0.75	100.00	100.00	0.00
Total	102.00	9.00	4.00	115.00	Overall tracker accuracy		63.59

Fractional values resulted when dung from more than one species (typically waterbuck and bongo) were collected at a given site. The category “Other” includes buffalo, and Ader’s duiker

led to an overestimate of bongo presence sites, resulting in models that generate falsely optimistic range or population estimates, and perhaps improperly informed conservation action.

Bongo antelope presence was confirmed in all four study areas (Table 1), indicating that this species, despite large reductions in its overall numbers, persists in at just over half of its historical range. Remote sensing data have shown that bongo habitat selection is presently shaped by the need to escape human pressure and vegetation structural characteristics at microhabitat (0.04–0.09 ha) and patch scales (20 ha) (Estes et al. 2008, 2010, and unpublished data). Since our sampling efforts were more intense in the Aberdares Mountains and Mount Kenya, we cannot infer habitat preference by bongo within or between these particular areas. Further, given that most of our sampling was restricted to localities within our four study areas previously suspected to hold bongo populations, it is possible that additional bongo herds may exist in other localities and habitat areas that were not surveyed. Interestingly, bongo and waterbuck samples were found together in ten sites from lower broadleaf forests in the Aberdares, in certain cases within just a few meters of each other, suggesting a much greater overlap in habitat requirements than we initially believed, and perhaps an inter-specific association. In the present study we did not individually identify bongos in order to estimate abundance. For that purpose, the use of other molecular markers, such as microsatellites, would be required.

Genetic variation

Analysis of the mtDNA control region sequences revealed the occurrence of only two haplotypes in the 102 samples analyzed, which were defined by a single nucleotide transition (haplotypes B01 and B02, accession numbers EU04246 and EU04245, respectively). Haplotype B01 was found in 30.39% of the bongo faecal samples while B02 was found in 69.61%. Haplotype B02 was predominant in all mountain areas sampled: 60.53% in the Aberdares samples, 95.23% in Mt. Kenya and 100% in Eburu and Mau samples. Haplotype diversity ranged from 0.000 in Eburu to 0.5020 in Aberdares, whilst nucleotide diversity (π) ranged from 0 in Eburu and Mau to 0.001 in the Aberdares (Table 3). A single PCR fragment on agarose gels, clear sequences with no ambiguities and sequence similarity with other species previously published on

Table 3 Haplotype absolute frequency (*Hap*), sample size (*N*), genetic diversity (*H*) and nucleotide diversity (π) in bongo and waterbuck samples from Kenya

	Bongo			Waterbuck		
	<i>Hap</i> (<i>N</i>)	<i>H</i>	π	<i>Hap</i> (<i>N</i>)	<i>H</i>	π
Mt Kenya	2 (21)	0.10	0.0002	3 (12)	0.3182	0.0067
Aberdare	2 (76)	0.5020	0.001	20 (51)	0.9286	0.0358
Eburu	1 (4)	0	0	–	–	–
Mau	1 (1)	0	0	–	–	–

Table 4 Comparison of diversity indices of mtDNA control region in antelope species

Species	<i>N</i>	<i>Hap</i>	<i>H</i>	π	Source
Mountain Bongo	102	2	0–0.502	0–0.001	Present study
Common eland	122	80	0.7–1.0	0.005–0.031	Lorenzen et al. (2010)
Impala	155	106	–	0.036	Nesting and Arctander (2001)
Greater kudu	90	46	–	0.032	Nesting and Arctander (2001)
Roan antelope	140	52	0–1.0	0–0.045	Alpers et al. (2004)
Saiga antelope	93	38	–	0.0036–0.039	Kholodova et al. (2006)
Tibetan antelope	57	53	0.982–0.997	0.0186–0.0247	Ruan et al. (2005)
Waterbuck	63	21	0.3182–0.9286	0.0067–0.0358	Present study

Sample size (*N*), number of haplotypes (*Hap*), genetic diversity (*H*) and nucleotide diversity (π)

GenBank indicate that the sequences amplified in this study were authentic mtDNA, and not nuclear copies. In addition, no individuals appeared to be heteroplasmic.

Conclusions about genetic diversity based exclusively on mtDNA and samples not attributable to separate and/or unrelated individuals have proved problematic, especially because the mitochondria represent a single locus and it has predominantly maternal inheritance. However, as microsatellite markers are not always available for the target species, several studies have focused on the use of mtDNA. Therefore, a wide dataset is available for comparisons of genetic diversity between species. Molecular studies using non-invasive sampling clearly require the application of multi-locus approaches to provide individual identification due to the potential of distinct samples originating from the same individual. This should be the next step towards a molecular census of these bongo populations which would allow mtDNA haplotype frequencies to be reliably estimated.

However, comparison of our results with those from others studies of antelope species suggest that the genetic variation in bongo antelopes from Kenya is low (see Table 4). The analysis of 63 waterbuck dung samples obtained during collection from the same areas inhabited by bongo, using the same sampling methodology, revealed 21 haplotypes. Assuming that our estimates of individual sampling bias apply similarly to waterbuck and bongo samples, the comparatively low bongo mitochondrial diversity could be a cause for conservation concern. Further examination using microsatellite markers is required to resolve this issue and also to understand the population structure of this species and possible gene flow disruption due to habitat fragmentation. Nonetheless, the discovery of bongo DNA sequences in all four mountain areas and the provision of a reliable species discriminatory method for application in unsurveyed areas is a positive development in mountain bongo conservation. Molecular techniques can potentially provide crucial new data for the management of this elusive and charismatic antelope.

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