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SHORT COMMUNICATION

Genetic testing of dung identification for antelope surveys in the Udzungwa Mountains, Tanzania

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Abstract Dung counts are frequently employed to infer abundance of antelope species in African forests, but the accuracy of dung identification has rarely been tested. We used non-invasive genetic methods to test the accuracy of both field identification and morphometrics for identifying dung samples collected in the Udzungwa Mountains, Tanzania. Species identity was established by sequencing part of the mitochondrial control region from faecal DNA. Field identification was found to be correct in only 58–76% of cases depending on the observer. Discriminant analysis of dung pellet length correctly classified 80% of samples but a larger reference sample size is needed before using this method to classify dung of unknown origin. The results of this study illustrate the potential inaccuracy of dung counts as a monitoring tool for sympatric forest antelope species when the probability of correct identification is unknown. We recommend molecular testing of species identity during forest antelope surveys before conclusions are drawn on the basis of other identification methods.

Keywords Forest antelope · Duiker · Mitochondrial d-loop · Genetic identification · Eastern Arc

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Introduction

Monitoring rare and elusive mammal species often involves counting indirect field signs such as faeces (Putman 1984). Faecal deposits (dung or scats) are often more easily counted than live animals and can provide a wealth of further information (Kohn and Wayne 1997). However, monitoring methods using dung counts rely on being able to identify dung to the taxonomic level of interest. Studies explicitly testing the accuracy of field identification using genetic data have found highly variable results (Davison et al. 2002; Prugh and Ritland 2005).

African forest antelope are difficult to monitor due to their cryptic behaviour and preference for dense forest undergrowth (Bowkett et al. 2006). However, accurate monitoring programmes are of great value to conservation managers as these species play a major role in understorey ecology and are a large component of the bushmeat trade with many species threatened with extinction (East 1999; Eaves 2000). Dung counts are often used to infer population abundance in forest antelope (Koster and Hart 1988; Plumptre and Harris 1995; Rovero and Marshall 2004; Nielson 2006). van Vliet et al. (in press) compared genetic results with field identification of dung in Gabon and found that only one of six sympatric species could be reliably identified in the field. These authors strongly recommended genetic identification when conducting duiker dung counts and encouraged further research.

This study aims to use molecular methods to test the accuracy of field identification of antelope dung to species in the Udzungwa Mountains, Tanzania. We also investigate differences in faecal pellet size between species. The study has important conservation implications as dung counts have been used to infer marked differences in abundance of antelope species between differently managed forests within the Udzungwas (Nielson 2006) and national surveys are urgently required for the seriously threatened but poorly known Tanzanian endemic, Abbott's duiker *Cephalophus spadix* (Rovero et al. in press).

Methods

Sample collection and field identification

Dung samples were collected along systematic 0.5 km reconnaissance walks, as well as opportunistically, in the Mwanihana Forest, Udzungwa Mountains National Park, between October and December 2007. This period covers the onset of the short rainy season. This area supports a diverse forest antelope community including bushbuck *Tragelaphus scriptus*, suni *Neotragus moschatus*, blue duiker *C. monticola* and Harvey's duiker *C. harveyi* in addition to Abbott's duiker (Dinesen et al. 2001, Rovero et al. 2005). Fresh dung piles were identified by their shiny surface and damp, greenish interior.

Dung piles were assigned to species in the field by AEB (Observer 1), with the assistance of other members of the field team, based on size and shape. All field workers, while not experts, had worked in the Mwanihana Forest previously and were familiar with the antelope species present. Samples were also identified to species (mostly post-collection) by a second observer, Ruben Mwakisoma (Observer 2). RM has 20 years of experience in Udzungwa forests and was considered to be typical of a local expert that would take part in dung count surveys in this area.

Diagnostic differences in faecal pellet size would allow future identification of antelope dung without genetic testing. Therefore, twenty pellets were collected from each sampled dung pile and the length and width measured using calipers accurate to 0.01 mm. The length: width ratio was also calculated as a measure of difference in pellet shape. Pellet size was considered the most obvious morphological character to measure as it has been used previously to distinguish duiker species of different body size (Bowland and Perrin 1994; Rovero and Marshall 2004).

Genetic identification

Fresh dung samples were collected and stored in approximately 1.5 ml of the nucleic acid preservative RNAlater (Ambion Ltd, Huntington, UK). DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Crawley, UK).

Molecular identity of dung samples was established by sequencing a ~600 bp fragment of the left-hand domain of the mitochondrial control region (d-loop) using the primers: N777 For 5′-TACACTGGTCTTGTAAACC (modified from Hoelzel et al. 1991) and H16498 Rev 5′-CCTGAAGTAGGAACCAGATG (Shields and Kocher 1991). This region was chosen rather than the 12S region used by van Vliet et al. (in press) as it is has previously been used to identify antelope from dung (Pitra et al. 2006) and because we had access to a large reference set for *Cephalophus* species (Anthony, N. unpublished data). Standard PCR conditions were followed and products were sequenced using Big Dye chemistry (Applied Biosystems, Foster City, CA, USA) and analysed on an ABI 3100 automated sequencer.

Dung sequences were aligned against new and published sequences from known sources: Harvey's duiker (EMBL: AM903087-90), Abbott's duiker (AM903083-86), blue duiker (AM903091), suni (AJ235323) and two Tanzanian bushbuck subspecies (EF138291 and EF138320) from Moodley and Bruford (2007). Species identity was established by visual inspection of aligned sequences, using ClustalX (Thompson et al. 1997), and confirmed using the BLAST program (NCBI, Bethesda, MD, USA).

Pairwise genetic distances between samples were calculated using the Kimura 2-parameter model (Kimura 1980) with PAUP* (Swofford 2001). The resulting genetic distance matrix was then analysed using multi-dimensional scaling with SPSS 14.0 (SPSS Inc., Chicago, IL, USA) in order to illustrate the discrete clustering of sequences within species. Nine randomly selected dung samples (17% of total) were re-extracted and sequenced for a second time to verify that our results were repeatable (following Davison et al. 2002).

Data analysis

Post-hoc discriminant analysis was employed in SPSS to test if morphological data could be used to classify dung samples correctly to species. Fischer's Exact Probability tests, as modified for 2 × 3 and 2 × 4 tables (Freeman and Halton 1951), were used to compare the identification results from molecular testing with those from the two observers and discriminant analysis (Probability tests executed at http://faculty.vassar.edu/lowry/VassarStats.html).

Results and discussion

Twenty six fresh dung samples were collected on 27 'recce' walks with an encounter rate of 1.93 km^{-1}. An

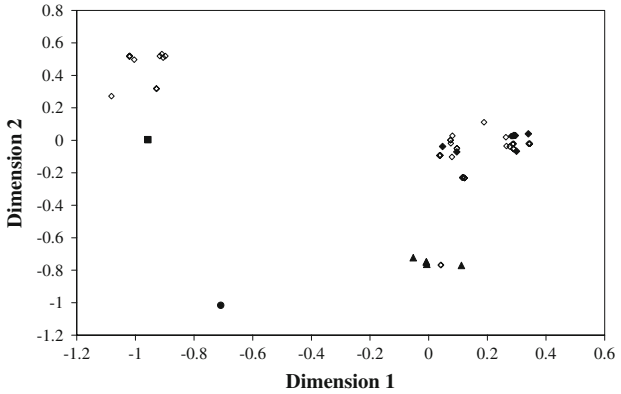

Fig. 1 Two dimensional MDS (multi-dimensional scaling) plot based on genetic distance (Kimura 2-parameter) between mitochondrial control region sequences from forest antelope found in the Mwanihana Forest. ◇ = sequences from dung of unknown origin, ◆ = *Cephalophus harveyi*, ▲ = *C. spadix*, ● = *C. monticola*, ■ = *Neotragus moschatus* (*Tragelaphus scriptus* not shown)

additional 31 samples were collected opportunistically and therefore randomly during fieldwork. The targeted mitochondrial control region was successfully sequenced for 54 samples (92%) with repeat extractions yielding identical sequences. Multi-dimensional scaling analysis reveals discrete clusters of dung sequences around known reference samples (Fig. 1). Harvey's duiker samples appear to cluster into two groups. However, both clusters include reference samples so, for the purposes of species designation, this variation is assumed to be intra-specific. This study shows that molecular testing provides an accurate method for identifying forest antelope dung for research and population surveys.

Table 1 shows the results from mtDNA compared to the other identification methods tested: field observation ($n = 54$) and pellet morphometrics ($n = 52$). Genetic results were significantly different from those of either observer (Fischer's Exact Test: Observer 1 [pooling *a priori* Abbott's and bushbuck], $P = 0.029$; Observer 2, $P > 0.001$). Direct identification in the field may vary between observers and, in this case, identification results were highly significantly different (Fischer's Exact Test: $P > 0.0001$). The most obvious difference here was the identification of Harvey's duiker dung as belonging to blue duiker by Observer 2. Contrary to previous reports (Dinesen et al. 2001), this species appears to be very scarce or possibly absent from Mwanihana Forest (Rovero et al. 2005, this study).

For other species, both observers had similar accuracy, a number of suni dung piles were misidentified due to the substantial overlap in pellet size with the larger-bodied Harvey's duiker. Similarly, van Vliet et al. (in press) found that the small-bodied blue duiker were frequently confused with the larger 'red duiker' species. Even the largest duiker species, such as Abbott's duiker, can still be confused with other forest antelope including bushbuck (this study) and, potentially, sitatunga *T. spekei* in central Africa (van Vliet et al. in press).

No diagnostic differences were found between Harvey's duiker and suni dung pellet measurements. Abbott's duiker pellets were larger than those of other species but sample size was too small to yield statistically meaningful results (Table 2).

Discriminant analysis was carried out for faecal pellet length as all pellet measurements were significantly intercorrelated ($r_s = 0.42$–0.55; $P < 0.005$) and this variable showed the greatest variation. Significant discrimination was found between species (Wilks' $\Lambda = 0.48$, $F_{2, 47} = 25.58$, $P < 0.001$) and 80% of samples were correctly classified (Table 1).

For the three species detected there was no significant difference between the results of the discriminant analysis and mtDNA (Fischer's Exact Test: $P = 0.215$). This

Table 1 Species identity of antelope dung piles from Mwanihana Forest, Tanzania, as recorded by two different observers and a discriminant analysis of faecal pellet length

Species	mtDNA	Observer 1	Observer 2	Discriminant analysis
Abbott's duiker	2	4 (50%)	5 (40%)	3 (67%)
Harvey's duiker	40	45 (80%)	31 (84%)	29 (97%)
Blue duiker	0	0	14 (0%)	NA
Suni	12	3 (100%)	2 (100%)	18 (57%)
Bushbuck	0	2 (0%)	0	NA
Total	54	54	52[a]	50[b]
Correct assignment (% of total)		41 (76%)	30 (58%)	40 (80%)

Species identity established with mitochondrial DNA (mtDNA) sequence data. Percentage of identifications matched with mtDNA results shown in brackets

[a] For two samples there were too few pellets following storage for identification by Observer 2

[b] For four samples there were too few pellets following storage for morphological measurements

Table 2 Faecal pellet measurements for forest antelope species as identified by mitochondrial DNA sequences

Species	Length (mm)	Width (mm)	L:W ratio
Abbott's duiker ($n = 2$)	13.59 (13.39–13.79)	9.02 (8.79–9.26)	1.51 (1.49–1.53)
Harvey's duiker ($n = 37$)	9.91 (7.54–12.28)	6.29 (4.87–8.27)	1.6 (0.98–1.95)
Suni ($n = 11$)	8.09 (6.48–10.23)	5.44 (4.81–6.13)	1.49 (1.26–1.76)

Values are the mean of n dung piles and the range of pile means (in brackets)

method shows promise for identifying forest antelope dung, particularly for the larger faecal pellets of Abbott's duiker. However, discriminant analysis cannot account for species not detected during sampling, such as bushbuck in this study. Therefore, larger sample sizes for all potential antelope species are required before using this method to identify unknown dung samples.

Although morphometrics are the primary means for identifying ungulate dung in the field (Chame 2003), other characteristics can also be valuable (e.g. Chapman 2004). We found that certain features were not useful for identifying duiker dung. For instance, counts of dung pellets per pile were not meaningful due to high rates of pellet removal by invertebrates. Additional factors, such as dung colour and smell, were considered too difficult to quantify under varied field conditions.

The results of this study raise doubts about the validity of dung counts as a monitoring tool for forest antelope in the Udzungwa Mountains and suggest caution should be taken when using this approach in any area with sympatric forest antelope species. This is because, as demonstrated here and in Gabon by van Vliet et al. (in press), there is no way to know *a priori* how reliable a designated 'expert' is going to be for dung identification and this problem is magnified if using multiple observers, as most monitoring programmes would need to do. However, to draw more robust conclusions as to the validity of dung counts for African forest antelope, a larger sample size of field observers would need to be tested.

In most cases it is desirable, or even critical, to be able to differentiate between antelope species during surveys. We recommend molecular testing of species identity for forest antelope dung counts to establish the probability of correct identification before conclusions are drawn on the basis of other identification methods.

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