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GENETIC PATTERNS IN FOREST ANTELOPE POPULATIONS IN THE UDZUNGWA MOUNTAINS, TANZANIA, AS INFERRED FROM NON-INVASIVE SAMPLING

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ABSTRACT

As for many tropical regions, the evolutionary and demographic status of antelope populations in the Udzungwa Mountains, Tanzania, are poorly resolved. We employed genetic information from 618 faecal samples to assess the status of forest antelope species in terms of their distribution, intraspecific diversity and population subdivision within the Udzungwa landscape. Most species were detected in the majority of forest fragments, except for *Philantomba monticola*. Phylogenetic analyses were consistent with traditional taxonomy with the exception of *Cephalophus harveyi* which was paraphyletic with respect to *C. natalensis*. There was strong support for three *C. harveyi* mtDNA clades within the Udzungwa Mountains although nuclear genetic variation did not partition strongly with these maternal lineages. Significant partitioning of genetic variation between sampling areas was detected for all species except the endangered *C. spadix*. Overall, our results demonstrate the value of non-invasive genetic sampling in studying the distribution and evolution of rarely observed species.

Keywords: duikers, suni, Harvey's duiker, Eastern Arc Mountains, East Africa

INTRODUCTION

Antelope constitute a major trophic guild in African forest and woodland ecosystems and play important roles as seed dispersal agents (Gautier-Hion *et al.*, 1980; Beaune *et al.*, 2013), understory browsers (Lunt, 2011) and as a food base for top predators (Boshoff *et al.*, 1994; Hayward *et al.*, 2006) and also human communities through the bushmeat trade (Nasi *et al.*, 2011). Identifying the products of evolutionary and demographic processes in forest antelope is therefore important for delineating appropriate conservation units, accurately documenting their role in ecosystems and predicting the ecological consequences of anthropogenic change. And yet forest antelope diversity is poorly defined at all levels from populations through to species and higher taxonomic ranks, particularly in comparison to savannah antelope (Du Toit & Cumming, 1999; Lorenzen *et al.*, 2012). This may seem surprising for relatively large mammal species but it could be argued that the diversity of forest-associated antelope was overlooked, if not obscured, by taxonomic revisions in the mid-twentieth century, such as Ellerman *et al.* (1953), with historical consequences which have only recently begun to be addressed by modern analyses (Colyn *et al.*, 2010; Groves & Grubb, 2011). At a local scale, forest antelope distributions and population dynamics are also poorly known primarily due to the difficulty of studying cryptic species in dense habitats leading to stark differences in how studies estimate demographic rates (van Vliet & Nasi, 2008).

Non-invasive genetic sampling from low-quantity DNA sources such as shed hair or faeces is potentially a highly informative tool for characterising populations in terms of taxonomy or conservation management (Kohn & Wayne, 1997; Beja-Pereira *et al.*, 2009). While ecological or behavioural data are often also required for robust conclusions regarding evolutionary or demographic status (Crandall *et al.*, 2000; Fraser & Bernatchez, 2001), non-invasive genetics provides information that would otherwise be very difficult or expensive to obtain.

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This study investigates genetic variation within forest antelope by non-invasive faecal DNA sampling across an Afrotropical mountain range, the Udzungwa Mountains in Tanzania. The Udzungwa Mountains ('the Udzungwas') are a high priority conservation landscape of global significance due to their remarkable levels of species diversity and endemism (Burgess *et al.*, 2007). This biodiversity, and associated ecosystem services, is under increasing threat from habitat fragmentation and extraction of natural resources, including hunting for bushmeat (Nielsen, 2006; Rovero *et al.*, 2010; Rovero *et al.*, 2012). Despite recent efforts to document the mammalian fauna (Rovero & De Luca, 2007; Rovero *et al.*, 2009), patterns of diversity within forest antelope species across the Udzungwas remain essentially unknown. We present the first multispecies genetic assessment and test predictions at three levels of antelope diversity.

The latest taxonomic treatment of hoofed mammals recognises 204 antelope species within the bovid subfamilies Bovinae and Antilopinae (Groves & Grubb, 2011), a substantial increase on the 92 species recognised by the World Conservation Union (IUCN, 2015). We test the prediction that forest antelope species are distributed uniformly throughout the major Udzungwa forested areas using genetic identification of faecal samples (Bowkett *et al.*, 2009). Regarding our genetic data we predict that mitochondrial haplotypes will be monophyletic with respect to other related species named by Groves & Grubb (2011) within those species groups (*i.e.* the traditionally recognised polytypic species).

Recognition of evolutionary diversity below the species level in antelopes has most often been attempted through the designation of subspecies. In some, but not all, cases this has led to separate conservation strategies for different subspecies, *e.g.* Pitra *et al.* (2006). Phylogeographic study of savannah antelopes has revealed regionally distinct evolutionary histories that in many cases are consistent with named subspecies (Lorenzen *et al.*, 2012). In contrast, very little is known about variation within forest antelopes and the apparent recent Pleistocene radiation of duiker species (Johnston & Anthony, 2012) brings into question at what point divergent lineages should be considered as species. We assess the genetic evidence for intraspecific diversity within the Udzungwas in the context of identifying Evolutionary Significant Units for conservation (Ryder, 1986; Fraser & Bernatchez, 2001). We predict that mitochondrial haplotypes will be monophyletic with respect to available conspecific sequences from other regions and we test representatives of strongly supported mitochondrial clades for significant genetic differentiation at nuclear loci *sensu* Moritz (1994).

Genetic data can be critical to identifying demographically independent populations within species, *e.g.* fish stocks (Moritz, 1994; Palsboll *et al.*, 2007). Management of such units may seek to conserve overall evolutionary potential by maintaining local adaptations but also has practical implications for achieving sustainable harvesting of populations – a situation highly relevant to forest antelope, many of which are hunted for bushmeat (van Vliet & Nasi, 2008). Conversely, managers may wish to identify populations that are not demographically or genetically viable without intervention in order to prioritise units for recovery. This may be the case where habitat fragmentation has resulted in population subdivision and reduced gene flow, *e.g.* Goossens *et al.* (2005). Relatively few studies have explored the genetic effects of fragmentation in African forests (Radespiel & Bruford, 2014), although examples include anthropoid apes (Bergl & Vigilant, 2006) and cloud forest birds (Callens *et al.*, 2011). Ruiz-Lopez *et al.* (2016) recently demonstrated that human habitat modification influences the genetic structure of colobus monkeys in the Udzungwas. We predict significant partitioning of forest antelope genetic variation across the Udzungwas and divergence in allele frequencies between discrete forest fragments.

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The intention of this study is to demonstrate the value of non-invasive genetic data by providing information that either conflicts with or supports our specific predictions concerning local populations and thereby identifies areas for further research or improves the evidence upon which management decisions are currently based. We do not seek to definitively resolve the taxonomy or phylogeography of those antelope species found in the Udzungwas as this would require range-wide information constituting multiple lines of evidence pertinent to the broader evolution of these taxa. Similarly, robust conclusions concerning meta-population dynamics would require greater sampling effort over a longer time period than feasible within this study. Nevertheless, the genetic patterns revealed by our non-invasive sampling, interpreted with the appropriate caveats, have important implications for forest antelope conservation in the Udzungwa Mountains.

MATERIAL AND METHODS

Study site and subjects

The Udzungwa Mountains are the southernmost and largest block of the Eastern Arc Mountains, a chain of ancient mountain ranges stretching from southern Kenya to south-central Tanzania. The mountains rise steeply from the Kilombero Valley on their eastern side (200–300 m) to the highest peaks (>2500 m). The Udzungwas encompass a total area of approximately 10 000 km^2 including 1600 km^2 of forest (Marshall *et al.*, 2010) representing the largest area of rainforest remaining in the Eastern Arc Mountains (Burgess *et al.*, 2007). Many of the main forests are within the Udzungwa Mountains National Park, together with the contiguous Kilombero Nature Reserve, although substantial forest patches outside of these areas are protected only nominally as Forest Reserves (figure1). The major forest fragments are described by Dinesen *et al*. (2001) and Marshall *et al*. (2010).

While it is possible that the Udzungwas were once covered by continuous forest this may have been disrupted by early human habitation (Rodgers, 1993) and has certainly been significantly impacted by humans for the last 100 years or more (Struhsaker *et al.*, 2004). Forest cover in the area had been reduced to 26 forest patches by the mid-1990s (Newmark, 1998). A habitat matrix of village agriculture, savannah woodland and extensive areas of fire-maintained grassland separates these forest fragments. The sampling sites included in this study include almost all the major forests in the central Udzungwas, an area of approximately 6500 km^2 excluding Image to the north (*ca*. 106 km^2) and fragmented forests on the Mufindi plateau to the west.

Rovero & De Luca (2007) list 12 species of antelope in the Udzungwas based on the taxonomy of Wilson & Reeder (2005). Their list includes three species only found in forest habitats: Abbott's duiker *Cephalophus spadix* "True, 1890" (figure 2), blue duiker *Philantomba monticola* (Thunberg, 1789) and suni *Neotragus moschatus* (Von Dueben, 1846) and two species that also occur in dry woodland: Harvey's duiker *Cephalophus harveyi* (Thomas, 1893) (figure 3) and bushbuck *Tragelaphus scriptus* (Pallas, 1776). The remaining species, including the grey or bush duiker *Sylvicapra grimmia* (Linnaeus, 1758), are almost entirely restricted to grassland and savannah woodland and are not considered further here.

The more recent taxonomy by Groves & Grubb (2011), and adopted by Wilson & Mittermeier (2011), splits three of the five traditionally recognised species found in the Udzungwas: *P. monticola* (into 10 species), *N. moschatus* (3 species) and *T. scriptus* (8 species). In all cases the new taxa were assigned names previously treated as subspecies or synonyms.

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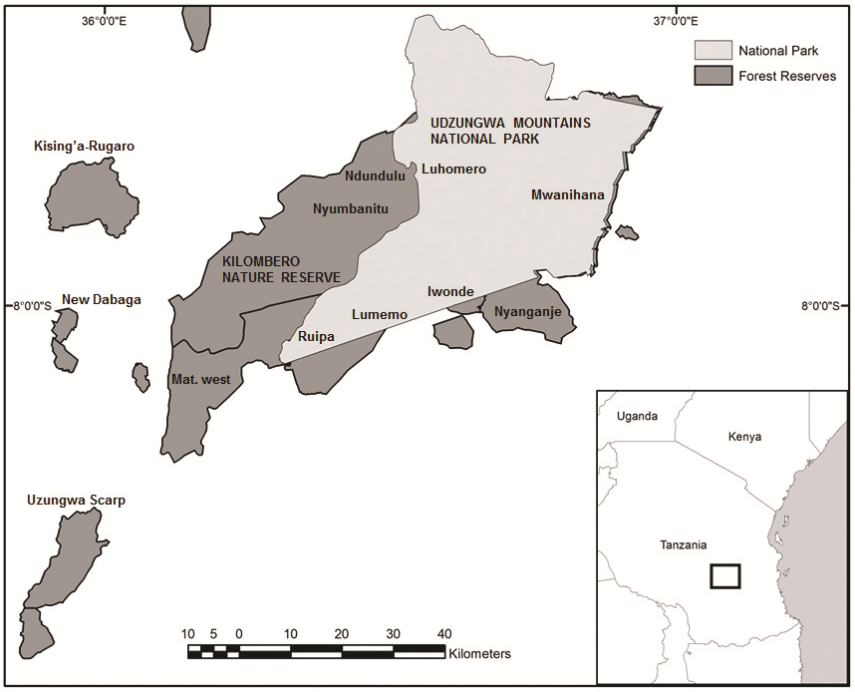

Figure 1. Protected areas in the Udzungwa Mountains, southern Tanzania. Non-invasive sampling locations for forest antelope are labelled in lower case.

Philantomba monticola within the Udzungwas are assigned to Tanzanian highland blue duiker, *P. lugens* (Thomas, 1898), together with those in the rest of the Eastern Arcs. The Southern Highlands and Kigoma district. *Neotragus moschatus* has been split into two species north of the Zambezi River, the nominate *moschatus* and *kirchenpaueri* (Pagenstecher, 1885), with a third species, *livingstonianus* (Kirk, 1865), to the south. *N. kirchenpaueri* is "not positively recorded" from the Udzungwas according to Groves & Grubb (2011), although given that *kirchenpaueri* is described from highland areas to the north and south we follow Wilson & Mittermeier (2011) in provisionally assigning Udzungwa populations to this species rather than *moschatus*. *T. scriptus* from central Tanzania, including the Udzungwas, are assigned to *T. sylvaticus* (Sparrman, 1780). As these species designations are provisional, we employ inverted commas when referring specifically to populations in the Udzungwas.

The two remaining species, *C. spadix* and *C. harveyi*, retain their previous mono-specific status under Groves & Grubb (2011). In contrast to other reference works, Groves & Grubb (2011) describe the southern limit of *harveyi* as north of the Udzungwas, although the area is included in the range map of Wilson & Mittermeier (2011) along with highlands in Malawi.

Sample collection and processing

We undertook transect-based surveys in all forest fragments in the central Udzungwas over 10 km^2, except for Iyondo, during the dry seasons between late 2006 and 2009 (figure 4 and 5). Total numbers of faecal samples collected from each location were as follows: Mwanihana (212); Luhomero (71); Ruipa (70); Uzungwa Scarp (56); New Dabaga-Ulang'ambi, hereinafter New Dabaga, (50); Lumemo (49); Iwonde (40); Nyanganje (34);

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Figure 2. Adult Abbott's duiker Cephalophus spadix*. Drawing by Jonathan Kingdon (Kingdon* et al., *2013)*.

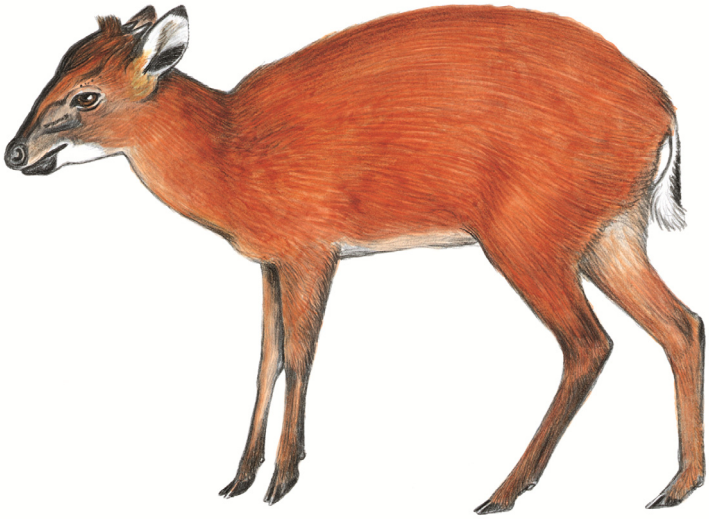

Figure 3. Harvey's duiker Cephalophus harveyi*. Drawing by Jonathan Kingdon (Kingdon* et al., *2013*).

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Ndundulu (27); Matundu West, hereinafter Mat. west, (16); Nyumbanitu (11); and Kising'a-Rugaro (3). In addition, thirteen tissue samples were opportunistically recovered from skins or dead animals found in poaching camps: Mwanihana (6), Luhomero (1), Uzungwa Scarp (2) and New Dabaga (4). Transects were walked in a triangular configuration (typically 1 km per side) and antelope samples were stored in RNAlater (Ambion Ltd, Huntington, UK). DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Crawley, UK) following the manufacturer's instructions but including an extended proteinase K digestion step of 20 to 30 minutes. For tissue samples we used the QIAGEN DNeasy Blood and Tissue Kit with overnight digestion for dried skin samples. Blank extractions were undertaken to control for contamination.

Genetic analysis

Antelope genetic diversity was characterised in the first instance using the mitochondrial control region (or D-loop), a highly variable non-coding marker that has been recommended for species identification in forest antelope (Ntie *et al.*, 2010a; Johnston *et al.*, 2011) and is often used in ungulate phylogeography (Lorenzen *et al.*, 2012). Primers and PCR conditions followed Ntie *et al.* (2010a). Sequences were edited in AutoAssembler (Applied Biosystems, Foster City, CA, USA) and aligned by MUSCLE (Edgar, 2004) in SEAVIEW (Gouy *et al.*, 2010). All unique haplotype sequences were submitted to GenBank/European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/LT220339-LT220454). Sequence entries can be identified by the sample names used in this study (listed under 'isolate').

For *Cephalophus* species we optimized a panel of nuclear microsatellite markers from those developed for central African species by Ntie *et al.* (2010b). Loci that amplified consistently for our target species were combined in three pre-PCR multiplexes: MPLX1 = INRA40 (Beja-Pereira *et al.*, 2004), BM1225, BM2113 and BRRIBO (Bishop *et al.*, 1994), MPLX2A = BM121 (Bishop *et al.*, 1994), and SR12 (Ntie *et al.*, 2010b; modified from Kogi *et al.*, 1995) and MPLX2B = BM143 (Bishop *et al.*, 1994) and INRA05 (Beja-Pereira *et al.*, 2004). MPLX2A and B were then combined for automated sequencer analysis. The majority of these loci failed to amplify consistently in the other antelope species tested. Therefore, microsatellite analysis for this study was restricted to *C. harveyi*, with the results for *C. spadix* published in Bowkett *et al.* (2014). Primers sequences and PCR conditions are given in appendix 1. Fragment analysis was undertaken on a Beckman Coulter capillary sequencer and allele sizes scored using CEQ 8000 software (Beckman Coulter, Fullerton, CA, USA).

Data quality

To check for consistency in sequencing from degraded DNA we re-extracted and re-sequenced just over 10% of all identified faecal samples. We also took a conservative approach to scoring microsatellite alleles, modified from Taberlet *et al.* (1996), whereby we only accepted heterozygous or homozygous genotypes at each locus if scored from three or four separate PCR reactions respectively. Negative PCR controls for both types of marker were used throughout.

Deviations from Hardy–Weinberg (HW) and linkage equilibria in the microsatellite dataset were tested for in GENEPOP 4.0.10 (Raymond & Rousett, 1995) with significance thresholds corrected for multiple tests using the False Discovery Rate (FDR) (Verhoeven *et al.*, 2005). The presence of null alleles, stutter and large allelic drop-out were tested for in MICROCHECKER (Van Oosterhout *et al.*, 2004).

Data analysis

Phylogenetic analysis of the control region haplotypes was employed to confirm species identification and explore intraspecific diversity. Separate analyses were carried out for each

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species. Trees were built using a maximum-likelihood approach in PhyML (Guindon & Gascuel, 2003) and a Bayesian Monte-Carlo Markov Chain (MCMC) approach in MrBayes 3.2 (Huelsenbeck & Ronquist, 2001). Trees included GenBank/EMBL entries for target taxa together with representative sequences for other genus members. For *Philantomba*, some published sequences contain a *ca*.100 bp insertion, as reported by Ntie *et al.* (2010a), and so were excluded. We were also able to sequence a number of tissue or faecal samples from outside of the study area including *C. natalensis* (Smith, 1834) from some other African countries (see appendix 2). Outgroup taxa from the sister genus to each of the species concerned, as presented in Hassanin *et al.* (2012), were used to root phylogenies, i.e. *Philantomba* for the *Cephalophus* trees, *Cephalophus* for the *Philantomba* tree and *Aepyceros* for the *Neotragus* tree.

Maximum-likelihood analysis (ML) was undertaken starting with an initial neighbour-joining tree and with default PhyML settings except for user-defined substitution models and parameters which were selected in jModelTest 0.1.1 (Posada, 2008), see table 1. The final choice of models was based on AIC values (all models were also ranked in the top three for AICc and BIC). The best tree topology was estimated from both NNI and SPR search options and branch-support was calculated with 1000 bootstrap repetitions.

Table 1. Best-fit evolutionary substitution models selected for Maximum-Likelihood analysis of forest antelope mitochondrial control region haplotypes from the Udzungwa Mountains, selected with jModelTest (Posada, 2008).

Species group	Model	Transition/ transversion ratio	Proportion of invariable sites	Gamma shape
C. harvey	HKY+I+G	16.38	0.33	0.64
C. spadix	TPM3uf+I+G	*	0.30	0.50
N. moschatus	HKY+I+G	11.55	0.41	0.44
P. monticola	TIM2+I+G	*	0.39	0.88

The Bayesian analysis implemented two Metropolis coupled MCMC searches each consisting of one cold and three heated chains. Instead of pre-selecting a substitution model we sampled across the entire general time reversible (GTR) model space (Huelsenbeck *et al.*, 2004) by averaging different models according to their posterior probability (*lset nst=mixed rates=gamma*) and checking for convergence across different runs with the *sump* command (Ronquist *et al.*, 2011). Convergence of MCMC runs was achieved by allowing the analysis to continue until the standard deviation of split frequencies reached the recommended threshold of 0.01 (between 1 and 1.5 million iterations in our case). The standard burn-in fraction of 25% was applied to the convergence diagnostic and the subsequent parameter and tree summaries. The *sump* command was used to check effective sample sizes, log-likelihood values and stationarity plots.

Phylogenetic relationships within *C. harveyi* were also explored using the median-joining algorithm in Network 4.6.1.0 (Fluxus Technology, Clare, UK). For the *C. harveyi* control region phylogeny we also calculated uncorrected genetic distances between major clades and other species in MEGA5 (Tamura *et al.*, 2011) including additional GenBank sequences (supplementary table 1). We also investigated microsatellite variation between samples grouped by their position in the mtDNA phylogeny (i.e. control region clades) with principal coordinates analysis in GenAlEx 6 (Peakall & Smouse, 2006).

Standard genetic diversity values were calculated in GenAlEx and Arlequin 3.5.1.2 (Excoffier *et al.*, 2005). To account for differences in number of samples per location we recalculated control region haplotype richness, microsatellite allelic richness and private allelic richness by rarefaction to the smallest sample size in Contrib (Petit *et al.*, 1998) and HP-Rare (Kalinowski, 2005).

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Partitioning of genetic variation within and amongst sampling locations was tested for using AMOVA, excluding sample sizes less than five. Pair-wise F_{ST} values between sampling locations were estimated with FDR correction applied to probability values. To test for an effect of isolation by distance, we carried out Mantel tests for the larger datasets ($>$ 5 sampling locations) in Arlequin.

RESULTS

We extracted DNA from 639 antelope faecal samples and 13 tissue samples from the Udzungwa Mountains, of which all but nine dung samples and one tissue sample yielded a sequence which could be assigned to one of the five traditionally recognised species. Samples that contained many ambiguous bases were excluded leaving a total of 618 Udzungwa sequence reads for genetic analysis plus sequences from other regions (appendix 2).

Distribution

T. 'sylvaticus' was only detected on six occasions during our study, three times in Ruipa and once in Iwonde, Lumemo, and New Dabaga. *C. harveyi* was recorded from all sampling locations and *C. spadix* and *N. 'kirchenpaueri'* from all but one each. *P. 'lugens'* had the most disparate distribution with no faecal DNA records from the central or eastern forests despite sampling effort being greatest in these areas (figure 4, table 2).

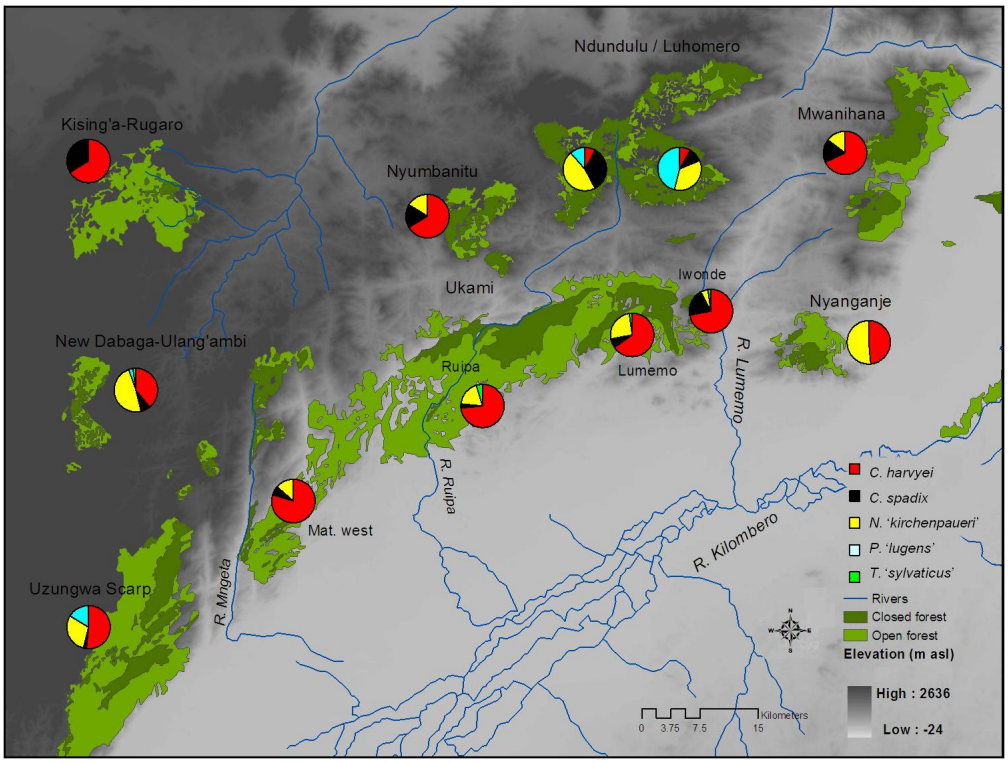

Figure 4 Forest antelope non-invasive sampling locations in the Udzungwa Mountains, Tanzania. Charts represent the proportion of samples assigned to each species (see table 2). See text for taxonomic notes.

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Table 2. Sample size (N), number of mitochondrial control region haplotypes, haplotypic richness and genetic diversity values for four species of antelope sampled in twelve locations in the Udzungwa Mountains, Tanzania. Haplotypic richness was rarefied to the smallest sample size (min. 5).

	Iw	Ki	Luh	Lum	Ma	Mw	Nd	Ne	Nya	Nyu	Ru	Uz	Total
C. harveyi													
N	28	2	6	30	12	137	1	21	16	4	47	29	334
Haplotypes	11	2	4	15	7	29	1	10	9	3	19	7	59
Haplotypic richness	3.740	-	3.000	3.959	2.999	4.009	-	3.811	3.857	-	4.257	2.827	23.922
Gene diversity	0.905	1	0.800	0.917	0.773	0.923	0	0.910	0.917	0.833	0.946	0.808	0.953
Nucleotide diversity	0.038	0.003	0.031	0.039	0.035	0.034	0	0.030	0.041	0.004	0.036	0.030	0.037
C. spadix													
N	8	1	8	3	1	35	9	2	0	1	2	1	73
Haplotypes	2	1	3	2	1	4	1	2	-	1	2	1	6
Haplotypic richness	1	-	2	-	-	1.049	0	-	-	-	-	NA	4.266
Gene diversity	0.429	1	0.464	0.667	1	0.266	0	1	-	1	1	1.000	0.381
Nucleotide diversity	0.010	0	0.010	0.001	0	0.003	0	0.017	-	0	0.017	0.000	0.006
N. 'kirchenpaueri'													
N	2	0	26	12	2	30	12	26	17	1	12	17	157
Haplotypes	2	-	7	6	2	8	5	5	5	1	6	7	28
Haplotypic richness	NA	-	3.639	5	-	4.82	4	2.63	3.676	-	5	4.794	16.366
Gene diversity	1	-	0.649	0.818	1	0.837	0.788	0.582	0.809	1	0.849	0.582	0.922
Nucleotide diversity	0.044	-	0.025	0.036	0.0178	0.033	0.029	0.010	0.043	0	0.038	0.010	0.039
P. 'lugens'													
N	0	0	34	0	0	0	3	2	0	0	0	9	48
Haplotypes	-	-	9	-	-	-	3	2	-	-	-	3	13
Haplotypic richness	-	-	1.594	-	-	-	2	-	-	-	-	1.226	12
Gene diversity	-	-	0.857	-	-	-	1	1	-	-	-	0.722	0.905
Nucleotide diversity	-	-	0.019	-	-	-	0.021	0.032	-	-	-	0.033	0.026

Key to forests: Iw = Iwonde, Ki = Kising'a-Rugaro, Luh = Luhomero, Lum = Lumemo, Ma =Mat. west, Mw = Mwanihana, Nd = Ndundulu, Ne = New Dabaga, Nya = Nyanganje, Nyu = Nyumbanitu, Ru = Ruipa, Uz = Uzungwa Scarp

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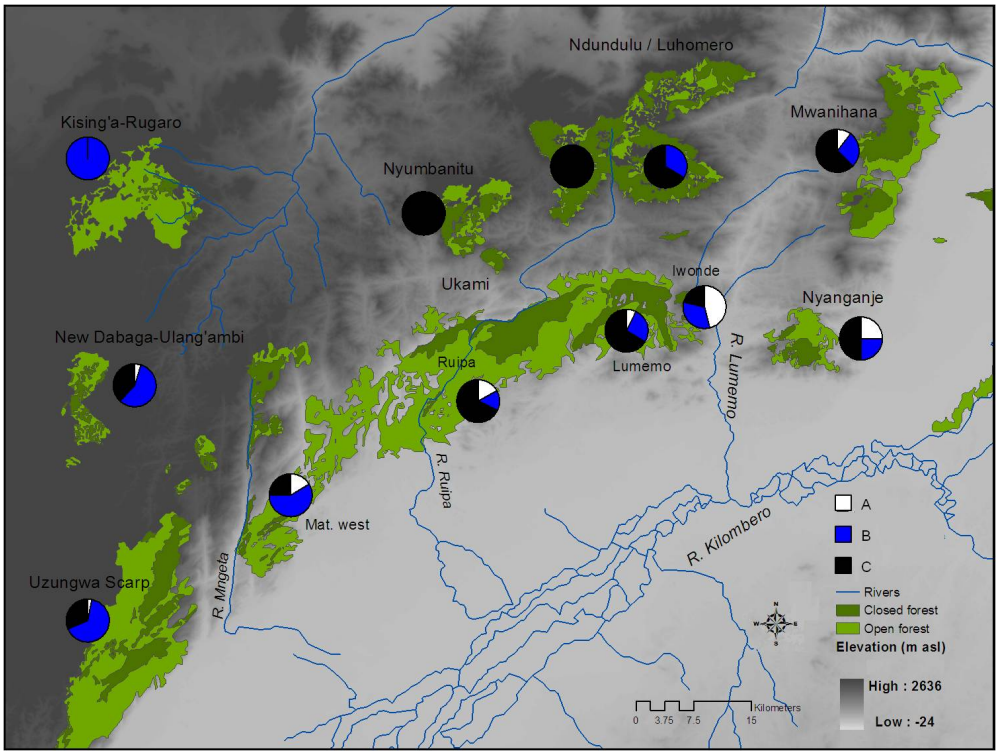

Figure 5. Forest antelope non-invasive sampling locations in the Udzungwa Mountains, Tanzania. Charts represent the proportion of major mtDNA control region phylogenetic clades in C. harveyi *samples (Figure 6a;* N = *334).*

Phylogenetic analysis

A total of 111 control region haplotypes were recovered from Udzungwa faecal and tissue samples. Sixty of 68 re-extracted faecal samples yielded identical sequences, while five failed to amplify target DNA on repeat and three samples had different sequences due to a mislabelling error that was subsequently corrected. We did not conduct phylogenetic analysis for *T. 'sylvaticus'* as we only recovered five haplotypes and an extensive CR phylogeny for this species group has been published previously (Moodley *et al.*, 2009). However, we note that our sequences were highly similar to haplotypes from the *sylvaticus* cluster, as opposed to *scriptus*, in BLAST (NCBI, Bethesda, MD, USA).

In comparison to published multi-locus phylogenies (Johnston & Anthony, 2012), Bayesian analysis recovered inter-species relationships more accurately than Maximum-Likelihood. Therefore, Bayesian trees are presented with bootstrap values (BS) for nodes supported by ML. Posterior probability values (PP) below 0.5 (or bootstraps below 50%) are not shown and some node values near terminal ends were omitted for clarity.

Three of the four species were potentially monophyletic with respect to other sequences (figure 6a, b, c, d). For *P. 'lugens'* and *N. 'kirchenpaueri'* this pattern is uncertain as the more strongly supported clades also included sequences from elsewhere in Tanzania but because precise localities are unknown these sequences could potentially originate from other taxa

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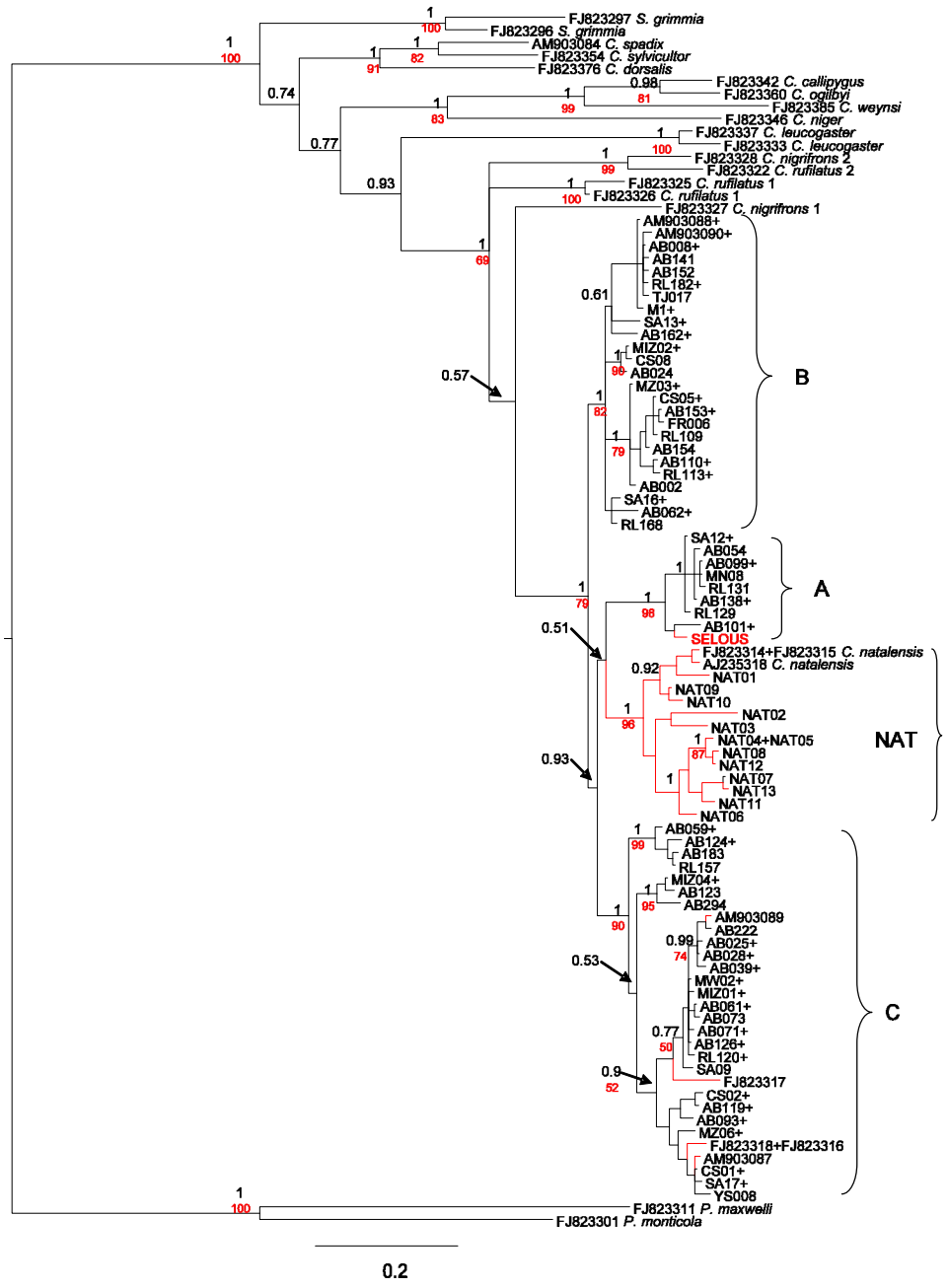

Figure 6a. Bayesian consensus phylogeny for Cephalophus harveyi *CR haplotypes from the Udzungwa Mountains, with representative sequences from other regions and species. Node support values are posterior probabilities (black) and bootstrap percentages (1000 iterations) from Maximum-Likelihood analysis (red).* C. harveyi *clades are labelled A–C with non-Tanzanian natalensis labelled NAT. Red clades represent haplotypes from outside of the Udzungwas. GenBank accession numbers and species names are given for previously published sequences.*

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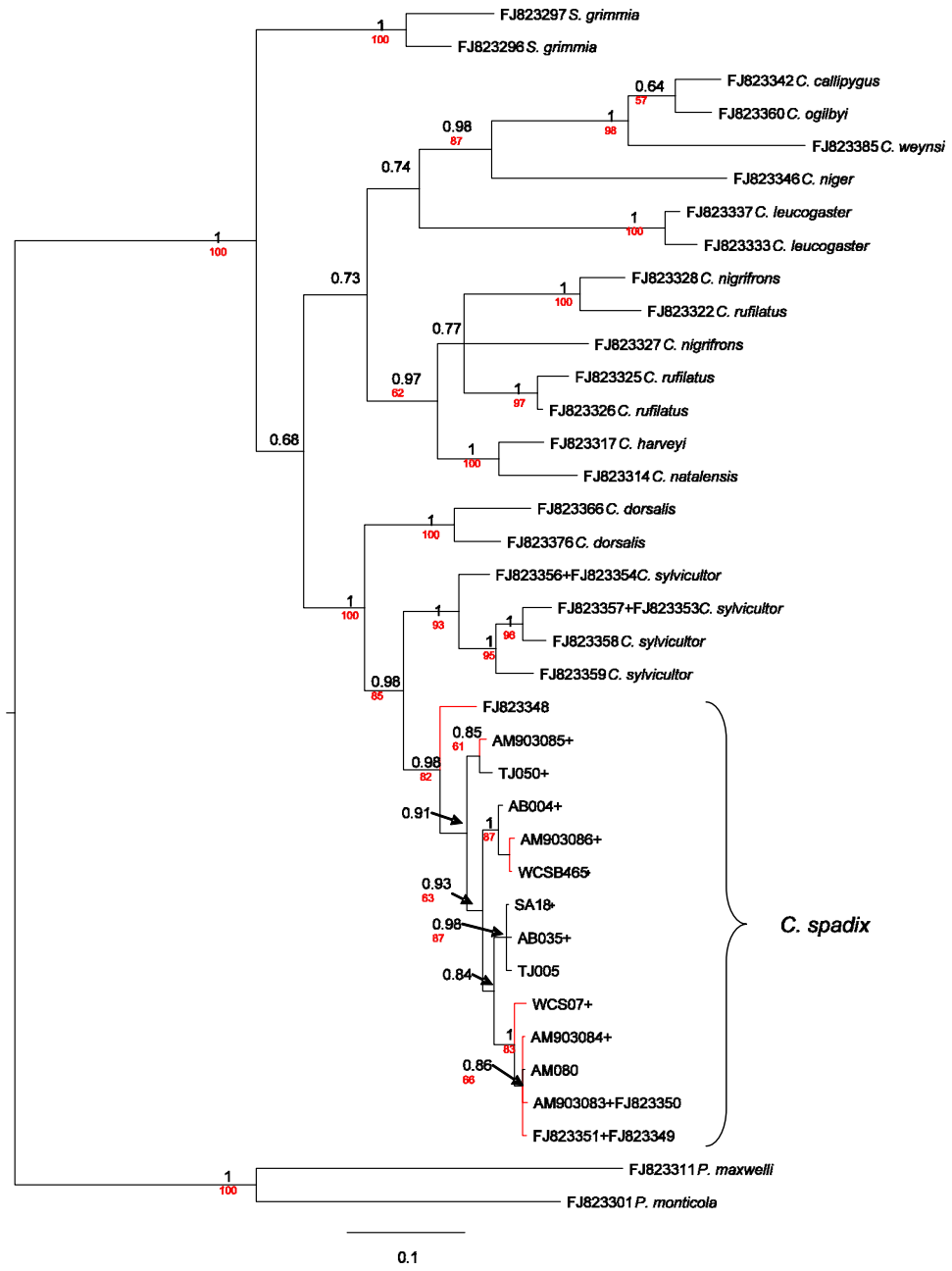

Figure 6b. Bayesian consensus phylogeny for Cephalophus spadix *CR haplotypes from the Udzungwa Mountains, with representative sequences from other regions and species. Node support values are posterior probabilities (black) and bootstrap percentages (1000 iterations) from Maximum-Likelihood analysis (red). Red clades represent* spadix *haplotypes from outside of the Udzungwas. GenBank accession numbers and species names are given for previously published sequences.*

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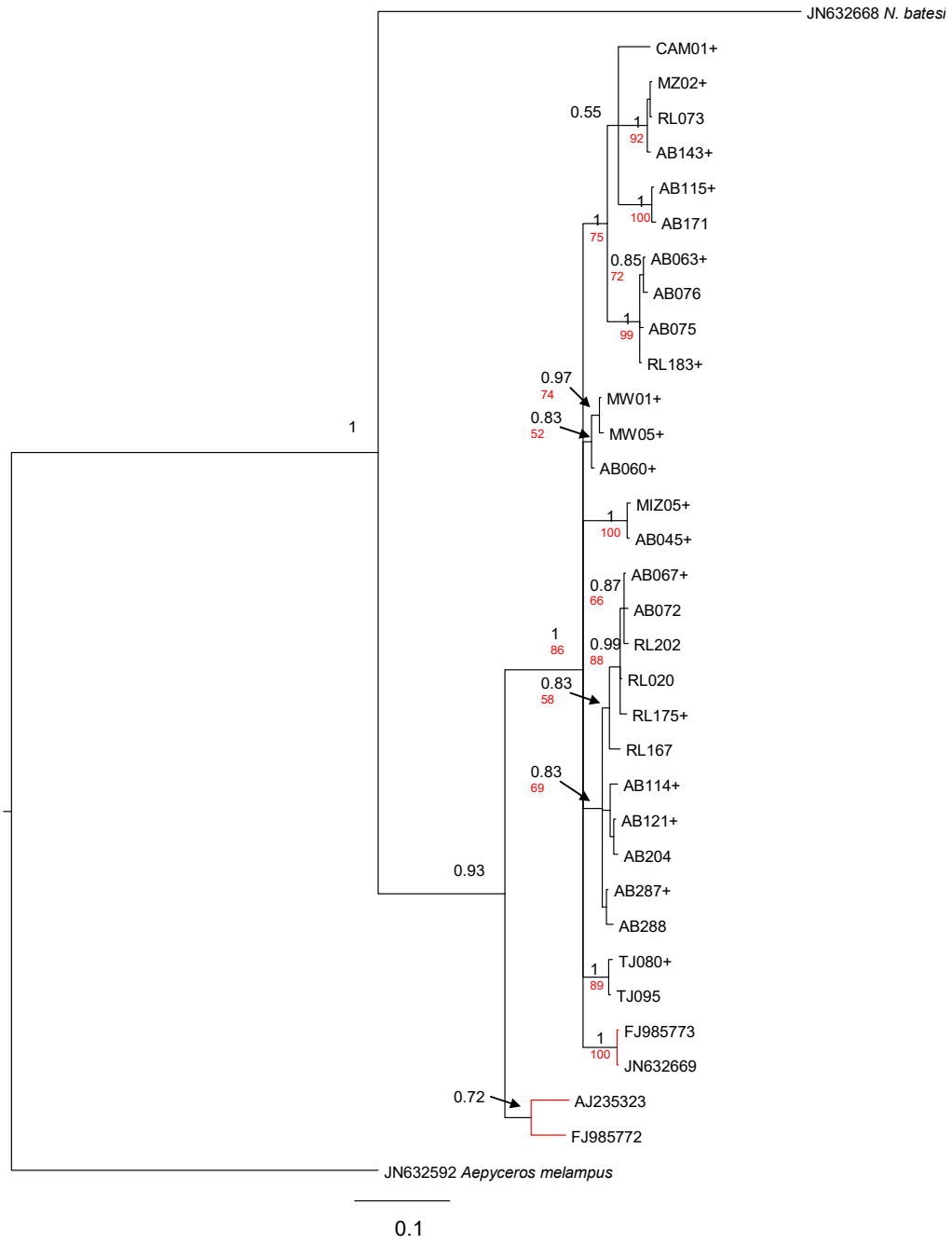

Figure 6c. Bayesian consensus phylogeny for putative Neotragus kirchenpauerii *CR haplotypes from the Udzungwa Mountains, with representative sequences from other regions and species. Node support values are posterior probabilities (black) and bootstrap percentages (1000 iterations) from Maximum-Likelihood analysis (red). Red clades represent suni haplotypes from outside of the Udzungwas. GenBank accession numbers and species names are given for previously published sequences.*

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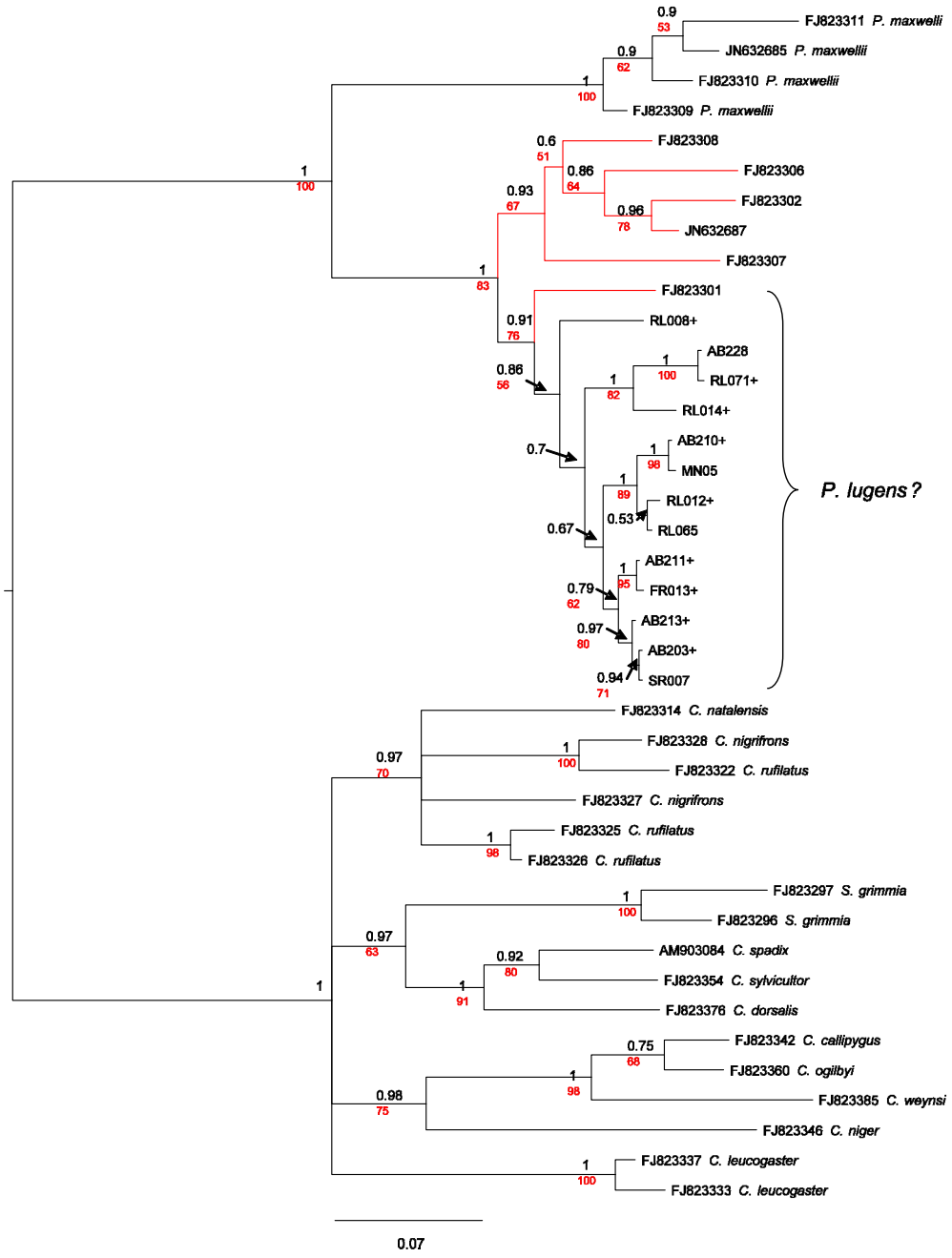

Figure 6d. Bayesian consensus phylogeny for putative Philantomba lugens *CR haplotypes from the Udzungwa Mountains, with representative sequences from other regions and species. Node support values are posterior probabilities (black) and bootstrap percentages (1000 iterations) from Maximum-Likelihood analysis (red). Red clades represent 'P. monticola' haplotypes from outside of the Udzungwas. GenBank accession numbers and species names are given for previously published sequences.*

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also elevated to species by Groves & Grubb (2011). Reciprocal monophyly was strongly supported for *C. spadix*, as was a sister relationship to *C. sylvicultor* (figure 6b).

In contrast, *C. harveyi* was paraphyletic with respect to *C. natalensis* (figure 6a). *C. harveyi* haplotypes from the Udzungwas fell into three strongly supported clades (figure 6a clades A–C; PP = 1, BS = 82–98%; figure 5), two of which appeared closer to *C. natalensis* than the third, although this deeper node was not supported by the ML tree. All three clades were recovered from the majority of sampling locations with the only exceptions being locations with minimal sampling (figure 5; table 2). Clade B was monophyletic with respect to sequences outside of the Udzungwas as were Udzungwa *P. 'lugens'* haplotypes although with less support (PP = 0.86, BS = 56%). Uncorrected genetic distances between clades within *C. harveyi* were equivalent to those between *C. harveyi* clades and *C. natalensis* (4.5–6.3%) but less than inter-species values for other red duiker (table 4).

The median-joining haplotype network for *C. harveyi* was largely congruent with the tree-based analyses although this approach highlights the long branch-lengths typically connecting haplotypes from other regions to those in the Udzungwas (figure 7a). The one sample from the Selous Game Reserve, to the east of the Udzungwas and mapped as part of the range of *C. natalensis* in East (1999), clustered closely with Clade A in both tree and network analyses (figure 6a and 7a).

Microsatellite variation did not partition strongly between mitochondrial clades in the Udzungwas for *C. harveyi* (AMOVA: Among clade variation 0.67%, within clade variation 99.33%, global F_{ST} = 0.007, P = 0.047) as illustrated by a plot of principal components (figure 7b). Therefore, we conclude that these lineages do not fulfil Moritz (1994)'s criteria for Evolutionary Significant Units whereby mitochondrial monophyletic groups should also exhibit corresponding significant divergence at nuclear loci.

Genetic diversity and differentiation

All eight microsatellite loci were polymorphic in *C. harveyi* with three loci deviating from Hardy-Weinberg expectations following FDR correction (BRRIBO, BM143 and INRA05). Furthermore, three loci exhibited homozygote excess indicating the potential presence of null alleles (BM2113, BRRIBO and BM143). There was no evidence of significant linkage disequilibrium within the data set.

Genetic diversity differed markedly between species with the most notable result being the low values for *C. spadix*. mtDNA results for *C. spadix* were typically less than half than those of other species (table 2). Analysis of genetic diversity within sampling locations was confounded by small sample size particularly for the more rarely encountered species. However, most locations had similar results within species with no obvious outliers (table 2 and 3).

Small sample sizes reduced the number of pair-wise F_{ST} estimates for *C. spadix* to six (F_{ST} = 0–0.16; P > 0.05) and one for *P. 'lugens'* (Luhomero *vs.* Uzungwa Scarp, F_{ST} = 0.37; P < 0.001). Most comparisons were statistically significant for *N. 'kirchenpaueri'* as were half for *C. harveyi* (table 5). For microsatellite loci, all but two significant comparisons for *C. harveyi* involved New Dabaga or Uzungwa Scarp (table 5). AMOVA results were highly significant for all species except *C. spadix* indicating potential population subdivision (table 6).

There was moderate support for isolation by distance across sampling locations for the control region: *N. 'kirchenpaueri'* (r = 0.47; P = 0.04) and *C. harveyi* (r = 0.25; P = 0.08) but a strong signal for microsatellite variation in *C. harveyi* (r = 0.54; P < 0.001).

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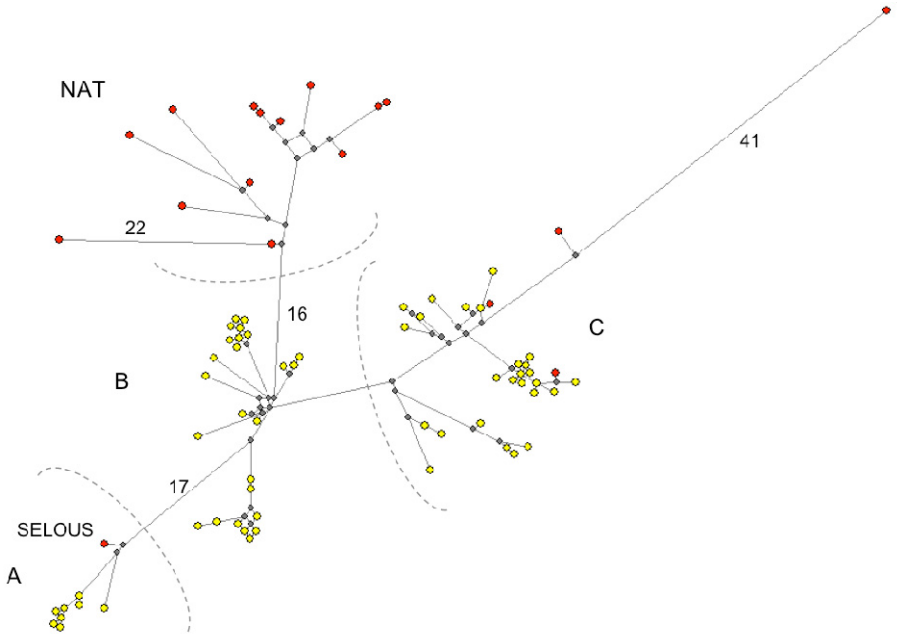
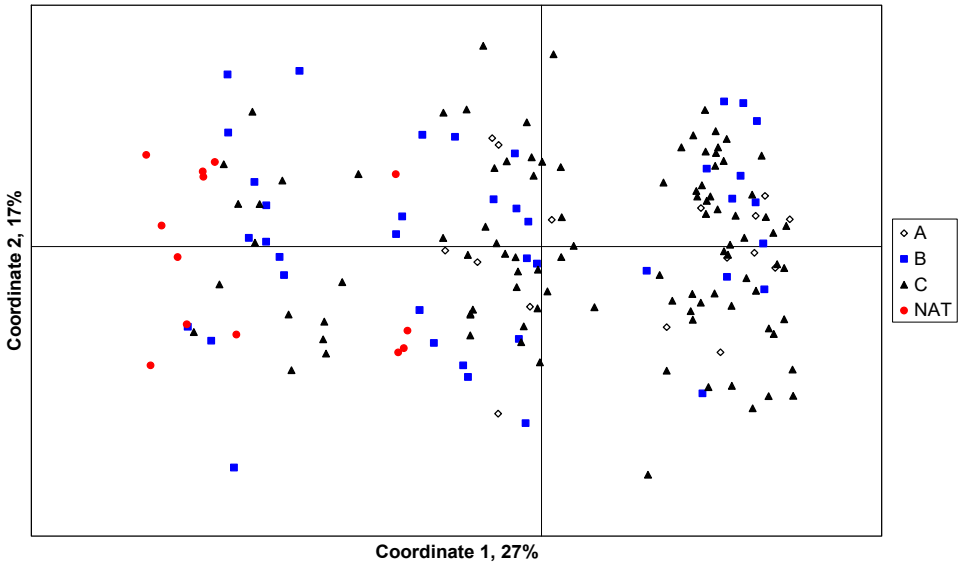


(a)



(b)

Figure 7. Genetic distance analyses for C. harveyi *in the Udzungwa Mountains and sister species* C. natalensis *(a) mtDNA control region median-joining network. Yellow nodes indicate haplotypes recovered from the Udzungwa Mountains, Tanzania, red nodes indicate haplotypes from elsewhere and grey nodes are inferred mutational steps. Long branches are labelled with the number of mutational steps. Major clades are labelled as in figure 6a; (b) two dimensional PCA plot of* C. harveyi / natalensis *microsatellite genotypes labelled by phylogenetic clade as in figure 6a.*

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Table 3. Sample size, polymorphism, unbiased expected heterozygosity (H_E) and allelic richness for C. harveyi *sampled in nine locations in the Udzungwa Mountains, Tanzania.*

	Iwonde	Luhomero	Lumemo	Mat. west	Mwanihana	New Dabaga	Nyanganje	Ruipa	Uzungwa Scarp	Total#
C. harveyi										
N	8	2	20	6	49	10	8	34	12	149
Polymorphic loci	8	8	8	7	8	8	7	8	8	8
Unbiased H_E	0.627	0.729	0.570	0.663	0.609	0.613	0.614	0.611	0.653	0.635
Allelic richness	4.875	2.625	6.500	4.375	7.250	5.250	4.500	6.875	4.500	8.375
Rarefied allelic richness*	4.05	-	3.82	4.07	3.86	3.97	3.77	3.83	3.6	-
Private allelic richness	0	0	0	0	0.13	0	0	0.25	0.13	-
Rarefied private allelic richness*	0.21	-	0.12	0.1	0.12	0.27	0.19	0.09	0.19	-

** Allelic richness values calculated with rarefaction to the smallest sample size for locations where* N > 5.

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Table 4. Pair-wise mitochondrial control region uncorrected genetic distances between red duiker clades and species. Values along the diagonal (in bold) are mean within-clade distances. Within-species clades (A-C; 1-2) are labelled on figure 4a. Number of haplotypes is given in parentheses.

	1	2	3	4	5	6	7	8	9	10
1 *C. harveyi* A(9)	**0.012**									
2 *C. harveyi* B (25)	0.051	**0.017**								
3 *C. harveyi* C (30)	0.063	0.048	**0.024**							
4 *C. natalensis* (14)	0.055	0.045	0.056	**0.021**						
5 *C. nigrifrons* 1 (2)	0.097	0.088	0.095	0.093	**0.000**					
6 *C. nigrifrons* 2 (2)	0.104	0.086	0.100	0.105	0.100	**0.000**				
7 *C. rufilatus* 1 (5)	0.080	0.083	0.093	0.079	0.079	0.087	**0.011**			
8 *C. rufilatus* 2 (2)	0.127	0.108	0.116	0.118	0.102	0.050	0.099	**0.000**		
9 *C. leucogaster* (4)	0.133	0.131	0.150	0.136	0.144	0.127	0.110	0.144	**0.022**	
10 *C. callipygus* (6)	0.146	0.154	0.148	0.148	0.145	0.157	0.140	0.173	0.137	**0.058**

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Table 5. Pair-wise F_{ST} values between sampling locations for two species of antelope in the Udzungwa Mountains, Tanzania. Values below the diagonal are for mtDNA control region and above for eight microsatellite loci (the latter not used for N. 'kirchenpaueri'*).*

	Iwonde	Luhomero	Lumemo	Mat. west	Mwani-hana	Ndundulu	New Dabaga	Nyanganje	Ruipa	Uzungwa Scarp
C. harveyi										
Iwonde		-	-0.002	0.019	0.024	-	0.099*	0.025	0.003	0.095*
Luhomero	0.200*		-	-	-	-	-	-	-	-
Lumemo	0.162*	-0.007		0.024	0.019*	-	0.118*	0.021	0.006	0.095*
Mat.west	0.126*	0.132	0.099*		0.019	-	0.066*	0.026	0.007	0.099
Mwanihana	0.160*	-0.008	0.000	0.117*		-	0.104*	0.001	0.028*	0.094*
Ndundulu	-	-	-	-	-		-	-	-	-
New Dabaga	0.204*	0.079	0.078*	-0.034	0.090*	-		0.086*	0.081*	0.065*
Nyanganje	0.050	0.032	0.007	0.076	0.010	-	0.103*		0.025	0.070*
Ruipa	0.155*	-0.010	0.003	0.121*	0.009	-	0.100*	0.015		0.069*
Uzungwa Scarp	0.223*	0.125	0.119*	-0.012	0.126*	-	-0.009	0.129*	0.146*	
N. 'kirchenpaueri'										
Iwonde										
Luhomero	-									
Lumemo	-	0.250*								
Mat.west	-	-	-							
Mwanihana	-	0.149*	0.253*	-						
Ndundulu	-	0.274*	0.309*	-	0.153*					
New Dabaga	-	0.558*	0.576*	-	0.459*	0.515*				
Nyanganje	-	0.230*	0.049	-	0.073*	0.190*	0.466*			
Ruipa	-	0.166*	-0.010	-	0.203*	0.249*	0.492*	0.058*		
Uzungwa Scarp	-	0.342*	0.291*	-	0.238*	0.328*	0.454*	0.206*	0.245*	

** Significant at α = 0.05 level after correction for False Discovery Rate.*

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Table 6. Analyses of molecular variance among and within sampling locations for four species of forest antelope in the Udzungwa Mountains, Tanzania.

	Control Region				Microsatellites
	C. harveyi	*C. spadix*	*N. 'kirchenpaueri'*	*P. 'lugens'*	*C. harveyi*
Sampling locations	9	4	8	2	8
Among population variation	8.02	4.92	29.36	36.62	4.26
Within population variation	91.98	95.08	70.64	63.38	95.74
Global F_{ST}	0.08	0.05	0.29	0.37	0.04
P *	< 0.001	0.12	< 0.001	< 0.001	< 0.001

** Probability values calculated with 1023 permutations*

DISCUSSION

Antelope taxonomy and species distribution

Our results are largely consistent with other published phylogenies (Jansen van Vuuren & Robinson, 2001; Hassanin *et al.*, 2012; Johnston & Anthony, 2012) in recovering major monophyletic species groups including the giant duikers [*C. spadix*, *sylvicultor* (Afzelius, 1815) and *dorsalis* Gray, 1846], dwarf duikers [*P. maxwelli* (C.H.Smith, 1827) and *monticola* including *lugens*], West African red duikers [*C. callipygus* Peters, 1876, *ogilbyi* (Waterhouse, 1838), *weynsi* Thomas, 1901 and *niger* Gray, 1846] and East African red duikers (*C. natalensis*, *harveyi*, *nigrifrons* Gray, 1871 and *rufilatus* Gray, 1846). Placement of *C. leucogaster* Gray, 1873, normally allied to the East African red duikers (Johnston & Anthony, 2012), was inconsistent in our trees. There was also only weak support for *S. grimmia* being sister to all other duiker species or just to *Cephalophus* (figure 6a, b, d). In fact, both Hassanin *et al.* (2012) and Johnston & Anthony (2012) place *Sylvicapra* as sister to the giant duiker clade making *Cephalophus* paraphyletic (figure 6d). Systematic resolution of this issue requires including *grimmia* in *Cephalophus* or splitting the genus into at least three groups with the name *Cephalophura* suggested for the red duiker clade by Hassanin *et al.* (2012).

At the species level, we have provisionally classified forest antelope in the Udzungwas according to the taxonomic revision of Groves & Grubb (2011). The name changes for three species reflect the tendency of these authors to split geographically widespread morphologically variable taxa, an approach which has proven controversial (Heller *et al.*, 2013; Zachos *et al.*, 2013; Cotterill *et al.*, 2014). In many cases, Groves & Grubb (2011) admit that a robust classification still requires more research and this may be the case for *P. monticola* and *N. moschatus* where populations have been separated based largely on pelage characters. Our genetic results are clearly not suitable for establishing the phylogenetic status of these putative species although they did not contradict our prediction that such taxa would be monophyletic at the mtDNA locus tested. Much more extensive sampling is needed to document morphological and genetic variation across the ranges of these proposed taxa.

The most interesting phylogenetic result was the mitochondrial paraphyly of *C. harveyi* with respect to *C. natalensis* (figure 6a). Although this has been reported before, based on a small number of sequences including nuclear loci (Ntie *et al.*, 2010a; Johnston & Anthony, 2012), the present study represents the most extensive genetic assessment of these species to date. Several authors have treated *C. harveyi* as a subspecies of *C. natalensis* (Ellerman *et al.*, 1953) or interpreted narrow genetic distance as support for this proposition (Jansen van

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Vuuren & Robinson, 2001; Hassanin *et al.*, 2012). However, differences between the two taxa in body size and coat pattern have led other authors and the IUCN Antelope Specialist Group to continue to recognise them as separate species (Kingdon, 1997; East, 1999; Groves & Grubb, 2011). Kingdon (1982) suggested that the two forms hybridise in southern Tanzania but, crucially, we lack samples from south and east of the Udzungwas except for the single Selous sample that grouped with *harveyi* clade A (figure 6a and 7a). Given the geographical and genetic evidence, it appears that the situation may be more complicated than a simple choice between one or two species or subspecies (see below).

Our prediction that these species of forest antelope would be distributed uniformly across the forested Udzungwas was fulfilled for *C. harveyi* and nearly so for *N. 'kirchenpaueri'* and *C. spadix* which were detected in all but one forest each. In the case of *N. 'kirchenpaueri'*, this is almost certainly a false absence due to under-sampling in Nyumbanitu, whereas the genuine absence of *C. spadix* from Nyanganje is possible. The exception to this pattern is *P. 'lugens'* which is almost certainly absent from Mwanihana, *contra* to earlier reports (Dinesen *et al.*, 2001), but may exist at low densities in other forests even though not detected here. This species not only went unrecorded during this study but also during intensive camera-trap surveys of the same areas (Rovero & Marshall, 2009). There is no obvious explanation for the patchy distribution of this duiker in the Udzungwas as the habitat and anthropogenic threats appear similar and blue duiker coexist with suni in the western Udzungwas as well as in east African coastal forests (Fitzgibbon *et al.*, 1995). Interestingly, there are other forest mammals that have highly localised distributions within the Udzungwas (Davenport *et al.*, 2008; Rovero *et al.*, 2008).

Intraspecific lineage diversity in antelope

There was little evidence for forest antelope populations in the Udzungwas constituting potential Evolutionary Significant Units. Udzungwa haplotypes did not form exclusive monophyletic clades, except for *P. 'lugens'* (weak support, figure 6d) and Clade B in *C. harveyi* (see below). We interpret this to mean that for the majority of species gene flow with other regions is ongoing or if populations are now isolated they have yet to undergo complete lineage sorting.

The lineage diversity within *C. harveyi* is more puzzling. On the one hand, the three strongly supported clades within the Udzungwas are as different from one another as they are to *C. natalensis* samples from a wide geographic area and exhibit equivalent genetic variation (table 5). On the other, the geographic and nuclear genetic data do not indicate any separation of individuals from different clades into different populations (or Evolutionary Significant Units).

Unexpected diversity in mitochondrial DNA can be caused by a variety of processes including inadvertent amplification of mitochondrial pseudo-genes from the nuclear genome (Numts), hybridisation with other species or incomplete sorting of ancestral lineages. Given the limited nature of our dataset we cannot rule out any of these explanations. However, in light of the climatic history of Africa (DeMenocal, 2004) and the radiation of *Cephalophus* during the Pleistocene (Johnston & Anthony, 2012) we speculate that an ancestral red duiker lineage may have diverged whilst populations were isolated during arid inter-pluvial periods and repeatedly undergone secondary contact in the Udzungwas (and elsewhere) when a wetter climate allowed forest refugia to reconnect. This is the opposite pattern to that shown by savannah antelope species (Lorenzen *et al.*, 2012). This secondary contact resulted in contemporary inter-breeding populations that show no segregation at microsatellite loci but retain the mitochondrial (and perhaps nuclear intron) signature of past isolation. This hypothesis requires testing with multi-

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locus data from throughout the range of *C. harveyi* and related species together with coalescent dating of lineage divergence, as in Bowie *et al.* (2006).

Genetic diversity and differentiation

Pair-wise comparisons and significant partitioning of molecular variation among sampling locations indicate substantial population subdivision in the Udzungwas at least for the two most sampled species (table 5 and 6). As the majority of sampling locations represent discrete forest patches this potential structure could be interpreted as reduced gene flow across unsuitable habitat and implicate deforestation as a threat to population persistence. Indeed, the majority of significant F_{ST} values for the *C. harveyi* microsatellite dataset involved the relatively isolated forests of Uzungwa Scarp and New Dabaga, which therefore stand out as priorities for conservation.

However, we also found a moderate-to-strong effect of isolation by distance, which may complicate interpretation of spatial genetic structure (Safner *et al.*, 2011). In our case, we note that many pair-wise F_{ST} values were highly significant over relatively short distances and that most comparisons between sampling locations within continuous forest were not significant (*e.g.* Lumemo and Ruipa). Therefore, we conclude that there is a likely effect of habitat fragmentation, in addition to isolation by distance, operating on forest antelope population structure.

Despite the potential impact of population subdivision, we found little evidence for negative effects on genetic diversity. Diversity values were generally high and microsatellite heterozygosity in *C. harveyi* was very similar to the mean for healthy bovid populations (Garner *et al.*, 2005). The stark exception to this pattern was the endangered Tanzanian endemic *C. spadix* for which we consistently estimated the lowest diversity values regardless of sample size (table 2), a similar result to the microsatellite data published elsewhere (Bowkett *et al.*, 2014). Being one of the largest duiker species, *C. spadix* typically exists at much lower population densities than other species and so may suffer disproportionately from threats such as hunting, *e.g.* Nielsen (2011).

Conservation and management implications

We suggest that the data presented here may be typical of non-invasive genetic surveys undertaken over a relatively short period, although few such multi-species studies have been published to date. Problems such as small sample size, DNA quality and failure of loci to cross-amplify in related species are likely to feature in similar attempts to non-invasively sample ecological guilds at a landscape scale. Nevertheless, we have attempted to demonstrate that such surveys can yield valuable information that can guide further research and inform conservation management.

The recognition of highland populations of suni and blue duiker as separate species, the latter endemic to Tanzania, attaches a far greater significance to the Udzungwas as a potential stronghold for what could now be considered range-restricted taxa largely confined to protected areas. The Udzungwas are one of the largest remaining areas of forest within either species' range. This designation did not depend on our genetic sampling but is informed by the knowledge that *N. 'kirchenpaueri'* is found throughout the Udzungwas, whereas *P. 'lugens'* is mainly found on the western and southern escarpments. Whether these taxa are referred to as evolutionary species, subspecies or Evolutionary Significant Units, their distinctness adds further weight to the case for effective conservation of the Udzungwas.

In contrast, *C. spadix* is recognised by all recent authorities as a unique species restricted to Tanzanian highlands and highly endangered (Moyer 2003, Bowkett *et al.* 2014). While

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detected in the majority of surveyed forests (Jones & Bowkett 2012), this study illustrates the very low relative abundance of this species. Maintaining this rare species across remote areas without further loss of genetic variation will be a major challenge to conservationists.

Given the potentially negative effect of habitat fragmentation on forest antelope gene flow, conservation management should aim to maintain connectivity between forest patches where it persists and potentially establish wildlife corridors to connect outlying areas as has been recommended for Uzungwa Scarp (MTSN 2007). While this would be very difficult for forests surrounded by agricultural land, many central forests are now within the Udzungwa Mountains National Park and contiguous Kilombero Nature Reserve. Corridors within these protected areas could be established principally by the control of fire and enforcing bans on cutting trees and hunting. For forests outside of the core protected area such as New Dabaga and Uzungwa Scarp the priority for antelope conservation is to control illegal hunting and halt current population declines (Nielsen 2006, Rovero *et al.* 2010, Nielsen & Treue 2012).

Non-invasive genetic sampling is an increasingly important tool for assessing the evolutionary and conservation status of rare or elusive species (Zhan *et al.*, 2006; Gebremedhin *et al.*, 2009). While expensive and technology-intensive, non-invasive genetic sampling provides a promising method to monitor the response of forest antelope populations to conservation efforts in terms of abundance, distribution and genetic health.

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Appendix 1. Microsatellite amplification from *Cephalophus harveyi* faecal samples.

Fourteen dinucleotide microsatellite markers from other bovid species were selected from those initially screened by Ntie *et al.* (2010). Only eight loci amplified consistently with Harvey's duiker *C. harveyi* samples and these were incorporated into two PCR multiplexes (table S1) and amplified under conditions modified from the QIAGEN Multiplex PCR kit (table S2). Difficulty in scoring stuttered peaks in BRRIBO was overcome by pig-tailing the reverse primer (Brownstein *et al.*, 1996). Optimal amplification in multiplexed reactions required adjustment of relative primer concentrations in pre-mixed solutions (table S3) except for MPLX2B primers (BM143 and INRA05), which were added directly to the PCR media at 0.9 µM each (table S2). PCR negative controls were run to test for contamination. Analogous information for Abbott's duiker *C. spadix* microsatellite amplification is given in the supplement to Bowkett *et al.* (2014) at www.int-res.com/articles/suppl/n024p105_ supp.pdf

Table S1. Microsatellite primers and multiplex design for use with C. harveyi *faecal DNA on a Beckman Coulter capillary sequencer platform (Beckman Coulter, Fullerton, CA, USA). F=forward, R=reverse, primer labelled with fluorescent tag in bold*.

Multiplex	Marker	Sequence	Dye	Size range	Source
MPLX1	BM2113	**F GCTGCCTTCTACCAAATACCC**	Blue	126–134	Bishop *et al.* (1994)
		R CTTCCTGAGAGAAGCAACACC			
	INRA40	**F TCAGTCTCCAGGAGAGAAAAC**	Green	158–170	Beja-Pereira *et al.* (2004)
		R CTCTGCCCTGGGGATGATTG			
	BM1225	F TTTCTCAACAGAGGTGTCCAC	Black	211–215	Bishop *et al.* (1994)
		R ACCCCTATCACCATGCTCTG			
	BRRIBO	F CACCCGTACCCTCACTGC	Blue	245–261	Bishop *et al.* (1994)
		R TCACAACCCTCTTCTCACCC			
MPLX2A	BM121	**F TGGCATTGTGAAAAGAAGTAAA**	Black	112–126	Bishop *et al.* (1994)
		R ACTAGCACTATCTGGCAAGCA			
	SR12	**F TGACCAGGTGACTAACAC**	Blue	232–244	Kogi *et al.* (1995)
		R AATCTGATTTCATTTCATG#			
MPLX2B	BM143	**F ACCTGGGAAGCCTCCATATC**	Green	87–107	Bishop *et al.* (1994)
		R CTGCAGGCAGATTCTTTATCG			
	INRA05	**F CAATCTGCATGAAGTATAAATAT**	Blue	153–159	Beja-Pereira *et al.* (2004)
		R CTTCAGGCATACCCTACACC			

Reverse primer designed by Ntie *et al.* (2010).

Table S2. Polymerase Chain Reaction (PCR) conditions for multiplexed microsatellite markers (MPLX) with C. harveyi *faecal DNA*.

	PCR media	PCR cycles
MPLX1	11 µl: 5 µl QIAGEN Multiplex PCR kit (inc. 2 mM MgCl2), 1 µl of 10x primer mix*, 2 µl of Q-solution, 1 µl of RNAase free water, and 2 µl DNA elute	Denaturation: 95°C for 15 min Amplification: 94°C for 30 s, 58°C for 3 min and 72°C for 60 s (35 cycles) Extension: 60°C for 30 min
MPLX2A#	As for MPLX1	As for MPLX1 (40 cycles)
MPLX2B#	As for MPLX1 except primers not pre-mixed (0.9 µM each)	As for MPLX1 (40 cycles)

* See Table S3.

Combined for automated sequencer analysis

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Table S3. Preparation of 10x primer mix for use with the QIAGEN Multiplex PCR Kit as recommended by the manufacturer (each primer at 2 μM) and relative primer concentrations used with C. harveyi *faecal DNA.*

	Recommended	Multiplex 1	Multiplex 2A
Primer stock concentration	50 μM	50 μM	50 μM
Each primer (forward and reverse)	20 μl (up to 12 pairs)	24 μl (BM1225, BRRIBO); 16 μl (INRA40, BM2113)	16 μl (BM121); 8 μl (SR12)
TE buffer or H_2O	Variable	340 μl	452 μl
Final volume	500 μl	500 μl	500 μl

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Appendix 2. Details of mitochondrial control sequences included in this study excluding those derived from faecal samples.

Sample name	Species	Geographic origin	Provider	GenBank
PhC20	*A. melampus*			JN632592
VV14*	*C. callipygus*	Republic of the Congo	B.J. van Vuuren	FJ823338
VV17*	*C. callipygus*	Republic of the Congo	B.J. van Vuuren	FJ823339
VV18*	*C. callipygus*	Republic of the Congo	B.J. van Vuuren	FJ823340
OK23*	*C. callipygus*	Okondja, Gabon	S. Touladjan	FJ823341
OK27	*C. callipygus*	Okondja, Gabon	S. Touladjan	FJ823342
OK18*	*C. callipygus*	Okondja, Gabon	S. Touladjan	FJ823345
N2274	*C. dorsalis*	Republic of the Congo	D. Pires	FJ823366
861ou198	*C. dorsalis*	Dja, Cameroon	M. Colyn	FJ823376
AB5 / HD01	*C. harveyi*	Uzungwa Scarp, Udz., Tanzania	F. Rovero	AM903088
AB36 / TIS03	*C. harveyi*	Rubeho Mts., Tanzania	F. Rovero	AM903089
AB105 /TIS02	*C. harveyi*	Mwanihana, Udz., Tanzania	A.E. Bowkett	AM903090
SUN125 / VV125	*C. harveyi*	Mt. Meru, Tanzania	B. Jansen van Vuuren	FJ823316
SUN117 / VV117	*C. harveyi*	Usambara Mts., Tanzania	B. Jansen van Vuuren	FJ823317
SUN115 / VV15	*C. harveyi*	Mt. Meru, Tanzania	B. Jansen van Vuuren	FJ823318
SUN130 / VV130	*C. harveyi*	Usambara Mts., Tanzania	B. Jansen van Vuuren	FJ823319 / AM903087
TIS01	*C. harveyi*	Mwanihana, Udz., Tanzania	A.E. Bowkett	
M1	*C. harveyi*	New Dabaga, Udz., Tanzania	M.R. Nielsen	
M2	*C. harveyi*	New Dabaga, Udz., Tanzania	M.R. Nielsen	
M3	*C. harveyi*	New Dabaga, Udz., Tanzania	M.R. Nielsen	
M4	*C. harveyi*	New Dabaga, Udz., Tanzania	M.R. Nielsen	
NAT14	*C. harveyi*?	Selous, Tanzania	K. Hecker	
VV16*	*C. leucogaster*	Republic of the Congo	B. Jansen van Vuuren	FJ823334
N22157*	*C. leucogaster*	Republic of the Congo	D. Pires	FJ823335
N22151*	*C. leucogaster*	Republic of the Congo	D. Pires	FJ823336
OK17	*C. leucogaster*	Okondja, Gabon	S. Touladjan	FJ823337
VV11	*C. leucogaster*	Republic of the Congo	B. Jansen van Vuuren	FJ823333
105483	*C. maxwelli*		AMNH	FJ823309
OR587013	*C. maxwelli*		San Diego Zoo	FJ823310

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Sample name	Species	Geographic origin	Provider	GenBank
OR837	*C. maxwelli*		San Diego Zoo	FJ823311
E11-9	*C. maxwelli*			JN632685
VV124	*C. monticola*	Tanzania	B. Jansen van Vuuren	FJ823301
KB15149	*C. monticola*	Cape Province, S. Africa	San Diego Zoo	FJ823302
86307M28	*C. monticola*	Kinsangani, DRC	M. Colyn	FJ823306
DIV009	*C. monticola*	Bamenda, Cameroon	M. Colyn	FJ823307
R16520	*C. monticola*	Lefini, Republic of the Congo	M. Colyn	FJ823308
Cameroon	*C. monticola*	Cameroon		JN632686
AJ235318	*C. natalensis*		E.J.P. Douzery	AJ235318
VV1470	*C. natalensis*	KwaZulu-Natal, S. Africa	B. Jansen van Vuuren	FJ823314
VV1467	*C. natalensis*	KwaZulu-Natal, S. Africa	B. Jansen van Vuuren	FJ823315
NAT01	*C. natalensis*	Zambezi Delta, Mozambique	K. Hecker	
NAT02	*C. natalensis*	Zambezi Delta, Mozambique	K. Hecker	
NAT03	*C. natalensis*	Zambezi Delta, Mozambique	K. Hecker	
NAT04	*C. natalensis*	Lorursberg, South Africa	K. Hecker	
NAT05	*C. natalensis*	KwaZulu-Natal, South Africa	K. Hecker	
NAT06	*C. natalensis*	KwaZulu-Natal? South Africa	K. Hecker	
NAT07	*C. natalensis*	Hluhluwe, South Africa	K. Hecker	
NAT08	*C. natalensis*	KwaZulu-Natal, South Africa	K. Hecker	
NAT09	*C. natalensis*	KwaZulu-Natal, South Africa	K. Hecker	
NAT10	*C. natalensis*	KwaZulu-Natal, South Africa	K. Hecker	
NAT11	*C. natalensis*	KwaZulu-Natal, South Africa	K. Hecker	
NAT12	*C. natalensis*	KwaZulu-Natal, South Africa	K. Hecker	
NAT13	*C. natalensis*	Zambezi Delta, Mozambique	K. Hecker	
OR2758	*C. niger*	Liberia	San Diego Zoo	FJ823346
N221004	*C. nigrifrons*	Republic of the Congo	D. Pires	FJ823327
VV12	*C. nigrifrons*	Republic of the Congo	B. Jansen van Vuuren	FJ823328
N2293*	*C. nigrifrons*	Republic of the Congo	D. Pires	FJ823329
VV24*	*C. nigrifrons*	Republic of the Congo	B. Jansen van Vuuren	FJ823331

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Sample name	Species	Geographic origin	Provider	GenBank
AJuin1995	*C. ogilbyi*	Brazzaville, Republic of the Congo	M. Colyn	FJ823360
GA172	*C. ogilbyi*	Malounga, Gabon	M. Colyn	FJ823363
OR2115*	*C. rufilatus*		San Diego Zoo	FJ823320
VV19*	*C. rufilatus*	Central African Republic	B. Jansen van Vuuren	FJ823321
VV22	*C. rufilatus*	Central African Republic	B. Jansen van Vuuren	FJ823322
KB11228*	*C. rufilatus*	Guinea	San Diego Zoo	FJ823323
KB13889*	*C. rufilatus*		San Diego Zoo	FJ823324
KB14034	*C. rufilatus*		San Diego Zoo	FJ823325
OR3182	*C. rufilatus*	Guinea	San Diego Zoo	FJ823326
N22224	*C. silvicultor*	Republic of the Congo	D. Pires	FJ823353
OR356	*C. silvicultor*	Liberia	San Diego Zoo	FJ823354
VV25	*C. silvicultor*	Republic of the Congo	B. Jansen van Vuuren	FJ823355
OR409	*C. silvicultor*	Liberia	San Diego Zoo	FJ823356
N220853	*C. silvicultor*	Republic of the Congo	D. Pires	FJ823357
DIE2	*C. silvicultor*	Diecke, Guinea	M. Colyn	FJ823358
NIM2	*C. silvicultor*	Mt. Nimba, Guinea	M. Colyn	FJ823359
AB6 /TIS08	*C. spadix*	W. Usambaras, Tanzania	J. Beraducci	AM903084
AB37 /TIS07	*C. spadix*	S. Highlands, Tanzania	T.R.B. Davenport	AM903085
SUN118 / VV118	*C. spadix*	Kilimanjaro, Tanzania	B. Jansen van Vuuren	FJ823348
SUN122 / VV122	*C. spadix*	Usambara Mts., Tanzania	B. Jansen van Vuuren	FJ823349
SUN121 / VV121	*C. spadix*	Usambara Mts., Tanzania	B. Jansen van Vuuren	FJ823350
SUN120 /VV120	*C. spadix*	Usambara Mts., Tanzania	B. Jansen van Vuuren	FJ823351
SUN126 / VV126	*C. spadix*	Usambara Mts., Tanzania	B. Jansen van Vuuren	FJ823352 / AM903083
ROV01	*C. spadix*	Mwanihana, Udz., Tanzania	F. Rovero	HG323846
ROV02	*C. spadix*	Mwanihana, Udz., Tanzania	F. Rovero	HG323846
ROV03	*C. spadix*	Mwanihana, Udz., Tanzania	F. Rovero	HG323846
ROV04	*C. spadix*	Mwanihana, Udz., Tanzania	F. Rovero	HG323846
TRD01	*C. spadix*	S. Highlands, Tanzania	T.R.B. Davenport	AM903086
TJ050	*C. spadix*	Luhomero, Udz., Tanzania	T. Jones	HG323852
AB107 / TIS05	*C. spadix*	S. Highlands, Tanzania	T.R.B. Davenport	AM903086
D456	*C. weynsi*	Rwanda	B. Jansen van Vuuren	FJ823385

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Sample name	Species	Geographic origin	Provider	GenBank
CAR86	*N. batesi*			JN632668
AJ235323	*N. moschatus*		E.J.P. Douzery	AJ235323
68	*N. moschatus*	Mozambique		FJ985772
108	*N. moschatus*	Tanzania	A.E. Bowkett	FJ985773
SUN	*N. moschatus*			JN632669
OR1502	*S. grimmia*		San Diego Zoo	FJ823296
VV26	*S. grimmia*	Central African Republic	B. Jansen van Vuuren	FJ823297

* Included in genetic distance matrix (table 4) but not in phylogenetic analysis.

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