Notes on the distribution and genetic diversity of Abbott's duiker *Cephalophus spadix* in the Udzungwa Mountains, Tanzania.

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ABSTRACT

Abbott's duiker Cephalophus spadix *is a forest antelope endemic to a very few highland forests in Tanzania. Apparently extinct over much of its historical range, the species is listed as Endangered by the IUCN based on its rarity and its likely current distribution in only four isolated upland areas: Kilimanjaro, Southern Highlands, West Usambara and Udzungwa Mountains. In contrast to the situation in the rest of its range Abbott's duiker is relatively well documented and locally abundant in parts of the Udzungwa Mountains, which may therefore be the only stronghold for the species. We review the distribution of Abbott's duiker within the Udzungwa Mountains and present new information based on the non-invasive genetic identification of dung piles collected from the majority of forest blocks between 2006 and 2009 (73 confirmed dung samples). Our results include new records from outlying forest blocks where the presence of Abbott's duiker was previously unknown. Moreover we present the first population-level analysis of genetic structure and diversity in this endangered species based on nuclear microsatellites and mitochondrial sequence data. While these genetic results should be considered preliminary due to small sample sizes they indicate some differentiation from other Abbott's duiker populations as well as low genetic diversity relative to sympatric antelope species. Finally we discuss threats to Abbott's duiker, and other antelope populations, in the context of our results and identify broad trends within the differently managed Udzungwa Mountain forests that suggest potentially successful conservation strategies for this neglected species.*

INTRODUCTION

The highland forests of Tanzania are amongst the most important areas in the world for biodiversity conservation due to the exceptional density of threatened and endemic species found there (Burgess et al. 2007). These forests are also of great value to the people of Tanzania through their provision of ecosystem services such as watershed protection and carbon sequestration (Burgess et al. 2009). The endemic species of Tanzania's highlands are valuable indicators of the health of these important ecosystems.

One of the most threatened highland endemic species of Tanzania is Abbott's duiker *Cephalophus spadix* a forest antelope found in only a few upland areas. This duiker species is notable for its head crest of pink or red hair and its large body size (Kingdon 1997). Despite these characteristics it is very rarely seen due to its secretive behaviour (often crepuscular or nocturnal), densely vegetated habitats and naturally low population density. The species is threatened by habitat loss, due to agricultural encroachment and selective logging, and hunting, particularly with snares, ongoing in many areas (Moyer et al. 2008).

Not much is known about the historical distribution of Abbott's duiker but the species has long gone unrecorded in many sites where it was formerly known including the Uluguru and East Usambara Mountains, the Gregory Rift forests, and the Poroto Mountains and Njombe escarpment in southern Tanzania (Moyer 2003). This apparent decline resulted in the species' IUCN Red List status being changed from Vulnerable to Endangered in 2008 (Moyer et al. 2008). This assessment considered Abbott's duiker to survive in just four isolated mountain ranges: Kilimanjaro, Udzungwa, West Usambara and Southern Highlands (Mount Rungwe and Livingstone forest). A small isolated population had also been discovered in the southern Rubeho Mountains in 2006. No information on abundance was available from Kilimanjaro or West Usambara, and the species was considered very rare in the Southern Highlands (Machaga & Davenport 2009), leaving the Udzungwa Mountains as the only known stronghold for the species.

The Udzungwa Mountains in south-central Tanzania are the southernmost and largest block of the Eastern Arc Mountains (Fig. 1). Many of the area's forests are protected by the Udzungwa Mountains National Park and the more recently gazetted Kilombero Nature Reserve however other forests are less well protected and threatened by illegal activities (e.g. Rovero et al. 2010). This variation in protected status was reflected in the Red List's assessment of Abbott's duiker within the Udzungwas with the species listed as "locally common" in Mwanihana, Luhomero and Ukami (the latter only 7 km^2) and "rare" or "scarce" in Matundu, Nyumbanitu and Uzungwa Scarp (Moyer et al. 2008). The status of Abbott's duiker in several other forests was unknown.

Knowledge of the status of Abbott's duiker in the Udzungwas has increased greatly since the last Red List assessment due to extensive survey work and the use of remotely triggered camera-traps (Rovero et al. 2005) and non-invasive genetics (Bowkett et al. 2009a; Bowkett et al; 2009b). These techniques not only provide more reliable survey methods than traditionally available but also a wealth of further information on abundance (Rovero & Marshall 2009), habitat-use (Bowkett et al. 2008), and, in the case of genetic analysis, population structure and genetic health

(Beja-Pereira et al. 2009). Here we present results from recent surveys for Abbott's duiker, including all major forest blocks in the Udzungwa Mountains, and an exploratory analysis of genetic information recovered from dung samples collected during this work.

MATERIALS AND METHODS

We surveyed 24 sites within 10 forests throughout the Udzungwas between 2006 and 2010 (Fig. 1; Table 1). Within each forest we walked reconnaissance transects in a triangular configuration (typically 3 km per day) using hip-chains to record distance. Suspected Abbott's duiker dung piles, encountered along transects or elsewhere, were recorded and collected for genetic analysis (unless desiccated). We also employed camera-traps in many forests both specifically to detect Abbott's duiker and as part of other research programmes (e.g. Bowkett et al. 2008; Rovero & Marshall 2009).

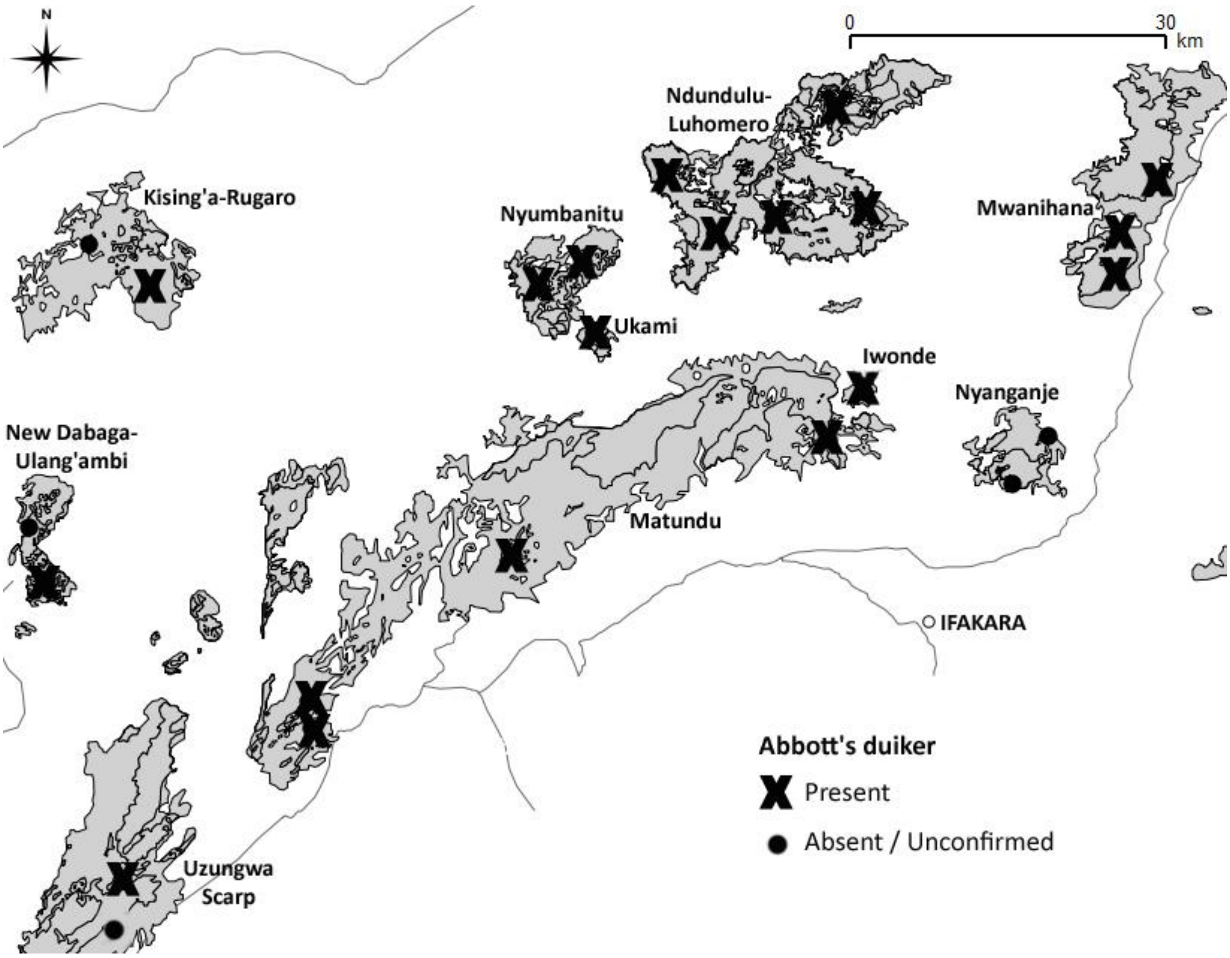

Fig. 1 Map of the Udzungwa Mountains, Tanzania, showing survey sites and recorded presence of Abbott's duiker (see Table 1 for more details). Outlined shapes represent closed- and open-canopy forest as identified from satellite imagery (Marshall et al. 2010)

Dung pellets were stored in RNAlater (Ambion Ltd) in the field and then kept refrigerated or frozen. DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN). To verify species identity and sample mitochondrial DNA variation we targeted a c.600 bp fragment of the left-hand domain of the control region using a combination of various primers (see Ntie et al. 2010a). Negative PCR controls were used throughout and PCR conditions followed Ntie et al. (2010a). To sample nuclear DNA variation we used seven microsatellite markers in two pre-PCR multiplexes: MPLX1 = INRA40 (Beja-Pereira et al. 2004), BM1225, BM2113 and BRRIBO (Bishop et al. 1994), and MPLX2 = BM143 (Bishop et al. 1994), INRA005 (Vaiman et al. 1994) and SR12 (Ntie et al. 2010b; modified from Kogi et al. 1995). We used the QIAGEN PCR Multiplex Kit (QIAGEN) following the manufacturer's instructions for degraded DNA including the addition of Q-solution and an extended annealing time of 3 minutes per cycle (35 – 40 cycles). PCR products were processed on a Beckman Coulter capillary sequencer and scored using CEQ 8800 software (Beckman Coulter, Fullerton, CA, USA). These markers have also been optimized for multiplex PCR and cross-species amplification in central African duiker species (Ntie et al. 2010b).

Sequence data were aligned using MUSCLE (Edger 2004) and checked in SEAVIEW (Galtier et al. 1996). Species identity was established by visual inspection of aligned sequences and confirmed using the BLAST programme (NCBI, Bethesda, MD, USA). For the preliminary analysis presented here a neighbour-joining (NJ) tree was constructed in PAUP* (Swofford 2001) based on Kimura 2-parameter corrected distances (Kimura 1980). The tree was rooted with two sequences for bay duiker *C. dorsalis* as a monophyletic outgroup sister to Abbott's duiker reflecting published duiker phylogenies (Jansen van Vuuren & Robinson 2001; Ntie et al. 2010a). The neighbour-joining analysis included all haplotypes recovered from dung and tissue samples in the Udzungwas (Table 1) and the Southern Highlands (S. Machaga & T. Davenport, Wildlife Conservation Society) plus all published control region sequences for Abbott's duiker and its sister species, the yellow-backed duiker *C. sylvicultor*.

For microsatellite loci we attempted to score each allele at least four times from separate PCRs to avoid the problems associated with reproducing consistent profiles from faecal DNA (Taberlet et al. 1999). We constructed a neighbour-joining tree based on Nei et al. (1983)'s genetic distance in POPULATIONS (Langella 1999) including all available genotypes from the Udzungwas and Southern Highlands and one from West Usambara (northern Tanzania). Standard genetic diversity values for both data sets, and deviations from Hardy–Weinberg and linkage equilibria in the microsatellite data, were tested for using Arlequin 3.5.1.2 (Excoffier et al. 2005). Samples from outside the Udzungwas were excluded when calculating diversity values.

RESULTS

We confirmed a total of 73 antelope dung samples from eight different forests as Abbott's duiker (Table 1). Many samples identified as Abbott's duiker in the field were in fact Harvey's duiker *C. harveyi* or bushbuck *Tragelaphus scriptus* and so were excluded from this study. In addition, we obtained camera-trap records from six forests including Ukami for which we were unable to collect dung samples.

While we included 14 control region haplotypes in our phylogenetic analysis, only five were recovered from the Udzungwas and the vast majority of samples shared one particular haplotype (Table 2). Almost all Udzungwa haplotypes were unique to one region (Table 2). There was strong bootstrap support for the monophyly of Abbott's duiker with respect to its sister species as expected (Fig 2). The microsatellite tree showed differentiation between regions with a distinct Southern Highlands clade (Fig 3).

Nineteen microsatellite genotypes from Udzungwa samples were included in our analysis of genetic diversity for this population (Table 3). Two of these samples did not meet the requirement of four repeats (but only at one locus) and a further sample had missing values at one locus. Identical genotypes were excluded to avoid including multiple samples from the same individual (one case). Two pairs of loci had significant Linkage Disequilibrium values, BM2113 with INRA40 ($P = 0.043$) and also with INRA005 ($P = 0.001$). BM1225 was monomorphic within the Udzungwas, although not in other regions. Overall heterozygosity values were relatively low (Table 3).

Table 1 Forest characteristics and Abbott's duiker survey results for ten forest blocks in the Udzungwa Mountains, Tanzania. CT = camera-trap; S = sightings.

Forest	**Size (km^2)**	**Elevation (m)**	**Walked transects (km)**	**Faecal DNA records**	**Other records**
Matundu	526	279-1,046	40	6	CT,
Uzungwa Scarp	314	290–2,144	25	1	S*
Luhombero-Ndundulu	231	1105–2,520	76	17	CT, S
Mwanihana	151	351–2,263	68	33	CT, S
Kising'a-Rugaro	116	1,627–2,322	30	1	-
Nyumbanitu	57	1,074–2,322	27	2	CT
Nyanganje	42	350–1,038	23	0	-
New Dabaga - Ulang'ambi	40	1,764–2,081	21	4	-
Ukami	7	902–1,651	10	NA**	CT
Iwonde	5	980-1,472	9	9	CT

* Sighting by Arafat Mtui, 25th March 2005.
** Dung samples were not collected from Ukami.

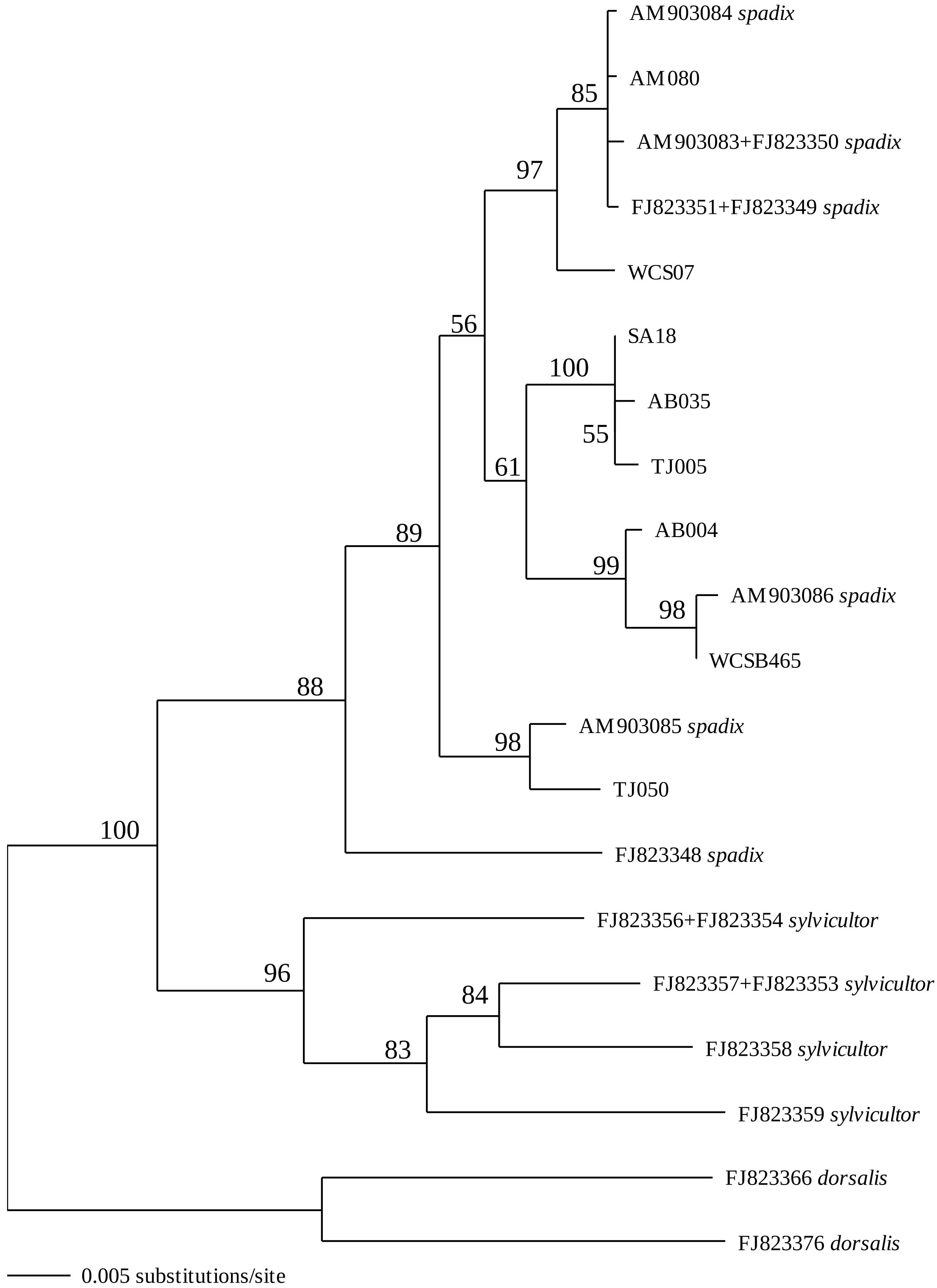

Fig. 2 Control region neighbour-joining bootstrap consensus phylogeny for *C. spadix* from the Udzungwa Mountains (c.600 bp). Bootstrap values are percentages of 1000 iterations (values below 50% not shown, results shown as polytomy).

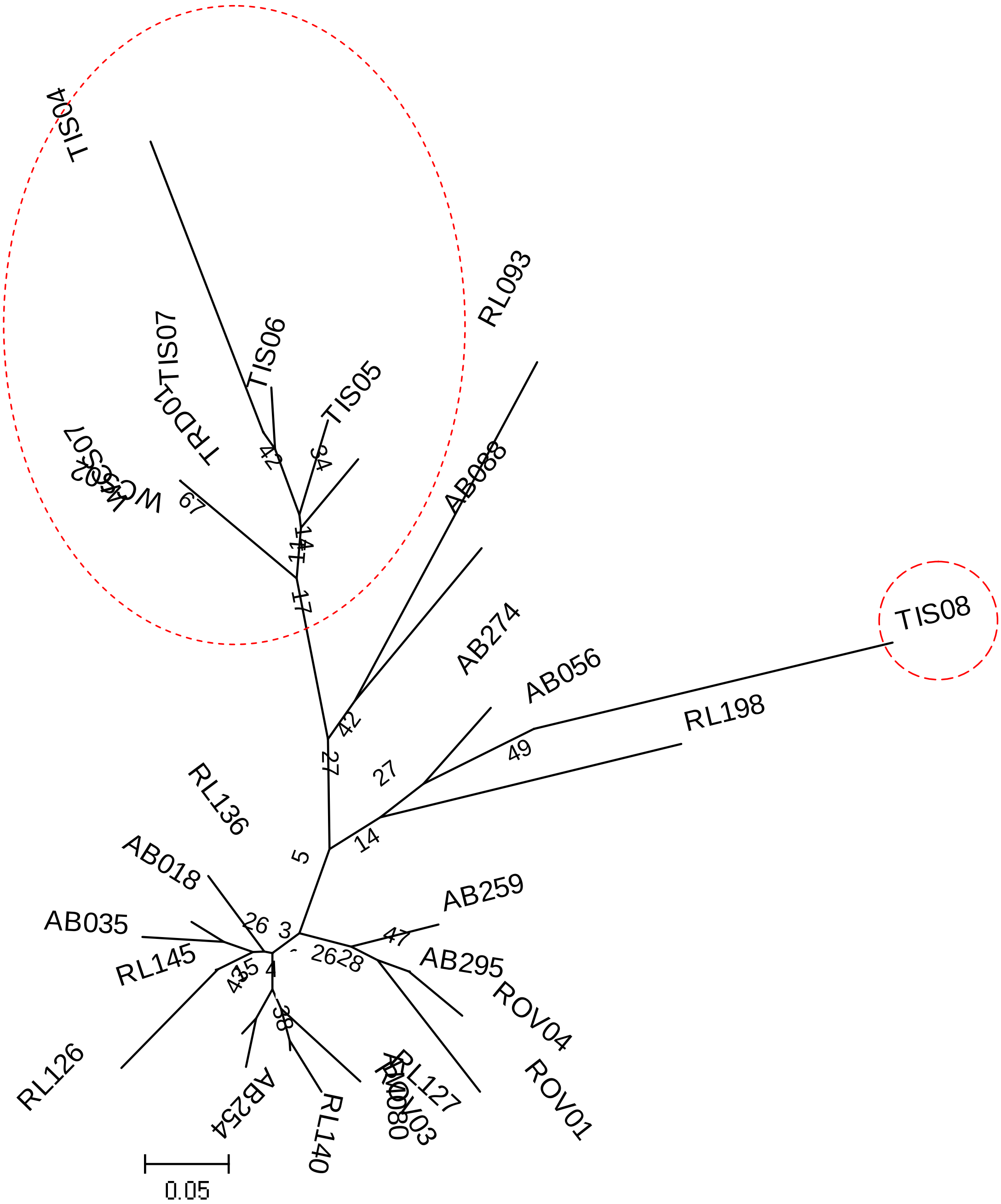

Fig 3. Neighbour-joining dendrogram showing relationships between Abbott's duiker genotypes using D_A distances (Nei et al. 1983). Bootstrap values are the result of 1000 pseudo-replicates (some values <5% not shown). Genotypes from the Southern Highlands (top left of figure) and West Usambara (TIS08 on right) are encircled.

Table 2 Frequency of Abbott's duiker mitochondrial control region haplotypes recovered from the Udzungwa Mountains with available data from other regions. MA = Matundu, LU = Luhomero-Ndundulu, UZ = Uzungwa Scarp, MW = Mwanihana, Kising'a-Rugaro, NY = Nyumbanitu, ND = New Dabaga-Ulang'ambi, I = Iwonde.

Haplotype	Udzungwa	Southern Highlands	West Usambara	Udzungwa forests
AM903084	0	0	2*	
SA18	57	0	0	MA, LU, MW, NY, ND, I
AB004	7	0	0	MA, UZ, MW, ND
AB035	3	0	0	MA, MW
AM903086	0	6	0	
WCS07	0	2	0	
WCSB465	0	2	0	
AM903085	0	2	0	
TJ005	1	0	0	KR
TJ050	4	0	0	LU, MW, I
AM080	1	0	0	LU
AM903083	0	0	2	
FJ823349	0	0	2	
FJ823348	0	0	1	

***** Also Ilole forest, Rubehos (x 1).

Table 3 Number of alleles (*Na*), observed (H_O) and expected (H_E) heterozygosities, and Hardy-Weinberg probability values (HW) for microsatellite loci and genetic diversity values for the mitochondrial control region in Abbott's duiker within the Udzungwa Mountains.

Locus	*Na*	H_o	H_e	HW (P-value)
BM2113	5	0.47	0.72	< 0.05
INRA40	4	0.37	0.47	NS
BRRIBO	5	0.68	0.67	NS
BM143	3	0.32	0.28	NS
INRA005	2	0.06	0.06	NS
SR12	3	0.11	0.10	NS
Mean	**3.67**	**0.33**	**0.38**	
Control Region	**Number of haplotypes**	**Polymorphic sites**	**Gene diversity**	**Nucleotide diversity**
	6	27	0.381	0.006

NS = non-significant.

DISCUSSION

As an endangered species found only in Tanzania our results for Abbott's duiker in the Udzungwa Mountains have global conservation significance. These surveys have confirmed Abbott's duiker from nine forests in the Udzungwa Mountains including three areas without molecular or camera-trap records prior to this study (Jones & Bowkett In press). While the species was already known from New Dabaga-Ulang'ambi (Nielsen 2006) we provide the first records from Iwonde, a small forest patch within the National Park, and Kising'a-Rugaro, a much larger outlying forest that has been heavily hunted and logged (Marshall et al. 2010; Jones unpubl. data).

Almost all the recovered control region haplotypes were unique to individual regions (Table 2) but several were too similar to be resolved by the neighbour-joining analysis and many clades with strong bootstrap support contained sequences from more than one region (Fig 2). This preliminary analysis therefore provides little evidence for geographic structuring of mitochondrial lineages. One possible explanation for this is incomplete lineage sorting whereby haplotypes may have undergone small sequence changes but there has not been sufficient time for groups of related haplotypes to become fixed in particular areas (Maddison & Knowles 2006).

In contrast our microsatellite analysis appears to differentiate genotypes from the three sampled regions (Fig 3). This result may reflect the more rapid evolution of microsatellite markers compared to mitochondrial DNA but caution should be taken in interpreting this preliminary analysis given the restricted sample sizes and marker limitations (see Results). Furthermore bootstrap support was generally low for the main clades although this may reflect the limited information available for reconstructing evolutionary relationships from such a small number of loci.

Overall genetic diversity values were low for Abbott's duiker compared to a similar analysis for Harvey's duiker in the Udzungwa Mountains (Bowkett et al. 2009b) or published values for other mammal species (see Appendix 1 of Gebremedhin et al. 2009). For Harvey's duiker thirty different control region haplotypes were recovered from 62 samples (Bowkett et al. 2009b) compared to just six for Abbott's duiker (73 samples). In addition, one of the microsatellite loci examined in this study appears to have undergone fixation within the Udzungwa Mountains but was polymorphic in the single Usambara sample. This lack of diversity may be the result of habitat fragmentation and the resulting isolation of small subpopulations.

While historical reduction in the size of forest fragments has likely affected Abbott's duiker in the Udzungwas the most serious current threat is almost certainly illegal hunting. Hunting occurs throughout the Udzungwas, including within the National Park, but is far more prevalent in the outlying forest reserves including Kising'a-Rugaro and Uzungwa-Scarp where our surveys suggest Abbott's duiker is much less abundant (Table 1). The threat to the large mammal communities of Uzungwa Scarp is particularly severe as outlined in a recent report documenting population declines in antelope and primates (Rovero et al. 2010).

However in those forests where conservation action has been taken there is some evidence that Abbott's duiker populations may be able to recover. Martin Nielsen reports, in Rovero et al. (2010), that hunted species have increased in abundance in

New Dabaga-Ulang'ambi following the successful introduction of domestic livestock schemes to local villages. Anecdotally there are also reports that Abbott's duiker has benefitted from anti-poaching patrols by National Park staff although Neilsen (2011) found that duiker dung densities remained approximately stable in Nyambanitu and Ndundulu forests between 2001 and 2008. These situations are in stark contrast to the example of the unprotected Uzungwa Scarp cited above.

While our survey results provide renewed hope for the survival of Abbott's duiker it is clear that the species remains threatened and potentially vulnerable to the negative impacts of small population size. We strongly recommend further survey work and non-invasive genetic sampling of Abbott's duiker in the Udzungwas and throughout the species' historical range. Prevention of further habitat loss and poaching is essential for the survival of the small isolated populations reported in this study and for the long-term viability of the species as a whole.

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