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Evolutionary Relationships Among Duiker Antelope (Bovidae: Cephalophinae)

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ABSTRACT

Duikers are a species rich subfamily of threatened African antelope whose recent origin poses a challenge to the molecular identification of taxa and estimation of their phylogeny. I test the ability of DNA barcodes to identify all taxa within this group. I then use mitochondrial and nuclear genes to estimate a multi-locus species tree and to date divergence times. DNA barcodes are unable to distinguish many sister taxa, calling into question the utility of barcodes for the regulation of duiker trade or in identification of field-collected feces. The multi-locus phylogeny provides support for the relationships among major duiker lineages and placement of two problematic taxa, but challenges the validity of the savanna genus and identifies hybridization between taxa. This study reveals that most duikers diverged during the Pleistocene, meriting further inquiry into the role that Pleistocene glacial cycling played in the diversification and population structuring of duikers.

Barcoding, Cytochrome *c* oxidase subunit 1 (*COX1*), Duiker, Antelope, Bushmeat, DNA barcoding

CHAPTER ONE: Evaluating DNA barcoding criteria using African duiker antelope (Cephalophinae) as a test case

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INTRODUCTION

One of the stated aims of DNA barcoding is to use sequences from a single universal gene fragment such as the cytochrome *c* oxidase subunit 1 (*COX1*) to identify unknown biological samples to species level (Hebert et al. 2003). This early barcoding study proposed that a 3% genetic distance between species could be used as a threshold for species delimitation. Hebert et al. (2004) later advocated a more flexible threshold known as the 10x rule in which species were delimited on the basis of inter-specific genetic distances being an order of magnitude greater than the average intra-specific variation within a given species pair. Other studies have not used distance thresholds, but instead relied on substantially differentiated or reciprocally monophyletic *COX1* lineages accompanied by supporting morphological and life history data (Hebert et al. 2004; Linares et al. 2009). Still other researchers have proposed the use of either pure (Eaton et al. 2009) or compound (DeSalle et al. 2005) DNA character state differences. Lastly, several groups have recently advocated using coalescent-based methods for species delimitation and identification (e.g. Abdo & Golding 2007; Carstens & Dewey 2010; O'Meara 2010; Pons et al. 2006). However, coalescent methods have yet to be widely applied to routine barcoding efforts (but see Dávalos & Porzecanski 2009; Monaghan et al. 2009; Tavares & Baker 2008).

Despite the need for a standardized method for species identification and delimitation, the Consortium for the Barcoding of Life (http://barcoding.si.edu) does not explicitly define what barcoding criterion should be adopted. As a result, the number of taxa and their distinction from one another may vary according to the method adopted. Species delimitation may also be very sensitive to the breadth of sampling both within and between species (Moritz & Cicero 2004). For example, poor geographic sampling would tend to underestimate intra-specific variation while exclusion of sister taxa would tend to overestimate inter-specific distances (Moritz & Cicero 2004). Furthermore, the over-representation of one or few dominant haplotypes through inadvertent re-sampling of the same individual or repeatedly

sampling closely related matrilines from the same site could also downwardly bias calculations of intra-specific variation and subsequent barcoding designations.

With these considerations in mind, the present study set out to evaluate the effectiveness of several traditionally used DNA barcoding criteria to delimit African duiker antelope (subfamily Cephalophinae). Duikers represent an interesting test case of DNA barcoding due to their high species richness, recent origin (Vrba 1995) and substantial intra-specific variation (Bowkett et al. 2009; Eaton et al. 2009; Ntie et al. 2010; van Vliet et al. 2008). Although a previous mitochondrial analysis of this group failed to resolve some species branches with high bootstrap or posterior support (Jansen van Vuuren & Robinson 2001; van Vliet et al. 2008), tree-based analyses using the mitochondrial control region were able to reliably distinguish most taxa, despite an apparent lack of monophyly in some species (Ntie et al. 2010). A recent *COX1*-based study by Eaton et al. (2009) also found that *COX1* DNA barcodes could be used to identify a subset of central African duikers. However, this study failed to include a complete taxonomic representation of this group, including many sister taxa. Consequently, no study to date has examined the ability of *COX1* DNA barcodes to differentiate species across the entire group or evaluated the performance of several traditionally employed DNA barcoding criteria to delimit these taxa. As several duiker species are listed under Appendices I and II of the Convention on the International Trade of Endangered Species (CITES) and are heavily hunted for their meat (Barnes 2002; Bennett et al. 2007; Newing 2001; Poulsen et al. 2009; Van Vliet & Nasi 2008; Wilkie & Carpenter 1999), studies aimed at understanding whether *COX1* barcodes could be used as an effective tool in wildlife forensics for this group are badly needed.

The goal of this study was therefore to generate a comprehensive set of *COX1* barcodes from all species currently recognized by the International Union of Conservation of Nature (IUCN) Species Survival Commission (East et al. 1990; East et al. 1988) within the subfamily Cephalophinae and assess which, if any, of the traditionally employed criteria are the most effective at differentiating taxa. Despite some minor discrepancies, the taxonomic scheme used by the IUCN is largely congruent with designations outlined by other mammalian taxonomists (Ansell 1971; Grubb 2005; Haltenorth & Diller 1977; Dorst & Danderlot 1970) and was therefore selected as a working framework for the present study.

METHODS

Tissue samples used in this study (n=34) were collected from bushmeat market surveys conducted in collaboration with the Wildlife Conservation Society in Gabon, or donated by zoos and scientific collectors (Table 1.1 for locality, material provider, and source material, Table 1.2 for number of samples per species, species common names, and conservation status). These genetic resources comprise samples used in the work of Jansen van Vuuren & Robinson (2001), Bowkett et al. (2009), van Vliet et al. (2008), Ntie et al. (2010) and Eaton et al. (2009). With the exception of the easily distinguishable blue duiker *Philantomba monticola* and the spiral horned antelope *Tragelaphus spekei*, a photographic record was used to verify the species identity of all bushmeat samples from Gabonese markets (Ntie et al. 2010). Tissue samples from the San Diego Zoo and a fecal sample taken from the *Cephalophus jentinki* at Gladys Porter Zoo were accompanied by species records. Details for the samples provided by Jansen van Vuuren are found in Jansen van Vuuren & Robinson (2001). These samples come from wildlife preserves, university collections or hunting concessions. In addition to these records, the identity of all samples sequenced in this analysis (excluding *C. jentinki*) was further supported by their placement in well-supported species clades within a reference control region phylogeny (Ntie et al. 2010). All GenBank sequences in the present study (n=68) are from a previous barcoding analysis by Eaton et al. (2009). The identity of these samples was verified by (i) photographic records of whole animals and (ii) branch placement on a maximum likelihood tree (Eaton et al. 2009). Fecal samples were obtained from several sites throughout Africa and were a subset of those sequenced in Ntie et al. (2010) and van Vliet et al. (2008).

All bushmeat and some San Diego Zoo tissues were extracted using a standard phenol-chloroform extraction method (Sambrook & Russell 2001). DNA provided by Jansen van Vuuren was extracted according to the methods described in Jansen van Vuuren & Robinson (2001). Other samples from San Diego Zoo were provided as genomic DNA extracts. Fecal DNA samples (n=10) were either extracted according to the methods described in van Vliet et al. (2008) or were extracted using the QIAamp DNA Stool Minikit (Qiagen). All fecal extractions were carried out in a designated room and a blank was included in each extraction series to control for DNA contamination.

Table 1.1 Sample list, species name, GenBank accession number, locality, material provider and source material of the samples used in this study. Note: Congo refers to The People's Republic of the Congo; DRC refers to The Democratic Republic of the Congo; CAR refers to The Central African Republic.

Sample ID	Species	GenBank Accession Number	Locality	Material Provider	Source Material
DKME 52	*T. spekei*	HQ644120	Okondja, Gabon	S. Touladjan	Muscle/skin tissue
OR25470	*C. zebra*	HQ644117	Liberia	San Diego Zoo	Muscle/skin tissue
VV119	*C. niger*	HQ644105	Ghana	B. Jansen van Vuuren	Muscle/skin tissue
OR2758	*C. niger*	HQ644106	Liberia	San Diego Zoo	Muscle/skin tissue
C.weynsi	*C. weynsi*	HQ644116	Rwanda	B. Jansen van Vuuren	Muscle/skin tissue
MJE 0410	*C. callipygus*	GQ144501.1	Ndoki, Congo	GenBank	GenBank
MJE 1468	*C. callipygus*	GQ144502.1	Ndoki, Congo	GenBank	GenBank
MJE 0402	*C. callipygus*	GQ144495.1	Ndoki, Congo	GenBank	GenBank
MJE 2121	*C. callipygus*	GQ144505.1	Ndoki, Congo	GenBank	GenBank
MJE 0412	*C. callipygus*	GQ144500.1	Ndoki, Congo	GenBank	GenBank
MJE 1453	*C. callipygus*	GQ144483.1	Ndoki, Congo	GenBank	GenBank
MJE 0910	*C. callipygus*	GQ144493.1	Ndoki, Congo	GenBank	GenBank
MJE 0003	*C. callipygus*	GQ144489.1	Ndoki, Congo	GenBank	GenBank
MJE 2823	*C. callipygus*	GQ144487.1	Ndoki, Congo	GenBank	GenBank
MJE 0417	*C. callipygus*	GQ144494.1	Ndoki, Congo	GenBank	GenBank
MJE 0367	*C. callipygus*	GQ144486.1	Ndoki, Congo	GenBank	GenBank
Nd07	Feces		Ndoki, Congo	E. Stokes, F. Maisels	Feces
Nd12	Feces		Ndoki, Congo	E. Stokes, F. Maisels	Feces
MJE 0920	*C. callipygus*	GQ144490.1	Ndoki, Congo	GenBank	GenBank
MJE 2232	*C. callipygus*	GQ144503.1	Ndoki, Congo	GenBank	GenBank
MJE 0351	*C. callipygus*	GQ144488.1	Ndoki, Congo	GenBank	GenBank
N22138	*C. callipygus*	HQ644090	Congo	D. Pires	Muscle/skin tissue
FR07	*C. ogilbyi*	HQ644109	Franceville, Gabon	S. Touladjan	Muscle/skin tissue
MJE 0400	*C. callipygus*	GQ144496.1	Ndoki, Congo	GenBank	GenBank
MJE 399	*C. callipygus*	GQ144497.1	Ndoki, Congo	GenBank	GenBank
MJE 0353	*C. callipygus*	GQ144499.1	Ndoki, Congo	GenBank	GenBank
OK18	*C. callipygus*	HQ644088	Okondja, Gabon	S. Touladjan	Muscle/skin tissue
LOPE102	Feces		Lope, Gabon	P. Mickala	Feces
OK27	*C. callipygus*	HQ644089	Okondja, Gabon	S. Touladjan	Muscle/skin tissue
MJE 2153	*C. callipygus*	GQ144504.1	Ndoki, Congo	GenBank	GenBank
MJE 0406	*C. callipygus*	GQ144492.1	Ndoki, Congo	GenBank	GenBank
MJE 0398	*C. callipygus*	GQ144498.1	Ndoki, Congo	GenBank	GenBank
MJE 0356	*C. callipygus*	GQ144485.1	Ndoki, Congo	GenBank	GenBank
MJE 0922	*C. callipygus*	GQ144491.1	Ndoki, Congo	GenBank	GenBank
MJE 2246	*C. callipygus*	GQ144484.1	Ndoki, Congo	GenBank	GenBank
MJE 1004	*C. nigrifrons*	GQ144550.1	Ndoki, Congo	GenBank	GenBank
MJE 0293	*C. nigrifrons*	GQ144546.1	Ndoki, Congo	GenBank	GenBank
MJE 2218	*C. nigrifrons*	GQ144549.1	Ndoki, Congo	GenBank	GenBank
MJE 2213	*C. nigrifrons*	GQ144548.1	Ndoki, Congo	GenBank	GenBank
MJE 2101	*C. nigrifrons*	GQ144547.1	Ndoki, Congo	GenBank	GenBank
N221004	*C. nigrifrons*	HQ644108	Congo	D. Pires	Muscle/skin tissue
VV24	*C. nigrifrons*	HQ644107	Congo	B. Jansen van Vuuren	Muscle/skin tissue
VV22	*C. rufilatus*	HQ644111	CAR	B. Jansen van Vuuren	Muscle/skin tissue

Table 1.1 continued

OR2115	*C. rufilatus*	HQ644110		San Diego Zoo	Muscle/skin tissue
VV117	*C. harveyi*	HQ644093	Tanzania	B. Jansen van Vuuren	Muscle/skin tissue
VV1467	*C. natalensis*	HQ644104	S. Africa	B. Jansen van Vuuren	Primary Culture
VV1470	*C. natalensis*	HQ644103	S. Africa	B. Jansen van Vuuren	Primary Culture
N22157	*C. leucogaster*	HQ644097	Congo	D. Pires	Muscle/skin tissue
VV11	*C. leucogaster*	HQ644096	Congo	B. Jansen van Vuuren	Muscle/skin tissue
VV16	*C. leucogaster*	HQ644095	Congo	B. Jansen van Vuuren	Muscle/skin tissue
MJE 2220	*C. leucogaster*	GQ144519.1	Ndoki, Congo	GenBank	GenBank
MJE 2157	*C. leucogaster*	GQ144518.1	Ndoki, Congo	GenBank	GenBank
MJE 0251	*C. leucogaster*	GQ144516.1	Ndoki, Congo	GenBank	GenBank
MJE 0234	*C. leucogaster*	GQ144515.1	Ndoki, Congo	GenBank	GenBank
N22151	*C. leucogaster*	HQ644098	Congo	D. Pires	Muscle/skin tissue
MJE 0854	*C. leucogaster*	GQ144517.1	Ndoki, Congo	GenBank	GenBank
F17Jan	Feces		Ivindo, Gabon	N. van Vliet	Feces
MJE 2191	*C. leucogaster*	GQ144521.1	Ndoki, Congo	GenBank	GenBank
MJE 2206	*C. leucogaster*	GQ144520.1	Ndoki, Congo	GenBank	GenBank
VV26	*S. grimmia*	HQ644119	CAR	B. Jansen van Vuuren	Primary Culture
OR1786	*S. grimmia*	HQ644118		San Diego Zoo	Muscle/skin tissue
C. jentinki	*C. jentinki*	HQ644094		Gladys Porter Zoo	Feces
N220853	*C. silvicultor*	HQ644112	Congo	D. Pires	Muscle/skin tissue
OR356	*C. silvicultor*	HQ644113	Liberia	San Diego Zoo	Muscle/skin tissue
Nd10	Feces		Ndoki, Congo	E. Stokes, F. Maisels	Feces
VV120	*C. spadix*	HQ644115	Tanzania	B. Jansen van Vuuren	Primary Culture
VV121	*C. spadix*	HQ644114	Tanzania	B. Jansen van Vuuren	Primary Culture
MCR14	Feces		Mts de Cristal,	R. Aba'a	Feces
MJE YF75	*C. dorsalis*	GQ144511.1	Ndoki, Congo	GenBank	GenBank
MJE 0901	*C. dorsalis*	GQ144513.1	Ndoki, Congo	GenBank	GenBank
MJE YF15	*C. dorsalis*	GQ144510.1	Ndoki, Congo	GenBank	GenBank
MJE	*C. dorsalis*	GQ144509.1	Ndoki, Congo	GenBank	GenBank
MJE YF42	*C. dorsalis*	GQ144506.1	Ndoki, Congo	GenBank	GenBank
MJE 1031	*C. dorsalis*	GQ144507.1	Ndoki, Congo	GenBank	GenBank
MJE 1010	*C. dorsalis*	GQ144512.1	Ndoki, Congo	GenBank	GenBank
N221022	*C. dorsalis*	HQ644092	Congo	D. Pires	Muscle/skin tissue
MJE 0887	*C. dorsalis*	GQ144514.1	Ndoki, Congo	GenBank	GenBank
MJE	*C. dorsalis*	GQ144508.1	Ndoki, Congo	GenBank	GenBank
OR 761	*C. dorsalis*	HQ644091		San Diego Zoo	Muscle/skin tissue
MCR35	Feces		Mts. de Cristal,	J.L. Dew	Feces
VV20	*C. adersi*	HQ644087	Zanzibar, Tanzania	B. Jansen van Vuuren	Muscle/skin tissue
VV21	*C. adersi*	HQ644086	Zanzibar, Tanzania	B. Jansen van Vuuren	Muscle/skin tissue
OR837	*P. maxwelli*	HQ644100		San Diego Zoo	Muscle/skin tissue
OR587013	*P. maxwelli*	HQ644099		San Diego Zoo	Muscle/skin tissue
KB15149	*P. monticola*	HQ644101		San Diego Zoo	Muscle/skin tissue
SA02	Feces		Salonga, DRC	B. Curran	Feces
F13Jan	Feces		Ivindo, Gabon	N. van Vliet	Feces
MJE 0394	*P. monticola*	GQ144527.1	Ndoki, Congo	GenBank	GenBank
MJE 0409	*P. monticola*	GQ144530.1	Ndoki, Congo	GenBank	GenBank
MJE 2156	*P. monticola*	GQ144538.1	Ndoki, Congo	GenBank	GenBank
MJE 5801	*P. monticola*	GQ144544.1	Ndoki, Congo	GenBank	GenBank
MJE 0941	*P. monticola*	GQ144523.1	Ndoki, Congo	GenBank	GenBank

Table 1.1 continued

MJE 2265	*P. monticola*	GQ144542.1	Ndoki, Congo	GenBank	GenBank
MJE 2148	*P. monticola*	GQ144535.1	Ndoki, Congo	GenBank	GenBank
MJE 2110	*P. monticola*	GQ144539.1	Ndoki, Congo	GenBank	GenBank
MJE 0912	*P. monticola*	GQ144545.1	Ndoki, Congo	GenBank	GenBank
MJE 2295	*P. monticola*	GQ144541.1	Ndoki, Congo	GenBank	GenBank
MJE 0420	*P. monticola*	GQ144534.1	Ndoki, Congo	GenBank	GenBank
MJE 0421	*P. monticola*	GQ144537.1	Ndoki, Congo	GenBank	GenBank
MJE 0947	*P. monticola*	GQ144536.1	Ndoki, Congo	GenBank	GenBank
MJE 0832	*P. monticola*	GQ144532.1	Ndoki, Congo	GenBank	GenBank
MJE 0396	*P. monticola*	GQ144526.1	Ndoki, Congo	GenBank	GenBank
MJE 0918	*P. monticola*	GQ144543.1	Ndoki, Congo	GenBank	GenBank
MJE 0946	*P. monticola*	GQ144533.1	Ndoki, Congo	GenBank	GenBank
MJE 0416	*P. monticola*	GQ144531.1	Ndoki, Congo	GenBank	GenBank
MJE 0395	*P. monticola*	GQ144524.1	Ndoki, Congo	GenBank	GenBank
DKME1	*P. monticola*	HQ644102	Gabon	S. Touladjan	Muscle/skin tissue
MJE 5802	*P. monticola*	GQ144525.1	Ndoki, Congo	GenBank	GenBank
MJE 0397	*P. monticola*	GQ144522.1	Ndoki, Congo	GenBank	GenBank
MJE 2222	*P. monticola*	GQ144540.1	Ndoki, Congo	GenBank	GenBank
MJE 0002	*P. monticola*	GQ144529.1	Ndoki, Congo	GenBank	GenBank
MJE 0363	*P. monticola*	GQ144528.1	Ndoki, Congo	GenBank	GenBank

Table 1.2. Species, number of individual specimens, number of unique haplotypes, common name and the IUCN 3.1 conservation status of the 19 antelope examined used in this study.

Species	Individuals	Haplotypes	Common name	Status
C. adersi	2	2	Aders' Duiker	Critically Endangered
C. callipygus	26	11	Peter's Duiker	Least Concern
C. dorsalis	11	4	Bay Duiker	Least Concern
C. harveyi	1	1	Harvey's Duiker	Least Concern
C. jentinki	1	1	Jentink's Duiker	Endangered
C. leucogaster	11	6	White-bellied Duiker	Least Concern
C. natalensis	2	2	Natal Red Duiker	Least Concern
C. niger	2	2	Black Duiker	Least Concern
C. nigrifrons	7	2	Black-fronted Duiker	Least Concern
C. ogilbyi	1	1	Ogilby's Duiker	Least Concern
C. rufilatus	2	2	Red-flanked Duiker	Least Concern
C. silvicultor	2	2	Yellow-backed	Least Concern
C. spadix	2	1	Abbott's Duiker	Endangered
C. weynsi	1	1	Weyns' Duiker	Least Concern
C. zebra	1	1	Zebra Duiker	Vulnerable
P. maxwelli	2	2	Maxwell's Duiker	Least Concern
P. monticola	26	15	Blue Duiker	Least Concern
S. grimmia	2	2	Grey Duiker	Least Concern
T. spekei	1	1	Sitatunga	Least Concern

A 710bp fragment of the mitochondrial cytochrome oxidase I gene (*COX1*) was amplified using the forward bovid_F1 5'-TTTTCAACCAACCACAAAGACATCGG-3' and reverse bovid_R1 5'-TATACTTCAGGGTGTCCAAAGAATCA-3' primers. These primers were based on the HCO/LCO primers originally published by Folmer et al. (1994) and modified to better match the GenBank sequence data from 27 species within the Bovidae family (Table 1.3).

Table 1.3. GenBank accession numbers and species identifications of the 27 GenBank sequences used to design the bovid_F1 and bovid_R1 primers.

GenBank accession number	Species
FJ171915	*Bubalus bubalis*
FJ171914	*Bos indicus*
EF494179	*Bos grunniens*
EF490455	*Ovis aries*
NC_009510	*Ammotragus lervia*
AF533441	*Capra hircus*
NC_006380	*Bos grunniens*
DQ191826	*Pantholops hodgs*
NC_010640	*Naemorhedus swinhoei*
DQ124371	*Bos taurus*
FJ207531.1	*Hemitragus jemlahicus*
FJ207537	*Pseudois nayaur*
FJ207538	*Rupicapra pyrenaica*
FJ207536	*Ovibos moschatus*
FJ207535	*Oreamnos americanus*
FJ207533	*Capricornis crispus*
FJ207532	*Naemorhedus griseus*
FJ207531	*Hemitragus jemlahicus*
FJ207530	*Damaliscus pygargus*
FJ207529	*Capra sibirica*
FJ207528	*Capra pyrenaica*
FJ207527	*Capra nubiana*
FJ207526	*Capra ibex*
FJ207525	*Capra falconeri*
FJ207524	*Budocras taxicolor*
FJ207523	*Hemitragus jayakari*
FJ207522	*Ammotragus lervia*

PCR reactions were carried out in 1X buffer (200 mM Tris pH8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2mM of each primer, 5mg Bovine Serum Albumin (BSA), 1.25 U of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA) and 1-2 mL of template DNA in a total volume of 20mL. Initial denaturation at 94°C for 4 min was followed by 35 cycles of denaturation at 94°C for 30 s, primer

annealment at 52°C for 30 s, and extension at 72°C for 30 s with a final extension step at 72°C for 10 min. PCR products were purified prior to sequencing using ExoAp (Glenn & Schable 2005) and then sequenced on both strands using the BigDye Terminator Cycle Sequencing Kit v1.1 (ABI).

Forward and reverse sequences were edited using the program SEQUENCHER v4.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Trace files of all sequences obtained from tissues other than field-collected feces (n=35) were submitted to the BOLD database (http://www.barcodinglife.org; Ratnasingham and Hebert 2007) under the general projects code CEP. GenBank sequences from Eaton et al. (2009) were combined with these sequences and aligned using Clustal X v2.06 (Thompson et al. 1997). For the purposes of the present study, only unique haplotypes within each taxon were used although a companion analysis including all sequences was also carried out. Sequences from fecal samples were included in tree-based analyses of the data but excluded from the distance and character-based analyses.

Phylogenetic analysis was carried out using the neighbor-joining (NJ) and maximum parsimony (MP) methods in PAUP 4.0b10 (Swofford 2000) and the Bayesian method implemented in MrBayes (Huelsenbeck et al. 2001). In all cases, *Tragelaphus spekei* was used as the outgroup. In NJ analysis, a Kimura-2-parameter (K2P) model of nucleotide substitution was used, as recommended for species-level barcoding analysis (Hebert et al. 2003). For MP analysis, heuristic searches were carried out using the tree-bisection-reconnection (TBR) algorithm and starting trees were obtained using the stepwise addition option. Character changes were considered un-weighted and unordered. Bayesian analyses adopted a general time-reversible model allowing for among site rate variation and a proportion of invariant sites. Prior probabilities for model parameters were left at default settings. Monte Carlo Markov Chains were run for 10,000,000 iterations and trees were sampled every 10,000 generations. A suitable burn-in period of 10% was discarded after examining trace files using TRACER (Drummond & A Rambaut 2007). Individual branch support was accepted for bootstrap values $\geq$ 75% and posterior probabilities $\geq$ 0.95.

Identification of simple, pure (DeSalle et al. 2005) synapomorphies was carried out in the program MEGA v4 (Kumar 2004). A simple, pure synapomorphy is a single base where the character state is fixed within a species but differs at the homologous position in all other species. Pair-wise K2P distances were calculated in MEGA v4 to determine which pair-wise species differences exceeded the 3% threshold criterion of Hebert et al. (2003). A 10x genetic distance threshold criterion was calculated in which the inter-specific distance for each candidate species pair was divided by the average intra-specific divergence between the two species under consideration. Pair-wise K2P distances between species were calculated firstly for unique haplotypes and secondly for all sequences.

RESULTS

Several species formed well-supported monophyletic clades in the Bayesian phylogenetic tree (Figure 1.1). However, this tree-based analysis was unable to distinguish the sister taxa *C. weynsi*, *C. callipygus* and *C. ogilbyi*. The *COX1* tree was also unable to distinguish the sister taxa *C. rufilatus* and *C. nigrifrons* or provide phylogenetic support for the *C. niger* clade. Lastly, the position of *C. zebra* was unresolved in the phylogeny as has been observed in earlier studies (Jansen van Vuuren & Robinson 2001). However, the phylogeny was able to recover the identity of fecal samples previously diagnosed to species level by Ntie et al. (2010) and van Vliet et al. (2008).

Several species had no conserved, species-specific diagnostic sites (Table 1.4). These included *C. nigrifrons*, *C. rufilatus*, *C. silvicultor, C. callipygus* and *C. ogilbyi*. In one case, *C. callipygus* shares an identical haplotype with *C. ogilbyi*. Other species possess only one (*C. niger*, *C. natalensis*, and *C. spadix*), two (*C. weynsi*, *C. harveyi*, *C. jentinki*, *C. adersi* and *P. maxwelli*), three (*P. monticola* and *C. dorsalis*), four (*C. leucogaster* and *C. zebra*), or seven (*S. grimmia*) diagnostic sites. In contrast, the sympatric outgroup taxon *T. spekei* possesses 30 diagnostic sites relative to the duikers under study. Although sequence ambiguities were occasionally encountered, these were not at positions where diagnostic sites were identified.

Using a 3% genetic distance criterion, seven out of 171 (4.1%) pair-wise comparisons failed to meet this threshold (Table 1.5; See Table 1.6 for pairwise comparisons calculated from duplicate haplotypes). Inter-specific distances between the sister taxa *C. ogilbyi* and *C. callipygus, C. ogilbyi* and *C. weynsi,* and *C. callipygus* and *C. weynsi* were very low (1.40%, 0.50%, and 1.20%, respectively). *Cephalophus nigrifrons* and its sister taxon *C. rufilatus* were also separated by a very low inter-specific distance (2.30%). Also failing to satisfy the 3% threshold are comparisons between *C. nigrifrons* and *C. natalensis* (3.00%), *C. natalensis* and *C. harveyi* (0.90%), and *C. silvicultor* and *C. spadix* (0.90%). The 10x threshold for species delimitation was even more widely violated in this group. Of the 153 comparisons for which average intra-specific variation could be assessed, 73 (47.7%) pairs failed to reach this threshold. In some cases, comparisons between genera did not exceed this threshold, suggesting that a 10x criterion is the least appropriate for defining these taxa.

Fig. 1.1 Bayesian phylogeny of Cephalophinae rooted with *Tragelaphus spekei*. Asterisks indicate > 75% bootstrap or 0.95 posterior support in Bayesian, Maximum Parsimony and Neighbor-joining analyses, respectively. Dashes indicate where this level of phylogenetic support was not obtained. Country label Congo refers to the People's Republic of the Congo.

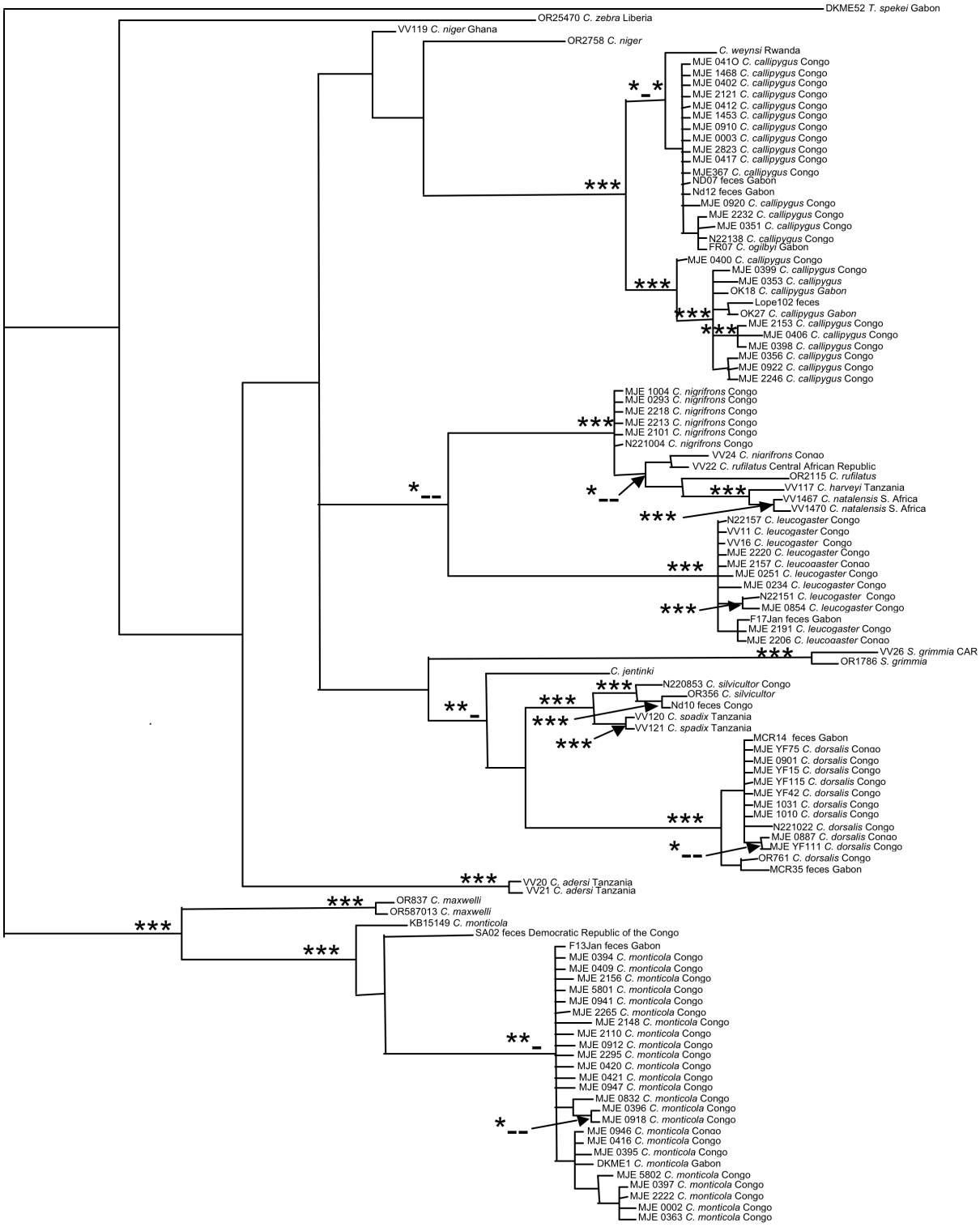


Table 1.4. Nucleotide position and character states of variable sites in 658 base pairs of *COX1* gene fragment examined in duiker antelope. A dot signifies a shared character state with the first line of sequence. Ambiguity codes summarize intra-specific variation. Sites that are diagnostic across all species are shown in white text on black background. A base is considered diagnostic if it is found only in that species and is fixed within that species.

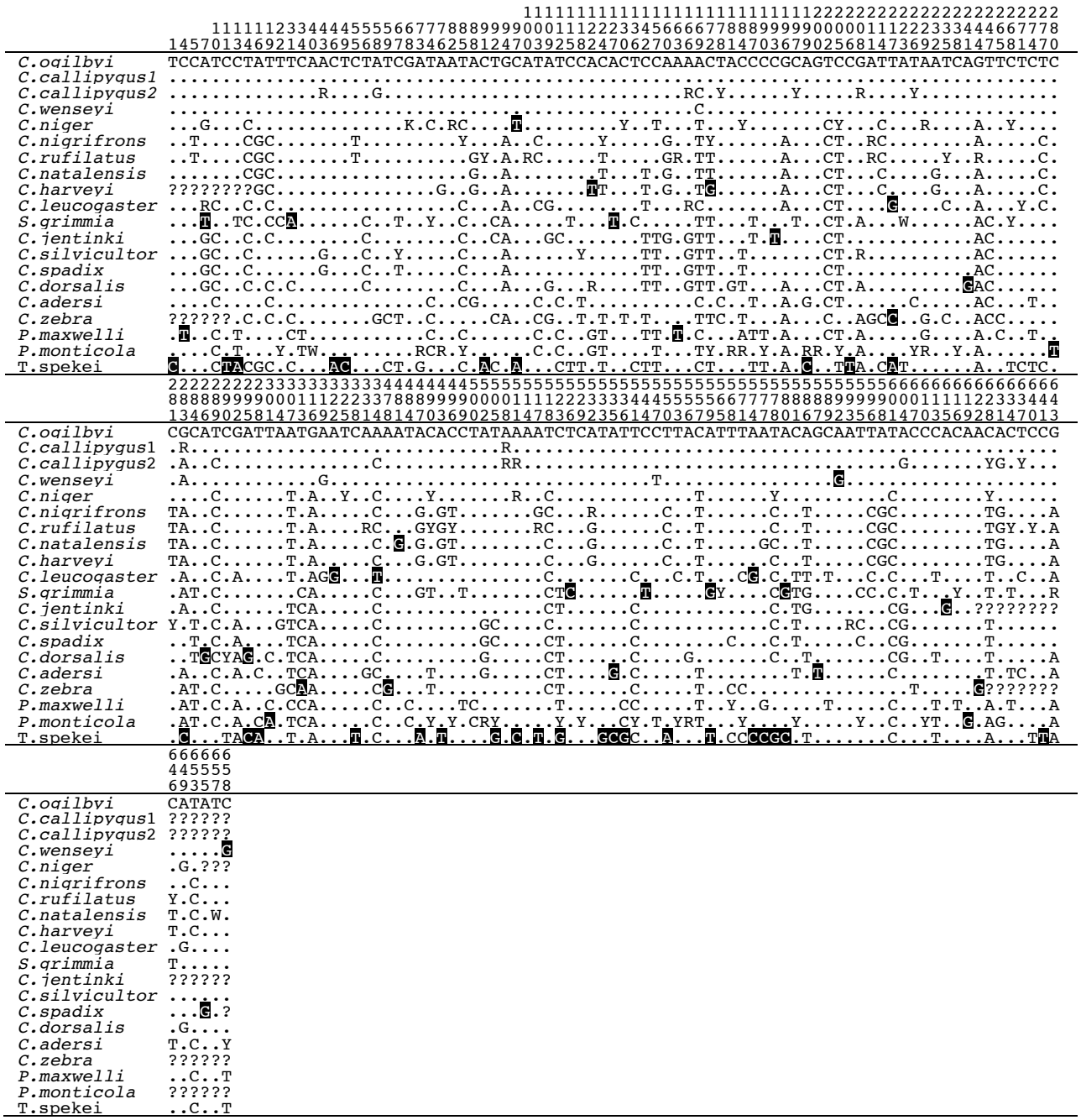


Table 1.5 Intra- and inter-specific nucleotide differences between unique haplotypes in the *COX1* gene of Cephalophinae species. The lower left triangular matrix depicts the ratio of inter- to average intra-specific distances between species. The upper right triangular matrix represents raw % K2P distance values. The diagonal values in bold depict intra-specific haplotypes diversity. Cells in grey represent species pairs that fail to meet their respective distance criterion. Specifically, grey cells in the lower left matrix are values less than 10 and grey cells in the upper right matrix are values 3% or less.

	1	2	3	4	5	6	7	8	9	10	11
1. *C. ogilbyi*	**n/c**	1.40%	0.00%	1.90%	0.50%	5.20%	5.00%	6.30%	5.20%	6.20%	5.90%
2. *C. callipygus*	2.333	**0.012**			1.20%	6.40%	6.20%	7.50%	6.40%	7.40%	6.50%
3. *C. callipygus*1			**0.000**	1.90%	0.50%	5.20%	5.00%	6.30%	5.20%	6.20%	5.90%
4. *C. callipygus*2	4.750		4.750	**0.008**	1.40%	6.90%	6.60%	7.90%	6.90%	7.90%	6.70%
5. *C. weynsi*		2.000		3.500	**n/c**	5.30%	5.00%	6.30%	5.30%	6.30%	5.40%
6. *C. niger*	2.737	2.560	2.737	3.000	2.789	**0.038**	5.50%	7.90%	6.30%	7.30%	6.90%
7. *C. nigrifrons*	7.143	4.769	7.143	6.000	7.143	2.115	**0.014**	2.30%	3.00%	4.00%	5.20%
8. *C. rufilatus*	4.500	3.750	4.500	4.389	4.500	2.394	1.095	**0.028**	3.30%	4.30%	7.00%
9. *C. natalensis*		10.667		17.250		3.316	4.286	2.357	**0.000**	0.90%	5.90%
10. *C. harveyi*		12.333		19.750		3.842	5.714	3.071		**n/c**	6.90%
11. *C. leucogaster*	29.500	8.125	29.500	11.167	27.000	3.286	5.778	4.375	29.500	34.500	**0.004**
12. *S. grimmia*	17.111	7.810	17.111	9.882	17.333	3.532	7.478	5.946	20.889	23.111	14.615
13. *C. jentinki*		13.167		20.250		3.842	10.143	6.786			33.000
14. *C. silvicultor*	6.316	3.935	6.316	4.593	6.105	2.035	4.303	4.043	7.158	8.211	5.304
15. *C. spadix*		10.167		15.500		2.789	9.286	6.357			30.500
16. *C. dorsalis*	80.000	12.000	80.000	17.200	80.000	3.750	9.625	6.800	75.000	84.000	20.667
17. *C. adersi*		11.333		17.250		3.842	8.571	5.214			29.500
18. *C. zebra*		20.333		31.000		5.579	14.000	7.571			48.000
19. *P. monticola*	20.667	9.905	20.667	12.706	20.667	3.830	7.913	5.676	22.000	21.111	15.385
20. *P. maxwelli*		18.833		29.000		4.789	13.000	7.714			42.500
21. *T. spekei*		25.333		38.750		7.368	20.143	11.357			64.000

	12	13	14	15	16	17	18	19	20	21
1. *C. ogilbyi*	7.70%	7.30%	6.00%	5.70%	8.00%	6.80%	11.70%	9.30%	10.40%	14.30%
2. *C. callipygus*	8.20%	7.90%	6.10%	6.10%	8.40%	6.80%	12.20%	10.40%	11.30%	15.20%
3. *C. callipygus*1	7.70%	7.30%	6.00%	5.70%	8.00%	6.80%	11.70%	9.30%	10.40%	14.30%
4. *C. callipygus*2	8.40%	8.10%	6.20%	6.20%	8.60%	6.90%	12.40%	10.80%	11.60%	15.50%
5. *C. weynsi*	7.80%	7.30%	5.80%	5.80%	8.00%	6.30%	11.70%	9.30%	9.90%	13.80%
6. *C. niger*	8.30%	7.30%	5.80%	5.30%	7.50%	7.30%	10.60%	9.00%	9.10%	14.00%
7. *C. nigrifrons*	8.60%	7.10%	7.10%	6.50%	7.70%	6.00%	9.80%	9.10%	9.10%	14.10%
8. *C. rufilatus*	11.00%	9.50%	9.50%	8.90%	10.20%	7.30%	10.60%	10.50%	10.80%	15.90%
9. *C. natalensis*	9.40%	6.80%	6.80%	6.30%	7.50%	6.80%	10.00%	9.90%	9.40%	13.20%
10. *C. harveyi*	10.40%	7.80%	7.80%	7.30%	8.40%	7.80%	11.10%	9.50%	9.90%	14.30%
11. *C. leucogaster*	9.50%	6.60%	6.10%	6.10%	6.20%	5.90%	9.60%	10.00%	8.50%	12.80%
12. *S. grimmia*	**0.009**	7.30%	7.30%	6.70%	8.40%	8.80%	12.70%	13.10%	10.90%	11.40%
13. *C. jentinki*	16.222	**n/c**	4.80%	4.30%	5.40%	7.90%	13.50%	12.10%	9.40%	13.20%
14. *C. silvicultor*	5.214	5.053	**0.019**	0.90%	3.90%	7.90%	11.70%	11.00%	8.90%	13.20%
15. *C. spadix*	14.889		0.947	**n/c**	3.90%	8.40%	11.70%	11.00%	9.40%	13.80%
16. *C. dorsalis*	15.273	54.000	3.714	39.000	**0.002**	9.60%	10.70%	10.80%	8.20%	13.90%
17. *C. adersi*	19.556		8.316		96.000	**0.000**	11.20%	8.90%	7.30%	13.20%
18. *C. zebra*	28.222		12.316		107.000		**n/c**	12.80%	11.10%	13.80%
19. *P. monticola*	14.556	26.889	7.857	24.444	19.636	19.778	28.444	**0.009**	6.30%	15.20%
20. *P. maxwelli*	24.222		9.368		82.000			14.000	**0.000**	11.40%
21. *T. spekei*	25.333		13.895		139.000			33.778		**n/c**

Table 1.6. Intra- and inter-specific nucleotide differences between all *COXI* sequences obtained from Cephalophinae species. The lower left triangular matrix depicts the ratio of inter- to average intra-specific distances between species. The upper right triangular matrix represents raw % K2P distance values. The diagonal values in bold depict intra-specific diversity. Cells in grey represent species pairs that fail to meet their respective distance criterion. Explicitly, grey cells in the lower left matrix are values less than 10 and grey cells in the upper right matrix are values 3% or less.

	1	2	3	4	5	6	7	8	9	10	11
1. *C. ogilbyi*	**n/c**	0.80%	0.00%	2.00%	0.50%	5.70%	4.90%	6.90%	5.70%	6.80%	6.40%
2. *C. callipygus*	1.455	**0.01**			0.90%	6.40%	5.60%	7.60%	6.40%	7.50%	6.80%
3. *C. callipygus1*			**0**	2.00%	0.50%	5.70%	4.90%	6.90%	5.70%	6.80%	6.40%
4. *C. callipygus2*	8.000		8.000	**0.01**	1.50%	7.40%	6.50%	8.60%	7.40%	8.50%	7.30%
5. *C. weynsi*		1.636		6.000	**n/c**	5.80%	4.90%	6.90%	5.80%	6.90%	5.80%
6. *C. niger*	2.780	2.462	2.780	3.217	2.829	**0.04**	5.90%	8.60%	6.90%	8.00%	7.50%
7. *C. nigrifrons*	24.500	7.467	24.500	14.444	24.500	2.622	**0.004**	2.60%	3.10%	4.20%	5.40%
8. *C. rufilatus*	4.452	3.619	4.452	4.778	4.452	2.389	1.486	**0.031**	3.60%	4.70%	7.50%
9. *C. natalensis*		11.636		29.600		3.366	15.500	2.323	**0**	1.00%	6.40%
10. *C. harveyi*		13.636		34.000		3.902	21.000	3.032		**n/c**	7.50%
11. *C. leucogaster*	42.667	9.714	42.667	18.250	38.667	3.409	15.429	4.412	42.667	50.000	**0**
12. *S. grimmia*	15.800	7.810	15.800	11.333	16.000	3.373	12.286	5.659	19.400	21.800	15.077
13. *C. jentinki*		15.091		34.800		3.902	37.500	6.710			48.667
14. *C. silvicultor*	6.000	3.935	6.000	5.040	5.800	1.902	5.833	3.843	6.900	8.000	5.391
15. *C. spadix*		10.909		25.600		2.537	32.000	5.935			41.333
16. *C. dorsalis*	162.000	13.833	162.000	28.667	162.000	3.571	30.400	6.563	150.000	172.000	31.000
17. *C. adersi*		13.455		29.600		3.902	32.000	5.226			42.667
18. *C. zebra*		22.727		51.600		5.366	51.500	7.097			66.000
19. *P. monticola*	30.333	11.529	30.333	19.455	30.333	3.745	17.400	5.622	32.667	30.667	21.778
20. *P. maxwelli*		19.455		45.600		4.293	45.000	6.839			53.333
21. *T. spekei*		28.364		65.200		7.220	75.500	10.839			89.333

	12	13	14	15	16	17	18	19	20	21
1. *C. ogilbyi*	7.90%	8.00%	6.00%	5.70%	8.10%	7.40%	12.20%	9.10%	10.30%	15.10%
2. *C. callipygus*	8.20%	8.30%	6.10%	6.00%	8.30%	7.40%	12.50%	9.80%	10.70%	15.60%
3. *C. callipygus1*	7.90%	8.00%	6.00%	5.70%	8.10%	7.40%	12.20%	9.10%	10.30%	15.10%
4. *C. callipygus2*	8.50%	8.70%	6.30%	6.40%	8.60%	7.40%	12.90%	10.70%	11.40%	16.30%
5. *C. weynsi*	8.00%	8.00%	5.80%	5.80%	8.10%	6.90%	12.30%	9.10%	9.70%	14.50%
6. *C. niger*	8.60%	8.00%	5.80%	5.20%	7.50%	8.00%	11.00%	8.80%	8.80%	14.80%
7. *C. nigrifrons*	8.60%	7.50%	7.00%	6.40%	7.60%	6.40%	10.30%	8.70%	9.00%	15.10%
8. *C. rufilatus*	11.60%	10.40%	9.80%	9.20%	10.50%	8.10%	11.00%	10.40%	10.60%	16.80%
9. *C. natalensis*	9.70%	7.50%	6.90%	6.30%	7.50%	7.50%	10.40%	9.80%	9.10%	13.90%
10. *C. harveyi*	10.90%	8.60%	8.00%	7.40%	8.60%	8.60%	11.60%	9.20%	9.70%	15.10%
11. *C. leucogaster*	9.80%	7.30%	6.20%	6.20%	6.20%	6.40%	9.90%	9.80%	8.00%	13.40%
12. *S. grimmia*	**0.01**	7.40%	6.80%	6.30%	8.60%	9.10%	13.50%	12.70%	10.80%	12.00%
13. *C. jentinki*	14.800	**n/c**	4.70%	4.10%	5.30%	8.60%	14.20%	12.20%	9.10%	13.90%
14. *C. silvicultor*	4.533	4.700	**0.02**	1.00%	3.10%	8.00%	11.70%	10.30%	8.00%	13.20%
15. *C. spadix*	12.600		1.000	**0**	3.10%	8.60%	11.70%	10.30%	8.50%	13.90%
16. *C. dorsalis*	15.636	106.000	2.952	62.000	**0.001**	9.90%	11.70%	10.20%	8.50%	15.20%
17. *C. adersi*	18.200		8.000		198.000	**0**	11.70%	8.60%	6.80%	13.90%
18. *C. zebra*	27.000		11.700		234.000		**n/c**	12.20%	11.50%	15.20%
19. *P. monticola*	15.875	40.667	7.923	34.333	29.143	28.667	40.667	**0.006**	5.80%	15.00%
20. *P. maxwelli*	21.600		8.000		170.000			19.333	**0**	11.90%
21. *T. spekei*	24.000		13.200		304.000			50.000		**n/c**

DISCUSSION

The present study set out to evaluate how well DNA barcoding sequence data could be used to delineate African duiker antelope within the subfamily Cephalophinae. This is the first *COX1* barcoding study to include all recognized species within this group. The recent divergence of species within this subfamily (Vrba 1995) presents a challenge to effective species delimitation because recently diverged taxa may fail to constitute reciprocally monophyletic groups. This could be attributed to incomplete lineage sorting and/or ongoing or recent gene flow between sister taxa (Crandall & Fitzpatrick 1996; Funk & Omland 2003; Goodacre & Wade 2001; Renoult et al. 2009).

Tree based analyses of the *COX1* data revealed a lack of monophyly for *C. callipygus*, *C. ogilbyi* and *C. weynsi*, as has been reported in earlier studies using other molecular markers (Jansen van Vuuren & Robinson 2001; Ntie et al. 2010). As has also been observed in other studies based on mitochondrial *COX1* or 12S ribosomal DNA sequences (Eaton et al. 2009; van Vliet et al. 2008), *C. callipygus* here appears to be composed of two distinct sub-clades that are sister to one another. In contrast, control region-based analysis revealed three clades within *C. callipygus*, two of which were sister to one another and a third clade that was basal to all duiker taxa in the phylogeny (Ntie et al. 2010). This phylogenetic pattern has not yet been identified in any other mitochondrial study of this group, including the present study, lending support to Ntie et al.'s (2010) speculation that the basal clade within the control region phylogeny is made up of nuclear integrations of mitochondrial DNA or numts (Lopez et al. 1994).

Neither *C. nigrifrons* nor its closely related sister taxon *C. rufilatus* are reciprocally monophyletic within the *COX1* phylogeny, recapitulating findings from earlier cytochrome *b* and control region analyses of this group (Ntie et al. 2010). However within the control region phylogeny, both species do cluster into statistically supported species-specific clades, making identification of these two species possible. Similarly, the monophyly of *C. niger* and its placement in the phylogenetic tree are supported in the control region analysis, whereas this species forms a non-monophyletic association with unresolved placement in *COX1* phylogenetic analysis. For these reasons, we advocate the use of the control region as the preferred marker for identifying Cephalophinae, especially for those working in areas where *C. nigrifrons* and *C. rufilatus* are sympatric.

The paucity of diagnostic sites in *C. callipygus, C. ogilbyi, C. rufilatus*, and *C. nigrifrons* mirrors the difficulty of differentiating these species using tree-based diagnostic methods and points to difficulties in the implementation of a species barcode based on only pure, simple character states. Furthermore,

ambiguous base calls could further complicate a character-based approach by potentially masking intra-specific variation. Further work should assess whether a diagnostic based on compound characters might represent a viable alternative (DeSalle et al. 2005). Compound character analysis is a relatively under-used method in DNA barcoding and can be implemented in the program Character Attribute Organization System (CAOS, Sarkar et al. 2008). Two previous case studies using CAOS indicate that this method performs poorly on polyphyletic species (Yassin et al. 2010) and can be prone to error (Kerr et al. 2009) making it unlikely to perform well in such a challenging group as the Cephalophinae.

Of the two genetic distance criteria evaluated, the 3% threshold cutoff traditionally used to delineate taxa was far more effective than the so-called 10x rule. The taxa that generally failed to meet both distance criteria were generally species that were sister to one another and often formed non-monophyletic associations in tree-based analyses. Not surprisingly, there is debate over the species status of many taxa in this group, including those that failed to satisfy distance-based DNA barcoding thresholds. *Cephalophus weynsi*, *C. callipygus, C. adersi, C. harveyi,* and *C. natalensis* have all been grouped as a red duiker super-species (Ansell 1971), although the same author has also argued that *C. callipygus*, *C. adersi*, and *C. natalensis* can be distinguished by morphological features. Evidence from the first phylogenetic analysis of the duiker group (Jansen van Vuuren & Robinson 2001) supported the placement of *C. harveyi* as a subspecies of *C. natalensis.* This designation was based on the finding that sequence divergence values between the two taxa were less than values distinguishing subspecies in two other closely related species. This finding is supported in the present study where it was observed that the sequence divergence between *C. harveyi* and *C. natalensis* was less than that observed in nearly all other species pairs (Table 1.5). Similarly, *C. spadix* has been considered a subspecies of *C. silvicultor* (Haltenorth 1963 cited in Grubb 2005) although (Haltenorth & Diller 1977) later treated these two taxa as separate species based on differences in dorsal markings and skeletal structure (Ansell 1971). Lastly, *C. weynsi* has also been considered a subspecies of *C. callipygus* (Kingdon 1997) and again, the low divergence values reported in this study support these hypotheses. In contrast, *C. ogilbyi* has never been merged with either *C. callipygus* or *C. weynsi* (Grubb 2005), yet cannot be readily differentiated using *COX1* data from either of these two allied taxa. Although the sister taxa *C. nigrifrons* and *C. rufilatus* have never been merged, Heyden (1969 cited in Ansell 1971) reported similarities in skeletal proportions between these taxa but nevertheless maintained their distinction on the basis of fur coloration and partial range overlap. As might be the case for *C. ogilbyi* and its allied taxa, the low divergence values observed between *C. nigrifrons* and its sister taxon *C. rufilatus* more likely reflect their recent origin and rapid speciation (Vrba 1995), although the possibility of hybridization in both these sister groups cannot be ruled out.

As the taxonomy of Cephalophinae presently stands, *COX1* barcodes fail to delimit all members of this group. Of those DNA barcoding methods tested here, tree-based analyses are the most effective method for delineating taxa and for identifying samples of unknown species origin. Other methods were less effective, with the 10x threshold rule performing the most poorly. The inability to effectively discriminate some sister-species coupled with a lack of information on range-wide genetic variation in many taxa poses a challenge to the future application of *COX1* barcodes in non-invasive genetic sampling and in the regulation of international trade of duiker meat. Since duiker meat confiscated at the international scale could have potentially originated from any taxa within the Cephalophinae, molecular forensics tools should include complete taxonomic sampling of this group, as has been done for the first time in this study. A comprehensive set of expert-identified reference material should also include a geographically comprehensive representation of sites across species' ranges and not depend exclusively on one locality. While we have tried to maximize geographic representation of samples in the present study, it is important that future reference material include additional sites across the ranges of geographically widespread taxa. Additional sampling of intra-specific variation from unrepresented areas may not only reveal non-monophyletic lineages not captured in the present study but also decrease the number of conserved diagnostic sites that could be used to differentiate taxa, further complicating the application of a simple, character-based diagnostic method for this group. Most importantly, additional sampling of under-represented, problematic taxa, such as *C. weynsi*, *C. ogilbyi* and *C. rufilatus* is needed to unambiguously confirm that the patterns observed in this study and previous studies using the same samples (Jansen van Vuuren & Robinson 2001; Ntie et al. 2010; van Vliet et al. 2008) are not the result of misidentification, mislabeling, or cross-contamination.

Advances in coalescent-based approaches to species delimitation and assignment (Abdo & Golding 2007; Carstens & Dewey 2010; Pons et al. 2006; Yang & Rannala 2010; O'Meara 2010) may be useful to future barcoding efforts, particularly in cases where taxa do not necessarily constitute monophyletic lineages (Knowles & Carstens 2007), as is the case here. Further work using additional nuclear markers is needed to better clarify the taxonomic status of some of the taxa within this group. Findings from such studies may then resolve the taxonomic status of CITES-protected species such as *C. ogilbyi* and help illuminate why some barcoding criteria fail to reliably differentiate species.

CHAPTER TWO: A multi-locus species phylogeny of African forest duikers in the subfamily Cephalophinae: evidence for a recent radiation.

INTRODUCTION

The duikers in the subfamily Cephalophinae (family Bovidae) are a species-rich group of African forest antelope of conservation concern (IUCN 2011). Although there have been several phylogenetic studies of this group, all have relied exclusively on single mitochondrial DNA markers to estimate their genealogical history (Jansen van Vuuren & Robinson 2001; Ntie et al. 2010; Johnston et al. 2011). While valuable in recovering major mitochondrial lineages, these studies have thus far failed to resolve both the deeper nodes in the tree or the placement of several taxa. Furthermore, no study of duiker antelope to date has used nuclear loci to assess relationships, as has been the case for other closely related African artiodactyls (Matthee & Davis 2001; Willows-Munro et al. 2005). Multi-species coalescent theory now provides a powerful computational approach to species tree estimation and can accommodate the conflicting signal that may be present in multiple, unlinked loci (e.g Maddison & Knowles 2006; Carstens & Knowles 2007; Edwards et al. 2007; Liu & Pearl 2007; Liu et al. 2008; Degnan & Rosenberg, 2009). However, these methods have not yet been applied to species rich groups of tropical artiodactyls like the subfamily Cephalophinae. Finally, while there is evidence from the fossil record and species divergence times to support a Late Miocene/Early Pliocene origin of this subfamily (Vrba 1995; Jansen van Vuuren & Robinson 2001), no studies to date have estimated the timing and subsequent radiation of the group. Pleistocene glacial cycling has been implicated in the diversification of many African mammalian taxa (deMenocal 1995; Querouil et al. 2003; Anthony et al. 2007; Trauth et al. 2009; Nicolas et al. 2011; Moodley & Bruford 2007) and might explain the high species diversity of the Cephalophinae.

Currently, three duiker genera are recognized within Cephalophinae: (a) the dwarf duiker genus *Philantomba* (b) the monotypic savanna specialist genus *Sylvicapra* and (c) the forest dwelling duiker genus *Cephalophus.* The placement of dwarf duikers into their own genus is contentious however, and some authors recognize *Philantomba* (Robinson et al. 1996; Jansen van Vuuren & Robinson 2001; Hard 1969; Groves & Grubb 1981; Grubb 2005; Pocock 1910), while others have merged *Philantomba* into *Cephalophus* (Ansell 1971; Nowak 1999). Species within *Philantomba* have been distinguished from *Cephalophus* and *Sylvicapra* on both morphological and molecular grounds. Species in *Philantomba* lack the inguinal glands (Pocock 1910) and true crest (Groves & Grubb 1981) present in species in the genus *Cephalophus*. Their untufted tail, coat color pattern and tendency to show reversal of the neck hair (Groves & Grubb 1981) further distinguish them from other species within the subfamily. At the chromosomal level, *Philantomba* is distinguished from the rest of the subfamily by variation in the

location of the centromere in the X chromosome (Matthee & Robinson 1999) and by their chromosomal banding patterns (Jansen van Vuuren & Robinson 2001). Phylogenies build using mitochondrial sequences further support the monophyly of this genus but provide little support for the proposed basal placement of this genus within the Cephalophinae (Jansen van Vuuren & Robinson 2001; Ntie et al. 2010; Johnston et al. 2011).

The monotypic savanna genus *Sylvicapra* has traditionally been distinguished from other duikers by habitat differences and horn morphology, which are absent in the females but sharply pointed in males (Ansell 1971). However, unlike *Philantomba*, cytogenetic data did not distinguish *Sylvicapra* from *Cephalophus* (Jansen van Vuuren & Robinson 2001). Moreover, mitochondrial gene trees have either placed *Sylvicapra* in a weakly supported position that is basal to the *Cephalophus* genus (Jansen van Vuuren & Robinson 2001), or in within *Cephalophus* (Ntie et al. 2010; Johnston et al. 2011).

Relationships between the major lineages previously recognized within *Cephalophus* are also contentious. Using data from the mitochondrial cytochrome *b* and 12S ribosomal RNA gene, Jansen van Vuuren & Robinson (2001) identified three weakly supported mitochondrial lineages: the giant duikers (*C. silvicultor, C. spadix, C. dorsalis,* and *C. jentinki*), the east African red duikers (*C. leucogaster*, *C. rufilatus*, *C. nigrifrons*, *C. natalensis*, *C. rubidus*, and *C. harveyi*) and the west African red duikers (*C. callipygus*, *C. weynsi*, *C. ogilbyi*, and *C. niger*). These three major lineages were strongly supported in a mitochondrial control region phylogeny (Ntie et al. 2010) but relationships among lineages were unresolved. Furthermore, the position of *C. adersi* and *C. zebra* relative to other taxa remains unresolved and appears to be highly labile among studies (Jansen van Vuuren & Robinson 2001; Ntie et al. 2010; Johnston et al. 2011).

Lastly, mitochondrial gene trees have indicated that several closely related species might be paraphyletic (Ntie et al. 2010; Johnston et al. 2011). These included the *C. callipygus*/*C. ogilbyi*/*C. weynsi* group, the *C. nigrifrons*/*C. rufilatus* group, the *C. natalensis/C. harveyi* group and the *P. monticola/P. maxwelli* group. While it is possible that these paraphyletic relationships could reflect poor taxonomy, their lack of resolution could also be attributed to either incomplete lineage sorting and/or mitochondrial introgression between recently derived taxa.

The goal of the present study is therefore to re-evaluate the evolutionary relationships of species within this group to test the monophyly of *Philantomba* and *Cephalophus*, measure support for the major lineages identified by Jansen van Vuuren & Robinson (2001) and their relationships to one another, and investigate the placement of *C. adersi* and *C. zebra*. This study builds on previous mitochondrial studies (Ntie et al. 2010; Johnston et al. 2011) by combining sequences from two mitochondrial markers with

four additional unlinked nuclear markers. These nuclear markers have previously been used to resolve species and genus level relationships within a group of closely related bovids believed to have undergone a similarly recent and rapid radiation (Willows-Munro et al. 2005). The multi-locus data from this study can also be used to assess whether the paraphyly observed in previous mitochondrial studies may have arisen due to mitochondrial introgression or incomplete lineage sorting. We also compare traditional methods of gene tree estimation based on a concatenated data matrix to species trees estimated using the coalescent-based approach implemented in the program *BEAST (Heled & Drummond 2010). Lastly, we use a fossil calibrated relaxed molecular clock (Drummond et al. 2006) in a Bayesian phylogenetic framework implemented in the program BEAST (Drummond & Rambaut 2007) to estimate divergence times for major lineages within this group and test the hypothesis that speciation of many duikers occurred during the Pleistocene epoch.

MATERIALS AND METHODS

In this study, we used tissue sampled from 28 individuals from Cephalophinae, representing all eighteen species recognized by the International Union for Conservation of Nature (IUCN 2011). These taxa and associated sample information (sample ID, providers, locality when available, and GenBank accession numbers) are listed in Table 2.1. Recent mitochondrial studies have suggested that the klipspringer (*Oreotragus oreotragus*) may be sister to the Cephalophinae (Hassanin & Douzery 1999; Agnarsson & May-Collado 2008). However, nuclear markers (Matthee & Davis 2001) and supertree analysis (Fernandez & Vrba 2005) do not provide support for this relationship, or for any consistent sister group to the Cephalophinae. Given the uncertainty of these relationships, we have included not only *O. oreotragus* but also two other closely related taxa within the subfamily Antelopini, the suni (*Neotragus moschatus*) and Kirk's dik-dik (*Madoqua kirkii*), and two more divergent species within the subfamily Bovinae, the bushbuck (*Tragelaphus scriptus*) and the sitatunga (*T. spekei*).

Table 2.1. Species, sample ID, country of origin where known, material donor, and GenBank accession numbers of all samples and sequences used in the present study. Highlighting of accession number corresponds to initial publication as follows: pink in Ntie et al. 2010; blue in Johnston et al. 2011; orange in Colyn et al. 2010; white in Jansen van Vuuren and Robinson, 2001; red in Matthee and Robinson 1999; yellow in Matthee et al. 2001; green in present study.

Species	Sample ID	Locality	Material Donor	cyt*b*	*COX1*	MGF	PRKCl	SPTBN	THY
C. adersi	VV20	Tanzania	B. Jansen van Vuuren	FJ807616	HQ644087	JN645565	JN645603	JN645639	JN645661
C. callipygus	N22138	Congo	D. Pires	FJ807573	HQ644090	JN645551	JN645589	JN645634	JN645647
C. callipygus	OK27	Gabon	S. Touladjan	FJ807620	HQ644089	JN645563	JN645601	JN645623	JN645659
C. dorsalis	OR761		San Diego Zoo	FJ807588	HQ644091	JN645548	JN645586	JN645614	JN645644
C. dorsalis	N221022	Congo	D. Pires	FJ807577	HQ644092	JN645552	JN645590	JN645616	JN645648
C. harveyi	VV117	Tanzania	B. Jansen van Vuuren	FJ807623	HQ644093	JN645569	JN645607	JN645626	JN645665
C. harveyi	AB05		A. Bowkett	FJ959388	JN645580	JN645573	JN645612	JN645631	JN645669
C. jentinki			Gladys Porter Zoo	JN645578	HQ644094	JN645577	JN645611	JN645627	JN645670
C. leucogaster	N22151	Congo	D. Pires	FJ807578	HQ644098	JN645553	JN645591	JN645635	JN645649
C. natalensis	VV1467	S. Africa	B. Jansen van Vuuren	FJ807610	HQ644104	JN645571	JN645609	JN645629	JN645667
C. natalensis	VV1470	S. Africa	B. Jansen van Vuuren	FJ807611	HQ644103	JN645572	JN645610	JN645630	JN645668
C. niger	OR2758	Liberia	San Diego Zoo	FJ807621	HQ644106	JN645561	JN645599	JN645621	JN645657
C. nigrifrons	N221004	Congo	D. Pires	FJ807572	HQ644108	JN645550	JN645588	JN645633	JN645646
C. nigrifrons	VV24	Congo	B. Jansen van Vuuren	FJ807627	HQ644107	JN645567	JN645605	JN645625	JN645663
C. ogilbyi	FR07	Gabon	S. Touladjan	FJ807618	HQ644109	JN645560	JN645598	JN645620	JN645656
C. rufilatus	VV22	CAR	B. Jansen van Vuuren	FJ807626	HQ644111	JN645566	JN645604	JN645640	JN645662
C. rufilatus	OR2115		San Diego Zoo	FJ807586	HQ644110	JN645555	JN645593	JN645617	JN645651
C. rubidus			Genbank	AF153900					
C. silvicultor	N220853	Congo	D. Pires	FJ807579	HQ644112	JN645549	JN645587	JN645615	JN645645
C. silvicultor	OR356	Liberia	San Diego Zoo	FJ807622	HQ644113	JN645562	JN645600	JN645622	JN645658
C. spadix	VV126	Tanzania	B. Jansen van Vuuren	FJ807608	HQ644115	JN645570	JN645608	JN645628	JN645666
C. weynsi		Rwanda	B. Jansen van Vuuren	FJ807614	HQ644116				
C. zebra	OR25470	Liberia	San Diego Zoo	FJ807601	HQ644117	JN645557	JN645595	JN645637	JN645653
P. walterii	BE52		GenBank	HM144009	HM144019				
P. walterii	BE118		Genbank	HM144014	HM144017				
P. walterii	BE119		GenBank	HM144013	HM144018				
P. maxwelli	OR837		San Diego Zoo	FJ807603	HQ644100	JN645558	JN645596	JN645638	JN645654
P. maxwelli	OR587013		San Diego Zoo	FJ807602	HQ644099	JN645559	JN645597	JN645619	JN645655
P. monticola	KB15149		San Diego Zoo	FJ807619	HQ644101	JN645564	JN645602	JN645624	JN645660
P. monticola	DKME1	Gabon	S. Touladjan	FJ807585	HQ644102	JN645554	JN645592	JN645636	JN645650
S. grimmia	VV26	CAR	B. Jansen van Vuuren	FJ807613	HQ644119	JN645568	JN645606	JN645641	JN645664
S. grimmia	OR1786		San Diego Zoo	FJ807613	HQ644118	JN645556	JN645594	JN645618	JN645652
M. kirkii	Sun71		B. Jansen van Vuuren	AF022070	JN645582	JN645574	AF165757	AF165758	AF165761
N. moschatus	Sun108		B. Jansen van Vuuren	FJ959387	JN645581	JN645575	AF210192	AF210214	AF210236
O. oreotragus	Sun266		B. Jansen van Vuuren	AF022052	JN645583	JN645576	AF210193	AF210215	AF210237
T. scriptus	T3553		Yoshan Moodley	FJ807604	JN645579	JN645547	JN645585	JN645613	JN645643
T. spekei	DKME 52	Gabon	S. Touladjan	FJ807594	HQ644120	JN645546	JN645584	JN645632	JN645642

Samples were either obtained from bushmeat market surveys conducted in collaboration with the Wildlife Conservation Society (WCS) in Gabon, or donated by zoos and other researchers (Table 2.1). With the exception of the easily distinguishable *P. monticola* and *T. spekei*, a photographic record was used to verify the species identity of all WCS collected bushmeat samples. Species records accompanied tissue samples of several species obtained from the San Diego Zoo and a fecal sample taken from *C. jentinki* at Gladys Porter Zoo. Details for all remaining samples are found in Jansen van Vuuren & Robinson (2001), Matthee & Davis (2001), Bowkett et al. (2008), Ntie et al. (2010) and Johnston (2011).

DNA from all bushmeat and some San Diego Zoo tissues was extracted using a standard phenol-chloroform extraction method (Sambrook & Russell 2001). DNA provided by Jansen van Vuuren was extracted according to the methods described in Jansen van Vuuren & Robinson (2001). Other samples provided by the San Diego Zoo were obtained as genomic DNA extracts. The *C. jentinki* fecal sample was extracted using the QIAamp DNA Stool Minikit (Qiagen) in a designated room and a blank was included to control for DNA contamination. The sample AB05 was extracted from blood using a salt-based extraction method (Aljanabi & Martinez 1997).

Portions of two coding mDNA genes were included in phylogenetic analyses: 514 bp of the cyt*b* gene and 658 bp of the cytochrome *c* oxidase subunit 1 (*COX1).* GenBank accession numbers for all previous studies and the current work are listed in Table 2.1. Most (n=36) of the cyt*b* sequences were previously published in Colyn et al. (2010), Jansen van Vuuren & Robinson (2001), Matthee & Robinson (1999) and Ntie et al. (2010). All Genbank sequences were trimmed to match the region used by Ntie et al. (2010). One sample of *C. jentinki* was amplified according to published primers and protocols (Ntie et al. 2010). Most (n=31) of the *COX1* sequences were previously published in Colyn et al. (2010) and Johnston (2011) and an additional five samples were amplified according to published protocols (Johnston et al. 2011). Four nuclear DNA markers were also amplified and sequenced using published primers and PCR conditions (Matthee & Davis 2001) that span introns within four genes: stem cell factor (MGF), protein-kinase-CI (PRKCl), B-spectrin non-erythrocytic (SPTBN1) and thyrotropin (THY). Internal primers were designed and used to amplify smaller fragments for samples that were highly degraded or difficult to amplify (Table 2.2). PRKCl, SPTBN1 and THY sequences for outgroup taxa *M. kirkii, N. moschatus*, and *O. oreotragus* were obtained from Matthee et al. (2001). Following amplification, all PCR products were purified using ExoAp (Glenn & Schable 2005) and then sequenced on both strands using the BigDye Terminator Cycle Sequencing Kit v1.1 (ABI). Resulting products were run on a 3100 ABI automated DNA sequencer.

Table 2.2. Internal primers designed to work in combination with the primers and protocols designed by Matthee et al. 2001 (indicated by an asterisk) to amplify overlapping fragments for degraded samples, using a modified [Mg 2+] and annealing temperature.

Primer	Target region	Sequence	Primer pair	[Mg^{2+}] (mM)	Annealing temperature (°C)
MGF_FF	Stem cell factor	5'-CCAGTCTTCTTAATGTCCC-3'	FF/RD*	2	54
MGF_RG	Stem cell factor	5'-GAAGATGAGAATTCATAACTC-3'	FE*/RG	2	51
PRKCl_FE	Protein-kinase-C	5'-TAAGATATATTTAAAGTTGTCTG-3'	FE/RD*	2.5	50
PRKCl_RG	Protein-kinase-C	5'-AAAGCTCAAGTACTCTCCC-3'	FC*/RG	2.5	52
THY_FC	Thyrotropin	5'-TGAGTATACGATGCATATCG-3'	FC/RG	2.5	54
THY_RG	Thyrotropin	5'-GAGATAAAAGTGACACTCCC-3'	FC/RG	2.5	54
SPTBN_FF	B-spectrin non-erythrocytic	5'-GCAGTGAACTTTGCCAGAC-3'	FF/RB*	2.5	56
SPTBN_RG	B-spectrin non-erythrocytic	5'-CTGAACCCGCACAAAACC-3'	FA*/RG	2.5	54

Forward and reverse sequences were edited using the program SEQUENCHER v4.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA). For nuclear loci, heterozygous individuals were verified by the presence of two similarly sized peaks in both sequencing directions and were coded using standard IUPAC ambiguity codes. Nuclear haplotypes were reconstructed manually for sequences with one heterozygous site. In cases of multiple heterozygous sites, the program PHASE v2.1 (Stephens et al. 2001) was used to reconstruct haplotypes with 0.95 or greater posterior probability. Sequences for each marker were aligned using the default settings of Clustal X v2.06 (Thompson et al. 1997).

The incongruence length difference (ILD; Farris et al. 1995) test implemented in PAUP* was used to evaluate incongruence between individual nuclear trees and between mitochondrial and nuclear phylogenies. These ILD tests used 1,000 randomizations and a heuristic search on each randomization to obtain the sum of tree lengths for each partition.

The models of nucleotide substitution that best fit the data were selected by jModelTest (Guindon & Gascuel 2003; Posada 2008) under the Bayesian information criterion (BIC; Schwarz 1978). Gene trees were estimated for each nuclear locus and the combined mitochondrial loci using maximum parsimony (MP), maximum likelihood (ML), and Bayesian (BA) methods (see below). Additionally, nuclear loci were concatenated with and without mitochondrial sequences into a single data matrix for species tree estimation using MP, ML, and BA methods. Nuclear sequences were not available for *C. weynsi, C. rubidus* and *P. walterii*.

All MP analysis were performed in PAUP* vers. 4.0b10 for UNIX (Swofford 2000). For each analysis, preliminary maximum parsimony searches were conducted using heuristic search methods with tree bisection reconnection (TBR) branch swapping, collapse of zero-length branches, all characters weighted equally, and 100 replicates of the random addition starting tree option. A nonparametric bootstrap test (Felsenstein 1985) was carried out using 300 replicates of these heuristic search settings. The "Max Trees" was set to 50,000 for both initial searches and for the bootstrap tests.

Maximum likelihood analyses using a single model of nucleotide substitution for individual genes and concatenated matrices were performed in PAUP* vers. 4.0b10 for UNIX. Heuristic searches were carried out using the TBR branch swapping algorithm, collapsing zero-length branches and using 100 replicates of the random addition option for the starting tree. Nonparametric bootstrap values were calculated from a consensus of the 300 replicate searches.

Two additional ML searches were conducted in RAxML vers. 7.0.4 (Stamatakis 2006) in which regions within the character matrix were partitioned, allowing genes to be assigned independent models of nucleotide substitution. The nuclear data were analyzed with each gene assigned its own partition and an additional analysis was preformed in which the mitochondrial data was included as an additional partition. Within each heuristic search, 500 discrete starting trees were used and a bootstrap consensus tree was estimated from the resulting trees. Each search used a GTR model of nucleotide substitution with the gamma model of rate heterogeneity initiated from a complete random starting tree. Model parameters were optimized to a likelihood difference of 0.00001. Each bootstrap analysis was repeated twenty times to explore tree space and ensure that each analysis converged on a similar likelihood score.

Bayesian analyses were carried out using the Metropolis-coupled Markov chain Monte Carlo methods implemented in MrBayes vers. 3.1.2 (Ronquist & Huelsenbeck 2003). Each analysis included two independent, simultaneous replicate runs. Each run consisted of four chains, one of which was the 'cold' chain and three of which were the chains heated according to the default heating method parameters of MrBayes. Each chain was run for up to 50 million generations, initiated from a random

starting tree. The chain was sampled every 1,000 generations for a total of up to 50,001 tree samples per run. Convergence was determined if the standard deviation of split frequencies between simultaneous runs was less than 0.01 as calculated by MrBayes. Additionally, trace files were evaluated with the program Tracer vers. 1.5 (Drummond & Rambaut 2007) and 10% of points collected prior to chain stationarity were discarded as burn-in. The parameter and tree samples from the two simultaneous runs were combined and summarized using the sump and sumt commands, respectively. For the first set of runs, BA searches assumed a single model of nucleotide substitution across the dataset. A second analysis was carried out in which nuclear genes were partitioned to allow each gene to be assigned its own model of nucleotide substitution. This analysis was repeated with the mitochondrial DNA included as an additional partition.

Bayes Factor (BF) analysis was used to investigate the effects of partitioning on the Bayesian analysis. Following Brandley et al. (2005), two times the natural logarithm of the Bayes Factor was calculated as $2 \ln BF_{(21)} = 2[\ln(hm_2)-\ln(hm_1)]$; where hm_2 and hm_1 are the harmonic means of the post-burn-in likelihood values for the partitioned and un-partitioned analyses, respectively as estimated using the sump command in Mr. Bayes. The threshold of $2 \ln BF > 10$ was taken as strong evidence for the partitioned model (Kass & Raftery 1995).

A multi-locus species tree was estimated with *BEAST (Heled & Drummond 2010) using the phased nuclear data with and without the concatenated mitochondrial data. *BEAST uses a multi-species coalescent framework to model lineage sorting and directly estimates the species tree using multi-locus data. The program was run with default settings, except where specified below. A relaxed uncorrelated lognormal clock model and a Yule tree prior was used in all *BEAST runs. For each analysis, we conducted two independent *BEAST runs for 50 million generations, sampling every 1000 generations. Convergence was determined when the combined independent chains yielded posterior probability effective sample sizes (ESS) greater than 200, calculated in Tracer vers. 1.6.1 (Drummond & Rambaut 2007). After examining trace files, the first 25% of the samples were discarded as burn-in. The remaining 37,501 samples from each run were combined in the program Logcombiner vers. 1.6.1 (Drummond & Rambaut 2007) for a total of 75,002 sample genealogies per analysis. Tree Annotator vers. 1.6.1 (Drummond & Rambaut 2007) was used to summarize the trees into a single maximum clade credibility tree.

Divergence times and tree topology were simultaneously estimated using the program BEAST vers. 1.6.1 (Drummond & Rambaut 2007). BEAST analyses were run with and without the mitochondrial

data because ILD tests indicate conflicting signal between nuclear and mitochondrial genomes. PAUP* was used to determine if a molecular clock hypothesis could be rejected for each locus.

Radiometrically dated fossil remains suggest that the earliest appearance of this group was between 6.31 – 5.65 million years ago mya (Vrba 1995). Secondly, Jansen van Vuuren & Robinson (2001) estimated the oldest speciation event within Cephalophinae at 5.3 million years (± 53,434 years), using a cyt*b* molecular clock calibration for the family Bovidae (Matthee & Robinson 1999). From this information, the prior on the age of the node uniting all taxa within the Cephalophinae was set as a lognormal distribution with an offset of 5.3 mya, a log mean of 0.32 and log standard deviation of 1 such that 95% of the prior probability encompassed the timeframe suggested by fossil and molecular evidence. We unlinked the substitution models across nuclear genes, but left the mitochondrial genes linked. Because a molecular clock hypothesis could be rejected for all loci (MGF: c^2= 150.96398, PRKCl: c^2= 60.019, STBN1: c^2= 115.41636, THY: c^2= 128.10964, mt: c^2= 223.16424, d.f.=30, $p < 0.05$), we used a relaxed, uncorrelated lognormal clock model and a Yule tree prior as implemented by the program. All other priors were left at their default settings. Two independent MCMC chains were run for 10 million generations and sampled every 1000 states, after which convergence was determined when the combined independent chains yielded posterior probability effective sample sizes (ESS) greater than 200 and by examining combined traces in Tracer. After examining trace files, the first 25% of the samples were discarded as burn-in and the remaining 7,501 samples from each run were combined in Logcombiner for a total of 15,002 sample genealogies per analysis. Tree Annotator summarized the trees into a single maximum clade credibility tree.

RESULTS

The final aligned data matrix contained four unlinked nuclear DNA regions and two mitochondrial DNA regions for a total of 4152 characters, of which 1172 were from mitochondrial and 2980 were from nuclear loci. Results from jModeltest for the best model of nucleotide substitution for each of the five partitions and the combined datasets are found in Table 2.3, along with the aligned sequence length, unaligned average length, percent variable sites, number of parsimony informative characters, consistency index (CI) and retention index (RI) values. As expected, the mitochondrial partition contained a greater proportion of variable sites (38%) relative to the nuclear matrix (15%). The CI and RI values in the mitochondrial partition (CI: 0.417, RI:0.621) are lower than those of the nuclear matrix (CI: 0.823, RI: 0.846), indicating higher levels of homoplasy in the mitochondrial dataset.

Table 2.3. Patterns of sequence variability among individual mitochondrial and nuclear markers, combined mitochondrial genes, combined nuclear loci, and combined mitochondrial and nuclear loci. These data comprise the number and percent of variable sites, the number and percent of parsimony informative characters (PIC), the consistency (CI) and retention (RI) indices, and the suggested model of nucleotide substitution, as selected under the Bayesian information criterion (BIC). GTR, HKY, G and I represent the General Time Reversible model, the Hasegawa-Kishino-Yano model, the alpha shape parameter describing gamma distributed rate variation, and a proportion of invariant sites, respectively.

Marker	Aligned length	Average unaligned length (Range)	Variable sites (%)	PIC (%)	CI, RI	BIC model
MGF	759	696 (617-727)	137 (18)	64 (8)	0.85, 0.871	HKY+G
PKRCl	552	535 (469-548)	88 (16)	54 (10)	0.892, 0.884	HKY+G
SPTBN1	894	843 (508-871)	130 (15)	64 (7)	0.873, 0.912	HKY+I
THY	775	694 (372-770)	104 (13)	46 (6)	0.891, 0.912	HKY+G
Mitochondrial	1172	1117 (514-1172)	447 (38)	368 (31)	0.417, 0.621	HKY+G+I
Nuclear	2980	2770 (2158-2895)	459 (15)	208 (7)	0.823, 0.846	HKY+G

The results of the pair-wise ILD tests reject hypotheses that the four nuclear genes recover the same topology ($p > 0.10$). The nuclear gene genealogies estimated using MP, ML and BA methods also recovered different topologies across individual nuclear loci (Figure 1), but were largely congruent across methods. Although there was generally little support for most of the branches within the individual nuclear genealogies, all of the nuclear genes significantly support the monophyly of the dwarf genus *Philantomba* (MGF: MP bootstrap=95, ML bootstrap=95 BA posterior probability=1.0; PRKCl: MP=96, ML=100, BA=1.0; STBN1: MP=97, ML=97, BA=1.0; THY: MP=83, ML=87, BA=1.0). The savanna genus *Sylvicapra* was sister to some or all of the giant duikers in three of the four nuclear trees (MGF: MP=66, BA=0.52; PRKCl: ML=54, BA=0.61; STNB1: MP=60, ML=56, BA=0.97) but this relationship lacked significant support. Within the forest genus *Cephalophus*, support was generally weak or lacking for the giant, east and west African red duiker lineages described by van Vuuren and Robinson (2001). The STBN1 genealogy supported the monophyly of the west African red duiker lineage (MP=85, ML=88, BA=1.0). The THY genealogy recovered the giant duiker lineage (MP=84, ML=85, BA=0.99). The position of *C. adersi* and *C. zebra* varied across genealogies and remained unresolved or weakly supported, with the exceptions of the MGF genealogy which supported *C. zebra* as basal to the *C. jentinki/C. dorsalis* clade (MP=94, ML=92, BA=1.0) and the THY genealogy that supported *C. adersi* as basal to the east and west African red duikers (MP=82, ML=80, BA=1.0).

Figure 2.1. Majority-rule consensus tree showing the Bayesian estimate of nuclear gene trees for a) MGF, b) PRKCl, c) SPTBN and d) THY. Thickened branches indicate support by both BA posterior probability (PP) values ≥0.95 and ML bootstrap support (BS) ≥ 75. Table 2.4 lists support values by node for this phylogeny.

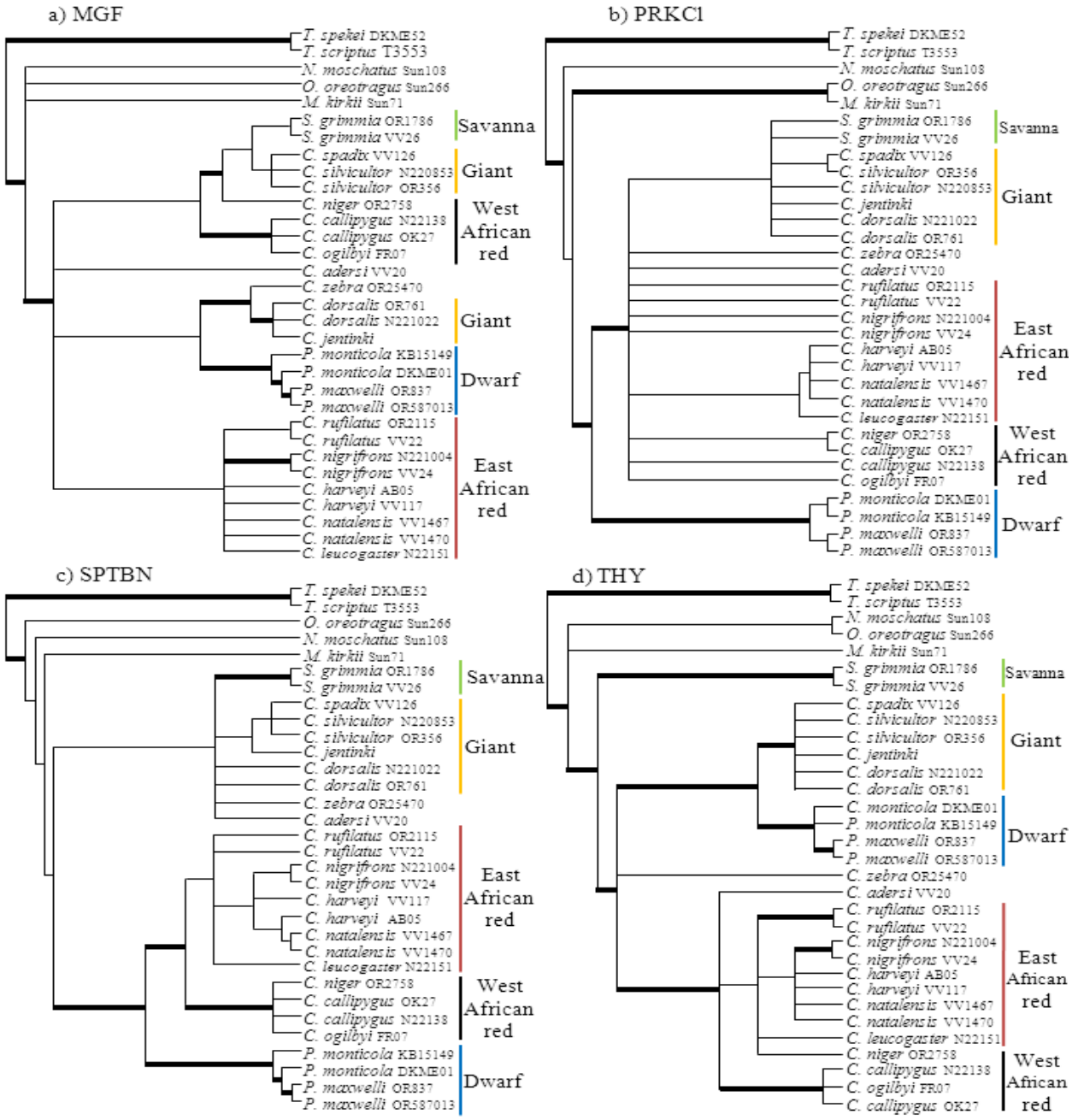


Table 2.4. Posterior probabilities (PP) or bootstrap support (BS) for the branches in Figures 1, 2, 3 and 4. Values in columns A are BEAST PP/*BEAST PP. Values in columns B are un-partitioned BA PP/partitioned BA PP. Values in columns C are un-partitioned ML BS/partitioned ML BS (MP BS). Values in columns D are BA PP/ML BS/MP BS. Values in bold are PP ≥ 0.95 or BS ≥ 75.

	Total Evidence			Mitochondrial	Nuclear			MGF	PRKCl	STBN1	THY
Branch Description	A	B	C	D	A	B	C	D	D	D	D
Stem	1.00/NA				**1.00/1.00**						
Unites Antelopinae and Cephalophinae	0.79/.055	**1.00/1.00**	**100/100 (100)**	**1.00/100/100**	0.54/0.72	**1.00/1.00**	**100/100 (100)**	**1.00/100/100**	**1.00/99/99**	**1.00/100/100**	**1.00/100/100**
Unites *Madoqua*, *Oreotragus* and Cephalophinae	**1.00/1.00**	0.93/**1.00**	**75/78** (39)	**1.00**/79/NA	0.46/0.93	0.50/0.49	40/59 **(80)**	0.94/**87/89**			
Unites *Madoqua* and Cephalophinae	0.64/0.49	0.84/0.57	62/63 (NA)			0.51/0.56	52/65 (NA)			**0.99**/72/72	
Unites Cephalophinae	**1.00/1.00**	**1.00/1.00**	**100/100 (100)**	**1.00/91/77**	**0.98/1.00**	**1.00/1.00**	**100/100 (100)**	**1.00/84**/72	**1.00/81/83**	0.93/51/50	**1.00/91/90**
Unites *Cephalophus* and *Sylvicapra*	**1.00/0.97**	**1.00/1.00**	**76/86 (77)**	**1.00/91**/68	**0.96**/0.65	0.87/0.83	26/46 (41)				
Unites *C. zebra*, savanna and giant duikers	0.68/0.74	0.40/0.47	23/NA (NA)		NA/0.70						
Unites *C. adersi,* east and west African red duikers	**0.99**/0.72	**1.00/0.98**	73/65 (61)	0.93/**1.00**	0.93/0.74	0.93/0.91	59/39 (60)				**1.00/80/82**
Unites giant and savanna duikers	**0.96/0.97**	0.81/**0.95**	50/80 (38)	0.84/58/32	NA/0.93				0.61/54/45		
Unites east and west African red duikers	**1.00/0.95**	**1.00/1.00**	**98/100 (95)**	0.94/58/52	**1.00**/0.82	**1.00/1.00**	**98/99 (95)**			**1.00/96/96**	
Unites *Philantomba*	**1.00/1.00**	**1.00/1.00**	**100/100 (100)**	0.76/53/**88**	**1.00/1.00**	**1.00/1.00**	**100/100 (100)**	**1.00/95/95**	**1.00/100/96**	**1.00/97/97**	**1.00/87**/83
Unites west African red duikers	**1.00/0.99**	**1.00/1.00**	**100/98 (96)**	**0.99/83/87**	**1.00**/0.93	**1.00/1.00**	**75/86** (67)			**1.00/88/85**	
Unites giant duikers	**1.00/0.99**	**1.00/1.00**	**95/99 (98)**	**1.00/97/98**	NA/0.75						**0.99/85**/84
Unites *Tregalophini*	**1.00/1.00**	**1.00/1.00**	**100/100 (100)**	**1.00/100/100**	**1.00**/NA	**1.00/1.00**	**100/100 (100)**	**1.00/100/100**			
Unites east African red duikers	**1.00/1.00**	**1.00/1.00**	**99/100 (99)**	**1.00/98/93**	**0.99/1.00**	**1.00/1.00**	**91/98 (91)**	0.91/59/56		0.77/63/74	
Monophyly of *P. monticola*	**1.00**/NA	0.73/0.57	57/48 (62)	0.92/61/62					**0.99**/44/51		
Unites *C. jentinki* and *C. dorsalis*	**1.00**/0.48	**1.00/1.00**	**92/99 (97)**	0.60/61/41	**1.00**/0.50	**1.00/1.00**	**97/98 (99)**	**0.99/90/93**			
Unites east African red duikers less *C. leucogaster*	**1.00/0.99**	**1.00/1.00**	**99/99 (100)**								

Table 2.4 continued

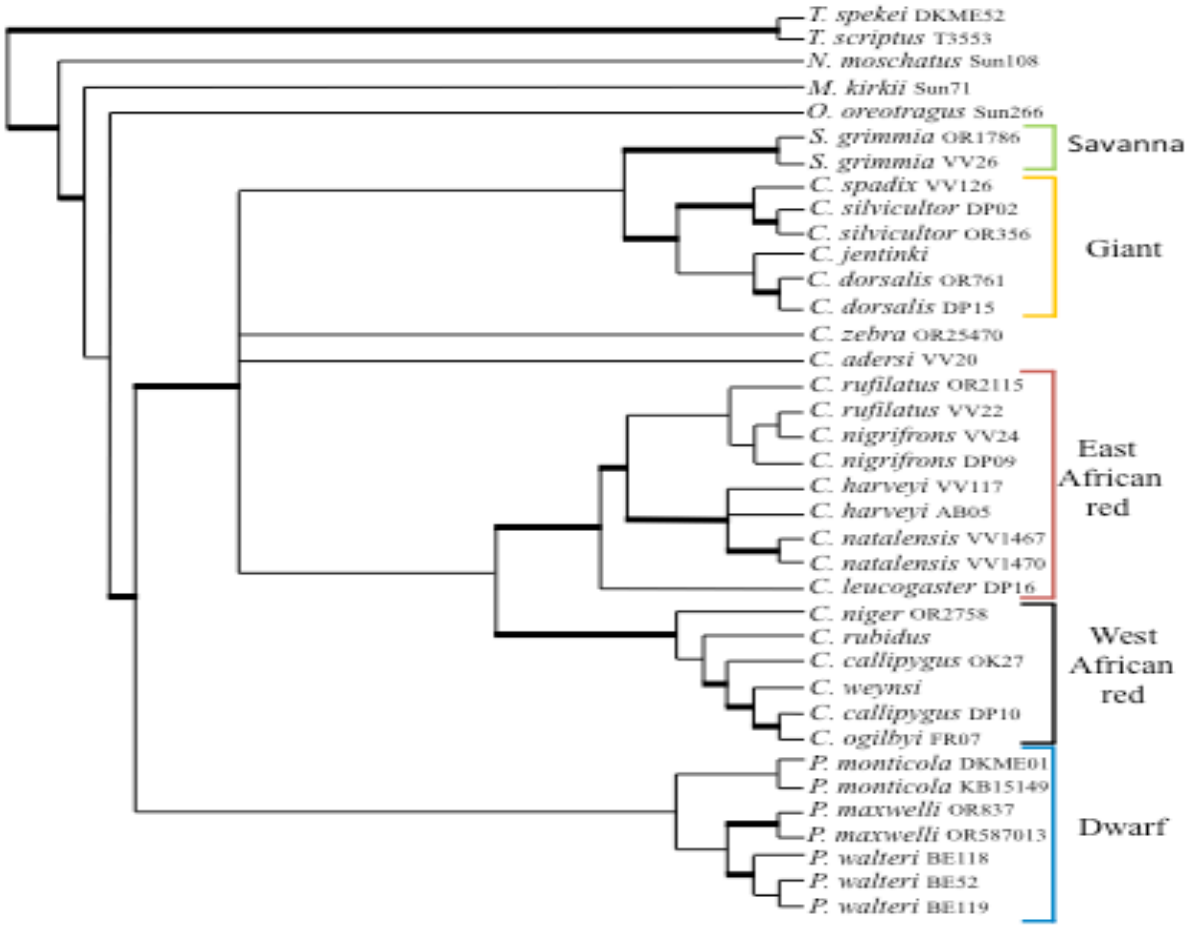
	Total Evidence			Mitochondrial	Nuclear			MGF	PRKCl	STBN1	THY
Branch Description	A	B	C	D	A	B	C	D	D	D	D
Unites *C. nigrifrons* and *C. rufilatus*	0.92/**1.00**	**0.99**/0.90	72/**80** (69)	0.86/52/63							
Unites *C. spadix* and *C. silvicultor*	**1.00/1.00**	**1.00/1.00**	**100/100 (100)**	**1.00/99/100**	**1.00/1.00**	**1.00/1.00**	**99/99 (99)**	0.52/46/60		0.57/29/35	
Unites *C. callipygus* and *C. ogilbyi*	**1.00/1.00**	**1.00/1.00**	**100/100 (100)**	**1.00/100/100**	**1.00/1.00**	**1.00/1.00**	**99/100 (98)**	**1.00/99/98**			**0.96**/54/58
Monophyly of *S. grimmia*	**1.00**/NA	**1.00/1.00**	**100/100 (100)**	**1.00/100/100**	**1.00**/NA	**1.00/1.00**	**100/100 (100)**	**0.95**/66/**76**		**0.99/83/79**	**1.00/99/89**
Paraphyly of *C. rufilatus* to *C. nigrifrons*	**1.00**/NA	**1.00**/NA	71/69 **(80)**	0.87/44/59							
Monophyly of *C. dorsalis*	**1.00**/NA	**1.00/1.00**	**100/100 (100)**	**1.00/100/100**	0.68/NA	0.56/0.56	**84/91** (NA)				
Unites *C. harveyi* and *C. natalensis*	**1.00/1.00**	**1.00/1.00**	**96/99 (95)**	**1.00/91/90**	**0.99/0.99**	**0.99/0.99**	**66/88 (79)**				
Paraphyly of *C. harveyi* to *C. natalensis*	0.66/NA	0.50/NA	29/48 (62)		0.52/NA	0.70/0.71	54/69 (49)			0.55/38/49	
Monophyly of *C. silvicultor*	**1.00**/NA	**1.00/0.99**	**83/78 (99)**	**1.00/99/99**							
Monophyly of *C. nigrifrons*	**1.00**/NA	**1.00/1.00**	**90/98 (83)**		**1.00**/NA	**1.00/1.00**	**99/100 (99)**	**1.00/97/94**		**0.98**/63/62	**0.95**/66/66
Monophyly of *C. maxwelli*	**1.00**/NA	**1.00/1.00**	**100/100 (100)**	**1.00/100/100**	**1.00**/NA	**1.00/1.00**	**100/100 (100)**	**1.00/98/98**	0.64/60/42	**1.00/99/99**	0.91/58/70
Paraphyly of *C. callipygus* to *C. ogilbyi*	**1.00**/NA	**1.00/1.00**	**97/97 (98)**	**1.00/87/99**							
Monophyly of *C. natalensis*	**1.00**/NA	**1.00/1.00**	**99/100 (99)**	**1.00/97/98**	0.94	0.70/0.69	34/91 (63)			0.78/68/56	
Unites *O. oreotragus* and Cephalophinae				0.74/51/NA							
Unites east African red duikers less *C. leucogaster*				**1.00/94/100**							
Paraphyly of *C. nigrifrons* to *C. rufilatus*				0.89/52/75							
Unites *C. nigrifrons*, *C. harveyi*, and *C. natalensis*					0.91/0.69	0.93/0.96	50/59 (57)			**0.99**/63/58	0.86/47/41
Unites *C. zebra*, *C. jentinki*, and *C. dorsalis*					**1.00**	**1.00/0.98**	59/70 **(77)**	**1.00/92/94**			
Unites *C. silvicultor*, *C. spadix*, *S. grimmia* and red duikers					0.53	0.56/0.52	13/4 (26)				

Table 2.4 continued

	Total Evidence			Mitochondrial	Nuclear			MGF	PRKCl	STBN1	THY
Branch Description	A	B	C	D	A	B	C	D	D	D	D
Unites *C. silvicultor*, *C. spadix* and *S. grimmia*					**1.00**	**1.00/0.98**	66/**75 (75)**	0.52/47/66			
Paraphyly of *C. silvicultor* to *C. spadix*					0.94	**0.98/0.97**	66/70 (63)		0.82/47/38		
Monophyly of *C. rufilatus*					**1.00**	**1.00/1.00**	**82/95 (86)**	0.88/**97**/21			**0.96**/69/64
Paraphyly of C. monticola to *C. maxwelli*					0.86	**0.98/0.97**	69/72 (67)	**0.97/78/86**		**0.99/87/90**	
Unites *N. moschatus* and *O. oreotragus*					0.69/NA						0.73/61/49
Unites *C. zebra*, *C. jentinki*, *C. dorsalis*, and *Philantomba*								0.69/38/42			
Unites *C. niger*, *C. spadix*, *C. silvicultor*, and *S. grimmia*								**0.99/75**/63			
Paraphyly of *C. callipygus* to *C. niger*									0.88/67/66		
Unites *O. oreotragus* and *M. kirkii*									**0.99/93/96**		
Unites *N. moschatus*, *M. kirkii* and Cephalophinae										0.63/67/63	
Unites *Sylvicapra*, giant duikers, *C. adersi* and *C. zebra*										**0.97**/56/60	
Unites *C. jentinki*, *C. silvicultor*, and *C. spadix*										0.67/54/50	
Unites *Philantomba* and red duikers										**1.00/76**/73	
Unites *Philantomba* and *Cephalophus*											**1.00/90/89**
Unites giant duikers and *Philantomba*											**1.00/93/89**
Unites east African red duikers and *C. niger*											0.92/61/64

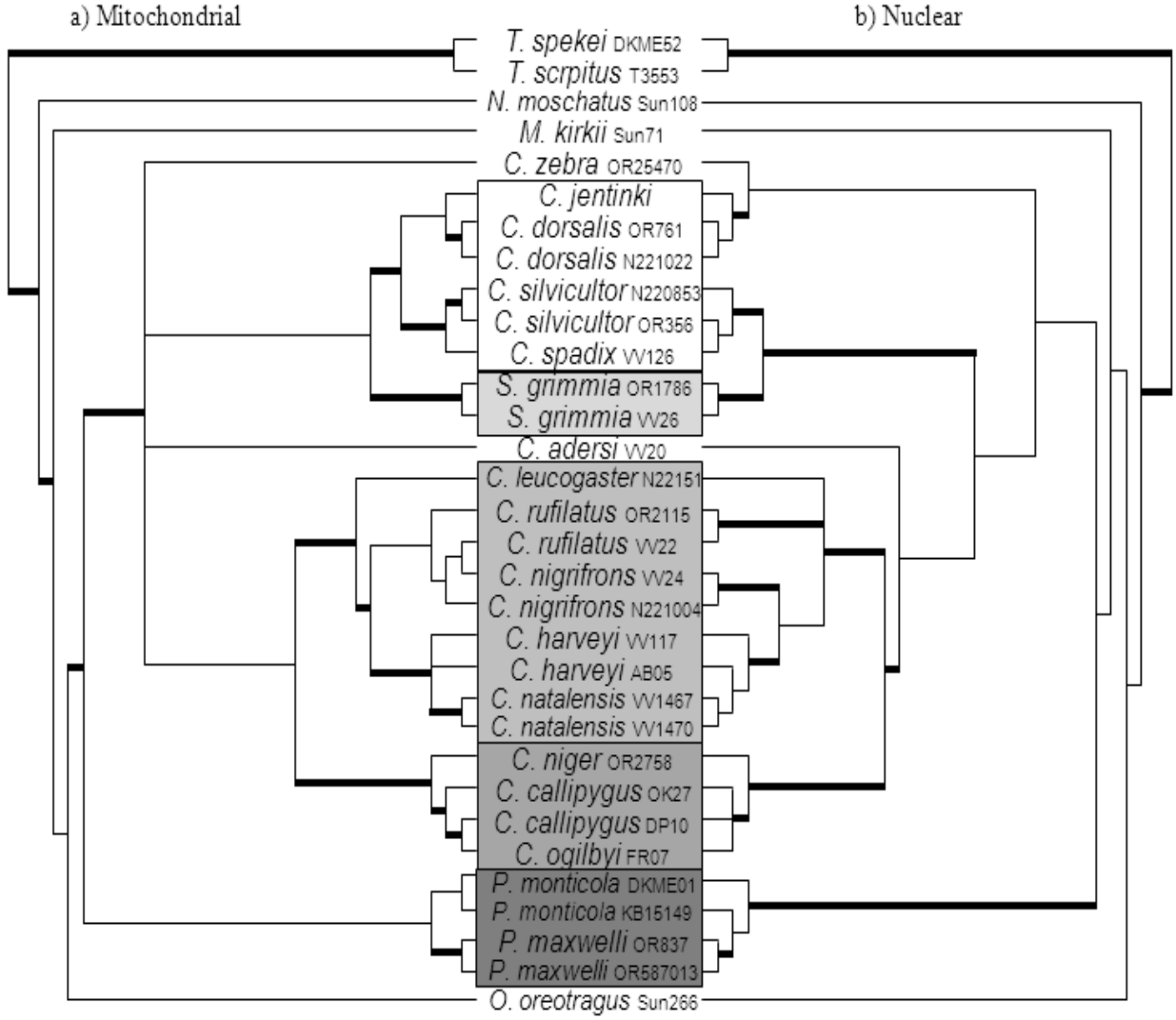
Figure 2.2 illustrates a complete mitochondrial phylogeny of all species within Cephalophinae. The mitochondrial tree has weak support for the monophyly of *Philantomba* (MP=88, ML=53, BA=0.76), but has strong support for the placement of these taxa as basal to all other taxa in the Cephalophinae (MP=68, ML=91, BA=1.0). *Sylvicapra* is sister to the giant duikers, although this branch has weak support (MP=32, ML=58, BA=0.84). Within *Cephalophus*, there is strong support for the monophyly of the giant duikers (MP=98, ML=97, BA=1.0), the east African red duikers (MP=93, ML=98, BA=1.0), and west African red duikers (MP=87, ML=83, BA=0.99), but weak support for their placement relative to one another. The position of *C. zebra* and *C. adersi* is unresolved. There is also weak support for the paraphyly of *C. rufilatus* relative to *C. nigrifrons* (MP=59, ML=44, BA=0.87) and strong support for the paraphyly of *C. callipygus* relative to *C. ogilbyi and C. weynsi* (MP=98, ML=97, BA=1.0).

Figure 2.2. Majority-rule consensus tree showing the Bayesian estimate of the complete mitochondrial dataset. Thickened branches indicate support by both BA posterior probability (PP) values ≥ 0.95 and ML bootstrap support (BS) ≥ 75. Table 2.4 lists support values by node for this phylogeny.



In nuclear concatenated matrices, the harmonic mean of the log likelihood of the partitioned combined mitochondrial and nuclear Bayesian analysis was hm_2=-15370.43 (compared to hm_1= –15851.64 for the un-partitioned analyses), giving a value of 2 ln BF=-962.42, strong evidence against a partitioned model. Alternatively, when the mitochondrial data are excluded from analyses, Bayes Factor analysis found strong evidence for the partitioned model (hm_2=-8088.25, hm_1=-7976.32, 2 ln BF=223.86). The species tree estimated using concatenated nuclear data is illustrated in Figure 2.3 and aligned with the corresponding mitochondrial gene tree. Table 2.4 illustrates support values for branches in each tree. The concatenated nuclear tree shows strong support for the monophyly of *Philantomba* (MP bootstrap=100, ML un-partitioned/partitioned bootstrap=100/100, BA un-partitioned/partitioned posterior probability=1.0/1.0). However, the basal position of this genus relative to the other duikers is not supported. Nuclear analyses support a sister relationship between the monotypic genus *Sylvicapra* and the *C. silvicultor/C. spadix* group (MP=75, ML=66/75, BA=1.0/0.98), making both the genus *Cephalophus* and the giant duiker lineage paraphyletic. There is also support for the monophyly of the east African red duiker lineage (MP=91, ML=91/98, BA=0.99/1.0), the west African red duiker lineage (MP=67, ML=75/86, BA=1.0/1.0), and a sister relationship between these two red African duiker lineages (MP=95, ML=1.00/1.00 BA=1.0/0.82). *Cephalophus adersi* is basal to both east and west African red duikers (MP=60, ML=93/91 BA=0.93/0.74) and *C. zebra* is basal to the *C. jentinki/C. dorsalis* group (MP=77, ML=59/70, BA=1.0/0.98). Unlike the mitochondrial tree, *C. rufilatus* and *C. nigrifrons* form reciprocally monophyletic clades (MP=86, ML=82/95, BA=1.0/1.0 and MP=99, ML=99/100, BA=1.00, respectively) in the nuclear tree. However, *C. harveyi* is paraphyletic with respect to *C. natalensis*, as is *P. monticola* to *P. maxwelli*. *Cephalophus callipygus and C. ogilbyi* form an unresolved polytomy.

Figure 2.3. Majority-rule consensus tree showing the Bayesian estimate of the species tree from either a) mitochondrial or b) nuclear concatenated datasets. Thickened branches indicate support by both BA posterior probability (PP) values ≥0.95 and ML bootstrap support (BS) ≥ 75. Table 2.4 lists support values by node for this phylogeny. Boxes show major lineages on a grey scale, starting with the giant duikers in white, then the savanna duiker, the east African red duikers, the west African red duikers, and the dwarf duikers in darkest grey.



Both coalescent-based and concatenation-based methods of species tree estimation recovered the same topology and were almost completely resolved (Figures 2.4a and 2.4b, respectively). *Philantomba* was monophyletic and basal to Cephalophinae. *Cephalophus* was paraphyletic, with the savanna genus *Sylvicapra* sister to the monophyletic giant duiker clade. The east and west African red duiker lineages are monophyletic and are sisters. While their placement is not strongly supported by all methods of estimation, Bayesian support places *C. adersi* basal to the east and west African red duiker lineages (*BEAST posterior probability=0.72, MP=61, ML=73/65 BA=1.0/0.98). Placement of *C. zebra* as basal to the giant and savanna duiker lineages is not supported in any trees. Furthermore, the sister relationship of *C. dorsalis* and *C. jentinki* was not supported by coalescent-based methods.

Analyses in BEAST recovered the same topologies as obtained by BA methods (figures 2.4b and 2.3 respectively). However, the tree estimated using both nuclear and mitochondrial data was better resolved with higher support and narrower confidence intervals than the tree estimated from nuclear analysis alone. For this reason, we report only the results of estimation from both nuclear and mitochondrial data, although ages for nodes recovered in both analyses are presented in Table 4. The split of the dwarf genus *Philantomba* from all other members of the *Cephalophinae* was estimated to be 5.45 mya (5.31-5.91 highest posterior density, HPD). This is followed by the divergence of the giant duiker and *Sylvicapra* lineage from the red duikers at 4.35 mya (3.83-4.95 HPD), and a subsequent split between the east and west African red duiker lineages at 3.08 mya (2.61-3.63 HPD). With the exception of *C. adersi, C. zebra*, and *S. grimmia*, all other extant duiker species are estimated to have originated in the Pleistocene (< 2.588 mya).

Figure 2.4. Estimation of the multi-locus species tree using either: a) coalescent-based methods implemented in *BEAST or b) Bayesian methods using a concatenated data matrix. Node numbers refer to divergence time estimations in Table 4. Thickened branches indicate support by both BA posterior probability (PP) values ≥0.95 and ML bootstrap support (BS) ≥ 75. Table 2.4 lists support values by node for this phylogeny.

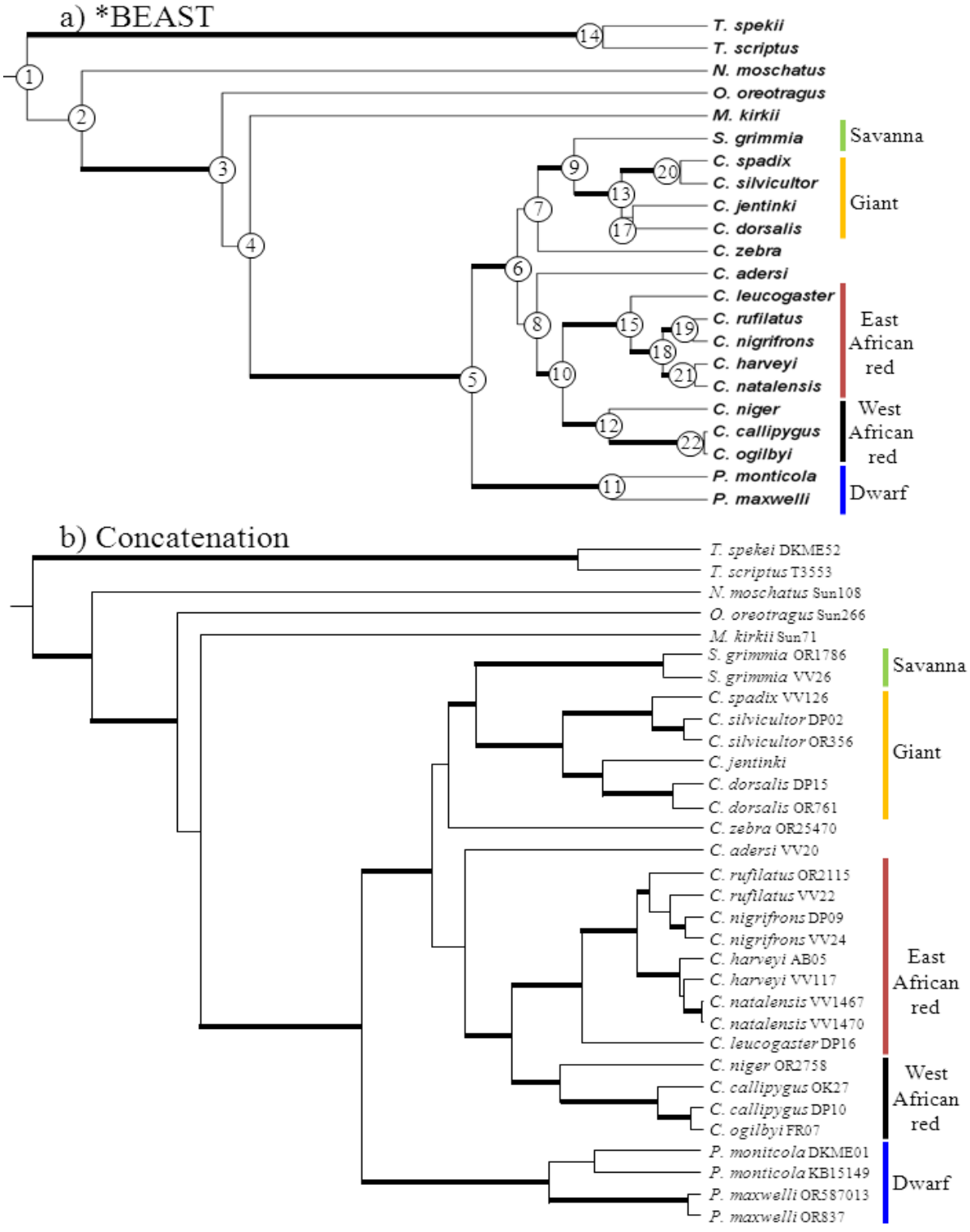


Table 2.5. Divergence times estimated by BEAST based on either total evidence (mitochondrial + nuclear) or nuclear only datasets. Divergence time estimates are the median age of the posterior distributions in million years of age and the 95% highest posterior density (HPD) intervals are indicated in brackets. Label numbers refer to the topology of Figure 4.

Label	Node Description	Total Evidence dataset	Nuclear dataset
1	Origin of branch leading to Tragelaphini	10.73 [9.06-12.45]	10.73 [6.41-15.93]
2	Origin of branch leading to *N. moschatus*	9.81 [8.48-11.31]	9.39 [6.90-12.45]
3	Origin of branch leading to *O. oreotragus*	8.43 [7.40-9.66]	
4	Origin of branch leading to *M. kirkii*	8.06 [7.08-9.19]	
5	*Philantomba/Sylvicapra+Cephalophus* lineages diverge	5.45 [5.31-5.91]	5.86 [5.35-7.11]
6	Giant and savanna/East and west African red duiker lineages diverge	4.35 [3.83-4.95]	
7	Origin of branch leading to *C. zebra*	4.09 [3.53-4.67]	3.06 [1.63-4.64]
8	Origin of branch leading to *C. adersi*	3.83 [3.30-4.44]	3.88 [2.54-5.25]
9	Giant/savanna duiker lineages diverge	3.63 [3.07-4.22]	
10	East/west African red duiker lineages diverge	3.08 [2.61-3.63]	2.55 [1.61-3.76]
11	*P. monticola/P. maxwelli* divergence	2.45 [1.98-2.95]	2.26 [1.17-3.47]
12	Origin of branch leading to C. niger	2.29 [1.83-2.78]	1.91 [1.03-2.93]
13	*C. spadix* + *C. silvicultor/C. jentinki* + *C. dorsalis* divergence	2.27 [1.87-2.74]	
14	*T. speckei/T.scriptus* divergence	1.99 [1.47-2.53]	1.26 [0.43-2.40]
15	Origin of branch leading to *C. leucogaster*	1.95 [1.52-2.38]	1.03 [0.39-1.80]
17	*C. jentinki/C. dorsalis* divergence	1.61 [1.25-1.99]	1.13 [0.50-2.07]
18	*C. rufilatus* + *C. nigrifrons/C. harveyi* + *C. natalensis* divergence	1.06 [0.82-1.32]	
19	*C. nigrifrons/C. rufilatus* divergence	0.86 [0.65-1.10]	
20	*C. spadix/C. silvicultor* divergence	0.81 [0.56-1.09]	0.43 [0.12-0.90]
21	*C. harveyi*/*C. natalensis* divergence	0.30 [0.17-0.49]	0.34 [0.07-0.72]
22	*C. ogilbyi/C. callipygus* divergence	0.18 [0.08-0.32]	0.74 [0.30-1.31]

DISCUSSION

The goal of this study was to construct a well-supported species tree for Cephalophinae to specifically investigate: i) the monophyly of *Philantomba* and *Cephalophus* ii) the placement of the major mitochondrial lineages first identified by Jansen van Vuuren & Robinson (2001), iii) the placement of *C. adersi* and *C. zebra* (iv) the monophyly of all species within this group and v) the timing of the radiation of this group.

Results illustrate the most well supported phylogeny for this challenging group to date. While the monophyly of *Philantomba* is well supported by previous mitochondrial DNA analyses (Jansen van Vuuren & Robinson 2001; Johnston et al. 2011; Ntie et al. 2010), the basal position of this genus relative to the rest of the subfamily were previously unresolved. Results provide convincing support for the basal

position of this genus within the subfamily and consequently provide support for the recognition of *Philantomba*, as recommended by Jansen van Vuuren & Robinson (2001). In contrast, results call into question the validity of the monotypic genus *Sylvicapra,* since this species forms a close affiliation with the giant duikers, leaving *Cephalophus* otherwise paraphyletic. It is instead suggested that *S. grimmia* is a savanna-dwelling member of the giant duiker lineage of *Cephalophus* that evolved from a forest-dwelling common ancestor, reinforcing Grubb's (1978) belief that habitat transitions occur primarily from forest to savanna. While Jansen van Vuuren & Robinson (2001) were correct in hypothesizing that the savanna duiker diverged early in the group's evolutionary history, this study shows its return to the savanna does not predate the appearance of other forest-dwelling taxa.

The present phylogeny also supports the validity of the three lineages within the genus *Cephalophus* and for the first time provides significant support for their placement. There is also limited support for the placement of two taxa that previous studies have thus far failed to have reliably assessed: (a) *C. adersi* now appears basal to the east and west African red duiker radiations whereas (b) *C. zebra* appears to be basal to the radiation that gave rise to the giant and savanna duikers. However, support for the placement of these two older taxa is still relatively weak, suggesting that they might have originated from a rapid divergence during the Pliocene that created extremely shallow branches lacking substantial phylogenetic signal.

With the exception of *C. adersi*, *C. zebra* and *S. grimmia*, divergence times of duikers all date to the Pleistocene, when the colder, drier temperatures during glacial maxima might have led to the isolation and subsequent diversification of tropical, forest-associated taxa (Haffer 1969). Although most tropical speciation events predate the Pleistocene (Moritz et al. 2000), this hypothesis remains largely untested in the Afro-tropics. However, an emerging pattern among studies of African birds is that climate-driven forest dynamics during the Pliocene, rather than the Pleistocene, are implicated as the driving force of Afro-tropical speciation (Voelker et al. 2010; Njabo et al. 2008; Roy et al. 2001). Although previous studies have speculated on the importance of Pleistocene glacial cycling in duiker diversification (Vrba 1995; Estes 1991), this study is the first to date the divergence times of most of the duikers to the Pleistocene epoch and provides an important starting point for further inquiry. Furthermore, isolation through glacial cycling is also believed to have influenced the population genetic structure of other tropical forest-dwelling taxa (Querouil et al. 2003; Anthony et al. 2007; Nicolas et al. 2011; Born et al. 2011; Telfer et al. 2003) and might have influenced the population structure of duiker species too. Further work using neutral nuclear microsatellite data is needed to better understand the role that Pleistocene glacial cycling played in shaping population structure within duiker species and may help to identify areas of priority for duiker conservation.

A comparison of the mitochondrial and nuclear DNA phylogenies also gives an interesting insight into evolutionary processes operating within this group. Because mitochondrial DNA has one quarter of the effective population size of nuclear DNA, mitochondrial haplotypes sort much more rapidly (Zink & Barrowclough 2008). Thus, the paraphyly observed in the mitochondrial DNA of recently diverged lineages should be reflected in the nuclear data (Zink & Barrowclough 2008), as observed for *C. natalensis/C. harveyi* and *C. ogilbyi/C. callipygus*. Incomplete lineage sorting would also explain the paraphyly observed in the nuclear DNA of species that exhibit reciprocally monophyletic relationships in mitochondrial analyses, as appears to be the case for *C. sylvicapra/C. spadix and P. monticola/P. maxwelli*. However, *C. nigrifrons* and *C. rufilatus* do not follow either of these patterns, exhibiting a paraphyletic relationship in mitochondrial analyses and a reciprocally monophyletic relationship in nuclear analyses. Mitochondrial introgression between *C. nigrifrons* and *C. rufilatus,* followed by extensive backcrossing to the original parental taxa, could have obscured mitochondrial relationships but maintained their monophyly at the nuclear level. These two taxa share a west/central African distribution and are partially sympatric, provided opportunity for hybridization. Interestingly, Bayesian analysis of the nuclear data also supports a sister relationship between *C. nigrifrons* and the *C. natalensis/C. harveyi* clade., indicating that *C. nigrifrons* and *C. rufilatus* may not be sister taxa, as previously mitochondrial analyses suggest (Jansen van Vuuren & Robinson 2001; Ntie et al. 2010; Johnston et al. 2011). Therefore, if *C. nigrifrons* is hybridizing with a more distant relative than its sister taxa, it might be geographic proximity and not phylogenetic distance that limits opportunities for hybridization in this group.

A well supported estimation of a species tree is often a useful precursor for guiding conservation and management decisions (Crandall et al. 2000). Phylogenetic analysis finds significant support for a west African red duiker lineage that contains *C. rubidus*, a geographically-restricted taxon that is commonly treated as a subspecies of *C. nigrifrons*, a east African red duiker. This observation lends additional strength to Jansen van Vuuren & Robinson's (2001) recommendation that this taxon should be managed independent form *C. nigrifrons* and its conservation status should be elevated from threatened to endangered. (Kingdon 1997). Furthermore, the relationship between *C. callipygus* and CITES protected species *C. ogilbyi* is problematic. Inclusion of nuclear data lends further support to the paraphyletic relationship of these taxa and suggests that *C. ogilbyi* may be a morphotype of *C. callipygus*, although hybridization and incomplete lineage sorting cannot be ruled out. It seems unlikely that any marker will be able to differentiate these two taxa, posing a challenge to the regulation of the bushmeat trade (Eaton et al. 2009, Johnston et al. 2011) or wildlife monitoring studies of field collected feces (Ntie et al. 2010).

Unlike earlier studies of the subfamily, this study is the first to use both nuclear and mitochondrial data to estimate a species tree and to date divergence times of duikers to the Pleistocene.

While inclusion of nuclear markers did not alter the topology of the mitochondrial genealogy, they did nevertheless increase the support for the deeper nodes and aid the placement of two challenging duiker taxa. Furthermore, this is the first molecular study to challenge the validity of the genus *Sylvicapra* and to suggest that *C. nigrifrons* may share a more recent common ancestor with *C. natalensis* and *C. harveyi* than it does with C. *rufilatus*. Further work should investigate the extent of gene flow between recently derived species and use a population genetics approach to assess the impact that Pleistocene glacial cycling may have had on the diversification and population structure of duikers.

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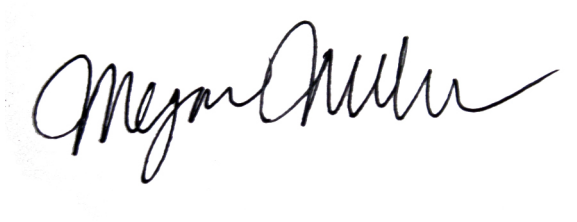
APPENDIX

I, Megan Morikawa, authorize Anne Johnston to use the work entitled ***Evaluating DNA barcoding criteria using African duiker antelope (Cephalophinae) as a test case*****,** which I co-authored, in her Masters thesis manuscript.

Signed,



I, Stephan Ntie, authorize Anne Johnston to use the work entitled ***Evaluating DNA barcoding criteria using African duiker antelope (Cephalophinae) as a test case*****,** which I co-authored, in her Masters thesis manuscript.

Signed, Stephan Ntie

VITA

Anne Roddy Johnston was born in Columbus, Ohio. She joined the Anthony lab in April, 2006 while earning her Bachelor of Science in Biology with a minor in Earth and Environmental Sciences from the University of New Orleans, which she completed in December, 2008. She enrolled for her Masters of Science at the University of New Orleans in January 2009. Anne is a member of the New Orleans Society for Conservation Biology and has served on the board and as the volunteer coordinator since 2006.