# Molecular Phylogeny of Duiker Antelope (Mammalia: Cephalophini)

by

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Submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
(Zoology)

in the
Faculty of Biological and Agricultural Science
University of Pretoria
Pretoria

October 1999

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## ABSTRACT

Molecular sequence data, comparative cytogenetics, and fluorescence *in situ* hybridization (FISH) were employed to study evolutionary relationships within the duiker antelope (tribe Cephalophini). The results of the different data sets are highly concordant. Mitochondrial DNA sequences (the full cytochrome *b* gene and a 767 bp portion of the 12S rRNA) were analyzed from all 19 recognized species. Complete species representation and good cytochrome *b* resolution allowed for the retrieval of four adaptive lineages, the conservative dwarfs *(Cephalophus monticola, C. maxwellii)* which were the most basal clade, the savanna specialist *(Sylvicapra grimmia)* which groups apart from all the forest duiker, the giant duiker group *(C. silvicultor, C. spadix, C. dorsalis, C. jentinki)*, and the red duiker lineage *(C. leucogaster, C. rufilatus, C. nigrifrons, C. natalensis, C. harveyi, C. callipygus, C. weynsi, C. ogilbyi, C. rubidus, C. niger)*. The placement of the endangered *C. zebra* and the enigmatic *C. adersi* remains obscure.

Conventional chromosome banding showed a 2n=60 complement in *C. spadix* extending previous observations that speciation in duiker antelope does not involve euchromatic rearrangements or variation in diploid number. At a finer level, fluorescence *in situ* hybridization with species specific satellite fragments derived from the chromosomal DNA of *C. maxwellii* and *C. monticola*, resulted in intense fluorescence to the centromeric regions of the autosomes of all species *(S. grimmia, C. dorsalis, C. maxwellii, C. monticola, C. natalensis, C. silvicultor, C. spadix)*. However, variation in hybridization to the X and Y chromosomes allowed for some distinction among taxa. These results are consistent with the delimitation of the four adaptive groups suggested by molecular analysis and the published morphological data which, when taken together, question and in some instances support several of the nomenclatural divisions in current use in duiker taxonomy. These include the recognition of *Philantomba* as genus name for *C. monticola* and *C. maxwellii*, an arrangement that would secure *Cephalophus* monophyly, and that *C. harveyi* be relegated to a subspecies of *C. natalensis*.

**Keywords** Duiker, Cephalophini, systematics, phylogeny, mitochondrial DNA, cytochrome *b*, 12S rRNA, biogeography, comparative cytogenetics, fluorescence *in situ* hybridization.

*For Mom, Dad, and Miriam*
*with love*

# ACKNOWLEDGMENTS

I am greatly indebted to my supervisor Professor T.J. Robinson for his guidance and sustained encouragement during the course of my postgraduate studies. Terry's unflagging enthusiasm for science in general, and for this project in particular, was truly inspirational. His constructive criticism and thoughtful comments laid the foundation for much of this work. I am also grateful for the opportunities he provided for me to participate in the Euro-American Mammal Congress held in Santiago de Compostela, Spain in 1998 and the 8th International Symposium on African Small Mammals, Paris, France, earlier this year.

My sincere appreciation is extended to the following people and institutions for time and assistance in obtaining duiker samples: Aarn (Australia), P. Arctander (University of Copenhagen, Denmark), P. Bishop (University of the Witwatersrand, South Africa), The Brownsville Zoological Gardens (United States of America), C. Claude (University of Zürich, Switzerland), E. Douzery (University of Montpellier, France), B. Fernholm (Swedish Museum of Natural History, Sweden), P. Grubb (London, United Kingdom), W. Harrison (University of Texas Medical School, United States of America), K. Heckler (Van Rooyen Taxidermists, South Africa), B. Herzig (Vienna Museum for Natural History, Austria), R. Hutterer (Alexander Koenig Museum, Germany), P. Jenkins (Natural History Museum London, United Kingdom), C. Powell (University of Port Harcourt, Nigeria), R. Sabin (Natural History Museum London, United Kingdom), E. Stockenstroom (Congo Safaris, South Africa), S. Strand (Swedish Museum of Natural History, Sweden), C. Stuart (Loxton, South Africa), M. Viljoen (Pretoria, South Africa), L. Vinciguerra (Safariroyal, Tanzania), A. Williams (Zanzibar Protected Areas Project, Tanzania), V. Wilson (Chipangali Wildlife Orphanage, Zimbabwe), and Kevin Kruickshank for assistance in the field.

My thanks go also to Wilbur Harrison (University of Texas Medical School, Houston) for assistance with fluorescence *in situ* hybridization. Most of the hybridization results were generated while Wilbur was a visiting scientist in Terry's laboratory. I also wish to thank Victor Rambau for his help and endless patience with the cytogenetic aspect of the study. Cloning experiments were carried out in the laboratories of Professors H. Huismans (University of Pretoria) and V. Miserahi (South African Medical Research Council). Pamela de Waal is thanked for her time and assistance. Oliver Wittekindt (Institute for Human

Genetics & Anthropology, University of Freiburg) cloned and sequenced the satellite fragments used in this study; I am greatly indebted to him for his time. Colin Groves, Peter Grubb, Ken Halanych, Conrad Matthee, and Marc Robinson are thanked for comments on various aspects of the study.

My sincere appreciation to Hanlie Engelbrecht and her family, Stefanie Freitag, Warren Funston, Conrad Matthee, Sonja Matthee, Sarita Maree, Ben Orban, and Victor Rambau for their continued friendship, interest, and words of encouragement. Babs Potgieter and Simon Maake are thanked for technical assistance, and for keeping me on my toes.

Lastly, my special thanks to Mom, Dad, and Miriam for loving and supporting me throughout.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

**Figure 5** The cytochrome *b* parametric bootstrap topology obtained from 1 000 simulated data sets created by the Siminator computer program (Huelsenbeck *et al.* 1996). The most parsimonious tree (Fig. 2) was used as the model tree, with the gamma shape parameter (0.168) and kappa value (27.167) estimated *via* maximum likelihood. The simulated data sets were developed under the HKY (Hasegawa *et al.* 1985) model of evolution. 50

**Figure 6** Strict consensus tree of 14 equally most parsimonious trees obtained from 767 bp of the 12S rRNA gene under a 2:1 ti/tv ratio. Nonparametric bootstrap values for nodes are presented above branches and the number of unambiguous changes that plot along them are indicated below the branches. No changes are shown on branches within polytomies. 53

**Figure 7** Neighbor joining tree constructed from 767 bp of the small ribosomal subunit in Paup 4.0b2a (Swofford 1999). The HKY correction (Hasegawa *et al.* 1985) was applied to the distance matrix. Branches are drawn proportionally to the amount of change occurring on them, and nonparametric bootstrap support for nodes is indicated. 55

**Figure 8** Maximum likelihood topology obtained from the 12S rRNA gene. The tree was constructed in Paup 4.0b2a (Swofford 1999) under the HKY (Hasegawa et al. 1985) model of sequence evolution with equal rates, the empirical ti/tv ratio (7:1), and nucleotide frequencies calculated from the data. Bootstrap support was obtained using 100 iterations. 56

**Figure 9** G-banded female karyotype of *C. spadix* (2n=60) from Tanzania. 70

**Figure 10** C-banded metaphase cell of a *C. spadix* female. Arrowheads indicate the X chromosomes. 71

**Figure 16** Results of the unordered parsimony analyses based on the combined molecular and cytogenetic data sets. (A) The bootstrap consensus tree (1 000 replicates) obtained when all duiker taxa are included. Nodes that received less than 50% support were collapsed. (B) The single most parsimonious tree obtained when only taxa for which cytogenetic data were available are included. The tree was produced with the heuristic search option in Paup 4.0b2a (Swofford 1999) with 100 random replacements. Bootstrap support obtained from 1 000 iterations is shown above the branches. **86**

# CHAPTER 1

# GENERAL INTRODUCTION

## AIMS AND OBJECTIVES OF THE STUDY

In the past, studies of systematic relationships among duiker antelope comprising the tribe Cephalophini have focused almost exclusively on the morphological and phenotypic variation evident among representatives of the group (St. Leger 1936, Heyden 1968, Groves & Grubb 1974, 1981, Kingdon 1982, 1997). This approach has, however, proved to be limited in its usefulness and the taxonomic status and relationships of many species remain unresolved. In an attempt to redress these deficiencies, the phylogenetic history of the cephalophine species is examined using techniques and approaches which have hitherto not been applied to this specious and phenotypically divergent group of African antelope.

The objectives were:

i) To assess the molecular phylogenetic relationships and taxonomic status of taxa comprising the Cephalophini based on cytochrome *b* and 12S rRNA nucleotide sequence data

ii) To ascertain by fluorescence *in situ* hybridization (FISH) whether highly repeated satellite sequences differ between the species of duiker antelope

iii) To determine the degree of concordance in the phylogenetic placement of taxa from three independent data sets (mitochondrial DNA nucleotide sequences, comparative cytogenetics, and the fluorescence *in situ* hybridization patterns of chromosomes)

iv) To correlate the phylogeny of the taxa with available climatic data in an attempt to clarify the events that have shaped the speciation and evolutionary events within the group.

# INTRODUCTION

## Natural history of duiker antelope

Members of the tribe Cephalophini are, with the exception of a single species *(Sylvicapra grimmia)*, adapted to an inconspicuous existence in forests. According to Kingdon (1982) there is not a single indigenous forest in Africa that does not provide habitat to at least one duiker species, a clear indication of their relative importance as the dominant medium-sized forest antelope. Several morphological and dietary adaptations enable them to successfully occupy this niche. All the *Cephalophus* taxa have a similar body form with short front legs and an arched back. Well developed hind quarters propel them through the thick undergrowth while the horns are short and project backwards from the skull (Heyden 1968, Bigalke 1972, Walker *et al.* 1975, Kingdon 1982).

Color variation within the tribe is unprecedented for antelope (for species descriptions see Happold 1973, Walker *et al.* 1975, Kingdon 1997) and there is speculation that this may account for species of similar size and habitat preference existing sympatrically (Kingdon 1982). Although the majority of species show quite considerable variation in coat and facial markings, Groves and Grubb (1974) recognize four independent trends towards melanism within the group with the completely black *C. niger* representing the extreme in phenotype.

The monotypic *Sylvicapra* is the only taxon within the tribe that is not a strict forest specialist. It prefers drier and relatively more exposed habitats, hence the more slender body form (Bigalke 1972, Walker *et al.* 1975, Groves & Grubb 1981). Moreover, this species has the widest distribution of all duiker, ranging from Senegal to east, central, and southern Africa (Walker *et al.* 1975). Apart from habitat differences distinguishing *Sylvicapra* from *Cephalophus*, Ansell (1971) delimits the taxa on the presence/absence of horns in the females (*Sylvicapra* females do not have horns), the plane of horn projection (in *Cephalophus* the horns project backwards while they are upright in *Sylvicapra)*, as well as the length of the ears.

As is typical for the majority of small antelope, all duiker are thought to be territorial and, in most species there is a close association between the sexes with territories shared by both males and females; males are, however, more active in patrolling and defending them (Kingdon 1997). It is generally thought that the length of horns in females are correlated

with the degree of active territorial defense. *Sylvicapra* males have territories that encompass the home ranges of several females.

**Higher order taxonomy**

The Cephalophini is one of 14 recognized tribes within the family Bovidae (Vrba 1985a). The higher order classification of bovids is problematic, in particular the intratribal, intertribal, and subfamilial relationships. Simpson (1945) noted that morphological convergence and rapid radiation, in conjunction with the questionable monophyly of some tribes/subfamilies, renders the classification of the Bovidae difficult, a point which is further underscored by the lack of agreement concerning the number of recognized species, tribes, and subfamilies within the family (Simpson 1945, Ansell 1971, Gentry 1978, Vrba 1985a, Gentry 1992).

Although the monophyly of the Cephalophini has never been questioned, the taxonomic placement of this tribe has proved problematic. Various authors argue that duiker antelope represent a distinct, early off-shoot within the bovid radiation (Bigalke 1972, Vrba 1985a, Georgiadis *et al.* 1990, Gentry 1992), while others propose a more recent origin based on the high degree of overall similarity between species (Kingdon 1982, 1997). Morphological data (Gentry 1992) indicate a close association between the Cephalophini and Tragelaphini, and between the Cephalophini and the Boselaphini/Bovini (the two latter tribes are closely related to each other). A basal placement of the Cephalophini is in agreement with allozyme data (Georgiadis *et al.* 1990), with the cephalophine representatives being only distantly related to all other tribes.

Phylogenetic trees constructed from mitochondrial DNA sequence data of both the large and small ribosomal subunits suggest a common ancestry for the tribes Cephalophini, Reduncini and Antelopini (Gatesy *et al.* 1992). In a similar study by Allard *et al.* (1992) using nucleotide sequences from the 12S rRNA gene, the minimum sequence divergence (uncorrected distances; Kraus & Miyamoto 1991) between any two taxa of these tribes (the Ovibovini, Peleini, and Rupicaprini were not available to these authors) was between *C. maxwellii* (Cephalophini) and *Kobus ellipsiprymnus* (Reduncini). Most recently, however, sequence data from the complete mitochondrial DNA cytochrome *b* gene (Matthee & Robinson 1999a) indicate a sister taxon relationship between the Cephalophini and a neotragine representative *(Oreotragus oreotragus)*.

## Taxonomy of the Cephalophini

The taxonomy of the tribe Cephalophini has been complicated by the inconsistent treatment of some taxa and the questionable taxonomic status of others. Currently, two genera are recognized (Grubb 1993), the monotypic *Sylvicapra* Ogilby, 1837 and the specious *Cephalophus* H. Smith, 1827. However, the validity of *Sylvicapra* has been questioned (Lydekker & Blaine 1914, Haltenorth 1963, Van Gelder 1977; but see Heyden 1968, Ansell 1971, Meester *et al.* 1986, Grubb 1993, Kingdon 1997). Haltenorth (1963) followed Lydekker and Blaine (1914) in treating *Sylvicapra* as a subgenus, while Van Gelder (1977) recognized only *Cephalophus*, and regarded *Sylvicapra* as a synonym based on evidence of hybridization between *S. grimmia* and *Cephalophus* spp. (*C. nigrifrons;* Anon. 1965 in Van Gelder 1977 and *C. natalensis;* Van Gelder 1977).

A third genus, *Philantomba* Blyth, 1840 is sometimes recognized for *C. monticola* and *C. maxwellii* with the distinction resting on the absence of inguinal glands in these species Pocock (1910). This was followed by later authors (Hard 1969; Groves & Grubb 1981). In spite of these considerations the recognition of *Philantomba* is not universal (see Ralls 1973, Haltenorth & Diller 1986, Kingdon 1997) and Grubb's (1993) most recent revision of the tribe submerged *Philantomba* in *Cephalophus*, a treatment which is followed in the present study.

*Cephalophula* has been proposed as the genus name for *C. zebra.* This species is a distinct member of the forest group and is characterized by several unique features. These include an unique, heavily boned skull with the nasals of both sexes being unusually thick and reinforced (Heyden 1968, Kingdon 1982). Moreover, the attachment of the horns to the skull and their plane of projection distinguishes *C. zebra* from other representatives of the Bovidae (Heyden 1968).

Dobzhansky (1970) and Van Gelder (1977) state that the boundaries of a genus are arbitrary constructs since the criteria for delimiting them are subjective. A number of definitions and criteria have been proposed to delineate species. These include taxonomic/morphological species, biological species, and phylogenetic species. Given that convergent evolution and local adaptations pose serious problems when using phenotypic characters, it is not surprising that molecular results often contradict species descriptions based on morphological grounds (see O'Brien & Mayr 1991). Although criticized (Wiley 1978, Frost & Hillis 1990, Wheeler & Nixon 1990), the most widely accepted definition is that of the

biological species which remains the baseline for the taxonomic description of our fauna and flora. Under this concept, a species is defined as a group of naturally (or potentially) interbreeding populations that are reproductively isolated from other such groups (Mayr 1963). Notwithstanding difficulties involved with determining reproductive compatibilities between alleged species (specifically allopatric forms; see Avise & Ball 1990), the biological species concept has several advantages (e.g. the recognition of variation within species that is often partitioned geographically or temporally by population subdivision into subspecies; O'Brien & Mayr 1991), and this definition of a species is followed herein.

The deep divisions that characterize duiker taxonomy are reflected by the lack of consensus on the number of species. If one excludes the monotypic *S. grimmia* which appears to enjoy overwhelming support (Ansell 1971, Bigalke 1972, Happold 1973, Eltringham 1979, Groves & Grubb 1981, Grubb 1993, Kingdon 1997), 10 forest species are recognized by Walker *et al.* (1975), 11 - 13 species by Haltenorth and Diller (1986), 15 species by Ansell (1971), Bigalke (1972) and Groves and Grubb (1981), 17 species by Kingdon (1997) and finally, 18 species by Grubb (1993) and Nowak (1999). The various taxonomic classifications that have been suggested and their synonyms are summarized in Table 1.

St. Leger (1936) examined all the duiker specimens of the subgenus *Cephalophus* available to her in the British, Tervueren, Berlin and Leyden Museums. Based mainly on the size of the false hoof and texture of hair on the shoulders she proposed two species groups. The first consisted of only *C. nigrifrons* and *C. rufilatus* (including *C. n. rubidus*, which is currently recognized as a valid species; see Grubb 1993 and Table 1); the second comprised the remaining taxa: *C. jentinki*, *C. silvicultor*, *C. spadix*, *C. niger*, *C. weynsi*, *C. w. harveyi* (currently recognized as a valid species; see Grubb 1993 and Table 1), *C. adersi*, *C. natalensis*, *C. zebra*, *C. dorsalis*, *C. callipygus*, *C. leucogaster* and *C. ogilbyi*.

This approach was further extended by Groves and Grubb (1981) who, after an evaluation of external and skeletal features, proposed the possible existence of three species groups or subgenera in *Cephalophus*[1]. The first comprised the monotypic *C. (Cephalophula) zebra*.

[1] Note that these authors recognize *Philantomba* as the genus name for *P. monticola* and *P. maxwellii* and that they are therefore not included in *Cephalophus*.

Table 1 Taxonomic classification of the tribe Cephalophini (Bovidae) following Grubb (1993). The synonyms listed in Grubb's revision are presented under earlier classifications.

| **Species (Grubb 1993)** | **Earlier classifications** | **Reference** |
|---|---|---|
| *C. adersi* | *C. harveyi adersi* | Haltenorth 1963 |
| | *C. natalensis adersi* | Heyden 1968 |
| *C. callipygus* | *C. ogilbyi callipygus* | Groves & Grubb 1981 |
| | *C. harveyi callipygus* | Haltenorth 1963 |
| | *C. natalensis callipygus* | Heyden 1968 |
| *C. dorsalis* | | |
| *C. harveyi* | *C. natalensis harveyi* | Ansell 1971, Heyden 1968, Ellerman *et al.* 1953, Groves & Grubb 1981 |
| *C. jentinki* | | |
| *C. leucogaster* | *C. dorsalis leucogaster* | Rode 1943 |
| *C. maxwellii* | *C. monticola maxwellii* | Haltenorth & Diller 1986 |
| | *P. maxwellii* | Pocock 1910, Groves & Grubb 1981 |
| *C. monticola* | *P. monticola* | Pocock 1910, Groves & Grubb 1981 |
| *C. natalensis* | | |
| *C. niger* | | |
| *C. nigrifrons* | | |
| *C. ogilbyi* | | |
| *C. rubidus* | *C. nigrifrons rubidus* | Ansell 1971, Groves & Grubb 1981 |
| *C. rufilatus* | | |
| *C. silvicultor* | | |
| *C. spadix* | *C. silvicultor spadix* | Haltenorth 1963 |
| *C. weynsi* | *C. natalensis weynsi* | Ansell 1971 |
| | *C. callipygus weynsi* | Kingdon 1997 |
| *C. zebra* | *Cephalophula zebra* | |
| | *C. doria* | Heyden 1968 |
| *S. grimmia* | *C. grimmia* | Van Gelder 1977 |
| | *C. (Sylvicapra) grimmia* | Haltenorth 1963 |

As emphasized previously, this species is characterized by many unique features. The second species group was thought to include four species of duiker *C. silvicultor*, *C. dorsalis*, *C. spadix* and *C. jentinki* and is currently regarded by Grubb (pers comm[2]) as paraphyletic. These duiker are generally larger, their horns are not thickened and, if present in females, are nearly as large as those of the males. Juveniles are characterized by a dark, speckled pelage. The third subgenus represents a monophyletic clade in which the front of the skull is thickened in males, the horns are thickened and present in females (although of smaller size than in the males), and the juvenile pelage is similar to that of the adults. This assemblage includes *C. niger*, *C. natalensis*, *C. nigrifrons*, *C. rufilatus*, *C. ogilbyi*, *C. leucogaster*, and *C. adersi* and is known as the *Cephalopia* species group.

Most recently, Kingdon (1997) recognized 17 species within a single genus, *Cephalophus*, which he subdivided into four adaptive lineages. Although the number of recognized taxa differ, there is good agreement with these four lineages and the three species-groups originally proposed by Groves and Grubb (1981). First, *C. monticola* and *C. maxwellii* are clustered as the conservative dwarfs. The second lineage, the so-called red duiker, are the most diverse in which the smaller sized duiker are seen as conservative (having retained some primitive adaptive traits), occupying peripheries or islands that are peripheral to the central habitat (e.g. *C. adersi* and *C. nigrifrons)*, while others have adapted to marginal environments within the forests. Three species are placed within the fibre duiker lineage *(C. ogilbyi*, *C. dorsalis* and *C. jentinki)* which is believed to have originated in Upper Guinea; these species have largely retained a western geographic bias in their present distribution. Interestingly, some of the red duiker i.e. *C. leucogaster* are characterized by much coarser diets and are seen as closely related to the fibre diet group. Lastly, the giant duiker lineage comprising the two largest species *(C. silvicultor* and *C. spadix)*, is regarded as being the evolutionary most derived.

## Speciation

Mammalogy provides exceptionally fertile grounds for advancing and testing evolutionary theory since, in many instances, it couples living forms to a particularly rich fossil record (Vrba 1992). Among the best studied groups in this regard is the family Bovidae, a large assemblage comprising African and Indian antelope, cattle sheep and goats which includes

[2] Peter Grubb. 35 Downhills Park Road, London N17 6PE. United Kingdom.

some 45 genera and 124 species, more than half of which are endemic to the grasslands and savannas of sub-Saharan Africa (Vrba 1985a).

The earliest known Eurasian bovid dates back approximately 20 million years ago (myr; Vrba 1985a). At much the same time the first sub-Saharan bovids also appeared, with subsequent early evolutionary activity restricted to 14 myr ago. Synchronous events among taxa presumed to be species are also concentrated near the Miocene-Pliocene border (5 myr ago), the late Pliocene (2.5 myr ago), as well as mid Pleistocene (0.9 and 0.7 myr ago; Vrba 1985b).

These temporally separated speciation events suggested by the fossil record are closely tied to glacial oscillations. Global temperature changes over the past 65 million years (based on deep sea records) show large fluctuations with a net cooling trend (Prentice & Matthews 1988). Smaller excursions in the form of periodic cycles, known as glacials and interglacials, are also evident. Evidence from the Pliocene and Pleistocene shows that the cycles were accompanied not only by large scale expansions and retractions of ice at the poles, but also by major climatic and vegetational changes in the tropics. During the downward extension of the snow caps on the mountains, the resulting precipitation extended the area of montane forest to almost a continuum (Van Zinderen Bakker 1962). During the interglacials, however, there was a southward shift in aridity which is thought to have caused a significant reduction in the size of both the Upper Guinea and the main Congo forest blocks (Moreau 1963), habitat to many of the duiker species. This change in habitat would result in severe fragmentation of tropical rainforest species possibly leading to speciation by distance (allopatric speciation), and to the high species diversity found in this biome (Grubb 1982, Myers 1982). Given the profound effect that climatic changes have had on the tropical forests, forest specialists such as the duiker antelope are useful models for furthering our understanding of the evolutionary events leading to speciation and diversity within this biome.

## Conservation status

The short to medium term survival of several duiker antelope species is threatened. Human invasion and deforestation are responsible for habitat loss. Additionally, hunting pressure on rare and endangered taxa is intensifying due to commercial bush-meat hunters (Kingdon 1982, Williams 1997). Moreover, several species show narrow endemicity, for example *C. rubidus* which is found only at high altitudes on the Ruwenzori Mountains, *C. jentinki* and

*C. zebra* which are limited to the high primary forest zone between Sierra Leone and the Niouniourou river, and *C. adersi* which occurs only on the Island of Zanzibar and in Kenya's Sokoke coastal forests. These considerations have contributed, in part, to *C. adersi*, *C. dorsalis*, *C. jentinki*, *C. monticola*, *C. ogilbyi*, *C. rubidus*, *C. silvicultor*, *C. spadix* and *C. zebra* as being listed by CITES and IUCN as deserving of endangered and/or vulnerable status (Grubb 1993).

Given this background, the need for a comprehensive review of the duiker species and their phylogenetic relationships is evident. Not only will an understanding of the relationships within the group allow for the clarification of the taxonomic status of many taxa, but the data may provide wildlife managers with an evolutionary framework that could prove useful in reassigning conservation priorities to some of the species.

# CHAPTER 2

# MOLECULAR PHYLOGENY OF THE CEPHALOPHINI

## OBJECTIVES

The aims of this aspect of the study were twofold. First, an attempt is made to determine evolutionary relationships between recognized duiker species using molecular sequence data from two mitochondrial DNA genes, cytochrome *b* and the small ribosomal subunit (12S rRNA). Secondly, by applying a molecular clock to date divergences, speciation within the tribe is correlated with biogeographical events which are known to have shaped forest biota.

## INTRODUCTION

The assessment of evolutionary relationships have, traditionally, rested on morphological and phenotypic character states (Avise 1994). These characters are, however, frequently obscured by parallelisms and convergent evolution as are molecular data (Hedges & Maxson 1996) which may lead to spurious relationships. The tribe Cephalophini is a case in point. Several genera and species groups have been described using characters such as the length and texture of hair on the shoulders, the presence of inguinal glands, the length of the false hoof, and juvenile pelage patterns (Pocock 1910, St. Leger 1936, Heyden 1968, Groves & Grubb 1981; see Chapter 1). The inconsistency regarding the number of recognized species and genera within the tribe is therefore not surprising, and even today, relationships among species are largely unresolved.

The discovery of the polymerase chain reaction (PCR; Mullis & Faloona 1987) has had a profound effect on phylogenetic studies leading to the extensive use of DNA sequences to describe diversity and evolutionary relationships in all life forms. Arguably, the most frequently targeted sequences used to retrieve phylogenetic relationships are those derived from the mitochondrion. The extranuclear mitochondrial molecule is a closed, circular gene system that replicates and transcribes within the organelle itself (Avise & Lansman 1983). It has no intervening sequences, spacers, or repetitive DNA. Moreover, several unique

features make it especially useful as a tracer of macroevolutionary events. First, it is usually maternally inherited (Lansman *et al.* 1983, Gyllensten *et al.* 1985, Avise & Vrijenhoek 1987, but see Avise 1991, Gyllensten *et al.* 1991) which allows for an effective haploid transmission across generations. Secondly, the mitochondrial DNA molecule is free from recombination events (Harrison 1989). Thirdly, variation in the evolutionary rate of different parts of the molecule permits the selection of genes which are appropriate to address taxonomic questions that span a wide evolutionary time scale (Harrison 1991, Hillis & Dixon 1991).

The phylogenetic value of a gene, or region of DNA, rests on the premise that the rate of evolution matches the divergence times of the taxa under consideration (Hillis & Huelsenbeck 1992). The most informative studies compare sequences that vary in amount of divergence but are not so different that patterns are obscured by multiple events at the same nucleotide site (Moritz *et al.* 1987). In the case of the Bovidae, sequence data from the mitochondrial cytochrome *b* and 12S rRNA genes have been widely used to address evolutionary questions (Allard *et al.* 1992, Groves & Shields 1996, Robinson *et al.* 1996, Gatesy *et al.* 1997, Hassanin & Douzery 1999, Matthee & Robinson 1999a), and the same loci were selected in this study to investigate relationships between the cephalophine species. In so doing it was hoped that cytochrome *b* with its faster mutation rate would possibly resolve relationships near the terminal branches of the phylogenetic tree relative to the slower 12S rRNA characters which, based on other investigations (Moritz *et al.* 1987, Halanych & Robinson 1999), could be predicted to be distributed more basally.

# MATERIAL AND METHODS

## Sample collection and storage

Total genomic DNA was extracted from representatives of all 19 recognized duiker species (Grubb 1993; Table 2). In instances where the species concerned was characterized by an extensive range (i.e. *S. grimmia, C. monticola)* an attempt was made to include two or more specimens from widely separated localities. Since these specimens formed monophyletic clusters in preliminary investigations, only a single representative per species was used in the assessment of phylogenetic relationships. In these instances the retention of a particular specimen for subsequent analyses was based on its sharing the same geographic region as that of the holotype or, alternatively, that it was geographically the closest of the competing specimens to the holotype's locality.

Fresh material was the preferred source for DNA extractions (either soft tissue preserved in a 20% DMSO/saturated salt solution, or cells harvested from fibroblast cultures). Given the difficulties encountered in obtaining fresh samples, as well as the scarcity and endangered status of several of the taxa, teeth or dried skins were used as alternatives for DNA extraction *(C. ogilbyi, C. rubidus, C. zebra).* Table 2 lists the collection localities as well as type of material used for DNA extraction from all specimens included in the phylogenetic analysis.

## Genomic DNA extraction and nucleotide sequencing

Total genomic DNA extraction from fresh material was done following standard protocols (Maniatis *et al.* 1982, Amos & Hoelzel 1991). These involved phenol/chloroform/iso-amyl alcohol steps with precipitation carried out overnight in ice-cold absolute ethanol. DNA pellets were re-suspended in dd$H_2O$ to a final concentration of 1 $\mu$g/$\mu$l.

Hagelberg (1994) described an extraction protocol for DNA from ancient bones. This protocol, with minor modifications, was followed for DNA extractions from duiker teeth. A Rotary Power Tool (Sears Best/Craftsman) fitted with 0.05 mm drill-bits was used to drill into the pulp cavity of a molar and the resulting powder collected for DNA extractions. Incubation in a lysis buffer was overnight and the DNA was subsequently allowed to precipitate in ice-cold absolute ethanol for one week. Extractions using skin pieces obtained from museum specimens followed conventional protocols (as described for fresh material),

Table 2 Duiker species used in the present study. Vernacular names, collection localities, and tissues used fo DNA extractions are provided. Where multiple specimens were sequenced per species, those included in th phylogenetic analyses are indicated by an * (see text for details).

| Species | Vernacular name | Locality | Source material | Material provided |
|---|---|---|---|---|
| *S. grimmia** | Gray duiker | Zimbabwe | Primary culture | V. Wilson[1] |
| *S. grimmia* | Gray duiker | CAR. | Soft tissue | E. Stockenstroom[2] |
| *C. adersi* | Ader's duiker | Zanzibar Island | Soft tissue | A. Williams[3] |
| *C. callipygus** | Peters' duiker | Gabon | Soft tissue | Aarn[4] |
| *C. callipygus* | Peters' duiker | Congo | Soft tissue | E. Stockenstroom[2] |
| *C. dorsalis* | Bay duiker | Ghana | Primary culture | V. Wilson[1] |
| *C. harveyi* | Harvey's red duiker | Tanzania | Soft tissue | L. Vinciguerra[5] |
| *C. jentinki* | Jentink's duiker | Brownsville Zoo. | Blood | Brownsville Zoo[6] |
| *C. leucogaster* | White-bellied duiker | Congo | Soft tissue | E. Stockenstroom[2] |
| *C. monticola** | Blue duiker | South Africa | Primary culture | V. Wilson[1] |
| *C. monticola* | Blue duiker | Cameroon | Soft tissue | P. Bishop[7] |
| *C. monticola* | Blue duiker | Gabon | Soft tissue | Aarn[4] |
| *C. maxwellii* | Maxwell's duiker | Ghana | Primary culture | V. Wilson[1] |
| *C. natalensis* | Natal red duiker | South Africa | Primary culture | V. Wilson[1] |
| *C. niger* | Black duiker | Ghana | Soft tissue | P. Arctander[8] |
| *C. nigrifrons* | Black-fronted duiker | Congo | Soft tissue | E. Stockenstroom[2] |
| *C. ogilbyi* | Ogilby's duiker | Nigeria | Dried skin | C. Powell[9] |
| *C. silvicultor* | Yellow-backed duiker | Philadelphia Zoo. | Primary culture | V. Wilson[1] |
| *C. spadix* | Abbot's duiker | Tanzania | Primary culture | L. Vinciguerra[5] |
| *C. rubidus* | Ruwenzori red duiker | Ruwenzori Mt. | Tooth | S. Strand[10] |
| *C. rufilatus* | Red-flanked duiker | CAR. | Soft tissue | E. Stockenstroom[2] |
| *C. weynsi* | Weyne's duiker | Rwanda | Soft tissue | C. Claude[11] |
| *C. zebra* | Zebra duiker | Liberia | Tooth | C. Claude[11] |

CAR=Central African Republic; 1=Chipangali Wildlife Orphanage, Zimbabwe; 2=Congo Safaris, Sou Africa; 3=Zanzibar Protected Areas Project, Commission for Natural Resources, Tanzania; 4=Aarn, Australi 5=Safariroyal, Tanzania; 6=Brownsville Zoological Garden, USA; 7=University of the Witwatersrand, Sou Africa; 8=University of Copenhagen, Denmark; 9=University of Port Harcourt, Nigeria; 10=Swedish Museu of Natural History, Sweden; 11=Zoologisches Museum, Universität Zürich, Switzerland.

however, the incubation and precipitation steps were prolonged and were similar to tooth extractions. In view of the possibility of contamination when working with degraded DNA typically retrieved from museum specimens, procedures routinely used in the laboratory were followed (Robinson *et al.* 1996, Matthee & Robinson 1999b, Robinson & Matthee 1999). Among others, these included the DNA extractions being performed in a separate DNA-free laboratory, multiple extractions, the amplification and sequencing of the museum specimens being separated temporally, and that the sequence data made phylogenetic sense (Austin *et al.* 1997).

Two mitochondrial DNA genes were targeted for phylogenetic inference. Cytochrome *b* (full gene) and 12S rRNA (approximately 800 bp on the 5' side of the subunit) were amplified by PCR using universal primers (cytochrome *b*: Pääbo & Wilson 1988, Kocher *et al.* 1989, Irwin *et al.* 1991, Matthee & Robinson 1999a; 12S rRNA: Allard & Honeycutt 1992). To allow for single stranded sequencing, end primers were biotin coated. PCR reactions were set up in a laminar flow hood to minimize the risk of contamination and negative controls, comprising all components except DNA, were always included. Given the degraded nature of DNA extracted from museum material, amplification was not always successful when using universal primers that spanned sections larger than 350 bp. Customized internal primers were therefore designed for both amplification and sequencing. Table 3 provides a summary of PCR and sequencing primers used in this study.

A 35 cycle PCR program was routinely followed (initial denaturation for 3 min at 94°C, denaturation for 45 sec at 94°C, annealing for 45 sec at 50°C, and extension for 1 min at 72°C with a final extension cycle of 5 min at 72°C). PCR products were separated in 1% agarose gels (Techcomp LTD), and fragments extracted using Nucleotrap Extraction Kit for Nucleic Acids (Macherey-Nagel).

Sequencing followed Sanger *et al.* (1977) using the Sequenase Kit v.2.0 (United States Biochemical Corporation) with [$\alpha$-$^{32}$P] dATP as the labeling agent. Single stranded template was obtained by treating the purified product with dynabeads-streptavidin (Dynal A.S.), a magnetic particle with an affection for biotin, followed by a denaturation step involving 0.1 M NaOH. Overlap in nucleotide sequences obtained from L and H sequencing primers provided verification of sequences.

Table 3 Primers used and their sequences for both amplification and sequencing of duiker DNA. Biotin coated end primers are indicated by *. Customized primer numbers correspond to *Bos taurus* (Anderson *et al.* 1982) and the sequences are reported 5' to 3'.

| Cytochrome b | Reference |
|---|---|
| *L14724 | Pääbo & Wilson 1988 |
| L14841 | Kocher *et al.* 1989 |
| L15162 | Pääbo & Wilson 1988 |
| L15408 | Irwin *et al.* 1991 |
| BL | Matthee & Robinson 1999a |
| AH | Matthee & Robinson 1999a |
| H15149 | Kocher *et al.* 1989 |
| *H15494 | Irwin *et al.* 1991 |
| *H15915 | Pääbo & Wilson 1988 |

| 12S rRNA | Reference |
|---|---|
| *L82 (A) | Allard & Honeycutt 1992 |
| L509 (C) | Allard & Honeycutt 1992 |
| L673 | AGCCACCGCGGTCATACG |
| L941 | CTCAAAGGACTTGGCGGTG |
| H708 | CACGTTTTACGCCGTATTCC |
| H618 (B) | Allard & Honeycutt 1992 |
| H975 | TTATAGAACAGGCTCCTC |
| *H900 (D) | Allard & Honeycutt 1992 |

## Sequence analysis

The inclusion of more than one outgroup taxon for phylogenetic analyses may limit the introduction of errors in the polarization of characters and, by implication, the retrieval of erroneous evolutionary relationships (Milinkovitch *et al.* 1996, Milinkovitch & Lyons-Weiler 1998). To test the robustness of ingroup topologies, and also to avoid possible biases introduced by using a single outgroup, three outgroup taxa of successive relatedness to the ingroup were selected. These comprised the klipspringer, *O. oreotragus* (tribe Neotragini: cytochrome *b;* Matthee & Robinson 1999a; 12S rRNA; Hassanin & Douzery 1999), cattle, *B. taurus* (tribe Bovini: Anderson *et al.* 1982) and the more distantly related pronghorn antelope, *Antilocapra americana* (Family Antilocapridae: cytochrome *b;* Irwin *et al.* 1991; 12S rRNA; Kraus & Miyamoto 1991). For illustrative purposes trees incorporating all three outgroups simultaneously are presented. It is noteworthy, however, that although some minor branch swapping occurred when the outgroups were used singly, the main phylogenetic conclusions remained unchanged.

Both cytochrome *b* and 12S rRNA gene sequences were aligned by eye. The cytochrome *b* sequences were translated into protein codons. Both nucleotide sequences and their corresponding amino acids were examined for irregularities (e.g. stopcodons in the reading frame and that the nucleotide substitution pattern followed that of conventional protein coding genes) which could indicate nonfunctional nuclear homologs (see Arctander 1995, Zhang & Hewitt 1996 and references therein). All nucleotide sequences resulting in a unique amino acid change were verified. The 12S rRNA segment was assembled against the secondary structure model for *B. taurus* (Springer *et al.* 1995). When aligning the 12S rRNA sequences for the ingroup taxa, a single 3 bp insertion was found in the variable loop region between stem positions 35 and 36 (see Springer *et al.* 1995) which was common to both *C. monticola* and *C. maxwellii*. Sequence alignment to the outgroup taxa revealed several other insertions (between 1 and 3 bp in length) but these were unique to the outgroup sequences (i.e. were not present in the duiker antelope). Since the aim was to determine ingroup relationships, these indels were deleted from the outgroup species in all phylogenetic analyses. Sequences generated in the present study were deposited in Genbank (accession numbers AF153883-AF153905, AF154247-AF154270), and the alignments will be available in EMBL Treebase (http://phylogeny.harvard.edu/treebase).

Saturation of nucleotide changes within the Cephalophini was determined by plotting the actual number of transitions and transversions against time, expressed as sequence

divergence. Since corrected divergences might be more linear with respect to time than uncorrected values (Hackett 1996), only uncorrected p-distances are shown. Moreover, the use of uncorrected p-distances further allowed for comparisons of saturation with other bovid tribes. Given that different portions of the genes evolve at different rates, and would thus exhibit different saturation curves, the three codon positions were plotted separately for cytochrome *b* as well as stem and loop changes in the 12S rRNA gene.

The effects of deviations from a molecular clock on phylogeny reconstruction is well documented (DeBry 1992, Takezaki *et al.* 1995, Robinson *et al.* 1998). The relative rates of evolutionary change for individual lineages within the tribe Cephalophini were determined by RRTree (Robinson *et al.* 1998). Since the amount of noise being introduced on the branch leading to the reference taxon could influence the results, the closest relative outgroup *(O. oreotragus)* was used as reference taxon. This test was chosen because it takes the phylogenetic branching pattern into account and corrects for unbalanced sampling by assigning weights proportionally to branches. Given the differences in evolutionary rates between synonymous and non-synonymous substitutions, these two classes were separated when calculating evolutionary rates.

The data were analyzed by distance (neighbor joining algorithm; Saitou & Nei 1987), parsimony, and maximum likelihood (Felsenstein 1981) using Paup 4.0b2a (Swofford 1999). The HKY correction model (Hasegawa *et al.* 1985) was applied to the distance matrix for neighbor joining analyses. This correction takes the unequal base frequencies observed for most mitochondrial DNA genes into account and adjusts for different rates of transition and transversion accumulation. To accommodate among site rate variation, gamma shape parameters were calculated *via* maximum likelihood in Paup 4.0b2a using the optimal parsimony trees as the reference topology (shape parameter for cytochrome *b*=0.168; shape parameter for 12S rRNA=0.164). Distance and maximum likelihood trees constructed with an adjustment for among site rate variation were compared to those constructed under the assumption of equal rates.

Parsimony trees were generated using the heuristic search option in Paup 4.0b2a with 100 random replicates. To account for the transitional bias, all analyses were carried out using unweighted characters, transversion (tv) weighted at 2:1 over transitions (ti), and the empirical ti/tv ratio for the ingroup (13:1 for cytochrome *b*; 7:1 for 12S rRNA). Empirical values were calculated in MacClade (Maddison & Maddison 1992) by counting the average number of changes over 1 000 random trees (Halanych 1996). In the case of maximum

likelihood analyses, the empirical ti/tv ratios were specified for the genes and base frequencies were calculated from the data.

The skewness of the tree-length distribution (g1; Hillis & Huelsenbeck 1992), an indication of the phylogenetic signal present in the data, was calculated for 10 000 random trees (Paup 4.0b2a). Since closely related taxa can contribute to the amount of overall signal, g1 values were estimated for subsets of the data excluding closely related taxa.

Without an appraisal of the support for a particular clade, a phylogeny is of limited value (Sanderson 1995). Although not without its detractors (e.g. Faith & Cranston 1991, Kluge & Wolf 1993, Sitnikova *et al.* 1995), the nonparametric bootstrap technique (Efron 1979, Felsenstein 1985) is the most frequently used statistical method to assess the reliability of specific groupings of taxa. Bootstrapping entails random resampling of characters with replacement and its usefulness stems, in part, from the fact that it can be used in conjunction with many different tree-building algorithms and types of data. To assess the robustness of topologies retrieved by this study one thousand bootstrap replicates were performed for distance and parsimony analyses; due to computational difficulties, however, only 100 replicates were done for maximum likelihood.

In contrast to conventional (nonparametric) bootstrapping where biases present in the original data matrix are retained in the pseudoreplicates, the parametric bootstrap creates independent replicates of a data matrix based on numerical simulations using parameters estimated from the original data (Efron 1985, Felsenstein 1988, Huelsenbeck *et al.* 1996). In addition to this attribute, parametric bootstrapping is also useful for examining possible branch-length problems that may result in artificial relationships (Huelsenbeck *et al.* 1996). Simulated data sets were created with the Siminator computer program (Huelsenbeck *et al.* 1996) using the cytochrome *b* parsimony topology as the model tree. One thousand simulated data sets were generated under an HKY model of sequence evolution, with gamma shape parameter (0.168), ti/tv ratio (kappa=27.167) and nucleotide frequencies estimated by maximum likelihood (Paup 4.0b2a). Tree reconstructions were performed in Paup 4.0b2a using parameters identical to those adopted in the cytochrome *b* parsimony analyses with the exception of an unordered weighting scheme and the "as-is" sequence addition as opposed to 100 random additions. Since the specification of a model tree is a prerequisite for simulating data matrices, the largely unresolved topologies obtained from the small ribosomal sequence data were considered inadequate as input models for parametric bootstrap analysis of this gene.

All cytochrome *b* analyses were carried out using the complete sequence for the gene. For the small ribosomal subunit, compensatory changes in stem regions have led various authors (Dixon & Hillis 1993, Springer *et al.* 1995) to down-weight paired characters. In the parsimony analyses of this study, stem characters were assigned a base weight of 0.6 relative to loop characters. The 3 bp indel found in the conservative dwarf lineage was treated as an additional single character state in parsimony analyses (characters 701 and 702 were excluded from the data matrix and only character 703 was included in analyses); when applying different weighting schemes for transitions and transversions, the indel was assigned the same weight as a transition (Jansen van Vuuren & Robinson 1997).

Several contrasting ideas have been proposed on how best to analyze multiple data sets (Bull *et al.* 1993, Huelsenbeck *et al.* 1994, De Queiroz *et al.* 1995). In the present investigation, the cytochrome *b* and 12S rRNA data sets were analyzed separately and, since the partition homogeneity test (Farris *et al.* 1995 in Paup 4.0b2a) resulted in no significant heterogeneity (P=0.226) between them, they were subsequently combined (Hillis 1987, Swofford 1991). For the combined parsimony analysis, the empirical ti/tv ratios for the two genes were retained, stem characters were down-weighted by 0.6, and the indel was treated as a fifth character in 12S rRNA. For maximum likelihood analyses, the combined empirical ti/tv ratio (11:1) was specified.

# RESULTS AND DISCUSSION

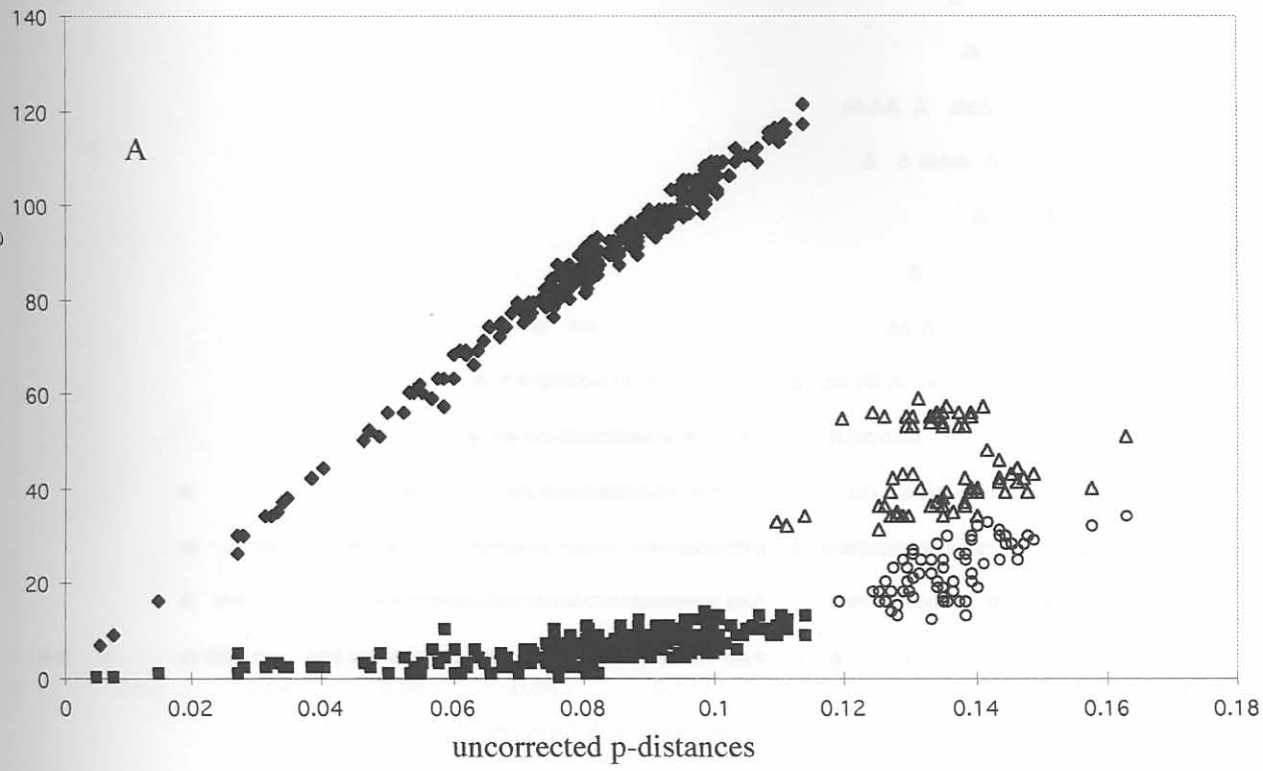
## Saturation analysis

No evidence of saturation was found for cytochrome *b* when the number of transitions and transversions were plotted against time (expressed as uncorrected sequence divergences; Fig. 1A). This is an interesting finding since there are reports, encompassing several mammalian families, of saturation occurring as sequence divergences approach ≥ 10%; this includes the Bovidae (Irwin *et al.* 1991, Griffiths 1997, Matthee & Robinson 1999a). In contrast, although several of the duiker species are separated by sequence divergences similar to this (the highest uncorrected p-distance between duiker species being 11.4% between *C. monticola* and *C. harveyi;* see Table 4) there was no evidence of saturation in the Cephalophini data set. The linear increase evidenced by the plots may reflect the low accumulation of transversions in the ingroup (see Fig. 1). When different codon positions are considered separately, no saturation was observed for either first (Fig. 1B) or second codon changes (Fig. 1C). Third codon transitions showed some leveling off for certain ingroup comparisons (Fig. 1D), mostly involving either *C. monticola* or *C. maxwellii*. However, the majority of changes are linear with time, thus indicating the general absence of saturation at the third codon position. The lack of any distortion introduced by third codon position substitutions is also evident when CI values are determined for all ingroup changes (CI=0.431) versus only third codon changes (CI=0.421).

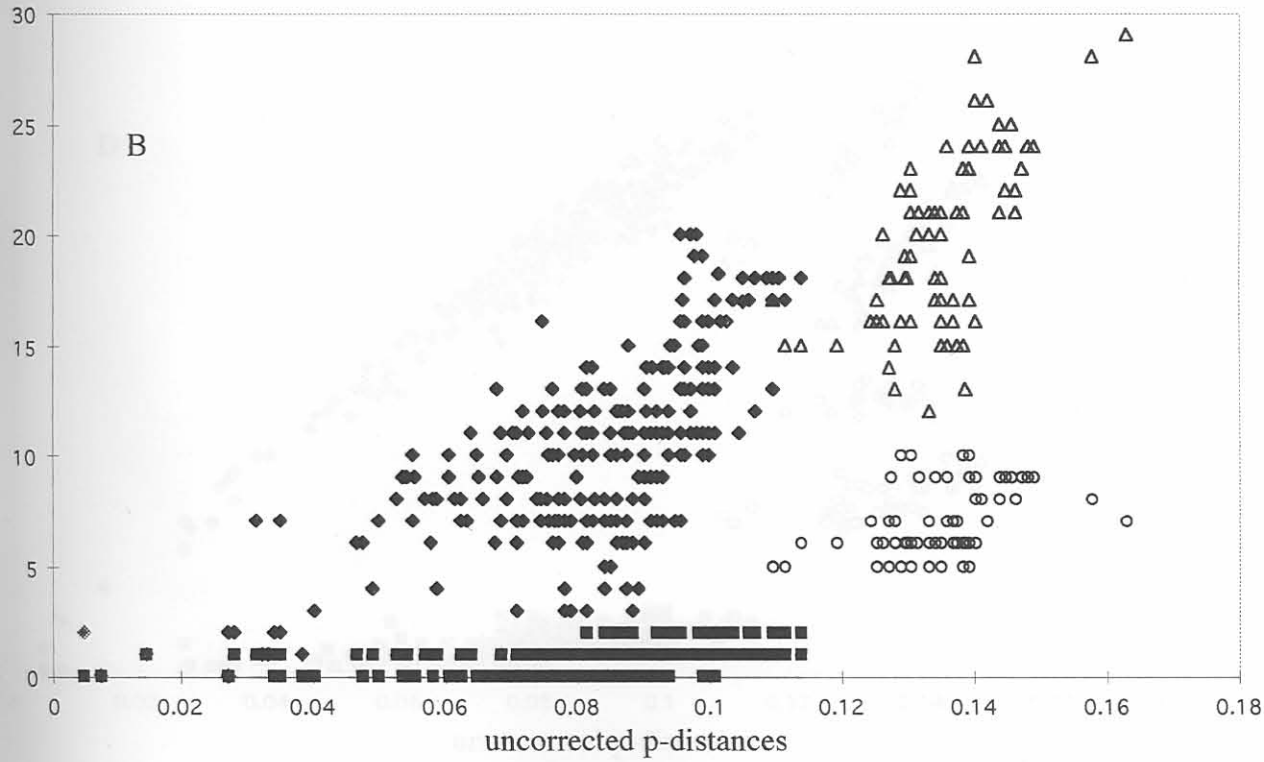
In the case of the 12S rRNA data, the highest uncorrected sequence divergence was 5.7% (between *C. maxwellii* and *C. nigrifrons;* see Table 5); not unexpectedly therefore, pairwise comparisons for this gene showed a monotonic linear increase over time (Fig. 1E). Considering paired and unpaired regions separately, loop characters may be expected to show some saturation given the weak evolutionary constraints acting on these domains (Springer *et al.* 1995). However, no evidence of this was found in the loop regions, either for transitions or transversions (Fig. 1F). Additionally, the CI values computed from the complete gene sequences obtained from the ingroup taxa versus those from the loop domain alone were similar (CI for complete data set=0.597; CI for loop domain=0.597). Given the conservative nature of the stems, the CI value for this region was, as expected, considerably higher (CI for stem domain=0.789).

Figure 1 Saturation plots for all duiker taxa included in the present study. The actual number of transitions and transversions are plotted against uncorrected p-distances. Closed symbols indicate ingroup comparisons while open symbols represent ingroup to outgroup comparisons. (A) Complete cytochrome *b* gene (B) Cytochrome *b* first codon position (C) Cytochrome *b* second codon position (D) Cytochrome *b* third codon position (E) Complete 12S rRNA gene (F) 12S rRNA loop domain.

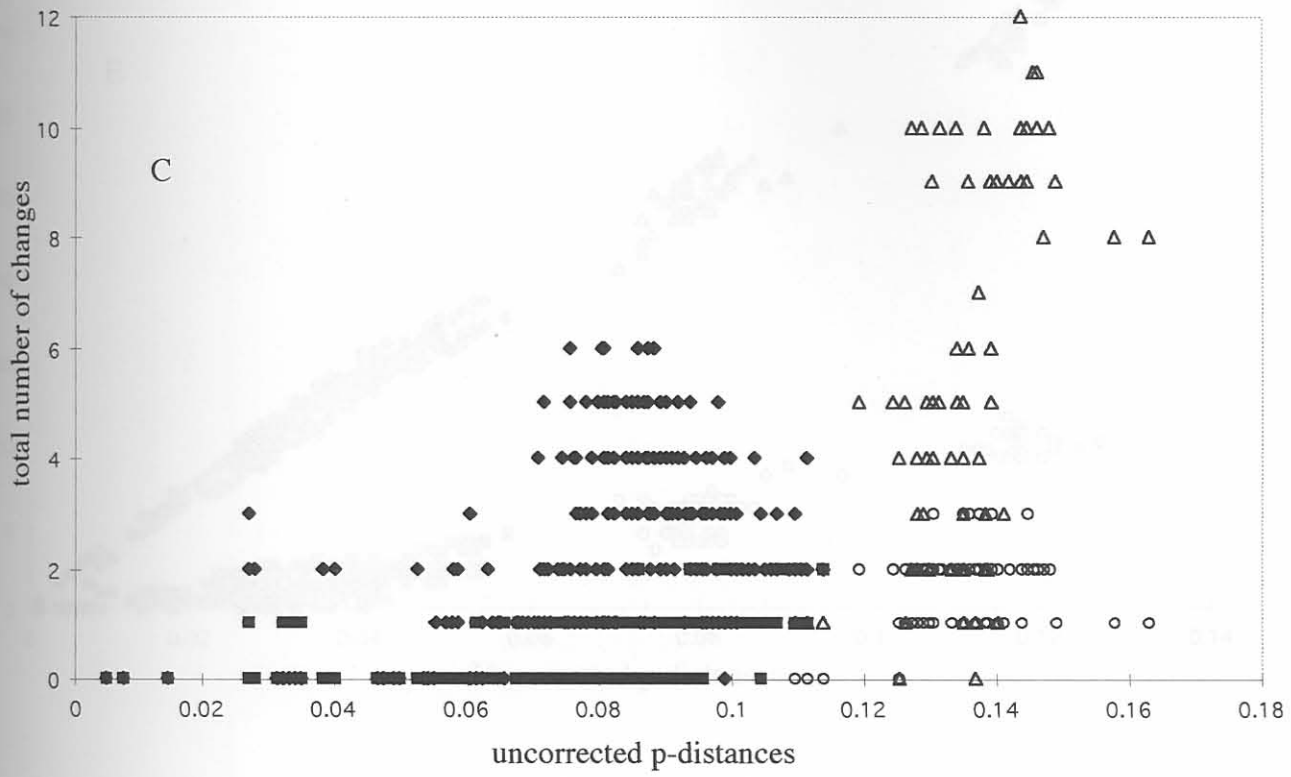
Complete cytochrome *b* gene



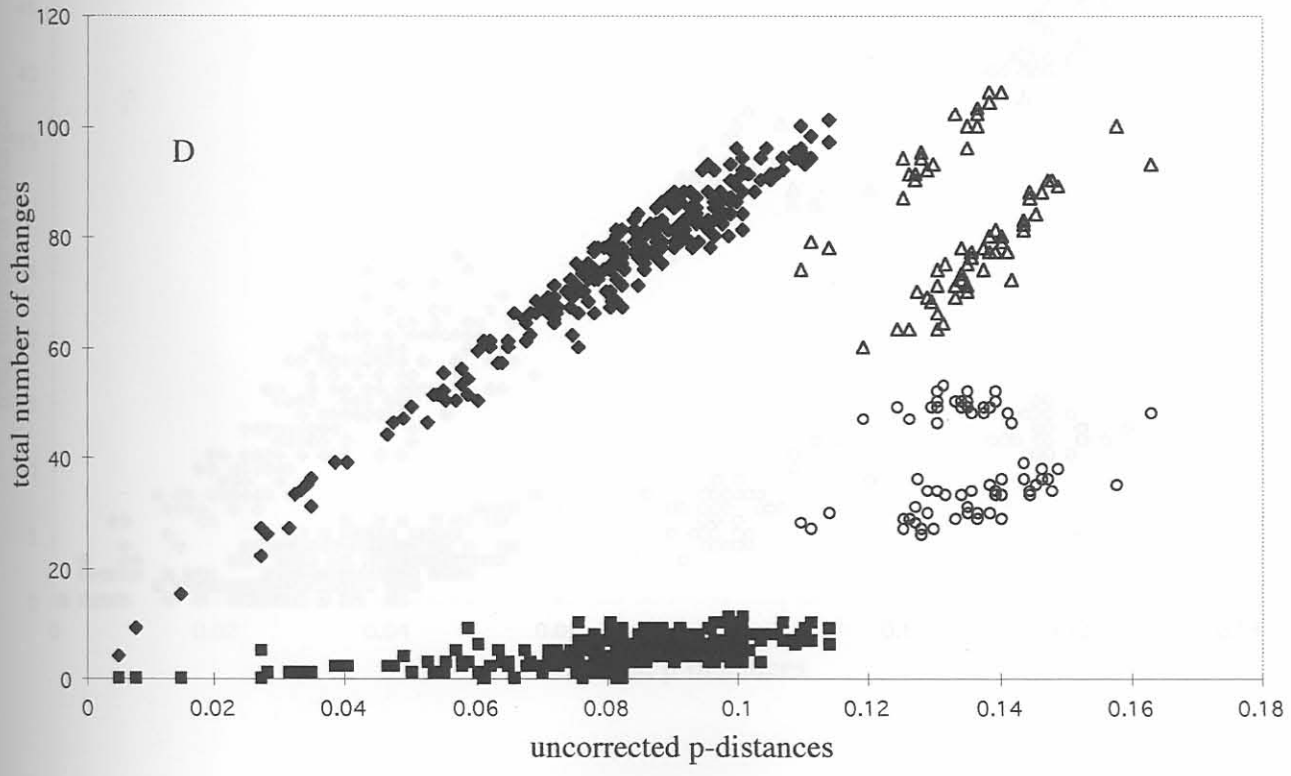

1st codon position

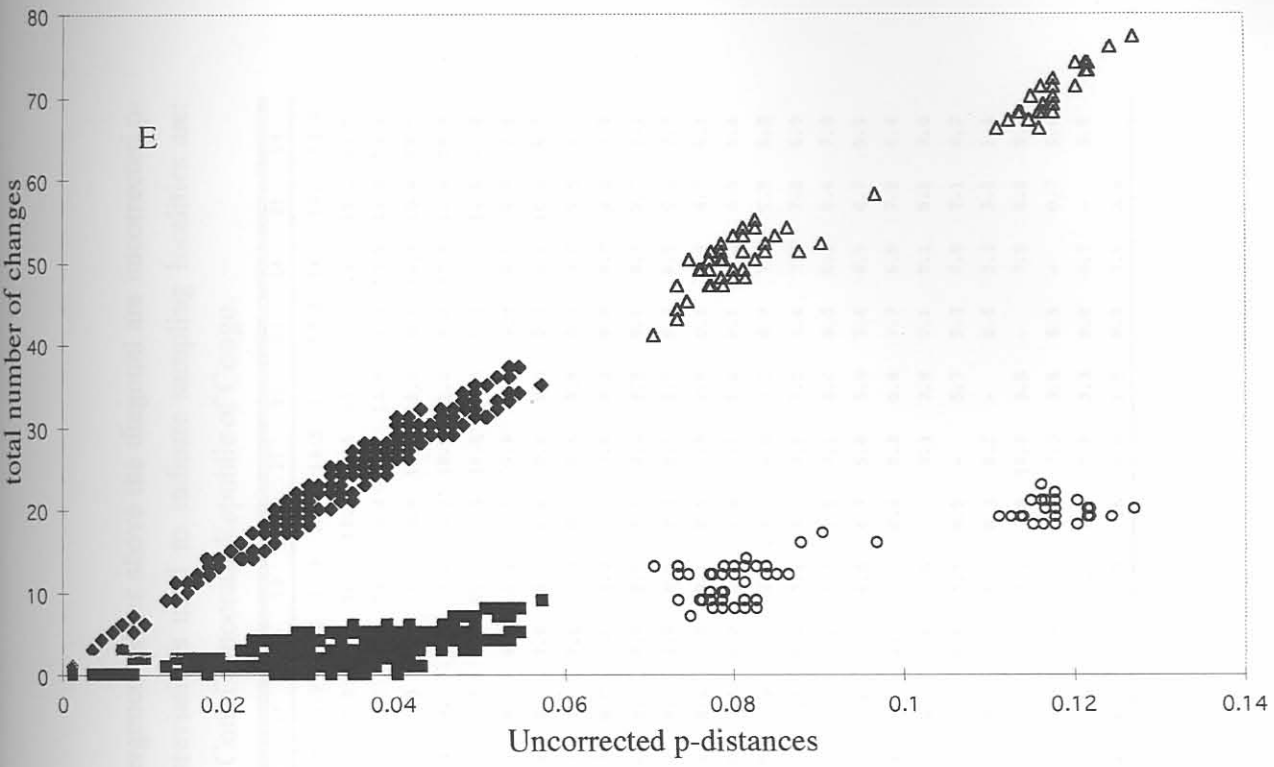


2nd codon position




3rd codon position






12S rRNA loop characters

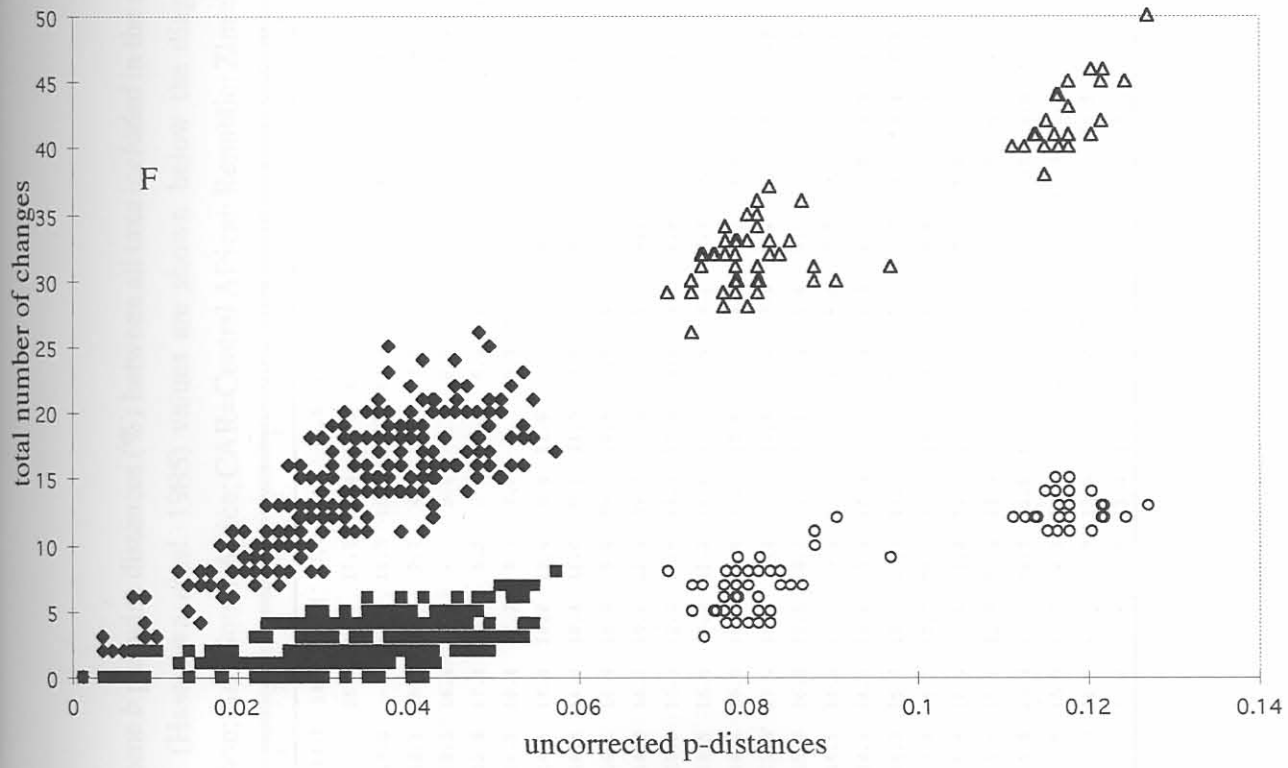


Table 4 Observed cytochrome *b* pairwise distances (%) between all taxa included in the study. Divergence values above the diagonal are uncorrected p-distances, while the HKY (Hasegawa *et al.* 1985) values are shown below the diagonal. Abbreviations used to indicate sampling localities are: Cam=Cameroon; Gab=Gabon; SA=South Africa; CAR=Central African Republic; Zim=Zimbabwe; Con=Democratic Republic of Congo.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Antilocapra* | - | 14.1 | 14.2 | 13.3 | 13.0 | 13.0 | 13.8 | 13.7 | 13.5 | 13.1 | 13.5 | 13.4 | 13.9 | 12.9 | 13.9 | 13.4 | 11.9 | 12.6 | 12.4 | 12.9 | 13.5 | 13.3 | 13.0 | 13.5 | 13.7 | 13.0 |
| *Oreotragus* | 15.9 | - | 16.3 | 10.9 | 11.1 | 11.4 | 12.5 | 12.8 | 12.8 | 12.5 | 12.7 | 13.5 | 12.8 | 12.6 | 13.8 | 13.5 | 12.8 | 13.0 | 12.7 | 13.3 | 13.6 | 13.6 | 13.8 | 13.6 | 14.0 | 13.5 |
| *Bos* | 16.1 | 19.0 | - | 14.0 | 14.3 | 14.9 | 15.7 | 12.8 | 13.0 | 14.3 | 14.6 | 14.5 | 14.6 | 14.0 | 14.8 | 14.4 | 12.7 | 13.1 | 13.8 | 13.4 | 14.7 | 13.9 | 14.3 | 13.9 | 14.4 | 13.5 |
| *monticola*(Cam) | 14.9 | 12.1 | 15.9 | - | 2.7 | 4.9 | 6.7 | 9.2 | 9.3 | 9.1 | 9.2 | 10.3 | 9.2 | 9.6 | 9.9 | 9.9 | 9.0 | 9.5 | 9.5 | 9.6 | 10.0 | 10.6 | 9.1 | 10.7 | 10.8 | 10.5 |
| *monticola*(Gab) | 14.6 | 12.3 | 16.4 | 2.7 | - | 5.8 | 7.7 | 9.3 | 9.9 | 9.8 | 10.0 | 11.1 | 10.0 | 9.5 | 9.7 | 9.3 | 9.0 | 9.4 | 9.2 | 9.2 | 10.0 | 11.1 | 8.8 | 10.8 | 11.4 | 10.9 |
| *monticola*(SA) | 14.5 | 12.6 | 17.1 | 5.1 | 6.2 | - | 7.1 | 9.3 | 8.5 | 8.8 | 8.8 | 10.0 | 9.6 | 10.0 | 10.7 | 10.7 | 9.3 | 9.8 | 10.0 | 9.9 | 10.9 | 10.5 | 9.9 | 11.0 | 11.4 | 10.2 |
| *maxwelli* | 15.6 | 14.1 | 18.4 | 7.2 | 8.3 | 7.6 | - | 10.3 | 10.0 | 9.7 | 10.0 | 10.9 | 9.9 | 9.7 | 10.1 | 10.0 | 9.1 | 9.7 | 9.4 | 9.5 | 9.9 | 9.9 | 9.0 | 9.5 | 9.8 | 9.6 |
| *grimmia*(CAR) | 15.5 | 14.4 | 14.4 | 10.2 | 10.4 | 10.4 | 11.6 | - | 2.7 | 8.5 | 9.2 | 8.2 | 8.6 | 8.2 | 9.0 | 9.0 | 8.0 | 7.6 | 8.1 | 8.4 | 9.4 | 9.0 | 9.0 | 9.6 | 10.0 | 8.7 |
| *grimmia*(Zim) | 15.2 | 14.4 | 14.6 | 10.4 | 11.0 | 09.4 | 11.3 | 2.8 | - | 8.2 | 8.4 | 7.4 | 8.8 | 7.9 | 8.7 | 8.7 | 7.7 | 7.6 | 7.8 | 8.5 | 9.5 | 8.9 | 8.7 | 9.0 | 9.5 | 8.5 |
| *silvicultor* | 14.6 | 14.1 | 16.4 | 10.0 | 10.9 | 09.7 | 10.8 | 9.3 | 9.0 | - | 2.8 | 6.0 | 7.1 | 8.5 | 8.5 | 8.7 | 8.0 | 7.5 | 8.0 | 8.0 | 7.6 | 8.2 | 8.2 | 9.0 | 9.2 | 7.8 |
| *spadix* | 15.1 | 14.3 | 16.7 | 10.2 | 11.1 | 09.7 | 11.3 | 10.2 | 9.2 | 2.8 | - | 5.7 | 7.7 | 9.0 | 9.1 | 9.2 | 8.7 | 8.0 | 8.5 | 8.5 | 9.0 | 8.5 | 8.1 | 9.3 | 9.7 | 8.1 |
| *dorsalis* | 15.0 | 15.3 | 16.7 | 11.6 | 12.5 | 11.2 | 12.3 | 9.0 | 8.0 | 6.4 | 6.0 | - | 7.1 | 8.5 | 8.9 | 8.7 | 8.0 | 8.0 | 8.5 | 8.4 | 8.5 | 8.1 | 8.7 | 9.1 | 9.2 | 7.8 |
| *jentinki* | 15.7 | 14.5 | 16.8 | 10.2 | 11.1 | 10.6 | 11.0 | 9.5 | 9.7 | 7.7 | 8.4 | 7.6 | - | 8.8 | 8.1 | 7.8 | 8.2 | 7.5 | 8.1 | 8.5 | 7.8 | 8.8 | 0.4 | 8.9 | 8.7 | 8.4 |
| *leucogaster* | 14.4 | 14.2 | 16.0 | 10.7 | 10.6 | 11.2 | 10.8 | 9.0 | 8.7 | 9.3 | 9.9 | 9.3 | 9.7 | - | 8.2 | 8.0 | 7.4 | 6.7 | 7.5 | 7.6 | 7.1 | 5.4 | 8.1 | 6.1 | 6.5 | 5.5 |
| *callipygus*(Gab) | 15.7 | 15.8 | 17.0 | 11.0 | 10.8 | 12.0 | 11.4 | 10.0 | 9.6 | 9.4 | 10.0 | 9.8 | 8.9 | 9.0 | - | 0.5 | 7.5 | 5.3 | 3.5 | 6.0 | 4.0 | 7.1 | 8.7 | 7.7 | 7.9 | 6.9 |
| *callipygus*(Con) | 15.0 | 15.3 | 16.5 | 11.0 | 10.3 | 12.0 | 11.1 | 10.0 | 9.6 | 9.6 | 10.2 | 9.6 | 8.4 | 8.8 | 0.5 | - | 7.3 | 5.0 | 3.1 | 5.5 | 3.8 | 7.1 | 8.4 | 7.5 | 7.8 | 6.9 |
| *zebra* | 13.1 | 14.5 | 14.2 | 10.0 | 10.0 | 10.3 | 10.1 | 8.8 | 8.4 | 8.8 | 9.5 | 8.7 | 9.0 | 8.1 | 8.2 | 8.0 | - | 7.2 | 6.3 | 7.8 | 7.1 | 8.0 | 8.0 | 8.2 | 8.6 | 7.8 |
| *rubidus* | 14.0 | 14.7 | 14.8 | 10.6 | 10.4 | 10.9 | 10.8 | 8.3 | 8.3 | 8.1 | 8.8 | 8.8 | 8.2 | 7.3 | 5.7 | 5.3 | 7.8 | - | 4.6 | 4.7 | 5.8 | 5.8 | 7.4 | 6.5 | 6.7 | 5.5 |
| *ogilbyi* | 13.8 | 14.3 | 15.7 | 10.5 | 10.1 | 11.2 | 10.5 | 8.9 | 8.6 | 8.7 | 9.4 | 9.4 | 8.8 | 8.2 | 3.6 | 3.2 | 6.7 | 4.9 | - | 6.2 | 5.2 | 6.4 | 7.7 | 6.8 | 7.2 | 6.4 |
| *niger* | 14.4 | 15.1 | 15.1 | 10.7 | 10.1 | 11.0 | 10.6 | 9.2 | 9.3 | 8.8 | 9.4 | 9.2 | 9.4 | 8.3 | 6.4 | 5.8 | 8.5 | 5.0 | 6.6 | - | 7.4 | 7.6 | 7.1 | 8.1 | 8.2 | 7.0 |
| *weynsi* | 15.1 | 15.6 | 16.9 | 11.1 | 11.2 | 12.3 | 11.0 | 10.5 | 10.6 | 8.2 | 9.9 | 9.4 | 8.4 | 7.6 | 4.2 | 4.0 | 7.7 | 6.1 | 5.5 | 8.1 | - | 5.7 | 9.3 | 6.8 | 7.1 | 6.2 |
| *rufilatus* | 14.9 | 15.6 | 15.8 | 11.9 | 12.6 | 11.8 | 11.0 | 10.0 | 9.8 | 9.0 | 9.4 | 8.9 | 9.7 | 5.7 | 7.7 | 7.7 | 8.8 | 6.2 | 6.9 | 8.3 | 6.1 | - | 8.6 | 3.3 | 3.2 | 1.4 |
| *adersi* | 14.6 | 15.8 | 16.4 | 10.0 | 09.7 | 11.0 | 09.9 | 10.0 | 9.6 | 9.0 | 8.9 | 9.6 | 11.7 | 8.9 | 9.6 | 9.2 | 8.8 | 8.1 | 8.4 | 7.8 | 10.4 | 9.5 | - | 7.8 | 8.2 | 8.0 |
| *natalensis* | 15.1 | 15.6 | 15.8 | 12.0 | 12.2 | 12.4 | 10.6 | 10.7 | 10.0 | 9.9 | 10.3 | 10.0 | 9.8 | 6.5 | 8.4 | 8.2 | 9.0 | 6.9 | 7.3 | 8.9 | 7.3 | 3.4 | 8.5 | - | 0.7 | 3.4 |
| *harveyi* | 15.4 | 16.0 | 16.5 | 12.2 | 12.9 | 12.9 | 10.9 | 11.2 | 10.6 | 10.1 | 10.8 | 10.3 | 9.6 | 7.0 | 8.7 | 8.5 | 9.5 | 7.3 | 7.8 | 9.0 | 7.6 | 3.3 | 9.0 | 0.7 | - | 3.5 |
| *nigrifrons* | 14.5 | 15.3 | 15.4 | 11.8 | 12.4 | 11.4 | 10.7 | 9.6 | 9.3 | 8.4 | 8.9 | 8.6 | 9.2 | 5.8 | 7.4 | 7.4 | 8.5 | 5.8 | 6.8 | 7.6 | 6.6 | 1.5 | 8.8 | 3.5 | 3.6 | - |

Table 5 Pairwise distances (%) calculated for the small ribosomal subunit. Divergence values above the diagonal line are uncorrected p-distances while HKY (Hasegawa *et al.* 1985) values are shown below the diagonal line. Abbreviations used are: Cam=Cameroon; Gab=Gabon; SA=South Africa; Zim=Zimbabwe; Con=Democratic Republic of Congo

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Antilocapra* | - | 12.9 | 13.6 | 12.2 | 12.1 | 12.1 | 12.5 | 13.0 | 13.0 | 12.9 | 13.7 | 13.3 | 12.7 | 12.7 | 12.3 | 12.1 | 12.8 | 12.5 | 12.5 | 12.4 | 12.5 | 12.8 | 12.5 | 12.9 | 13.2 | 12.5 |
| *Oreotragus* | 14.4 | - | 13.0 | 11.6 | 11.5 | 11.3 | 11.1 | 10.4 | 10.2 | 11.2 | 11.0 | 11.0 | 10.4 | 11.1 | 11.1 | 11.0 | 11.2 | 11.2 | 10.2 | 11.2 | 10.7 | 11.9 | 11.2 | 11.9 | 11.9 | 11.9 |
| *Bos* | 15.5 | 14.6 | - | 7.8 | 7.9 | 7.9 | 7.7 | 7.7 | 7.6 | 7.6 | 8.2 | 8.3 | 8.3 | 7.8 | 8.1 | 8.2 | 7.9 | 8.0 | 7.9 | 8.2 | 7.8 | 7.9 | 7.3 | 7.8 | 7.9 | 7.7 |
| *monticola*(Cam) | 13.8 | 12.8 | 8.4 | - | 0.3 | 1.4 | 2.3 | 5.0 | 5.0 | 5.2 | 5.3 | 5.0 | 4.8 | 4.4 | 4.3 | 4.4 | 4.4 | 4.3 | 4.4 | 4.8 | 3.7 | 4.9 | 4.1 | 5.1 | 5.4 | 5.2 |
| *monticola*(Gab) | 13.6 | 12.7 | 8.6 | 0.3 | - | 1.8 | 2.7 | 4.7 | 4.7 | 5.0 | 5.2 | 4.9 | 4.7 | 4.3 | 3.9 | 4.0 | 4.3 | 4.1 | 4.3 | 4.7 | 3.6 | 4.8 | 4.3 | 4.9 | 5.3 | 5.1 |
| *monticola*(SA) | 13.6 | 12.5 | 8.6 | 1.4 | 1.8 | - | 1.9 | 4.7 | 4.9 | 5.1 | 5.4 | 4.7 | 4.7 | 4.0 | 4.1 | 4.3 | 4.0 | 4.1 | 4.3 | 4.9 | 3.6 | 4.8 | 4.0 | 4.9 | 5.3 | 5.1 |
| *maxwellii* | 14.1 | 12.2 | 8.3 | 2.4 | 2.8 | 1.9 | - | 4.8 | 4.5 | 5.4 | 5.6 | 5.1 | 4.8 | 4.4 | 4.5 | 4.7 | 4.7 | 4.8 | 4.7 | 5.1 | 4.0 | 5.4 | 4.4 | 5.6 | 5.4 | 5.7 |
| *grimmia*(Cam) | 14.8 | 11.4 | 8.3 | 5.3 | 4.9 | 4.9 | 5.0 | - | 1.3 | 4.1 | 3.7 | 4.0 | 4.1 | 4.0 | 3.5 | 3.6 | 4.5 | 3.5 | 3.6 | 3.7 | 3.2 | 4.4 | 3.7 | 4.3 | 4.1 | 4.4 |
| *grimmia*(Zim) | 14.8 | 11.0 | 8.2 | 5.3 | 4.9 | 5.2 | 4.7 | 1.3 | - | 3.9 | 3.5 | 3.5 | 3.6 | 4.0 | 3.2 | 3.4 | 4.3 | 3.7 | 3.1 | 3.7 | 3.0 | 4.7 | 3.7 | 4.5 | 4.4 | 4.7 |
| *silvicultor* | 14.7 | 12.3 | 8.2 | 5.5 | 5.3 | 5.3 | 5.8 | 4.3 | 4.0 | - | 1.9 | 2.7 | 2.6 | 3.0 | 3.0 | 3.1 | 3.5 | 3.0 | 2.8 | 2.7 | 2.7 | 3.9 | 3.7 | 3.2 | 3.6 | 3.9 |
| *spadix* | 15.7 | 12.0 | 8.9 | 5.6 | 5.5 | 5.8 | 5.9 | 3.9 | 3.6 | 2.0 | - | 2.6 | 2.7 | 3.9 | 3.1 | 3.2 | 3.6 | 3.4 | 2.7 | 3.1 | 3.1 | 4.3 | 4.1 | 3.9 | 4.0 | 4.3 |
| *dorsalis* | 15.2 | 12.0 | 9.1 | 5.3 | 5.2 | 4.9 | 5.3 | 4.2 | 3.6 | 2.8 | 2.6 | - | 2.7 | 3.4 | 2.8 | 3.0 | 3.4 | 3.1 | 2.7 | 3.4 | 2.6 | 3.7 | 3.6 | 3.6 | 3.7 | 3.7 |
| *jentinki* | 14.3 | 11.4 | 9.1 | 5.0 | 4.9 | 4.9 | 5.0 | 4.3 | 3.8 | 2.6 | 2.8 | 2.8 | - | 3.2 | 2.4 | 2.3 | 3.0 | 2.7 | 1.8 | 2.4 | 2.2 | 4.1 | 3.7 | 3.5 | 3.4 | 4.1 |
| *leucogaster* | 14.3 | 12.2 | 8.5 | 4.6 | 4.4 | 4.2 | 4.6 | 4.2 | 4.2 | 3.1 | 4.0 | 3.5 | 3.3 | - | 2.3 | 2.4 | 3.0 | 1.8 | 2.4 | 2.8 | 2.0 | 2.6 | 3.1 | 2.3 | 2.7 | 2.8 |
| *callipygus*(Gab) | 13.8 | 12.2 | 8.8 | 4.5 | 4.0 | 4.3 | 4.7 | 3.6 | 3.3 | 3.1 | 3.2 | 2.9 | 2.5 | 2.4 | - | 0.1 | 2.3 | 1.7 | 0.9 | 1.8 | 0.5 | 3.0 | 2.3 | 2.6 | 2.7 | 3.2 |
| *callipygus*(Con) | 13.6 | 12.0 | 8.9 | 4.6 | 4.2 | 4.5 | 4.9 | 3.8 | 3.5 | 3.2 | 3.3 | 3.1 | 2.4 | 2.5 | 0.1 | - | 2.2 | 1.8 | 0.7 | 1.9 | 0.6 | 3.1 | 2.4 | 2.7 | 2.8 | 3.4 |
| *zebra* | 14.5 | 12.3 | 8.6 | 4.6 | 4.5 | 4.2 | 4.9 | 4.8 | 4.5 | 3.6 | 3.8 | 3.5 | 3.1 | 3.1 | 2.4 | 2.2 | - | 3.2 | 1.7 | 3.6 | 2.3 | 3.5 | 3.0 | 3.7 | 4.1 | 3.7 |
| *rubidus* | 14.2 | 12.3 | 8.6 | 4.4 | 4.3 | 4.3 | 5.0 | 3.6 | 3.9 | 3.1 | 3.5 | 3.2 | 2.8 | 1.8 | 1.7 | 1.8 | 3.3 | - | 1.9 | 2.2 | 1.4 | 1.7 | 2.8 | 0.9 | 1.0 | 1.9 |
| *ogilbyi* | 14.1 | 11.1 | 8.6 | 4.6 | 4.5 | 4.5 | 4.9 | 3.8 | 3.2 | 2.9 | 2.8 | 2.8 | 1.8 | 2.5 | 0.9 | 0.7 | 1.7 | 2.0 | - | 2.2 | 0.9 | 3.4 | 2.7 | 3.0 | 3.1 | 3.6 |
| *niger* | 14.0 | 12.4 | 8.9 | 5.0 | 4.9 | 5.2 | 5.3 | 3.9 | 3.9 | 2.8 | 3.2 | 3.5 | 2.5 | 2.9 | 1.8 | 2.0 | 3.8 | 2.2 | 2.2 | - | 1.5 | 3.2 | 2.8 | 2.8 | 3.0 | 3.2 |
| *weynsii* | 14.1 | 11.7 | 8.5 | 3.9 | 3.7 | 3.7 | 4.2 | 3.3 | 3.1 | 2.8 | 3.2 | 2.6 | 2.2 | 2.1 | 0.5 | 0.6 | 2.4 | 1.4 | 0.9 | 1.5 | - | 2.7 | 2.3 | 2.3 | 2.4 | 3.0 |
| *rufilatus* | 14.4 | 13.1 | 8.6 | 5.2 | 5.0 | 5.0 | 5.7 | 4.6 | 4.9 | 4.0 | 4.5 | 3.9 | 4.3 | 2.6 | 3.0 | 3.2 | 3.6 | 1.7 | 3.5 | 3.3 | 2.8 | - | 3.5 | 1.5 | 1.7 | 0.5 |
| *adersi* | 14.1 | 12.3 | 7.8 | 4.3 | 4.5 | 4.2 | 4.6 | 3.9 | 3.9 | 3.9 | 4.3 | 3.8 | 3.9 | 3.2 | 2.4 | 2.5 | 3.1 | 2.9 | 2.8 | 2.9 | 2.4 | 3.6 | - | 3.6 | 4.0 | 3.7 |
| *natalensis* | 14.6 | 13.1 | 8.4 | 5.3 | 5.2 | 5.2 | 5.9 | 4.5 | 4.7 | 3.3 | 4.0 | 3.7 | 3.6 | 2.4 | 2.6 | 2.8 | 3.9 | 0.9 | 3.0 | 2.9 | 2.4 | 1.5 | 3.7 | - | 0.3 | 1.3 |
| *harveyi* | 15.0 | 13.1 | 8.6 | 5.7 | 5.6 | 5.6 | 5.7 | 4.3 | 4.6 | 3.7 | 4.2 | 3.9 | 3.5 | 2.8 | 2.8 | 2.9 | 4.3 | 1.0 | 3.2 | 3.0 | 2.5 | 1.7 | 4.2 | 0.3 | - | 1.4 |
| *nigrifrons* | 14.1 | 13.1 | 8.3 | 5.4 | 5.3 | 5.3 | 6.0 | 4.6 | 4.9 | 4.0 | 4.5 | 3.9 | 4.3 | 2.9 | 3.3 | 3.5 | 3.9 | 2.0 | 3.7 | 3.3 | 3.0 | 0.5 | 3.9 | 1.3 | 1.4 | - |

## Rate heterogeneity

The relative rate test (Robinson *et al.* 1998) identified a single species, *C. monticola*, as having a different substitution rate relative to its congeners. Four pairwise comparisons involving the three *C. monticola* lineages resulted in significant P values for synonymous changes. These were *C. monticola* vs.: *C. callipygus* (P=0.005); *C. weynsi* (P=0.004); *C. harveyi* (P=0.005); *C. adersi* (P=0.002). Since no rate variation was detected between the three *C. monticola* specimens analyzed it would appear that the lineage itself is characterized by a different rate. When excluding transitions, and considering transversional changes in isolation, none of the 19 duiker lineages are characterized by significant rate heterogeneity. In contrast to the cytochrome *b* gene, analysis of the small ribosomal sequences revealed only a single case of rate heterogeneity involving *C. natalensis* and *C. ogilbyi* (P=0.006). This is not a novel finding since different rates of evolution between cytochrome *b* and the small ribosomal subunit have been reported (Zhang & Ryder 1995, Matthee & Robinson 1997), perhaps reflecting the different evolutionary constraints under which these loci have evolved. As was the case with cytochrome *b*, no rate heterogeneity was detected when analyses were based on transversional changes alone.

## Cytochrome *b*

The cytochrome *b* data set consisted of 1 140 bp of which 311 characters were variable and 243 were parsimony informative for the ingroup. As is typical for protein coding genes, most of these variable sites were at the third codon position (259 variable sites, 207 parsimony informative sites), with the second codon position being the most conserved (14 variable sites, 8 parsimony informative sites). First codon positions had 38 variable and 28 parsimony informative characters, six of which comprised leucine changes. Synonymous changes far outnumbered non-synonymous changes, resulting in only 28 variable and 18 parsimony informative amino acids for the ingroup.

The nucleotide composition for cytochrome *b* is given in Table 6. As is typical for most mammalian lineages (Irwin *et al.* 1991), the duiker lineages display an overall bias against guanine (13.2%), with the selection against this nucleotide most pronounced at third codon positions (3.5% guanine). Transitional changes outnumbered transversions 6.3 to 1 when counting all changes across 23 ingroup and 3 outgroup taxa. This ratio increased to 13.2 to 1 when the outgroups were excluded, and when considering a single representative for each cephalophine species, the ratio was 13.7 to 1.

Table 6 Base composition for the complete cytochrome *b* gene, as well as first, second, and third codon positions. Base frequencies were calculated in Paup 4.0b2a (Swofford 1999). Abbreviations used to indicate sampling localities are: Cam=Cameroon; Gab=Gabon; SA=South Africa; CAR=Central African Republic; Zim=Zimbabwe; Con=Democratic Republic of Congo.

| | Complete gene | | | | First | | | | Second | | | | Third | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Taxon | A | C | G | T | A | C | G | T | A | C | G | T | A | C | G | T |
| *Antilocapra* | 0.325 | 0.298 | 0.128 | 0.247 | 0.302 | 0.260 | 0.221 | 0.215 | 0.200 | 0.239 | 0.136 | 0.423 | 0.473 | 0.394 | 0.028 | 0.102 |
| *Oreotragus* | 0.317 | 0.276 | 0.130 | 0.275 | 0.310 | 0.250 | 0.213 | 0.226 | 0.202 | 0.247 | 0.136 | 0.413 | 0.439 | 0.331 | 0.042 | 0.186 |
| *Cow* | 0.312 | 0.301 | 0.134 | 0.251 | 0.286 | 0.260 | 0.228 | 0.223 | 0.205 | 0.252 | 0.136 | 0.405 | 0.444 | 0.392 | 0.036 | 0.126 |
| *monticola*(Cam) | 0.314 | 0.293 | 0.127 | 0.264 | 0.294 | 0.255 | 0.231 | 0.218 | 0.205 | 0.247 | 0.134 | 0.413 | 0.444 | 0.378 | 0.015 | 0.160 |
| *monticola*(Gab) | 0.314 | 0.289 | 0.131 | 0.264 | 0.294 | 0.260 | 0.231 | 0.213 | 0.202 | 0.244 | 0.136 | 0.415 | 0.447 | 0.363 | 0.026 | 0.163 |
| *monticola*(SA) | 0.313 | 0.291 | 0.128 | 0.267 | 0.294 | 0.255 | 0.228 | 0.221 | 0.205 | 0.247 | 0.134 | 0.413 | 0.439 | 0.371 | 0.021 | 0.168 |
| *maxwellii* | 0.311 | 0.285 | 0.133 | 0.269 | 0.297 | 0.247 | 0.228 | 0.226 | 0.202 | 0.247 | 0.136 | 0.413 | 0.434 | 0.363 | 0.034 | 0.168 |
| *grimmia*(CAR) | 0.314 | 0.300 | 0.128 | 0.256 | 0.297 | 0.271 | 0.223 | 0.207 | 0.205 | 0.252 | 0.136 | 0.405 | 0.439 | 0.378 | 0.026 | 0.155 |
| *grimmia*(Zim) | 0.317 | 0.298 | 0.126 | 0.257 | 0.297 | 0.271 | 0.223 | 0.207 | 0.210 | 0.252 | 0.134 | 0.402 | 0.444 | 0.371 | 0.021 | 0.163 |
| *silvicultor* | 0.313 | 0.298 | 0.128 | 0.260 | 0.297 | 0.257 | 0.226 | 0.218 | 0.207 | 0.244 | 0.134 | 0.413 | 0.434 | 0.392 | 0.023 | 0.150 |
| *spadix* | 0.313 | 0.298 | 0.129 | 0.258 | 0.302 | 0.260 | 0.223 | 0.213 | 0.207 | 0.250 | 0.134 | 0.407 | 0.428 | 0.384 | 0.031 | 0.155 |
| *dorsalis* | 0.314 | 0.296 | 0.127 | 0.262 | 0.302 | 0.257 | 0.221 | 0.218 | 0.207 | 0.252 | 0.134 | 0.405 | 0.431 | 0.378 | 0.026 | 0.163 |
| *jentinki* | 0.314 | 0.284 | 0.128 | 0.272 | 0.302 | 0.257 | 0.223 | 0.215 | 0.200 | 0.250 | 0.142 | 0.407 | 0.439 | 0.344 | 0.021 | 0.194 |
| *leucogaster* | 0.309 | 0.299 | 0.135 | 0.256 | 0.297 | 0.255 | 0.226 | 0.221 | 0.205 | 0.250 | 0.136 | 0.407 | 0.426 | 0.392 | 0.042 | 0.139 |
| *callipygus*(Gab) | 0.306 | 0.295 | 0.137 | 0.260 | 0.300 | 0.257 | 0.223 | 0.218 | 0.205 | 0.247 | 0.136 | 0.410 | 0.413 | 0.381 | 0.052 | 0.152 |
| *callipygus*(Con) | 0.306 | 0.297 | 0.137 | 0.258 | 0.300 | 0.257 | 0.223 | 0.218 | 0.205 | 0.247 | 0.136 | 0.410 | 0.413 | 0.386 | 0.052 | 0.147 |
| *zebra* | 0.313 | 0.305 | 0.129 | 0.249 | 0.300 | 0.263 | 0.223 | 0.213 | 0.202 | 0.250 | 0.139 | 0.407 | 0.436 | 0.402 | 0.026 | 0.126 |
| *rubidus* | 0.318 | 0.295 | 0.126 | 0.258 | 0.294 | 0.260 | 0.228 | 0.215 | 0.205 | 0.247 | 0.136 | 0.410 | 0.456 | 0.377 | 0.013 | 0.147 |
| *ogilbyi* | 0.310 | 0.304 | 0.131 | 0.253 | 0.292 | 0.273 | 0.228 | 0.205 | 0.205 | 0.247 | 0.136 | 0.410 | 0.434 | 0.392 | 0.028 | 0.144 |
| *niger* | 0.314 | 0.295 | 0.130 | 0.259 | 0.300 | 0.260 | 0.223 | 0.215 | 0.205 | 0.247 | 0.136 | 0.410 | 0.436 | 0.378 | 0.031 | 0.152 |
| *weynsi* | 0.306 | 0.289 | 0.137 | 0.266 | 0.300 | 0.255 | 0.223 | 0.221 | 0.205 | 0.247 | 0.136 | 0.410 | 0.413 | 0.365 | 0.052 | 0.168 |
| *rufilatus* | 0.302 | 0.305 | 0.140 | 0.251 | 0.297 | 0.257 | 0.223 | 0.221 | 0.205 | 0.250 | 0.136 | 0.407 | 0.405 | 0.407 | 0.060 | 0.126 |
| *adersi* | 0.309 | 0.295 | 0.133 | 0.260 | 0.297 | 0.257 | 0.226 | 0.218 | 0.205 | 0.252 | 0.136 | 0.405 | 0.426 | 0.376 | 0.036 | 0.157 |
| *natalensis* | 0.307 | 0.300 | 0.137 | 0.253 | 0.300 | 0.252 | 0.223 | 0.223 | 0.207 | 0.250 | 0.136 | 0.405 | 0.415 | 0.400 | 0.052 | 0.131 |
| *harveyi* | 0.304 | 0.298 | 0.141 | 0.256 | 0.300 | 0.252 | 0.223 | 0.223 | 0.207 | 0.250 | 0.136 | 0.405 | 0.405 | 0.392 | 0.063 | 0.139 |
| *nigrifrons* | 0.306 | 0.307 | 0.137 | 0.249 | 0.300 | 0.252 | 0.223 | 0.223 | 0.205 | 0.250 | 0.136 | 0.407 | 0.413 | 0.418 | 0.052 | 0.115 |
| Mean | 0.311 | 0.296 | 0.132 | 0.259 | 0.298 | 0.258 | 0.225 | 0.217 | 0.205 | 0.248 | 0.136 | 0.409 | 0.432 | 0.381 | 0.035 | 0.150 |

The evaluation of 10 000 random trees showed that the data contained significantly more signal than expected at random (26 taxa: g1=-0.699; P<0.01; Hillis & Huelsenbeck 1992). Since phylogenetic signal is not necessarily evenly distributed throughout the topology (Hillis 1991, Hillis & Huelsenbeck 1992), g1 values were recalculated for subsets of the data. The g1 values were significant even when various combinations of taxa, including species at the well supported tips, were excluded.

## Cytochrome *b* phylogeny

Using all three outgroups, and the empirical ingroup ti/tv ratio (13:1) and heuristic search option in Paup 4.0b2a, parsimony analyses of the 22 taxa (three outgroup taxa and a single representative of each duiker species) resulted in a single tree of 2739 steps (Fig. 2). Four duiker clades were retrieved: the conservative dwarf lineage *(C. monticola, C. maxwellii)*, the savanna specialist *(S. grimmia)*, the giant duiker group *(C. silvicultor, C. spadix, C. dorsalis, C. jentinki)* and the red duiker lineage. Within the latter, two distinct maternal clusters are evident: an east African red duiker clade (sensu Kingdon 1982) comprising *C. leucogaster, C. rufilatus, C. nigrifrons, C. natalensis, C. harveyi* and a west African red duiker clade (sensu Kingdon 1982) comprising *C. callipygus, C. weynsi, C. ogilbyi, C. rubidus,* and *C. niger*. There is good agreement between these mitochondrially defined groups and those which have previously been delimited on phenotypic grounds, principally the length and texture of hair on the shoulders (Kingdon 1982). The only exceptions are *C. leucogaster* which Kingdon (1982) places in the west African group, and *C. rubidus* which is allocated to the east African group. Analysis of the data using a 2:1 and unordered weighting scheme resulted in several equally most parsimonious solutions (three and eight trees respectively); however, the consensus trees (not shown) were near identical to the empirical tree, differing only with respect to associations that received less than 50% bootstrap support. Both of the consensus trees indicate a sister group relationship between *C. adersi* and *C. niger*, with *C. zebra*, the red duiker lineage, and the giant duiker forming a trichotomy.

Two trees, one step longer than the most parsimonious solution (Fig. 2), were recovered. The consensus of these differed from Figure 2 in the positioning of *C. zebra* and *C. adersi* (which clustered as sister taxa basal to the red duiker lineage as opposed to being basal within the forest duiker), and in the unresolved placement of *C. niger* within the red duiker lineage. However, nonparametric bootstrap values for nodes supporting *C. zebra*, *C. adersi*, and *C. niger* were invariably low (<50%) and the resolution of these species in the

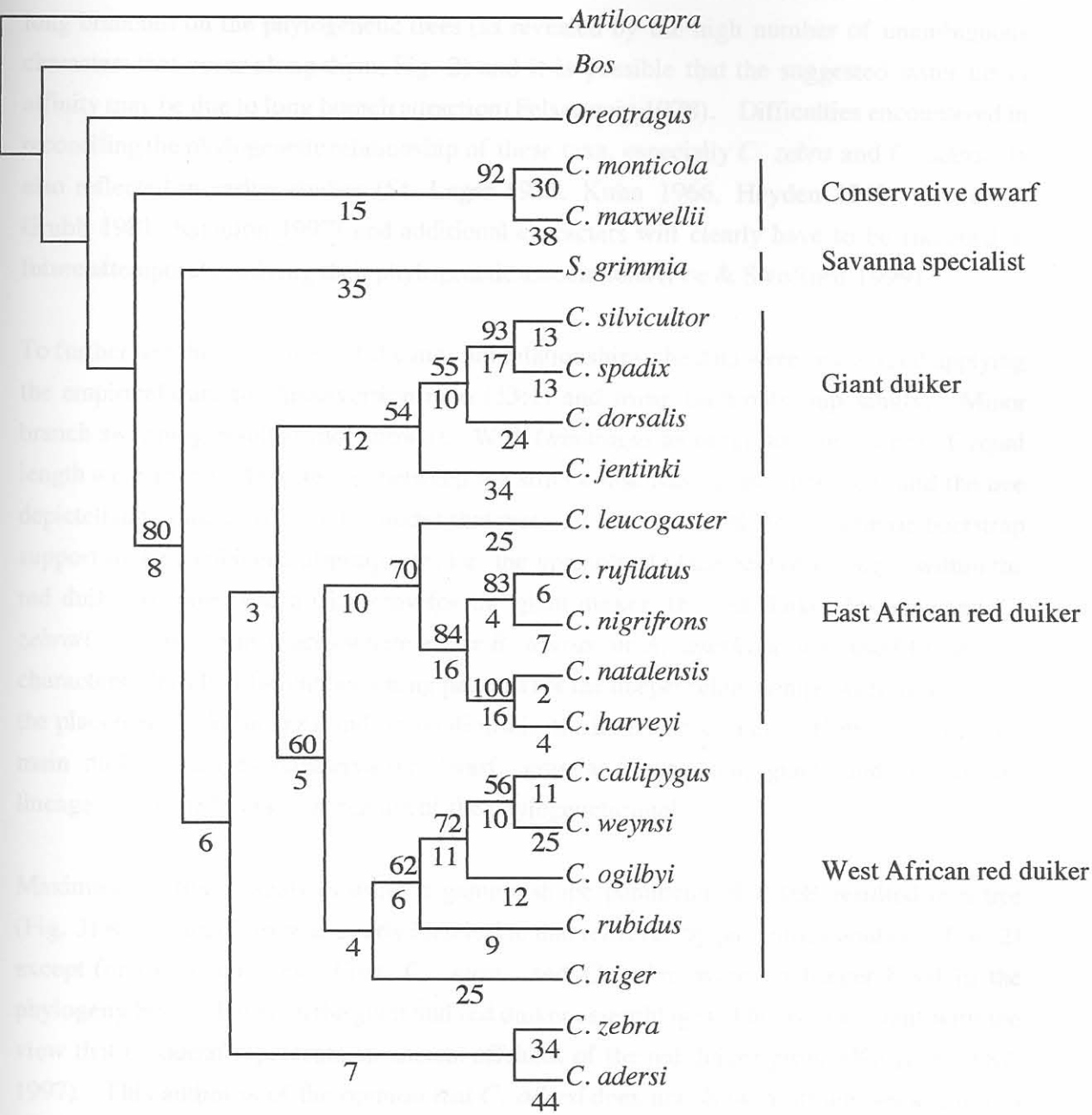



Figure 2 The most parsimonious tree obtained under the empirical cytochrome *b* ti/tv weighting of 13:1. This tree is based on 1 140 bp and has a length of 2 739 steps. Nonparametric bootstrap values obtained after 1 000 iterations are shown above branches while the number of unambiguous characters that occur along branches are shown below them. Bootstrap values for nodes that received less than 50% support are not indicated.

duiker phylogeny unfortunately remains obscure irrespective method of analyses or weighting scheme used. Moreover, *C. zebra* and *C. adersi* are characterized by relatively long branches on the phylogenetic trees (as revealed by the high number of unambiguous characters that occur along them; Fig. 2) and it is possible that the suggested sister taxon affinity may be due to long branch attraction (Felsenstein 1978). Difficulties encountered in reconciling the phylogenetic relationship of these taxa, especially *C. zebra* and *C. adersi*, is also reflected in earlier studies (St. Leger 1936, Kuhn 1966, Heyden 1968, Groves & Grubb 1981, Kingdon 1997) and additional characters will clearly have to be included in future attempts at resolving their phylogenetic associations (Poe & Swofford 1999).

To further test the robustness of the ingroup relationships, the data were reanalyzed applying the empirical transition/transversion ratio (13:1) and using each outgroup singly. Minor branch swapping resulted (not shown). With *Oreotragus* as outgroup, three trees of equal length were found. Differences between the strict consensus of the three trees and the tree depicted in Figure 2 involved the nodes that received less than 50% nonparametric bootstrap support in the combined outgroup tree, i.e. the unresolved placement of *C. niger* within the red duiker lineage, and a tricotomy for the giant duiker, the red duiker lineage, and *C. zebra*/*C. adersi*. In instances where either *B. taurus* or *A. americana* was used to polarize characters, slightly different branching patterns for the deeper relationships were found (e.g. the placement of *Sylvicapra* and its relationship to the forest duiker). However, the four main duiker lineages (conservative dwarf, grassland specialist, giant, and red duiker lineages) remained a constant feature of the phylogenetic analyses.

Maximum likelihood analysis using a gamma shape parameter of 0.168 resulted in a tree (Fig. 3) whose topology was nearly identical to that retrieved by parsimony analysis (Fig. 2) except for two disparities. First, C. *adersi* and *C. zebra* were no longer basal in the phylogeny but fell between the giant and red duiker assemblages. This is consistent with the view that *C. adersi* represents an ancient offshoot of the red duiker group (Kingdon 1982, 1997). This author is of the opinion that *C. adersi* does not show a strong association to either of the two red duiker clades (east or west African) but is rather central to their radiation.

Secondly, contrary to the parsimony result (although admittedly with no bootstrap support) *C. adersi* and *C. zebra* are not grouped as sister taxa by maximum likelihood. Since this analysis does not suffer from the effects of long branch attraction (Huelsenbeck 1995, Swofford *et al.* 1996) their association by parsimony may be misleading. The tree generated

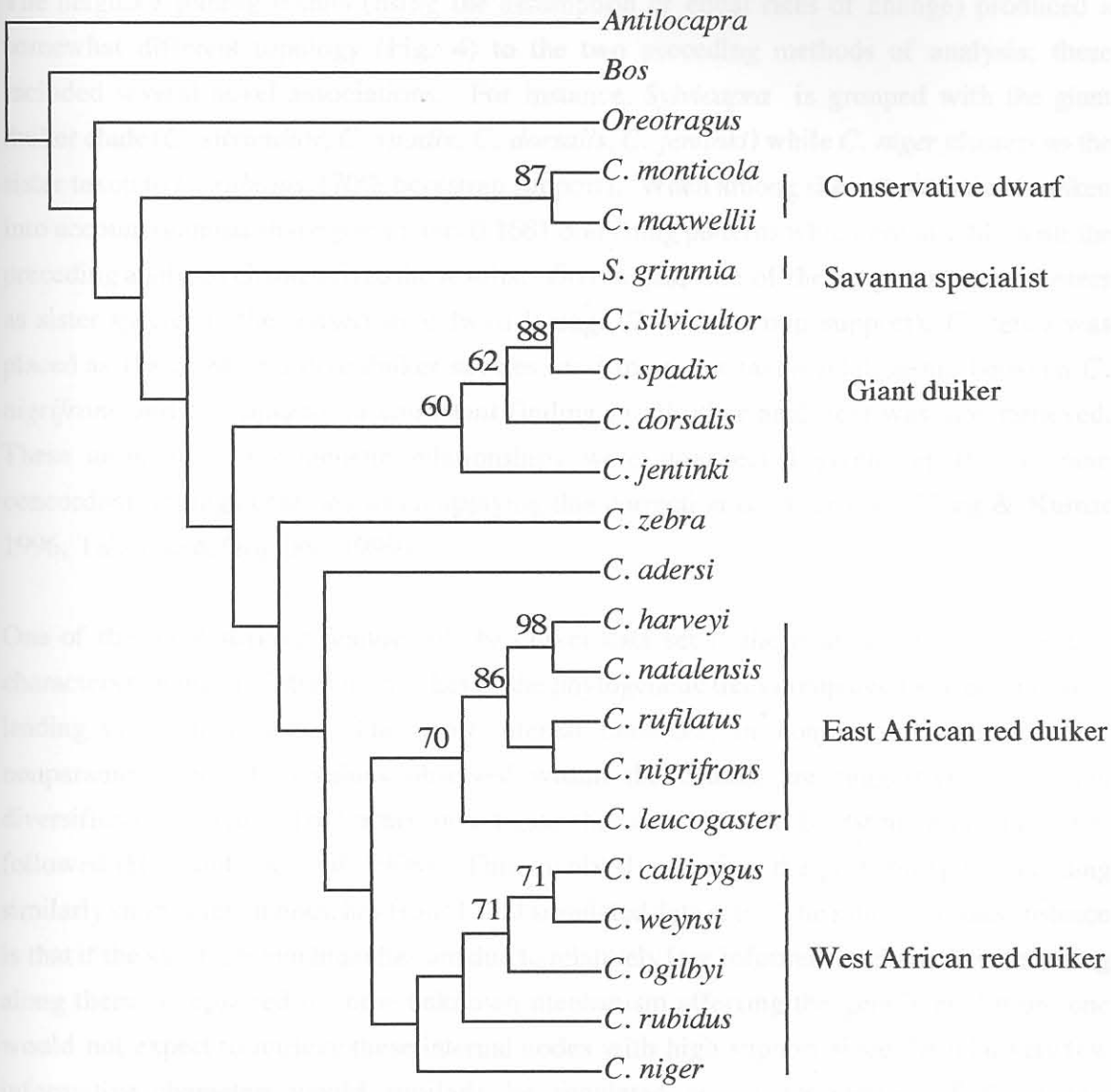



Figure 3 The optimal maximum likelihood topology inferred from the cytochrome *b* data. The tree was constructed in Paup 4.0b2a (Swofford 1999) under the HKY (Hasegawa *et al.* 1985) model of sequence evolution with rate heterogeneity (gamma shape parameter=0.168), empirical ti/tv ratio (13:1), and nucleotide frequencies calculated from the data. Bootstrap support, obtained from 100 iterations, is shown.

by maximum likelihood analysis under the assumption of equal rates of change (not shown) was identical to that presented in Figure 3 with the exception of a sister taxon relationship for *C. adersi* and *C. zebra* which received less than 50% bootstrap support.

The neighbor joining results (using the assumption of equal rates of change) produced a somewhat different topology (Fig. 4) to the two preceding methods of analysis; these included several novel associations. For instance, *Sylvicapra* is grouped with the giant duiker clade *(C. silvicultor, C. spadix, C. dorsalis, C. jentinki)* while *C. niger* clusters as the sister taxon to *C. rubidus* (70% bootstrap support). When among site rate variation is taken into account (gamma shape parameter=0.168) branching patterns which are at odds with the preceding analyses characterized the results. *Oreotragus*, one of the outgroup taxa, clusters as sister species to the conservative dwarf lineage (74% bootstrap support), *C. zebra* was placed as the most primitive duiker species, and the sister taxon relationship between *C. nigrifrons* and *C. rufilatus* (a consistent finding in all other analyses) was not retrieved. These anomalous phylogenetic relationships were unexpected given reports of more concordant findings obtained when applying this correction to other taxa (Yang & Kumar 1996, Takezaki & Gojobori 1999).

One of the most striking features of the duiker data set is the relatively few informative characters that map to internal branches of the phylogenetic trees compared to larger numbers leading to terminal taxa. The short internal branches, in conjunction with the low nonparametric bootstrap values observed within the clades, are suggestive of a rapid diversification event. To further investigate this a parametric bootstrap procedure was followed (Huelsenbeck *et al.* 1996). This involved assessing the probability of obtaining similarly short internal branches from 1 000 simulated data sets. The rational in this instance is that if the short internal branches are due to relatively few informative characters occurring along them, as opposed to some unknown mechanism affecting the gene's evolution, one would not expect to retrieve these internal nodes with high support since the relatively few informative characters would similarly be simulated as an assumption of the model (Halanych & Robinson 1999). Importantly for the present investigation, this approach led to a parametric bootstrap topology which is identical to the cytochrome *b* parsimony topology. High bootstrap support (Fig. 5) is found for the main duiker lineages (conservative dwarf, giant duiker, and red duiker lineage). In sharp contrast, the internal branches received low support, indicating the relatively few characters defining earlier speciation events within the group.

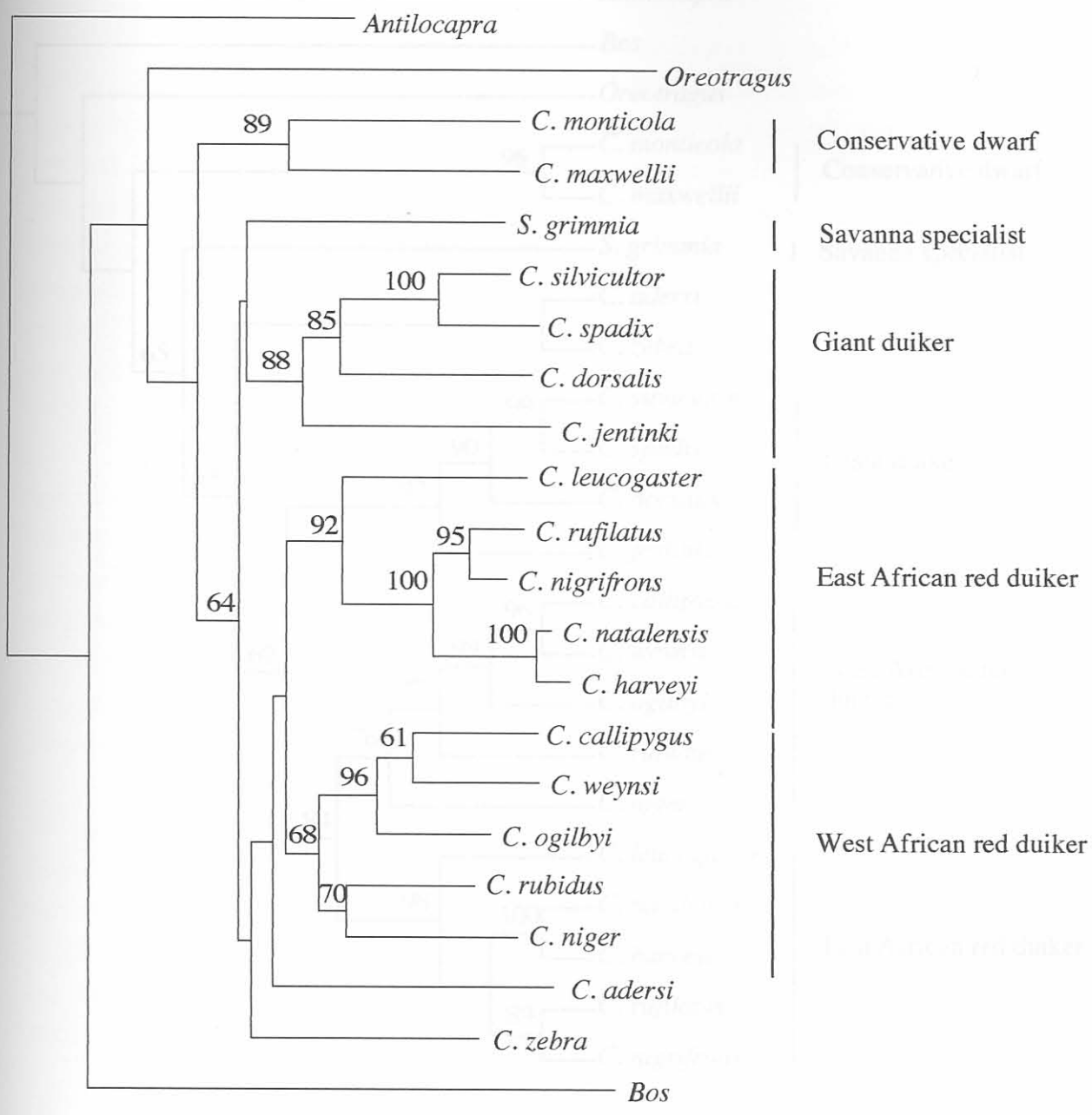



Figure 4 Neighbor joining tree constructed in Paup 4.0b2a (Swofford 1999) from 1 140 bp of the cytochrome *b* gene. The HKY (Hasegawa *et al.* 1985) correction was applied to the distance matrix, with equal rates of evolution assumed for all lineages. The branch lengths are drawn proportionally to the amount of change occurring along them, and nonparametric bootstrap support is indicated.

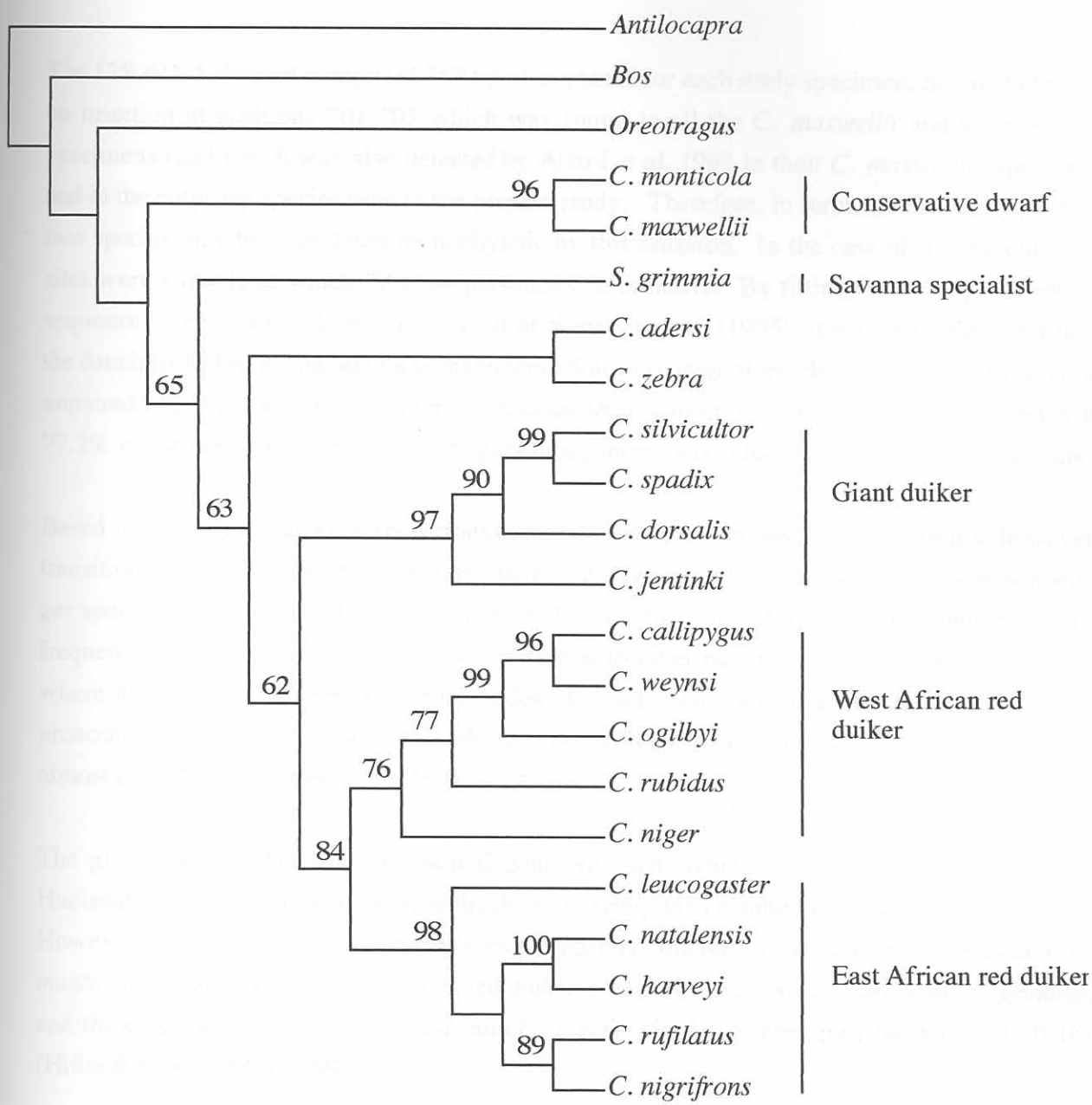



Figure 5 The cytochrome *b* parametric bootstrap topology obtained from 1 000 simulated data sets created by the Siminator computer program (Huelsenbeck *et al.* 1996). The most parsimonious tree (Fig. 2) was used as the model tree, with the gamma shape parameter (0.168) and kappa value (27.167) estimated *via* maximum likelihood. The simulated data sets were developed under the HKY (Hasegawa *et al.* 1985) model of evolution.

## 12S rRNA

The 12S rRNA data set comprised 767 bp of sequence for each study specimen; this included a 3 bp insertion at positions 701-703 which was found in all the *C. maxwellii* and *C. monticola* specimens (and which was also detected by Allard *et al.* 1992 in their *C. maxwellii* specimen) and in the outgroup species used in the present study. Therefore, in terms of the ingroup, these two species may be considered monophyletic by this criterion. In the case of the ingroup, 110 sites were variable of which 72 were parsimony informative. By fitting the 767 bp 12S rRNA sequence to the secondary structure model of Springer *et al.* (1995), it was possible to partition the data into 359 stem and 408 loop characters. Since compensatory changes are not involved in unpaired regions, the loop characters were considerably more variable than those of stems with 77.2% of the variable and 81.9% of the parsimony informative characters found in loop regions.

Based on the 23 ingroup taxa, transitions outnumbered transversions 7.1:1. In stems, however, transitions were 9.8 times more frequent than transversions. When only a single representative per species was included (19 taxa), the overall ti/tv ratio was 7.3:1. The distribution of base frequencies across all characters is in accordance with other mammals (see Springer *et al.* 1995) where adenines outnumber other nucleotides (37.8%). This unequal base frequency is most pronounced in unpaired regions (43.1% adenine) with paired regions being characterized by an almost equal base occurrence (Table 7).

The g1 statistic for the small ribosomal gene was significant (g1=-0.853; P<0.01; Hillis & Huelsenbeck 1992). The exclusion of the three outgroup taxa resulted in a g1=-1.019 (P<0.01). However, the inclusion of a single species per adaptive duiker lineage (conservative dwarfs: *C. monticola*, giant duiker: *C. silvicultor*, red duiker: *C. natalensis*; savanna specialist: *S. grimmia*, and the enigmatic *C. zebra*, *C. adersi*, and *C. niger)* yielded a nonsignificant value of -0.101 (Hillis & Huelsenbeck 1992).

## 12S rRNA phylogeny

Parsimony analysis of the 12S rRNA sequences with a 2:1 weighting resulted in 14 equally most parsimonious trees of 386 steps. The consensus tree is presented in Figure 6. Moreover, when weakly supported nodes (<50%) were collapsed, the resultant topology was identical to the 12S rRNA bootstrap trees obtained under an unordered and empirical 7:1 weighting scheme. Treating the 3 bp indel as missing data, or as a fifth character, did not influence the result although in the latter instance marginally higher bootstrap support for the basal placement of

Table 7 Base composition for the small ribosomal subunit. The composition of the stem and loop domains are also indicated. Frequencies were calculated in Paup 4.0b2a (Swofford 1999). Abbreviations used to indicate sampling localities are: Cam=Cameroon; Gab=Gabon; SA=South Africa; CAR=Central African Republic; Zim=Zimbabwe; Con=Democratic Republic of Congo.

| | Complete gene | | | | Stem | | | | Loop | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Taxon | A | C | G | T | A | C | G | T | A | C | G | T |
| *Antilocapra* | 0.364 | 0.226 | 0.185 | 0.223 | 0.298 | 0.224 | 0.246 | 0.230 | 0.422 | 0.232 | 0.127 | 0.218 |
| *Oreotragus* | 0.371 | 0.228 | 0.177 | 0.222 | 0.310 | 0.229 | 0.229 | 0.229 | 0.423 | 0.235 | 0.127 | 0.213 |
| *Bos* | 0.381 | 0.222 | 0.176 | 0.219 | 0.308 | 0.227 | 0.235 | 0.229 | 0.444 | 0.223 | 0.120 | 0.211 |
| *monticola*(Cam) | 0.379 | 0.221 | 0.169 | 0.229 | 0.318 | 0.221 | 0.224 | 0.235 | 0.428 | 0.227 | 0.119 | 0.224 |
| *monticola*(Gab) | 0.378 | 0.224 | 0.170 | 0.226 | 0.318 | 0.221 | 0.224 | 0.235 | 0.425 | 0.232 | 0.122 | 0.220 |
| *monticola*(SA) | 0.382 | 0.221 | 0.165 | 0.229 | 0.317 | 0.227 | 0.224 | 0.230 | 0.437 | 0.224 | 0.110 | 0.227 |
| *maxwellii* | 0.378 | 0.227 | 0.171 | 0.223 | 0.314 | 0.227 | 0.227 | 0.230 | 0.433 | 0.232 | 0.117 | 0.217 |
| *grimmia*(CAR) | 0.379 | 0.226 | 0.174 | 0.219 | 0.321 | 0.221 | 0.221 | 0.235 | 0.428 | 0.238 | 0.127 | 0.204 |
| *grimmia*(Zim) | 0.379 | 0.226 | 0.175 | 0.218 | 0.321 | 0.218 | 0.221 | 0.237 | 0.428 | 0.240 | 0.130 | 0.200 |
| *silvicultor* | 0.379 | 0.226 | 0.172 | 0.221 | 0.313 | 0.224 | 0.229 | 0.232 | 0.436 | 0.236 | 0.118 | 0.209 |
| *spadix* | 0.373 | 0.226 | 0.179 | 0.221 | 0.313 | 0.224 | 0.229 | 0.232 | 0.421 | 0.236 | 0.132 | 0.209 |
| *dorsalis* | 0.380 | 0.231 | 0.172 | 0.214 | 0.318 | 0.227 | 0.224 | 0.229 | 0.433 | 0.243 | 0.122 | 0.200 |
| *jentinki* | 0.379 | 0.223 | 0.172 | 0.223 | 0.316 | 0.221 | 0.227 | 0.235 | 0.433 | 0.233 | 0.120 | 0.212 |
| *leucogaster* | 0.380 | 0.225 | 0.171 | 0.222 | 0.313 | 0.227 | 0.227 | 0.232 | 0.438 | 0.231 | 0.118 | 0.212 |
| *callipygus*(Gab) | 0.380 | 0.229 | 0.172 | 0.217 | 0.321 | 0.221 | 0.221 | 0.235 | 0.431 | 0.243 | 0.125 | 0.200 |
| *callipygus*(Con) | 0.379 | 0.229 | 0.174 | 0.217 | 0.318 | 0.221 | 0.224 | 0.235 | 0.431 | 0.243 | 0.125 | 0.200 |
| *zebra* | 0.380 | 0.221 | 0.171 | 0.226 | 0.313 | 0.218 | 0.227 | 0.240 | 0.438 | 0.231 | 0.118 | 0.212 |
| *rubidus* | 0.377 | 0.226 | 0.173 | 0.222 | 0.316 | 0.221 | 0.224 | 0.237 | 0.429 | 0.236 | 0.123 | 0.210 |
| *ogilbyi* | 0.380 | 0.225 | 0.172 | 0.221 | 0.318 | 0.221 | 0.224 | 0.235 | 0.433 | 0.236 | 0.122 | 0.207 |
| *niger* | 0.382 | 0.222 | 0.170 | 0.225 | 0.321 | 0.224 | 0.221 | 0.232 | 0.433 | 0.228 | 0.120 | 0.216 |
| *weynsi* | 0.382 | 0.227 | 0.171 | 0.218 | 0.321 | 0.221 | 0.221 | 0.235 | 0.433 | 0.240 | 0.122 | 0.202 |
| *rufilatus* | 0.378 | 0.227 | 0.170 | 0.223 | 0.313 | 0.221 | 0.227 | 0.237 | 0.433 | 0.238 | 0.115 | 0.212 |
| *adersi* | 0.384 | 0.221 | 0.170 | 0.223 | 0.316 | 0.221 | 0.227 | 0.235 | 0.443 | 0.228 | 0.115 | 0.212 |
| *natalensis* | 0.374 | 0.230 | 0.174 | 0.221 | 0.313 | 0.221 | 0.227 | 0.237 | 0.426 | 0.243 | 0.122 | 0.207 |
| *harveyi* | 0.371 | 0.229 | 0.176 | 0.222 | 0.313 | 0.221 | 0.227 | 0.237 | 0.421 | 0.240 | 0.127 | 0.209 |
| *nigrifrons* | 0.376 | 0.227 | 0.171 | 0.223 | 0.310 | 0.221 | 0.229 | 0.237 | 0.433 | 0.238 | 0.115 | 0.212 |
| Mean | 0.378 | 0.226 | 0.173 | 0.222 | 0.315 | 0.223 | 0.226 | 0.234 | 0.431 | 0.235 | 0.121 | 0.210 |

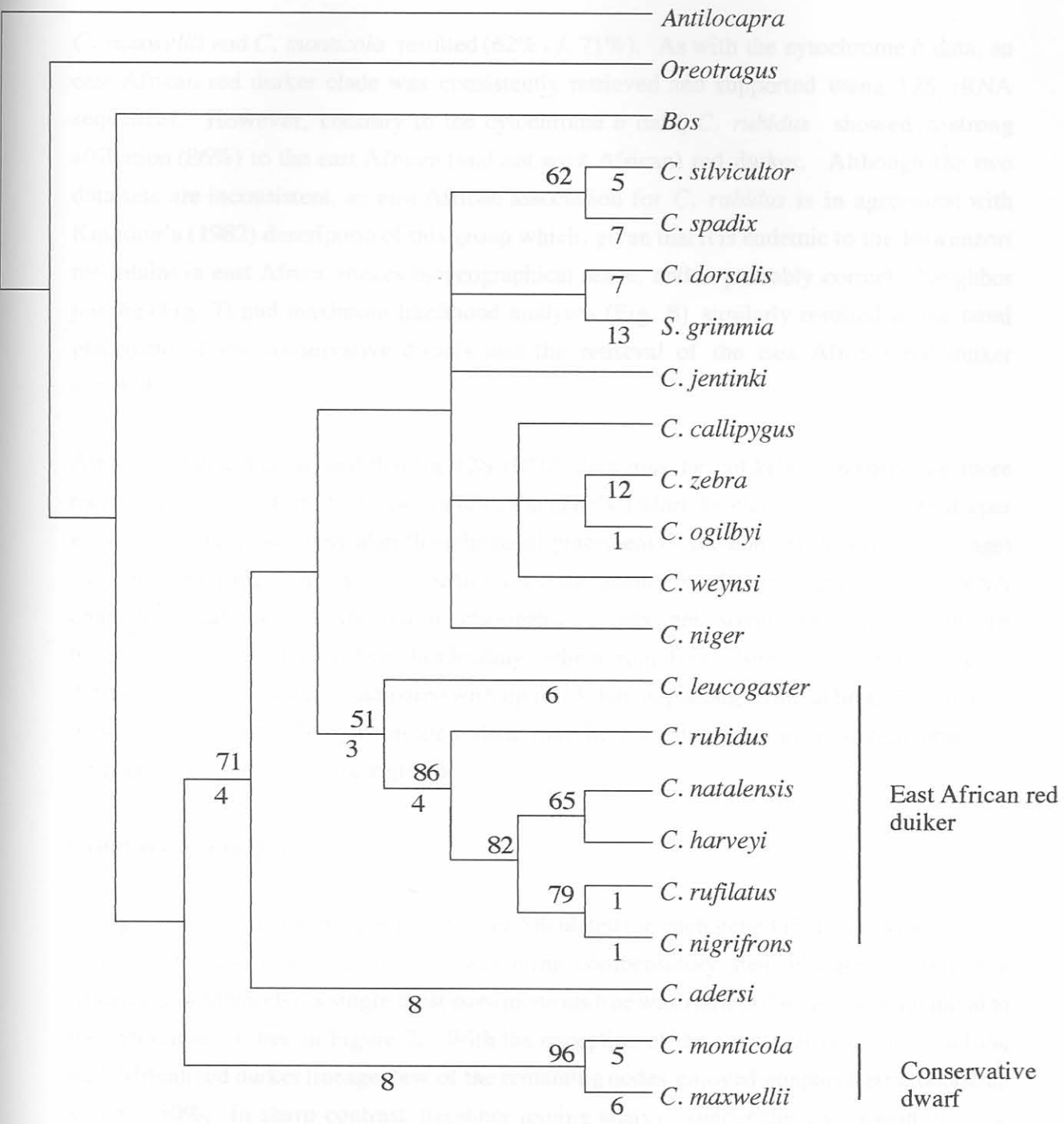



Figure 6 Strict consensus tree of 14 equally most parsimonious trees obtained from 767 bp of the 12S rRNA gene under a 2:1 ti/tv ratio. Nonparametric bootstrap values for nodes are presented above branches and the number of unambiguous changes that plot along them are indicated below the branches. No changes are shown on branches within polytomies.

*C. maxwellii* and *C. monticola* resulted (62% *cf.* 71%). As with the cytochrome *b* data, an east African red duiker clade was consistently retrieved and supported using 12S rRNA sequences. However, contrary to the cytochrome *b* data, *C. rubidus* showed a strong affiliation (86%) to the east African (and not west African) red duiker. Although the two data sets are inconsistent, an east African association for *C. rubidus* is in agreement with Kingdon's (1982) description of this group which, given that it is endemic to the Ruwenzori mountains in east Africa, makes biogeographical sense, and is probably correct. Neighbor joining (Fig. 7) and maximum likelihood analyses (Fig. 8) similarly resulted in the basal placement of the conservative dwarfs and the retrieval of the east African red duiker assemblage.

Although it could be argued that the 12S rRNA data may be unlikely to resolve the more recent radiations within the Cephalophini, the gene's failure to clarify many of the deeper evolutionary relationships (other than the basal placement of the conservative dwarf lineage) was disappointing. As with cytochrome *b*, the numbers of unambiguous 12S rRNA characters that support the basal cladogenic events are significantly less than the unambiguous characters on branches leading to the terminal taxa (between 3 and 4 characters define the deeper branches compared with up to 13 that map along terminal branches) and, as was speculated with the cytochrome *b* data, may be indicative of a near contemporaneous divergence for the main duiker groups.

**Combined analysis**

Using the empirical ti/tv weighting schemes calculated for each gene (13:1 for cytochrome *b* and 7:1 for 12S rRNA), and down weighting compensatory stem changes by 0.6 (see Material and Methods), a single most parsimonious tree was retrieved which was identical to the cytochrome *b* tree in Figure 2. With the exception of the conservative dwarfs and the east African red duiker lineage, few of the remaining nodes enjoyed nonparametric bootstrap values >50%. In sharp contrast, neighbor joining analysis (under the assumption of equal rates) resulted in a well supported tree whose topology was identical to that presented in Figure 4. The maximum likelihood tree (estimated by applying equal rates of change and the empirical ti/tv ratio of 11:1 for the combined data set) was identical to that retrieved using parsimony (see Fig. 2).

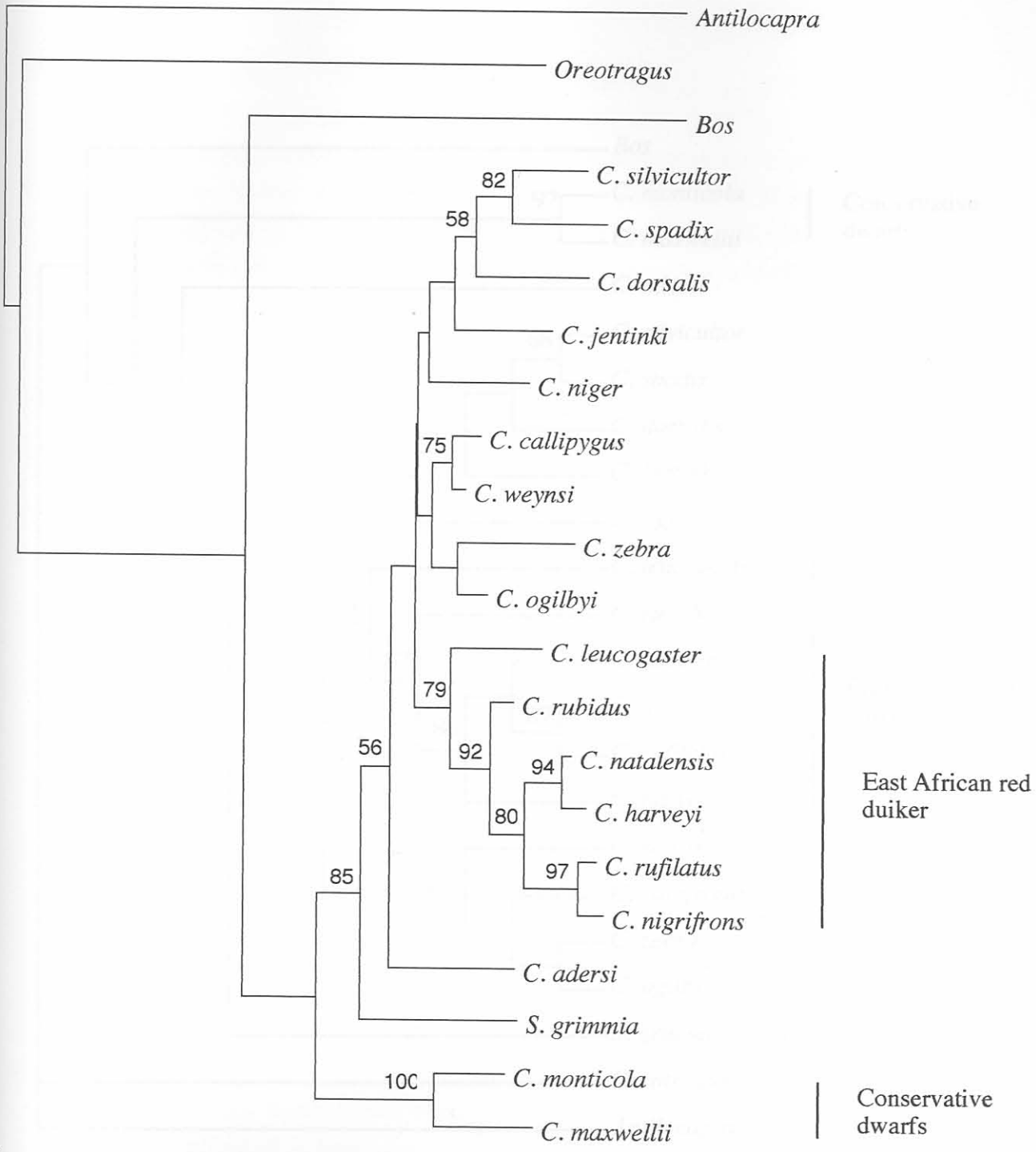



Figure 7 Neighbor joining tree constructed from 767 bp of the small ribosomal subunit in Paup 4.0b2a (Swofford 1999). The HKY correction (Hasegawa *et al.* 1985) was applied to the distance matrix. Branches are drawn proportionally to the amount of change occurring on them, and nonparametric bootstrap support for nodes is indicated.

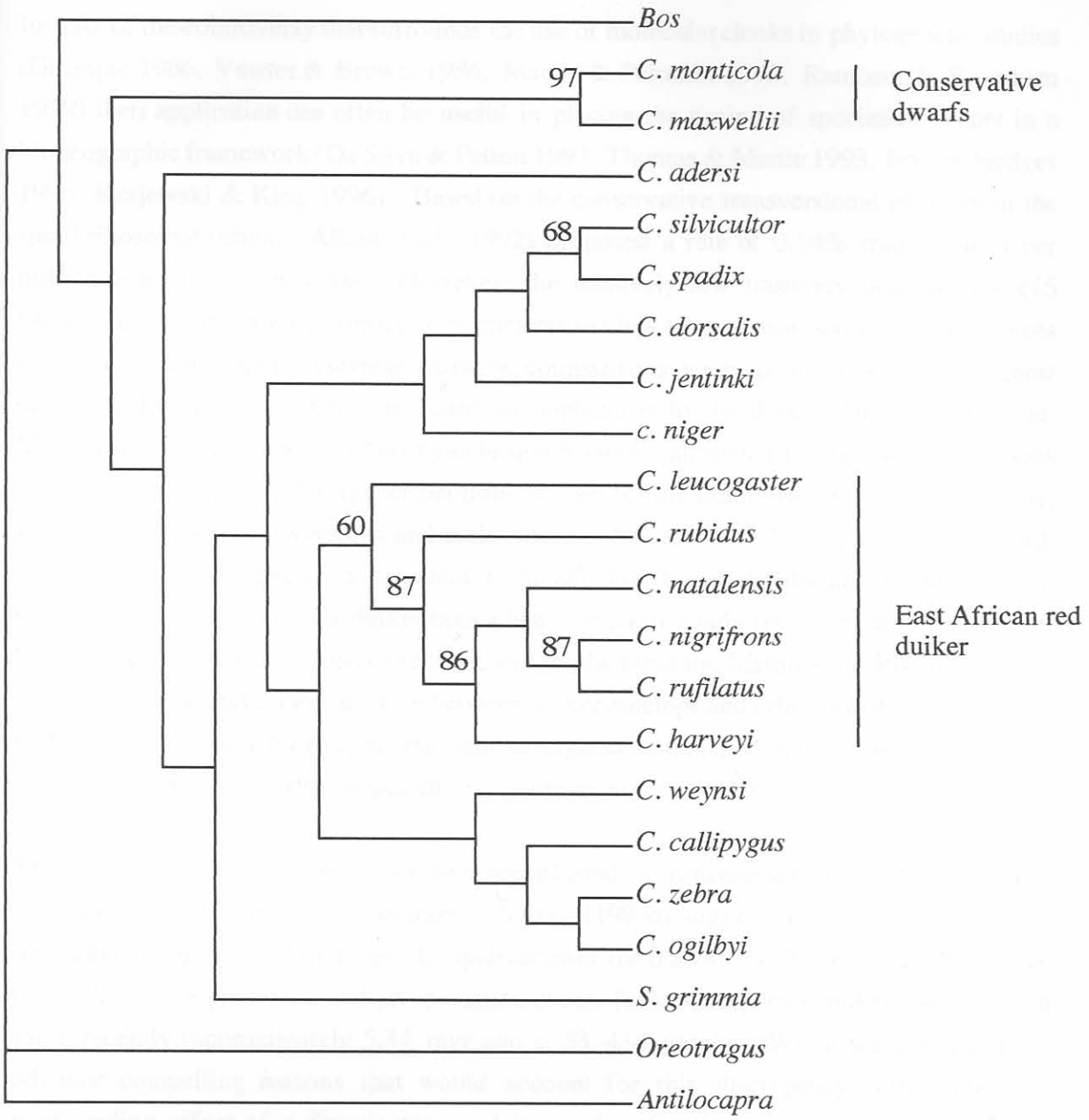



Figure 8 Maximum likelihood topology obtained from the 12S rRNA gene. The tree was constructed in Paup 4.0b2a (Swofford 1999) under the HKY (Hasegawa *et al.* 1985) model of sequence evolution with equal rates, the empirical ti/tv ratio (7:1), and nucleotide frequencies calculated from the data. Bootstrap support was obtained using 100 iterations.

## Speciation events

In spite of the controversy that surrounds the use of molecular clocks in phylogenetic studies (Gillespie 1986, Vawter & Brown 1986, Martin & Palumbi 1993, Rambaut & Bromham 1998) their application can often be useful in placing the timing of speciation events in a biogeographic framework (Da Silva & Patton 1993, Thomas & Martin 1993, Frye & Hedges 1995, Krajewski & King 1996). Based on the conservative transversional changes in the small ribosomal subunit, Allard *et al.* (1992) proposed a rate of 0.14% transversions per million years for the Bovidae. However, the relatively few transversional changes (15 variable and 8 parsimony informative characters) and low transversion sequence divergences within the Cephalophini (several pairwise comparisons were identical, with the highest sequence divergence=1.18%) precluded its application to the duiker data set. Instead, Matthee and Robinson's (1999a) cytochrome *b* clock calibration is used which suggests 0.23% ± 0.01 sequence divergence per million years for the family Bovidae. This estimate, which is based on transversions and makes use of the extensive Alcelaphini fossil record, included two duiker species *(S. grimmia, C. monticola).* Given the absence of rate variation in the cytochrome *b* gene for duiker taxa when considering only transversions, the obvious linear increase of transversions over time, and the fact that the Matthee and Robinson study showed no meaningful rate variation between duiker antelope and other bovid taxa, suggests that it is not unreasonable to apply the same divergence rate to the Cephalophini in an attempt to explain the processes that shaped the evolutionary history of these antelope.

The highest cytochrome *b* sequence divergence based on transversions was 1.23% (between *C. monticola* and *C. rubidus).* Contrary to Vrba's (1985a) suggestion that the Cephalophini originated roughly 12-14 myr ago, the application of the 0.23% ± 0.01 sequence divergence per million years places the earliest speciation events for contemporary duiker species much more recently (approximately 5.34 myr ago ± 53 434 years). While we are unable to advance compelling reasons that would account for this discrepancy (other than the confounding effect of a fragmentary and incomplete fossil record) it is striking that at roughly this time (5 myr ago) several other bovid tribes similarly make their first appearance in the fossil record (Vrba 1985a). Hamilton (1982) and Boaz (1985) have speculated that climatic fluctuations became more marked towards the end of the Miocene (5 myr ago) which, coupled to an increase in the ice sheet covering Antarctica and subsequent lowering in temperatures (Lovett 1993), brought about a more arid climate with a possible reduction of the forest biome.

In addition to the climatic changes at the end of the Miocene, rifting in east Africa caused the uplift of the central African plateau leading to the separation of the main Guineo-Congolian forest block from the east African forests and the development of an arid north-south corridor between them (Lovett 1993). It is generally agreed (Grubb 1978, Livingstone 1982) that in contrast to the more stable west African environment, no single large refugium could have existed in east Africa but rather that the region comprised an archipelago of montane and lowland/coastal forests, with volcanic activity and rainshadows further contributing to the instability of forest habitat (Livingstone 1982, Wasser & Lovett 1993). If duiker speciation has been driven predominantly by habitat fragmentation and the concomitant disruption of gene flow between demes, one may anticipate that this would be reflected in the occurrence of taxa. Indeed, the giant duiker lineage (with its west African distribution) comprises only four species compared to the diverse red duiker lineage which includes 11 of the 18 forest duiker species. Although clearly speculative, one could also suggest that the species inhabiting the more stable west African forests should be of older age compared to east African species, where forest fragmentation during the more recent past (Moreau 1963, Livingstone 1975, Cooke 1978, Hamilton 1982) would have presented ample opportunities for allopatric speciation. This biogeographic scenario is well supported by the mitochondrial data in which the oldest Cephalophini lineages are those with a west African distribution. The average cytochrome *b* transversion sequence divergence within the almost exclusively west African giant duiker clade would suggest that the group is approximately 2.03 million years old, compared to both the west-central (sensu Kingdon 1982) and east African (sensu Kingdon 1982) red duiker species groups which are far more recent (985 506 and 695 625 years old respectively).

There has been much debate concerning the abilities of forest adapted species to adjust to life in an open savanna habitat. Dubost (1968) has argued that forest habitats may enforce such a strong and specialized adaptation on taxa that it significantly impedes the colonization of other ecotypes by them. This view is also held by Kingdon (1982) who posits that *Sylvicapra* has always been a savanna species which never entered the African forests. In sharp contrast, however, Grubb (1978) believes (based mainly on the derived nature of savanna species compared to their forest dwelling conspecifics and congeners) that the transition between habitats has primarily been from forest to savanna. This view is consistent with the mitochondrial DNA data which show *Sylvicapra* to be more derived than the conservative dwarf lineage *(C. monticola, C. maxwellii).* However, the divergence of *Sylvicapra* from the forest clade occurred early in the group's evolutionary history, since it predated the appearance of the other forest dwelling taxa.

## Taxonomic implications

The results of this investigation question, and in some instances support, several of the nomenclatural divisions in current use in the Cephalophini taxonomy but which have traditionally been a source of contention.

*Recognition of the genus* Philantomba

The recognition of *Philantomba* as valid genus name for the two dwarf species has been hotly debated (Hard 1969, Groves & Grubb 1981, Smithers 1983, Robinson *et al.* 1996, Kingdon 1997). One of the most striking and strongly supported phylogenetic associations to emerge from the mitochondrial DNA analysis is that both *C. monticola* and *C. maxwellii* are basal within the tribe, a finding which is consistent with their retention of several primitive morphological (constricted rostrum, lack of inguinal glands) and phenotypic characters (small size; Grubb 1978, Kingdon 1982). Moreover, sequence divergence values separating the conservative dwarfs from the rest of the forest taxa are of similar magnitude to those between *Sylvicapra* and *Cephalophus* which, when taken together with the morphological (Grubb 1978) and cytogenetic evidence (Robinson *et al.* 1996), would argue for the reinstatement of *Philantomba* as the valid genus name for *C. monticola* and *C. maxwellii*. The recognition of a separate generic status for these two taxa would secure *Cephalophus* monophyly.

*Phylogenetic placement of* C. zebra *and* C. adersi

The evolutionary relationships of two duiker species *(C. adersi, C. zebra)* remains problematic. Ader's duiker is confined entirely to the Island of Zanzibar and the Sokoke coastal forest in Kenya while *C. zebra*, one of the most distinct taxa within the forest group, has a restricted west African distribution (Sierra Leone, Liberia, Ivory Coast). Kingdon (1982) recognizes *C. adersi* as a primitive member of the red duiker lineage, not really closely allied to either the east or west African species groups. In the present study, this species showed a novel association, grouping for the most part as a sister taxon to *C. zebra.* However, whether this is a true reflection of their evolution history or merely an artifact of long branch attraction is moot. In turn, *C. zebra* has been described as a modified survivor of an early phase in the duiker evolution (Kingdon 1982) and has even been placed in its own genus or subgenus *(Cephalophula zebra*; Heyden 1968, Groves & Grubb 1981).

Mitochondrial DNA analyses fail to unequivocally place this taxon in any of the recognized groups, with different genes and methods of analyses either clustering the zebra duiker basally to the red duiker lineage or, alternatively, outside of the forest duiker clade altogether. Both genetic and morphological data would therefore seem to suggest an independent evolutionary trajectory for this species.

*Subspecies status of* C. harveyi

The lowest sequence divergence detected between any of the duiker species using either the cytochrome *b* or 12S rRNA data is between *C. natalensis* and *C. harveyi* (cytochrome *b*=0.7%; 12S rRNA=0.3%). These values are comparable to those distinguishing the *S. grimmia* (cytochrome *b* =2.8%; 12S rRNA=1.3%) and *C. monticola* (cytochrome *b* =6.2%; 12S rRNA=1.8%) subspecies and clearly point to an extremely close evolutionary relationship between them. The fact that *C. harveyi* and *C. natalensis* hybridizes in their regions of sympatry (Kingdon 1982) comes as no surprise. Mayr (1940) defines a subspecies as "a geographically defined aggregate of local populations which differ taxonomically from other subdivisions of the species". In the light of genetic data (present study), their phenotypic and morphological resemblance (Heyden 1968 and references therein, Ansell 1971, but see Grubb 1993, Kingdon 1982, 1997) and the hybridization evidence (Kingdon 1982), it is proposed that *C. harveyi* be relegated to a subspecies of *C. natalensis (C. n. harveyi)* as has been suggested by other authors (Ansell 1971, Heyden 1968, Ellerman *et al.* 1953, Groves & Grubb 1981).

# CHAPTER 3

# COMPARATIVE AND MOLECULAR CYTOGENETICS

## OBJECTIVES

The aims of this aspect of the investigation were twofold. First, G-banded and C-banded data are presented for *C. spadix*, a species which has not previously been reported in the literature. In contrast to the conserved autosomes, considerable variation in the X chromosomes of this group have been documented and these have been used in support of some of the earlier taxonomic treatments. The cytogenetic analyses contained herein extend previous observations of autosomal conservativeness in duiker antelope and allow for the comparison of the *C. spadix* X chromosome to that of six other cephalophid species. Second, by using satellite DNA repeat sequences as fluorescence *in situ* hybridization (FISH) probes, an attempt is made to further resolve the evolutionary relationships between seven species representative of the main duiker groups (conservative dwarfs, the savanna specialist, giant duiker, and the red duiker lineage). The relationships based on hybridization patterns were subsequently overlaid on the mitochondrial DNA phylogeny (Chapter 2) to determine the degree of concordance between these independent genetic data sets.

## INTRODUCTION

Bovids are characterized by extensive karyotypic variation with respect to diploid number (ranging from 2n=30 to 2n=60; Wurster & Benirschke 1968, Wallace 1977, Buckland & Evans 1978, Gallagher & Womack 1992). In sharp contrast, however, the number of autosomal arms (NAA) is relatively constant varying, with few exceptions, between 56 and 58. This has led authors to argue that bovids are, in general, a cytogenetically conservative family (Wurster & Benirschke 1968, Robinson *et al.* 1998). Moreover, it is likely that the 2n=60, NAA=58, karyotype configuration is the primitive condition for the Bovidae (Gallagher *et al.* 1994).

Previous reports on Cephalophini representatives (Koulischer *et al.* 1967, Hard 1969, Robinson *et al.* 1996) document a constant autosomal complement (2n=60), with the X chromosome morphology varying between acrocentric in *C. maxwellii* and *C. monticola* to metacentric in *C. rufilatus*. Although based on unbanded preparations, Hard (1969) was nonetheless struck by the high degree of morphological variation in the X chromosomes of several species *(C. dorsalis, C. jentinki, C. maxwellii, C. niger, C. silvicultor, C. rufilatus)*. The six duiker species *(S. grimmia, C. dorsalis, C. maxwellii, C. monticola, C. natalensis, C. silvicultor)* examined by Robinson and co-workers (1996) were also characterized by NAA=58 and possessed an invariant G-banded autosomal compliment. As with Hard's study, these authors noted considerable variation in X chromosome morphology with both *C. monticola* and *C. maxwellii* being characterized by an acrocentric X while the remaining species all have bi-armed X chromosomes of various configurations. Changes in the morphology of the X chromosome between species is due primarily to heterochromatic short arm additions *(S. grimmia, C. dorsalis, C. natalensis, C. silvicultor)* and to a pericentromeric heterochromatic inversion *(C. maxwellii, C. monticola)*.

The technique known as *in situ* hybridization (ISH) was introduced in 1969 when three independent groups described a method for detecting highly repetitive nucleic acid sequences in cell preparations (Buongiorno-Nardelli & Amaldi 1969, Gall & Pardue 1969, John *et al.* 1969). A more sensitive molecular method, fluorescence *in situ* hybridization, which is similarly based on the double stranded nature of DNA, makes use of non-radioactive hybridization. As a further refinement on ISH, FISH permits the localization of much shorter probe homologies (see Fan *et al.* 1990, Lichter & Ward 1990). DNA probes can be defined according to their target DNAs i.e. chromosome painting probes, satellite DNA probes, and locus specific DNA probes (Reilly 1994).

FISH has been used with success to determine whether highly repetitive satellite sequences, typically localized to constitutive heterochromatic regions, differ between species (Hamilton *et al.* 1990, Modi 1992, Houseal *et al.* 1995, Volobouev *et al.* 1995, Robinson *et al.* 1998). These localized DNA sequences are known to comprise substantial portions of higher eukaryotic genomes, they are not transcribed and, as a result, are susceptible to rapid evolutionary change (Modi 1992). Given the high turn-over rate of satellite sequences, analysis of these regions can be used to explore genome organization and evolution at many taxonomic levels. Since satellite DNA arrays tend to be homogenized through the effects of concerted evolution (Dover 1982, Arnheim 1983), sequence homogeneity should be greater within species than among members of different species (Houseal *et al.* 1995).

Considering that the changes in X chromosome morphology in the Cephalophini were primarily heterochromatic in nature, Robinson *et al.* (1996) used total genomic DNA as hybridization probes to determine whether highly repeated satellite sequences differ among duiker species. The amount and distribution of heterochromatin (as revealed by both C-banded karyotypes and FISH, Robinson *et al.* 1996) are consistent with several previously suggested duiker relationships, for instance the pericentromeric inversion in the X chromosome separating *C. monticola* and *C. maxwellii* from the rest of the forest species would support the recognition of *Philantomba* as a valid genus for the conservative dwarfs (Groves & Grubb 1981). Similarly, the absence of a large G-band negative area around the centromeres of the X chromosomes in *C. silvicultor* and *C. dorsalis* indicate a close relationship between them, an association also suggested by morphological features (Groves & Grubb 1981, Kingdon 1982, 1997). These findings led Robinson and co-workers to speculate that comparative genome painting using heterochromatic sequences as probes might provide a further means of identifying evolutionary closely related taxa within this group, an approach followed in the present investigation.

# MATERIAL AND METHODS

## Specimens

Fibroblast cultures were established for seven duiker species. Species names and their collection localities are listed in Table 8. These specimens are representative of the main duiker lineages (conservative dwarfs: *C. maxwellii, C. monticola;* red duiker: *C. natalensis;* grassland specialist: *S. grimmia;* giant duiker: *C. dorsalis, C. silvicultor, C. spadix)* previously identified on morphological and phenotypic characters (Groves & Grubb 1981, Kingdon 1997) and molecular sequence data (present study).

Ear clippings from field specimens were collected into tissue culture medium (MaCoy's 5A; Highveld Biological) supplemented with 15% fetal calf serum. After these were vigorously cleaned with 70% ethanol, primary cultures were established in the laboratory. Cultures were allowed to grow to confluence at 37°C in the presence of 5% $CO_2$ and harvested for analyses using standard techniques (Schwarzacher *et al.* 1974).

## Comparative cytogenetics

### *Harvesting and slide preparation*

Cell division was blocked in metaphase using 50 $\mu$l Colcemid (10 $\mu$g/$\mu$l; Gibco BRL). In addition, 50 $\mu$l 5-bromo-2-deoxyuridine (BrdU; 3 mg/ml) was added to cultures 14 to 15 hours prior to harvesting. The cells were trypsinized and resuspended in a pre-warmed hypotonic solution (0.075 M KCl) for 15 to 17 min at 37°C, and fixed in modified Carnoy's fixative (3 methanol: 1 glacial acetic acid). Slides were prepared by dropping the cell suspension onto fogged microscope slides. The mitotic index was assessed using a phase-contrast microscope (Nikon, Alphaphot YS).

### *Banding of chromosomes*

Giemsa banding (G-banding) followed standard techniques (Seabright 1971). Metaphase cell preparations were aged for 1 to 2 weeks at 37°C to ensure crisp bands. Enzymatic digestion of the A+T rich regions was done by treating slides with Enzar-T trypsin (Intergen) and the bands detected using a 2% phosphate buffered Giemsa solution. For comparative

Table 8 The seven duiker species used in comparative and molecular cytogenetic analysis. Vernacular species names and their collection localities are provided.

| Species | Vernacular name | Locality | Material provided |
|---|---|---|---|
| *S. grimmia* | Gray duiker | Zimbabwe | V. Wilson[1] |
| *C. dorsalis* | Bay duiker | Ghana | V. Wilson[1] |
| *C. maxwellii* | Maxwell's duiker | Ghana | V. Wilson[1] |
| *C. monticola* | Blue duiker | South Africa | V. Wilson[1] |
| *C. natalensis* | Natal red duiker | South Africa | V. Wilson[1] |
| *C. silvicultor* | Yellow-backed duiker | Philadelphia Zoo. | V. Wilson[1] |
| *C. spadix* | Abbot's duiker | Tanzania | L. Vinciguerra[2] |

1=Chipangali Wildlife Orphanage, Zimbabwe; 2=Safariroyal, Tanzania.

purposes, G-banded chromosomes were numbered and arranged according to Robinson *et al.* (1996) and the standard cattle G-banded karyotype (Ford *et al.* 1980).

Staining of constitutive heterochromatic regions (C-banding; Sumner 1972) is optimal when slides are freshly prepared. The metaphase spreads were subjected to a 5% barium hydroxide solution at 55°C for 1.5 min followed by 1 hour in 2 X SSC at 60°C. The cells were stained in a 4% phosphate buffered Giemsa solution.

**Molecular cytogenetics**

*Isolation of satellite repeat sequences for FISH*

Total genomic DNA of the seven duiker species listed in Table 8 was extracted from fibroblast cultures following standard procedures (Maniatis *et al.* 1982). A suite of 6-base recognition endonucleases were used to screen the DNA of the seven duiker species for the presence of satellite bands following conditions recommended by the manufacturers (AEC Amersham, Boehringer Mannheim, Promega). Digestion products were electrophoretically separated on 1% agarose gels stained with ethidium bromide. Fragments of highly repetitive DNAs form distinct bands visible under UV light; the selection of probes for FISH analyses (and subsequently Southern blot analysis; see below) was based on the unique occurrence of bands in the digestion profiles of species. All DNA digestions were repeated to verify the reproducibility of fragment patterns. Four species specific satellite bands released by *Eco*RI, *Pst*I, and *Pvu*II digestion were eluted from the gels, purified using the Cleanmix kit (Talent), and used as FISH probes. For ease of presentation these probes are referred to as *Eco*RI-Blue, *Eco*RI-Max, *Pst*I-Blue, *Pvu*II-Max (names are derived from the restriction enzyme and duiker species from which the fragments were isolated).

*Fluorescence* in situ *hybridization*

The four purified satellite fragments isolated from the duiker species were hybridized to metaphase spreads of all seven duiker species included in the present study, and to an outgroup species *(B. taurus)*.

The satellite bands were labeled by nick translation (BioNick Labeling System, Gibco BRL) using biotin-14-dATP. Labeled DNA and 5 $\mu$g Bovine COT-1 DNA were denatured in 2 X SSC with 50% formamide and 10% dextran sulfate by heating to 75°C for 6 min prior to

hybridization. The metaphase preparations were similarly denatured for 2 min at 75°C in 70% formamide, 2 X SSC and then dehydrated successively in 70%, 80%, 95% and 100% ethanol. Slides were flooded with probe DNA, covered with a glass cover slip and the edges sealed with rubber cement. Hybridization was conducted overnight in a moist chamber incubated at 37°C.

Following post hybridization washes at 75°C in 2 X SSC, hybridization events were detected using fluorescein isothiocyanate (FITC)-conjugated avidin. The chromosomes were counter stained with propidium iodide (0.5 $\mu$g/ml) in an antifade solution. Signals were amplified by flooding the slides with anti-avidin for 30 min. An Olympus BX60 microscope equipped for epifluorescence was used to view and photograph metaphase cells.

*Southern blot hybridizations*

Southern blot analyses (Southern 1975) were performed on eight species *(B. taurus* and seven duiker species; Table 8) following standard procedures (Maniatis *et al.* 1982). Total genomic DNA of the eight species was digested to completion with *Cfo*I and transferred to Hybond N nylon membranes (AEC Amersham). Agarose gels were denatured (1.5 M NaCl, 0.5 M NaOH) to ensure the transfer of single stranded DNA, and then neutralized (1.5 M NaCl, 0.001 M EDTA, 0.5 M tris-base). The gels were briefly rinsed in 10 X SSC after which capillary blots were prepared using standard techniques (Maniatis *et al.* 1982).

The four satellite bands *(Eco*RI-Blue, *Eco*RI-Max, *Pst*I-Blue, *Pvu*II-Max) selected for use as FISH probes were oligolabeled to high specific radioactivity with [$\alpha$-$^{32}$P] dATP using the Klenow fragment (Feinberg & Vogelstein 1983). To determine whether the fragments are derived from a common satellite family, the probes were hybridized to the DNAs of the seven duiker and one outgroup species following their digestion with *Cfo*I. This enzyme has previously been shown to produce a Type A pattern in cattle (Modi *et al.* 1993, Modi *et al.* 1996).

*Cloning and sequencing*

The *Eco*RI-Max satellite fragment was selected for cloning and sequencing. A shotgun approach was followed (Boehm & Nehls 1995). Approximately 100 ng of the excised *Eco*RI-Max fragment was self-ligated using 10 units T4-ligase in a volume of 15 $\mu$l.

Random fragments were generated by annealing the primer RAN-1 (5' CTG CAG GGT ACA TAT ATN NNN NNN 3') to the concatenerised fragments. Primer extension was achieved in the presence of 1 mM dNTPs and 3 units T7-Sequenase (Amersham) for 1 hour at 37°C. The reaction was stopped by denaturing at 96°C for 3 min followed by chilling on ice.

This reaction mixture (25 $\mu$l) was used to amplify the random fragments by PCR using random primers (5' CTG CAG GGT ACA TAT AT 3'). A 35 cycle PCR program was followed (initial denaturation for 3 min at 96°C, denaturation for 15 sec at 96°C, annealing for 15 sec at 55°C, and extension for 1 min at 72°C with a final extension cycle of 10 min at 72°C). PCR products were separated on 1% GTG agarose gels and fragments of approximately 0.8 kb were eluted from the gel and purified using the QIAquick gel extraction kit (QIAGEN). The fragments were cloned into the vector pCR2.1-TOPO (Invitrogene) using the TOPO-TA cloning kit version D (Invitrogene) and transformed into *E. coli* TOP10Fë (Invitrogene). Transformed cells were plated on LB-plates containing kanamycine and recombinants were selected by blue/white screening (Maniatis *et al.* 1982).

Single white colonies were transferred to LB-plates containing ampicillin and after incubating overnight at 37°C colonies were transferred to 3 ml 2YT-medium containing ampicillin and cultured overnight at 37°C. Plasmid DNA was extracted using the GFX Micro plasmid prep kit (Pharmacia). Plasmids were screened for inserted foreign DNA fragments by *Eco*RI digestion and positives were sequenced using the M13 universal primers and the BigDye ready reaction kit (Perkin Elmer) and an ABI 310 automated sequencer.

# RESULTS AND DISCUSSION

## *C. spadix* karyotype analysis

### *G-banded karyotype and X chromosome morphology*

A representative G-banded karyotype for *C. spadix* is presented in Figure 9. As with previous reports on other duiker antelope (Koulischer *et al.* 1967, Hard 1969, Robinson *et al.* 1996), *C. spadix* is characterized by a diploid number of 2n=60 with all the autosomes acrocentric in morphology. The G-banded compliment of *C. spadix* is identical, at least at this level of resolution, to that of *C. maxwellii* (Robinson *et al.* 1996) extending the observation that speciation in duiker antelope does not involve euchromatic rearrangements or variation in diploid number.

The *C. spadix* X chromosome is bi-armed in morphology, a configuration mirrored by *C. silvicultor*, *C. natalensis*, *S. grimmia*, and *C. dorsalis* (see Robinson *et al.* 1996). In sharp contrast to the conserved Xq, the short arm of the X chromosome in these species is noteworthy for the varying amounts of heterochromatin that comprise it. Comparisons between species reveal *C. spadix* to have the longest Xp, being almost identical in size to the Xq, while *C. silvicultor* represents the other extreme (see Fig. 2B in Robinson *et al.* 1996). Notwithstanding this size difference, the X chromosome of *C. spadix* is similar to that of *C. silvicultor* and *C. dorsalis* in that it lacks the large G-negative band around the centromere. The conspicuous absence of a large non-staining pericentromeric region in these three species is an important phylogenetic character, supporting the previously suggested close relationship between *C. spadix*, *C. silvicultor*, and *C. dorsalis* based on morphology (thin horns and a speckled juvenile pelage; Groves & Grubb 1981) and molecular sequence data (present study). Given that only a single female specimen was available for analysis, the morphology of the *C. spadix* Y chromosome remains unknown.

### *C-banding*

C-banding (Fig. 10) showed that constitutive heterochromatin is restricted to the areas around the centromeres in all the autosomes - a pattern in keeping with previous reports on duiker antelope (Robinson *et al.* 1996). In addition, the short arm of the *C. spadix* X chromosome is entirely heterochromatic although not as darkly stained as the centromeric regions on the autosomes. This would suggest that the C-banded material constituting the

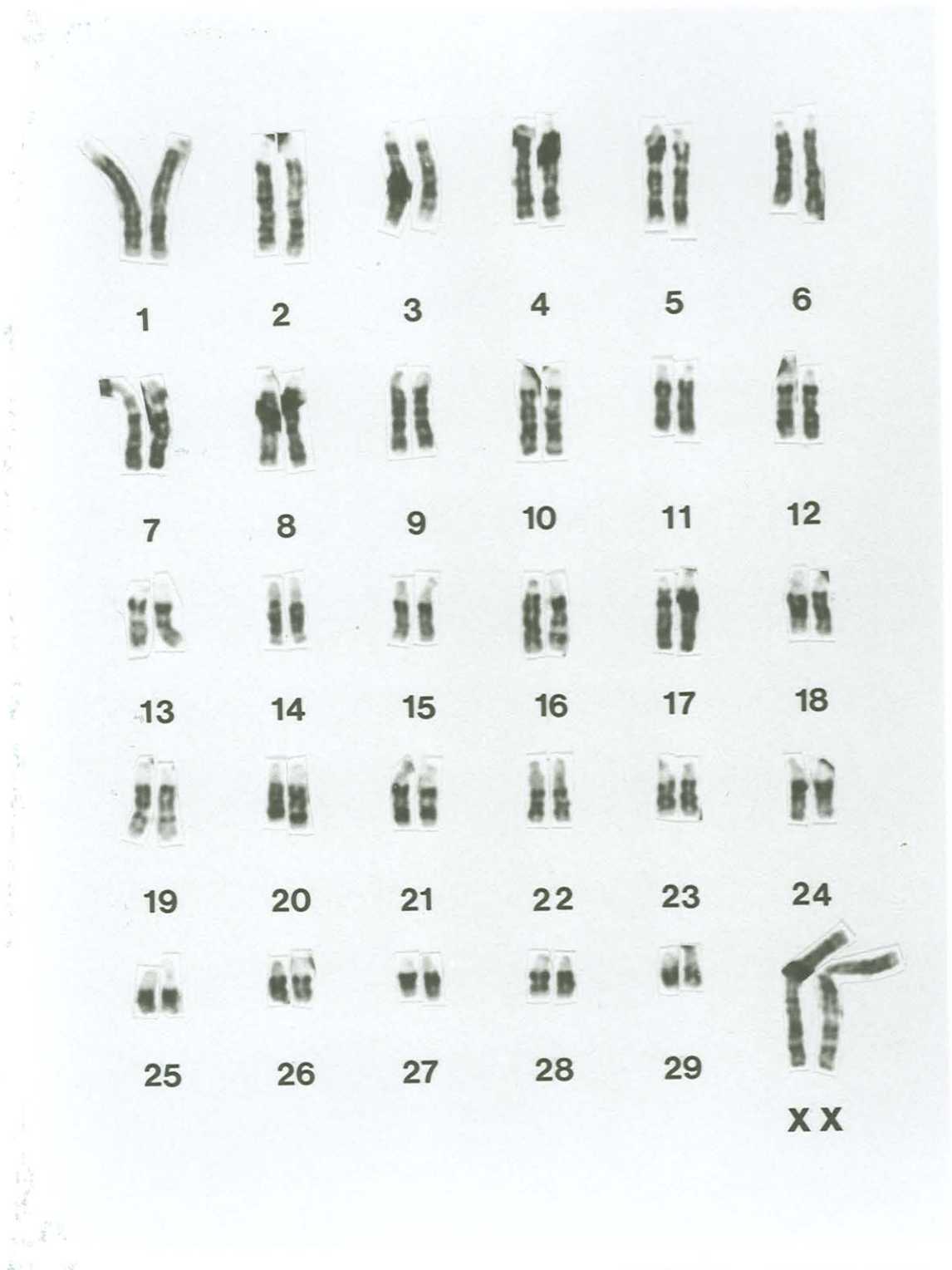



Figure 9 G-banded female karyotype of *C. spadix* (2n=60) from Tanzania.

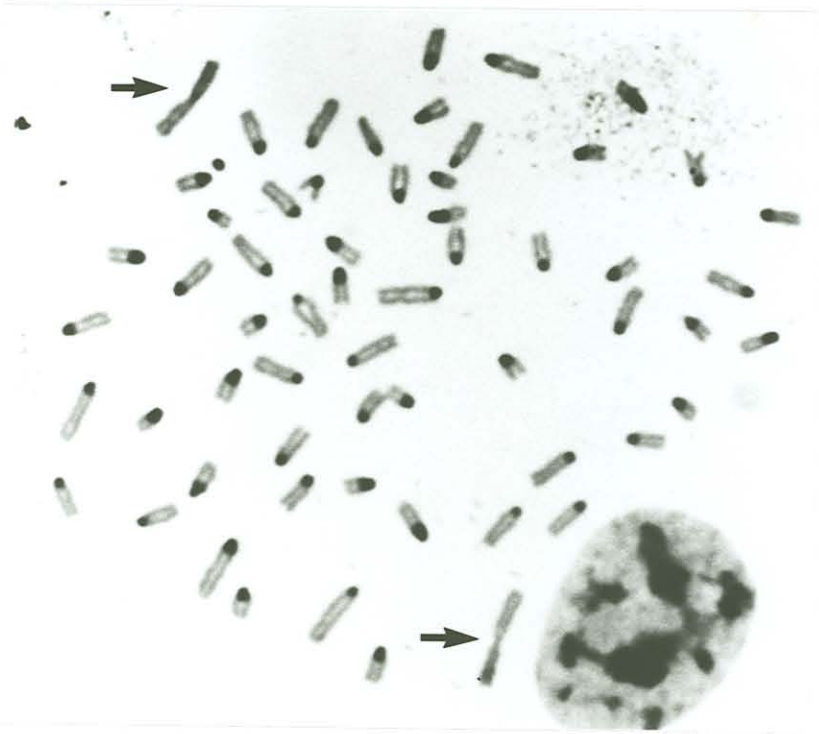


Figure 10 C-banded metaphase cell of a *C. spadix* female. Arrowheads indicate the X chromosomes.

Xp is of a different sequence composition to the autosomal centromeric heterochromatin, extending Robinson and co-workers' (1996) earlier duiker study.

## Molecular cytogenetics

*Satellite fragments*

Four unique satellite fragments were selected for use as FISH probes after digestion with restriction enzymes. These intense bands were observed in either *C. monticola* or *C. maxwellii*, but not in the DNAs of the other duiker species included in this study: digestion with *Eco*RI produced a fragment of 2.8 kb in both *C. monticola* and *C. maxwellii* (*Eco*RI-Blue and *Eco*RI-Max respectively); a unique fragment of 2.8 kb was released in *C. maxwellii* after digestion with *Pvu*II (*Pvu*II-Max); *Pst*I produced a band of 1.4 kb in *C. monticola* (*Pst*I-Blue). The restriction profiles of the cephalophine species after digestion with the three restriction enzymes are shown in Figure 11.

*FISH*

Both orthologous and heterologous FISH chromosome painting schemes involving all seven duiker species revealed identical hybridization patterns for the four satellite probes with no individual variation among them. The centromeric regions of all the autosomes showed intense fluorescence (yellow) while the chromosome arms were typified by a conspicuous absence of signal (red; Fig. 12). Hybridization of satellite DNAs to the pericentromeric sites in duiker antelope tracks the distribution of constitutive heterochromatin as revealed by C-banding (see Fig. 10).

In sharp contrast to the autosomes, there is considerable variation in the FISH signals involving the sex chromosomes of the seven duiker species examined. Three hybridization patterns are evident which support previously suggested evolutionary relationships between species.

First, a unique pattern, common only to *C. monticola* and *C. maxwellii*, resulted in intense hybridization to the centromeres of both the X and Y chromosomes in these species (Fig 12A); the signals were indistinguishable from the autosomes. Secondly, a FISH pattern common to both *S. grimmia* and *C. natalensis* is exhibited where the juxtacentromeric regions of the X chromosomes show intense hybridization to all four probes, while the Y

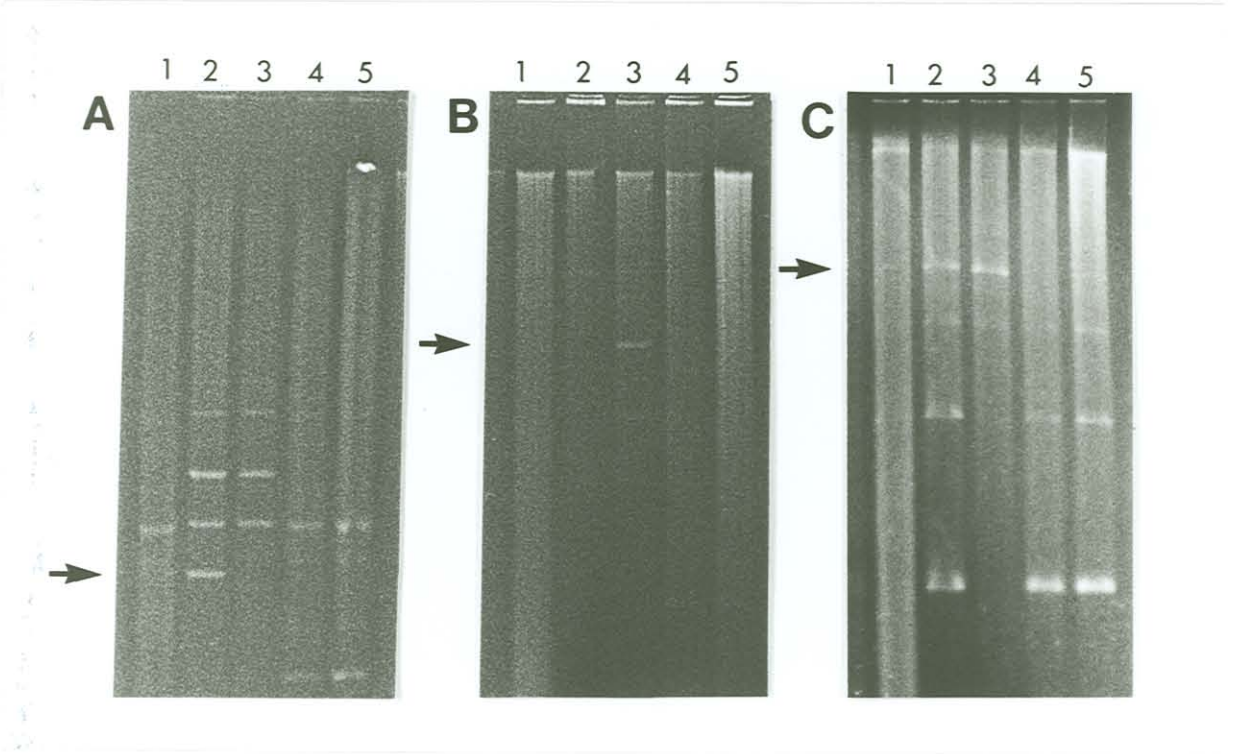
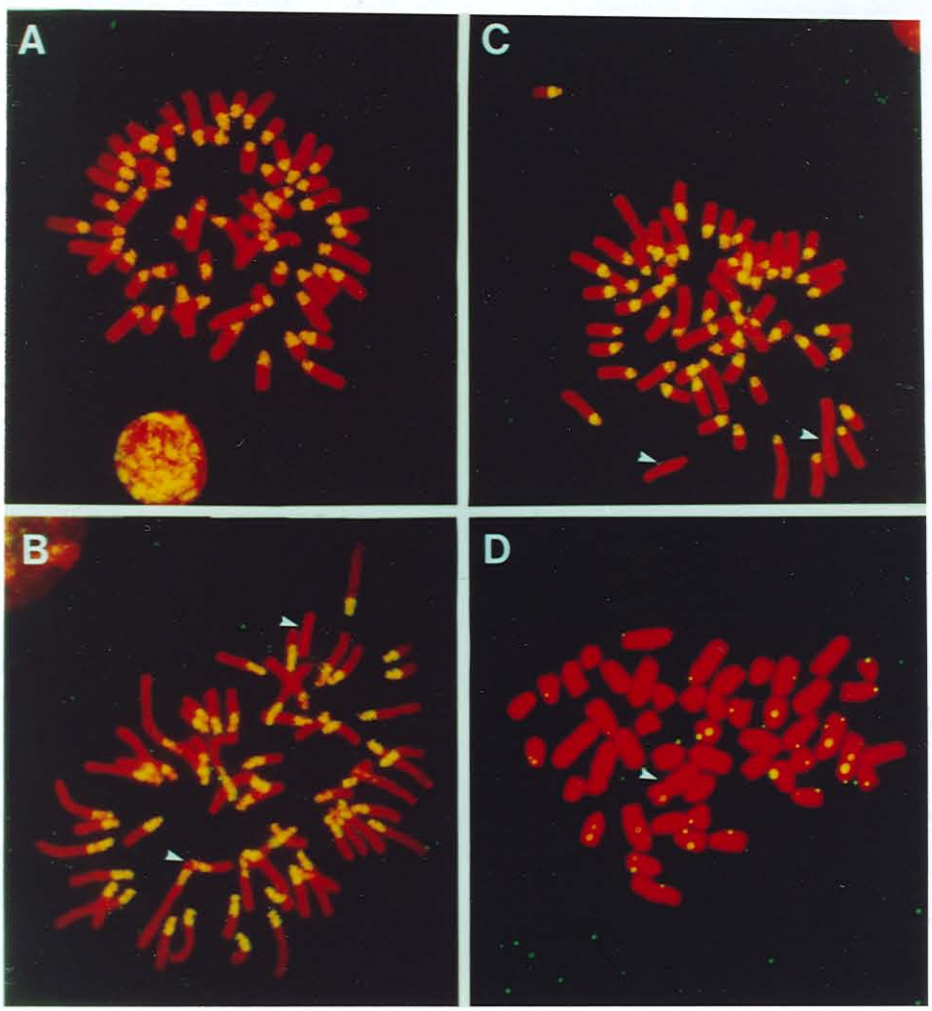



Figure 11 Digestion profiles of 5 duiker species. The unique satellite fragments that were selected as FISH probes are indicated by the arrows. (A) 1.4 kb fragment (lane 2) released in *C. monticola* by *Pst*I digestion, (B) 2.8 kb satellite fragment isolated from *C. maxwellii* (lane 3) after digestion with *Pvu*II, (C) two 2.8 kb fragments (lanes 2 and 3) isolated from *C. monticola* and *C. maxwellii* after digestion with *Eco*RI.

Figure 12 FISH results showing hybridization patterns of the four probes isolated from *C. monticola* and *C. maxwellii*. Identical hybridization patterns were found using all four painting probes (see text). As a representative, the *Eco*RI-Max probe results are illustrated here. The X and Y chromosomes, where present and identifiable, are indicated by arrows. (A) *C. maxwellii* and *C. monticola* (shown) had identical FISH patterns; (B) FISH patterns characteristic of *S. grimmia* and *C. natalensis* (shown); (C) FISH patterns characteristic of *C. silvicultor, C. spadix* and *C. dorsalis* (shown); (D) absence of signal following FISH to the metaphase chromosomes of cattle (outgroup). Only a partial spread for *B. taurus* is presented.



chromosomes showed a lack of signal (see Fig. 12B). The lack of hybridization to the X centromere is particularly striking and is reflected in the species' C-bands where two well delimited dark C-bands bracket the centromere (see Figure 3F in Robinson *et al.* 1996). Given that there is hybridization to the repeat elements surrounding the centromere, but not to those located in the centromeric region itself, this may suggest that the former reflects a more recent heterochromatic addition. Finally, the species within the giant duiker lineage *(C. silvicultor, C. spadix, C. dorsalis)* were united by a common hybridization pattern wherein neither the X nor the Y chromosomes showed any fluorescence signal to any of the four FISH probes used (Fig 12C).

A pattern to emerge from these investigations is that although C-banding revealed the short arms of the X chromosomes of *C. spadix*, *C. dorsalis*, *C. natalensis*, *C. silvicultor* and *S. grimmia* to be entirely heterochromatic (Fig. 10; see Fig. 3 in Robinson *et al.* 1996), the FISH data clearly show a lack of sequence complimentarity among the satellite DNAs comprising the short arms of the X chromosomes and those localized within the centromeric/juxtacentromeric regions. These results confirm previous suggestions based on staining intensity that the constitutive heterochromatin around the duiker autosomal centromeres is different to that comprising the short arms of the X chromosomes (Robinson *et al.* 1996).

In a final FISH experiment, all four probes (*Eco*RI-Blue, *Eco*RI-Max, *Pst*I-Blue, *Pvu*II-Max) were hybridized to the chromosomes of the outgroup species *(B. taurus)* to test the species specificity of the fragments. No hybridization resulted to either the autosomes or sex chromosomes of *Bos* (Fig 12D) indicating a lack of sequence complimentarity between representatives of the Bovini and Cephalophini. From the FISH results, it seem likely that the satellite family contained in the FISH probes evolved after the divergence of the Bovini and Cephalophini from a common ancestor.

*Southern blot analysis*

Southern hybridization of the four probe DNAs (*Eco*RI-Blue, *Eco*RI-Max, *Pst*I-Blue, *Pvu*II-Max) to the DNAs of seven duiker species digested with *Cfo*I yielded identical restriction patterns for all the probes. A ladder (as is typically observed for tandemly arranged satellite DNAs) resulted in which the satellite was cleaved to a unit length of approximately 800 bp (Fig. 13). Contrary to the FISH results, hybridization (albeit very weak) is evident following long autoradiographic exposures to the genomic DNA of *B. taurus*.

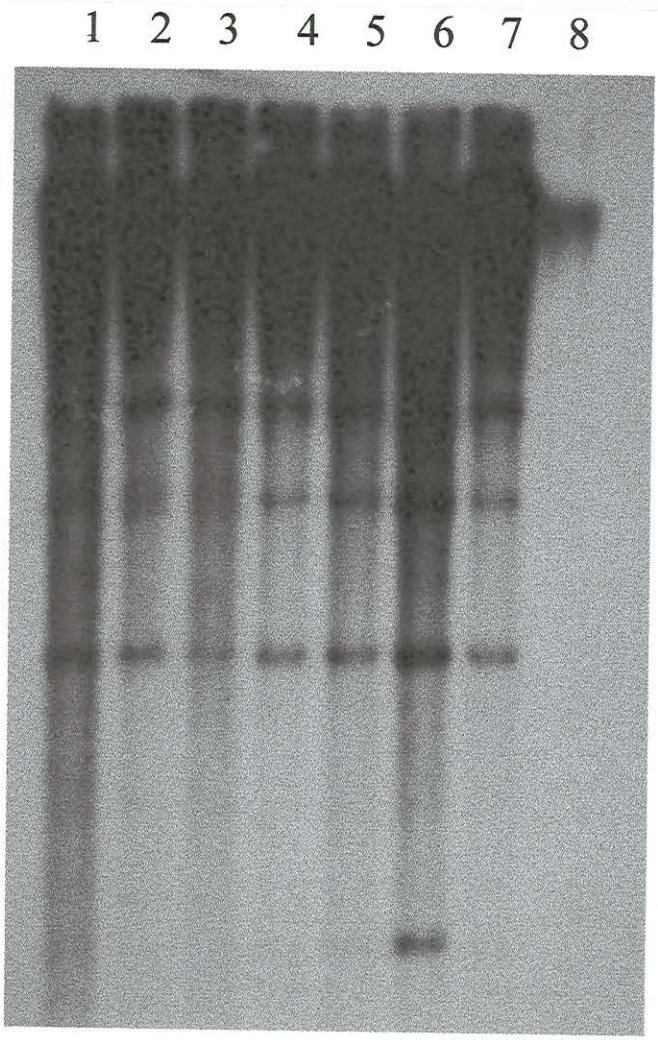



Figure 13 Southern blot of *Cfo*I digested DNAs of seven duiker species and an outgroup species after probing with the *C. maxwellii Eco*RI fragment. Lane 1=*S. grimmia*; lane 2=*C. maxwellii*; lane 3=*C. monticola*; lane 4=*C. natalensis*; lane 5=*C. dorsalis*; lane 6=*C. silvicultor*; lane 7=*C. spadix*; lane 8=*B. taurus*.

This reflects the difference in sensitivity between these two methods. Rapid sequence turn-over and concerted evolution has probably altered sequence homology to such an extent that the Bovini and Cephalophini elements are characterized by such low levels of sequence homology that analyses by FISH (under relatively stringent conditions) failed to detect it.

*Characterization of the satellite family*

The similarity among the four probes in terms of fluorescence hybridization signals, Southern blot patterns, as well as unit repeat length suggest extensive sequence homology between them. To further investigate the structure and sequence composition of this satellite repeat family a representative probe (*Eco*RI-Max) was chosen for sequence analyses.

Cloning of the *Eco*RI-Max fragment followed a shotgun approach (Boehm & Nehls 1995). Positive clones were chosen based on the presence of inserts ranging in size from 0.6 kb to 0.8 kb (see Fig. 14). Four clones (OLI 1-4) were randomly selected for amplification and sequenced. The partial sequences obtained from these four clones are presented in Figure 15. No sequence overlap was found between these four fragments, indicating that they are from different regions of the original 2.8 kb fragment. In general, the satellite sequences are A+T rich (57% to 72% A+T), with only a single clone (OLI 2) being characterized by a higher G+C content (G+C=57%). No internal repeat motif was evident in any of the sequenced fragments. Although unexpected, this is not an unusual finding. For example, satellite fragments with no obvious internal repeat have been documented for both the giant panda (Wu *et al.* 1990) as well as for the 2.6 kb 1.711b bovine satellite family (Streeck 1981).

Sequences obtained from the four clones were compared with the Genbank data base using the blast search option. No significant homologies were found to three of the sequences, however, OLI 4 showed extensive homologies (approximately 90% homology) to an artificial consensus sequence constructed from 13 cow, 2 goat, 3 sheep and 2 muntjac

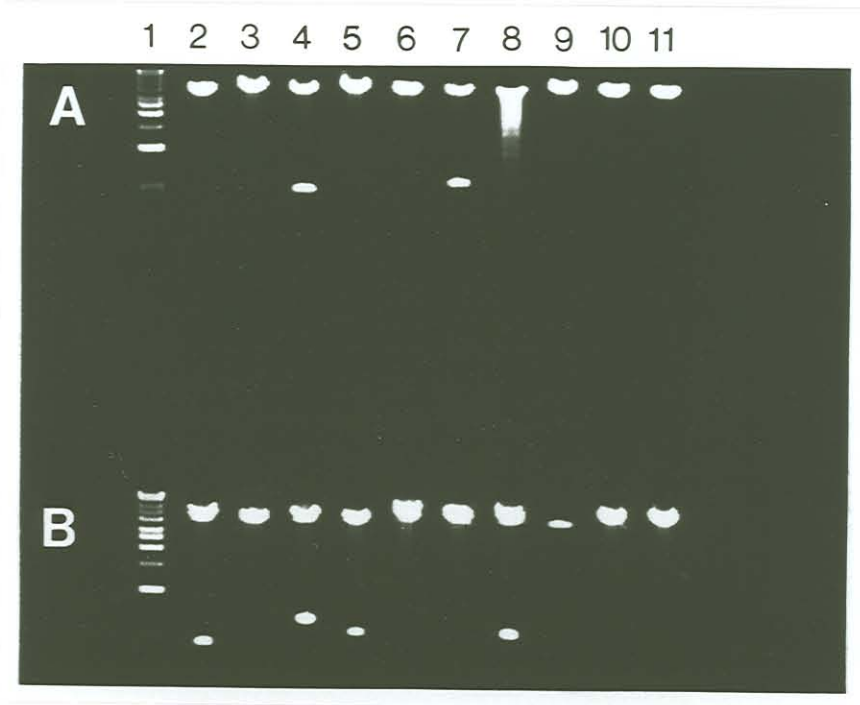



Figure 14 Cloned *Eco*RI-Max fragments released after digestion by *Eco*RI. Clones with an insert size spanning approximately 0.6 kb to 0.8 kb were selected for sequencing (lanes A7, B4, B5, and B8). The Pharmacia kilobase DNA marker (lanes A1 and B1) was used as the size standard.

OLI 1

TAGGGGAACTGTCCATGTTGTTCAATATTGTGATAGCATGTTATTTAATTTTCATATAACGA
TCCATCATCTTTCCATGTGTGCATGCTAAGTCACTTCAATCCTGTCTGACTCTTTGAGAACA
TATGGACCATAGCCCTCCAGGCTCCTCTGTCCATGGGATTTCCCAGGCAAGAATACTGTAGT
GGGTTGCCATTTACTCCTCCAGGGGATTTTCCTGACTCAGGGATTGAACCCAAGTCTCTTGC
ATCTAATACTGGTGGCTATAACTGGATAGGGCTGGAGAGATTGAAGGTAATTGTTATTTTCC
CAAATTTATAAACACATATTACTGGTTAATTTTACCTGATTTTTTAAATTGGAGG

OLI 2

CGGGGAATTTCCTGAGTTGCAGCAAGGGTGGGAAGGACCCTTGCGAGGTACAAGAGAGAAAG
CATGAATGCCCTCGAGATGCCGCTGCAGAAAACGGCCTGATTTCGCCTGGAGGTTAGATCCT
CCTGGTTTTTCTCGAGTTGTGGCAGGTTATTCTCAAGTTACAACATGGCCCTCAAGGACCCG
CATGCGT?GCCGCAGGAAGGTGATTCGCTGAGCGAGTTGAGAGTAGCCCCTCGGGATTCGT?
?CCAGTCAGTGCCGGTCCTAACTCCCGTCTGAGCGAAACTGACCTCGTGTTTCGGCTCCAGT
GCTGGCATTGGTCTGGGTTCCTCTGAGTCTCCAGGGAGCCAGCCTGTCCCCTGTGTTATGGT
GCAC

OLI 3

TTACTCTTGCTGGGAGTCTGGTTTATAAATGGTTTACTGATCAAGAGAAGGCAAGTTGTTAA
TTATTAAATAAATTATAGTTATAGGTATTATTGTTTCTAATGTTTTTATAGAACAATTATTT
CATCAGTCACATTCCCTTCCCCCTTTAAATTGAACCTCAGATTCTAATGTTAAATT?TTTTT
CAAATGAAGTCAATTATTTTTTTAGGGTTAATATGGAAGTTGTTACAAGGGTAATTATGGAA
ACTAAATACTTT?ATTGAGATAGAGAAAAAATAAGTCCTATTGATTCTTCTTG?GATTATAC
T

OLI 4

GAGAGATGCTGGGCTGGAAGAAACACAAGCTGGAATCAAGATTGCAGGAGAAATATCAATAA
CCTCAAATATGCAGATGACACCACCCTTATGGCAGAAAGTGAAGAGGAACTAAAAAGCCTCT
TGATGAAAGTGAAAGAAGAGAGTGAAAAAGTTGGCTTAAAGCACAACATTCAGAAACGAAGA
TCATGGCATCCGGTCCCATCACTTCATGGGAAATAGATGGTGAAACAGTGGAAACAGTGTCA
GACTTTATTTTGGGGGGCTCTAAACCATTGCAGATGGTGACGGCAGCCATGAATTAGAGACG
CTACTCCTTGAAGAAAGGTTATTGACCACTGATAGCATATTCAAAAGCAGAGACATTACTTT
GCCCGACCTTAAGGTCC

Figure 15 Partial sequence of four cloned *Eco*RI-Max fragments.

sequences (Kelsell pers comm[3], accession number X82879) and a *B. taurus* repeat fragment (Leguina pers comm[4], accession number AF060172).

*Phylogenetic implications*

In the past, *C. monticola* and *C. maxwellii* have been assigned separate generic status *(Philantomba;* Pocock 1910, Groves & Grubb 1981), recognized as separate species within *Cephalophus* (Grubb 1993), as well as placed in synonymy *(C. monticola;* Haltenorth & Diller 1986; see Chapter 1). The FISH results draw these two species together to the exclusion of the remaining taxa. Moreover, their close evolutionary relationship is further underscored by a shared pericentromeric heterochromatic inversion in the X chromosome (Robinson *et al.* 1996). When these findings are taken together with the morphological, phenotypic, and sequence data (Groves & Grubb 1981, Kingdon 1997, Chapter 2) there is clearly strong support for the resurrection of a separate genus *Philantomba* for the blue and Maxwell's duiker (Pocock 1910, Groves & Grubb 1981).

Although there are obvious differences in habitat preference and morphology between *Sylvicapra* and *Cephalophus* (Chapter 1), the FISH results could not distinguish *Sylvicapra* from the forest species. Similarly, no conventional cytogenetics markers were found that separated *Sylvicapra* from the forest species (Robinson *et al.* 1996). This is in marked contrast to the molecular sequence data (see Chapter 2) and underscores the differences inherent in the two markers' abilities to discriminate between closely related species where autosomal rearrangement is constrained.

It has been hypothesized (Groves & Grubb 1981) that *C. silvicultor*, *C. dorsalis*, and *C. spadix* belong to the same subgenus *Cephalophus*, characterized by little sexual dimorphism in horn shape and size, and a dark speckled juvenile pelage. The close evolutionary association between these taxa is strongly supported by the FISH data (absence of fluorescence at the centromeres of both the sex chromosomes) as well as by conventional banding (absence of a large G-negative juxtacentromeric band). In conclusion, Zoo-FISH using satellite DNAs as painting probes proved to be a useful and cost effective means to

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identify related species and to establish some of the evolutionary relationships within these antelope. Although the technique could not distinguish between species within adaptive lineages, the major duiker lineages were clearly delimited.

# CHAPTER 4

# COMBINED APPROACH, CONSERVATION GENETICS, AND GENERAL CONCLUSIONS

## TOTAL EVIDENCE

Independent genetic markers can provide different levels of resolution which, in turn, allow for the accurate reconstruction of evolutionary events ranging from the most distant to the most recent. For example, mitochondrial DNA sequence data are frequently used to reconstruct recent evolutionary events (although the use of appropriate genes would allow resolution of deep nodes), whereas changes in diploid number and intrachromosomal rearrangements may be invariant among closely related taxa, as is the case for duiker antelope (see Chapter 3). To clarify evolutionary relationships between duiker antelope, data were gathered using three independent approaches: the sequencing of selected mitochondrial genes (cytochrome *b* and 12S rRNA), comparative cytogenetics, and fluorescence *in situ* hybridization of satellite DNA sequences.

There has been an increase in the number of studies designed to test hypotheses based on combining independent data sets (e.g. De Queiroz *et al.* 1995). Two approaches are generally followed. The first involves the separate analysis of each data set where the resultant topologies are examined for congruence among them. Secondly, a search for the single most parsimonious solution using all available data, the so-called "total evidence" approach (Kluge 1989), can be performed. The rationale is that different data sets may provide phylogenetic signal at different hierarchical levels so that in combination they may improve the overall resolution of a phylogeny (Hillis 1987). In the present investigation, a "total evidence" approach was adopted in which the comparative and molecular cytogenetic characters were combined with the molecular sequence data after a partition homogeneity test (Farris *et al.* 1995 in Paup 4.0b2a) showed no significant conflict among them (P=0.51).

Initially the cytochrome *b* and 12S rRNA sequences from all 19 recognized duiker taxa were combined with the FISH and comparative cytogenetic data which are unfortunately limited to only seven of the 19 species. The cytogenetic characters were coded as present or absent

and are presented in Table 9. Due to the large number of taxa for which there was missing data a largely unresolved topology resulted (Fig. 16A). A reanalysis of the data including only taxa for which both sequence and chromosomal information are available resulted in a single tree of 909 steps (Fig. 16B). The inclusion of the cytogenetic and FISH characters significantly improved the support for most of the nodes. These finding would suggest that the three independent data sets (molecular sequence data, conventional cytogenetics, and FISH) compliment each other in the combined analysis. In addition, a high degree of correspondence was found between the results of the combined analysis and those from markers analyzed independently.

## GENETICS AND WILDLIFE CONSERVATION

The role of genetics in conservation biology centers mostly around the description of biodiversity and the patterns of genetic variation among populations and species. The data presented herein have implications beyond simply providing an assessment of the duiker phylogeny and provide wildlife managers with an evolutionary framework that may be useful in reassigning conservation priorities to some of the species, in particular the Ruwenzori red duiker and Weyne's duiker.

The Ruwenzori red duiker *(C. rubidus)* has commonly been regarded as a subspecies of the wide spread black-fronted duiker, *C. nigrifrons* (St. Leger 1936, Ansell 1971, Groves & Grubb 1981) and has therefore enjoyed little attention as a conservation priority in its own right. However, the results of this study suggest otherwise. The Ruwenzori duiker groups for most part within the west African red duiker clade and is quite distinct from the black-fronted duiker which clusters as the sister taxon to *C. rufilatus* in the east African red duiker lineage (Chapter 2). This association is maintained irrespective of the method of analysis or weighting scheme used. Moreover, the sequence divergence separating *C. rubidus* and *C. nigrifrons* approximates that delimiting all recognized species; should this hold (the inadequacies of sample size withstanding) it is reasonable to assume that the Ruwenzori red duiker may in fact represent a distinct species and, if valid, clearly raises the conservation profile of this species. This finding is all the more important given its restricted distribution (it occurs only on the Ruwenzori mountains at altitudes above 3 000 meters), and diminishing population numbers (Kingdon 1982, 1997).

Table 9 Five FISH and comparative cytogenetic characters used in the combined analyses. The characters are coded as presence/absence data. The cytogenetic data of Robinson *et al.* (1996) is included.

| | A | B | C | D | E |
|---|---|---|---|---|---|
| Outgroup | 0 | 0 | 0 | 0 | 0 |
| *C. monticola* | 1 | 0 | 1 | 0 | 0 |
| *C. maxwellii* | 1 | 0 | 1 | 0 | 0 |
| *C. natalensis* | 0 | 0 | 0 | 1 | 0 |
| *C. silvicultor* | 0 | 1 | 0 | 0 | 1 |
| *C. spadix* | 0 | 1 | 0 | 0 | 1 |
| *C. dorsalis* | 0 | 1 | 0 | 0 | 1 |
| *S. grimmia* | 0 | 0 | 0 | 1 | 0 |

A=Pericentromeric heterochromatic inversion of the X chromosome, B=Absence of a G-negative juxtacentromeric band on the X chromosome, C=FISH hybridization of satellite probes (*Eco*RI-Max, *Eco*RI-Blue, *Pst*I-Blue, *Pvu*II-Max) to both the X and Y chromosome, D=FISH hybridization of satellite probes (*Eco*RI-Max, *Eco*RI-Blue, *Pst*I-Blue, *Pvu*II-Max) to the X chromosome but not the Y chromosome, E=Absence of FISH hybridization of satellite probes (*Eco*RI-Max, *Eco*RI-Blue, *Pst*I-Blue, *Pvu*II-Max) to both the X and Y chromosomes.

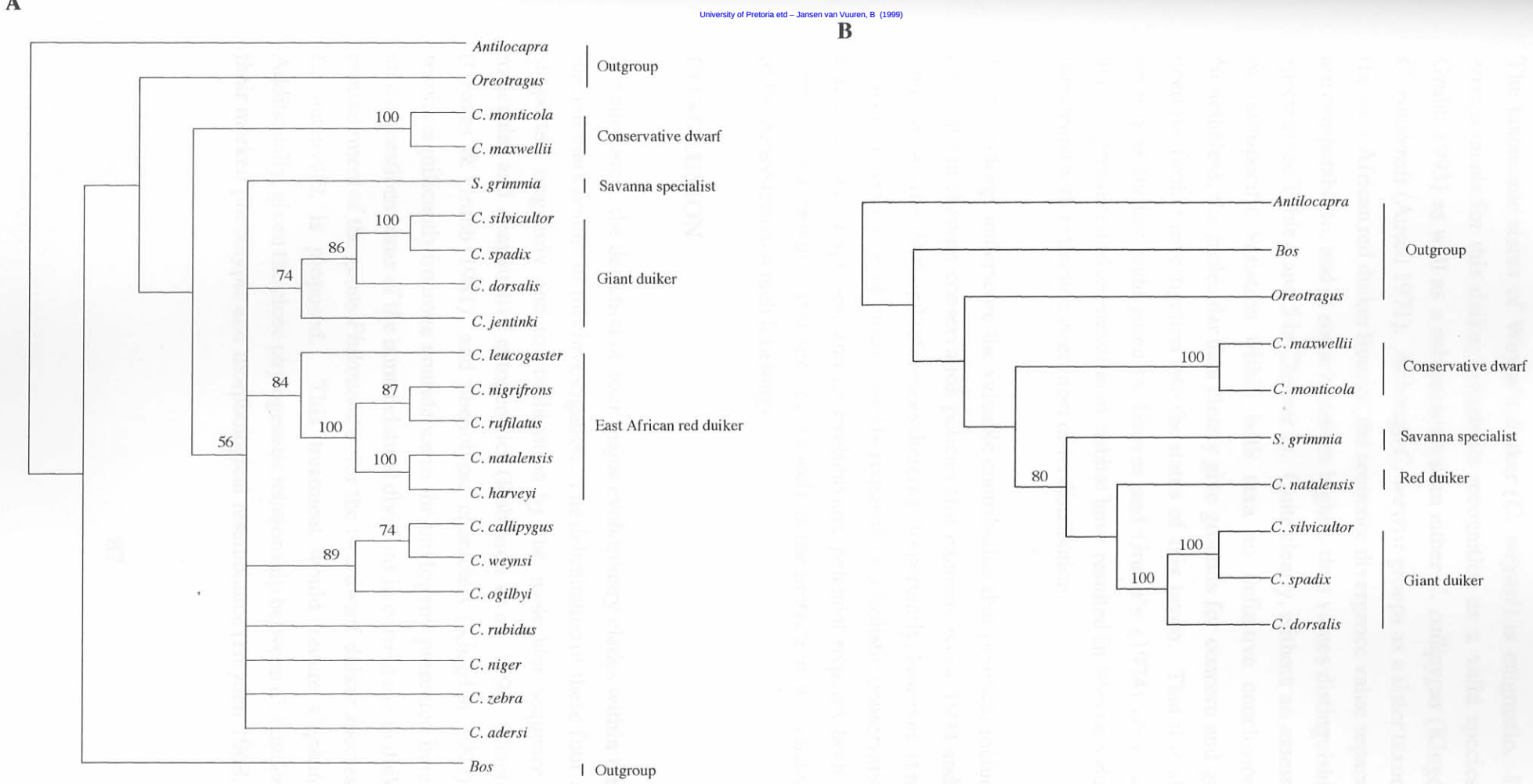


Figure 16 Results of the unordered parsimony analyses based on the combined molecular and cytogenetic data sets. (A) The bootstrap consensus tree (1 000 replicates) obtained when all duiker taxa are included. Nodes that received less than 50% support were collapsed. (B) The single most parsimonious tree obtained when only taxa for which cytogenetic data were available are included. The tree was produced with the heuristic search option in Paup 4.0b2a (Swofford 1999) with 100 random replacements. Bootstrap support obtained from 1 000 iterations is shown above the branches.

The taxonomic status of Weyne's duiker *(C. weynsi)* is enigmatic. Previous taxonomic arrangements for this duiker include its recognition as a valid species (St. Leger 1936, Grubb 1993) as well as a subspecies within either *C. callipygus* (Kingdon 1982, 1997) or *C. natalensis* (Ansell 1971). Although *C. weynsi* groups as a sister taxon to *C. callipygus* in the west African red duiker lineage, the sequence divergence value separating these two taxa are comparable to, and in some instances higher, than values distinguishing well recognized species (see Table 4 and 5 in Chapter 2). Quite clearly, without an assessment of the degree of intraspecific variation within both taxa no definitive conclusions can be drawn. Nonetheless, the molecular data clearly give grounds for concern and give emphasis to the need for further investigation into the status of this taxon. That this should enjoy a high priority is further highlighted by Groves and Grubb's (1974) observation that increased hunting pressure and degradation of habitat have resulted in Weyne's duiker being severely threatened with extinction over most of its distribution.

These findings underscore the valuable contribution that genetics, including phylogenetics, can make in shaping conservation policies (for example Avise 1994 and references therein, Smith & Wayne 1996 and references therein). Importantly, however, it must be realized that genetics is only one of many aspects required in a holistic conservation approach. The conservation of a species, and its evolutionary potential requires both the preservation of genetic variation within that species, as well as the protection of its habitat and the integrity of the ecosystem to which it belongs.

## CONCLUSION

In conclusion, the detection of four major evolutionary clades within the Cephalophini was the hall mark feature of this investigation. The delimitation of these four adaptive lineages is supported singularly and in combination by the molecular sequence data (Chapter 2), molecular and comparative cytogenetics (Robinson *et al.* 1996, Chapter 3), morphology (Groves & Grubb 1981), and phenotypic characters (Kingdon 1997). The concordant results significantly improve confidence in the phylogeny presented herein. Moreover, this study questions some of the nomenclatural divisions in current use in duiker taxonomy. The reinstatement of the genus *Philantomba* for the two dwarf duiker species, *C. monticola* and *C. maxwellii,* is proposed. This treatment would secure *Cephalophus* monophyly. Additionally, given the close phylogenetic relationship between *C. natalensis* and *C. harveyi,* their marked phenotypic and morphological resemblance (Heyden 1968, Ansell 1971), and

evidence of hybridization between them (Kingdon 1982), it is not unreasonable to suggest that *C. harveyi* be relegated to a subspecies of the Natal red duiker, *C. natalensis* .

# SUMMARY

Duiker antelope constitute a distinct tribe within one of the most diverse mammalian families, the Bovidae. Current taxonomy allows for the recognition of the monotypic *Sylvicapra* (a grassland specialist) and the highly diverse *Cephalophus* which includes 18 forest species. Although the monophyly of the group has never been questioned, the placement of taxa within the tribe has proved problematic. The aim of this investigation was therefore to reconstruct the group's evolutionary history using three investigatory parameters: nucleotide sequences from two mitochondrial DNA genes, comparative cytogenetics, and fluorescence *in situ* hybridization (FISH).

Nucleotide sequences were obtained from the complete cytochrome *b* gene and a 767 bp portion of 12S rRNA from all 19 recognized duiker species. Scatter plots showed no significant evidence of cytochrome *b* saturation even though several of the species are characterized by relatively high sequence divergences; pairwise comparisons based on 12S rRNA data showed a monotonic linear increase over time. A consistent finding of the molecular sequence study is the retrieval of four adaptive lineages. The most basal group comprises the conservative dwarf species *(C. monticola, C. maxwellii)*, with the savanna specialist *(S. grimmia)* placed apart from all forest duiker. The giant duiker group includes *C. silvicultor*, *C. spadix*, *C. dorsalis*, and *C. jentinki*. Within the red duiker lineage, a further subdivision was evident; an east African red duiker clade *(C. leucogaster, C. rufilatus, C. nigrifrons, C. natalensis, C. harveyi)* and a west African clade *(C. callipygus, C. weynsi, C. ogilbyi, C. rubidus, C. niger)*. From these data it is proposed that duiker speciation has been driven largely by habitat fragmentation which probably led to the disruption of gene flow between geographic isolates. This is reflected by the relative abundance of species with 11 of 18 forest species associated predominantly with the east African forest which has a history of climatically driven fragmentation.

The G-banded compliment of *C. spadix* was shown to be identical to that of six other species previously reported, extending the observation that speciation in duiker antelope has occurred in the absence of euchromatic chromosomal rearrangement. FISH, involving seven duiker species representative of the four adaptive lineages (conservative dwarfs: *C. maxwellii, C. monticola;* red duiker: *C. natalensis;* grassland specialist: *S. grimmia;* giant

duiker: *C. dorsalis, C. silvicultor, C. spadix)* revealed centromeric hybridization to all the autosomes. However, variation was found in the hybridization patterns of the sex chromosomes which supported the delimitation of the four adaptive groups based on molecular sequence data and morphology.

The results from three independent data sets (sequence data, comparative cytogenetics, FISH) question, and in some instances support, several of the nomenclatural divisions in current duiker taxonomy. These include the recognition of *Philantomba* as genus name for *C. monticola* and *C. maxwellii*, an arrangement that would secure *Cephalophus* monophyly, and the relegation of *C. harveyi* to a subspecies of *C. natalensis*.

# OPSOMMING

Duikers verteenwoordig 'n unieke tribus binne een van die mees uiteenlopende soogdier families, die Bovidae. Huidige taksonomie laat toe vir die herkenning van die monotipiese *Sylvicapra* ('n savanna spesie) en die diverse *Cephalophus* wat 18 woudbewonende spesies insluit. Alhoewel die monofilie van die groep nog nooit bevraagteken is nie, is die plasing van taksa binne die tribus nog problematies. Die doel van hierdie ondersoek was dus die rekonstruksie van die groep se evolusionêre verwantskappe deur die gebruik van drie parameters: nukleotieddata van twee mitokondriale DNS gene, vergelykende sitogenetika, en fluoreserende *in situ* hibridisasie (FISH).

Nukleotieddata is verkry vir die volledige sitokroom *b* geen en 767 karakters van die 12S rRNS geen vir al 19 erkende duiker spesies. Verstrooingsgrafieke dui geen noemenswaardige bewys van versadiging aan vir die sitokroom *b* geen nie alhoewel verskeie van die spesies gekenmerk word deur relatiewe hoë nukleotied verskille, tweeledige vergelykings gebaseer op die 12S rRNS data dui op 'n deurlopende monotoniese lineêre toename oor tyd. 'n Deurlopende bevinding van die molekulêre nukleotied studie is vier evolusionêre lyne. Die mees primitiefste groep bestaan uit die konserwatiewe dwergspesies *(C. monticola, C. maxwellii)*, met die savanna spesialis *(S. grimmia)* wat apart geplaas word van alle woud duikers. Die reuse duiker groep sluit *C. silvicultor, C. spadix, C. dorsalis*, en *C. jentinki* in. Binne die rooi duiker evolusionêre lyn is daar 'n verdere onderverdeling sigbaar: die Oos-Afrika rooi duiker groep *(C. leucogaster, C. rufilatus, C. nigrifrons, C. natalensis, C. harveyi)* en 'n Wes-Afrika rooi duiker groep *(C. callipygus, C. weynsi, C. ogilbyi, C. rubidus, C. niger)*. Gegrond op hierdie data word voorgestel dat duiker spesiasie hoofsaaklik aangedryf word deur habitatsfragmentasie, wat lei tot die onderbreking van genevloei tussen geografiese isolate. Hierdie bevinding word weerspieël deurdat 11 van die 18 woudbewonende spesies hoofsaaklik geassosieer word met reenwoude in Oos-Afrika. Die Oos-Afrikaanse reenwoude word veral gekenmerk deur klimaatfragmentasie.

Die G-band kompliment van *C. spadix* is identies aan die ses spesies wat tans in die literatuur beskryf is. Dit ondersteun die waarneming dat spesiasie in duikers plaasgevind het in die afwesigheid van eukromatiese herrangskikkings. FISH, op sewe duiker spesies verteenwoordigend van die vier hoof evolusionêre lyne (konserwatiewe dwerge: *C.*

*maxwellii, C. monticola;* rooi duiker: *C. natalensis;* savanna spesialis: *S. grimmia;* reuse duikers: *C. dorsalis, C. silvicultor, C. spadix)*, dui op hibridisasie van die sentromere van al die outosome. Desnieteenstaande is variasie gevind in die hibridisasiepatrone van die sekskromosome ter stawing van die afbakening van die vier evolusionêre lyne gebaseer op molekulêre nukleotieddata en morfologie.

Ooreenstemming in die bevindinge van die drie onafhanklike datastelle (molekulêre nukleotieddata, vergelykende sitogenetika, FISH) bevraagteken, en in sommige gevalle ondersteun, verskeie van die nomenklature afdelings tans in gebruik is in duiker taksonomie. Hierdie sluit in die erkenning van *Philantomba* as die genus naam vir *C. monticola* en *C. maxwellii*, 'n skikking wat *Cephalophus* monofilie verseker, en die relegasie van *C. harveyi* tot 'n subspesie van *C. natalensis*.

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