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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).

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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).

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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.

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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 μl Colcemid (10 μg/μl; Gibco BRL). In addition, 50 μl 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

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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.

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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 μg 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

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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 μg/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 [α-^{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 μl.

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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 μl) 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.

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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

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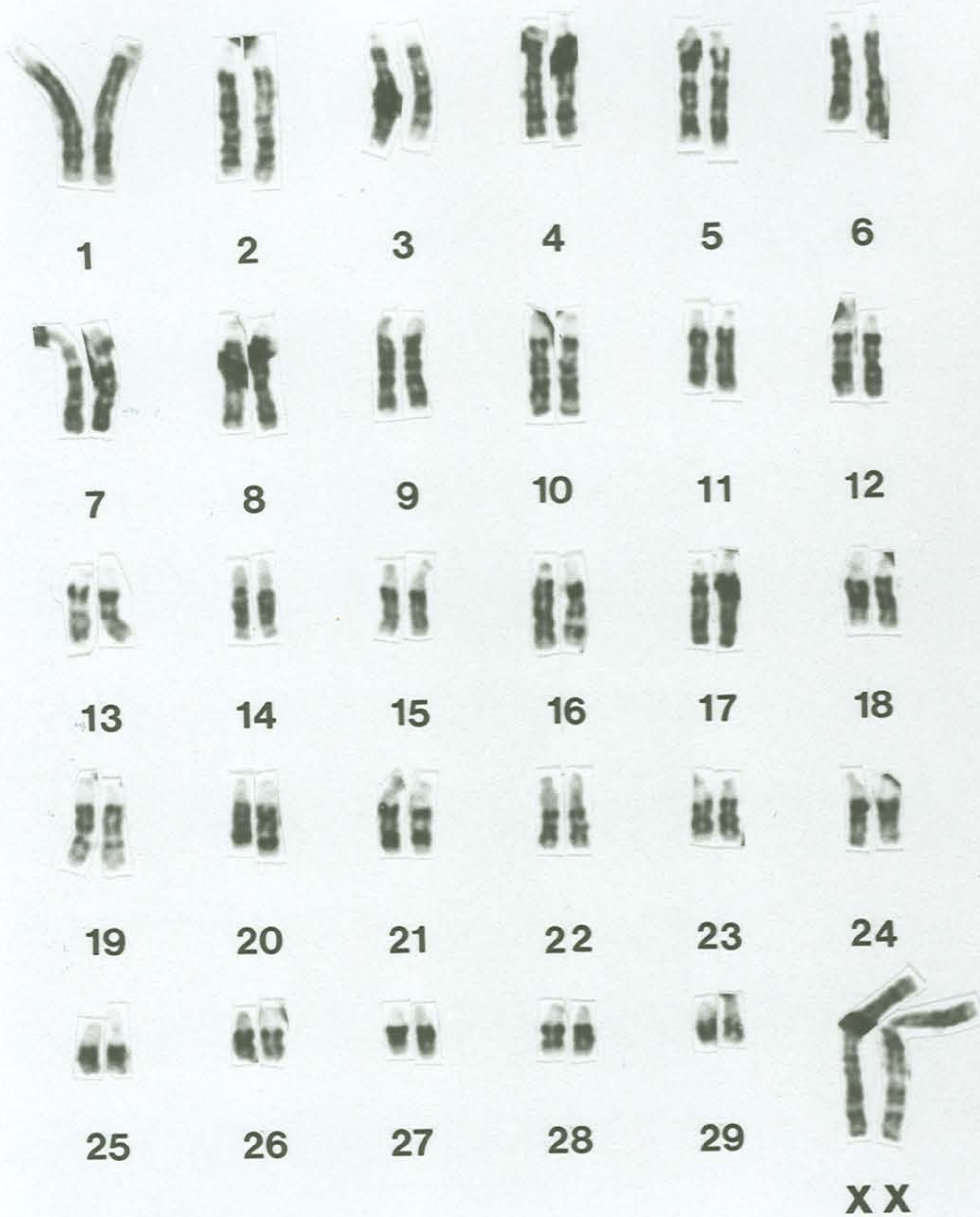

Figure 9 G-banded female karyotype of *C. spadix* (2n=60) from Tanzania.

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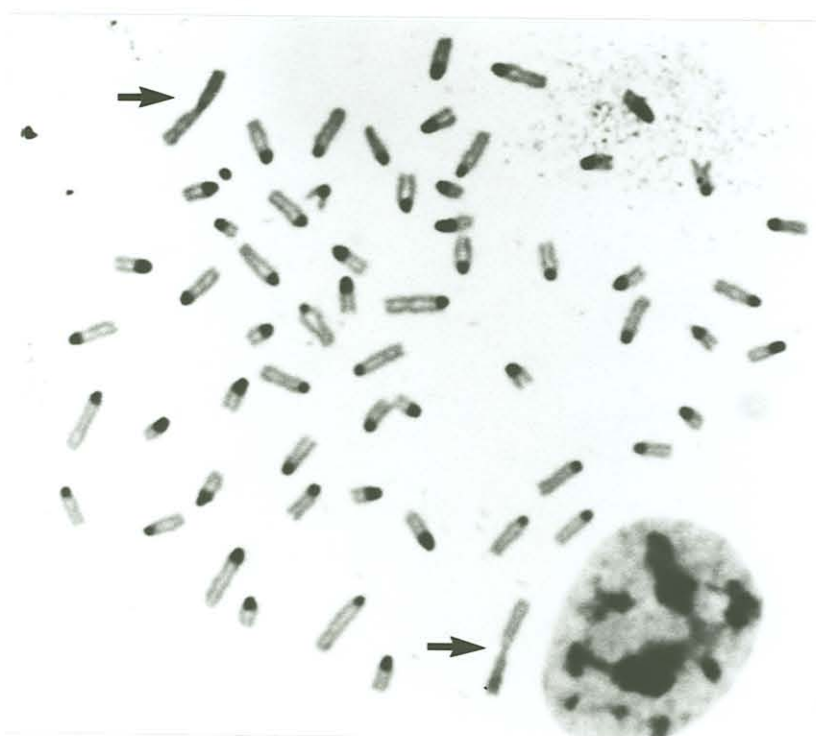

Figure 10 C-banded metaphase cell of a *C. spadix* female. Arrowheads indicate the X chromosomes.

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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

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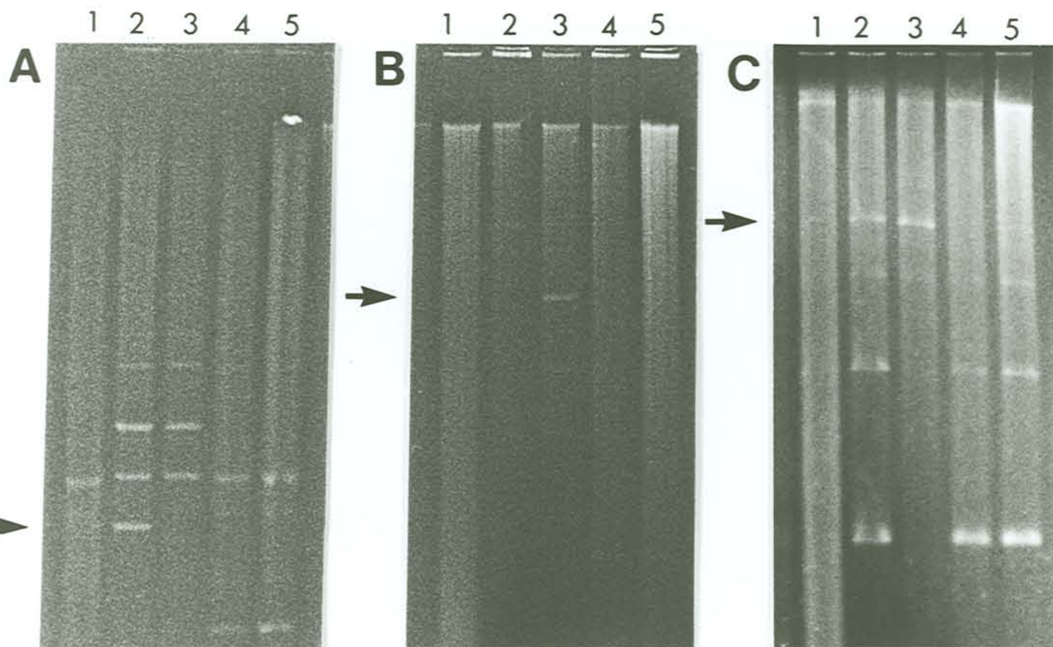

Figure 11 Digestion profiles of 5 duiker species. The unique satellite fragments that were selected as FISH probes are indicated by the arrows. (A) 1.4 kb fragment (lane 2) released in *C. monticola* by *Pst*I digestion, (B) 2.8 kb satellite fragment isolated from *C. maxwellii* (lane 3) after digestion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

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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.

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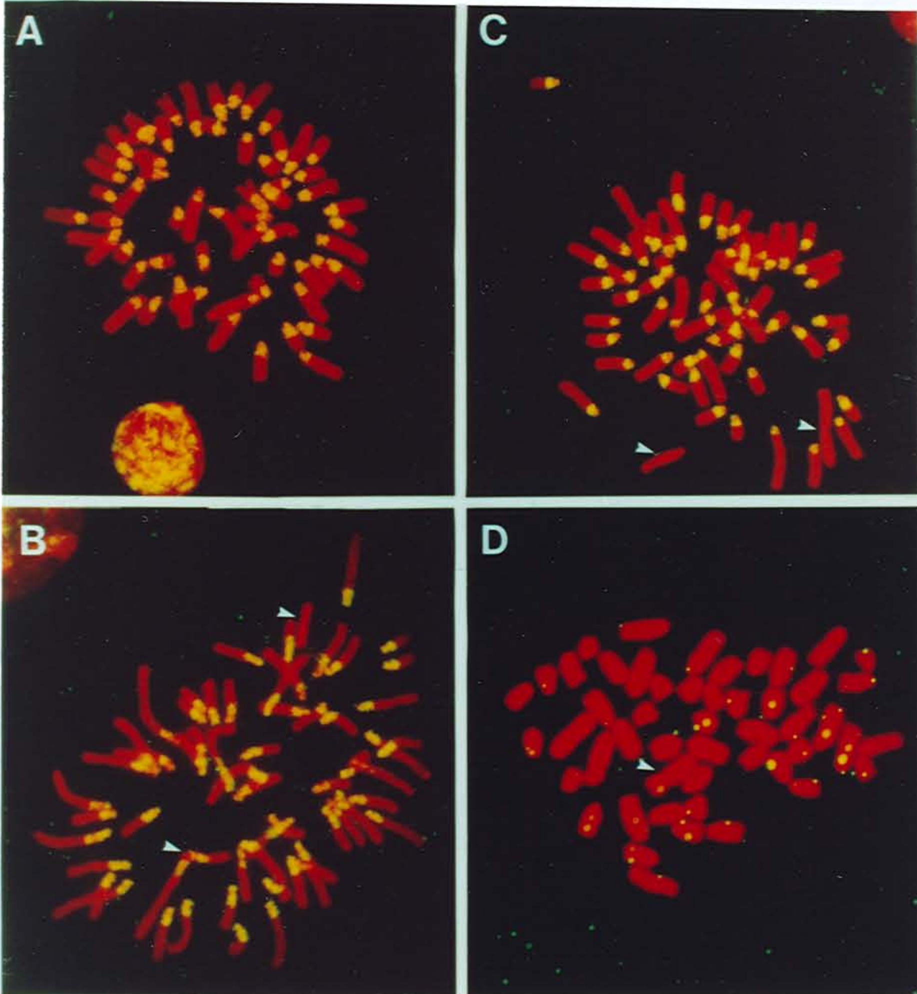

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chromosomes showed a lack of signal (see Fig. 12B). The lack of hybridization to the X centromere is particularly striking and is reflected in the species' C-bands where two well delimited dark C-bands bracket the centromere (see Figure 3F in Robinson *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*.

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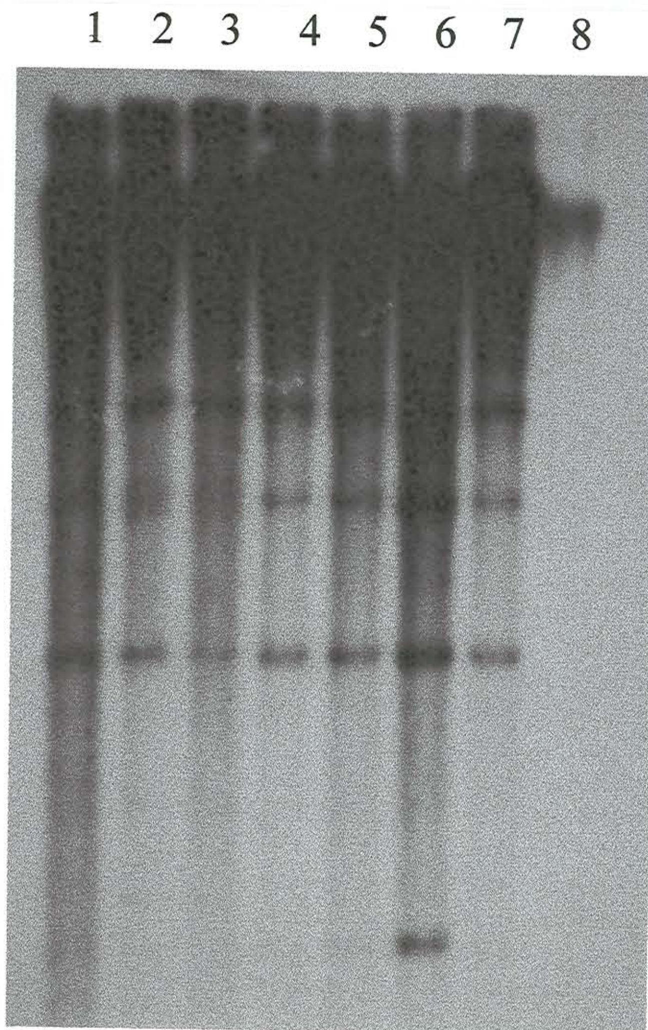

Figure 13 Southern blot of *Cfo*I digested DNAs of seven duiker species and an outgroup species after probing with the *C. maxwellii Eco*RI fragment. Lane 1=*S. grimmia*; lane 2=*C. maxwellii*; lane 3=*C. monticola*; lane 4=*C. natalensis*; lane 5=*C. dorsalis*; lane 6=*C. silvicultor*; lane 7=*C. spadix*;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

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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

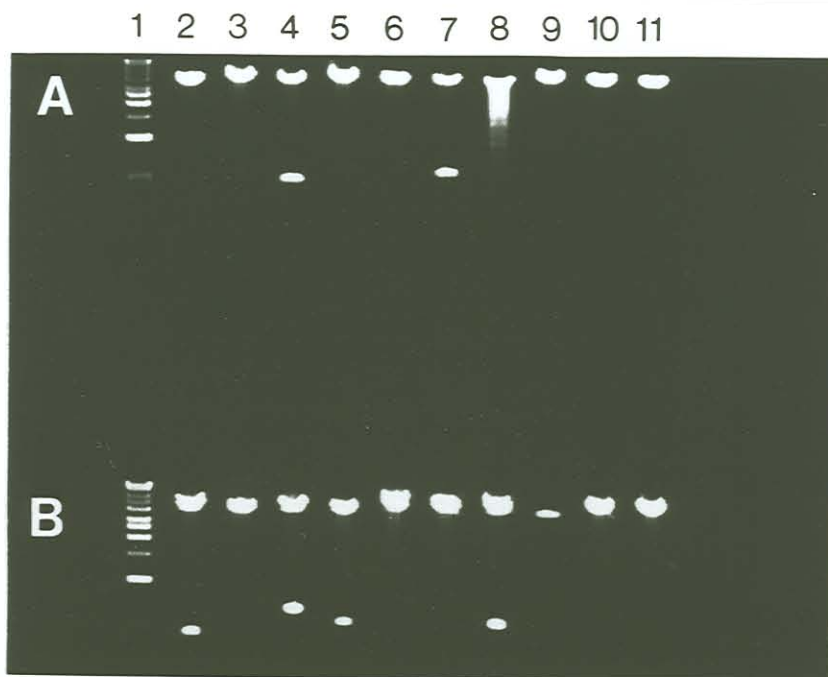

Figure 14 Cloned *Eco*RI-Max fragments released after digestion by *Eco*RI. Clones with an insert size spanning approximately 0.6 kb to 0.8 kb were selected for sequencing (lanes A7, B4, B5, and B8). The Pharmacia kilobase DNA marker (lanes A1 and B1) was used as the size standard.

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OLI 1

TAGGGGAACTGTCCATGTTGTTCAATATTGTGATAGCATGTTATTTAATTTTCATATAACGA
TCCATCATCTTTCCATGTGTGCATGCTAAGTCACTTCAATCCTGTCTGACTCTTTGAGAACA
TATGGACCATAGCCCTCCAGGCTCCTCTGTCCATGGGATTTCCCAGGCAAGAATACTGTAGT
GGGTTGCCATTTACTCCTCCAGGGGATTTTCCTGACTCAGGGATTGAACCCAAGTCTCTTGC
ATCTAATACTGGTGGCTATAACTGGATAGGGCTGGAGAGATTGAAGGTAATTGTTATTTTCC
CAAATTTATAAACACATATTACTGGTTAATTTTACCTGATTTTTTAAATTGGAGG

OLI 2

CGGGGAATTTCCTGAGTTGCAGCAAGGGTGGAAGGACCCTTGCGAGGTACAAGAGAGAAAG
CATGAATGCCCTCGAGATGCCGCTGCAGAAAACGGCCTGATTTCGCCTGGAGGTTAGATCCT
CCTGGTTTTTCTCGAGTTGTGGCAGGTTATTCTCAAGTTACAACATGGCCCTCAAGGACCCG
CATGCGT?GCCGCAGGAAGGTGATTCGCTGAGCGAGTTGAGAGTAGCCCCTCGGGATTCGT?
?CCAGTCAGTGCCGGTCCTAACTCCCGTCTGAGCGAAACTGACCTCGTGTTTCGGCTCCAGT
GCTGGCATTGGTCTGGGTTCCTCTGAGTCTCCAGGGAGCCAGCCTGTCCCCTGTGTTATGGT
GCAC

OLI 3

TTACTCTTGCTGGGAGTCTGGTTTATAAATGGTTTACTGATCAAGAGAAGGCAAGTTGTTAA
TTATTAAATAAATTATAGTTATAGGTATTATTGTTTCTAATGTTTTTATAGAACAATTATTT
CATCAGTCACATTCCCTTCCCCCTTTAAATTGAACCTCAGATTCTAATGTTAAATT?TTTTT
CAAATGAAGTCAATTATTTTTTTAGGGTTAATATGGAAGTTGTTACAAGGGTAATTATGGAA
ACTAAATACTTTT?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.