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Phylogenomic study of spiral-horned antelope by cross-species chromosome painting

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Abstract

Chromosomal homologies have been established between cattle (*Bos taurus*, 2n = 60) and eight species of spiral-horned antelope, Tribe Tragelaphini: Nyala (*Tragelaphus angasii*, 2n = 55♂/56♀), Lesser kudu (*T. imberbis*, 2n = 38♂,♀), Bongo (*T. eurycerus*, 2n = 33♂/34♀), Bushbuck (*T. scriptus*, 2n = 33♂/34♀), Greater kudu (*T. strepsiceros*, 2n = 31♂/32♀), Sitatunga (*T. spekei*, 2n = 30♂,♀) Derby eland (*Taurotragus derbianus* 2n = 31♂/32♀) and Common eland (*T. oryx* 2n = 31♂/32♀). Chromosomes involved in centric fusions in these species were identified using a complete set of cattle painting probes generated by laser microdissection. Our data support the monophyly of Tragelaphini and a clade comprising *T. scriptus*, *T. spekei*, *T. euryceros* and the eland species *T. oryx* and *T. derbianus*, findings that are largely in agreement with sequence-based molecular phylogenies. In contrast, our study suggests that the arid adaptiveness of *T. oryx* and *T. derbianus* is recent. Finally, we have identified the presence of the rob(1;29) fusion as an evolutionary marker in most of the tragelaphid species investigated. This rearrangement is associated with reproductive impairment in cattle and raises questions whether subtle distinctions in breakpoint location or differential rescue during meiosis underpin the different outcomes detected among these lineages.

Introduction

The family Bovidae (Artiodactyla) is a highly heterogeneous clade comprising approximately 49 recent genera and 140 species (Nowak 1999) whose evolutionary relationships are often obscure, in large part owing to morphological convergence among species. Recent molecular investigations (Gatesy *et al*. 1997, Hassanin & Douzery 1999a, 2003, Matthee & Davis 2001, Hassanin & Ropiquet 2004,

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Ropiquet & Hassanin 2005a,b) have confirmed the presence of two main lineages, the Bovinae and Antilopinae. The former includes cattle, buffalos, nilgai and spiral-horned antelopes within three tribes (Hassanin & Douzery 1999b), while the latter contains sheep, goats and non-bovine antelopes in nine tribes (Bronner *et al*. 2003).

Tragelaphini (spiral-horned antelope) is represented by a group of medium- to large-bodied species that are widely distributed through forested and bush-savannah regions of Africa south of the Sahara. The nine extant species are assigned to two genera (Grubb 2005): *Tragelaphus* containing Greater kudu (*T. strepsiceros*, TST), Lesser kudu (*T. imberbis*, TIM), Nyala (*T. angasii*, TAN), Mountain nyala (*T. buxtoni*, TBU), Sitatunga (*T. spekei*, TSP), Bushbuck (*T. scriptus, TSC*) and Bongo (*T. eurycerus*, TEU), and *Taurotragus* containing Common eland (*T. oryx, TOR*) and Derby eland (*T. derbianus*, TDE). These genera were conventionally identified based on morphology and fossil evidence (Ansell 1971). However, there has been considerable disagreement regarding their classification (see reviews by Nowak 1999, Skinner & Chimimba 2005). Cranial similarities suggest a sister relationship between *T. spekei* and *T. angasi* and between *T. imberbis* and *T. strepsiceros* respectively (Kingdon 1982, Alden *et al*. 1995), while the presence of horns in both sexes of *T. euryceros* and the two *Taurotragus* species, *T. oryx* and *T. derbianus*, are thought to indicate a close evolutionary affinity among these species (Gentry 1992). In sharp contrast, recent studies using mtDNA data, or combined nuclear intron and mitochondrial DNA sequences, in conjunction with comprehensive taxon representation failed to provide genetic support for the recognition of three tragelaphid genera, or for any of the sister species relationships outlined above (Matthee & Robinson 1999, Willows-Munro *et al*. 2005; see also Georgiadis *et al*. 1990, Essop *et al*. 1997, Gatesy *et al*. 1997).

Given the conflict characterizing the relationships suggested by morphology and molecules we wished to determine whether the analysis of chromosomal rearrangements among species would resolve some of the phylogenetic confusion within Tragelaphini. At first glance, chromosomes may be considered unlikely markers for resolving relationships among species that are known to have undergone a rapid radiation during the past 15 My (Vrba 1985, Matthee & Robinson 1999, Hassanin & Douzery 2003, Willows-Munro *et al*. 2005). However, we were encouraged by the variability in Tragelaphini diploid number ($2n = 30$ to $2n = 56$), and the sometimes remarkably rapid rate of karyotypic diversity in species such as the house mouse (*Mus musculus*) which appears to have occurred independently of adaptive processes, reflecting rather geographic isolation and drift (Britton-Davidian *et al*. 2000). We therefore examined karyotypic change with the Tragelaphini using conventional and molecular cytogenetic techniques that relied on whole-chromosome and subchromosomal painting probes developed from cattle using laser microdissection technology.

Material and methods

Species analysed

Peripheral blood samples were taken from captive-born specimens held in the Dvur Kralove animal facility (Czech Republic): *T. strepsiceros* (Greater kudu, 8 animals), *T. imberbis* (Lesser kudu, 15 animals), *T. angasii* (Nyala, 12 animals), *T. spekei* (Sitatunga, 9 animals), *T. eurycerus* (Bongo, 3 animals, one specimen from the Prague Zoo) and *T. oryx* (Common eland, 16 animals). Blood samples of *T. derbianus* (Derby eland, 6 animals) were obtained from the Bandia reserve in Senegal. Fibroblast lines of *T. scriptus* (Bushbuck, 2 animals) were established from a specimen provided by the National Zoological Gardens, Pretoria, South Africa, while the other was collected in the Western Cape Province of South Africa.

Culture conditions, metaphase preparation and banding

Whole blood (2.7 ml) was added to 22 ml of RPMI 1640 culture medium (Sevapharma, Prague, Czech Republic) supplemented with 20% of fetal calf serum (Sigma-Aldrich Corp., St Louis, MO, USA), glutamine (0.5 mg/ml, Sevapharma) and pokeweed mitogen (0.15 mg/ml, Sigma). The cultures were harvested after 72 h of incubation at 38°C following a 40 min colcemid (Sigma) block at a final concentration of 0.1 μg/ml. Cells were fixed in acetic acid– methanol [1:3] and air-dried metaphase preparations were made (Verma & Babu 1989). The Bushbuck chromosome preparations were obtained

from fibroblast cultures grown from skin explants using conventional procedures. Metaphase chromosomes were GTG- and CBG- banded following Seabright (1971) and Sumner (1972) respectively.

DNA probes

We used a complete set of whole-chromosome painting probes derived from cattle (*Bos taurus*, BTA 1–29, X and Y) for cross-species chromosome painting among the Tragelaphini. Additionally, subchromosomal probes were developed from the distal or proximal euchromatic ends of all the BTA autosomes (i.e. BTA 1–29) so as to orientate the synteny blocks within the various species. Arm-specific Xp and Xq painting probes were similarly prepared. A generic probe that hybridizes to pericentromeric heterochromatin was prepared from template DNA obtained from the pooled centromeric regions of selected acrocentric autosomes from different tragelaphine species. All DNA probes were prepared using laser microdissection techniques (Kubickova *et al*. 2002). The probes were labelled by DOP-PCR with SpectrumOrange-dUTP, SpectrumGreen-dUTP (Abbott, IL, USA), digoxigenin-dUTP and biotin-dUTP (Roche Diagnostics GmbH, Mannheim, Germany). The assignment of painting probes was validated by two different cytogenetic laboratories against GTG-banded bovine chromosomes, and subsequently verified by molecular markers (data not shown) of known chromosomal location (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene)

Fluorescence in-situ hybridization

Hybridization mixture (10 μl) containing 50% formamide, 2× SSC, 10% dextran sulfate, 5 μg salmon sperm DNA, 1.5 μg calf thymus DNA (Sigma) and 1 μl probe (DOP PCR labelled product) was denatured at 72°C for 10 min and preannealed at 37°C for 80 min. In instances where we applied two-colour FISH, 0.5–0.7 μl of red-labelled probe (Spectrum Orange, Abbott) and 0.5–0.8 μl of green-labelled probe (Spectrum Green, Abbott) were added to the probe mix. Metaphase spreads were denatured in 70% formamide, 2× SSC (pH 7.0) at 72°C for 2 min, dehydrated, and hybridized overnight in a moist chamber at 37°C. Slides were washed twice in 0.4× SSC (pH 7.0) at 72°C for 2 min. Non-specific binding sites were blocked with 1% Blocking reagent (Roche Diagnostics) in TN buffer for 10 min. Biotin- and dioxigenin-labelled probes were detected with avidin-CY3 (Amersham Pharmacia Biotech, Piscataway, USA) and antidioxigenin-fluorescein (Roche Diagnostic). Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 0.24 μg/ml) and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). An Olympus BX60 fluorescent microscope was used for visualizing probe signals on metaphase spreads and digital images were captured with ISIS 3 software (Meta-Systems, Altlussheim, Germany).

Identification of chromosomal fusions

The identification of the arms that form the different bi-armed chromosomes detected in the karyotypes of the investigated species was by GTG-banding following the cattle standard presented by the ISCNDB2000 (2001). The different combinations of chromosome arms were checked by double-colour FISH; the orientation of the various fusion products was determined by subchromosomal microdissected painting probes (see above). Additionally, the orientation of the BTA 22 orthologue in the various species was further refined using a probe to the histamine receptor H1 (HRH1) gene which is localized at distal 22q2.4 (GeneID:281231).

Parsimony analysis

Chromosomal rearrangements (characters) were scored by their presence or absence (characters states) in the eight ingroup species based on comparison with *B. taurus* (the outgroup) following Dobigny *et al*. (2004a). The resulting matrix of characters (Supplementary Table S1) was analysed using Maximum Parsimony in PAUP* vs 4.0b10 by applying the exhaustive search option with characters unweighted; the robustness of each node was assessed by 100 bootstrap iterations.

Results and discussion

The karyotypes of the eight species studied are in agreement with those previously reported in the literature and are therefore not included here, but they are available from the corresponding author. Chromosome numbers range from 30 (♂/♀) in

T. spekei to 56/55 (♂/♀) in *T. angasii*. The only recognized species within the Tragelaphini that was not included in our investigation is the Mountain nyala (*T. buxtoni*), which was not available to us.

The first studies of the chromosomes of species of Tragelaphini date back to the late 1960s, with the karyotypic characteristics of most species determined between 1968 and 1977 (reviewed by Wallace 1978). G-banded karyotypes have subsequently been published in the *Atlas of Mammalian Chromosomes* (O'Brien *et al*. 2006). Our study, however, is the first to systematically and unequivocally identify all fusions characterizing eight of the nine recognized species using FISH, and to subject these to parsimony analysis to further clarify their phylogenetic relationships, as well as to examine the patterns and processes that have shaped the evolution of their genomes.

Autosomes and autosomal rearrangements

Fifty-seven different autosomal fusions (53 centric and 4 tandem fusions) were identified among the eight ingroup taxa (Table 1). The largest autosome among all species examined is (with the exception of the Lesser kudu, *T. imberbis*) a submetacentric element that on FISH painting was shown to comprise three fused BTA chromosomes (Figure 1). The most parsimonious explanation for the origin of this compound chromosome is that there was an initial centric fusion of BTA 22 and BTA 2 which is shared by all the ingroup species except *T. imberbis* (TIM). This was followed by tandem fusion with the equivalent of BTA 3; this rearrangement (BTA 2;22;3) is common to *T. scriptus*, *T. spekei*, *T. eurycerus*, *T. oryx* and *T. derbianus*. Uniquely derived (autapomorphic) rearrangements of the BTA 22;2 fusion entails fusion with BTA 11 in the Nyala (*T. angasii*), and BTA 24 in the Greater kudu (*T. strepsiceros*). Support for this was suggested by the subchromosomal painting probes BTA 3qdist and BTA 2qdist in conjunction with the HRH1 locus that maps to BTA 22q2.4 (Figure 1A). Additionally, the Lesser kudu (*T. imberbis*) carries a unique autapomorphy involving a tandem fusion between chromosomes of BTA 22 and BTA 15 which shows the same telomere:centromere orientation as the BTA 22;3 fusion. All other autosomal rearrangements involve simple Robertsonian fusions, and their identification in the various species is given in Table 1.

One species in particular deserves more detailed discussion. An earlier analysis of the Greater kudu (*T. scriptus*) was done by Gallagher & Womack (1992) and relied on QFH-banding to infer homology with cattle. Our results are in accordance with their findings except for the identification of chromosomes 22, 25, 27 and 29, which on painting data correspond to pairs BTA 27, 22, 29 and 25 respectively.

Table 1. Centric and tandem chromosome fusions in different species of Tragelaphinia

Lesser kudu	Nyala	Greater kudu	Bushbuck	Sitatunga	Bongo	Common eland	Derby eland
t (1; 5)	t (**11; 22**; 2)	t (**24; 22**; 2)	t (**3; 22**; 2)	t (**3; 22**; 2)	t (**3; 22**; 2)	t (**3; 22**; 2)	t (**3; 22**; 2)
t (2; 10)		t (4; 5)	t (6; 10)	t (5; 10)	t (5; 10)	t (6; 11)	t (6; 11)
t (4; 7)		t (3; 10)	t (1; 29)	t (4; 12)	t (1; 29)	t (5; 10)	t (5; 10)
t (3; 11)		t (1; 29)	t (4; 15)	t (1; 29)	t (4; 19)	t (4; 12)	t (4; 12)
t (6; 16)		t (6; 20)	t (5; 17)	t (8; 15)	t (6; 21)	t (1; 29)	t (1; 29)
t (12; 18)		t (7; 18)	t (8; 16)	t (6; 24)	t (7; 28)	t (9; 20)	t (9; 20)
t (8; 20)		t (8; 17)	t (11; 20)	t (11; 18)	t (8; 15)	t (8; 24)	t (8; 24)
t (9; 27)		t (12; 16)	t (7; 28)	t (7; 26)	t (9; 23)	t (15; 16)	t (15; 16)
t (**15; 22**)		t (11; 23)	t (9; 26)	t (16; 20)	t (11; 20)	t (7; 28)	t (7; 28)
t (14; 29)		t (9; 27)	t (23; 12)	t (14; 19)	t (14; 27)	t (18; 19)	t (18; 19)
		t (19; 21)	t (18; 21)	t (9; 28)	t (16; 17)	t (14; 26)	t (14; 26)
		t (14; 26)	t (19; 25)	t (17; 27)	t (18; 26)	t (21; 23)	t (21; 23)
		t (15; 28)		t (21; 23)		t (17; 27)	t (17; 27)
t (X; 13)	X	X	X	t (X; 13)	X	X	X
t (Y; 13)	t (Y; 13)	t (Y; 13)	t (Y; 13)	t (Y; 13)	t (Y; 13)	t (Y; 13)	t (Y; 13)

[a]Chromosomes were numbered following the standard karyotype of cattle (*Bos taurus*) presented by the ISCNDB2000. Bold numbers indicate tandem fusions. Nyala (*Tragelaphus angasii*), Lesser kudu (*T. imberbis*), Bongo (*T. eurycerus*), Bushbuck (*T. scriptus*), Greater kudu (*T. strepsiceros*), Sitatunga (*T. spekei*), Derby eland (*T. derbianus*) and Common eland (*T. oryx*).

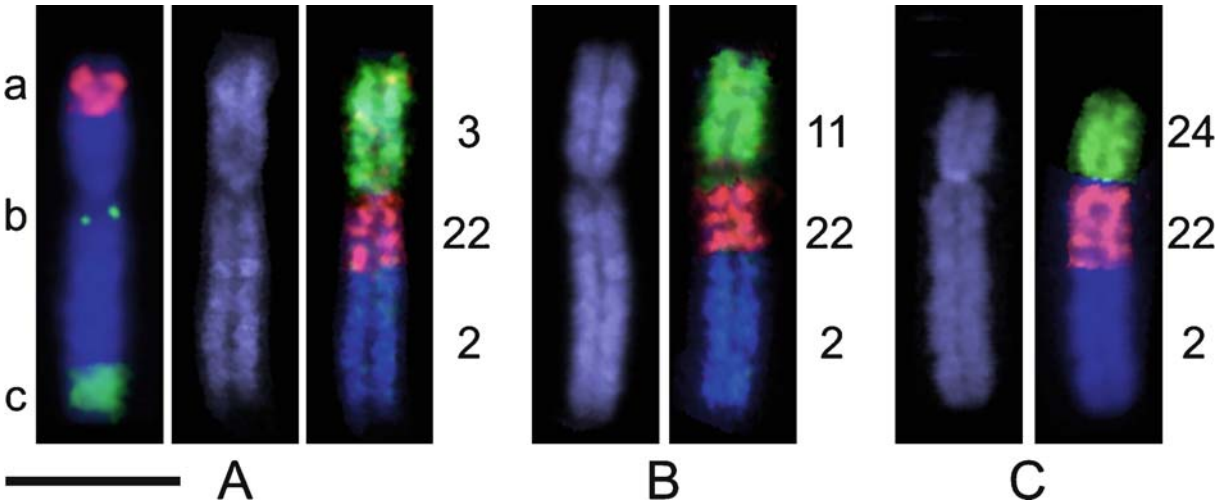

Figure 1. Different compound chromosomes detected in the Tragelaphini that comprise three orthologous BTA chromosomes in each instance shown on the right. (**A**) The fusion BTA 3/22/2 found in *Tragelaphus scriptus, Tragelaphus spekei, Tragelaphus eurycerus*, *Taurotragus oryx* and *Taurotragus derbianus*. Letters a, b, c to the left in panel (A) show: (a) the position of subchromosomal probes for distal part of BTA 3, (b) the localization of the gene HRN1 in subtelomeric region of BTA 22q2.4 and (c) probe for distal part of BTA 2. (**B**) Cross-species chromosome painting showing the presence of the BTA 11/22/2 in *Tragelaphus angasii*. (**C**) Cross-species chromosome painting showing the presence of the BTA 24/22/2 in *Tragelaphus strepsiceros*. BTA 2 is stained with DAPI (blue). Scale bar represents 5 μm.

Gallagher *et al*. (1999) correctly documented (without presenting supporting data) the BTA homology of all fusions present in the Common eland (*T. oryx*).

Sex chromosomes

Y chromosome

All species of Tragelaphini exhibit a Y-autosomal translocation involving BTA 13 (Figure 2) which would result in an imbalance in diploid numbers between sexes (i.e., X1X2Y sex chromosome system). This sex–autosome fusion product can be classified into one of three morphological types within the tragelaphines: (i) submetacentric chromosome with both chromosomes fused at the centromere (*T. spekei*, *T. strepsiceros* and *T. scriptus* (Figure 2A–C); (ii) submetacentric chromosome characterized by a centromere shift to Y chromosome resulting from transposition of Y-specific heterochromatin (*T. eurycerus*, *T. derbianus* and *T. oryx*; Figure 2D–F); (iii) large acrocentric chromosome with the Y chromosome forming the proximal one-third of the fusion chromosome (*T. imberbis*, *T. angasii*; Figure 2G,H). In *T. scriptus* (Figure 2C), a part of Y heterochromatic p arm is inserted in the proximal part of chromosome 13, where it forms a band that probably originated from paracentric inversion of the more submetacentric types shown in panels (D)–(F) (Figure 2).

A Y-autosomal fusion in tragelaphines was detected by Wallace in 1977 in bushbuck (*T. scriptus*). The autosome was later identified as a chromosome homologous to BTA 13 in a variety of different species (*T. eurycerus*, Benirschke *et al*. 1982; *T. strepsiceros*, Gallagher & Womack 1992; *T. oryx* and *T. spekei*, Petit *et al*. 1994). It was originally hypothesized (Wallace 1978, 1980) that the tragelaphine ancestor may have had a metacentric Y;A fusion and that a single pericentric inversion subsequently resulted in the acrocentric Y;A chromosome observed in *T. angasii*. It is noteworthy that the acrocentric Y/autosome fusion is detected only in *T. imberbis* and *T. angasii*, the basal species in our chromosomally derived PAUP tree (an observation consistent with the analysis of DNA sequences: Matthee & Robinson 1999, Willows-Munro *et al*. 2005).

These findings suggest another, possibly more likely, scenario. We hypothesize that the Y;13 chromosome resulted from a telomere (corresponding to the cattle Yq telomere)–centromere (of BTA 13) fusion—an orientation still retained in the Lesser kudu (*T. imberbis*) and Nyala (*T. angasii*) based on our FISH data. We show that the proximal part of

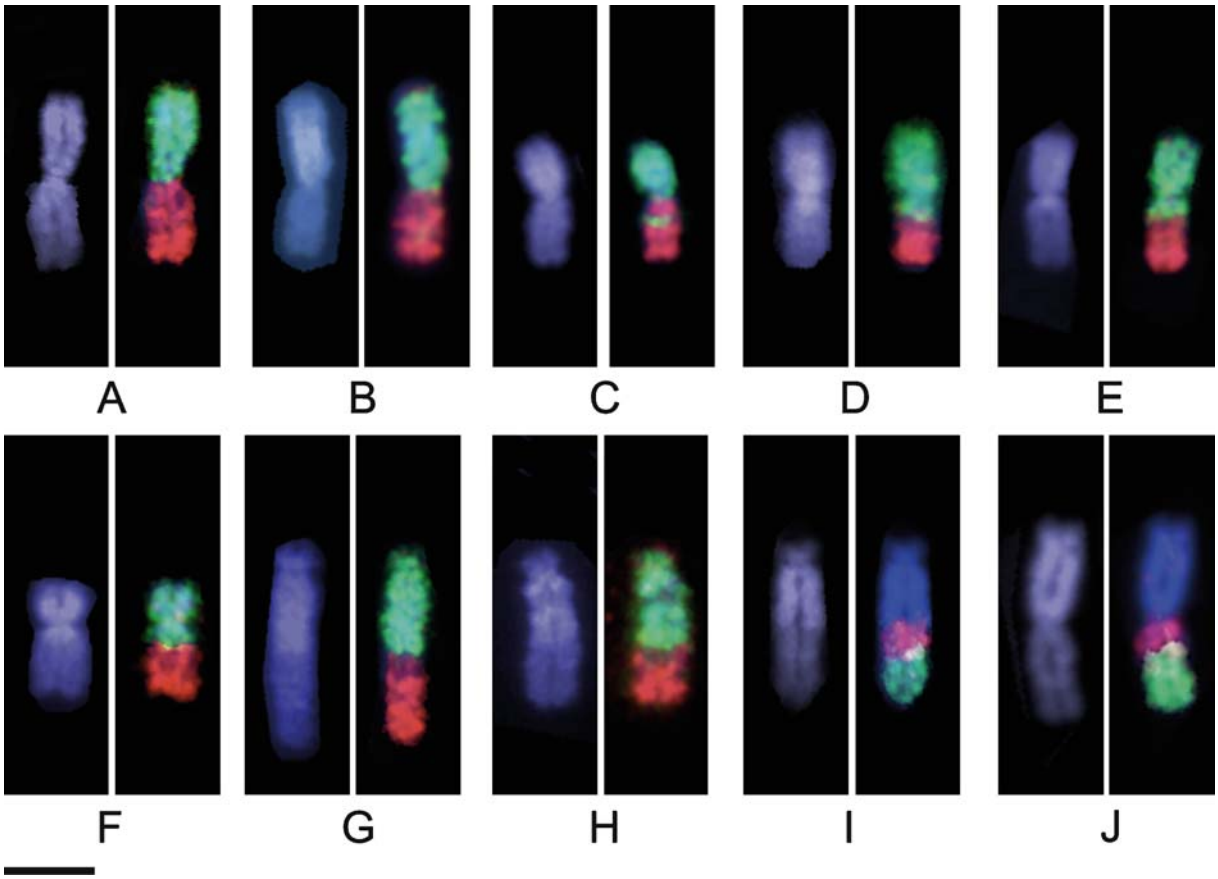

Figure 2. Y chromosomes of Tragelaphini. (**A**–**H**) Y chromosome is green and the translocated BTA 13 red. The DAPI images are shown to the left in each instance. (**A**) *Tragelaphus spekei*; (**B**) *Tragelaphus strepsiceros*; (**C**) *Tragelaphus scriptus* (a part of Y heterochromatic p arm is inserted in the proximal part of chromosome 13 where it forms a green band); (**D**) *Tragelaphus eurycerus*; (**E**) *Taurotragus oryx*; (**F**) *Taurotragus derbianus*; (**G**) *Tragelaphus angasii*; (**H**) *Tragelaphus imberbis*. (**I, J**) Orientation of chromosome 13 towards chromosome Y (blue). Proximal part of chromosome 13 is red and the rest of chromosome is green. (**I**) *Tragelaphus angasii*; (**J**) *Tragelaphus spekei*. Scale bar represents 5 μm.

chromosome 13 is oriented towards chromosome Y in both the acrocentric and submetacentric Y;13 fusion products (Figure 2I,J). Although we cannot unequivocally illustrate this with the probes at hand, the telomere–centromere orientation seems likely given that the pseudoautosomal region is located at the terminal end of the p arm of the submetacentric Y;13 fusion, on short arms of acrocentric type Y;13 chromosome, and at the tip of the p arm in cattle (Di Meo *et al*. 2005). Improved resolution of the orientation of the Y is dependent on more refined data such as those obtained from mapping subchromosomal BACs to this chromosome. However, should this hold, the submetacentric Y;13 fusion observed in *T. spekei*, *T. strepsiceros*, *T. scriptus*, *T. eurycerus*, *T. oryx* and *T. derbianus* can be derived from the acrocentric chromosome referred to above through pericentric inversions, or through transpositions as suggested by Gallagher *et al*. (1999).

X chromosome

Using 17 FISH markers, Iannuzzi *et al*. (2000) identified a series of intrachromosomal rearrangements that distinguish cattle (*Bovinae*) and sheep (*Caprinae*) X-chromosomes. Further work on the bovid X is that of Gallagher *et al*. (1999), who demonstrated that the bovine submetacentric X chromosome found in species of *Bos* and *Bison* is derived from the acrocentric condition by transposition of the centromere, and the detailed work of Chaves *et al*. (2005), which interpreted the variation

in the hybridization patterns of satellite DNA markers in a phylogenetic context.

All species of Tragelaphini included in our study possess an acrocentric X chromosome (which is fused with an autosomal element in *T. spekei* and *T. imberbis*), also referred to as the 'eland acrocentric type' by Robinson *et al.* (1997, 1998). The use of arm-specific probes and probes localized at the distal end of p arm and proximal end of q arm of the cattle X chromosome showed that the parts orthologous to bovine Xp and Xq are in the same position and orientation in all Tragelaphini (Figure 3). Additionally, our data show variation in X chromosome morphology due to (i) heterochromatic addition/deletion (in Bongo, *T. euryceros*) and (ii) an X;BTA 13 autosomal translocation in *T. spekei* and *T. imberbis* (i.e., XY1Y2 sex chromosome system). This rearrangement is the reciprocal of the Y;13 fusion discussed above, and results in even diploid numbers for the sexes in these species. A large intercalary heterochromatic block (IHB) is present between chromosomes X and 13 in these species (i.e., *T. spekei* and *T. imberbis*; Figure 4A,B), which, although by no means a universal finding (Veyrunes *et al.* 2004), has been noted for several other mammals (Viegas-Péquignot *et al.* 1982, Ratomponirina *et al.* 1986, Pack *et al.* 1993, Dobigny *et al.* 2002, Veyrunes *et al.* 2004 and Deuve *et al.* 2006 in rodents; Vassart *et al.* 1995, Yang *et al.* 1997 in Artiodactyls; Fredga 1972 in Carnivores; Tucker 1986 in Chiroptera), where it is thought to prevent the spread of X inactivation to the translocated autosome (reviewed by Dobigny *et al.* 2004b). It is thought that without this heterochromatic barrier complications with replication timing and X inactivation would negatively affect the establishment of

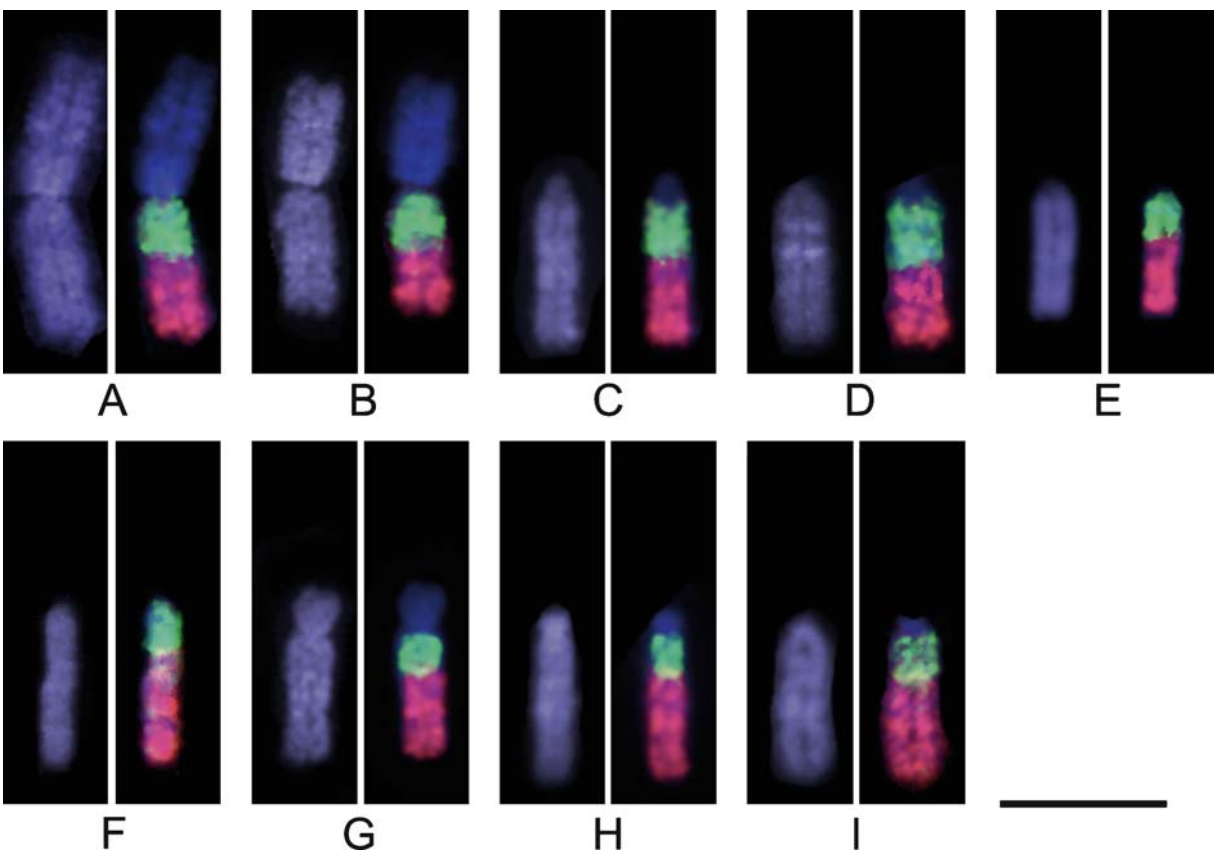

Figure 3. X chromosomes of Tragelaphini labelled by BTA X arm-specific probes: Xp (green), Xq (red) and DAPI (blue). (**A**) *Tragelaphus spekei*; (**B**) *Tragelaphus imberbis*; (**C**) *Tragelaphus angasii*; (**D**) *Tragelaphus strepsiceros*; (**E**) *Tragelaphus scriptus*; (**F, G**) *Tragelaphus eurycerus*; (**H**) *Taurotragus oryx*; (**I**) *Taurotragus derbianus*. Scale bar represents 5 μm.

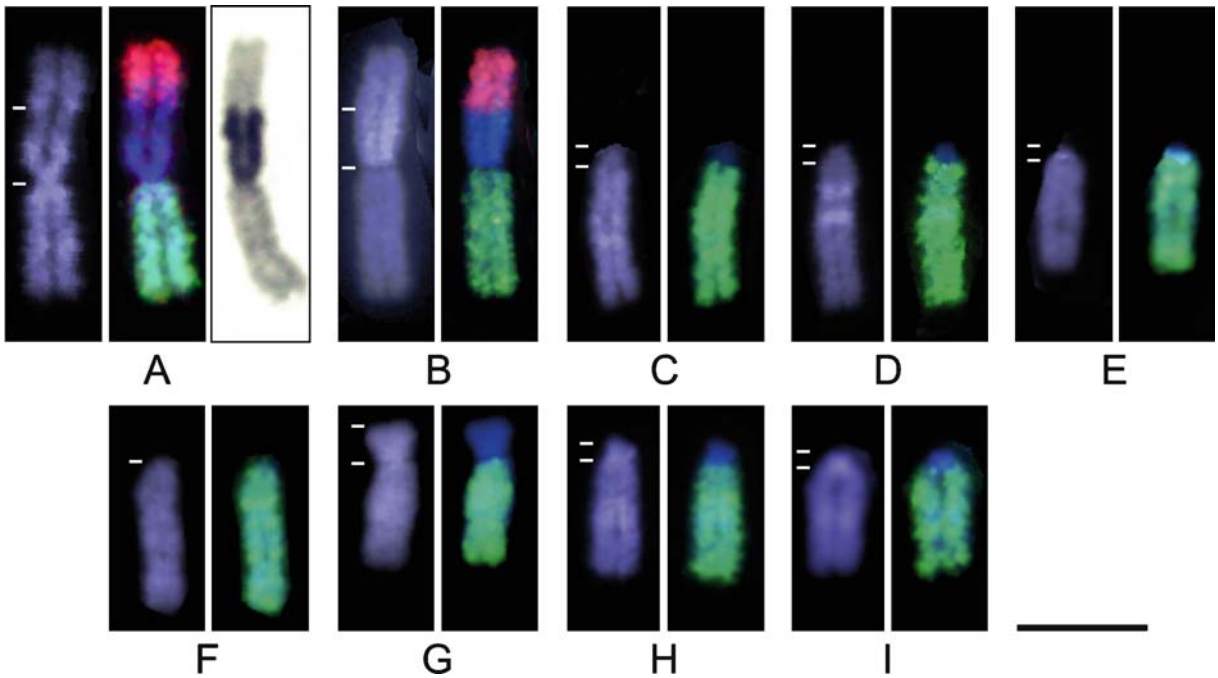

Figure 4. X chromosomes of Tragelapinae. X chromosome (green) and the translocated BTA 13 (red). The DAPI images are shown to the left in each instance. (**A**) *Tragelaphus spekei* (C-banding is on the right); (**B**) *Tragelaphus imberbis*; (**C**) *Tragelaphus angasii*; (**D**) *Tragelaphus strepsiceros*; (**E**) *Tragelaphus scriptus*; (**F, G**) *Tragelaphus eurycerus*: (**H**) *Taurotragus oryx*: (**I**) *Taurotragus derbianus*. The dashed white lines indicate heterochromatic blocks. Scale bar represents 5 μm.

this type of rearrangement in the evolutionary process (Ashley 2002), and where it is absent, another type of repeat may fulfil this role.

Benirschke *et al*. (1982) described two types of X chromosomes in Bongo (*T. eurycerus*): one that is acrocentric and the other submetacentric. The three Bongo specimens analysed by us similarly exhibited two morphologically different X chromosomes: one specimen (a male) possessed an X with acrocentric morphology, and the two remaining specimens (both female) possessed submetacentric X chromosomes (Figure 4F,G). In this species, however, the variation in morphology is due to heterochromatic addition/deletion. The entire p arm of the submetacentric X chromosome is C-band-positive (Figure 5A), a situation lacking in the acrocentric morph (Figure 5B).

Heterochromatic variation

Hybridization with a heterochromatin-specific probe resulted in strong fluorescent signal at centromeric regions of all acrocentric autosomes. Interestingly, the unfused BTA 13 (X2 in the X1X2Y sex autosome translocation discussed below) has a large heterochromatin block at centromeric region (see also Adega *et al*. 2006), which is absent in the chromosome Y;13 (Figure 5C). In contrast, the bi-armed autosomes of *T. strepsiceros* and *T. imberbis*, *T. spekei* and *T. eurycerus* show weak hybridization. However, this is not the situation in *T. scriptus*, *T. derbianus* and *T. oryx*, where several bi-armed chromosomes show strong fluorescent signal. Loss of centromeric heretochromatin often occurs in conjunction with the translocation process. Interestingly, a reduction of the heterochromatin is considered to reflect the age of the fusion event—the more recent, the more heterochromatin is present (Buckland & Evans 1978, Iannuzzi *et al*. 1987, Chaves *et al*. 2000, Di Meo *et al*. 2006). This is borne out by our data, since the fusions that show strong hybridization (e.g.17;27, 21;23, 8;24 and 18;19) are all in the recently derived species (see below, Figure 6). In this regard it is noteworthy that earlier work by Chaves *et al*. (2005) and Adega *et al*. (2006) using various satellite DNAI families (1.709, 1.714 and 1.715) as phylogenetic markers has similarly shown

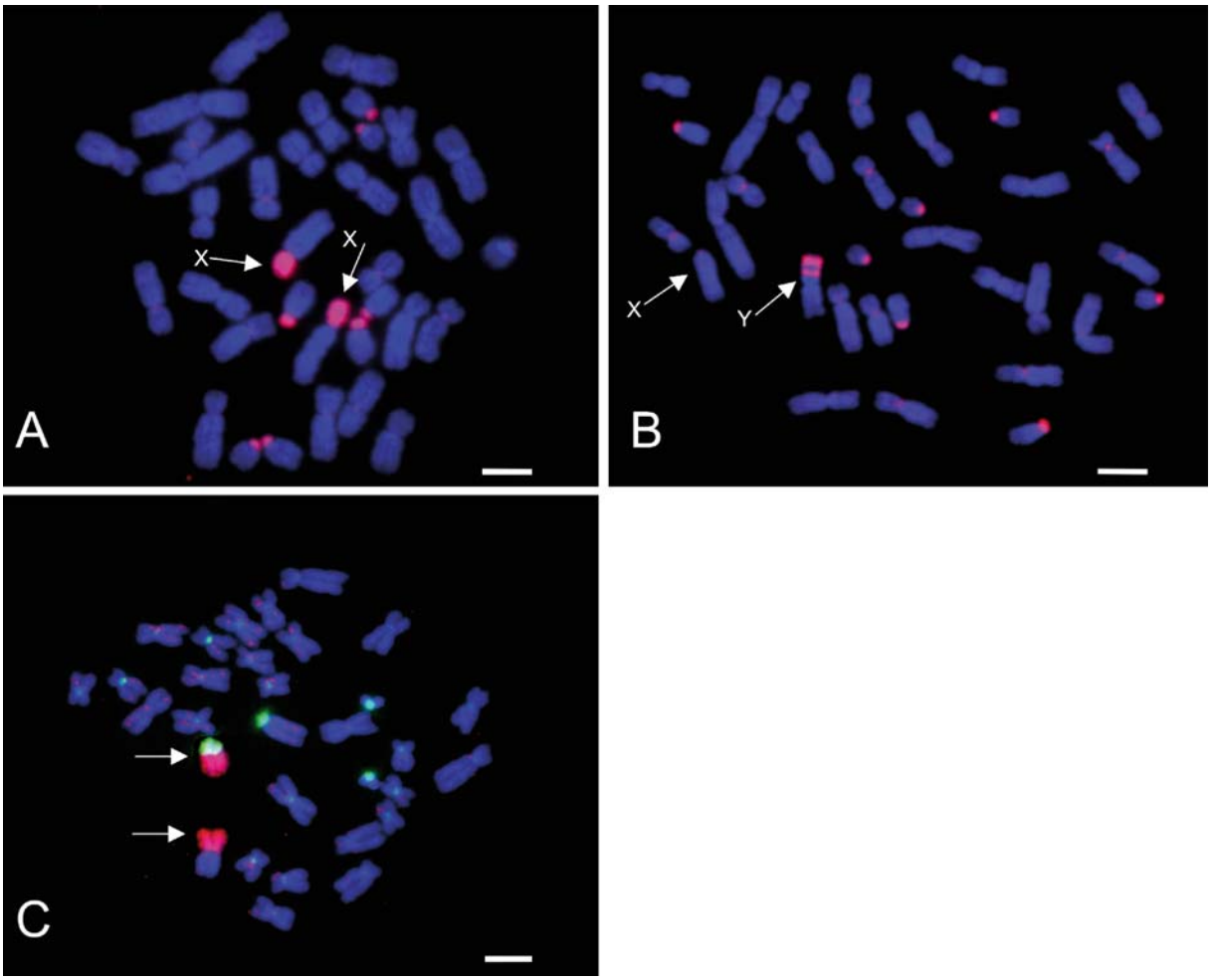

Figure 5. (**A, B**) Chromosomes of Bongo labelled by a heterochromatin specific probe (red) showing variation in the morphology of the X chromosomes. (**A**) Female (2n = 34) with two submetacentric X chromosomes (arrows) showing that the entire Xp is heterochromatic in this morph. (**B**) Male (2n = 33) with an acrocentric X chromosome lacking pronounced C-band positive short arm. The Y portion of the submetacentric Y;13 chromosome translocation has two distinct heterochromatic bands on the Y. The sex chromosomes are arrowed. (**C**) Chromosomes of Greater kudu male (2n = 33) showing deletion of heterochromatin in the fused chromosome 13 in comparison with its unfused homologue. Painting probe for the BTA 13 is red and the heterochromatin specific probe is green. Scale bar represents 5 μm.

the usefulness of heterochromatic variation as markers in determining phylogenetic relationships within Bovidae.

Parsimony analysis

Our chromosomal binary matrix comprised 59 characters of which 18 are parsimony informative. An exhaustive search of 135 135 possible topologies resulted in two equally parsimonious trees of 65 steps (CI = 0.908; RI = 0.793). The 50% majority rule consensus tree is shown in Figure 6. In this data set, one of the rob fusions (e.g., 14;26, Table 1) is best explained as having transcended successive speciation nodes in a polymorphic state (i.e., hemiplasic, see Avise & Robinson 2008), while the X/13 translocation is probably homoplasic, given the peculiarities of this type of rearrangement. The same tree was recovered when the four tandem fusions (corresponding to BTA 11;22;2, 24;22;2, 3;22;2 and 15;22) were weighted at 2:1 on the grounds that these are intuitively more underdominant than rob fusions which are common within the Bovidae. The data confirms the Tragelaphini as a monophyletic lineage, underpinned in our study by the Y;13 translocation.

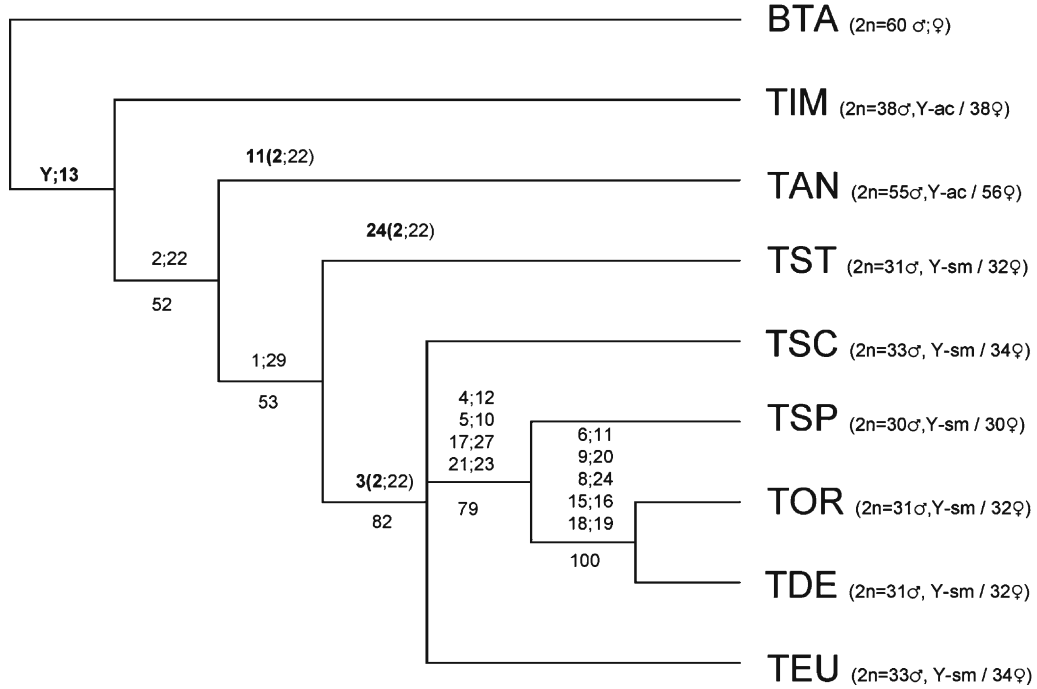

Figure 6. Phylogenetic reconstruction of the Tragelaphini based on the majority rule consensus of two unweighted equally parsimonious trees. Greater kudu (*T. strepsiceros*, *TST*), Lesser kudu (*T. imberbis*, *TIM*), Nyala (*T. angasii*, *TAN*), Sitatunga (*T. spekei*, *TSP*), Bushbuck (*T. scriptus*, *TSC*), Bongo (*T. eurycerus*, *TEU*), (*Taurotragus oryx*, *TOR*) and Derby eland (*T. derbianus*, *TDE*). Diploid numbers (2n) are shown for each species as is the morphology of the Y/autosome translocation (ac = acrocentric, sm = submetacentric). Synapomorphies are shown above branches and percentage bootstrap values below branches. Tandem fusions (centromere:telomere) are presented in bold.

Tragelaphus imberbis and *T. angasi* were, respectively, the most basal taxa within this assemblage. Both the monophyly of the Tragelaphini and the basal placement of *T. imberbis and T. angasi* were similarly recovered in the combined analysis of mtDNA and nuclear sequences by Willows-Munro *et al*. (2005). Additionally, our data show strong bootstrap support (82%) for a monophyletic *T. scriptus, T. spekei* + (*T. oryx* + *T. derbianus*) + *T. eurycerus* that is supported by the BTA 3;2;22 fusion (i.e. two characters in our matrix, see Table S1 in Supplementary Material). Interestingly, this clade includes the so-called 'Closed forest' group of species, with the exception of *T. buxtoni* (which was not available to us), thus mimicking the results of the sequence analysis. Importantly, however, *T. oryx* and *T derbianus* are shown as a recently derived arid group within this clade, suggesting that the ability to survive in extreme arid habitats is a recently derived trait. The ecological scenario that could be invoked to describe the evolutionary relationships suggested by the FISH data is that there was a basal divergence between the bushland specialists *T. angasi* and *T. imberbis* (dated at approximately 10.89 Mya on sequences, Willows-Munro *et al*. 2005). This was followed by the rapid cladogenesis of species confined to moist forest environments and the recent arid adaptation of *T. oryx* and *T. derbianus*, which is in marked contrast to their placement as basal arid specialists in the sequence-based tree.

Although the node distinguishing *T. strepsiceros* in the parsimony analysis is not supported by bootstrapping (53%), it is noteworthy for defining a group of species all of whom share the BTA 1;29 fusion (*T. strepsiceros*, *T. scriptus*, *T. spekei*, *T. eurycerus*, *T. oryx*, and *T. derbianus*,). The translocation rob(1;29) is the most frequently studied translocation in domestic cattle because of it is global presence in different cattle breeds (Popescu 1996) and its association with reduced fertility in bulls (Schmutz *et al*. 1996, Dyrendahl & Gustavsson 1997) and cows (Maurer & Vogt 1988, Schmutz *et al*. 1991). Consequently, what appears to be a shared rearrangement that on one hand defines close evolutionary relationships among several bovid species, on the other manifests as a veterinary clinical disorder with

reproductive impairment. As interesting as this juxtaposition appears, whether the breakpoints are truly equivalent remains moot since the different outcomes could reflect, for example, (i) differences in exact breakpoint location given that fragile regions and segmental duplications span vast stretches of genomic sequence, or (ii) that there is differential rescue of derivative chromosomes by the meiotic apparatus (Froenicke & Lyons 2008).

In conclusion, we have shown that cross-species chromosome painting provides a novel approach for determining phylogenetic affinities within the African spiral-horned antelope. Our data provide support for the monophyly of Tragelaphini and for the grouping *T. scriptus*, *T. spekei* and *T. euryceros*. However, in contrast to a basal placement of the arid-adapted *T. oryx* and *T. derianus* in the molecular studies (Matthee & Robinson 1999; Willows-Munro *et al.* 2005), the chromosomal phylogeny suggests rather that this is a recent adaptation within the clade. The character defining the monophyly of the spiral-horned antelope (a Y:autosome 13 translocation) results in an imbalance in the diploid numbers between sexes, the exceptions being the Lesser kudu (*T. imberbis*) and the Sitatunga (*T. spekei*) in which the reciprocal X;13 translocation is present. This rearrangement (X;13) in two distinct, but distant branches of the phylogenetic tree, is probably homoplasic, suggesting independent origins in the two lineages. Finally, our data show that the rob(1;29) fusion, which impacts on the fertility of cattle (Bonnet-Garnier *et al.* 2008), unexpectedly occurs as an evolutionary marker in most of the tragelapine species investigated. While this could be construed as providing some basis for a role in reproductive isolation and hence speciation, a detailed molecular analysis might reveal additional rearrangement (or different breakpoints) in cattle, clearly prompting further inquiry into what has contributed to the different outcomes among these lineages.

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