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Phylogenetic position of the saola *(Pseudoryx nghetinhensis)* inferred from cytogenetic analysis of eleven species of Bovidae

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Abstract. Previous morphological and molecular analyses failed to resolve the phylogenetic position of the critically endangered saola (*Pseudoryx nghetinhensis*) with respect to its placement in Bovina (cattle, bison, and yak) or Bubalina (Asian and African buffaloes). In the present study, G- and C-banding, Ag-staining and FISH with 28S and telomeric probes was undertaken for 17 bovid species. An analysis of these data allowed us to identify 49 structural rearrangements that included autosomes, gonosomes and 17 different NOR sites. The combined data set was subjected to a cladistic analysis aimed at: (i) providing new insights on phylogenetic relationships of the saola and other species within the subfamily Bovinae, and (ii) testing the suitability of different classes of chromosomal characters for phylogenetic reconstruction of the family Bovidae. The study revealed that nucleolar organizing regions (NORs) are phylogenetically informative. It was shown that at least one, or sometimes two of these characters punctuate divergences that include nodes that are the most basal in the tree, to those that are the most recent. In this context, the shared presence of three NORs in saola and species of *Syncerus* and *Bubalus* strongly suggests the saola's placement within the subtribe Bubalina. This contrasts with Robertsonian rearrangements which are informative only at the generic level. These findings suggest that NORs are an important and frequently overlooked source of additional phylogenetic information within the Bovidae that may also have applicability at higher taxonomic levels, possibly even for Pecora.

The saola (*Pseudoryx nghetinhensis*), until recently unknown to science (Dung et al., 1993), joins several other bovid species as being regarded as critically endangered by IUCN (2007). Estimates of its population size vary from several hundreds within the restricted habitat in the north of central Vietnam (Dung et al., 1993) to upward of 1500 individuals, of which 70–700 are distributed in Laos (Mallon and Kingswood, 2001). The most recent estimates suggest that less than 250 mature individuals survive in the wild (IUCN, 2007).

The unusual combination of 'caprine' and 'bovine' morphological characters make it difficult to determine the taxonomic position of saola with certainty, and two opposing morphological hypotheses on its phylogenetic affinities have been proposed. The anatomical study (cranial and dental characteristics) of Thomas (1994) suggested that the saola is attributable to the Antilopinae, and more precisely to the tribe Caprini sensu lato which includes species closely related to goat and sheep (Ropiquet and Hassanin, 2005).

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Other morphological studies, however, support its placement within the Bovinae (cattle, buffaloes, spiral-horned antelopes, nilgai, etc.) (Schaller and Rabinowitz, 1995; Robichaud, 1998).

In contrast, mitochondrial cytochrome *b* sequences allied the saola with Bovinae and a provisional placement in the tribe Boselaphini (the latter represented by the nilgai *Boselaphus tragocamelus*) (Dung et al., 1993). This initial finding was further refined by the phylogenetic analyses of combined mitochondrial and nuclear sequences which similarly place this enigmatic species within the subfamily Bovinae, but, importantly, within the tribe Bovini (Hassanin and Douzery, 1999b; Gatesy and Arctander, 2000; Hassanin and Ropiquet, 2004). Hassanin and Douzery (1999b) have defined three subtribes within Bovini: (i) Bovina which incorporates species of the genera *Bos* and *Bison*; (ii) Bubalina which contains species of the genera *Bubalus* and *Syncerus*; and (iii) the subtribe Pseudoryina, represented by *P. nghetinhensis*. The position of *Pseudoryx* with respect to Bovina and Bubalina, however, remains unresolved (Hassanin and Douzery, 1999b; Hassanin and Ropiquet, 2004).

In some mammalian taxa, particularly rodents, comparative chromosome banding studies have been extremely helpful in clarifying the systematic position of species which do not display clear affinities when assessed by morphological or/and molecular genetic characters (Taylor, 2000; Volobouev et al., 2002a, b, 2007; Dobigny et al., 2003a). In the case of the Bovidae, however, in spite of the large interspecific variation in diploid numbers and chromosome morphology, chromosome banding has convincingly shown that this variation is largely attributable to Robertsonian (Rb) rearrangements (Wurster and Benirschke, 1968; Buckland and Evans, 1978; Gallagher and Womack, 1992) most of which are species-specific (i.e. autapomorphies), and this, combined with a scarcity of the other types of structural changes, limits the usefulness of these data in bovid phylogenetic reconstructions. In contrast, molecular cytogenetic studies of the bovid X chromosome have unambiguously demonstrated the importance of changes involving this chromosome in resolving problematic evolutionary relationships (Ponce de Leon et al., 1996; Robinson et al., 1996, 1997, 1998; Gallagher et al., 1999). Additionally, preliminary data on the chromosomal distribution of NORs among bovids suggested that they too may prove useful in a phylogenetic context (Gallagher et al., 1999).

Cytogenetic information on the saola is limited due to accessibility of live animals (cultured fibroblasts exist from a single female specimen worldwide). The first analysis (Nguyen et al., 2005) provided the G-banded karyotype of the species and the hypothesis that the 2n = 50 chromosomal complement resulted from a series of Rb fusions of *Bos*-like ancestral acrocentric chromosomes. However, interspecies comparisons were not performed. Subsequently, G- and Q-banding for both saola and cattle was undertaken by Ahrens et al. (2005) who provided for the identification of chromosomes involved in Rb translocations using FISH mapping of 32 BAC probes (i.e. one marker locus per chromosome and two for pair 13 and X). No supportive banding data was provided. Therefore, the questions on the extent of whole-arm conservation between saola and other bovid species remained unanswered.

In the present investigation cytogenetic data including G- and C-banding, NOR location and number, and FISH using ribosomal 28S and telomeric probes obtained for saola were compared for 16 bovid species. These were subjected to cladistic analysis with three caprine species, *Capra ibex*, *Rupicapra rupicapra* and *Ovis aries*, as the outgroup. Our findings provide further insights on the phylogenetic position of *Pseudoryx nghetinhensis* within the subfamily Bovinae, and demonstrate the utility of different classes of chromosomal markers in phylogenetic reconstruction within the family Bovidae.

Materials and methods

Samples

The list of the species studied and their main karyotype characteristics are given in Table 1. For saola, skin samples were collected from a young female captured in the Bach Ma National Park, Thua Thien Hue province, Vietnam in 1994. They were dipped in 1.5 M DMSO and 0.2 M sucrose and immediately frozen in liquid nitrogen to be used subsequently for cell culture. The fibroblast cell cultures for other species were available as cryopreserved cells in the collection of the *Muséum National d'Histoire Naturelle* (Paris).

Chromosome preparation and banding techniques

Chromosome preparations were obtained from fibroblast cell cultures following standard protocols. G- and C-banding was carried out as described by Seabright (1971) and Sumner (1972) respectively with minor modifications. NORs were visualized by Ag-staining (Bloom and Goodpasture, 1976). To identify NOR-bearing chromosomes, Ag-staining was followed by Q-banding (Caspersson et al., 1972) of the same metaphase spreads. At least 10 quality metaphases were analyzed for each species.

Nomenclature

Chromosomes of the species studied as well as all chromosomal rearrangements identified were expressed in accordance to the domestic cattle (*Bos taurus*, BTA) standard nomenclature adopted by International System for Chromosome Nomenclature of Domestic Bovids (ISCNDB, 2000).

Fluorescent in situ hybridization

FISH using ribosomal 28S and telomeric probes was performed on saola metaphase chromosomes as described by Gerbault-Serreau et al. (2004). Hybridization results were examined and analyzed using a Zeiss fluorescent microscope and the ISIS software package (Metasystems, Altlussheim, Germany). Metaphases were photographed with a cooled CCD camera system Quantix II (Photometrics, Tucson, AZ).

Cladistic analysis

The cladistic treatment of chromosomal data followed the principles detailed in Dobigny et al. (2004). Chromosomal changes were identified on the basis of comparative analysis of banding patterns between a set of 14 bovine species (the ingroup) and three caprine species, namely *Capra ibex, Rupicapra rupicapra* and *Ovis aries* that were used as outgroup. In addition to structural rearrangements of both autosomes and gonosomes, we added available data on chromosomal location of the NORs identified for most species by both FISH with 28S ribosomal probe and Ag-staining. The chromosome changes and NORs were used as characters and their presence/absence as character

Table 1. Karyotype characterization of the studied species

Species, their common and abbreviated names[a]	Sex	2n[b]	NFa[c]	X[d]	Rearrangements identified[e]
Bos taurus (Cattle), BTA (1)	F	60	58	Sm	–
Bos indicus (Zebu cattle), BIN (1)[f]	M	60	58	Sm	–
Bos frontalis (Gaur), BFR (1)	M	58	58	Sm	t(2;28)
Bos javanicus (Banteng), BJA (1)	F	60	58	Sm	–
Bison bonasus (European bison), BBO (1)	F	60	58	Sm	–
Bison bison (American bison), BBI (1)[f]	M	60	58	Sm	–
Bubalus bubalis (Swamp buffalo), BBU (1)	F	48	56	A	t(5;28;7), t(1;27), t(2;23), t(8;19), t(16;25)
Bubalus depressicornis (Lowland anoa), BDE (1)	M	48	58	A	t(1;27), t(2;23), t(8;19), t(5;28), t(11;20), t(17;25)
Bubalus mindorensis (Tamaraw), BMI (2)[g]	M, F	46	56	A	t(5;28;11), t(2;23), t(8;19), t(4;14), t(16;29)
Syncerus caffer (African buffalo), SCA (1)[h]	M	52	58	A	t(1;13), t(2;3), t(5;20), t(11;29)
Pseudoryx nghetinhensis (Saola), PNG (1)	F	50	58	A	t(1;10), t(8;13), t(6;19), t(4;18), t(11;12), inv12
Taurotragus oryx (Eland), TOR (1)	F	32	56	St	t(3;22;2), t(5;10), t(6;11), t(1;25), t(4;12), t(8;24), t(9;20), t(7;27), t(15;16), t(18;19), t(14;26), t(21;23), t(17;28)
Taurotragus derbianus (Giant eland), TDE (1)	M	31	56	St	t(3;22;2), t(5;10), t(6;11), t(1;25), t(4;12), t(8;24), t rcp (9; 20dis)[i], t(7;27), t(15;16), t(18;19), t rcp (14;20prx;26)[i], t(21;23), t(17;28)
Boselaphus tragocamelus (Nilgai), BTR (4)[j]	M, F	46	56	Cm	t(1;5), t(2;3), t(6;13), t(8;12), t(19;27), t(24;25)
Capra ibex (Alpine ibex), CIB (1)	M	60	58	A	t rcp (9;14)
Rupicapra rupicapra (Chamois), RRU (1)	M	58	58	A	t(1;3), t rcp (9;14)
Ovis aries (Domestic sheep), OAR (1)[f]	M	54	58	A	t(1;3), t(2;8), t(5;11), t rcp (9;14)

[a] The numbers in brackets denote the number of specimens examined.
[b] 2n: diploid number.
[c] Number of autosomal arms.
[d] Sm: submetacentric, A: acrocentric, St: subtelocentric and Cm: compound metacentric chromosome resulting from gonosome – autosome translocation (see text).
[e] Numbered in accordance with cattle standard chromosome nomenclature (ISCNDB 2000).
[f] Gallagher et al. (1999).
[g] Tanaka et al. (2000).
[h] Gallagher and Womack (1992).
[i] See text for explanation.
[j] Gallagher et al. (1998).

state coded '1' or '0' respectively. The missing data on the NORs distribution for *Taurotragus derbianus* was coded as '?'. The data set was analyzed using Maximum Parsimony (MP) in PAUP 3.1.1 (Swofford, 1993). Parsimony analyses were carried out with the following options: heuristic search with unlimited number of trees to be saved, swapping with the tree-bisection-reconnection (TBR) algorithm, and random addition of sequences with 100 replicates in order to increase the chance of finding the most optimal tree(s). We performed successive weighting using the retention index to reweight each character (similar results were obtained with consistency index or rescaled consistency index; data not shown). Both Acctran and Deltran optimizations (accelerated and delayed transformations, respectively) were compared to identify unambiguous apomorphies on the branches. To examine the support for inferred relationships, bootstrap analyses were done using 1000 replicates with the closest stepwise addition option.

Results

Diploid numbers, chromosome morphology and the karyotypes of the species analyzed correspond to earlier descriptions (see Table 1 for reference). The only exceptions to this are *Taurotragus derbianus* and *Pseudoryx nghetinhensis* where our data suggested positional changes in the karyotypes with respect to earlier descriptions (see Tables 1 and 2 for references).

Comparison of autosomal G-banding patterns

Genus *Pseudoryx*. The karyotype of the single female saola analyzed (2n = 50, NFa = 58) comprises five pairs of biarmed and 19 pairs of acrocentric autosomes; the two X chromosomes are the largest acrocentric elements in the set (Fig. 1). All the saola acrocentric chromosomes and the chromosomal arms of its biarmed autosomes (except 5p) correspond closely to their G-band homologues in karyotype of the cattle (see Table 2 for cattle equivalents involved in the various rearrangements). The short arm of the pair 5 appears to be modified by paracentric inversion making band-by-band comparisons difficult.

Genus *Bos*. Domestic cattle (*Bos taurus*) and banteng (*B. javanicus*) have similar karyotypes with 2n = 60 consisting of 29 pairs of acrocentric autosomes. The gaur (*B. frontalis*) has 2n = 58, the result of an Rb translocation involving ancestral chromosomes equivalent to BTA2 and 28. Of these three species only the haploid set of the cattle is presented as part of Fig. 1. All these taxa possess similar submetacentric X chromosomes.

Genus *Bison*. The G-banded karyotype of *B. bonasus* is similar to that of the cattle and consequently not shown herein.

Genus *Bubalus*. The swamp buffalo (*B. bubalis*) and the anoa (*B. depressicornis*) share the same diploid number

Table 2. Comparison of G-banding patterns between 11 species studied

BTA	BFR	BJA	BBO	BBU	BDE	PNG	TOR	TDE	CIB	RRU
1	1	1	1	2q	1q	1q	4q	4q	1	1q
2	2q	2	2	3q	2q	6	1q prx	1q prx	2	2
3	3	3	3	6	7	9	1p	1p	3	1p
4	4	4	4	7	8	4q	5q	5q	4	4
5	5	5	5	1p	4q	7	2q	2q	5	5
6	6	6	6	8	9	3q	3q	3q	6	6
7	7	7	7	1q dis	10	8	8q	8q	7	7
8	8	8	8	4q	3q	2q	6q	6q	8	8
9	9	9	9	9	11	10	7q	7q[a]	9[b]	9[b]
10	10	10	10	10	12	1p	2p	2p	10	10
11	11	11	11	11	5q	5q	3p	3p	11	11
12	12	12	12	12	13	inv 5p	5p	5p	12	12
13	13	13	13	13	14	2p	[c]	[c]	13	13
14	14	14	14	14	15	15	11q	11q[a]	14[b]	14[b]
15	15	15	15	15	16	14	9q	9q	15	15
16	16	16	16	5q	17	12	9p	9p	16	16
17	17	17	17	16	6q	17	13q	13q	17	17
18	18	18	18	17	18	4p	10p	10p	18	18
19	19	19	19	4p	3p	3p	10q	10q	19	19
20	20	20	20	18	5p	11	7p	7p[a]	20	20
21	21	21	21	19	19	16	12q	12q	21	21
22	22	22	22	20	20	18	1q dis	1q dis	22	22
23	23	23	23	3p	2p	19	12p	12p	23	23
24	24	24	24	21	21	22	6p	6p	24	24
25	25	25	25	5p	6p	13	4p	4p	25	25
26	26	26	26	22	22	21	11p	11p[a]	26	26
27	27	27	27	2p	1p	23	8p	8p	27	27
28	2p	28	28	1q prx	4p	20	13p	13p	28	28
29	28	29	29	23	23	24	15	15	29	29

[a] Involved in another reciprocal translocation (see text).
[b] Involved in reciprocal translocation (see text).
[c] Translocated onto Y chromosome (see Fig. 3).

(2n = 48) but differ in NFa, with 56 in the former, and 58 in the latter species. The reduction in diploid number is attributable to five Rb translocations in the swamp buffalo karyotype and six in the anoa, four of which are common to both species: t (1;27), (2;23), (8;19) and (5;28). In *B. bubalis*, one Rb translocation (Rb 5;28) was involved in a tandem translocation with cattle chromosome 7 leading to the reduction of both 2n and NF (Fig. 2, Table 1). The X chromosomes of both species are acrocentric.

Genus *Taurotragus*. The karyotype of the female eland (*T. oryx*) studied corresponds to earlier descriptions (Buckland and Evans, 1978; O'Brien et al., 2006). The sex chromosome system in this species is $X_1X_1X_2X_2/X_1X_2$neoY as a result of the Y-autosome (BTA13) translocation, and thus the females possess an even diploid number whereas the males have one chromosome less. The male of giant eland (*T. derbianus*) analyzed in our study had 2n = 31 and an X_1X_2Y sex chromosomes system and it was also heterozygous for a reciprocal translocation involving pairs 7 (BTA9;20) and 11 (BTA14;26) in the eland karyotype (Fig. 3). Given that *T. oryx* and *T. derbianus* have previously been reported to possess similar karyotypes (O'Brien et al., 2006), it is possible that the giant eland here is a carrier of spontaneous reciprocal translocation. Further studies are needed to clarify the origin of this rearrangement and its frequency in natural populations of *T. derbianus*.

Genera *Capra* and *Rupicapra*. The alpine ibex (*C. ibex*) and chamois (*R. rupicapra*), with 2n = 60 and 58 respectively, differ from each other by one Rb translocation involving cattle equivalents BTA1 and BTA3 (karyotypes not shown). In addition, both are characterized by reciprocal translocations between cattle equivalents 9 and 14, a rearrangement characteristic of numerous non-bovine species. The X chromosomes are acrocentric.

C-banding

Blocks of constitutive heterochromatin were detected in pericentromeric regions of all acrocentric chromosomes in all species studied (Fig. 4). However, these varied in size from pair to pair and from species to species and are thus difficult to quantify. Compared to acrocentrics, the amount of C heterochromatin on the biarmed autosomes and submetacentric X chromosomes in *Bos* and *Bison* is reduced, and in some species totally absent. Comparisons of the C-banding patterns failed to yield clear-cut interspecies differences.

X chromosomes

There are three main types of the X chromosomes present among the species studied (one submetacentric in morphology and two acrocentric types). A submetacentric X is characteristic of all species of the genera *Bos* and *Bison*. Of the two acrocentric X chromosome variants, one type is found in *Capra* and *Rupicapra*, whereas the second is shared by saola, swamp buffalo, anoa and, except minor differences, also by the two eland species (Fig. 5). Data from comparative banding and the integration of molecular cytogenetic studies of the X chromosomes in bovids (Ponce de Leon et al., 1996; Robinson et al., 1996, 1998; Gallagher et al., 1999) allowed us to identify the rearrangements (Fig. 6) that have shaped the morphological diversity of the X chromosome and the sex chromosome systems in modern Bovidae.

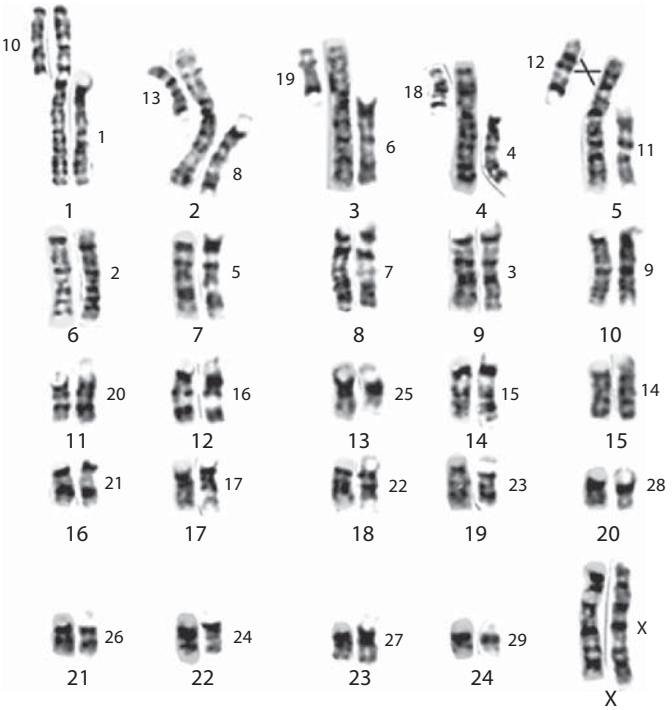

Fig. 1. Comparison of G-banded chromosomes between saola *Pseudoryx nghetinhensis* (numbered below) and cattle *Bos taurus*.

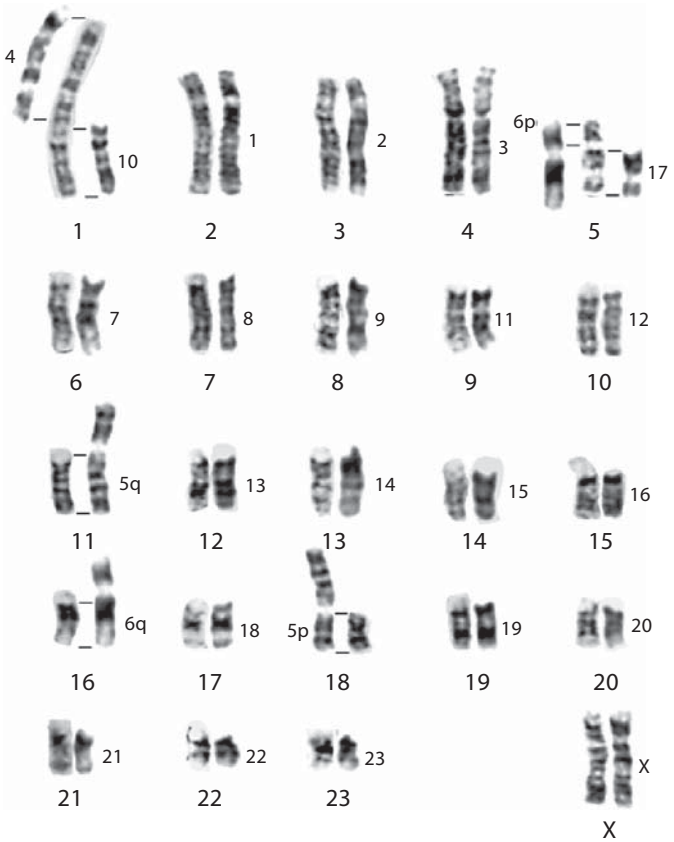

Fig. 2. Comparison of G-banded chromosomes between swamp buffalo *Bubalus bubalis* (numbered below) and lowland anoa *Bubalus depressicornis*.

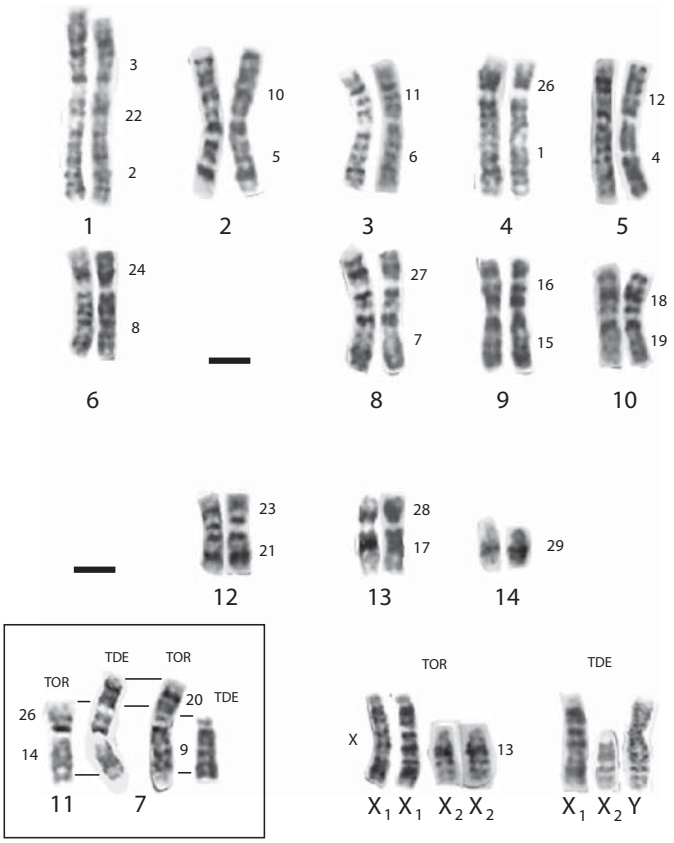

Fig. 3. Comparison of G-banded chromosomes between eland *Taurotragus oryx* (left) and giant eland *Taurotragus derbianus* (right), and their correspondence to the cattle chromosomes. Insert: chromosome pairs 7 and 11 involved in translocation.

4

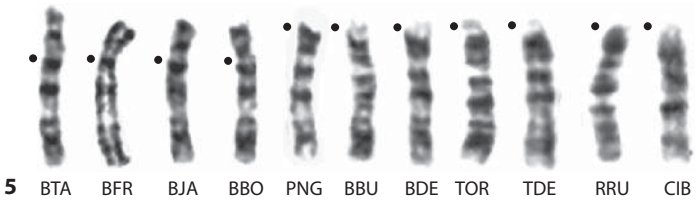

Fig. 5. Three main types of the X chromosome among the species studied. Black dots indicate the centromere position (see text for details).

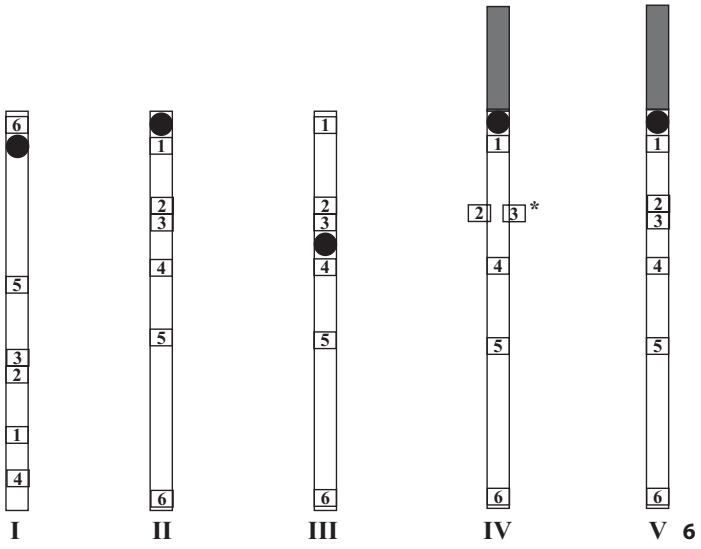

Fig. 6. Schema of the X chromosome transformation in bovids following molecular cytogenetic data by Robinson et al. (1998) and Gallagher et al. (1999). I – Caprini type, II – *Bubalus* type, III – *Bos* type, IV – *Tragelaphus* type and V – *Boselaphus* type. Numbers on the chromosomes show position of the six BACs mapped. I → II – 3 inversions, II → III – centromere transposition, III → IV – gonosomes-BTA13 translocation, III → V – gonosomes-BTA14 translocation. * Two BACs in eland (IV) are co-localized (for details see Gallagher et al., 1999).

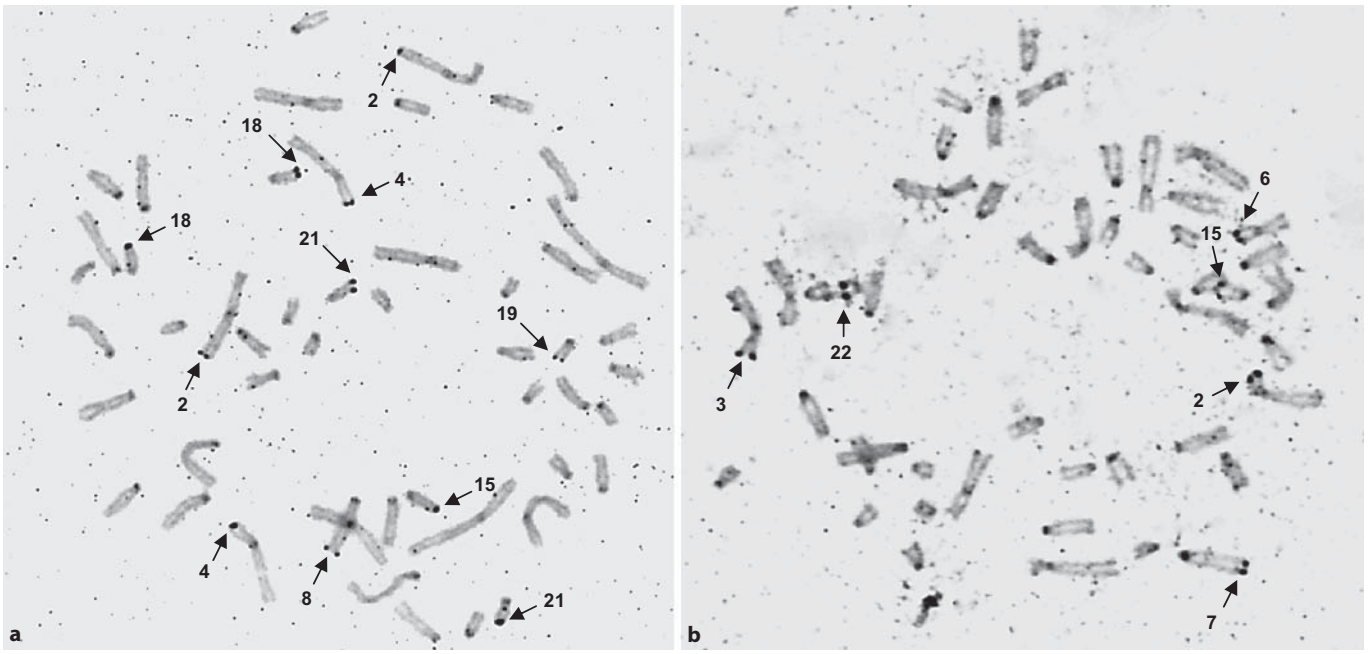

Fig. 7. Silver staining of saola *P. nghetinhensis* (**a**) and anoa *B. depressicornis* (**b**) metaphase spreads. Arrows show the NOR-bearing chromosomes.

NORs

The number and chromosomal location of the NORs was determined for *P. nghetinhensis* and *B. depressicornis* (Fig. 7) since these species were not included in Gallagher et al. (1999). With Ag-staining, *P. nghetinhensis* shows seven NOR bearing chromosome pairs, the highest number recorded in Bovidae. All the NORs are located in telomeric regions and were present on both homologues of pairs 2, 4, 18 and 21 (corresponding to BTA8, 18, 22 and 26), and only one homologue of pairs 8, 15 and 19 (corresponding to BTA7, 14 and 23). NORs are also telomerically located in the anoa being detected on one homologue of six autosomal pairs corresponding to BTA3, 14, 19, 23, 25 and 26 (Fig. 7).

Fig. 4. C-banded karyotypes of (**a**) *Bos taurus*, (**b**) *Bos frontalis*, (**c**) *Bos javanicus*, (**d**) *Bison bonasus*, (**e**) *Bubalus bubalis*, (**f**) *Pseudoryx nghetinhensis*, (**g**) *Bubalus depressicornis*, (**h**) *Taurotragus oryx*, (**i**) *Taurotragus derbianus*, (**j**) *Capra ibex* and (**k**) *Rupicapra rupicapra*.

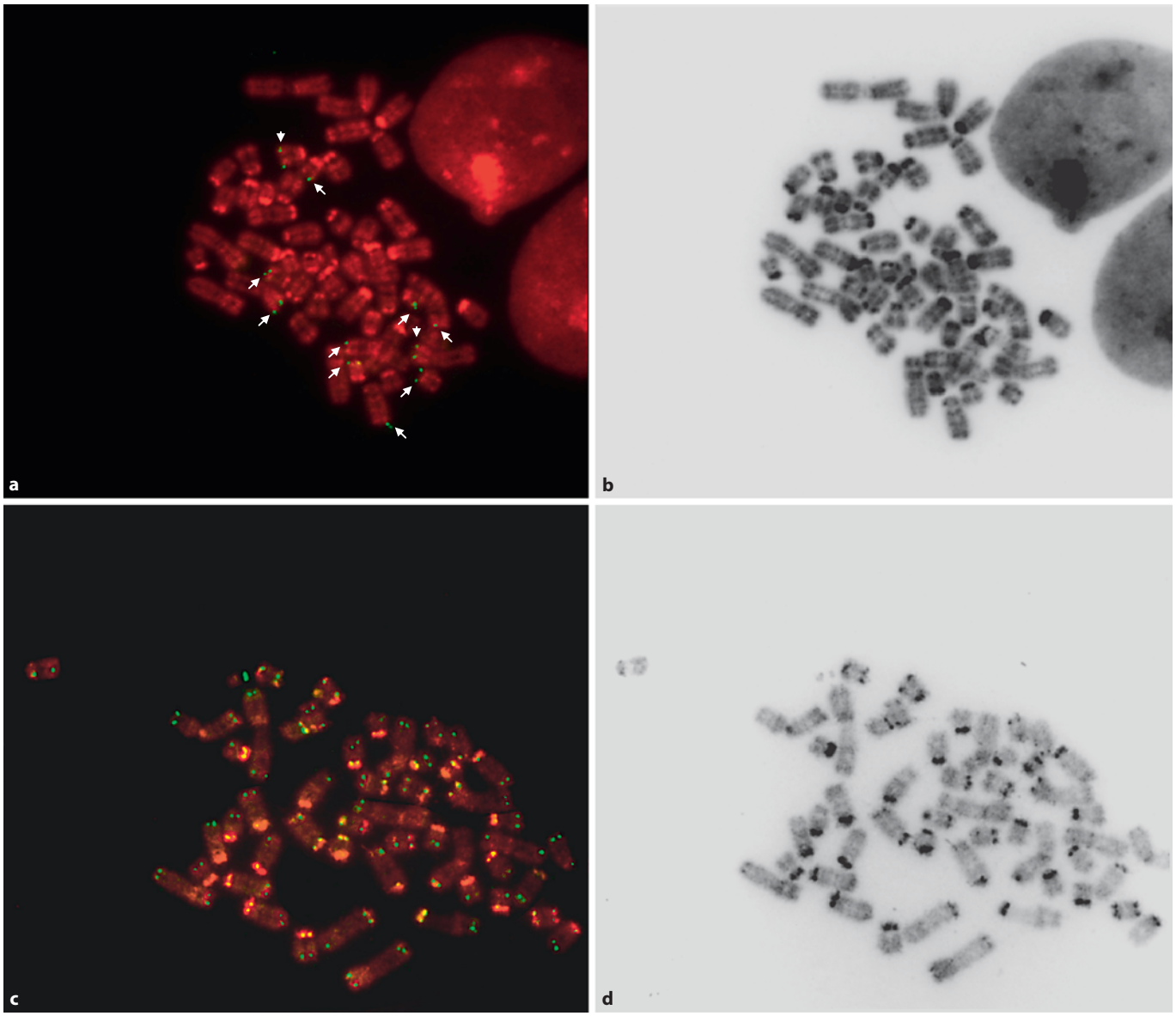

Fig. 8. Representative FISH images of ribosomal 28S (**a**) and telomeric (**c**) probes on saola metaphases. Chromosomes were identified using reverse PI banding (**b** and **d**). Arrows indicate sites of probe hybridization.

FISH analysis

The 28S ribosomal probe revealed the same number and chromosomal location of 28S genes as those detected by silver staining (Fig. 8a, b). This means that saola was heterozygous for the presence/absence of NORs on three autosomal pairs corresponding to BTA7, 14 and 23.

FISH using the $(TTAGGG)_n$ telomeric probe produced signals exclusively at the terminal ends of all chromosomes (Fig. 8c, d). The intensity of hybridization signals was constant and each chromosome generally exhibited four signals confirming the telomeric pattern previously found in most bovid species (Meyne et al., 1990; de la Seña et al., 1995; Tanaka et al., 2000).

Chromosomal phylogeny

We complemented our comparative banding analysis of the 11 bovid species presented here through the inclusion of published information from six additional taxa: *Bos indicus*, *Bison bison*, *Boselaphus tragocamelus*, *Syncerus caffer*, *Bubalus mindorensis* and *Ovis aries* (see Table 1 for references). Using three caprine species as outgroup, we identified all putative euchromatic autosomal homologies and thus all structural rearrangements that occurred during the evolution of the ingroup species. These comprise 39 Robertsonian rearrangements, three tandem and two reciprocal translocations and one paracentric inversion (Table 1). Additionally, we included four rearrangements of the sex chromosomes and 17 additional characters based on chro-

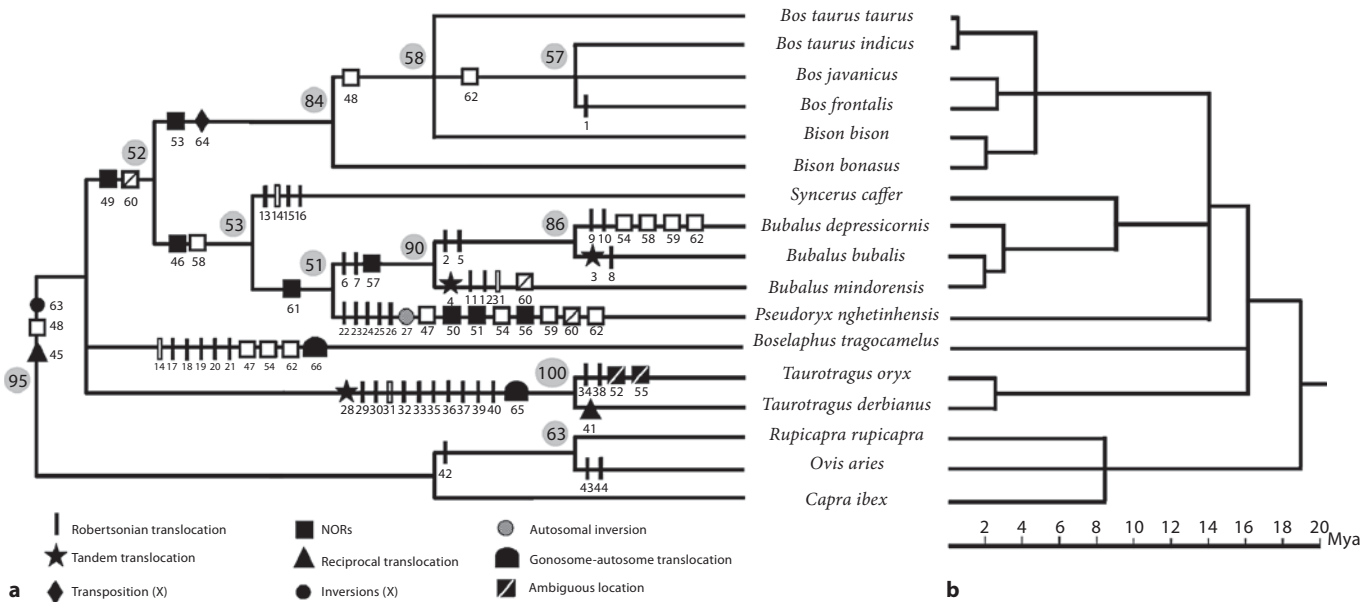

Fig. 9. Phylogenetic relationships between 17 species of Bovidae based on chromosomal (**a**) and molecular (**b**) data. The tree on the left (**a**) is the strict consensus tree of three equally parsimonious trees of 79 steps (consistency index = 0.892), obtained from the treatment of the chromosomal characters matrix (66 characters) by maximum parsimony (MP). The symbols on the branches represent chromosomal rearrangements and distribution of NORs. The numbers below the symbols correspond to chromosomal character numbers (see Appendix), and the empty symbols correspond to homoplasic characters. The numbers in grey circles are % bootstrap values (1000 replicates). The tree on the right (**b**) is obtained from the analyses of complete mitochondrial cytochrome *b* (1143 bp), 12S rRNA (956 bp) genes, non-coding regions from the nuclear genes for aromatase cytochrome P-450 (199 bp) and lactoferrin (338 bp) (Hassanin and Douzery, 1999b), and from sequences of the promoter of the lactoferrin (*Lf*), and two mitochondrial genes, i.e., the cytochrome *b* and the subunit II of cytochrome *c* oxidase (*CO2*) (Hassanin and Ropiquet, 2004) using Bayesian and MP methods. The data for *Bubalus* species are from Tanaka et al. (1996). The molecular time scale presented is calculated according to Hassanin and Ropiquet (2004).

mosomal distribution of the NORs (see Table 3 for references).

The treatment of the chromosomal character matrix (see Appendix) by the MP analysis resulted in three equally parsimonious trees of 79 steps (CI = 0.892, RI = 0.883). We performed successive weighting using the retention index to reweight each character and obtained the single tree presented in Fig. 9a. We retrieved the same topology when using consistency index or rescaled consistency index for successive weighting. The bootstrap (BP) analysis showed the presence of five robust nodes: (1) the separation between Bovinae and Caprini (BP = 95%); (2) the grouping of *Bos* and *Bison* species (subtribe Bovina, BP = 84%); (3) the monophyly of *Bubalus* (BP = 90%); (4) the sister-group relationships between *B. bubalis* and *B. depressicornis* (BP = 86%); and (5) the monophyly of *Taurotragus* (BP = 100%). All other nodes are supported by BP values <70%. We examined distribution of the parsimony-informative characters category by category to gain a further insight on their relevance to the tree topology, as well as to the cladogenetic process.

Of 66 chromosomal characters analyzed, 34 are phylogenetically informative. These comprise 16 Rb rearrangements, 12 NORs, one tandem and one reciprocal translocation involving autosomes, and four rearrangements of the sex chromosomes. Among 16 Rb rearrangements, 10 were shared by two species of *Taurotragus* (characters 29 to 33, 35 to 37, 39 and 40), four by three species of *Bubalus* (characters 2, 5, 6 and 7), one by *Boselaphus* and *Syncerus* (character 14) and one by *Taurotragus* and *Bubalus mindorensis* (character 31). When mapped to the tree 12.5% of the Rb rearrangements are homoplasic characters.

The analysis of the NORs, the second largest category of characters (n = 17) used, showed that 12 (70.6%) are phylogenetically informative, some of which display remarkable stability being preserved in many taxa since the Bovidae/Cervidae split (characters 47 and 48) (see Gallagher et al., 1999 for data on Cervidae) or the Bovinae/Antilopinae split (character 46). Others mark more recent events (characters 53 and 58) and sometimes are characteristic of individual species thus being autapomorphies for these lineages (50, 51, 52, 55 and 56). Although the loss of ancient NORs and the emergence of new chromosomal sites bearing NORs often occur in concert (i.e., chars. 46, 48, 53, 58, 61), their occurrence in the taxa belonging to unrelated lineages (i.e., chars. 54 and 62) was frequently found. As a consequence, NOR characters exhibit higher levels of homoplasy than do Rb translocations (53.8% vs. 12.5%). In contrast to autosomal characters, all changes of the X chromosome appeared to be strong phylogenetic signals. No instances of homoplasy were detected.

Table 3. Chromosome location of the NORs in the species of the families Bovidae and Cervidae

Species	NOR-bearing chromosomes[a]																References
Bovidae																	
Bos taurus	2	3	4				11						25		28		Gallagher et al. 1999
Bos frontalis	2	3	4				11						25				Ibid
Bos javanicus	2	3	4				11						25				Ibid
Bos indicus	2	3	4				11						25				Ibid
Bison bison	2	3	4				11						25		28		Ibid
Bison bonasus	2	3					11						25		28		Ibid
Syncerus caffer		3									22		25		28		Ibid
Bubalus bubalis		3								19	22		25	26	28		Ibid
Bubalus depressicornis		3						14		19		23	25	26			This study
Bubalus mindorensis		3								19	22			26	28		Tanaka et al. 2000
Pseudoryx nghetinhensis					7	8		14		18		22	23		26		This study
Boselaphus tragocamelus	2			5				14									Gallagher et al. 1998, 1999
Tautrotragus oryx	2	3		5			10		16						28		Gallagher et al. 1999
Ovis aries	2	3	4	5											28		Ibid
Capra ibex	2	3	4	5											28		Mayr et al. 1987
Rupicapra rupicapra	2	3	4	5											28		Ibid
Cervidae																	
Cervus nippon		3	4														Gallagher et al. 1999
Odocoileus virginianus		3	4														Gallagher et al. 1999

[a] Numbered in accordance to cattle standard chromosome nomenclature (ISCNDB 2000).

Discussion

Chromosomal changes in evolution of the family Bovidae

Comparative banding analysis involving 17 bovid taxa revealed extensive inter-species monobrachial homologies, a conclusion reached in earlier studies (Buckland and Evans, 1978; Gallagher and Womack, 1992; Gallagher et al., 1999). Indeed, except for some rare chromosomal changes (see below), the karyotypes of all bovine species studied may be easily derived from that of a *Bos*-like ancestor by means of numerous Rb and fewer tandem translocations (36 vs. 3), i.e., rearrangements that have not disrupted ancient synteny. Among Robertsonian rearrangements, shared Rb combinations may occur in closely related as well as phylogenetically distant species. Thus, in the absence of rigorous cladistic analysis it is difficult to decide which of these characters are synapomorphic and which are homoplasic (i.e., Rb (2;3) shared by *Syncerus* and *Boselaphus*, or Rb (1;25) which is shared by two species of eland and the tamaraw). The Rb (1;10) translocation which appears as a derived character in the saola has also been identified in two species of gazelles (*G. dorcas* and *G. gazella*, tribe Antilopini) (Vassart et al., 1995), and two species of *Damaliscus*, *D. lunatus* and *D. pygargus*, tribe Alcelaphini (Kumamoto et al., 1996). Yet more confusing is the shared presence of the Rb (1;25) in tamaraw (Bovini), eland (Tragelaphini), gazelle (Antilopini) and goral (Caprini). We conclude that Rb rearrangements may be useful phylogenetic characters but, in many cases, they may be homoplasic and thus their contribution to defining cladogenetic events in the family Bovidae may be rather modest. By not taking their rather high independent occurrence into account, these chromosomal changes can lead to misinterpretation of phylogenetic relationships in the absence of appropriate analysis, as was previously stated by Robinson et al. (1997).

Tandem translocations are well known for their severe effect on hybrid fertility and viability which explains the rarity of this chromosomal mutation in karyotype evolution of mammals (King, 1993). However, as soon as this rearrangement is fixed in a population it may lead to rapid reproductive isolation (Taylor, 2000). In our study one tandem translocation (BTA3;22;2) was identified in two species of eland and one (BTA7;5;28) in the swamp buffalo (*Bubalus bubalis*). It is noteworthy that chromosome 1 of the tamaraw (*B. mindorensis*) results from a tandem translocation involving Rb chromosome (BTA5;28; Tanaka et al., 2000) which is similarly present in the swamp buffalo, but which is fused with BTA11 in the former and BTA7 in the latter species. However, Tanaka et al.'s interpretation is likely erroneous since the same chromosome was matched twice – once as chromosome R6, and then as chromosome R12 of river buffalo; in the latter case it was presumably homologous to the tamaraw's chromosome 1q (Fig. 3 in Tanaka et al., 2000). Similar errors occur elsewhere in their comparison and it seems that this disagreement results from a partial lack of correspondence in chromosome numbering using G- and R-banded chromosome nomenclature, and the

mismatching of at least two chromosomal pairs in the Tanaka et al. (2000) publication. Should this hold, the tandem translocation (BTA7;5;28) unites *Bubalus bubalis* and *B. mindorensis* rather than *Bubalus bubalis* with *B. depressicornis* (as presented in Fig. 9a), a finding which is consistent with the molecular phylogenetic analysis (Fig. 9b).

Despite an elevated occurrence of de novo autosomal reciprocal translocation in man (Rousseaux et al., 1995) and domestic animals (Pinton et al., 1998), usually associated with the production of aneuploid gametes and sometimes leading to full sterility in mammals, this kind of rearrangement is rarely detected in wild mammalian species (King, 1993; Searle, 1993 and references therein). One of the few well documented cases involves *Arvicanthis*. These rodent species show de novo autosomal reciprocal translocation at the node marking the divergence of the two main evolutionary lineages (Volobouev et al., 2002a). Similarly, the reciprocal translocation (BTA9;14) appears to be an important event in the Bovidae where, together with the changes in X chromosome morphology, it marks the basal split between the subfamilies Bovinae and Antilopinae (species within these groups are characterized by 'bovine' and 'caprine' X chromosomes, and the presence/absence of the BTA9;14 reciprocal translocation) (Buckland and Evans, 1978; Gallagher and Womack, 1992; Gallagher et al., 1999). Furthermore, the detection of a paracentric inversion in the saola is noteworthy as this type of rearrangement has not previously been detected in Bovidae. This finding needs to be confirmed by analysis of more animals to exclude the possibility of mutation occurrence during cell culturing.

In contrast to the remarkable conservation of autosomal banding patterns, three structural variants of the X chromosome were detected among bovids studied supporting previous findings (Buckland and Evans, 1978; Robinson et al., 1997, 1998; Gallagher et al., 1999). Importantly, however, several bovid lineages show compound sex chromosomes resulting from gonosome-autosome translocations (see O'Brien et al., 2006 for references) as typified by the X_1X_2Y eland system which is similarly shared by bongo *Tragelaphus eurycerus* and lowland nyala *T. angasii*. After translocation of the same autosome onto X, the sex chromosome system evolved into neoXneoX/neoXneoY system as such found in the lesser kudu (all three species from the tribe Tragelaphini) (O'Brien et al., 2006) as well as in the nilgai *Boselaphus tragocamelus* of the tribe Boselaphini, although in this case another autosome (BTA14) is involved in translocation (Gallagher et al., 1998). All modifications of the X chromosome, especially X-autosome translocations, are potentially powerful reproductive isolating mechanisms due to their impact on sex determination and/or on the X-inactivation process (King, 1993).

NORs are a very different category of genetic marker from the structural rearrangements considered above. In brief, the evolutionary dynamics of NOR variation involve changes in rDNA copy number (amplification – deletion) and the chromosomal location of these simple multigene families. In addition to their main function – production of ribosomes – these genes are also involved in other important cellular activities such as regulation of rRNA transcription, formation of microtubule-associated proteins and nucleolar cortical skeletal proteins (Sumner, 1990, 2003 and references therein). The changes in NOR number and/or chromosomal location are not known to directly influence the reproductive performance and viability of their carriers. Most mammalian species possess 1 to 5 chromosome pairs that bear rDNA loci. There are, however, notable exceptions to this. For example, most bat species of the genus *Myotis* possess 1 to 4 NOR bearing chromosomal pairs but this extends to 14 chromosomal pairs in *M. myotis* (Volleth, 1987). Although 1 to 2 of these pairs might be shared by congeneric species, as well as the species from the evolutionarily close *Eptesicus*, *Nyctalus* and *Vespertilio*, no clear phylogenetic inference could be drawn from analysis of NOR chromosomal distribution in the above bat taxa (Volleth, 1987). Likewise, NOR distribution in the gerbils (*Taterillus*, Dobigny et al., 2003b and references therein) was similarly phylogenetically uninformative. In other words, the lack of NOR phylogenetic context spans species whose karyotype evolution is markedly different. Bats are highly conserved and usually the species differ from each other by a few, easily detectable changes of G-banding patterns, whereas the gerbil species show extensive and rapid karyotypic repatterning. In contrast, however, there are examples of surprisingly long-term conservation of NOR chromosomal locations. For example, FISH analysis of several 18S+28S and 5S rRNA genes revealed their retention on homeologous chromosomes or chromosomal segments in genomes of babirusa (Suidae) and collared peccary (Tayassuidae), taxa that diverged at least 35 Myrs ago and which have undergone extensive subsequent karyotype repatterning (Zijlstra et al., 1997; Bosma et al., 2004). Furthermore, two NOR bearing chromosomes found in cervids are shared by many bovid species (Gallagher et al., 1999), families that diverged about 28 Myrs ago (Hassanin and Douzery, 2003).

As it was discussed in detail by Dobigny et al. (2004), the use of NORs to determine phylogenetic relationships should be done with caution. Indeed the inability of Ag-staining to detect transcriptionally non-active rRNA genes and the nonspecific binding of silver nitrate with chromatin (Sumner, 1990; Dobigny et al., 2002) may lead to errors in estimation of NOR number and/or their chromosomal location. Another problem concerning their identification is related to the high variability of rRNA copy numbers within individuals and among species (Sumner, 1990, 2003; Gallagher et al., 1999). This variability can impact on the detection of low-copy rDNA loci due to limited sensitivity of FISH. These data make it clear that in addition to technical limitations, an appropriate sample size is crucial in accurately determining the number of rDNA loci and their chromosomal location within a particular species.

Although Gallagher et al. (1999) were the first to suggest that the chromosomal distribution of NORs among Bovidae may be phylogenetically useful, their data were not subjected to a rigorous phylogenetic analysis. This is supported by our cladistic analysis of the chromosomal distribution of NORs in 16 bovid species. In fact, 70.6% of NORs are phy-

logenetically informative. We show that one to two of these characters mark practically all divergence events from the most basal of nodes to the most recent. This differs markedly from Robertsonian rearrangements which appear to be variable only at a generic level (Fig. 9).

The finding of NOR heterozygosity (i.e. only one chromosome of a pair bears an NOR) on three chromosomal pairs in saola mimics an earlier observation of double heterozygosity in *Bos indicus* (Gallagher et al., 1999). This would seem to suggest that difference in the copy number of 28S genes among bovid karyotypes is not an unusual phenomenon. Given that the NOR data for our study were obtained from single specimen by species (with the exception of nilgai and tamaraw), it is highly probable that at least some NOR sites were overlooked, and this missing data may contribute to the elevated frequency of homoplasic characters. We are thus of the opinion that adequate sampling of specimens may further increase the performance of the NORs in resolving phylogenetic relationships in Bovidae, possibly also at higher taxonomic levels.

The availability of dated divergences within Bovidae (Hassanin and Douzery, 1999a, b) allowed us to place the NOR data in a temporal framework and to highlight why these data are phylogenetically useful in some taxonomic groups, and not in others. In our opinion the data on the chromosomal distribution of NORs can be valuable phylogenetic characters when at least three conditions are met. First, their number per karyotype should not be very low, and their chromosomal location should vary among species. Secondly, NOR variability should show a diversity of evolutionary ages and, finally, the group of taxa under study should be characterized by a low to moderate rate of karyotype reorganization. When these conditions are met, NORs become phylogenetically useful characters comparable to structural rearrangements.

Chromosomal vs. molecular phylogeny

Molecular phylogenetic studies involving concatenations of mitochondrial and/or nuclear genes all provide a strong support for monophyly of the subfamily Bovinae and the recognition of three tribes, the Bovini (cattle and buffaloes), Tragelaphini (African spiralled-horned bovids) and Boselaphini (nilgai and chousingha) (Hassanin and Douzery, 1999a, b; Hassanin and Ropiquet, 2004 and references therein). However, the intertribal relationships remain poorly resolved with the most recent analysis suggesting two alternative hypotheses for these associations, one a sister-group relationship between Bovini and Tragelaphini, and another one favoring an association of Boselaphini and Tragelaphini (Hassanin and Ropiquet, 2004).

In terms of the saola's phylogenetic affinities, all molecular studies agree on its placement within the subfamily Bovinae (Dung et al., 1993; Hassanin and Douzery, 1999b; Gatesy and Arctander, 2000; Hassanin and Ropiquet, 2004). There is no consensus, however, in terms of its tribal affinities with Dung et al. (1993) suggesting a placement in Boselaphini, whereas Hassanin and Douzery (1999b), Gatesy and Arctander (2000) and Hassanin and Ropiquet (2004) find an evolutionary affinity with Bovini. The latter authors define three subtribes within Bovini, (i) Bovina which includes all species of *Bos* and *Bison*, (ii) Bubalina which groups *Bubalus* and *Syncerus,* and (iii) Pseudoryina, which is represented only by the saola, *P. nghetinhensis*. The interrelationships among the Bovini subtribes remain uncertain and only tentatively saola was considered as sharing closer phylogenetic affinities with the subtribe Bovina (Hassanin and Douzery, 1999b; Gatesy and Arctander, 2000; Hassanin and Ropiquet, 2004). The topological conflicts outlined above are summarised on the consensus phylogenetic tree (Fig. 9b).

Despite a rather limited number of phylogenetically informative chromosomal characters identified and relatively weak support of some groupings on the chromosomal tree (Fig. 9a), the cytogenetic data nevertheless allow an evolutionary scenario which, although largely congruent with that inferred from molecular analyses (Fig. 9b), differs in certain important aspects allowing an interpretation that suggests the following sequence of events. After the Antilopinae/Bovinae split, three main lineages emerged within the subfamily Bovinae: one leading to *Boselaphus*, another one to Tragelaphini, and the last to Bovini; the three tribes differ from each other by their particular X:autosome configurations. The ancient type of the bovine X (Fig. 6, II) was modified by a gonosome-autosome translocation involving BTA14 (Fig. 6, V) in *Boselaphus* (unfortunately the sex chromosome constitution of chousingha *Tetracerus quadricornis* remains unstudied), and BTA13 in the species of the tribe Tragelaphini (Fig. 6, IV). Since the establishment of these potentially negatively heterotic rearrangements, chromosomal evolution in these lineages occurred independently. The chromosomal data suggest that the first lineages to diverge were *Boselaphus* and the Tragelaphini since both have NORs on BTA5 (character 49) which is shared with the more distant Caprini, but which is lost in all other in-group taxa. This divergence also predated the emergence of the tribe Bovini with its two monophyletic clades, one corresponding to the subtribe Bovina sensu Hassanin and Ropiquet (2004) which is strongly supported by modification of the X chromosome (Fig. 6, III) and the emergence of the new NORs site (character 53), and another uniting species of the subtribe Bubalina sensu Hassanin and Ropiquet (2004) plus the saola, *P. nghetinhensis*. The last grouping is supported by emergence of a new NOR site on BTA22 (character 58), and the loss of the old one on BTA2 (char. 46). Although interspecies relationships within the subtribe Bovina are unresolved in our analysis, they are clearly defined within Bubalina where *S. caffer* was the first species to diverge, an event that is underscored by a series of Rb fusions (characters 13 to 16) that are likely to have resulted in an effective genetic isolation. The remaining species, *Bubalus bubalis*, *B. depressicornis*, *B. mindorensis* and *P. nghetinhensis*, share a newly acquired NOR site on BTA26 (character 61), but were subsequently differentiated by a series of 5 Rb translocations that are specific to the saola (characters 22 to 26), two (characters 2 and 5) that are unique to two of the three species of the genus *Bubalus* studied, and two charac-

ters (6 and 7) that are shared by all three genera. Additionally, the saola could be definitely excluded from the subtribe Bovina on the morphology of its X chromosome. In this context, the three NOR characters shared by saola and species of the subtribe Bubalina, i.e., 58 (all but *Bubalus depressicornis*), 59 (with *Bubalus depressicornis*) and 61 (all but *Syncerus caffer*), strongly suggest its placement within this subtribe, and the recognition of two rather than three subtribes within the tribe Bovini.

In conclusion, our study highlighted the usefulness of NORs for phylogenetic reconstructions within Bovidae, while at the same time emphasizing the importance of appropriate sample sizes to rule out variation in their numbers and chromosomal location. We outline the conditions under which these chromosomal structures may result in additional phylogenetic information. Finally our data suggest the importance of X chromosome rearrangements in evolution of the species studied herein and raise the possibility that detailed molecular cytogenetic analysis of the sex chromosomes may provide additional insights on evolution and phylogenetic relationships within Bovidae and probably Ruminantia.

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Appendix

Chromosomal characters identified in Bovidae. Rob = Robertsonian translocation, t = tandem fusion, inv = inversion, t rcp = reciprocal translocation, NOR = nucleolus-organizing region bearing chromosomes. Chromosomes of studied species were numbered according to cattle standard nomenclature (ISCNDB2000).

1. rob (2;28); 2. rob (5;28); 3. t (7;5;28); 4. t (11;5;28); 5. rob (1;27); 6. rob (2;23); 7. rob (8;19); 8. rob (16;25); 9. rob (11;20); 10. rob (17;25); 11. rob (4;14); 12. rob (16;29); 13. rob (1;13); 14. rob (2;3); 15. rob (5;20); 16. rob (11;29); 17. rob (1;5); 18. rob (6;13); 19. rob (8;12); 20. rob (19;27); 21. rob (24;25); 22. rob (1;10); 23. rob (8;13); 24. rob (6;19); 25. rob (4;18); 26. rob (11;12); 27. inv para12; 28. t(3; 22;2); 29. rob (5;10); 30. rob (6;11); 31. rob (1;25); 32. rob (4;12); 33. rob (8;24); 34. rob (9;20); 35. rob (7;27); 36. rob (15;16); 37. rob (18;19); 38. rob (14;26); 39. rob (21;23); 40. rob (17;28); 41. t rcp [(9;20);(14;26)]; 42. rob (1;3); 43. rob (2;8); 44. rob (5;11); 45. t rcp (9;14); 46. NOR BTA2; 47. NOR BTA3; 48. NOR BTA4; 49. NOR BTA5; 50. NOR BTA7; 51. NOR BTA8; 52. NOR BTA10; 53. NOR BTA11; 54. NOR BTA14; 55. NOR BTA16; 56. NOR BTA18; 57. NOR BTA19; 58. NOR BTA22; 59. NOR BTA23; 60. NOR BTA25; 61. NOR BTA26; 62. NOR BTA28; 63. inv (X); 64. transposition (X); 65. t (Y;13); 66. t (X;14), t (Y;14)

Matrix of chromosomal characters

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            1111111111222222222233333333334444444444555555555566666666
    12345678901234567890123456789012345678901234567890123456789012345678
BTA 000000000000000000000000000000000000000000000000000111000010000001011100
BFR 100000000000000000000000000000000000000000000000000111000010000001001100
BJA 000000000000000000000000000000000000000000000000000111000010000001001100
BIN 000000000000000000000000000000000000000000000000000111000010000001001100
BBO 000000000000000000000000000000000000000000000000000110000010000001011100
BBI 000000000000000000000000000000000000000000000000000111000010000001011100
SCA 000000000000011110000000000000000000000000000000001000000000101011000
BBU 011011110000000000000000000000000000000000000000001000000000011011111000
BDE 010011101100000000000000000000000000000000000000001000000010011011101000
BMI 000101100011000000000000000000010000000000000000010000000001100111000
PNG 000000000000000000000011111100000000000000000000000011001010110101000
BTR 000000000000010011111000000000000000000000000010010000100000000001001
TOR 000000000000000000000000000011111111111110000011010010010000000011010
TDE 000000000000000000000000000011111101110111000??????????????????1010
RRU 000000000000000000000000000000000000000000001001111100000000000010000
CIB 000000000000000000000000000000000000000000000011111000000000000010000
OAR 000000000000000000000000000000000000000000001111111100000000000010000
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