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Comparative analysis (Hippotragini versus Caprini, Bovidae) of X-chromosome's constitutive heterochromatin by in situ restriction endonuclease digestion: X-chromosome constitutive heterochromatin evolution

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Abstract

The Bovidae X-chromosome shows a considerable variation, in contrast to the preservative autosomal conservatism. The X-chromosome variation is mostly a consequence of the constitutive heterochromatin (CH) variation; in what respect to its amount and position. This is especially common among the non-Bovinae subfamilies and tribes. In order to characterize the X-chromosome CH in non-Bovinae species – Hippotragini and Caprini tribes – we have used restriction endonuclease digestion on fixed chromosomes and sequential C-banding. With these techniques we were able to distinguish between the two X-chromosome types (Hippotragini and Caprini) CH, in what respect to its position and molecular nature. Moreover, we define at least, six subclasses of CH in both X-chromosome types analyzed. Evolutionary considerations were draw based on the results obtained. The technology used here for the analysis of the Bovidae X-chromosome CH showed to be more evolutionary informative than the classical approaches.

Abbreviations: BAC – bacterial artificial chromosomes; CBP-banding – C-bands by barium hydroxide using propidium iodide; CH – constitutive heterochromatin; Chr – chromosome; DAPI – 4¢-6-diamidino-2-phenylindole; DNA – deoxyribonucleic acid; FISH – fluorescent in situ hybridization; GTD-banding – G-bands by trypsin with DAPI; MYBP – million years ago before present; NAA – number of autosomal arms; PBS – phosphate-buffered saline; RE-banding – restriction endonuclease banding; RE-bands – restriction endonuclease bands; RE – restriction endonuclease; REs – restriction endonucleases; SSC – saline sodium citrate.

1. Introduction

In general, the Bovidae are chromosomal relatively conservative. Although the diploid chromosome number ranges from 2n ¼ 30 to 2n ¼ 60, the number of autosomal arms (NAA) has remained constant at 56–58 for most species. Chromosomal change in the Bovidae has occurred primarily through centric fusion (Wurster & Benirschke, 1968; Effron et al., 1976; Buckland & Evans, 1978a; Gallagher & Womack, 1992; Gallagher, Derr & Womack, 1994). Although there is good evidence of tandem rearrangements in some species (Gallagher & Womack, 1992) and paracentric inversions in others (Hayes, Petit & Dutrillaux,1991; Robinson et al., 1998), these seem to be the exception rather than the rule. In contrast to the preservative autosomal conservatism evident in the Bovidae, the X-chromosome is reported to show considerable variation between subfamilies, and often between tribes within subfamilies (Buckland & Evans, 1978b; Gallagher & Womack, 1992). The X-chromosome variation in the bovids is due to disruption of euchromatic regions, centromere placement, heterochromatic variation, and autosomal translocation (Robinson et al., 1998; Gallagher et al., 1999; Iannuzzi et al., 2000). Several studies have been done to delineate the structural rearrangements that characterized the evolution of bovid X-chromosome and to determine the primitive and ancestral condition of Bovidae X-chromosome. These studies include G- and C-banding comparisons, in situ hybridizations with X paint probes and bacterial artificial chromosomes (BAC) clones to localize the homologous regions between the X-chromosomes of several species (Hayes, Petit & Dutrillaux, 1991; Kaftanovskaya & Serov,1994; Robinson et al., 1997; Piumi et al., 1998; Iannuzzi et al., 2000). A substantial proportion of the higher eukaryote genome consists of constitutive heterochromatin (CH). This genomic fraction includes, among other repetitive sequences, satellite deoxyribonucleic acid (DNAs) that usually reside in the pericentromeric regions of chromosomes (Chaves et al., 2000).

The C-banding technique is extremely useful to identify CH, and its size differences. On the other

hand, other analytical techniques are necessary to obtain better information on the nature and origin of the CH. Among these technologies, the use of restriction endonucleases (REs) (which cleave DNA in specific sequences) and the base-specific fluorochromes (which delimit chromosome regions rich in different types of DNA bases) are techniques that proven to be very useful in improving the understanding of the mechanisms involved in the evolution of CH in different genomes (Pieczarka et al., 1998; for a revision see Gonzalez et al., 1997).

The use of RE techniques has also the ability to demonstrate the C-heterochromatin heterogeneity (Babu, 1988).

In this study, we used a panel of seven different restriction enzymes and sequentially C-banding to verify whether the X-chromosome CH of species from the Hippotragini and Caprini tribes presents, or not, the same characteristics in terms of its base nature or DNA composition. This technique proved to be more informative in the study of Bovidae X CH than the classical approach of single C-banding.

2. Materials and methods

Chromosome preparations

The material analyzed consists of chromosome preparations made from short-term lymphocyte cultures of whole blood samples obtained from animals of the Lisbon Zoo, Portugal: Addax nasomaculatus (2n ¼ 58, female), Oryx dammah (2n ¼ 58, male), Oryx leucoryx (2n ¼ 58, male); and of the University of Tras-os-Montes and Alto Douro, Portugal: Ovis aries (2n ¼ 54, male).

Briefly, peripheral blood was cultured at 37 C using standard protocols (Chaves et al., 2002). Cell suspension swelling and fixation procedures used a 75 mM KCl hypotonic treatment at 37 C for 20 min, and then chromosomes were fixed three times with methanol:acetic acid solution (3:1) and left overnight at)20 C.

GTD-banding

Air dried slides were aged at 65 C for 5 h or overnight and then were submitted to standard procedures of G-banding with trypsin (Verma & Babu, 1995). Since the chromosomes preparations proceed next to sequential C-banding techniques; they were not stained at this stage. Instead, they were first fixed with paraformaldehyde as described by Chaves et al. (2002). Briefly, dry slides were placed in a 1 · PBS solution (2 · 5 min) before fixation in 4% paraformaldehyde (Sigma)/1 · PBS (room temperature). After fixation for 10 min, slides were dehydrated for 2 min each in 70, 90 and 100% chilled ethanol and air dried. At this step, slides were stained with DAPI (instead of routine Giemsa) for a better contrast (Chaves et al., 2002), and for the identification of the respective Bovidae species chromosomes. The inversion of the DAPI colour in Adobe Photoshop revealed the chromosomes G-banding (GTD-banding, G-bands by trypsin with DAPI) for its identification. Karyotyping followed standardization of the Domestic Bovids karyotypes (ISCNDB, 2000), and Hippotragini karyotypes were also according to Claro, Hayes and Cribiu (1996) and Kumamoto et al. (1999) descriptions.

In situ RE digestion

Air dried slides were aged at 65 C for 6 h and then were submitted to in situ RE digestion. The restriction enzymes used (Hinf I, Pst I, Dde I, Hae III, Rsa I, Mbo I, and Apa I) were diluted in buffers indicated by the manufacturer (Invitrogen, Life Technologies), and final concentrations of 30 U were obtained per 100 ll. The 100 ll of each one of these solutions were placed on slides and covered with coverslips. These slides were incubated in a moist humid chamber for 16 h at 37 C. Control slides were submitted to the same treatment as described above but incubated only with buffer. The slides were then washed in distillated water and air dried. Since, the chromosomes preparations proceed next to sequentially C-banding techniques; they were not stained at this stage. Instead they were first fixed with paraformaldehyde as described by Chaves et al. (2002), and briefly described above (cf. GTD-banding). Finally, the slides were stained with DAPI. The residual bands obtained after the endonuclease digestion were adequate for chromosome identification, namely the X-chromosome. Karyotyping followed standardization of the Domestic Bovids karyotypes (ISCNDB, 2000), and Hippotragini karyotypes were also according to Claro, Hayes and Cribiu (1996) and Kumamoto et al. (1999) descriptions.

CBP-banding sequential to G-bands or RE-bands

The C-banding sequential to G-bands or to restriction enzyme bands was performed by distaining the slides and submitting them to C-banding technique. CBP-banding (C-bands by barium hydroxide using propidium iodide) was done following the standard procedure of Sumner (1972) but with propidium iodide as a counterstain. Briefly, the slides were submitted to routine C-banding with classical treatment times reduced, approximately, to half of it: hydrochloric acid (0.2 M) 10 min, barium hydroxide (5% solution) 4.5 min and 2 · saline sodium citrate (2SSC:0.3 mol/l NaCl, 0.03 mol/l sodium citrate) at 60 C only 20 min.

Chromosome observation

Chromosomes were observed with a Zeiss Axioplan 2 Imaging microscope coupled to Axiocam digital camera and AxioVision software (version 2.0.5 – Zeiss). Digitized photos were prepared for printing in Adobe Photoshop (version 5.0); contrast and colour

optimization were the functions used and all affected the whole of the image equally.

3. Results

Karyotypes description

The diploid number of the Hippotragini species – Addax nasomaculatus, Oryx leucoryx, and Oryx dammah – is 58. All chromosomes are acrocentric, except for the first pair of autosomes, which is a submetacentric one. The Ovis aries (Caprini tribe) diploid number is 54, in which all the autosomes are acrocentric, except for the first three pairs that are biarmed, according to ISCNDB (2000). The X-chromosomes from the Hippotragini species are morphological acrocentrics (Claro, Hayes & Cribiu, 1996). The X-chromosome from Ovis aries is a submetacentric one, with the short arm very small (ISCNDB, 2000).

In situ RE digestion and sequential C-banding

We used seven different restriction enzymes in chromosome preparations of Hippotragini and Caprini species. The CH of the X-chromosomes was analyzed with sequential C-banding techniques.

The Figure 1 presents demonstrative images of the sequential procedures used in this work for the identification of chromosomes (RE-banding with Dde I, Figure 1a and b), and C-banding treatment (Figure 1c and d) in Oryx dammah. In Figure 1b is possible to observe that the residual RE bands are appropriate for the identification of most chromosomes, particularly the X-chromosome. The G-banding technique was only used for the identification of chromosomes in the control experiment of C-banding (without previous treatment with in situ RE digestion).

The action of all seven different REs + C-banding in the X-chromosomes of Hippotragini (Addax nasomaculatus, Oryx leucoryx and Oryx dammah) and Caprini (Ovis aries) species are all presented in Figure 2, and are demonstrative of at least 35 metaphases analyzed (for each species), in no less than five experiments accomplished during different times. In the left column of Figure 2, we present control single C-banding X-chromosomes; these X-chromosomes single C-banded are the comparative controls for the analysis of the seven different RE actions. The Hippotragini C-bands are centromeric (in Figure 2 identified as a band) and interstitial (b band); Addax has a supplementary interstitial band (c) and a telomeric one (d). Moreover, the Caprini species has a pericentromeric C-band (identified as a) and a very small interstitial band (b).

When we applied REs and sequential C-banding it was possible to verify that there was heterogeneity in the C-bands of the X-chromosomes in analysis (Figure 2). This CH heterogeneity revealed to be present at two different levels: intra-band CH variation (disclosing subbands or minor bands in a major control C-band); and inter-band CH variation (showing that different bands have distinct subclasses of CH or a different molecular nature, and so a different molecular composition in its CH).

The effect of the seven REs in the various X-chromosomes (Figure 2) is classified in Table 1, and for that we used the following rules (always comparing the RE + C-banding results to the respective X-chromosome single C-banded control, cf. left column of Figure 2): presence (+) or absence ()) of a specific RE + C-band; RE-+ C-band of reduced size (r); and finally, some RE + C-bands demonstrated to have larger size than the control C-band, and for these cases we used the notation (++). These special cases (see Figure 2 and Table 1), where bands demonstrated to be larger after the RE treatments (compared to the control C-bands), could be explained by the fact that RE digestions can increase the capacity of

the stain (in the present work propidium iodide) to bind to a specific chromosome region (Gosalvez et al., 1997; Nieddu et al., 1999). Other point that is worth to mention is that there is no agreement with the correlation between the loss of DNA extraction (after RE treatment) and the reduction in the staining (Gosalvez et al., 1991). Therefore, during the present work we do not mention that a specific chromosome C-band is susceptible to DNA extraction by means of a particular RE 'attack'. Instead, we refer the action of a RE in a specific C-band related to band staining size (see Table 1, band +,), r, or ++).

The C-band designed by a, in Hippotragini X-chromosomes, can be observed with a reduced size (cf. Figure 2 and Table 1) in all the REs treatments.

Nevertheless, different RE blocks can be distinguished in band a (intra-band CH variation), with different REs (arrows in Figure 2). For instance, in band a of Addax X-chromosome is possible to identify different positive heterogeneous C-sub-bands for the various REs; with Pst I, two sub-bands are observed. Therefore, it is evident that the a C-band of Hippotragini X-chromosome contains heterogeneous types of CH. Band b (as band a), from Hippotragini X-chromosomes, present the same behavior for the seven REs (band with reduced size for all REs, except for Apa I where this band is absent), in all the three Hippotragini species (cf. Figure 2 and Table 1). This b C-band is heterogeneous in its heterochromatin content, being negative after Apa I treatment, and positive with all the others REs tested. Therefore, this b C-band exhibits at least, two distinct sub-bands of CH. Bands c and d, from Addax X-chromosome reveals heterogeneous CH after the seven REs treatments, since each one of the bands (c and d), and between the two bands being analyzed, show different digestion RE + C-banding patterns with the seven REs tested (Figure 2 and Table 1); that is, there is an intra and inter-band CH variation, respectively. Finally, all the Hippotragini X-chromosomes C-bands, a, b, c and d, have a different molecular composition (Table 1) because they show different digestion patterns for the

same REs panel used in this work. However, corresponding bands (by its chromosome localization) exhibit similar reactions among the three species, which is demonstrative of a common origin and phylogenetic proximity.

In the Caprini X-chromosome, and after the REs treatments, both a and b C-bands showed heterogeneity of minor C-bands (cf. arrows in Figure 2 and Table 1), and reveal a different CH composition between bands (inter-bands CH variation could be observed in Ovis aries C-bands and with all Hippotragini C-bands, see Figure 2 and Table 1). Notably, one of the most aggressive RE (Apa I) for the Hippotragini C-bands is the RE that in the Caprini C-bands X-chromosome results, apparently, in more pronounced C-bands. Specifically, Apa I + C-bands in Caprini X-chromosome are positive. The b Apa I + C-band seems even larger than the corresponding control C-band. The use of Apa I (and Mbo I), lay in evidence a CH band, that by classical C-banding was hardly seen.

In summary, consistently with the X-chromosomes CH heterogeneity results presented above, it was possible to draw three evolutionary diagrams of the non-Bovinae X-chromosome (Figure 3). The non-Bovinae X-chromosomes seem to evolve through CH addition, elimination or molecular nature change (mechanisms A, B and C in Figure 3).

4. Discussion

The study of CH heterogeneity with in situ RE digestion and subsequent C-banding in Bovidae chromosomes, as far as we know, is only described in bovine chromosomes (Hidas, 1992). The same author tested two different REs (Hinf I and Taq I) and sequentially C-banding (with Giemsa staining) in cattle chromosomes, and reported that some CH blocks have been removed, as a consequence of the prior RE digestion; moreover, some CH blocks revealed minor subsets, and so forth the CH was heterogeneous. Hidas (1992) also concluded that this type of experimental approach is a useful tool for the study of CH heterogeneity and composition. Later in 1998, Fernandez-Garcıa et al., applied three REs (Alu I, Taq I, and Hae III) in fixed sheep chromosomes for the CH analysis (with Giemsa and fluorochromes); however, they did not use sequential C-banding. The conclusions drawn around the CH analysis did not seem very accurate, as they did not use sequentially C-banding for specific revelation of CH.

The present paper demonstrated that the in situ RE digestion and sequentially C-banding (Figures 1 and 2) could be an alternative tool for the study of Bovidae chromosomes CH heterogeneity, namely when there are not available other specific techniques as fluorescence in situ hybridization (FISH) with repetitive sequences (isolated from each species in analysis). The RE digestion + C-banding technique allows an expedite characterization of the CH (in terms of its localization, detection of different CH subclasses, and revelation of the CH molecular composition); much more accurately than the classical single C-banding (that only reveal the localization of the major CH blocks). This technique has also the advantage to be reproducible, at least for mammalian chromosomes (Chaves et al., 2002). However, there are some technical limitations that must be avoided. For example, the intensity of the bands in RE treatments seems related with the type of counterstain used (e.g. Giemsa or fluorochroms) (Gosalvez et al., 1991); therefore, it is not advisable the comparison of results that used different counterstains.

The Bovidae X-chromosome shows a considerable variation, in contrast to the preservative autosomal conservatism (Buckland & Evans, 1978b; Gallagher & Womack, 1992). The X-chromosome variation is mostly a consequence of CH variation; in what respect to its amount and position (Robinson et al., 1998; Gallagher et al.,1999). This is especially common among the non-Bovinae subfamilies and tribes (Robinson et al.,1998; Gallagher et al., 1999). In order to characterize the X-chromosome CH in non-Bovinae species (Hippotragini and Caprini tribes) we applied the technique discussed above: in situ RE digestion (with a panel of seven different REs) on fixed chromosomes and sequential C-banding (Figure 2 and Table 1).

In a general observation of RE + C-bands chromosomes (Figure 2), the C-bands in centromeric regions are remarkable (as was expected by previous work with C-banding Bovidae chromosomes, see Buckland & Evans, 1978b); moreover it is also possible to detect some interstitial C-bands in some chromosomes (e.g. Figure 2). These C-bands could be interpreted as residual RE bands because of an incomplete denaturation of the C-band technique (we used half of routine time required for the C-banding denaturation step, with barium hydroxide, for better chromosome mophology preservation in the sequential banding procedures, cf. materials and methods). However, in control experiments (where we performed single C-banding), with the complete denaturation step with barium hydroxide, the majority of this bands were still observed. Moreover, in Claro, Hayes and Cribiu (1996) work, is also possible to observe interstitial and telomeric C-bands in several chromosomes in an image from Addax C-banding metaphase.

The CH heterogeneity detected in the Bovidae chromosomes in analysis was demonstrated to be present in the X-chromosomes at two different levels (Figure 2 and Table 1): intra-band CH variation and inter-band CH variation (none of these two levels of CH heterogeneity can be observed with single C-banding). The CH intra-band variation disclosed minor bands (in a major classical single C-band). On the other hand, the inter-band CH variation allows the definition of different subclasses of CH, among the X-chromosomes of the species in study, that certainly reveal the major differences in CH molecular composition (different repetitive DNA families) between the Hippotragini and Caprini X-chromosomes.

The inter-band CH variation is more useful for evolutionary studies, since it allows specifically a comparison between, or among, species. In Table 2 we summarize the CH subclasses detected and identified during this work, with the RE digestion and C-banding technique, in Hippotragini and Caprini X-chromosomes. In Hippotragini X-chromosome (cf. Figure 2, Tables 1 and 2), it was possible to identify at least, four subclasses of CH, that are revealing of a different CH composition, demonstrated by a different RE digestion in the respective X-chromosomes bands (Table 2): (a) centromeric (Addax and Oryx species), (b) intersticial (Addax and Oryx species), (c) intersticial (Addax species), and (d) telomeric (Addax species). In the Caprini X-chromosome (cf. Figure 2, Tables 1 and 2) we were able to demonstrate the additional existence of at least, two types of CH (Table 2): (a¢) pericentromeric, and (b¢) intersticial. In conclusion, the RE + C-bands from Hippotragini and Caprini X-chromosomes demonstrated the existence of at least six CH subclasses (Table 2). This suggests that the two types of X-chromosomes (Hippotragini and Caprini), already diverged in what respect to its CH content; besides, the different morphological X-chromosome types (submetacentric for Ovis aries, and acrocentric for Hippotragini species) enable also that conclusion, with most probable X-chromosome rearrangements (centromeric transposition or pericentric inversions). This observation leads to the assumption that the CH heterogeneity found between the Hippotragini and Caprini X-chromosomes is ancient, or it is a consequence of a rapid turnover of repetitive DNA families, that deserve further investigations at the molecular level. Nevertheless, and for both suppositions, it seems reasonable that the presence of CH facilitates the occurrence of chromosome rearrangements, as it is in accordance with several authors (Yunis & Yasmineh, 1971; Peacock, Dennis & Gerlach, 1982; John, 1988).

Alternatively, similar RE reactions against all the seven enzymes suggests a similar base composition, and is probable that the CH of, for instance, X-chromosome band a from Addax and Oryx species (Tables 1 and 2) have a common origin.

Hence, the primitive X-chromosome for Hippotragini tribe could be one, of the two, hypothesis: an X-chromosome type-Oryx or type-Addax. If the first assumption is true, we have to assume CH addition during Hippotragini X-chromosome evolution; quite the opposite, for the second hypothesis the elimination of CH seems the most probable event (cf. Figure 3). In none of the two hypotheses, seems reasonable the CH homogenization of DNA repetitive sequences among different CH bands, because the CH base nature is different for the four types of Hippotragini CH bands. These results are in accordance with previous ones, which described CH addition or elimination, during the Bovidae X-chromosome rearrangements (e.g. centromeric transpositions) (Piumi et al., 1998; Robinson et al., 1998; Gallagher et al., 1999; Iannuzzi et al., 2000). However, the data cited before was obtained with the comparison between different morphological X-chromosomes (metacentric, submetacentric and acrocentric) where X-chromosome rearrangements are obvious, and some of that rearrangements were consistently confirmed with microdissected paints or BAC probes. Data related to Bovidae species with different CH patterns, as is the case of Hippotragini species studied here, are very scarce. Consequently, as the X-chromosomes from Addax and Oryx are morphologically similar (acrocentric ones), but with a different CH pattern (representing at least four CH subclasses), as demonstrated during the present work, it will be very useful, in the future, the application of that tools (namely BAC probes) in X-chromosomes like the ones of Hippotragini tribe. Until then, it will be unreasonable to deduce which chromosome rearrangements occurred during the evolution of Hippotragini X-chromosome, and which of the two hypotheses is the most probable: CH addition or elimination. Nevertheless, these results highlight the importance of analyzing similar morphological Bovidae X-chromosomes, with different CH patterns, and reveal once more the complexity of the mechanisms in Bovidae X-chromosome evolution.

In summary, the characterization of CH in Bovidae X-chromosome, namely of the Hippotragini and Caprini tribes, with in situ RE digestion and sequential C-banding, demonstrated the existence of an enormous variation in its CH, not only in terms of its position, but also as this work reveal, in its different CH molecular nature. In Figure 3 is possible to observe the three possible mechanisms (A, B and C in Figure 3) that resume the evolutionary considerations about the most primitive X-chromosome condition with the species studied in this work. First, it is important to note, that the evolutionary step named by CH molecular change (indicated by III in Figure 3) was only possible to add to the diagram due to the methodology used. Second, it seems evident that the X-chromosome evolution among Hippotragini species is an issue of CH addition or elimination, which is evident in all three possible mechanisms (A, B and C in Figure 3). Third, the X-chromosome evolution between Hippotragini and Caprini species is a process that certainly involve CH molecular nature change (most likely accompanied by centromeric transpositions or pericentric inversions) (see Figure 3). Finally, is unreasonable to certainly assume which is the primitive X-chromosome type (Addax, Oryx or Ovis, see Figure 3). However, it seems that the mechanism C (Figure 3) is apparently the less probable to happen. Several authors have proposed the presumably primitive condition for the Bovidae X-chromosome, to be an acrocentric X-chromosome with several CH bands which could facilitate intra-chromosomal rearrangements during the X-chromosome evolution (Robinson et al., 1998; Gallager et al., 1999; Iannuzzi et al., 2000). From this same point of view, the mechanism A (Figure 3) seems to be the most plausible, and the X-chromosome type-Addax would be the better representative X-chromosome primitive condition of the species analyzed in this work, with the X-chromosome type-Ovis being the most recent derivative state.

However, as was already mention, in a near future, Bovidae X-chromosomes, as the ones from Hippotragini species, should be analyzed by other molecular techniques in comparative studies with other non-Bovinae species (e.g. Ovis aries), in order to determine possible chromosome rearrangements involved in X-chromosome evolution, and to establish the CH evolutionary process (elimination or addition). Furthermore, and as Gallagher et al. (1999) stated, the analysis of X-chromosome evolution within Bovidae (and specifically in non-Bovinae subfamilies), should also focus on the characterization of CH because there is a considerable variation in both amount and position, and from the present work, also a considerable variation in CH molecular composition. With these both strategies of work, in a large number of Bovidae species analyzed, probably it will be possible to determine the most primitive and derivative conditions for the Bovidae X-chromosome.

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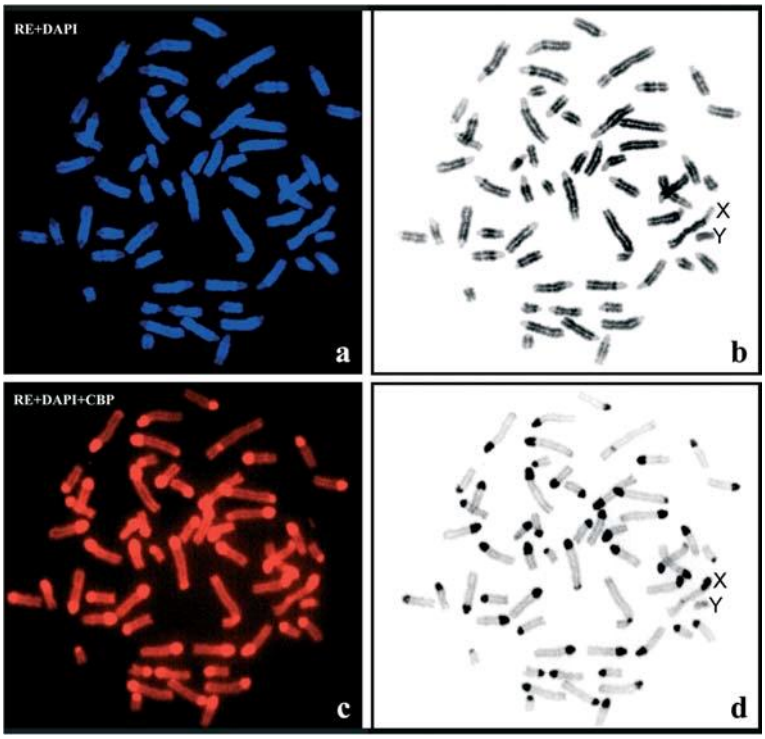

Figure 1. This figure presents demonstrative metaphase from Oryx dammah (male, 2n ¼ 58) of the sequential procedures used in this work for the identification of chromosomes (RE-banding with Dde I, a, b), and C-banding treatment (c, d). (a) RE-banding stain with DAPI. (b) Same image as (a), but with the inversion of the DAPI staining image (a) in Adobe Photoshop for a better contrast of the Dde I bands. (c) Sequential C-banding (CBP) to RE-bands; chromosomes stained with propidium iodide. (d) Same image as (c), but with the inversion of the propidium iodide staining image (c) in Adobe Photoshop for a better contrast of the Dde I + C-bands.

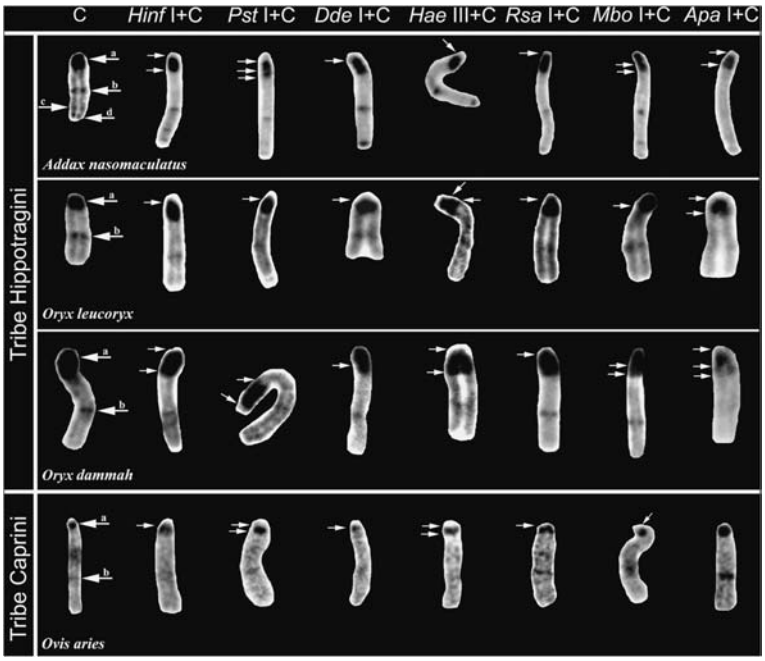

Figure 2. This figure summarizes the RE + C-banding pattern X chromosome's (with the seven RE panel used in this work) from Hippotragini (Addax nasomaculatus, Oryx leucoryx, and Oryx dammah) and Caprini (Ovis aries) species. In the left column of figure, we present control single C-banding X chromosomes; these X chromosomes single C-banded are the comparative controls for the analysis of the seven different RE actions. The CH-bands, in each species were named by letters (a, b, c and d) from the centromere to the telomere. Arrows in the RE + C-banding chromosomes indicates regions where the RE action is more evident, disclosing minor CH bands (intra-band CH variation).

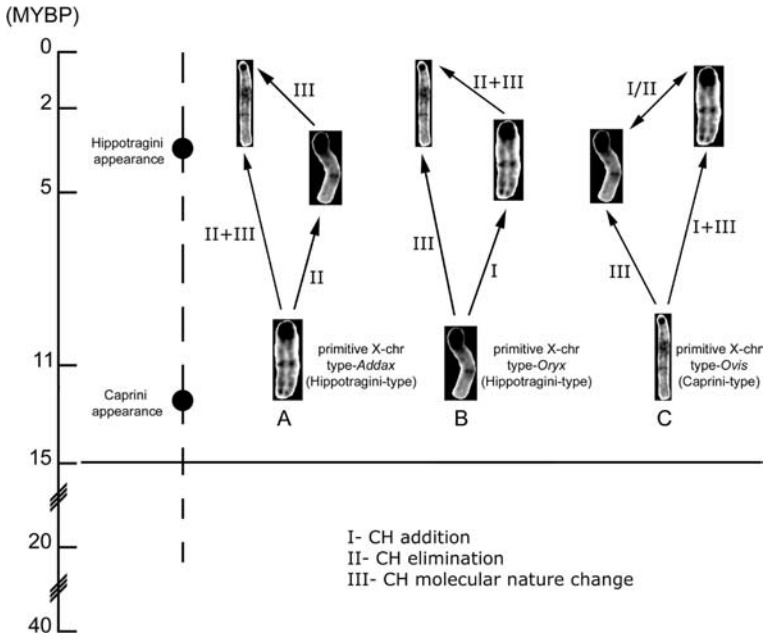

Figure 3. Possible evolutionary diagrams of the non-Bovinae X-chromosome (chr) depicted through the species studied during the present work. Three possible mechanisms are proposed for the most primitive X-chr condition (A, B and C). The CH molecular nature change (indicated by III, in the figure) was only possible to add in this diagram because of the methodology used with RE digestion and sequential C-banding (compared to the classical single C-banding). The divergence times at the tribal level are from Gentry (1992) and Vrba (1985).

Table 1. In situ RE digestion and sequentially C-banding

Species	Bands	Hinf I + C	Pst I + C	Dde I + C	Hae III + C	Rsa I + C	Mbo I + C	Apa I + C
Addax nasomaculatus	a	r	r	r	r	r	r	r
	b	r	r	r	r	r	r	)
	c	+	)	)	)	)	r	)
	d	r	r	+	+	r	r	)
Oryx leucoryx	a	r	r	r	r	r	r	r
	b	r	r	r	r	r	r	)
Oryx dammah	a	r	r	r	r	r	r	r
	b	r	r	r	r	r	r	)
Ovis aries	a	r	r	r	r	r	r	+
	b	r	+	+	r	+	++	++

The effect of the seven REs in the various X chromosomes, presented in Figure 3, is classified in the present table. (+) presence and ()) absence of a specific RE + C-band; (r) RE + C-band of reduce size; and finally, some RE + C-bands demonstrated to have larger size than the control C-band, and for these cases we used the notation (++).

Table 2. X-chromosome constitutive heterochromatin

CH band	Localization	Species name
a	Centromeric	Addax nasomaculatus; Oryx leucoryx; Oryx dammah
a′	Pericentromeric	Ovis aries
b	Interstitial	Addax nasomaculatus; Oryx leucoryx; Oryx dammah
b′	Interstitial	Ovis aries
c	Interstitial	Addax nasomaculatus
d	Telomeric	Addax nasomaculatus

In this table we summarize the CH subclasses detected and identified during this work, with the RE digestion and C-banding technique, in Hippotragini (Addax nasomaculatus, Oryx leucoryx, and Oryx dammah) and Caprini (Ovis aries) X chromosomes.