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Brief Report

Polymorphic organization of constitutive heterochromatin in *Equus asinus* (2n = 62) chromosome 1

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In the karyotype of *Equus asinus* (domestic donkey, 2n = 62), non-centromeric heterochromatic bands have been described in sub-centromeric and telomeric positions. In particular, chromosome 1 is characterised by heterochromatic bands in the proximal region of the long arm and in the short arm; it has been shown that these regions are polymorphic in size. Here we investigated the variation in the intensity and distribution of fluorescence signals observed on donkey chromosome 1 after *in situ* hybridization with two DNA probes containing fragments from the two major equine satellite DNA families. Our results show that, in *Equus asinus* chromosome 1, the amount and distribution of large clusters of satellite DNA can define at least nine polymorphic variants of the constitutive heterochromatin that cannot be detected by C-banding alone.

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Satellite DNA is composed of long stretches of tandemly repeated sequences and is usually located at regions of pericentromeric and telomeric heterochromatin (Ugarkovic and Plohl 2002). Although the results of early studies suggested that this abundant component of the mammalian genome corresponds to junk DNA, more recent evidence supports the notion that satellite DNA has specific functions in chromosome dynamics and segregation. Different families of satellite DNA co-exist in the genome of each species; however, both their copy number and nucleotide sequence tend to evolve rapidly, possibly contributing to the speciation process. Different mechanisms, such as unequal crossing over, DNA replication slippage and gene conversion, drive the dynamics of satellite DNA evolution and are responsible for the copy number variation and for the spreading of new mutations (Ugarkovic and Plohl 2002). In vertebrates, large amounts of tandem repeats are typically localized at centromeres, however, we recently showed that, in the genus *Equus,* satellite DNA is not required for centromere function (Wade et al. 2009). The current idea is that centromeric function is mainly determined by epigenetic factors (Marshall et al. 2008).

We previously isolated two centromeric satellite DNA sequences, 37cen and 2PI (Piras et al. 2010), from a horse genomic library in lambda phage (Anglana et al. 1996). The 37cen sequence (accession number: AY029358) is 93% identical to the horse major satellite family (Wijers et al. 1993; Sakagami et al. 1994), while the 2PI sequence (accession numbers: AY029359S1 and AY029359S2) belongs to the e4/1 satellite family (Broad et al. 1995a, 1995b) and shares 83% identity with it. We investigated the chromosomal distribution of these satellite tandem repeats in different species of the genus *Equus* and observed that several chromosomes, while lacking satellite DNA at their centromeres, as revealed by fluorescence *in situ* hybridization (FISH), contain such sequences at one non-centromeric terminus, probably corresponding to the relic of an ancestral now inactive centromere (Piras et al. 2009, 2010).

In the karyotype of *Equus asinus* (2n = 62), non-centromeric heterochromatic bands have been described

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in a number of chromosomes (Ryder et al. 1978, Gadi and Ryder 1983, Houck et al. 1998, Raudsepp et al. 2000, Di Meo et al. 2009). In particular, *Equus asinus* chromosome 1 (EAS1) is characterised by heterochromatic bands in the proximal q-arm and in the p-arm, being both these C-bands polymorphic in size (Alaoui et al. 2004; Di Meo et al. 2009). In this work, the analysis of the distribution of the two major *Equus* satellite families on EAS1 allowed us to identify several polymorphic variants that were undetectable by C-banding.

MATERIAL AND METHODS

Cell cultures and chromosome preparation

Metaphase spreads were prepared from peripheral blood cultures or fibroblast cultures of 13 non-related and phenotypically normal *Equus asinus* male individuals from Amiata and Ragusana breeds raised in southern Italy.

Peripheral blood cultures were set up in RPMI1640 medium (Euroclone) enriched with L-glutamine, foetal bovine serum (10%), penicillin-streptomycin (1%) and lectin (1.5%, Sigma, from *Phytolacca americana*) as mitogen.

Fibroblasts were isolated and established from skin biopsies and cultured in Dulbecco's modified Eagle's medium (Celbio), supplemented with 20% foetal bovine serum (Celbio), 2 mM glutamine, 2% non essential amino acids, 1 × penicillin/streptomycin.

For metaphase spread preparation, cell cultures were treated with Colcemid (30 ng ml^{-1}, Roche) for 2 h. Chromosome preparations were performed with the standard air-drying procedure.

C-banding

CBA (C, Barium, Acridine orange) banding was performed essentially following the method described by Sumner (1972), as modified by Iannuzzi and Di Berardino (2008). The slides were stained with acridine orange and analysed under a fluorescence microscope (Zeiss Axioplan) equipped with a cooled CCD camera (Photometrics). In order to increase image definition we analyzed computer-generated reverse images.

Fluorescence in situ hybridisation

Lambda phage 37cen and 2PI DNA clones, were extracted from 10 ml of bacteria cultures with the Quantum Prep Plasmid miniprep kit (BioRad), according to supplier instructions. The probes were labelled by nick translation with Cy3-dUTP or Cy5-dUTP (Perkin Elmer) and hybridized to metaphase spreads of primary fibroblasts as previously described (Carbone et al. 2006). Briefly, for each slide 250 ng of each satellite were used. Hybridization was carried out overnight at 37°C in 50% formamide and post-hybridization washes were performed at 42°C in 2 × SSC, 50% formamide. Chromosomes were counterstained with DAPI. Digital grey-scale images for Cy3, Cy5 and DAPI fluorescence signals were acquired as already described. Pseudocoloring and merging of images were performed using the IpLab software.

RESULTS AND DISCUSSION

In Fig. 1a, a schematic representation of the most frequently observed EAS1 C-banding pattern is shown together with all the possible target sites for each satellite DNA probe. The 37cen satellite covers both the extended heterochromatic regions visualized by C-banding on the p-arm and on the proximal q-arm, including the centromeric heterochromatin (Fig. 1a). The two hybridization signals vary in size (Fig. 1b-I chromosome type 1 vs 2, Fig. 1b-II chromosome type 3 vs 4, Fig. 1b-III chromosome type 2 vs 5), and, in some instances, this size variation is related to changes in the extension of the C bands; as an example, the 37cen telomeric signal is missing from chromosome type 4 (Fig. 1b-II), while the p arm C band is fainter with respect to the corresponding band on the homologous chromosome. As shown in Fig. 1a, the 2PI satellite can be localized: 1) on the distal end of the p-arm, at the distal border of the 37cen signal; 2) on the p-arm proximal region; 3) on the proximal border of the subcentromeric heterochromatin; and 4) on the distal border of the subcentromeric heterochromatin. The p-arm terminal and/or the q-arm proximal signals are missing in some instances (Fig. 1b-I chromosome type 1 and 2, Fig. 1b-III chromosome type 2 and 5); however, none of these variants can be identified at the C band level.

Besides the mentioned types of polymorphic chromosomes, among the analysed animals we observed an extended deletion involving a large portion of EAS1 short arm (Fig. 1c, del). As expected in this particular case, the loss of the 37cen and 2PI FISH signals was associated to the absence of the corresponding C-band.

In summary, if all the possible combinations of FISH signal variation observed here were considered, eight different polymorphic forms of chromosome EAS1 can be described (Fig. 1d), the most frequent of which is chromosome type 3 that has been observed in seven out of 26 cases.

It should be pointed out that we probably underestimated the variability of these regions because minor differences in signal intensity were also observed suggesting that these satellite repeat clusters can be organized in a wide range of sizes: it might very well be that in the donkey population a continuum of size and intensity variants exists. In addition, this analysis should be applied to a larger number of

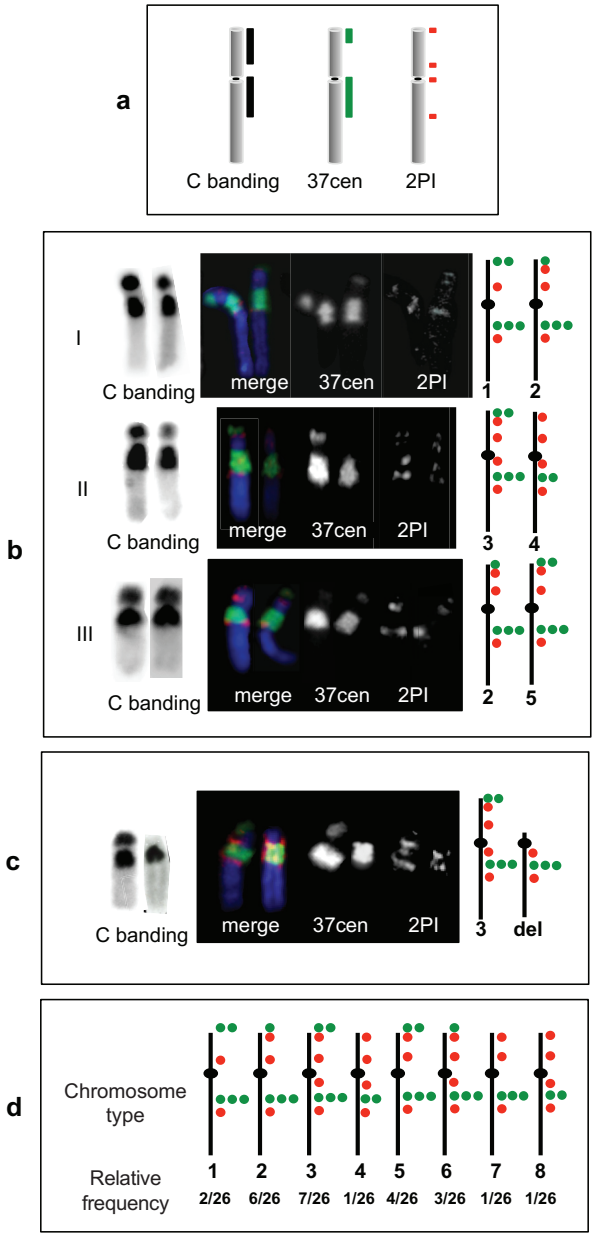

Fig. 1a–d. (a) The C-banding pattern of EAS1 (left) is shown together with the FISH pattern obtained with the 37cen probe (middle) and with the FISH pattern obtained with the 2PI probe (right). **(b)** Results of C-banding and FISH in 3 of the 13 individuals analysed. For each individual (rows I to III) the computer-generated reverse image obtained after CBA banding (left) is directly compared with the two colour FISH pattern (middle panel); on the right, a diagrammatic representation of the distribution and intensity of the FISH signals observed on each chromosome 1 with each probe is reported: green dots indicate the 37cen probe, red dots indicate the 2PI probe; the number of dots at each site relates to the intensity of the FISH signal. **(c)** Chromosomes 1 in an individual carrying a large deletion of the p arm. **(d)** Summary of the results obtained from the analysis of 13 individuals representing all the EAS1 types observed. The frequency of each variant is reported under each chromosome type.

individuals belonging to different breeds to fully evaluate the variability of these regions. However, an interesting point emerging from the present work is that, even if some polymorphic variants can be identified by C-banding, other variants can only be observed using FISH with the satellite probes. A number of data, dealing with C-banding and heterochromatin polymorphism in humans have been published so far (Craig-Holmes and Shaw 1971; Yunis and Yasmineh 1971; Ferguson-Smith 1974; Atkin and Brito-Babapulle V. 1981; Yurov et al. 1987). However, to our knowledge, no direct comparison between FISH analysis of satellite sequences and C-banding polymorphism has been established. In conclusion, our results indicate that the analysis of heterochromatin localization by means of C-banding alone gives only a partial view of the polymorphic state of large clusters of repetitive DNA sequences and that FISH analysis with satellite probes should be applied when a detailed characterization of constitutive heterochromatin has to be performed.

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