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Phylogeny of Horse Chromosome 5q in the Genus *Equus* and Centromere Repositioning

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Abstract
Horses, asses and zebras belong to the genus *Equus* and are the only extant species of the family Equidae in the order Perissodactyla. In a previous work we demonstrated that a key factor in the rapid karyotypic evolution of this genus was evolutionary centromere repositioning, that is, the shift of the centromeric function to a new position without alteration of the order of markers along the chromosome. In search of previously undiscovered evolutionarily new centromeres, we traced the phylogeny of horse chromosome 5, analyzing the order of BAC markers, derived from a horse genomic library, in 7 *Equus* species *(E. caballus, E. hemionus onager, E. kiang*, *E. asinus*, *E. grevyi, E. burchelli* and *E. zebra hartmannae)*. This analysis showed that repositioned centromeres are present in *E. asinus* (domestic donkey, EAS) chromosome 16 and in *E. burchelli* (Burchell's zebra, EBU) chromosome 17, confirming that centromere repositioning is a strikingly frequent phenomenon in this genus. The observation that the neocentromeres in EAS16 and EBU17 are in the same chromosomal position suggests that they may derive from the same event and therefore, *E. asinus* and *E. burchelli* may be more closely related than previously proposed; alternatively, 2 centromere repositioning events, involving the same chromosomal region, may have occurred independently in different lineages, pointing to the possible existence of hot spots for neocentromere formation. Our comparative analysis also showed that, while *E. caballus* chromosome 5 seems to represent the ancestral configuration, centric fission followed by independent fusion events gave rise to 3 different submetacentric chromosomes in other *Equus* lineages.

The order Perissodactyla (odd-toed ungulate mammals) includes 3 extant families: Equidae (horses, asses and zebras), Tapiridae (tapirs), and Rhinocerotidae (rhinoceroses). The Tapiridae and Rhinocerotidae are placed in the suborder Ceratomorpha, while the Equidae are in the suborder Hippomorpha. Modern Perissodactyla are

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the remnant of a once exceptionally successful order, while today, with the exception of domestic horses and donkeys, all species are declining. The Equidae are represented by only 1 extant genus, *Equus*, comprising 8 species: 2 horses *(E. caballus* and *E. przewalskii)*, 2 Asiatic asses *(E. hemionus onager* and *E. kiang)*, 1 African ass *(E. asinus)* and 3 zebras *(E. grevyi, E. burchelli* and *E. zebra hartmannae)*. The *Equus* species shared a common ancestor about 2–3 MYa, and the extant species emerged very recently [Oakenfull and Clegg, 1998]. Although the phylogenetic relationships within the genus *Equus* have been investigated using morphological [Bennet, 1980; Harris and Porter, 1980], molecular [Lowenstein and Ryder, 1985; George and Ryder, 1986; Flint et al., 1990; Oakenfull and Clegg, 1998; Oakenfull et al., 2000] and cytogenetic [Trifonov et al., 2008] data, they are still a matter of debate.

Perissodactyla have widely variable chromosome numbers with 2n = 32–66 in Equidae [Ryder et al., 1978], 2n = 52–80 in Tapiridae [Houck et al., 2000], and 2n = 82–84 in Rhinocerotidae [Wurster and Benirschke, 1968; Houck et al., 1994]. Trifonov and co-workers [2008] calculated the rate of genome reshuffling in each branch of the phylogenetic Perissodactyla tree. The rate of evolutionary chromosomal rearrangement in the Ceratomorpha branches and in the branch connecting the Perissodactyl ancestor (56 MYa) with the equid ancestor (2.4 MYa) was very low; consequently, karyotype evolution was extremely slow. The scenario changed remarkably during the radiation of the genus *Equus*. Speciation events in the genus *Equus* occurred very rapidly in the evolution time scale and were accompanied by extensive karyotype rearrangements, the estimated rate of change being 80 times faster than that observed in the other Ceratomorpha; this figure makes the evolution of the equid genome one of the most rapid so far observed in non-rodent mammals.

The karyotype of the extant species of Ceratomorpha is characterized by mostly acrocentric elements; this arrangement is believed to correspond to the Perissodactyl ancestral karyotype [Trifonov et al., 2008]. On the other hand, equid species display variable numbers of meta- and submetacentric chromosomes that presumably arose by fusion of ancestral acrocentric elements. The possible occurrence of centromere repositioning in the donkey was proposed by Yang et al. [2004] based on chromosome painting and G-banding comparisons. We then demonstrated, by marker order analysis, that the exceptionally rapid evolution of equid karyotypes is accompanied by a surprisingly high frequency of centromere repositioning events [Carbone et al., 2006].

Centromere repositioning consists in the shift of the centromeric function without chromosome rearrangement: a chromosomal region, lacking the satellite DNA sequences that typically characterize eukaryotic centromeres, becomes able to assemble a functional kinetochore; in the meantime, the old centromere is inactivated and progressively loses its repetitive DNA sequences. Centromere repositioning profoundly affects chromosomal architecture and may play a key role in driving karyotype evolution. In our previous work [Carbone et al., 2006], we compared the centromere position and marker order arrangement among homologous chromosomes of Burchell's zebra *(E. burchelli)*, domestic donkey *(E. asinus)*, and horse *(E. caballus)*. We could demonstrate that at least 8 centromere repositioning events took place during the evolution of the genus *Equus* and that 5 of the resulting evolutionarily new centromeres occurred in the donkey after its divergence from the zebra, that is, in very short evolutionary time (approximately 1 MYa).

While neocentromeres lacking repetitive DNA sequences have been found in a number of human clinical cases, all the evolutionarily new centromeres described to date carry variable amounts of satellite DNA. The exact mechanism by which centromere repositioning during evolution occurs is unknown, but it is believed that a partial loss of function of the original centromere may be followed by the formation of a new centromere at a favorable site via epigenetic mechanisms not involving alterations of the DNA sequence. While analysing the chromosomal distribution of satellite tandem repeats in *E. caballus*, *E. asinus*, *E. grevyi* and *E. burchelli*, we recently observed [Piras et al., submitted] that several centromeres, including the evolutionarily new centromeres previously described by us in these species, seem to be devoid of satellite DNA. Moreover, we observed that satellite repeats are often present at non-centromeric termini, probably corresponding to relics of ancestral, now inactive, centromeres.

In this paper we describe the phylogeny of horse chromosome 5q in the genus *Equus* and show that 2 new examples of neocentromeres are present in the homologous chromosomes of *E. asinus* and *E. grevyi*. These evolutionarily new centromeres seem devoid of (or extraordinarily poor of) satellite DNA and presumabily represent 'immature' centromeres.

Table 1. BAC clones

Code	BAC	NCBI EquCab2.0 ECA5	NCBI human36.3 HSA 1
A	CH241-29K24	49,889,446–50,078,906	119,711,220–119,881,220
B	CH241-102I9	53,023,949–53,184,772	116,058,861–116,228,861
C	CH241-10N21	64,283,602–64,470,308	102,079,675–102,280,578
D	CH241-25F11	81,403,324–81,621,335	82,094,926–82,264,926
E	CH241-107O3	84,774,407–84,931,834	78,136,904–78,306,904
F	CH241-25H7	98,031,303–98,224,666	62,046,123–62,317,592

Materials and Methods

Cell Cultures and Chromosome Preparations

E. caballus, *E. asinus, E. h. onager, E. kiang* and *E. z. hartmannae* fibroblasts were isolated and established from skin biopsies. *E. grevyi* and *E. burchelli* fibroblasts were purchased from Coriell Repositories (http://ccr.coriell.org/). Cells were grown in Dulbecco's modified Eagle's medium (GIBCO, http://www.invitrogen.com/site/us/en/home/brands/Gibco.htm) supplemented with 20% fetal calf serum (CELBIO, http://www.euroclonegroup.it/).

Cells were harvested following colcemid treatment for 2 h, centrifuged and incubated with 0.075 M KCl at 37°C for 20 min, then fixed in methanol:acetic acid (3:1) overnight. The fixative was changed 2 times and cells were spread onto microscope glass slides.

DNA Probes

Six horse BAC clones (CH241-29K24, CH241-102I9, CH241-10N21, CH241-25F11, CH241-107O3, CH241-25H7), belonging to the CHORI-241 library, were extracted from 10 ml of bacteria cultures using the Quantum Prep Plasmid miniprep kit (BioRad Laboratories, http://www.bio-rad.com/) according to the supplier's instructions. In table 1 the letter used to identify each clone, the identification library code and the NCBI map viewer location (http://www.ncbi.nlm.nih.gov/) on horse chromosome 5 and human chromosome 1 are reported.

Two repetitive DNA sequences, 37cen and 2PI, belonging to the major horse satellite DNA families, were previously isolated from a horse genomic library in lambda phage [Anglana et al., 1996] and subcloned in plasmid vector. 37cen (Accession number: AY02935), consisting of a 221-bp repeat, is 93% identical to a previously described centromeric DNA repeat [Wijers et al., 1993; Sakagami et al., 1994]; 2PI (Accession numbers: AY02935951 and AY02935952), consisting of a 23-bp repeat, belongs to the e4/1 family [Broad et al., 1995a, b].

FISH Experiments

The equine BAC clones and the 37cen and 2PI satellites were labeled by nick translation with Cy3-dUTP, Cy5-dUTP (Perkin Elmer, http://www.perkinelmer.com/) or Alexa Fluor 488-5-d-UTP (Invitrogen, http://www.invitrogen.com/site/us/en/home.html) and hybridized to metaphase spreads from the 7 *Equus* species. For each slide, 250 ng of the labeled probe 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, 0.2 μg/ml. Digital grey-scale images for Cy3, Cy5, Alexa 488 and DAPI fluorescence signals were acquired with a fluorescence microscope (Zeiss Axioscope.A1, http://www.zeiss.com/) equipped with a cooled CCD camera (Photometrics, http://www.photomet.com/). Pseudo-coloring and merging of images were performed using the IpLab software. Chromosomes were identified by computer-generated reverse DAPI banding according to the standard karyotypes.

Results and Discussion

In a previous paper [Carbone et al., 2006], we performed a marker order comparison between the chromosomes of horse, donkey and Burchell's zebra and demonstrated that centromere repositioning is a frequent occurrence in the genus *Equus*; in fact, we showed that at least 8 new centromeres were formed during the evolution of this genus (ECA11, EAS8, EAS9, EAS11, EAS13, EAS15, EAS18/EBU20 and EAS19), domestic donkey being the species more prone to centromere shift. However, the number of neocentromeres detected in our previous study was underestimated because cell lines from only 3 species were available to us at that time and appropriate probes for some of the chromosomes were missing.

In order to identify other possible neocentromeres, we compared the position of the primary constriction in chromosomes from different *Equus* species that were shown to be homologous by cytogenetic studies [Yang et al., 2003, 2004; Trifonov et al., 2008]. This analysis allowed us to unravel that the entire long arm of *E. caballus* chromosome 5 corresponds to small submetacentric chromosomes in *E. asinus* and *E. burchelli* (EAS16 and EBU17), suggesting that centromere repositioning events may have occurred.

To test this hypothesis, we investigated the evolutionary history of horse chromosome 5q in 7 species belonging to the genus *Equus* (*E. caballus* or ECA, *E. asinus* or EAS, *E. burchelli* or EBU, *E. grevyi* or EGR, *E. z. hartman-*

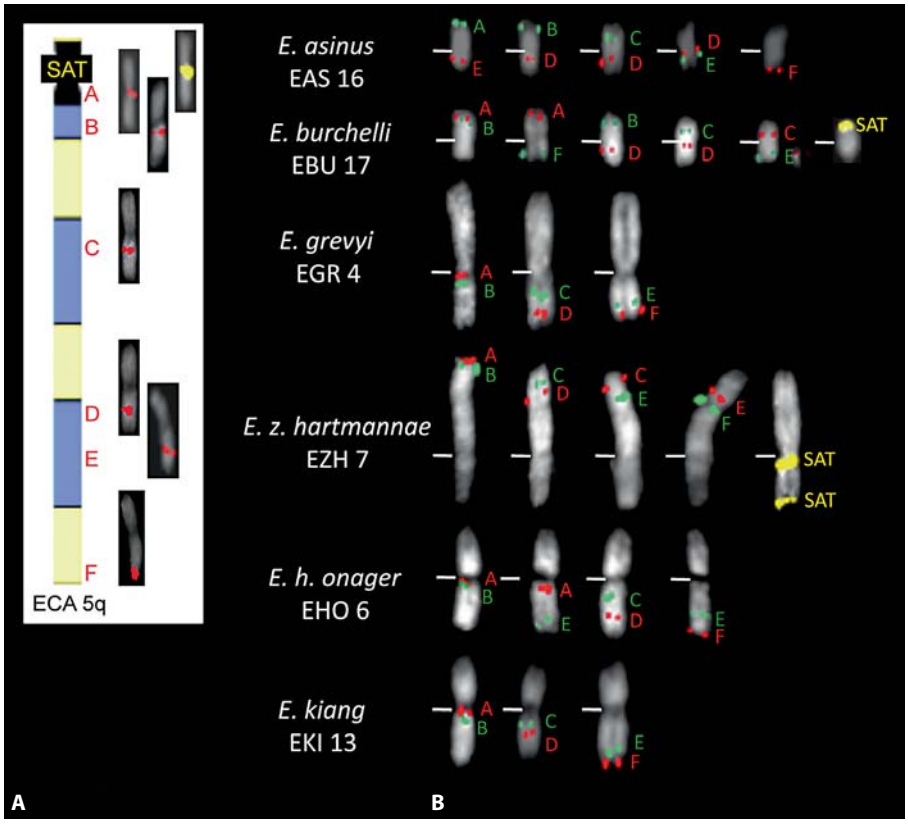

Fig. 1. Localization of BAC clones and satellite DNA on the chromosomes of *Equus* species. **A** Left: map position of the 6 BAC clones (A–F, red signals) and of the major horse satellite DNA (SAT, yellow signal) on the long arm of horse chromosome 5 are reported on an ideogram of ECA5q. Right: results of 1-color FISH experiments on horse chromosome 5. **B** Results of 2-color FISH experiments with couples of BAC probes (red and green signals) on ECA5 orthologous chromosomes in the 6 non-horse *Equus* species. The results of 1-color FISH with horse satellite DNA probe are also shown (yellow signals). Yellow FISH signals were evident only on EBU17 and on EZH7.

nae or EZH, *E. h. onager* or EHO and *E. kiang* or EKI), using 6 horse BAC clones, spanning the long arm of chromosome 5. The clones were selected from the CHORI-241 horse BAC library on the basis of their published map position [Leeb et al., 2006]. Map position and clone distance was verified by BLAST alignment against the human genome (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid = 9606). In silico mapping of the clones on horse chromosome 5 and human chromosome 1 database sequences (http://www.ncbi.nlm.nih.gov/) are reported in table 1.

In figure 1A, the cytological localization of the BAC clones, obtained by FISH, and their position are shown on an ECA5q ideogram. Different combinations of BAC clones were labeled with distinct fluorochromes and used as probes in 2-color FISH experiments on metaphase chromosomes from *E. asinus, E. burchelli, E. grevyi, E. z. hartmannae, E. h. onager* and *E. kiang* (fig. 1B). Metaphase spreads from all the species were also hybridized with plasmid clones containing fragments from the 2 major horse satellite DNA families (yellow in fig. 1).

In figure 2A, a schematic representation of the entire horse chromosome 5 (ECA5) and its corresponding segment on human chromosome 1 is shown. In this figure, all the chromosomes homologous to ECA5q in the other *Equus* species are also drawn and the positions of the 6 markers are indicated by letters.

The correspondence between horse chromosome 5 and human chromosome 1 was previously demonstrated by comparative mapping and chromosome painting [Raudsepp et al., 1996; Milenkovic et al., 2002; Chowdhary et al., 2003; Murphy et al., 2003, 2005; Yang et al., 2004]. The results of these studies demonstrated that horse chromosome 5p corresponds to a proximal region of human chromosome 1q, while horse chromosome 5q corresponds to a proximal region of human chromosome 1p. In addition, ECA5p/q is conserved as a single synteny block in humans and in Perissodactyla as well as in all boreoeutherians, but this synteny is disrupted in all other equids [Trifonov et al., 2008]. We then searched conserved syntenic segments on horse chromosome 5 and on human chromosome 1 using the published horse and human sequences (http://www.ncbi.nlm.nih.gov/

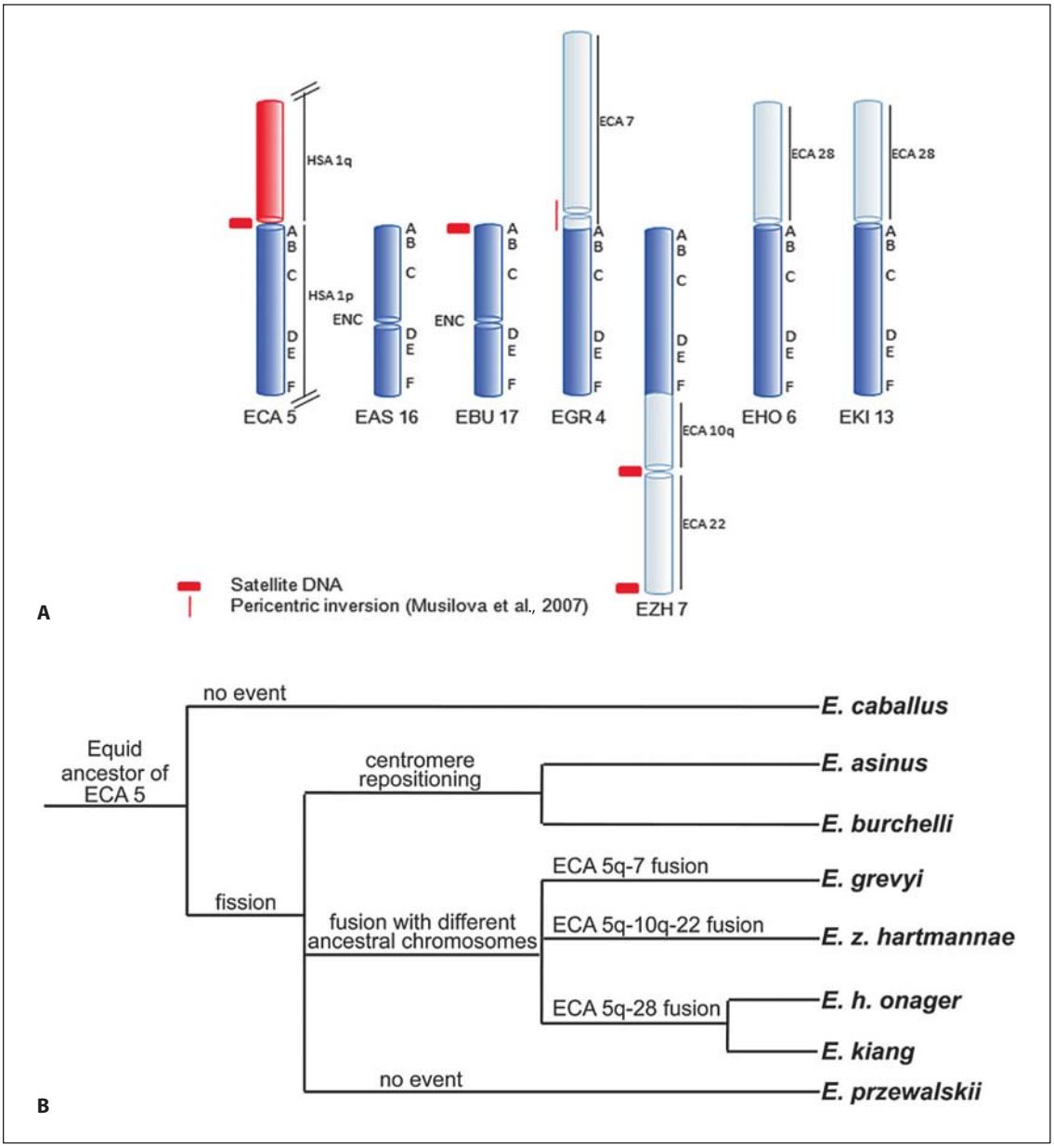

Fig. 2. Phylogeny of horse chromosome 5. **A** Diagrammatic representation of BAC (A–F) marker order on ECA chromosome 5 and on its homologous counterparts in the other equid species. ENC indicates Evolutionarily New Centromere. The black bars on the right of ECA5 show its conserved synteny with HSA1. Bars on the right of non-horse chromosome arms indicate synteny with horse chromosomes other than 5q. The red bar on the left of EGR4 represents a pericentric inversion that was reported by Musilova et al. [2007]. **B** Cladogram representing the phylogeny of ECA5 as inferred by the result of the present research and for *E. prewalskii* by the results of other groups [Ahrens and Stranzinger, 2005; Trifonov et al., 2008].

Genomes/). This analysis allowed us to verify, point by point, the uninterrupted alignment of horse 5p/q and of human chromosome 1 proximal long arm/proximal short arm and to determine the approximate borders of ECA5 homologous segments on HSA1 (nt 58,595,000 and nt 20,201,000). It is tempting to propose that the centromeres of HSA1 and ECA5 are also located at corresponding sites.

Our marker order comparison (fig. 2A), suggests that the ECA5 centromere is conserved in 3 other equids

(E. grevyi, E. h. onager and *E. kiang)*; in fact, EGR4, EHO6 and EKI13 are submetacentric chromosomes with one chromosome arm corresponding to ECA5q. This observation supports the hypothesis that a fission event occurred in the ancestral ECA5-like submetacentric chromosome (fig. 2B), giving rise to 2 acrocentric chromosomes now present only in *E. przewalskii*: EPR23 and EPR24 [Myka et al., 2003; Yang et al., 2003; Ahrens and Stranzinger, 2005; Trifonov et al., 2008]. As shown in figure 2A, the ancestral ECA5q-like acrocentric underwent independent fusion events: the first fusion, that generated EGR4, involved an ancestral acrocentric chromosome now corresponding to ECA7; the second fusion, that generated a chromosome now present both in *E. h. onager* and in *E. kiang* (EHO6 and EKI13), involved an ancestral chromosome corresponding to ECA28. In *E. z. hartmannae*, the large submetacentric chromosome EZH7 originated from complex fusion events involving ECA5q, ECA10q and ECA22 ancestors. In this chromosome, the ECA5 centromere was inactivated, possibly as a consequence of the chromosome rearrangements. The satellite repeats that we observed at the EZH7 centromere might correspond to those present at the centromere of the ECA10 ancestor, while those observed at the EZH7p terminus might be the relic of the centromere of the ECA22 ancestor. It should be pointed out that the centromeres of EGR4, EHO6 and EKI13 did not hybridize with the horse satellite probes. This observation suggests that the satellite sequences from the ancestral centromere, that are still present in the corresponding horse chromosomes [fig. 1A and B; Piras et al., submitted], were partially or completely lost during the fusion events.

In both *E. asinus* and *E. burchelli*, a small submetacentric chromosome (EAS16 and EBU17) is homologous to the entire long arm of ECA5. In these 3 chromosomes (ECA5, EAS16 and EBU17), the order of the markers (A–F) is conserved, but the position of the centromere is shifted; in particular, in the horse, all the markers are distal, whereas in the other 2 species, the centromere is located in an apparently identical interstitial position, between markers C and D. Thus, centromere shift is not due to chromosome rearrangement but to repositioning. Our results strongly suggest that in EAS16 and EBU17, the centromeres are evolutionarily new, while the ancestral centromere configuration is the one now present in ECA5. This conclusion is strongly supported by the localization of satellite DNA; in fact, satellite DNA, a typical mark of mammalian centromeres, is present on ECA5 cen, and on the corresponding terminal position of EBU17, whereas it is not detectable on the 2 evolutionarily new centromeres (fig. 1A and fig. 2A). Although we recently demonstrated by sequence analysis that 1 horse neocentromere, on ECA11, is completely devoid of satellite DNA [Wade et al., submitted], we cannot exclude that short arrays of tandem repeats, not detectable by FISH, or new families of satellites, not detectable with the probes used in this study, may be present at the EAS16 and EBU17 neocentromeres. The absence of C-banding positive regions at the neocentromeres of ECA11, EAS16 and EBU17 [Ryder et al., 1978] supports the hypothesis that they are devoid of highly repetitive tandem arrays.

The results reported here are in agreement with the hypothesis that, during evolution, the centromeric function can move to a new location lacking satellite DNA and that the old inactivated centromere may maintain satellite DNA sequences as a relic of the ancient centromere position. As mentioned above, we have recently shown [Piras et al., submitted] that in the genus *Equus*, other evolutionarily new centromeres seem to be devoid of satellite DNA and that satellite repeats are often present at non-centromeric termini, probably corresponding to relics of ancestral, now inactive, centromeres. The results presented here corroborate the hypothesis that in the genus *Equus*, centromere function is uncoupled from satellite DNA, and therefore, the acquisition of centromeric function during evolution seems to occur independently of satellite DNA recruitment.

The most parsimonious phylogenetic tree explaining the origin of present-day ECA5 and of its counterparts in the other extant equid species is reported in figure 2B. Since chromosomes corresponding to ECA5 are conserved in all boreoeutherians [Murphy et al., 2003, 2005], we propose that ECA5 represents an ancestral equid chromosome. The idea that ECA5 corresponds to an ancestral eutherian chromosomal segment is also supported by the recently published second generation RH map of the horse genome [Raudsepp et al., 2008]. Moreover, the localization of satellite DNA strongly suggests that the centromere position in ECA5q is ancestral as well. We then suppose that a centric fission event may have separated the long and the short arms of the ancestral ECA5 precursor (fig. 2B). Whereas nowadays ECA5 would correspond to the equid common ancestor, in the other *Equus* species the initial fission of the ECA5 precursor would have been followed by different independent events: (i) centromere repositioning in the ancestor of ECA5q that gave rise to a small submetacentric chromosome, now present in *E. asinus* and *E. burchelli* (EAS16

and EBU17); (ii) independent fusion events that occurred between the precursor of ECA5q and other different ancestral chromosomes giving rise to EGR4, EZH7 and to a submetacentric chromosome which is now present in *E. h. onager* and in *E. kiang* (EHO6 and EKI13); (iii) in the Przewalski's horse, no further rearrangement occurred; in fact, EPR24 and EPR23 are free acrocentric elements corresponding to ECA5p and ECA5q, respectively [Ahrens and Stranzinger, 2005; Trifonov et al., 2008]; thus, they may represent the living evidence of the original fission event. The presence of satellite DNA at the centromere of EPR24 and EPR23 observed by Ryder et al. [1978] may represent the relic of the repetitive DNA originally located at the centromere of the ancestral submetacentric chromosome.

Phylogenetic relationships within the genus *Equus* have been traced so far using different approaches based on the analysis of morphological traits [Bennet, 1980; Harris and Porter, 1980], of protein and DNA polymorphic markers [Lowenstein and Ryder, 1985; George and Ryder, 1986; Flint et al., 1990; Oakenfull and Clegg, 1998; Oakenfull et al., 2000] and of cytogenetic data [Trifonov et al., 2008]. The resulting cladograms are quite different, the main discrepancies concerning the evolutionary distance between asses and zebras. According to the cladogram reported in figure 2B, the domestic ass and the Burchell's zebra are more closely related than the Burchell's and Grevy's zebras; this conclusion derives from the observation that the evolutionarily new centromeres seem to be present in the same cytological position in EAS16 and in EBU17. Although molecular analyses would be necessary to determine whether the centromeres of EAS16 and EBU17 are actually confined to the same chromosomal region and totally devoid of satellite DNA sequences, the possibility that the same centromere is shared between the 2 chromosomes seems the most plausible. If this is the case, an alternative model should also be considered, that is, that 2 centromere shift events, involving the same chromosomal region, occurred independently during the radiation of the different *Equus* lineages, in agreement with the hypothesis that some chromosomal regions may be particularly prone to neocentromere formation [Choo, 2000].

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