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SCIENCE DIRECT

Genomics 87 (2006) 777 – 782

GENOMICS

www.elsevier.com/locate/ygeno

Evolutionary movement of centromeres in horse, donkey, and zebra

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Received 15 October 2005; accepted 22 November 2005
Available online 18 January 2006

Abstract

Centromere repositioning (CR) is a recently discovered biological phenomenon consisting of the emergence of a new centromere along a chromosome and the inactivation of the old one. After a CR, the primary constriction and the centromeric function are localized in a new position while the order of physical markers on the chromosome remains unchanged. These events profoundly affect chromosomal architecture. Since horses, asses, and zebras, whose evolutionary divergence is relatively recent, show remarkable morphological similarity and capacity to interbreed despite their chromosomes differing considerably, we investigated the role of CR in the karyotype evolution of the genus *Equus*. Using appropriate panels of BAC clones in FISH experiments, we compared the centromere position and marker order arrangement among orthologous chromosomes of Burchelli's zebra (*Equus burchelli*), donkey (*Equus asinus*), and horse (*Equus caballus*). Surprisingly, at least eight CRs took place during the evolution of this genus. Even more surprisingly, five cases of CR have occurred in the donkey after its divergence from zebra, that is, in a very short evolutionary time (approximately 1 million years).These findings suggest that in some species the CR phenomenon could have played an important role in karyotype shaping, with potential consequences on population dynamics and speciation.

Keywords: Centromere repositioning; Centromere; Donkey; Horse; Evolution

Evolutionary studies on primate chromosomes 9, X, 6, and 3 have disclosed the unprecedented centromere repositioning (CR) phenomenon, that is, the displacement of the centromere along the chromosome without disruption of the gene order [1–4]. The old centromere is inactivated and a new one produced, which then becomes fixed in the population. The phenomenon has been also reported in nonprimate mammals [5], in marsupials [6], and in birds [7]. A CR event has been invoked to explain the unusual intermediate state of heterochromatization of the chromosome 8 centromere of rice [8]. Altogether these data suggest that the phenomenon could be widespread in eukaryotes.

The inactivation of the old centromere is accompanied by the rapid loss of centromeric satellite DNA and by the dispersal of the pericentromeric duplicons over a relatively wide area. A well-known example of a recently inactivated centromere is present in the human species at 2q21, where the centromere of the ancestral chromosome IIq was inactivated after the telomere-telomere fusion that generated human chromosome 2 [9,10]. Few remains of alphoid centromeric repeats are still present at 2q21. More ancient inactivated centromeres appear to be completely devoid of centromeric satellite repeats, while duplicon clusters, typical of pericentromeric regions [11], were retained [3,4]. Successively, the new centromeres acquire the structural complexity of normal centromeres and become

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doi:10.1016/j.ygeno.2005.11.012

Table 1
Horse BAC clones used in FISH experiments

Chromosome	Code	BAC	UCSC (May 2004)
ECA5/HSA1	A	CH241-308A9	Chr 1: 90,112,182–90,112,217
	B	CH241-253I21	Chr 1: 99,778,499–99,778,534
	C	CH241-282K16	Chr 1: 142,726,734–142,726,769
	D	CH241-402G1	Chr 1: 150,977,815–150,977,850
	E	CH241-368I23	Chr 1: 152,865,292–152,865,327
	F	CH241-306L5	Chr 1: 158,835,250–158,835,285
	G	CH241-324G8	Chr 1: 184,841,974–184,842,009
ECA6p/HSA2q35-q37	A	INRA-272B8 (FN1)	Chr 2: 216,051,094–216,065,921
	B	INRA-193E12 (INHA)	Chr 2: 220,262,521–220,265,932
	C	INRA-234E11 (UGT1A1)	Chr 2: 234,450,918–234,463,937
ECA8/HSA22-12-18	A	CH241-510E21	Chr 22: 26,879,366–27,048,339
	B	CH241-211L22	Chr 12: 116,154,247–116,154,282
	C	INRA-391C5 (HPD)	Chr 12: 120,740,154–120,759,471
	D	CH241-236K22	Chr 12: 132,003,528–132,003,563
	E	CH241-445J22	Chr 18: 12,687,372–12,687,412
	F	INRA-27D9 (NPC1)	Chr 18: 19,365,461–19,420,399
	G	CH241-474A14 (LPIN2)	Chr 18: 2,906,999–3,001,945
	H	INRA-116H6 (CDH2)	Chr 18: 23,784,933–24,011,092
	I	CH241-345B23	Chr 18: 50,283,486–50,283,526
	J	CH241-298M6	Chr 18: 50,698,959–50,698,999
	K	CH241-232O2	Chr 18: 75,760,067–75,760,107
ECA9/HSA8	A	CH241-471D2	Chr 8: 90,068,408–90,068,443
	B	CH241-252J14	Chr 8: 71,478,574–71,478,609
	C	CH241-372G1	Chr 8: 52,914,714–52,914,749
	D	CH241-264L20	Chr 8: 131,814,574–131,814,609
ECA11/HSA17	A	CH241-297E22	Chr 17: 705,656–705,691
	B	CH241-336K9	Chr 17: 7,858,693–7,858,728
	C	CH241-222C23	Chr 17: 17,008,174–17,008,209
	D	CH241-236G12	Chr 17: 22,658,877–22,658,912
	E	CH241-362A20	Chr 17: 33,144,675–33,144,710
	F	CH241-212C17	Chr 17: 47,970,022–47,970,057
	G	CH241-236P15	Chr 17: 55,115,852–55,115,887
	H	CH241-340H10	Chr 17: 65,612,540–65,612,575
	I	CH241-212N12	Chr 17: 78,387,453–78,387,488
ECA12/HSA11	A	CH241-199O13	Chr 11: 35,985,378–35,985,413
	B	CH241-199F11	Chr 11: 46,836,445–46,836,480
	C	CH241-202H13	Chr 11: 56,649,209–56,649,244
ECA13/HSA7-16	A	CH241-214K20	Chr 7: 1,041,531–1,041,566
	B	CH241-415C2	Chr 7: 19,971,418–19,971,453
	C	CH241-295C2	Chr 7: 64,863,283–64,863,318
	D	CH241-212K23	Chr 16: 28,856,152–28,856,187
	E	CH241-296G6	Chr 16: 18,728,010–18,728,045
	F	CH241-298F24	Chr 16: 8,917,233–8,917,268
ECA14/HSA5	A	INRA-454H3 (HMGCR)	Chr 5: 74,687,922–74,691,721
	B	CH241-361A12	Chr 5: 80,643,902–80,643,937
	C	CH241-444O5	Chr 5: 105,044,806–105,044,841
	D	INRA-620C9 (CSF2)	Chr 5: 131,437,383–131,439,757
	E	CH241-465E13	Chr 5: 155,146,893–155,146,928
	F	CH241-348E4	Chr 5: 178,731,550–178,731,585
ECA15/HSA2p	A	CH241-392J11	Chr 2: 4,382,017–4,382,052
	B	CH241-302E10	Chr 2: 22,930,282–22,930,317
	C	CH241-310C7	Chr 2: 33,660,684–33,660,719
	D	CH241-263A21	Chr 2: 43,325,731–43,325,766
	E	CH241-246G11	Chr 2: 63,152,530–63,152,565
	F	CH241-289G16	Chr 2: 73,564,550–73,564,585
	G	CH241-146D15	Chr 2: 88,984,817–88,984,852
ECA17/HSA13	A	CH241-474K9	Chr 13: 19,498,765–19,498,804
	B	CH241-274D19	Chr 13: 23,044,760–23,044,799
	C	CH241-244I22	Chr 13: 30,615,981–30,616,020
	D	CH241-251G23	Chr 13: 33,296,083–33,296,122
	E	CH241-314H18	Chr 13: 42,367,197–42,367,236
	F	CH241-359D17	Chr 13: 64,874,777–64,874,816
	G	CH241-303L7	Chr 13: 77,670,695–77,670,734

Table 1 (*continued*)

Chromosome	Code	BAC	UCSC (May 2004)
ECA17/HSA13	H	CH241-463N7	Chr 13: 88,246,713–88,246,752
	I	CH241-300J2	Chr 13: 114,045,480–114,045,519
ECA20/HSA6	A	INRA-304G13 (SERPINB1)	Chr 6: 2,778,734–2,787,080
	B	INRA-45I1 (BMP6)	Chr 6: 7,672,028–7,826,726
	C	INRA-83G9 (TNFA)	Chr 6: 31,652,524–31,652,570
	D	INRA-66C7 (ITPR3)	Chr 6: 33,697,133–33,771,686
	E	INRA-360A12 (MUT)	Chr 6: 49,506,956–49,538,811
	F	INRA-391E2 (COL9A1)	Chr 6: 71,046,186–71,069,494
	G	INRA-924B12 (TKY321[a])	FISH mapping
ECA22/HSA20	A	CH241-312N4	Chr 20: 2,964,226–2,964,261
	B	CH241-501B17	Chr 20: 10,221,646–10,221,681
	C	CH241-324O21	Chr 20: 11,175,220–11,175,255
	D	CH241-311K16	Chr 20: 15,225,942–15,225,977
	E	CH241-380G19	Chr 20: 23,323,793–23,323,828
	F	CH241-241N14	Chr 20: 30,247,236–30,247,271
	G	CH241-12N13	Chr 20: 33,998,928–33,998,963
	H	CH241-345B18	Chr 20: 46,971,834–46,971,869
	I	CH241-499I10	Chr 20: 62,266,508–62,266,543
ECA26/HSA3-21	A	CH241-324G17	Chr 3: 89,164,694–89,164,734
	B	CH241-446K19	Chr 3: 76,837,658–76,837,698
	C	CH241-356L9	Chr 21: 14,767,387–1,476,7422
	D	CH241-444L13	Chr 21: 21,457,434–21,457,469
	E	CH241-508E17	Chr 21: 28,928,095–28,928,130
ECA24/HSA14	A	CH241-221F11	Chr 14: 56,725,481–56,725,520
	B	CH241-422I22	Chr 14: 72,624,820–72,624,855
	C	CH241-204P19	Chr 14: 88,161,177–88,161,216
	D	CH241-503D2	Chr 14: 104,921,784–104,921,823
ECA28/HSA12-22	A	CH241-219F9	Chr 12: 87,368,202–87,368,237
	B	CH241-198J21	Chr 22: 31,166,200–31,166,235
	C	CH241-403H2	Chr 22: 35,783,104–35,783,139
	D	INRA-926B4 (IGF1)	Chr 12: 101,313,740–101,376,808

All the BAC clones used in the FISH experiments are listed. Most of them belong to the CHORI-241 BAC library (http://bacpac.chori.org). The fourth column reports the position in the human sequence (UCSC May 2004 release) of the overgo probes used to screen them. INRA BAC clones [12] were identified using, as a probe, oligos from specific genes (reported in parentheses in column 3). The code letters identify these clones in Figs. 1 and 2.

[a] TKY321 is a horse-specific microsatellite.

indistinguishable from the other centromeres, at least at the molecular cytogenetic level [3,4,12].

It has been recently shown that two human neocentromeres at 15q24-q26, described in clinical cases, map to duplicons that flanked an ancestral inactivated centromere [4]. Furthermore, Ventura et al. [12] have reported that, in a patient, a neocentromere was seeded in the 3q26 region, which corresponds to the normal centromere in the Old World monkey (OWM) chromosome 3, and that this OWM centromere, in turn, was generated as a consequence of a CR event in an OWM ancestor. This scenario deeply affects our understanding of karyotype changes during evolution and strongly suggests that the present-day neocentromeres occurring in human clinical cases are better understood if viewed in an evolutionary frame.

Burchelli's zebra (*Equus burchelli*; EBU) and the donkey (*Equus asinus*; EAS) are two Equidae species that diverged about 0.9 million years ago (MYA), while their common ancestor diverged from the horse (*Equus caballus*; ECA) around 2 MYA [13,14]. Donkey, Burchelli's zebra, and horse have 62, 44, and 64 chromosomes, respectively, and show a large number of chromosomal differences that have been pointed out using classical and fluorescence in situ hybridization (FISH) techniques [15,16]. These data suggest that the karyotypes of these species are rapidly evolving. Spurred by the observation that, despite their greatly divergent karyotypes, horses, asses, and zebras show remarkable morphological similarity and capacity to interbreed, we investigated whether CR events were involved in the rapid chromosomal changes in the genus *Equus*. Toward this goal we performed FISH experiments on the chromosomes of these species to compare the marker order arrangement along orthologous chromosomes.

Results

To identify CR events, we performed a systematic marker order comparison between the chromosomes of donkey, zebra, and horse using an appropriate panel of horse BAC clones for each chromosome. The primary constriction was used as a marker for centromere localization. The clones specific for chromosomes for which detailed and informative results were obtained are reported in Table 1. Precise marker order definition for the remaining chromosomes was precluded by (i) the complex rearrangements that differentiated some donkey chromosomes from their zebra and/or horse homologs, (ii) the very small size of some chromosomes, and (iii) the occasional lack of appropriate probes. Seventy-seven informative BAC clones were selected by screening the CHORI-241 horse BAC library using locus-specific "overgo" probes designed on the

most conserved regions of human, mouse, and rat genomes (Table 1) [17]. This approach has been previously described [12] and was aimed at facilitating marker order comparison with respect to humans. Seventeen additional informative BAC clones, 2 from the same CHORI-241 library and 15 from the INRA horse BAC library [18], were also used. Sixteen of them were previously screened using exonic sequences from specific genes (Table 1, third column, in parentheses), while one of them, TKY321, is a microsatellite. The UCSC coordinates of these loci are reported in the last column (http://genome.ucsc.edu; May 2004 release). Different combinations of 2 or 3 BAC clones, labeled with distinct fluorochromes, were used in cohybridization FISH experiments on metaphases of the three species. Examples of FISH experiments are reported in Fig. 1. Orthologous chromosomes showing different centromere locations, and thus suggestive of CR occurrence, are shown in Fig. 2.

Donkey and zebra are more closely related to each other than either is to horse, since they diverged about 2 MY after the split of horse from their common ancestor [13,14]. This view is strongly supported by cytogenetic data [15,16]. In the present analysis, therefore, the horse was used as an outgroup with respect to donkey and zebra (see Fig. 1c).

Figs. 2a-2c clearly show CR events in donkey chromosomes EAS8, 9, and 11, respectively. In fact, marker order along orthologous chromosomes is conserved in all the three species, while in all three cases the position of the centromere in donkey is different from those of zebra and horse, which share the same centromere position.

The evolutionary history of EAS15, EBU12, and ECA22 appeared initially elusive. However, the arrangement of ECA22 (Fig. 2d), orthologous to human chromosome 20, has been recently shown to be ancestral to mammals [19]. Therefore, the centromere of EAS15 can be reasonably considered as repositioned. The donkey-specific inversion in EAS15, encompassed by a red line in Fig. 2d, does not affect this conclusion. The position of the centromere in EBU12 can be ascribed to an additional zebra-specific CR or to a small inversion involving only the centromere and marker E.

EAS13 is orthologous to ECA11 and to the long arm of EBU10 (Fig. 2e). These three chromosomes show a colinear marker order but a different centromere position. Hence, we cannot infer which centromere arrangement was ancestral, but we can conclude that at least two of the three centromeres are repositioned.

The donkey EAS18 and zebra EBU20 are orthologous to the horse acrocentric ECA26 (Fig. 2f). The three chromosomes share the same marker order, but the location of the horse centromere is different. These chromosomes derive from the fission of the mammalian ancestral 3/21 association, which is thought to maintain the human marker arrangement in the portion containing human HSA21 sequences [20] (sequences orthologous to HSA3 are in silver, arranged according to Yang et al. [16]). The emergence of centromeres at the telomere or at the breakpoint after fission, as hypothesized in Fig. 2f, is very plausible. Indeed this is not a rare event in mammalian evolution [21]. As a consequence, it is likely that a CR event occurred in a common ancestor of donkey and zebra.

The entire donkey chromosome 19 (Fig. 2g) corresponds to the telomeric part of human chromosome 2q (2q35–q37) [15],

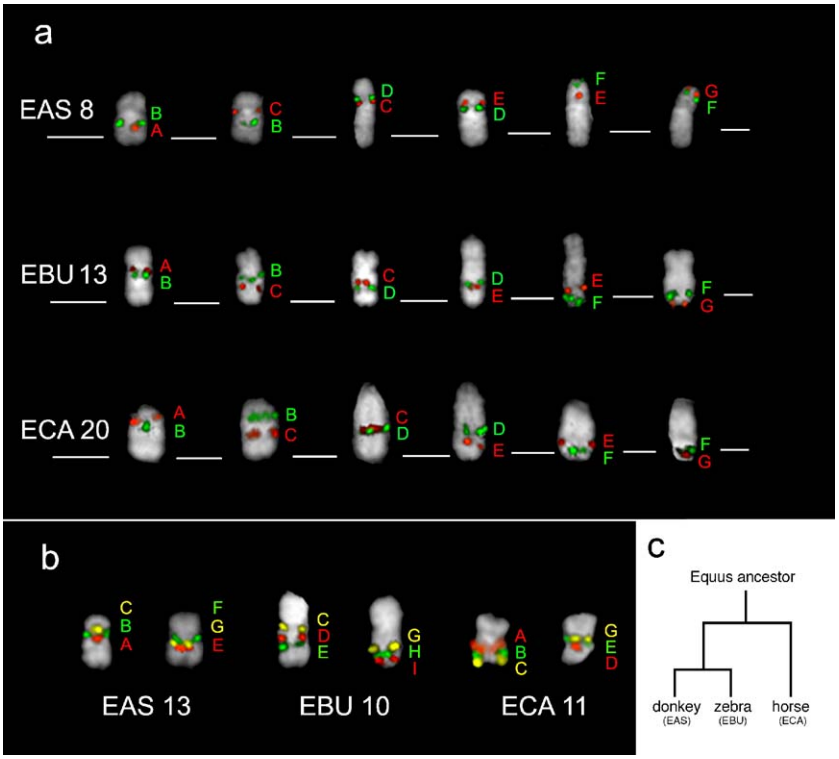

Fig. 1. Examples of FISH cohybridization experiments using (a) two or (b) three BAC clones. The letters correspond to specific BAC clones, as reported in Table 1. See also the legend to Fig. 2. (c) The evolutionary relationship of the three species under study.

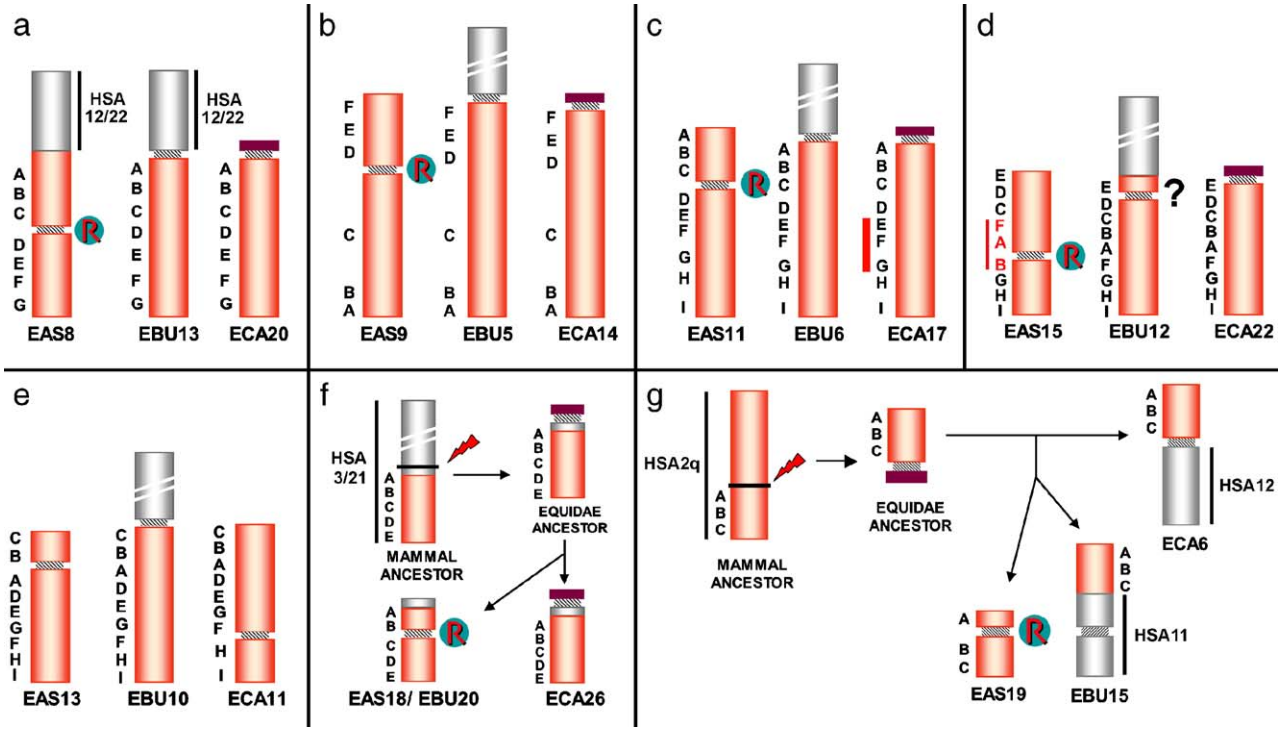

Fig. 2. Diagrams summarizing FISH results on selected chromosomes of donkey (EAS), zebra (EBU), and horse (ECA). Some chromosomes have been inverted to facilitate comparison. Letters on the left of each chromosome refer to specific BAC clones reported in Table 1. Letters were chosen according to the ordered map position, in humans, of the overgo probes or STS sequences used to screen the horse library, as reported in the last column of Table 1. Letters, therefore, help in marker order conservation analysis with respect to humans. Chromosome regions in silver indicate additional components of the chromosome. The "R" in a circle indicates a repositioned centromere. Chromosome length ratio among different sets of images is approximate. For details see text.

the marker order of which has been hypothesized to be conserved in mammal ancestors with respect to humans [20]. This segment is fused with different patterns in horse and in zebra to form ECA6 and EBU15, respectively. Therefore, ECA6p most probably represents the ancestral *Equus* form generated by the chromosome 2q fission, and the position of the centromere in the EAS19 chromosome can be reasonably assumed to be the consequence of a CR event.

Our analysis excluded CR events in donkey chromosomes EAS4, 7, 12, 14, 17, 25, 26, and X and their orthologs in zebra and horse (data not shown). FISH experiments using pericentromeric BAC clones of chromosomes EAS4, 7, and X showed that the shape variations of these chromosomes in donkey were due to a small pericentric inversion previously described for the X chromosome [22].

Discussion

We have investigated the position of the centromere, with respect to flanking markers, in donkey, zebra, and horse chromosomes. On each chromosome the position of the centromere was assumed to coincide with the primary constriction, that is, the structure linking together the chromatids of a metaphase chromosome. It is worth remembering that in the three species each chromosome is characterized by one well-defined primary constriction that is stable and clearly identifiable using standard cytogenetic techniques. It is widely accepted that the functional centromere of vertebrates coincides with the primary constriction.

The results showed that at least eight CR events have occurred in the last 3 MY in the genus *Equus*. Surprisingly, at least five of these events appear to have arisen in the donkey after its divergence from the zebra, which took place approximately 1 MYA [13,14]. It appears, therefore, that in some lineages, the CR phenomenon can be very frequent.

The chromosomal organization of the horse that we have assembled was tested against published papers, in particular with the recently published work of Murphy et al. [21]. Our results agree with horse chromosomal arrangements they propose. Yang et al. [16] performed a comparison of horse and donkey karyotypes using G-banding and painting probes. They noticed centromere position discrepancies between horse chromosomes ECA14, 15, 17, and 22 with respect to the corresponding donkey chromosomes EAS9, 6, 11, and 15. Our data support a CR on ECA14/EAS9, ECA17/EAS11, and ECA22/EAS15 (Figs. 2a, 2b, and 2d), whereas a CR event was excluded for ECA15/EAS6 (data not shown).

The relatively high number of CR events we have documented in donkey is puzzling. These events, like other gross chromosomal changes, are expected to affect the fitness of heterozygous carriers negatively, since an odd number of meiotic exchanges within the chromosomal region encompassed by the old and the new centromeres leads to the formation of dicentric and acentric chromosomes. Meiotic drive in females in favor of the repositioned chromosome is a possible explanation, as reported for Robertsonian fusion in humans [23]. Genetic drift could have also played an important role in neocentromere fixation [13,14]. The recent finding by

Stefansson et al. [24] of a positive selection acting on a small inversion in the human species adds a further point of discussion on how chromosomal changes can be fixed in the population.

As stated, CR events can be disclosed only if a detailed marker order arrangement is established. However, this approach can be inadequate in the case of very small chromosomes or when complex rearrangements differentiate the chromosomes under study. Another limitation is the lack of a comprehensive and detailed evolutionary history of mammalian chromosomes. Due to these limitations, the number of CR events that we detected in the present study is probably underestimated.

Finally, our data add a further twist to the debate on mule and hinny sterility: the presence of at least seven repositioned centromeres between these two species certainly represents an additional potential cause of meiotic disturbances that result in sterility.

Material and methods

Cell lines

Metaphase preparations were obtained from fibroblast cell lines of Burchelli's zebra (*E. burchelli*), donkey (*E. asinus*), and horse (*E. caballus*).

Library screening

Overgo probes of 36–40 bp each were designed on sequences conserved between the human and the mouse genomes according to the HomoloGene database (http://www.ncbi.nlm.nih.gov/HomoloGene/), as described by McPherson et al. [17]. The probes were hybridized to high-density filters of the horse CHORI-241 BAC library (see Results) and the images were analyzed with ArrayVision version 6.0 (Imaging Research, Inc.). The names of the horse BAC clones and their positions in the human sequence (UCSC May 2004 release) of the overgo probes used to screen them are reported in Table 1.

FISH experiments

DNA extraction from BACs has already been reported [2]. FISH experiments were performed essentially as described by Lichter et al. [25]. Digital images were obtained using a Leica DMRXA2 epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments, NJ, USA). Cy3–dCTP, FluorX–dCTP, DEAC, Cy5–dCTP, and DAPI fluorescence signals, detected with specific filters, were recorded separately as gray-scale images. Pseudocoloring and merging of images were performed using Adobe PhotoShop software.

Acknowledgment

Support from the Ministero dell'Istruzione, dell'Università, e della Ricerca is gratefully acknowledged.

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