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Cross-species chromosome painting in the Perissodactyla: delimitation of homologous regions in Burchell's zebra *(Equus burchellii)* and the white *(Ceratotherium simum)* and black rhinoceros *(Diceros bicornis)*

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Abstract. Conserved chromosomal segments in the black rhinoceros, *Diceros bicornis* (DBI, 2n = 84), and its African sister-species the white rhinoceros, *Ceratotherium simum* (CSI, 2n = 82), were detected using Burchell's zebra (*Equus burchellii*, EBU, 2n = 44) chromosome-specific painting probes supplemented by a subset of those developed for the horse (*Equus caballus,* ECA, 2n = 64). In total 41 and 42 conserved autosomal segments were identified in *C. simum* and *D. bicornis* respectively. Only 21 rearrangements (20 fissions and 1 fusion) are necessary to convert the Burchell's zebra karyotype into that of the white rhinoceros. One fission distinguishes the *D. bicornis* and *C. simum* karyotypes which, excluding heterochromatic differences, are identical in all respects at this level of resolution. Most Burchell's zebra chromosomes correspond to two rhinoceros chromosomes although in four instances (EBU18, 19, 20 and 21) whole chromosome synteny has been retained among these species. In contrast, one rhinoceros chromosome (DBI1, CSI1) comprises two separate Burchell's zebra chromosomes (EBU11 and EBU17). In spite of the high diploid numbers of the two rhinoceros species their karyotypes are surprisingly conserved offering a glimpse of the putative ancestral perissodactyl condition and a broader understanding of genome organization in mammals.

The higher level classification of eutherian mammals has undergone extensive scrutiny since the advent of molecular genetic phylogenies (reviewed in Novacek, 2001). Most recent studies would group the Perissodactyla together with Pholidota, Carnivora, and Cetartiodactyla (Waddell et al., 1999) as part of the so-called Laurasiatheria, one of four supraordinal groups of modern mammals (Murphy et al., 2001a, b). Within this assemblage the family Rhinocerotidae comprises five species for which cytogenetic data are available for the white rhinoceros (*C. simum* 2n = 82; Heinichen, 1968; Houck et al., 1994), black rhinoceros (*D. bicornis* 2n = 84; Hungerford et al., 1967; Houck et al., 1994), Indian rhinoceros (*R. unicornis* 2n = 82; Wurster and Benirschke 1968), and the Sumatran rhinoceros (*Dicerorhinus sumatrensis* 2n = 82; Houck et al., 1994). The fifth species, the Javanese rhinoceros, *R. sondaicus* has not been karyotyped. Most authors agree that the two African species are closely related (Groves, 1983) with new molecular data clearly showing a basal rhinocerotid divergence between the African and Asian species, with *D. sumatrensis* the sister-group to the genus *Rhinoceros* (Tougard et al., 2001).

Although G-banding and C-banding data are limited only to two subspecies within the white rhinoceros (*C. s. simum* and

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C. s. cottoni, Houck et al., 1994), that all taxa have either 2n = 82 or 2n = 84 suggests that the Rhinocerotidae is karyotypically highly conserved. This is in marked contrast to most Perissodactyla, particularly the horses, zebras and asses comprising the family Equidae and the tapirs, family Tapiridae. In the case of the former, diploid numbers vary from a low of 2n = 32 in Hartmann's zebra *(E. zebra)*, to 2n = 66 in Przewalski's horse (*E. przewalskii*; Ryder et al., 1978), and in the latter from 2n = 52 in *Tapirus indicus* to 2n = 80 in both *T. bairdii* and *T. terrestris* (Houck et al., 2000). The extreme chromosomal evolution within equids is underscored both by changes in the diploid chromosome number as well as by extensive intrachromosomal rearrangements evident by comparing regions of sequence homology detected by FISH with corresponding G-band patterns (Raudsepp and Chowdhary, 1999).

Given these contrasting patterns of chromosomal evolution, cross-species FISH within the Perissodactyla is pertinent to studies of comparative genome organization in mammals and, at a finer scale, to elucidating the mode and speed of chromosomal change distinguishing the Rhinocerotidae and Equidae. Moreover, the availability of limited human (HSA) vs. horse (ECA) painting data (Raudsepp et al., 1996; Raudsepp and Chowdhary, 2001) allow for the indirect identification of several segmental homologies that exist between the Rhinocerotidae, which have among the highest chromosome number in mammals, and man. (Higher diploid numbers have been recorded for the tetraploid rodent, *Tympanoctomys barrerae* 2n = 102, Contreras et al., 1990; Gallardo et al., 1999; the fish-eating rat, *Anotomys leander* 2n = 92, Gardner, 1971; the semiaquatic rodent *Ichthyomys pittieri* 2n = 92, Schmid et al., 1988; and the sigmodont rodent *Zygodontomys* 2n = 84–86, Mattevi et al., 2002). These data thereby provide a molecular cytogenetic basis to the correspondence within these species.

Materials and methods

Tissue culture, chromosome preparations and conventional banding

Skin biopsies were taken from white and black rhinoceroses under permit to E. Harley, Department of Chemical Pathology, University of Cape Town. Fibroblast cultures were established using routine procedures; chromosome banding was by trypsin (G-banding) and BaOH treatment (C-banding).

Painting probes

Chromosome-specific painting probes were made for the horse *(E. caballus)* and Burchell's zebra *(E. burchellii)* by degenerate oligonucleotide PCR (DOP-PCR) of flow-sorted chromosomes of both species (Yang et al., in press). The *E. burchellii* flow-sorts were characterized and found to correspond to EBU1, 2, 3, 4, 5, 6, 7, 8 + X, 9, 10 + 12, 11, 13 + 14, 15, 16, 17, 18, 19, 19 + 20 and chromosome 21. In an attempt to resolve those instances where more than a single chromosome was isolated in a specific flow peak, a subset of *E. caballus* chromosome paints was used: ECA4 and 31 which correspond to EBU8, and ECA22, 29, 30 which correspond to the *E. burchellii* chromosome 12. The flow peak containing EBU8 + X was further defined through the use of an ECA X painting probe. We were unable to resolve EBU13 + 14.

Fluorescence in situ hybridization

G-banding was performed on black and white rhinoceros chromosomes prior to FISH. Selected metaphases were digitally recorded following which the chromosomes were destained and treated with 1% formaldehyde in PBS to prevent subsequent over-denaturing. In situ hybridization using equid whole chromosome painting probes followed routine procedures. In brief, following a pepsin pretreatment (0.01% in 10 mM HCl at 37°C for 5 min), 10 µl of a probe solution (150 ng biotin- or digoxigenin-11-dUTP-labeled probe DNA in 50% formamide, 2× SSC and 10% dextran sulfate) was denatured for 5 min at 75°C. It was subsequently incubated 30 min at 37°C to suppress repeated sequences. Slides were denatured in 70% formamide, 2× SSC at 71°C for 3 min followed by dipping in a cold alcohol series (70%, 80%, 95%). Probes were sealed under a 22 × 22 mm glass coverslip and hybridization was carried out in a moist chamber at 37°C for 48 h. Following this the coverslips were removed, the slides washed twice in 50% formamide, 2× SSC at 42°C, twice in 2× SSC at 42°C, and then finally in 0.2× SSC at 42°C for 3 min. Signals were detected with avidin-Cy3 (biotin) or fluorescein isothiocyanate (FITC)-conjugated antidigoxigenin (digoxigenin). Slides were counterstained with DAPI. Hybridization signals were captured using "Genus" software (Applied Imaging) and assigned to specific chromosomes, or chromosome regions, using the G-banding patterns obtained prior to in situ hybridization.

Results and discussion

C. simum

The chromosomal complement of the white rhinoceros (a female) comprised 40 pairs of acrocentric autosomes and pair of metacentric X chromosomes (2n = 82). The G-banded and C-banded karyotypes of this species are published in Houck et al. (1994) and not repeated here.

D. bicornis

The karyotype of the black rhinoceros (male) comprised 41 pairs of acrocentric autosomes, a metacentric X chromosome and acrocentric Y chromosome (2n = 84, Fig. 1). The chromosomes were arranged following the format published by Houck et al. (1994) for the white rhinoceros. C-banding revealed large heterochromatic short arms in most large autosomes (1–7) as well as chromosomes 20 and 39 (Fig. 2). C-band heteromorphism was evident in the single specimen available to us and was most pronounced in pair 21.

Comparative cytogenetics and cross-species chromosome painting

G-banding revealed a close correspondence between the larger autosomes of both species, but the determination of G-band homology among the small autosomes is, in many instances, equivocal and in the case of the smallest dot-like chromosome (41), impossible.

The *E. burchellii* painting probes produced identical hybridization patterns in both rhinoceros species with the exception of a single chromosome pair in the white rhinoceros (CSI2), which is present as two independent chromosome pairs (DBI2 and DBI41) in the black rhinoceros (Table 1). It is this rearrangement that accounts for the difference in 2n characterizing the African rhinoceros species. The painting probe that contained both the *E. burchellii* X and chromosome 8 was found to hybridize to three chromosomes in the white rhinoceros (CSIX, CSI2 and CSI37, Fig. 3a), and to four in the black rhinoceros (DBIX, DBI2, DBI37 and DBI41). The X was identified using the ECAX painting probe allowing confirmation of the homologues of EBU8 in *C. simum* (CSI2 + CSI37) and *D. bicornis* (DBI2 + DBI37 + DBI41) respectively. Moreover, by using horse probes ECA4 and ECA31 (homologous to EBU8, Ta-

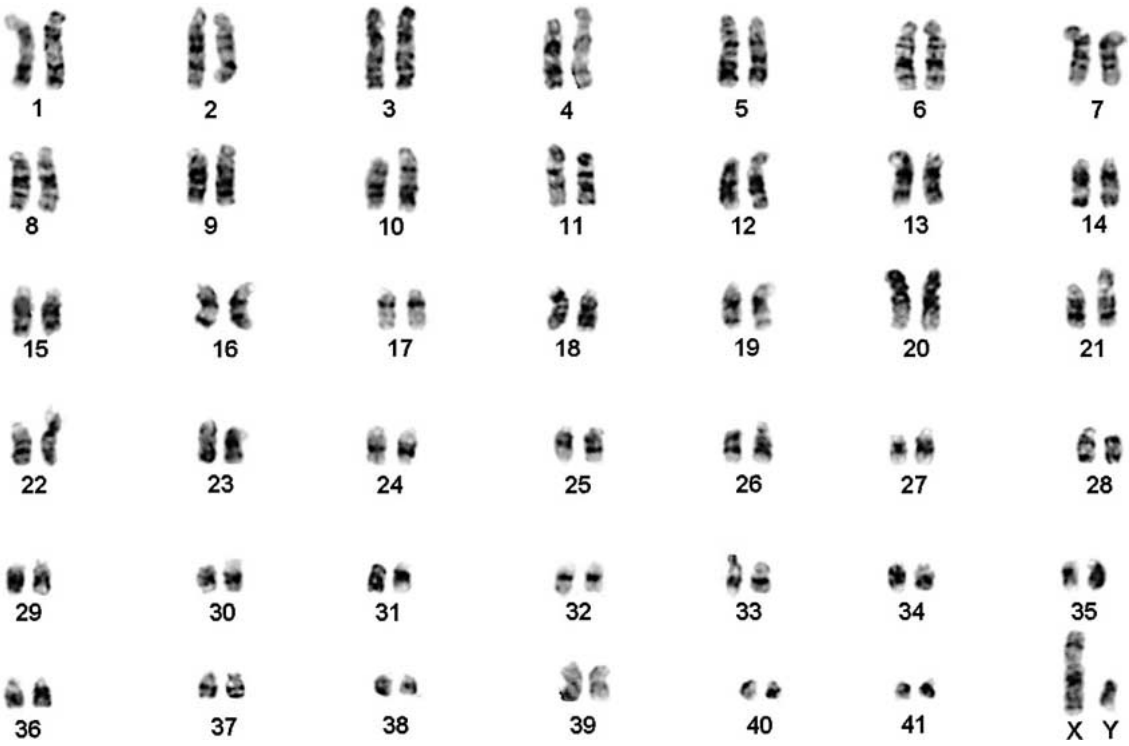

Fig. 1. G-banded karyotype of a male black rhinoceros, *D. bicornis* (2n = 84).

Fig. 2. C-banded karyotype of a male black rhinoceros, *D. bicornis* (2n = 84). Sequential G/C-banding was used to identify specific chromosomes.

Table 1. Chromosomal homologies among the white *(C. simum)* and black rhinoceroses *(D. bicornis)*, Burchell's zebra *(E. burchellii)* and selected horse chromosomes *(E. caballus)* identified by cross-species chromosome painting

Burchell's zebra (EBU)	White rhinoceros (CSI)	Black rhinoceros (DBI)
1	4, 19, 30	4, 19, 30
2	3, 6	3, 6
3	9, 13	9, 13
4	7, 16	7, 16
5	5, 23	5, 23
6	10, 18	10, 18
7	11, 20, 40	11, 20, 40
8 + X	X, 2, 37	X, 2, 37, 41
9	15, 21	15, 21
10 + 12	12, 17, 25, 29, 36	12, 17, 25, 29, 36
11	1proximal, 32, 33	1proximal, 32, 33
13 + 14	8, 14, 34, 39	8, 14, 34, 39
15	31, 38	31, 38
16	29, 35	29, 35
17	1distal	1distal
18	22	22
19 + 20	24, 27	24, 27
20	27	27
21	26	26
Horse (ECA)		
4 (EBU8)	2	2, 41
31 (EBU8)	37	37
22 (EBU12)	25	25
29 (EBU12)	29	29
30 (EBU12)	36	36
X	X	X

ble 1) we showed that ECA31 corresponds to CSI37 and DBI37, and ECA4 paints CSI2 and DBI2 + DBI41.

Although we could resolve the hybridization patterns resulting from the use of some paints that comprised more than a single chromosome (EBU8 + X - see above, EBU10 + 12, and EBU19 + 20) we were unable to do so in the case of EBU13 + 14. In this instance we can only state that Burchell's zebra chromosomes 13 and 14 correspond to four pairs of chromosomes in the African rhinoceros species (Table 1, Fig. 3b). The hybridization patterns obtained with the zebra painting probe EBU11 were more complex. This *E. burchellii* chromosome was shown to comprise the entire euchromatic portion of the African rhinoceros species' chromosomes 32 and 33, as well as the proximal portion of chromosome 1 (Fig. 3c). The distal part of rhinoceros chromosome 1 was painted by probe EBU17 (Fig. 3d). Four *E. burchellii* painting probes (EBU18, 19, 20 and 21) each hybridize to single chromosomes in both the white and black rhinoceros genomes. Nine *E. burchellii* chromosome probes (EBU2, 3, 4, 5, 6, 9, 10, 15, 16) each detect two chromosomes in *D. bicornis* and *C. simum*, and a further three *E. burchellii* chromosomes (EBU1, 7 and 12) each delimit three homologous rhinoceros chromosomes in both species.

The results of the hybridization of the 19 flow-sorted Burchell's zebra probes (EBU) and six horse probes (ECA) to metaphase chromosomes of *D. bicornis* and *C. simum* are presented in Table 1 and summarized in Fig. 4. When taken together, our data reveal 41 autosomally conservative segments between Burchell's zebra and the white rhinoceros, and 42 between Burchell's zebra and the black rhinoceros (Fig. 4). In total 20 fissions and one fusion are necessary to convert Burchell's zebra karyotype to that of the white rhinoceros, with 21 fissions and one fusion accounting for that of the black rhinoceros.

Elements of the likely ancestral perissodactyl karyotype and correspondence with man

Traditionally the extant Perissodactyla are thought to comprise two suborders, the Hippomorpha (horses and their relatives, i.e. the Equidae) and the Ceratomorpha (the tapirs and rhinoceroses, i.e. the Tapiridae and Rhinocerotidae). This dichotomy is generally well supported by molecular data (Tougard et al., 2001) with clock calibrations indicating a divergence between the Ceratomorpha and Hippomorpha lineages at approximately 47 MYA (Tougard et al., 2001). Although far from having complete taxon representation for the Perissodactyla our data do, nonetheless, encompass both evolutionary lineages, the Hippomorpha (represented by *E. burchellii* and *E. caballus*) and the Ceratomorpha (represented by the Rhinocerotidae). This allows for the identification of several chromosomes that are likely perissodactyl ancestral states.

Our data show that there are at least five rhinoceros autosomes (2, 25, 29, 36, 37) that are conserved in the horse (ECA4, 22, 29, 30, 31). Furthermore, there are five rhinoceros chromosomes conserved as single chromosomes in Burchell's zebra and both rhinoceros species (CSI, DBI17, 18, 19, 20, 21). These data suggest the likely presence of all ten chromosomes in the ancestral perissodactyl karyotype. Moreover, rhinoceros chromosome 25 is homologous to horse chromosome 22 (part of zebra chromosome 12). This chromosome (ECA22) corresponds to donkey *(E. asinus)* chromosome 15 and human chromosome (HSA) 20 (Raudsepp et al., 1996; Raudsepp and Chowdhary, 2001; Richard et al., 2001). HSA20 has been retained in toto in different mammalian orders, including the rearranged karyotypes of rodents (Serikawa et al., 1998) and the dog (Yang et al., 1999), and is clearly ancestral to all eutherian mammals (see Murphy et al., 2001c, Yang et al., 2003).

Other interspecific homologies identified in our study include ECA4 and ECA31 which correspond to EBU8 of Burchell's zebra (Table 1). ECA4 is homologous to a large part of HSA7 (Raudsepp et al., 1996; Milenkovic et al., 2002) and to CSI2 in the white rhinoceros and DBI2 and DBI41 in the black rhinoceros (discussed in more detail below). ECA31 on the other hand corresponds to part of HSA6q (Milenkovic et al., 2002) and to autosomal pair 37 in both the black and white rhinoceros. Of the remaining *E. caballus* paints at our disposal, ECA29 corresponds to chromosome 29 in both rhinoceros species as well as to part of HSA10 (Raudsepp et al., 1996). Finally, ECA30 (which paints chromosome 36 in the rhinoceros species) corresponds to part of HSA1, probably HSA1q (Milenkovic et al., 2002). Quite clearly, however, the correspondence between human and rhinoceros chromosomes suggested herein should be corroborated using direct chromosome painting or gene mapping approaches.

Tempo of chromosomal evolution in the Rhinocerotidae

Our data indicate that the karyotypes of two rhinoceros species differ by a single fission event, which caused the differences in 2n between them. Determination of the polarity of this

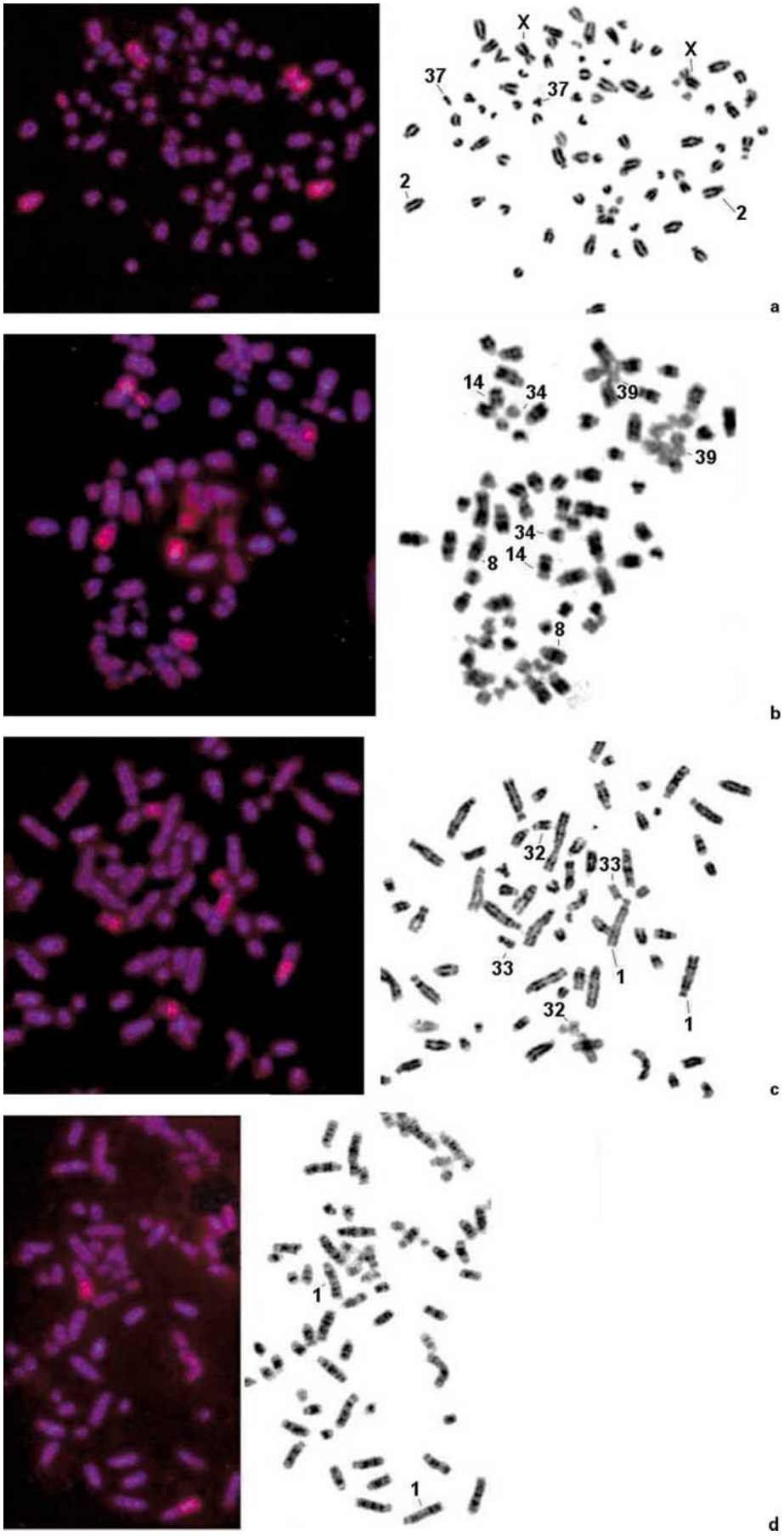

Fig. 3. Examples of FISH to rhinoceros chromosomes using painting probes derived from Burchell's zebra (EBU). (**a**) EBU8 + X hybridized to chromosomes CSIX, 2, and 37. (**b**) The EBU flow-sort comprising chromosomes 13 + 14 showing hybridization to four pairs of autosomes in the white rhinoceros (CSI8, 14, 34, 39). (**c**) EBU11 hybridized to rhinoceros chromosomes CSI1 (proximal part), 32, and 33. (**d**) EBU17 hybridized to the distal part of white rhinoceros CSI1.

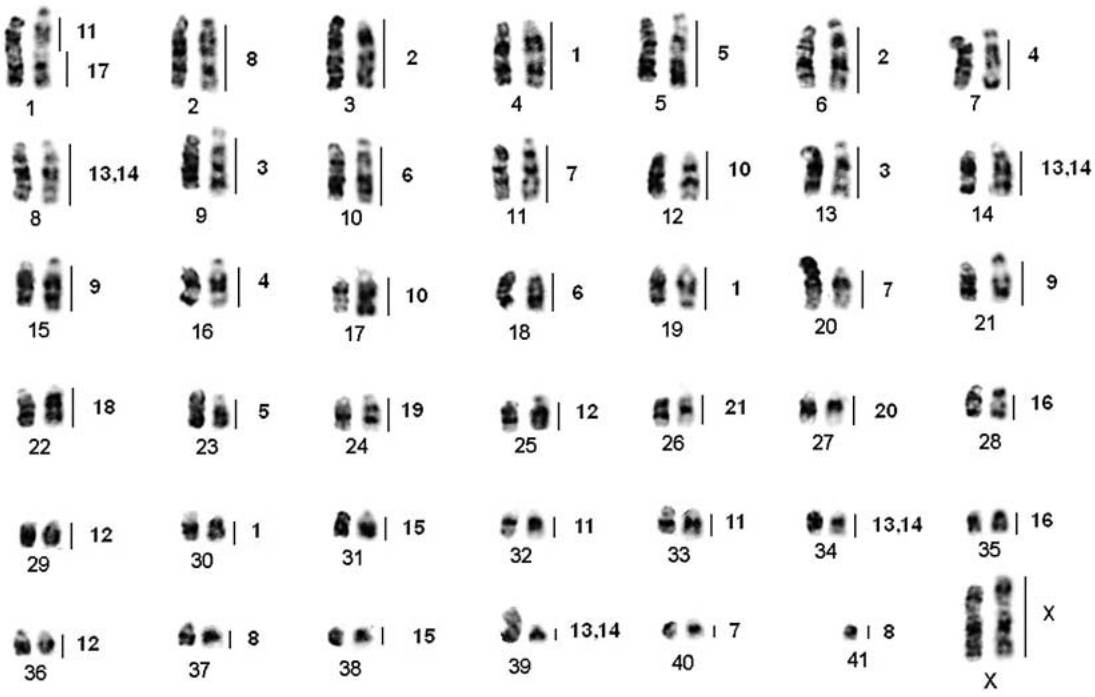

Fig. 4. G-banded chromosomes of the black (left) and white rhinoceros (right) species. The approximate regions of homology to Burchell's zebra and selected horse chromosomes are shown to the right of each pair of rhinoceros chromosomes.

change is evident from outgroup comparisons. ECA4 and 31 are syntenic in Burchell's zebra EBU8. In turn ECA4 paints to the whole of white rhinoceros CSI2 but to two chromosomes in the black rhinoceros (DBI2 and 41) and this arrangement is therefore unique to this species (i.e. an autapomorphy). Should this hold, a karyotype similar to that of the white rhinoceros may have been retained in the Rhinocerotidae for approximately 30 million years with the single rearrangement (disruption of chromosome 2 in *C. simum* into chromosomes 2 and 41 in *D. bicornis*) punctuating the evolutionary trajectory of the African species at approximately 17 MYA (see Tougard et al., 2001 and references therein for molecular clock estimates). In sharp contrast to this, the Equidae have undergone extensive karyotypic evolution (diploid numbers range from 32 to 66), with the horses' divergence from the zebra/ass ancestor occurring as recently as ~2.4 MYA, and the rapid radiation of the zebra and ass species at ~0.9 MYA (Oakenfull and Clegg, 1998). These data emphasize extremes in the rate of change both within one evolutionary lineage (the Perissodactyla), and in mammals in general where previous estimates of the rate of chromosomal evolution across several eutherian lineages suggest 1–10 changes per million years (O'Brien et al., 1999).

In conclusion, our data, which span approximately 50 million years of eutherian evolution, contribute significantly to ongoing studies of chromosome evolution and genome organization in the Perissodactyla. Further progress will be achieved by extending the cross-species chromosome painting schemes to include the human and the full suite of horse (ECA) painting probes. These endeavors will result in a detailed comparative map that will link an economically important species (horse) to one of the most karyotypically fractured eutherian karyotypes (rhinoceros) and, through the use of human as index species, to other eutherian lineages.

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