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Karyotypic relationships of horses and zebras: results of cross-species chromosome painting

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Abstract. Complete sets of chromosome-specific painting probes, derived from flow-sorted chromosomes of human (HSA), *Equus caballus* (ECA) and *Equus burchelli* (EBU) were used to delineate conserved chromosomal segments between human and *Equus burchelli*, and among four equid species, *E. przewalskii* (EPR), *E. caballus, E. burchelli* and *E. zebra hartmannae* (EZH) by cross-species chromosome painting. Genome-wide comparative maps between these species have been established. Twenty-two human autosomal probes revealed 48 conserved segments in *E. burchelli*. The adjacent segment combinations HSA3/21, 7/16p, 16q/19q, 14/15, 12/22 and 4/8, presumed ancestral syntenies for all eutherian mammals, were also found conserved in *E. burchelli*. The comparative maps of equids allow for the unequivocal characterization of chromosomal rearrangements that differentiate the karyotypes of these equid species. The karyotypes of *E. przewalskii* and *E. caballus* differ by one Robertsonian translocation (ECA5 = EPR23 + EPR24); numerous Robertsonian translocations and tandem fusions and several inversions account for the karyotypic differences between the horses and zebras. Our results shed new light on the karyotypic evolution of Equidae.

The family Equidae (horses, zebras and asses) comprises seven extant species (Nowak, 1999) that shared a common ancestor ~1.9–2.3 million years ago, with the extant species emerging at approximately 0.89–1.07 million years ago according to the latest estimate (Oakenfull et al., 2000). The equids are remarkable both for their rapid karyotypic diversification as well as variation in diploid numbers which range from 2n = 32 in Hartmann's mountain zebra (*Equus zebra hartmannae*; Benirschke and Malouf, 1967) to 2n = 66 in Przewalski's horse (*E. przewalskii*; Benirschke at al., 1965). Although early conventional chromosome banding comparisons made it possible to identify several likely homologues among extant species, the complexity of the genomic rearrangements confounded attempts to provide a genome-wide view of the modes and tempo of chromosomal change in the various equid lineages (Ryder et al., 1978).

Cross-species chromosome painting (Scherthan et al., 1994) in combination with chromosome sorting, comparative chromosome banding and digital imaging microscopy, offers an extremely powerful approach for delimiting true regions of chromosomal homology in mammals, which are essential to attempts to develop genome-wide homology maps among mammalian species (Yang et al., 1995). It is particularly pertinent to comparisons between distantly related species, species with highly rearranged karyotypes, as well as taxa for which mapping and other genomic data are rare or absent (for review see Chowdhary and Raudsepp, 2001). We have reexamined the karyotypic relationships among the domestic horse *(E. caballus)*, Przewalski's horse *(E. przewalskii)*, Burchell's zebra

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(E. burchelli) and Hartmann's mountain zebra *(E. z. hartmannae*) by cross-species chromosome painting and present here the first genome-wide comparative chromosome maps of these four equid species. The taxonomy of the equids is a subject of debate. For ease of presentation we follow Ryder et al. (1978) in recognizing *E. przewalskii* as a distinct species although some authors include *E. przewalskii* in *E. caballus*; Wilson and Reeder (1993), Nowak (1999). Most recently Groves and Ryder (2000) designate the domestic and Przewalski's horses as *E. ferus*]. In addition, we provide a comparative map between human and Burchell's zebra.

Materials and methods

Metaphase preparations

Fibroblast cell lines of four equid species were used in this study. Cell lines of *E. caballus* and *E. burchelli* were provided respectively by the Kunming Cell Bank of the Chinese Academy of Sciences and University of Cape Town. Cell lines for *E. przewalskii* and *E. z. hartmannae* were obtained from the Zoological Society of San Diego Center for Reproduction of Endangered Species. Metaphase chromosomes were prepared from fibroblast cultures grown at 37 °C in Dulbecco's modification of minimal essential medium (GIBCO) enriched with 10 % fetal bovine serum (GIBCO), penicillin (100 units/ml) and streptomycin (100 mg/ml). Chromosome preparations were made following standard procedures that included a 15-min hypotonic treatment in 0.4 % KCl, fixation in 3:1 methanol:glacial acetic acid, and air-drying.

Flow sorting and generation of chromosome-specific painting probes for E. burchelli

Chromosomes of *E. burchelli* were sorted on a dual laser cell sorter (FACStar Plus, Becton Dickinson) as previously described (Yang et al., 1995). Chromosome-specific painting probes were made by degenerate oligonucleotide-primed PCR (DOP-PCR) amplification of flow-sorted chromosomes (Telenius et al., 1992). DOP-PCR amplified chromosome-specific DNAs were labeled during the secondary PCR by either incorporating biotin-16-dUTP (Roche), fluorescein-12-dUTP (Roche) or Cy3-dUTP (Amersham). The generation and characterization of human and *E. caballus* chromosome painting probes have been previously described (Ferguson-Smith, 1997; Yang et al., in press).

Nomenclature

The *E. caballus* chromosomes were identified according to the international standard nomenlature for *E. caballus* (Bowling et al., 1997); *E. przewalskii* chromosomes were arranged and numbered in most part following Ryder et al. (1978) and the international standard nomenclature for *E. caballus* (ISCNH, 1997). The *E. z. hartmannae* karyotype follows that of Richard et al. (2001). The *E. burchelli* chromosomes were arranged according to decreasing length.

Fluorescence in situ hybridization

Comparative chromosome painting between *E. burchelli* and human followed Yang et al. (1997, 2003). For comparative painting among the equid species hybridization time was 16–24 h and the temperature of the post-hybridization washes was 45 °C. No equid competitor DNA was used in the hybridization protocol. In cases where identification of chromosomes by DAPI (4′,6-diamidino-2-phenylindole) banding was ambiguous, sequential trypsin G-banding (Seabright, 1972) and 2–7 color FISH experiments were performed. Briefly, metaphase slides were baked at 65 °C for 3 h and then treated with 0.005 % trypsin for 8–12 min before staining with 2 % Giemsa for 10 min. After image capture of G-banded metaphases using the CytoVision system, the immersion oil and Giemsa stain were removed by serially washing the slide for 5 min in 100 % ethanol followed by 100 % methanol. The slides were then baked at 65 °C for at least 1 h. The G-banded slides were subsequently denatured in a 70 % formamide/30 % 2× SSC (v/v) solution at 60 °C for 20–30 s. The hybridization, post-hybridization washes and detection conditions follow the procedure outlined above. In the case of multicolor FISH, probes were labeled with biotin-, FITC- and Cy3-dUTP according to the combinatorial labeling procedure proposed by Ried et al. (1992) and visualized with avidin-Cy5 and rabbit anti-FITC and FITC-conjugated goat anti-rabbit antibodies.

Results

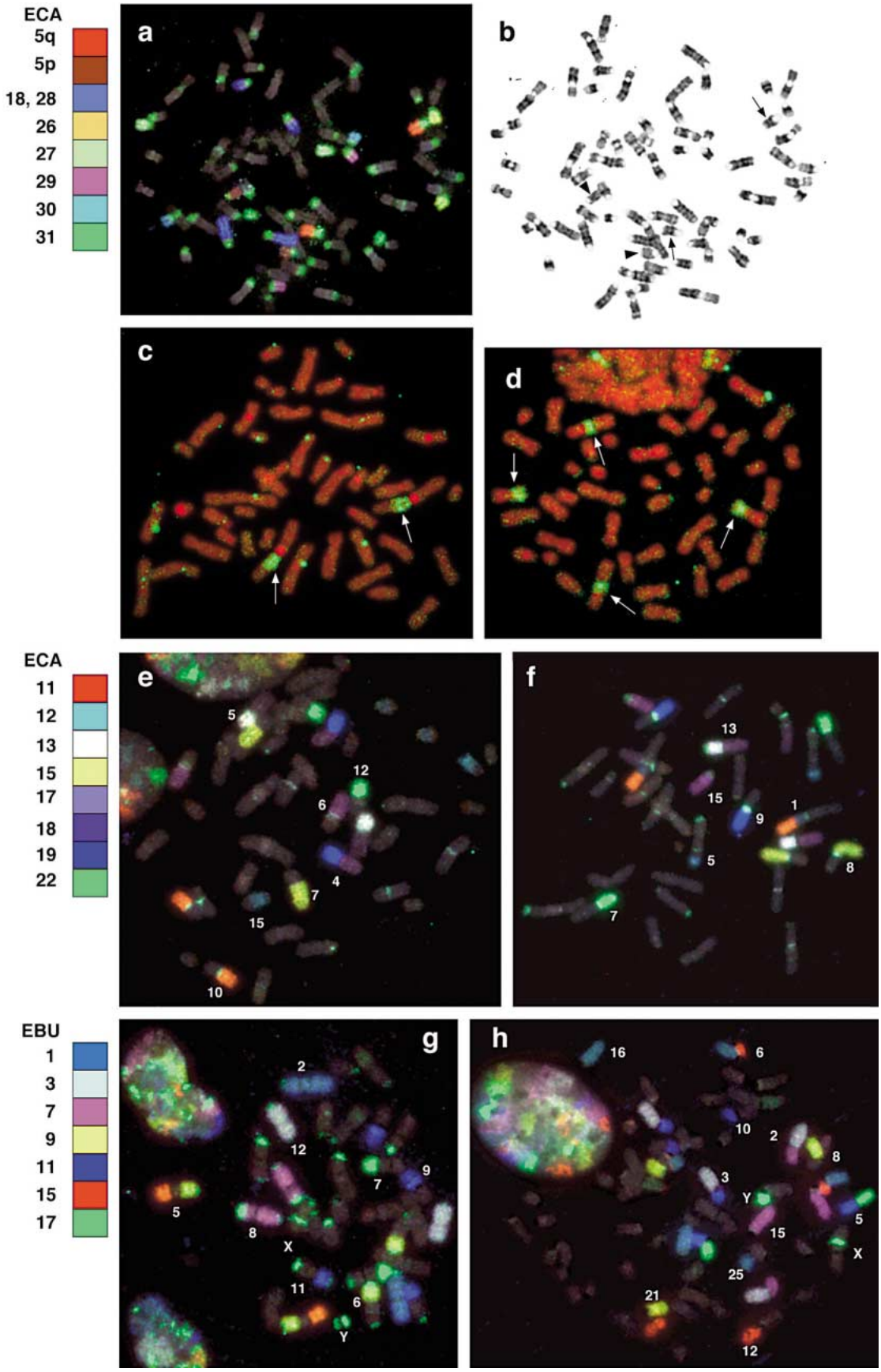
E. caballus – E. przewalskii comparison

To establish genome-wide homologies between *E. caballus* (2n = 64) and *E. przewalskii* (2n = 66) we hybridized the full complement of ECA painting probes (ECA1–31, X) onto *E. przewalskii* metaphases. Examples of comparative painting are shown in Fig. 1a and b and the summary of genome-wide correspondence between these two species is presented in Fig. 2. Our results confirm earlier investigations that showed that one Robertsonian translocation differentiates the karyotypes of these species (Benirschke et al., 1965; Short et al., 1974; Ryder et al., 1978). Our results provide for the unequivocal identification of the chromosomes that have been involved in the karyotypic divergence of these two horse species (i.e. ECA5 and EPR23 and EPR24). ECA5 can be reconstructed from the acrocentrics EPR23 and 24 via one centric fusion which accounts for the observed difference in 2n between them.

Reciprocal chromosomal painting between E. caballus and E. burchelli

We were able to make chromosome-specific painting probes for 15 of the 22 *E. burchelli* chromosome pairs (EBU1–7, 9, 11, 15–19, and 21). EBU8 and X were found in the same flow-peak, as were EBU10 and EBU12, EBU13 and 14, and EBU20 and one homologue of EBU19 (Fig. 3). Paints derived from EBU1, 8 + X, 10 + 12, and 17–21 show strong cross-hybridization to the heterochromatic regions of these chromosomes. In particular, this was most marked at 1pter, 12pter, 17–21qter and the interstitial heterochromatic region of Xq (data not shown) and is likely to be due to the existence of homologous repetitive sequences in these regions.

Fig. 1. Examples of cross-species chromosome painting. (**a**, **b**) Simultaneous painting of a G-banded metaphase of *Equus przewalski* (EPR) with probes for eight *E. caballus* (ECA) chromosomes by multicolor FISH. The color for each probe is shown to the left. Note that probe for *E. burchelli* (EBU) chromosome 17 (= ECA5q) was added to differentiate the ECA5q from ECA5p. The painting result demonstrates that ECA5q = EPR23 (arrows) and ECA5p = EPR24 (arrowheads). (**c**) Hybridization of EBU17 probe to the proximal region of human (HSA) 1p. (**d**) Hybridization of HSA9 probe onto the proximal part of EBU1q and to EBU6p. (**e**, **f**) Simultaneous hybridization of ECA11–13, 15, 17–19 and 22 probes onto metaphases of *E. burchelli* (**e**) and *E. z. hartmannae* (**f**) by multicolor FISH, with the color of each probe given to the left. In both instances only the identity of one of the two homologues is shown. Note that EZH13 is painted by probes from ECA13 and 18 (**f**), and that EBU4 is painted by probes from ECA18 and 19. (**g**, **h**) Simultaneous hybridization of EBU1, 3, 7, 9, 11, 15 and 17 probes onto metaphases of *E. z. hartmannae* (**g**) and *E. caballus* (**h**) by multicolor FISH, with the color of each probe indicated to the left. On the metaphase spread the identity of one of the two painted homologues plus the X and Y are shown. EBU17 probe (in green) shows strong cross-hybridization to the telomeric regions of several EZH chromosomes. It also hybridizes to heterochromatic regions on Xq and Y in both *E. burchelli* and *E. z. hartmannae.*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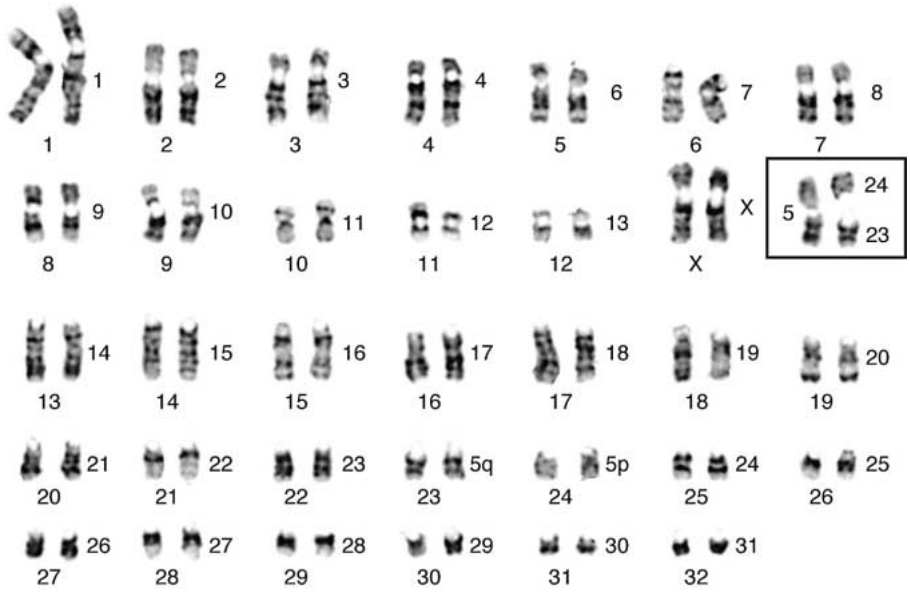

Fig. 2. Summary of genome-wide chromosomal correspondence between *E. caballus* and *E. przewalskii* with G-banded karyotype of *E. przewalskii* as the reference. The identities of *E. przewalskii* chromosomes are shown below each homologous pair; the numbers of corresponding *E. caballus* chromosomes are shown to the right. The insert demonstrates that ECA5 can be reconstructed from EPR23 and EPR24 via a centric fusion.

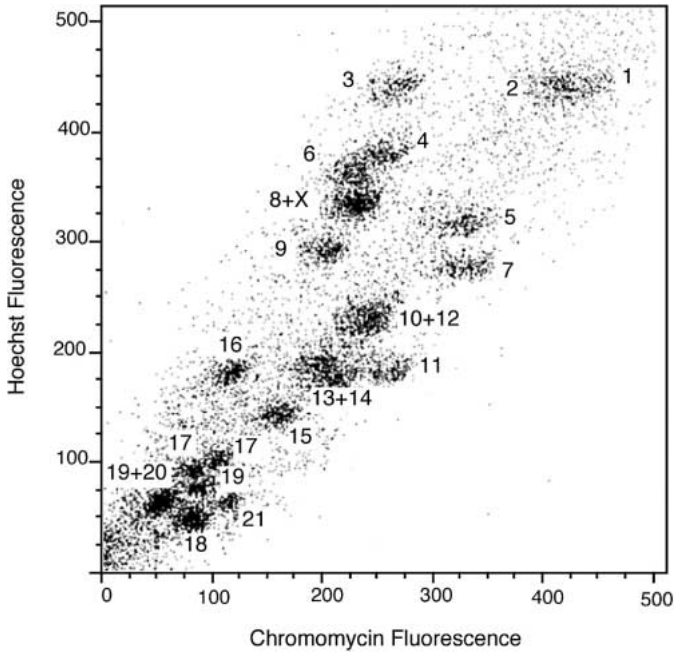

Fig. 3. Bivariate flow karyotype of *E. burchelli.* Note that EBU8 and X were found in the same flow-peak, as were EBU10 and EBU12, EBU13–14, and EBU20 and one homologue of EBU19.

Reciprocal painting was used to define unambiguously the genome-wide homologies that exist between *E. caballus* and *E. burchelli*. Our approach was, first, to hybridize the complete set of *E. caballus* paints (ECA1–31, X) to metaphase chromosomes of *E. burchelli*. Six autosomal probes (ECA2, 3, 5, 6, 8, and 10) each produced signals on two pairs of *E. burchelli* chromosomes; the remaining 25 autosomal painting probes and the X each hybridized to a single pair of EBU chromosomes. In total, the 31 ECA autosomal probes delimited 37 homologous segments in the *E. burchelli* genome (Fig. 4). Secondly, we hybridized paints derived from all the *E. burchelli* flow-peaks (including those that contain two types of chromosomes) to *E. caballus* metaphases to resolve the sub-chromosomal homologies of *E. caballus* that correspond to multiple *E. burchelli* chromosomes (or chromosomal segments) and vice versa. Examples of the reciprocal painting are shown in Fig. 1e and h and the chromosomal correspondence between these two species is summarized in Fig. 4. Although four probes each represent two types of EBU chromosomes (i.e. EBU8 and X, EBU10 and 12, EBU13 and 14, EBU19 and 20), the reciprocal painting results allow for the establishment of one-to-one correspondence between conserved chromosomal segments in the genomes of *E. caballus* and *E. burchelli* (Fig. 4). In brief, seven *E. burchelli* chromosomes (EBU2, 14, 16, 18–21) are each homologous to one entire ECA chromosome. EBU17 is homologous to ECA5q; EBU1, 11 and 12 are each homologous to three ECA

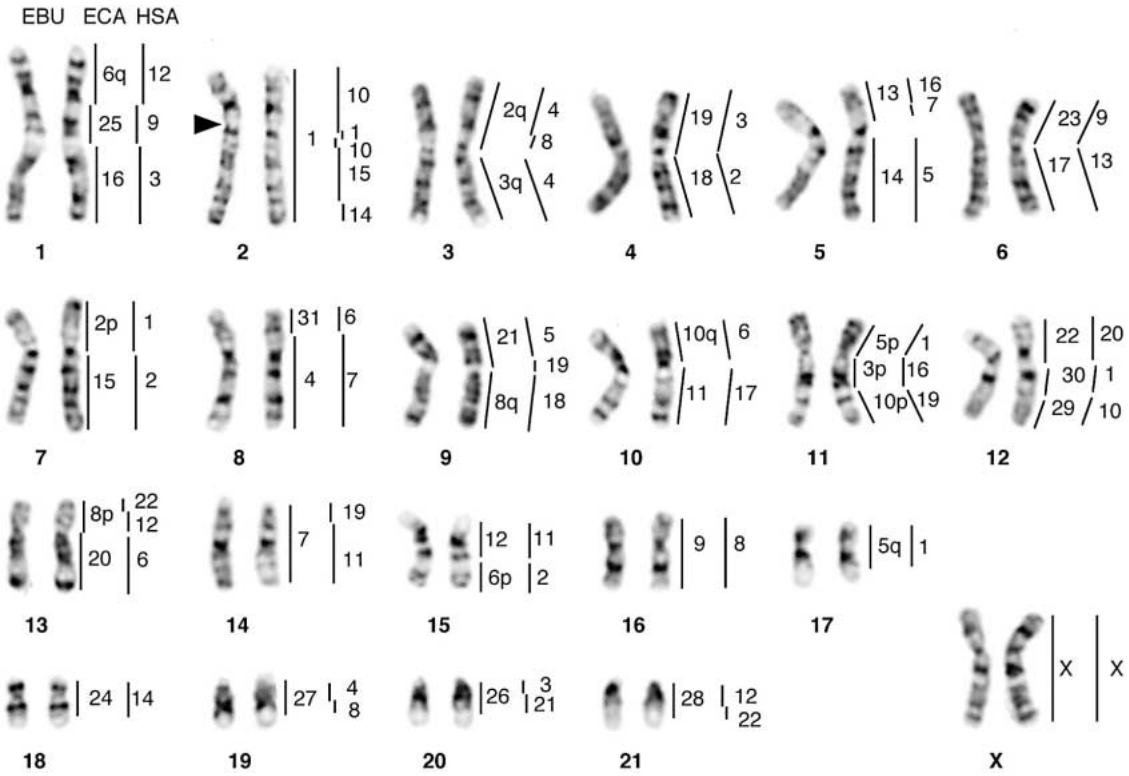

Fig. 4. Summary of genome-wide chromosomal correspondence among *E. burchelli, E. caballus* and human with the G-banded karyotype of *E. burchelli* as reference. The numbers below each homologous pair identify the chromosomes of *E. burchelli*; homology with *E. caballus* (ECA) and human (HSA) is indicated to the right of each EBU chromosomal pair. Note that reciprocal painting enabled the extension of homology to the subchromosomal level. Arrowhead indicates the position of the centromere on EBU2.

chromosomes and/or chromosomal arms. The remaining 10 EBU autosomes (3–10, 13 and 15) each correspond to two ECA chromosomes and/or chromosome arms. Most of the interspecies homologues show a high degree of conservation in G-banding patterns. A notable exception to this is EBU14 and its horse homologue ECA7 (= EPR6; Fig. 2) which differ in banding pattern, probably as a result of a pericentric inversion.

Reciprocal painting between human and E. burchelli

Cross-species reciprocal painting was used to map the evolutionarily conserved segments between *E. burchelli* and human genomes. Examples of chromosome painting are shown in Fig. 1c and d and hybridization patterns of all probes are summarized against a G-banded karyotype of *E. burchelli* (Fig. 4) as well as on the human idiogram (Fig. 5).

The twenty-two human autosomal paints defined 49 conserved segments in the zebra genome. Paints derived from the 21 EBU autosomes detected 60 conserved segments in the human genome. EBU16, 17 and 18 are each homologous to one human chromosomal segment. The remaining 18 EBU autosomes correspond to 2–5 homologous segments in the human genome. Six human chromosomes (HSA13, 15, 17, 18, 20 and 21) each correspond to one chromosomal segment in *E. burchelli*, indicative of complete synteny conservation.

Painting E. z. hartmannae chromosomes with probes from E. caballus and E. burchelli

To establish the genome-wide correspondence among *E. z. hartmannae, E. burchelli* and *E. caballus*, the complete complement of *E. caballus* and *E. burchelli* painting probes were hybridized onto the metaphase chromosomes of *E. z. hartmannae*. Examples of the FISH results are shown in Fig. 1f and g and a summary of the hybridization patterns is presented in Fig. 6. The 31 *E. caballus* autosomal paints revealed 38 homologous segments in *E. z. hartmannae.* Seven painting probes (ECA2, 3, 4, 5, 6, 8 and 10) each painted two chromosomes or chromosomal segments, with the remaining 24 probes corresponding to a single chromosome or chromosomal segment in *E. z. hartmannae*. The painting probes derived from the 21 autosomal chromosomes of *E. burchelli* detected 30 conserved segments in the *E. z. hartmannae* genome. In addition, the paints derived from ECAX and Y, EBU1, 8 + X, 10 + 12, 13 + 14, 17–21 and X show strong cross-hybridization to the heterochromatic regions on EZHXq and Y as well as the telomeric regions of several EZH autosomes including EZH1, 2, 4, 7, 8, 10–12 presumably indicating that these chromosomes share similar repeats.

The integration of hybridization results of human probes (Richard et al., 2001) and *E. caballus* and *E. burchelli* probes (this study) onto *E. z. hartmannae,* together with reciprocal

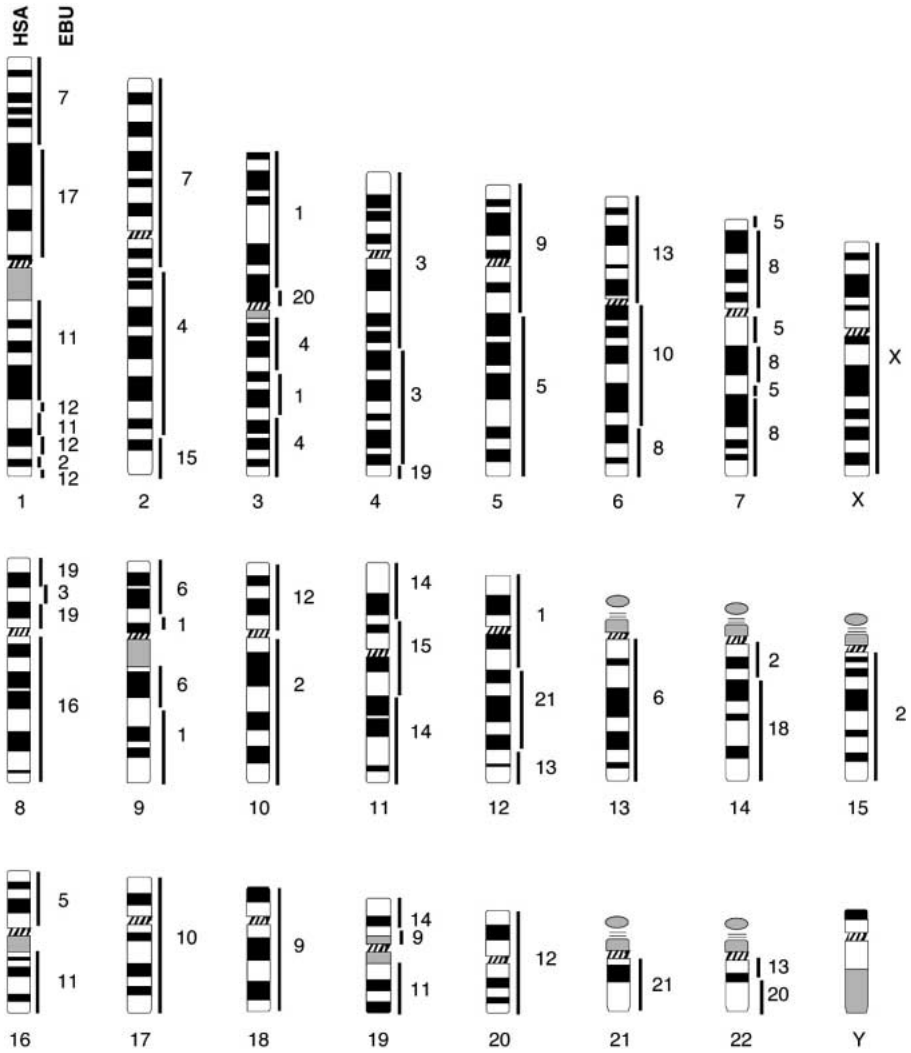

Fig. 5. Summary of hybridization patterns of *E. burchelli* (EBU) probes on the human idiogram. Although EBU8 and X were sorted together, as were EBU10 and EBU12, EBU13 and 14, and EBU20 and one homologue of EBU19 (Fig. 3), we were able to determine their correspondence to human chromosomes by integrating the data from the EBU probes onto human chromosomes and human probes on EBU chromosomes (Fig. 4).

painting data generated for *E. burchelli* and *E. caballus* (this study), allows for the deduction of one-to-one correspondence between the homologous segments conserved in *E. caballus, E. burchelli* and *E. z. hartmannae*. This does not require further reverse painting of *E. caballus* and *E. burchelli* chromosomes with probes derived from the flow-sorted chromosomes of *E. z. hartmannae* (Fig. 6). Comparisons of G-banding patterns in the regions of sequence homology revealed by FISH demonstrates that most interspecific homologues are characterized by conserved banding patterns. Importantly, however, although previous banding comparison suggested that ECA1 = EBU2 = EZH1 (Ryder et al., 1978), our results show that EZH3 (and not EZH1) is homologous to ECA1 and EBU2 respectively.

Discussion

The completion of the human genome sequencing project has made the human genome the standard reference for comparative genomic studies of mammals. Additionally, rapid progress in the horse genome project (Chowdhary et al., 2003) makes this species a useful adjunct for comparative genomic and cytogenetic studies of the equids. Among the equids, genome-wide comparative maps exist for *E. caballus* and *E. z. hartmannae* both of which have been established by comparative painting with human painting probes (Raudsepp et al., 1996; Richard et al., 2001). Additionally, paints derived from the twelve *E. caballus* metacentric autosomes (ECA1–12) and the sex chromosomes have been used to investigate the karyotypic relationships between the horse and donkey (Raudsepp and Chowdhary, 1999). Our study provides the first genome-wide comparative maps between human and *E. burchelli*, and provides comparative genome maps among *E. przewalskii*, *E. caballus*, *E. burchelli*, and *E. z. hartmannae*. Such genome-scale chromosomal correspondences have been impossible based on conventional cytogenetic approaches, the only exception being the karyotypic difference between *E. przewalskii* and *E. caballus* which involves a single Robertsonian translocation. The integration of our comparative chromosome maps with those of Raudsepp et al. (1996), Raudsepp and Chowdhary (1999) and Richard et al. (2001) sheds new light on the genome organization and karyotype evolution of Equidae.

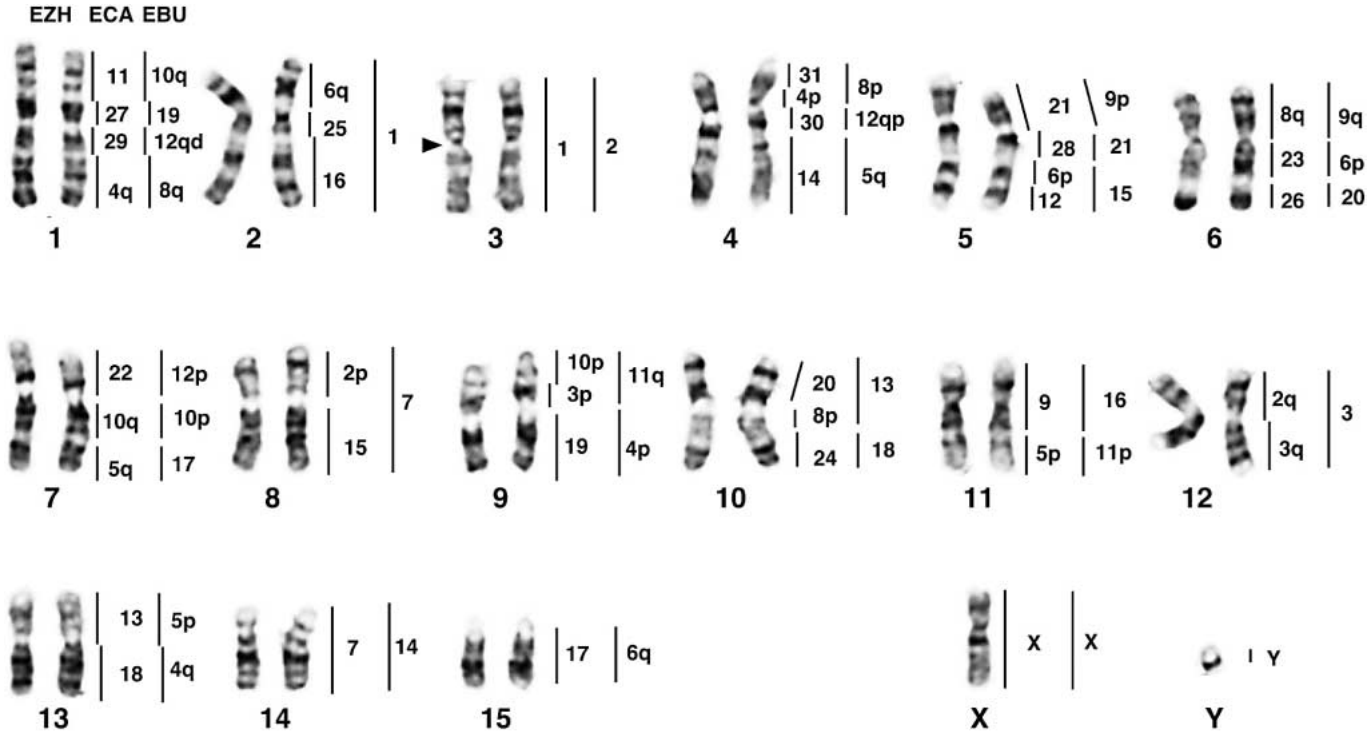

Fig. 6. Genome-wide chromosomal correspondence of *E. z. hartmannae* (EZH), *E. caballus* (ECA) and *E. burchelli* (EBU) with the G-banded karyotype of *E. z. hartmannae* as reference. The EZH chromosome numbers are given below each homologous pair; the homology to chromosomes of *E. caballus* and *E. burchelli* are indicated to the right. The subchromosomal correspondence among the three equid species was established by integrating with the comparative map between *E. caballus* and *E. burchelli* (Fig. 4). Arrowhead indicates the position of the centromere on EZH3.

Conservation of ancestral eutherian syntenies in the Equidae

Comparative chromosome painting between human and representative species of twelve of the extant 18 eutherian orders has led to proposals for the composition of ancestral karyotypes for eutherian mammals (Murphy et al., 2001; Yang et al., 2003). Our results demonstrate that in the case of *E. burchelli* ancestral syntenies equivalent to HSA1, 2pq, 2q, 5, 6, 7a (= 7p11-21 + 7q21 + 7q31-36), 8q, 10q, 9, 11, and 19p have each broken into multiple segments, but the ancestral syntenies HSA10p, 13, 17, 18, 20 and X have all been conserved. Of the proposed ancestral syntenic associations, 7b (= 7p22 +7q11 + 7q22)/16p, 12q-distal/22q-proximal and 16q/19q have been conserved in their entirety while HSA3/21, 4/8p, 12pq-/22q-distal and 14/15 have been partially conserved. Similar patterns showing the conservation and disruption of ancestral syntenies have been found in the genome of *E. z. hartmannae* (Richard et al., 2001) and to a lesser extent in *E. caballus* (Raudsepp et al., 1996). Notably, however, previous comparative painting schemes between human and horse (Raudsepp et al., 1996) failed to demonstrate the presence of the HSA3/21 and 4/8 syntenies in the *E. caballus* genome. Our comparative painting results among equid species suggest the retention of the HSA3/21 and HSA4/8 syntenies on ECA26 and ECA27, respectively. In addition, Raudsepp et al. (1996) reported that ECA1p-q = HSA22/10-cen-2/15/12/15/14 while our data indicate that ECA1p-q = HSA10-cen-1/10/15/14. Interestingly, the most recent radiation map shows that ECA1p-q = 22/10-cen-15/14 suggesting that final determination will be dependent on the development of a high-resolution comparative gene map.

Chromosomal mechanisms underlying the karyotype differences of E. caballus, E. burchelli and E. z. hartmannae

Our results demonstrate that most of the *E. caballus* chromosomes have been retained in toto, or as chromosome arms or parts of chromosome arms, in the zebras. Exceptions include the six *E. caballus* chromosomes (ECA2, 3, 5, 6, 8 and 10) for which the corresponding p and q arms were found on different chromosomes in the genomes of *E. burchelli* and *E. z. hartmannae*. In addition, ECA4 has been conserved in *E. burchelli* but is broken into two segments in *E. z. hartmannae.* Most of the homologous segments shown to be shared among the equid species by cross-species painting display conserved banding patterns. Exceptions to this involve the homologues of ECA1 and ECA7. The different morphologies of ECA1 and ECA7 and their corresponding homologues in the donkey and zebra species suggest the influence of intrachromosomal rearrangement such as inversions. Numerous centric fissions, centric fusions and tandem fusions, together with a small number of inversions underlie the karyotypic differences of the three equid species. For instance, the *E. z. hartmannae* karyotype can be reconstructed from the *E. caballus* karyotype through seven centric fissions, eleven centric fusions, twelve tandem fusions and at least two pericentric inversions. In turn, the karyotype of the

E. burchelli can be reconstructed from that of *E. caballus* through six centric fissions, eleven centric fusions, five tandem fusions and at least one inversion. It will require six centric fissions, seven centric fusions, nine tandem fusions and at least one inversion to reconstruct the *E. z. hartmannae* karyotype from that of the *E. burchelli*.

Cytogenetic signatures

Further analysis of the three human-equid maps, as well as of those using the horse chromosomes as reference, reveal several cytogenetic changes that may be signatures for certain phylogenetic lineages. For example, the HSA1/10 and 11/19 contiguous combinations are present in all equid species studied thus far and are likely cytogenetic signatures for the Equidae. In addition, the syntenic association of the HSA5/19 found on EBU9p is also present in the pig, Indian muntjac, cattle and dolphin. Therefore, the HSA5/19 association appears to be a synapomorphy that supports the arrangement of the Cetartiodactyla + Perissodactyla in the Euungulata (true ungulates) and in so doing gives additional credence to Waddell's et al. (2001) suggestion that this is a natural grouping. Similarly, the syntenic association ECA2q/3q is present in the zebras and donkey suggesting that this may be a synapomorphy uniting these lineages. This finding is consistent with their grouping as sister clades in a Maximum likelihood tree based on mtDNA control region and 12 rRNA sequences (Oakenfull et al., 2000). The ECA6q/25/16, 2p/15, 4p/31/, 3p/10p, 6p/12, and 8p/20 associations appear to be unique (an autapomorphy) to the zebras.

Ancestral karyotype and phylogeny of the Equidae

An ultimate aim of many comparative cytogenetic and genomic studies is to reconstruct the ancestral karyotype and develop a karyotypic phylogeny for species of interest (Neusser et al., 2001; Nie et al., 2002). To achieve this, full taxon representation is useful and appropriate outgroup comparisons critical, since it is only by documenting primitive and derived character states within the karyotypes along cladistic principles that determining the magnitude of change that has occurred within each lineage becomes possible. In the case of the Equidae, however, comparable data are lacking for *E. greyvi*, *E. kiang*, and *E. onager* [species recognition follows Nowak (1999)]. The most closely related outgroup species are the tapirs, followed by the rhinoceroses (Tougard et al., 2001), all of which have relatively high, or high diploid numbers (tapirs 2n = 52–80, Houck et al., 2000; rhinoceroses 2n = 82–84, Houck et al., 1994; Trifonov et al., in press). The data from comparative chromosome painting among five equid species (i.e. the ECA-EAS comparison, Raudsepp and Chowdhary, 1999; Yang et al., submitted; the ECA-EPR-EBU-EZH comparison, this study) demonstrate that these five equid species share 37 evolutionary conserved segments equivalent to ECA1, 2p, 2q, 3p, 3q, 4p, 4q, 5p, 5q, 6p, 6q, 7, 8p, 8q, 9, 10p, 10q and 11–31. We believe that these 37 conserved segments originated during the divergence of the common ancestor of the modern equid species and that the ancestral karyotype of the Equidae is likely to have had a high diploid number. The nearly random distribution of the 37 conserved segments in the *E. burchelli* and *E. z. hartmannae* genomes suggest that independent fusion (centric fusions and tandem fusions) combinations of the 37 ancestral segments gave rise to the karyotypes of the extant equid species. Hybridization of the *E. caballus* paints onto the uncharted genomes of the *E. greyvi*, *E. kiang* and *E. onager*, as well to the rhinoceros and tapirs, should finally allow for the development of a well-resolved chromosomal phylogeny for the extant equids.

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