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Construction of a medium-density horse gene map

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Summary

A medium-density map of the horse genome (*Equus caballus*) was constructed using genes evenly distributed over the human genome. Three hundred and twenty-three exonic primer pairs were used to screen the INRA and the CHORI-241 equine BAC libraries by polymerase chain reaction and by filter hybridization respectively. Two hundred and thirty-seven BACs containing equine gene orthologues, confirmed by sequencing, were isolated. The BACs were localized to horse chromosomes by fluorescent *in situ* hybridization (FISH). Overall, 165 genes were assigned to the equine genomic map by radiation hybrid (RH) (using an equine RH_{5000} panel) and/or by FISH mapping. A comparison of localizations of 713 genes mapped on the horse genome and on the human genome revealed 59 homologous segments and 131 conserved segments. Two of these homologies (ECA27/HSA8 and ECA12p/HSA11p) had not been previously identified. An enhanced resolution of conserved and rearranged chromosomal segments presented in this study provides clarification of chromosome evolution history.

Keywords comparative mapping, gene mapping, horse.

Introduction

Genome mapping in domestic animals is used to reveal the structure and evolution of the genome and to identify markers for genes of interest that might be used in selection. Construction of genetic linkage maps for the horse (*Equus caballus*) was initiated using anonymous microsatellite markers (Guérin *et al.* 2003; Penedo *et al.* 2005), but these maps did not allow for interspecific comparisons except for a few microsatellite-flanking sequences (Farber & Medrano 2004). A major step towards comparative mapping occurred with chromosome painting of human chromosomes on the equine karyotype (Raudsepp *et al.* 1996). Further refinement of the horse–human comparative map occurred through the localization of genes using somatic cell hybrids (SCH) and by radiation hybrid (RH) and cytogenetic (FISH) mapping (Shiue *et al.* 1999; Milenkovic *et al.* 2002; Chowdhary *et al.* 2003).

A likely comparative evolution of chromosomes in mammals from a common ancestor was first described by Chowdhary *et al.* (1998), who used chromosome painting data from eight species belonging to five different orders. Comparisons across species have also been performed with genes mapped to specific locations (Milenkovic *et al.* 2002; Chowdhary *et al.* 2003; Gustafson-Seabury *et al.* 2005). Dense gene maps covering the entire horse genome are still needed, but once available, these will allow for a general comparison of genomes (Murphy *et al.* 2005). Specific algorithms for studying mammalian chromosome evolution are

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being developed (Bourque & Pevzner 2002; Pevzner & Tesler 2003).

The present work was aimed at constructing a medium-density map of the horse genome with evenly distributed gene-specific markers assigned using RH and cytogenetic mapping approaches. These maps will allow studies of conservation and evolution of chromosomes and chromosomal regions between the horse and other mammalian genomes.

Materials and methods

BAC library screening

Gene selection and primer design Five hundred and twenty-five genes evenly distributed at an average interval of 5.7 Mb on human autosomes and the X chromosome were selected. Bovine or equine intra-exonic primers based on expressed sequence tag sequences in consensus human-cattle and human-horse regions were designed with the ICCARE (Muller *et al.* 2004; http://bioinfo.genopole-toulouse.prd.fr/iccare/) and Primer 3 algorithms to amplify DNA fragments of approximately 200 bp.

DNA amplification Primers were used to amplify genomic DNA from cattle and horse by PCR using the following conditions: 10–20 ng DNA in a total volume of 15 μl containing 1.5 mM $MgCl_2$, 125 μM each dNTP, 1 μM primer and 0.5 U *Taq* polymerase. Amplifications were performed in PTC100 thermocyclers (MJ Research, Bio-Rad Laboratories, Hercules, CA, USA) with 5-min denaturation at 94 °C and then 35 cycles of 30-s denaturation at 94 °C, 30-s primer hybridization at 55 °C and 30-s elongation at 72 °C. Fragments were analyzed on 2% agarose gels.

Hybridization screening Three high-density filters, each containing 18 432 duplicated clones of the equine CHORI-241 BAC library (BACPAC Resources, Oakland, CA, USA) and representing about three genome equivalents, were screened by hybridization using bovine or equine probes produced by PCR amplification on whole genomic DNA with the primers mentioned above. The PCR products were purified using Wizard SV Gel and PCR Clean-Up System columns (Promega, Madison, WI, USA). For each hybridization, 25–30 probes (2 ng per probe) were mixed together in a maximum volume of 30 μl and labelled by random priming with 50 μCi of (α^{32}P)-dCTP and 2 U Klenow enzyme for 1 h at 37 °C. The reaction was stopped by adding 0.5 M EDTA (pH 8.0), and the labelled products were purified with commercial columns (Nick™ Column; Amersham Bioscience AB, Uppsala, Sweden) in conditions recommended by the manufacturer. Homologous and heterologous hybridization procedures followed standard protocols (Sambrook *et al.* 1989). Autoradiography using Kodak X-OMAT AR films (E.I.S., Massy, France) were visually interpreted to identify clone addresses. Pools of positive clones were distributed in a two-dimension scheme, and each BAC was further verified by PCR with the appropriate primers.

PCR screening, sequencing and gene identification Genes that could not be identified in the equine CHORI-241 BAC library were then screened on the equine INRA BAC library by PCR as described previously (Milenkovic *et al.* 2002). DNA from each isolated BAC clone was amplified by PCR with the corresponding gene primer pair and the products purified as above. One to three nanograms of purified PCR product was directly sequenced on one strand with the forward primer using either an ABI 377 (Applied Biosystems, Foster City, CA, USA) or a MegaBACE (Molecular Dynamics, Sunnyvale, CA, USA) automatic sequencer. Sequences were queried against homologous sequences using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov) to confirm the identity of the gene in the isolated clone ($E < e^{-05}$).

Genome mapping

FISH mapping One hundred and thirty BAC clones chosen in regions that were poorly defined in the equine map (corresponding to human chromosomes 6, 9, 10, 11, 12, 18, 19 and 21) were localized on equine metaphase chromosomes by fluorescent *in situ* hybridization (FISH). R and G banding was as described elsewhere (Hirota *et al.* 2001; Lear *et al.* 2001; Chowdhary *et al.* 2003; Perrocheau *et al.* 2005). The international horse chromosome banding standard (ISCHN 1997) was used as a reference.

RH mapping Genes reported here and those in Perrocheau *et al.* (2005) were localized on the horse RH_{5000} panel (Chowdhary *et al.* 2002) by PCR or SSCP using the corresponding primers. The PCR components included 50 ng panel DNA, 2 mM $MgCl_2$, 250 μM each dNTP, 0.36 μM each primer and 1.12 U *Taq* polymerase in a final volume of 15 μl. Amplification conditions were identical to those described above. Fragments were analyzed on 2% agarose gels and visually interpreted. The ExcelGel DNA Analysis kit (Amersham) was used for SSCP analysis (Laurent *et al.* 2000). Each gene was incorporated into the first version of the whole-genome map (Chowdhary *et al.* 2003). A two-point analysis was then performed with a program developed in our laboratory. Markers with a LOD score ≥6 were included in the same synteny group and considered to belong to the same chromosome. Multipoint analysis ordering the genes within linkage groups was carried out with the Defalgo option of the CarthaGène software (http://www.inra.fr/bia/T/CarthaGene/).

Comparative mapping A comparative horse–human map was constructed using the most recent gene locations on the horse and human maps (Chowdhary *et al.* 2003; Lee *et al.* 2004; Raudsepp *et al.* 2004; Gustafson-Seabury *et al.*

2005). First, a backbone map for horse was made using the RH mapping data alone. Then, FISH-mapped genes were added on the horse map in positions that minimized rearrangements with the human genome. Orthologous gene locations on the human genome were obtained from sequencing data available at Ensembl (http://www.ensembl.org; version dated April 2005). Genes were classified into homologous segments and conserved segments according to their comparative location in the human and equine genomes. Homologous segments between the horse and human genomes were defined as chromosome segments containing one or more homologous genes. Conserved segments were defined as smaller units of homologous segments containing at least two genes, and segments were assumed to be uninterrupted in the two species. Within a segment, the order of more than two genes had to be preserved. Slight differences in gene order in small intervals were accepted for conserved segments. In these cases, conserved segments were defined according to the parsimony hypothesis, which limits the number of rearrangements between the horse and human genomes.

Results

Primer panel

Consensus sequences between human–bovine (428) and human–equine (97) ESTs were generated from intra-exonic fragments of each chosen gene. Among these, 323 primer pairs (226 bovine and 97 equine) amplified horse genomic DNA (Table S1). The corresponding fragments had an average length of 175 bp (ranging from 82 to 300 bp) and were used as hybridization probes.

BAC library screening

Three filters, which corresponded to about 25% of the CHORI-241 BAC library and represented three equine genome equivalents, were screened by hybridization with 323 probes (226 bovine and 97 equine) resulting in the identification of 151 and 66 genes respectively. Overall, 217 genes were obtained, representing a 67% yield. The INRA-LGBC library was screened by PCR with 113 primer pairs, which led to the isolation of 83 genes, with a 73% yield. BLASTN analysis of sequences from 300 putative genes confirmed the identity of 237 unique genes (Table S2). These genes were distributed, on average, at 12.7-Mb interval in the horse genome. An average of 2.1 BAC clones per gene was obtained, but this figure is an underestimate because the genes were not sequenced in all retrieved BACs.

RH map

Two hundred and thirty-seven genes from this study and 32 genes from Perrocheau *et al.* (2005) were typed on the RH_{5000} panel (Table S2). Among these, 205 genes were scorable. The two-point linkage analysis assigned 167 genes at a LOD score ≥6.0. The RH location was in agreement with that determined by FISH and/or with what was expected from the horse–human comparative map. Among the remaining 38 genes, 19 were assigned because their RH localization agreed with those obtained by FISH even though their two-point linkage LOD scores were <6.0. The other 19 were discarded (LOD < 6.0 and a discrepancy with FISH). In total, 186 genes were added to the 255 previously mapped on the RH map (Fig. 1). The RH map now includes 441 type I markers and 470 type II markers. These 911 markers are clustered in 101 linkage groups distributed on the 31 autosomes and on the X chromosome. Twenty-six markers remain as singletons. On average, three linkage groups were found on each chromosome with a range of one to eight per chromosome. For seven chromosomes, enrichment of the RH map has allowed merging two to five linkage groups into a single group. The cumulative size of the RH map obtained by summing all linkage groups was 17 039 cR. Thus, the map provides on average one gene every 2.5 Mb, estimated at 18.7 cR.

FISH mapping

One hundred and thirty BACs were localized on equine chromosomes by FISH mapping (Table S2). Among these, 87 had chromosomal locations in agreement with RH data and/or with those expected from comparative mapping. The remaining 43 assignments were not conclusive. Thus, there are 511 cytogenetic gene localizations including those recently located on ECA17, ECA22 and ECAX (Lee *et al.* 2004; Raudsepp *et al.* 2004; Gustafson-Seabury *et al.* 2005). In this study, at least one gene was mapped on each equine chromosome, except for eight chromosomes (ECA16, ECA17, ECA18, ECA19, ECA25, ECA28, ECAX and ECAY). For the first time, two genes were mapped by FISH on the short arm of chromosome 12 and three on chromosome 27, which previously contained only one gene by RH mapping (Perrocheau *et al.* 2005). Moreover, the map was enriched with genes in previously poorly covered regions on ECA6, ECA8, ECA12, ECA27, ECA29 and ECA30. These new gene localizations allowed anchoring of a fifth RH linkage group on ECA8 and the orientation of 11 RH linkage groups (Fig. 1).

Comparative mapping

Our work adds 165 genes to the equine gene map, which now contains 713 genes (441 on the RH map, 511 genes on the cytogenetic map and 238 on both maps). Genes mapped as a singleton, by a single technique or in total disagreement with the comparative map were removed from this calculation. These 713 genes are distributed into 59 homologous segments between the horse and

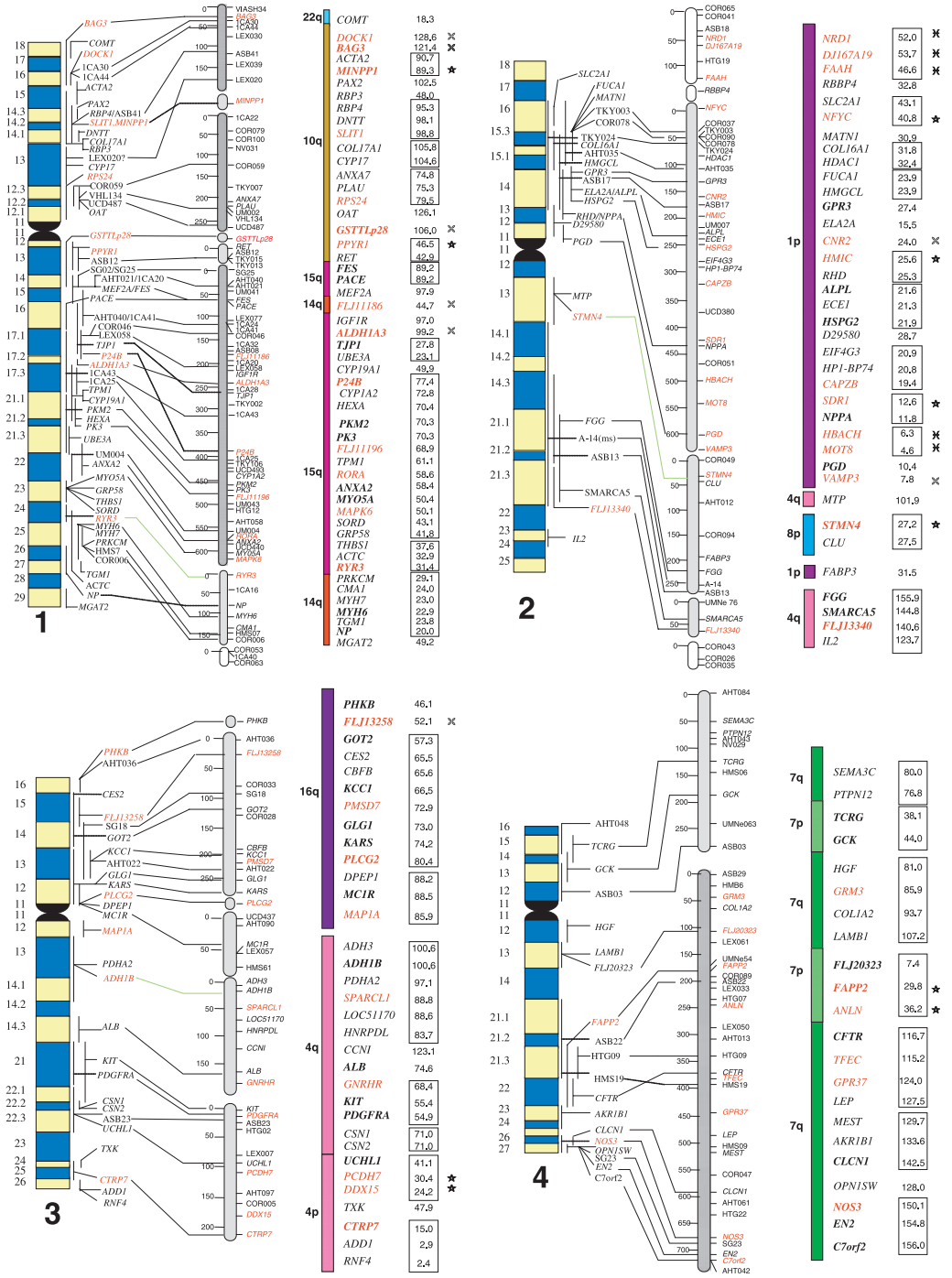


Figure 1 Integrated equine map and comparative horse–human map. Equine chromosomes are represented to the left of the figure as G-banded idiograms. Markers located by fluorescent *in situ* hybridization (FISH) are represented to the right of the idiograms. Radiation hybrid (RH)-mapped markers are aligned in the most likely order to the right of vertical rounded bars representing RH linkage groups. Shaded bars are anchored to the chromosomes by at least one marker while white bars are ordered according to the linkage map data. RH linkage groups anchored during this study are depicted with a green line and striped bars correspond to merged RH linkage groups. The distances are indicated in cR. Comparative mapping to the human genome is depicted as rectangular vertical bars showing homologous segments with, to their right, the most likely order of the genes based on their equine localizations. The physical locations of the genes on the human genome are indicated in megabases (http://www.ensembl.org). Segments considered as conserved appear in blocks. Gene symbols (in italics) of the present study are shown in red and those located by both FISH and RH are in bold face.

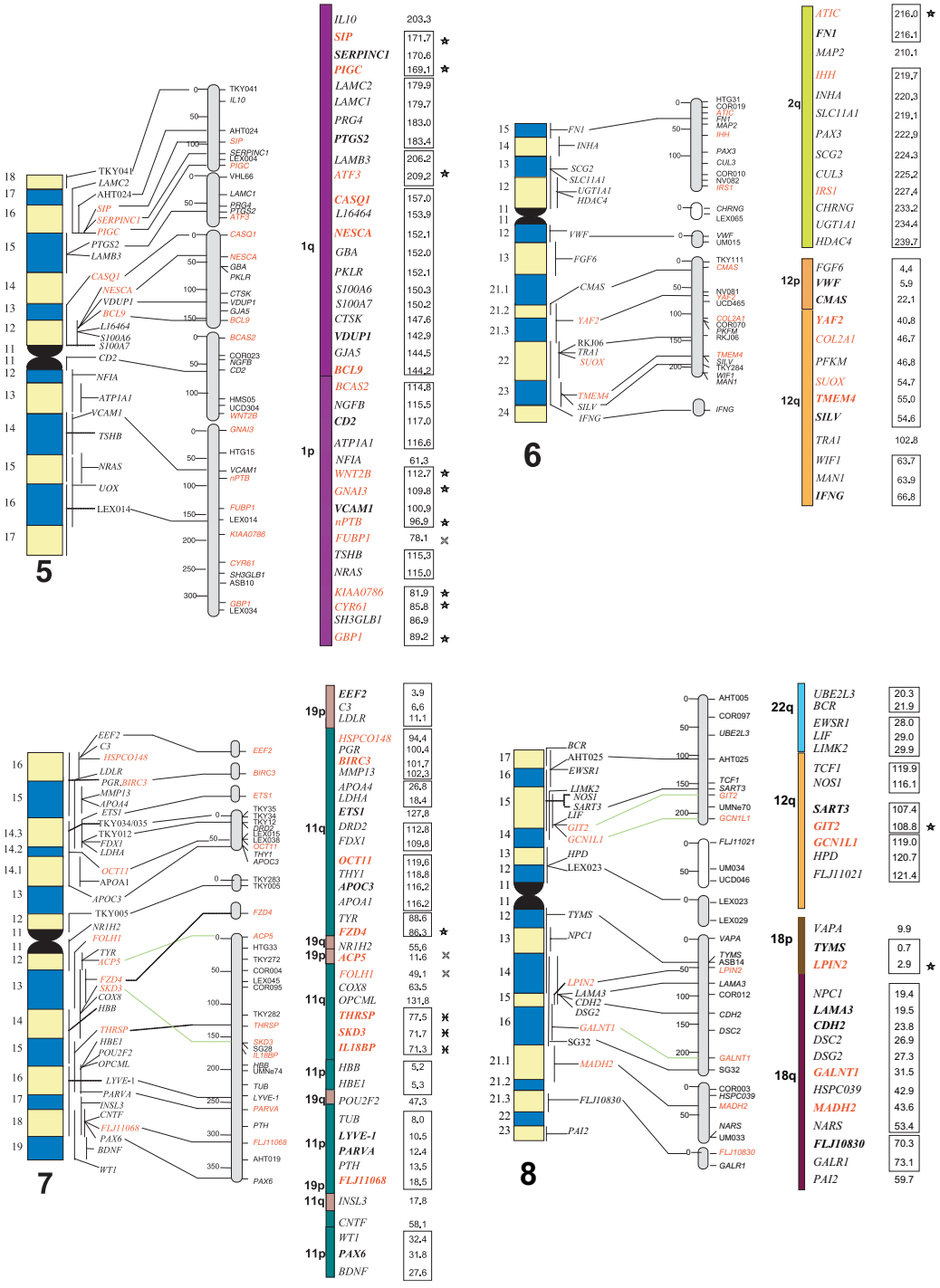
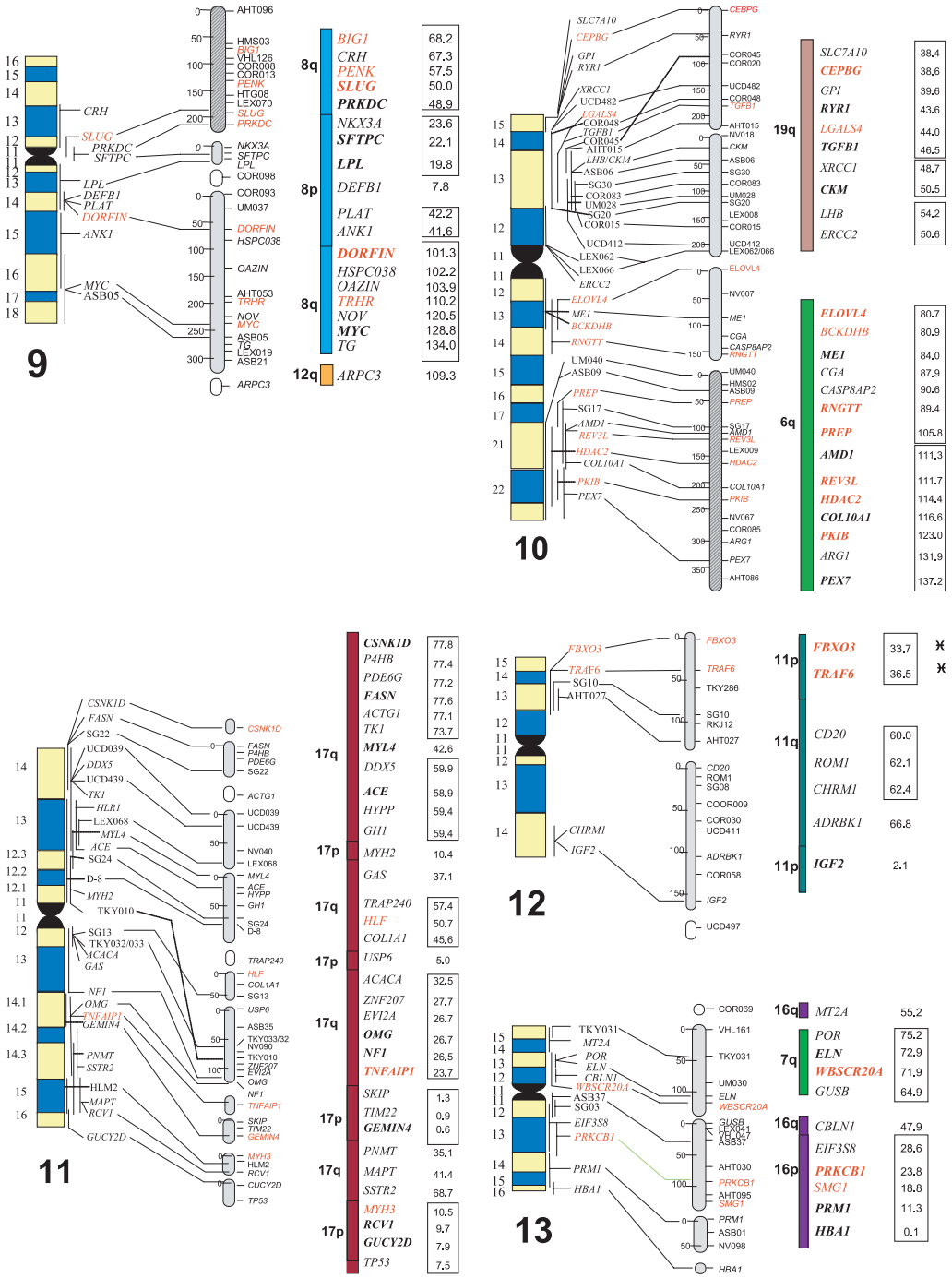


Figure 1 (Continued).

human genomes. Among these, only nine contain a single gene. This work confirmed the existence of two homologous segments, ECA2/HSA8 and ECA7/HSA19, which were previously suggested by the location of a single gene (Lindgren *et al.* 2001; Mariat *et al.* 2001; Chowdhary *et al.* 2003). Moreover, the mapping of *FLJ20036* confirmed the homology between ECA27 and HSA4, which was previously identified with a microsatellite linked to the *F11* gene (Chowdhary *et al.* 2003) and by the FISH localization of *FRG1* (Perrocheau *et al.* 2005).

Two additional homologies between horse and human chromosomes, ascertained by both FISH and RH mapping, were revealed: (1) a homologous segment between ECA27



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and HSA8 with the mapping of *PDGFRL* and *LOC84549* and (2) a homologous segment between the p arm of ECA12 and the p arm of HSA11 with the mapping of *FBXO3* and *TRAF6*. In addition, a segment on ECA1 homologous to HSA15q may be interrupted with genes on HSA14q as revealed by RH mapping of one gene, *FLJ11186*. Within these 59 horse–human homologous segments, we were able to define 131 conserved segments. These conserved segments include 87% of the compared loci and contain an average of four to five genes, revealing a noticeable level of chromosomal rearrangements within homologous segments. For example, although ECA23 is completely homologous with HSA9, the chromosome is composed of four different conserved segments containing genes alternatively located on the p and q arms of HSA9. In addition, 33 conserved segments

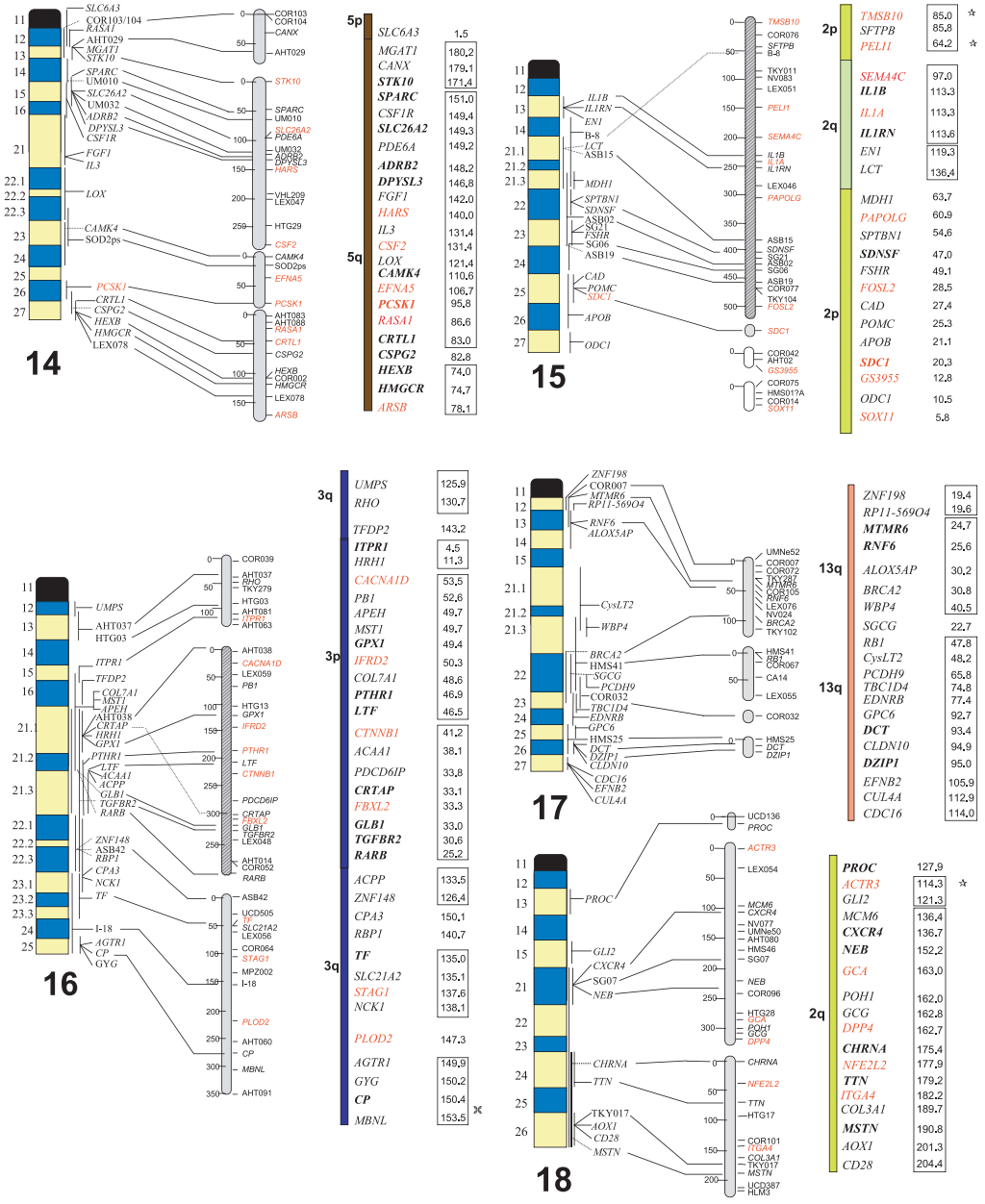

Figure 1 (Continued).

were newly identified. Two of these introduced insertions in previously defined homologous segments, one on ECA4 corresponding to the distal part of HSA7p (*FLJ20323*, *FAPP2* and *ANLN*) and the other on ECA12 with a segment of HSA11p (*FBXO3* and *TRAF6*). Thirty-seven localizations converted homologous segments that were previously specified by one gene into conserved segments (Fig. 1☆). Identification of new conserved segments was made using 10 loci (Fig. 1✠) and 16 isolated loci (Fig. 1⌘). This represents 38% of the newly localized genes, whereas 62% (102/165) of the genes were included in previously defined conserved segments.

Discussion

The 323 primer pairs amplifying horse DNA were used to screen the INRA and the CHORI-241 equine BAC libraries. Screening efficiency of equine probes was inferior to that of bovine probes, possibly because of the presence of horse-specific repeated sequences in the equine probes. The PCR screening identified more positive clones than hybridization screening, but after sequencing, the yields of positive clones were the same (61% and 59% respectively). Overall, the number of confirmed genes (237) relative to the number of searched genes (323) was 73%, which was comparable

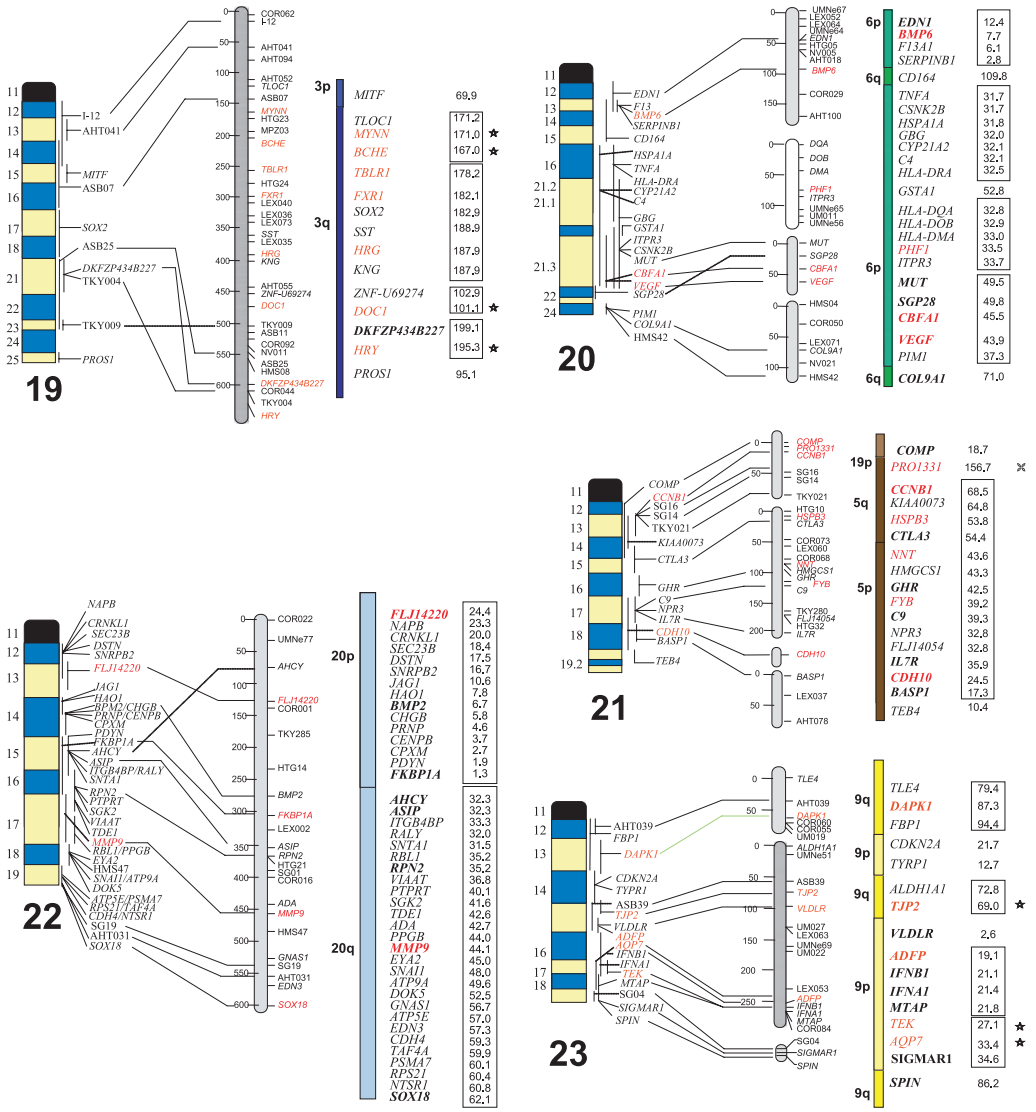

Figure 1 (Continued).

with other studies (Cai *et al.* 1998; Lindgren *et al.* 2001; Raudsepp *et al.* 2002). In this study, the number of assigned genes on the horse genomic map increased by 31% over the previous version of the map.

The comparative map between horse and human containing 713 genes was developed based on RH and FISH data. We identified 59 homologous segments between horse and human, confirming close genome organization between the two species. However, intra-chromosomal rearrangements of 131 conserved segments occurred. It is not known whether these intra-chromosomal rearrangements are because of repeated horse sequences present at a higher density in certain chromosomes. These observations support the hypothesis of a first phase of differentiation because of inter-chromosomal rearrangements followed by a second phase with the intra-chromosomal reshuffling as suggested by the observed difference between the number of conserved (131) and homologous (59) segments. Heterogeneity in the conservation of horse–human chromosomal regions was illustrated by the comparison of ECA24/HSA11 and ECA11/HSA17. ECA24 represents a single conserved segment on HSA11, whereas ECA11 is divided into at least six conserved segments on HSA17. Conversely, for some equine chromosomes, gene order is strongly conserved, as observed for ECA17, ECA22 and ECAX (Lee *et al.* 2004; Raudsepp *et al.* 2004; Gustafson-Seabury *et al.* 2005). The cumulative length of all conserved segments is about half of the human genome.

Our mapping data can also be used for understanding the evolution of the human and horse genomes as initiated by Chowdhary *et al.* (1998) with chromosome painting. The eutherian ancestral karyotype (EUT) was tentatively reconstructed with 50 chromosomes, of which 40–42 were acrocentric (Murphy *et al.* 2001; Richard *et al.* 2003).

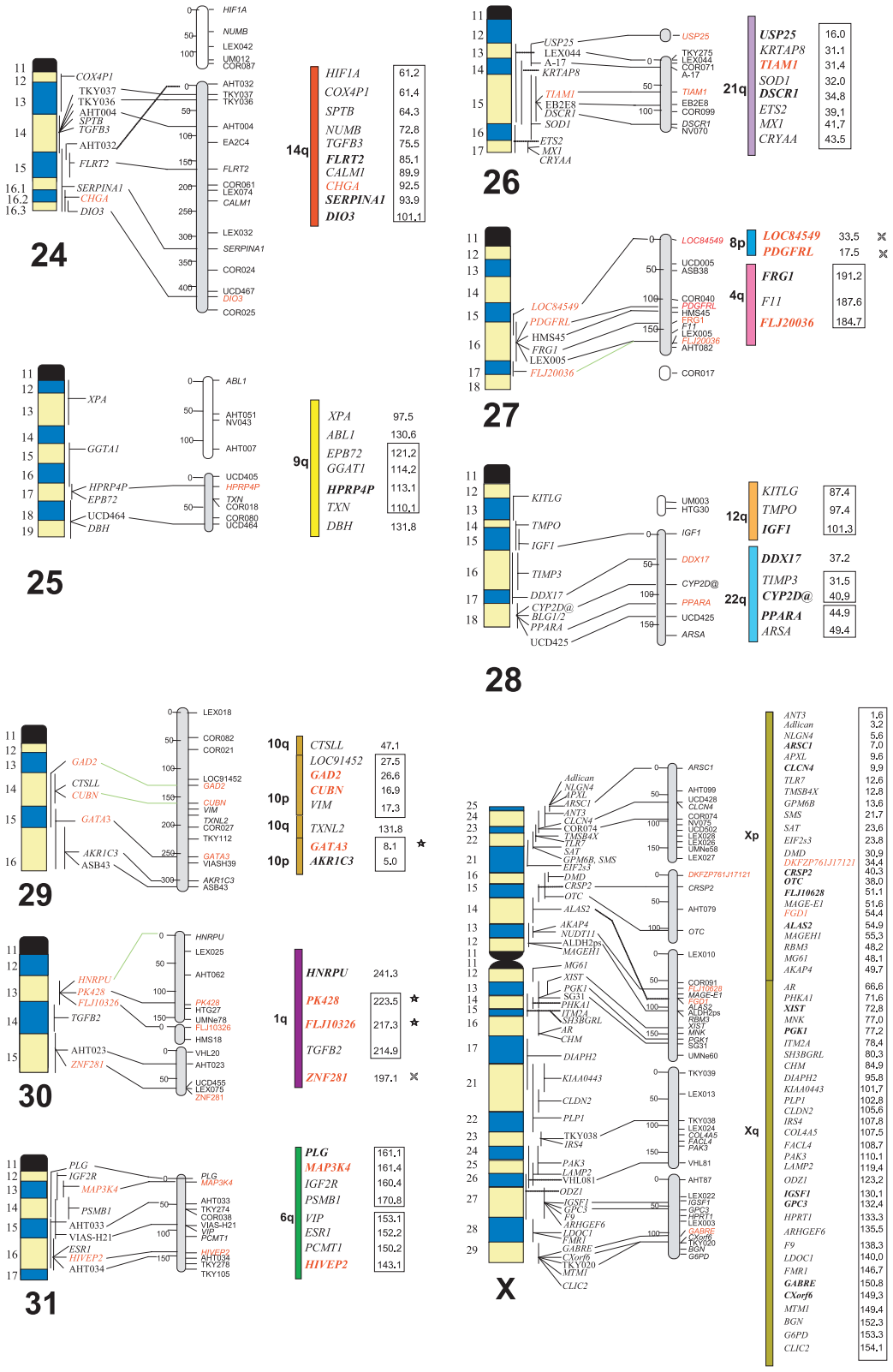

Figure 1 (Continued).

Compared with the EUT, the equine genome has undergone more inter-chromosomal rearrangements than the human genome simply because of the existence of a larger number of chromosomes. For example, EUT3 is homologous to HSA6 but splits into three equine chromosomes or chromosome arms (ECA10q/ECA20/ECA31). In some instances, the one-to-one reciprocal relation between ECA and HSA also stands for the eutherian karyotype; for example, EUT19, 17, 21, X, and Y correspond to ECA11, 17, 22, X, Y and HSA17, 13, 20, X and Y respectively. An interesting situation is given by EUT6, which is homologous to HSA3 and HSA21, but HSA3 split into ECA16 and ECA19 after the human-horse lineage diverged.

The construction of a medium-density map is considered as a step toward high-density maps, which will refine the horse map and the comparative maps with other species. These data will also contribute to ongoing studies aimed at the identification of genes of interest and the construction of an equine physical map.

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Supplementary Material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com

Table S1. Gene symbols, human FISH localizations, sequence accession numbers and bovine and equine primers.

Table S2. Genes identified in horse BAC clones (for BAC libraries, 1 = CHORI-241 and 2 = INRA-LGBC) and horse localizations of the genes by RH and FISH mapping.