

# Towards the delineation of the ancestral eutherian genome organization: comparative genome maps of human and the African elephant (*Loxodonta africana*) generated by chromosome painting

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This study presents a whole-genome comparison of human and a representative of the Afrotherian clade, the African elephant, generated by reciprocal Zoo-FISH. An analysis of Afrotheria genomes is of special interest, because recent DNA sequence comparisons identify them as the oldest placental mammalian clade. Complete sets of whole-chromosome specific painting probes for the African elephant and human were constructed by degenerate oligonucleotide-primed PCR amplification of flow-sorted chromosomes. Comparative genome maps are presented based on their hybridization patterns. These maps show that the elephant has a moderately rearranged chromosome complement when compared to humans. The human paint probes identified 53 evolutionary conserved segments on the 27 autosomal elephant chromosomes and the X chromosome. Reciprocal experiments with elephant probes delineated 68 conserved segments in the human genome. The comparison with a recent aardvark and elephant Zoo-FISH study delineates new chromosomal traits which link the two Afrotherian species phylogenetically. In the absence of any morphological evidence the chromosome painting data offer the first non-DNA sequence support for an Afrotherian clade. The comparative human and elephant genome maps provide new insights into the karyotype organization of the proto-afrotherian, the ancestor of extant placental mammals, which most probably consisted of  $2n = 46$  chromosomes.

**Keywords:** chromosomal homology; Zoo-FISH; synteny conservation; phylogeny; Afrotheria

## 1. INTRODUCTION

In recent years our knowledge about mammalian genome evolution has been greatly increased on different levels: (i) by DNA sequence comparisons or whole genome sequencing efforts (Waterston *et al.* 2002); (ii) by comparative gene mapping studies in bio-medical model species and farm animals (for review see Clark 1999); and (iii) by comparative molecular cytogenetics. In comparative molecular cytogenetics the most widely used approach is cross-species (or comparative) chromosome painting (Wienberg *et al.* 1990), which allows the generation of global comparative genome maps at a cytogenetic resolution limit of about 5 Mbp. Since the first report of cross-species chromosome painting between distantly related species (Zoo-FISH) (Scherthan *et al.* 1994) about 25 mammalian species have been analysed by inter-ordinal chromosome painting (see Wienberg *et al.* (2000) for review). The majority of these studies employed human whole chromosome painting probes. However, the combination of degenerate oligonucleotide-primed PCR (DOP-PCR) and chromosome sorting by bivariate flow cytometry facilitated the generation of high-quality chromosome painting

probes for an increasing number of species (Carter 1994; Ferguson-Smith 1997).

These probe sets have enabled reciprocal Zoo-FISH experiments, which define the chromosomal homologies at a higher resolution and allow the identification of evolutionary chromosome breakpoints in the karyotypes of both analysed species. Inter-ordinal reciprocal Zoo-FISH studies have been published for the pig, the dog, the cat, the rabbit and the tree shrew (Goureau *et al.* 1996; Wienberg *et al.* 1997; Breen *et al.* 1999; Korstanje *et al.* 1999; Müller *et al.* 1999; Yang *et al.* 1999; Sargan *et al.* 2000). A closer analysis of the conserved features in these genomes allowed for a preliminary reconstruction of ancestral eutherian karyotypes (Chowdhary *et al.* 1998; Haig 1999; Wienberg *et al.* 2000; Murphy *et al.* 2001b).

However, mammalian genome evolution needs to be interpreted in a phylogenetic context. Recent comprehensive comparisons of nuclear DNA sequences representing all eutherian orders resulted in a remarkably revised view of supraordinal mammalian phylogeny (Eizirik *et al.* 2001; Madsen *et al.* 2001; Murphy *et al.* 2001a). These studies identify four principal eutherian clades and agree that Afrotheria (including elephants, manatees, hyraxes, aardvark, elephant-shrews, tenrecs and golden moles (Springer *et al.* 1997)) represent the oldest extant eutherian clade. They do not rule out however, a basal phylogenetic position for Xenarthra.

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Until very recently, comparative gene mapping data or comparative chromosome painting data for Afrotherian species (as well as for Xenarthra) were missing. This lack reduces the significance of previous attempts to define the ancestral eutherian karyotype, because data from the taxa closest to the root of the evolutionary tree are missing. The present work was initiated to help fill this gap and to test previous reconstructions of mammalian genome evolution. We have performed reciprocal Zoo-FISH using paint probes derived from flow-sorted African elephant (*Loxodonta africana africana*) chromosomes (LAF) and human chromosomes (HSA) to identify the segment-to-segment homologies between the two karyotypes. The African elephant is the largest terrestrial animal. The habitat of this endangered species is restricted to the sub-Saharan Africa, where it is subjected to ongoing destruction (Roca *et al.* 2001). There are several classical cytogenetic reports published on African elephant chromosomes (e.g. Hungerford *et al.* 1966; Thurig 1970) but only recently have elephant karyotypes been analysed by chromosome banding (Houck *et al.* 2001). Houck and collaborators reported a high level of chromosome band homology between the African elephant and the Asian elephant (*Elephas maximus*). Recent genetic studies have identified a second species of elephant in Africa: the smaller forest elephants (*Loxodonta cyclotis* (Roca *et al.* 2001)). The forest elephants have not yet been studied cytogenetically. The existing chromosome banding studies (Houck *et al.* 2001) indicate, however, a strong conservation of elephant karyotypes since the evolutionary separation of *Elephas maximus* and *Loxodonta africana* 4–7 Myr ago (Thomas *et al.* 2000; Vignaud *et al.* 2002). After the submission of this manuscript, a Zoo-FISH study of armadillo and elephant genomes was published (Yang *et al.* 2003). This study allows the comparison of our data with a second Afrotherian species and the delineation of cytogenetic traits common to Afrotherian karyotypes.

## 2. MATERIAL AND METHODS

### (a) Metaphase preparations

African elephant metaphases were prepared from a female fibroblast cell culture (LAF-12) stored in the cell bank of the Laboratory of Genomic Diversity (NCI-Frederick). The cell line was originally prepared in 1983 from a skin biopsy sampled in the National Zoo (Washington, DC) from an elephant originating from the Serengeti National Park (Tanzania). Human chromosomes were prepared from lymphoblastoid cell lines (e.g. GM00130; Coriell Cell Repositories). Chromosome preparations were harvested by conventional cytogenetic hypotonic and fixation treatments.

### (b) Flow sorting of chromosomes and generation of painting probes

Chromosome suspensions of both human and elephant were prepared and stained as described previously (Rabbitts *et al.* 1995). The preparations were analysed on a dual laser cell sorter (FACS DiVa; Becton Dickinson Immuno-Cytometry Systems). About 300 chromosomes from each peak of the resulting flow karyotypes were sorted directly into PCR tubes containing 30 µl of sterile deionized water. Chromosome specific probes were generated from flow sorted chromosomes by DOP-PCR (Telenius *et al.* 1992) using 6MW primers (Telenius *et al.* 1992)

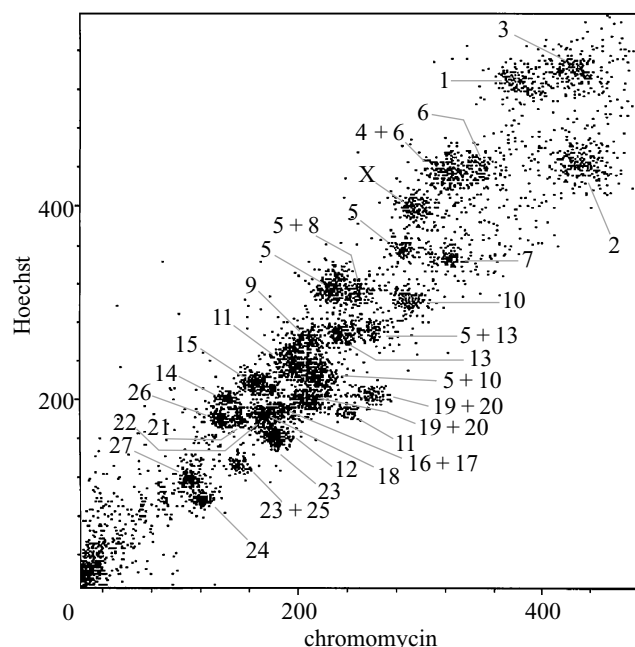


Figure 1. Flow karyotype of the African elephant (*Loxodonta africana*). The dot plot of the bivariate flow karyotype of the elephant is shown for 10 000 events. Chromosomes were sorted for DNA content and (A + T)/(G + C) base pair ratio into 31 peaks after staining with Hoechst 22358 (vertical axis) and chromomycin-A3 (horizontal axis). The numbers identify the African elephant chromosomes represented in the individual peaks.

for human and G1-4 primers (5'-GAGGATGAGGTT-GAGNNNNNTGG-3') for elephant chromosomes.

Tests with various primers showed that G1-4/G2 primers produced better paint probes for the elephant than the standard 6MW primers. With the G1-4/G2 primer, elephant paint probes displayed a lower amplification of satellite sequences in hybridizations to elephant metaphases and a higher signal to background ratio in cross-species hybridizations.

### (c) In situ hybridization, image processing and mapping of FISH signals

The probes were labelled by the incorporation of biotin-dUTP or digoxigenin-dUTP (both Roche Molecular Biochemicals) during the secondary PCR reaction using 6MW primers for the human probes and G2 primers (5'-GTGAGTGAGAGGATGAGGTTGAG-3') for the elephant probes. For labelling, the dTTP concentration in the PCR reaction mixture was reduced to 150 µM (dATP, dCTP and dGTP, 200 µM each) and either 35 µM biotin-dUTP or 25 µM digoxigenin-dUTP were added. For the hybridization of elephant probes onto human metaphases, 500 ng of the painting probes were co-precipitated with 10 µg of human Cot-1 DNA, 10 µg of sonicated salmon sperm DNA (both Invitrogen) and 5 µg sonicated genomic elephant DNA, and dissolved in 15 µl of hybridization solution (50% formamide, 2 × SSC, pH 7.0, 10% dextran sulphate). For hybridizations of human paint probes the genomic elephant DNA was omitted. The probes were denatured at 70 °C for 10 min, allowed to preanneal for 90 min at 37 °C, dropped on denatured chromosome preparations and mounted with 18 mm × 18 mm coverslips. Chromosome preparations were denatured in 70% formamide, 2 × SSC at 70 °C for 1 min 30 s, rinsed in ice-cold 70% ethanol, and dehydrated

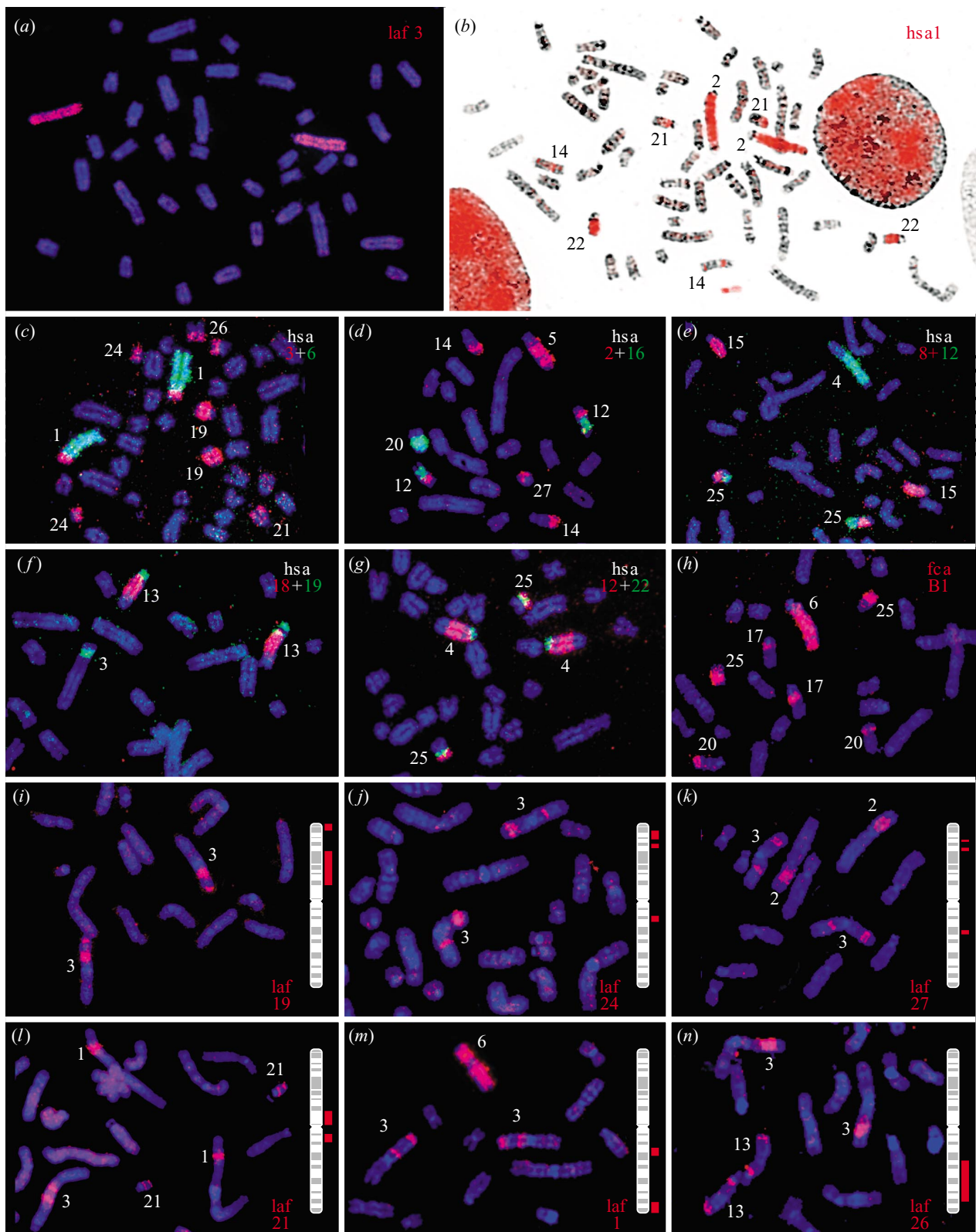


Figure 2. Examples of reciprocal Zoo-FISH hybridizations between humans and the African elephant and of chromosome painting with elephant probes. In all cases chromosomes were counterstained with DAPI and appear as either blue or, in the case of (b), as computer enhanced greyscale images. The probes employed are indicated on each picture in the same colour as the corresponding FISH signals. The target chromosomes are identified by the numbers beside them. (a) Chromosome painting with the LAF 3 probe on a partial African elephant metaphase. (b–h) Zoo-FISH signals of human (b–g) and cat (h) paint probes on elephant chromosomes. (i–n) Hybridizations of elephant painting probes to human metaphases reveal a complex hybridization pattern on human chromosome 3. The location of the FISH signals on HSA 3 is indicated by red bars to the right of the HSA 3 ideogram in each picture.

through a 70%, 90% and 100% ethanol series. *In situ* hybridization was performed for at least 72 h at 37 °C. Detection of the hybridization signal was as published (Pinkel *et al.* 1986; Wienberg *et al.* 1997). Post-hybridization stringency washes were performed at 42 °C (1 × SSC for 1 min, 50% formamide/2 × SSC twice for 5 min, 2 × SSC twice for 5 min). The digoxigenin labelled chromosome paints were detected with Rhodamine labelled goat-anti-digoxigenin antibodies (Roche), and biotin labelled probes were detected with Avidin-FITC (Vector). The slides were mounted in antifade solution containing 0.5 µg ml<sup>-1</sup> 4',6-diamidino-2-phenylindole (DAPI) and 5 µg ml<sup>-1</sup> actinomycin D (AMD). The addition of the nonfluorescent AMD enhances the contrast of the DAPI banding pattern (Fröncke & Scherthan 1997). Microscopy and digital imaging were as described before (Fröncke & Wienberg 2001).

Chromosome numbering followed the G-banded karyotype of Houck *et al.* (2001). For each probe, hybridizations to at least 20 metaphase spreads were analysed. Hybridization signals were assigned to specific chromosome regions defined by the DAPI/AMD-banding patterns.

### 3. RESULTS

#### (a) *Flow sorting of elephant chromosomes and generation of chromosome painting probes*

The African elephant has a predominantly acrocentric karyotype consisting of 2n = 56 chromosomes containing only one subtelocentric and two metacentric autosomes. The G-banding pattern of chromosomes derived from the elephant cell culture used in this study matches that of the published karyotype (Houck *et al.* 2001). The cell line displayed polymorphisms in the length of the short arms of the acrocentric chromosomes LAF 2, 5, 6, 7, 12, 19 and 23. LAF 19 showed an additional polymorphism at the long arm telomere. In the flow karyotype of the African elephant 31 distinct peaks were identified (figure 1). Although the same species was analysed, the present work displays remarkable differences in the flow karyotype compared with the one presented by Yang *et al.* (2003). These differences are most probably due to polymorphisms of the heterochromatic short arms of the acrocentric elephant chromosomes.

Painting probes were made by DOP-PCR from chromosomes sorted from each peak. To assign the content of each peak to particular chromosomes, the painting probes were hybridized to metaphase preparations of *L. africana*. Single chromosomes were found in 23 peaks, whereas eight peaks contained two or three chromosomes (LAF 4 + 6, 5 + 8, 5 + 10, 5 + 13, 16 + 17, 19 + 20 [2×], 23 + 25; figure 1). Chromosome 5 appeared to be especially difficult to resolve and was present in four peaks. Representative images of hybridization signals obtained during our study are presented in figure 2.

#### (b) *Cross-species chromosome painting*

The two sets of human and elephant painting probes produced hybridization signals covering almost the entire euchromatin of the target species in the Zoo-FISH experiments. The cross-species hybridization results are presented in table 1 and mapped onto ideograms of both human and elephant chromosomes (figure 3a,b).

The comparative maps resulting from the reciprocal hybridizations are in good agreement with each other

(figure 3). The Zoo-FISH experiments delineated matching syntenic associations and comparable sizes of conserved segments in both genomes. The only discrepancy was noted for the hybridization efficiencies of HSA 1 and LAF 14 probes. Whereas the LAF 14 probe produced a strong signal on human chromosome 1, the reverse experiment resulted only in very weak signals in the subcentromeric and telomeric region of the elephant chromosome. The latter signals were included in the comparative map, because of the proof of homology provided by the LAF 14 probe.

As in other Zoo-FISH studies, no hybridization signals were recorded in heterochromatic regions (e.g. the short arms of the acrocentric elephant chromosomes) during hybridizations in either direction. The Zoo-FISH signals tended to be weaker towards the telomeres and the telomeres were sometimes devoid of signals. This phenomenon was reported previously for other species (Jauch *et al.* 1992; Fröncke *et al.* 1996; Nash *et al.* 1998; Breen *et al.* 1999) and has been attributed to the presence of subtelomeric repeat sequences and their high evolutionary mutation rate. For the purpose of comparing Zoo-FISH maps of different species, unhybridized gaps between FISH signals of the same probe are disregarded. Similarly, syntenic associations that are not contiguous but separated by unhybridized regions or centromeres were registered.

#### (c) *Painting of human chromosomes with probes derived from the African elephant*

The elephant paint probes delineated 68 homologous segments in the human karyotype. The elephant probe signals covered the complete human euchromatin except HSA 1q31.2-31.3, 8p23 and 19p13.1. Elephant chromosomes 10, 11, 23 and X probes delineated whole chromosome homologies to human chromosomes 9, 17, 20 and X, respectively. The LAF 12 probe, which produced FISH signals on eight segments distributed over four human chromosomes, exemplifies the other extreme. Material homologous to human chromosome 3 seems to have undergone particular evolutionary rearrangements, as it displayed the most complex hybridization pattern. Elephant probes delineated an extremely high number of 11 evolutionary conserved segments (ECS) on HSA 3. Elephant chromosome pairs 16 + 17 and 19 + 20 were never separated in the flow karyotype. For these chromosomes the human homologies could be identified because of the reciprocal Zoo-FISH data.

#### (d) *Painting of African elephant chromosomes with human probes*

The reciprocal Zoo-FISH analysis with paint probes for all human chromosomes except the Y identified a total of 53 conserved segments in the elephant, comprising 52 autosomal hybridization sites and a further ECS on the X chromosome (figure 3a). In addition to the above-mentioned chromosomes displaying complete conservation, the synteny of human chromosomes 5, 6 and 18 has been maintained in the elephant genome, although the homologous elephant chromosomes display additional signals from HSA 21, 3 and 19, respectively. None of the human paint probes produced FISH signals in a small region below the centromere on LAF 23.

Table 1. Chromosomal homologies between human and African elephant karyotypes.

(The conserved segments are ordered according to the numbering of elephant chromosomes (LAF) and to their position on elephant chromosomes from top to bottom.)

LAF	human chromosome homologies	LAF	human chromosome homologies	LAF	human chromosome homologies
1	3q21, 3q27-qter, 6pter-qter	11	17pter-qter	21	1q32.1-q32.3, 3p12-q13.1, 21q12, 21q22.1-q22.2, 3p12-q13.1
2	1pter-q22, 19p13.3-p13.2	12	2pter-p23, 2p14-13, 11q12, 11q23.2-qter, 16p11, 16pter-p11 7pter-p22, 7q11.1-11.2, 7q21.2-21.3	22	1q23-31, 1q41-qter
3	21q21, 5pter-qter	13	18pter-qter, 19q13, 19q13.3-qter	23	20pter-q13.2
4	10pter-cen, 12 pter-q23 22q12.2-13.1	14	1q32.1, 11q22.3-23.2, 2p13-q13 1q32.1	24	3p25-24.1, 3p23-22, 3q13.2-13.3
5	2q14.2-qter	15	8cen-qter	25	8p22-cen, 22q11-q12.3, 12q23-qter
6	4pter-q31.3	16	13q11.3-q32	26	3q22-q27, 13q11, 13q32-qter
7	11pter-cen, 11q12-22.3	17	4q31.3-32, 15q21.1-qter	27	3p24.1, 3p22, 3q21, 2p23-p15
8	7p21-cen, 7q11.3-21.1	18	10cen-qter		
9	15q11-21.1, 14cen-qter	19	3pter-p25, 3p21.3-p13		
10	9pter-qter	20	19q13.1, 19q13.2-13.3, 16p11-qter 4q32-35		

To confirm our mapping data for the elephant karyotype, all identified human syntenic associations were checked by two-colour hybridizations with the respective human probes onto elephant metaphases (see figure 2). To determine the homologies of human chromosomes 4 and 8 with precision, we also hybridized the cat chromosome B1 and F2 probes on elephant metaphases which are the homologues to these human chromosomes (Wienberg *et al.* 1997). The B1 probe hybridized to the same regions as HSA 4 and in addition to the HSA 8 homologous region on LAF 25. Cat chromosome F2 showed homology to the LAF 15 long arm.

#### 4. DISCUSSION

A comparison of the present maps with the elephant Zoo-FISH data of Yang *et al.* (2003) shows agreement for all large conserved blocks. However, the new study provides more details regarding smaller conserved segments. Our data delineate eight additional conserved segments in the elephant genome and 12 further segments in the human genome. The higher number of conserved segments in the human genome is in part due to the differences in the flow karyotype. Our sorts were able to separate the previously unresolved elephant chromosomes 24 and 25. Two further differences have most probably been caused by the difficult identification of some elephant chromosomes as the mapping data differ for two elephant chromosome pairs. We have mapped the FISH signals of HSA 2 and 4 to LAF 5 and 6 instead of 6 and 5, respectively, and signals of HSA 3 and 1 to LAF 19 and 22 instead of the reverse assignment.

##### (a) Genome conservation

Comparison with other Zoo-FISH studies shows that the elephant karyotype is moderately rearranged. The number of 53 conserved segments identified by human paint probes in the elephant is a little higher than average. This number varies in other Zoo-FISH studies, from 29 (dolphin (Bielec *et al.* 1998)) and 30 (harbour seal

(Frönicke *et al.* 1997)) to 74 (fox (Yang *et al.* 1999)). In part due to the smaller size of the elephant chromosomes, the elephant probes delineate the chromosomal homologies at a higher resolution of 68 conserved segments. They do not only pinpoint evolutionary translocations but also delineate inversions on human chromosomes 1, 2, 3, 7, 11, 13 and 21 by alternating hybridization patterns. For HSA 3 and 7 these inversions can be clearly assigned to the human evolutionary lineage as our findings are in agreement with previous reports involving probe sets from different species (Goureau *et al.* 1996; Korstanje *et al.* 1999; Müller *et al.* 2000). The reciprocal painting experiments allowed us to identify the segment-to-segment homologies between human and elephant karyotypes (table 1).

##### (b) Afrotherian karyotype characteristics

Whereas the classification of mammals into 18 orders has remained mostly undisputed over the last few decades (Novacek 1992), the supraordinal phylogeny of placental mammals has remained the focus of intense discussions in both morphological and molecular systematics and has witnessed the development of varying hypotheses.

The supraordinal grouping Afrotheria was introduced on the base of DNA sequence comparisons (Springer *et al.* 1997; Stanhope *et al.* 1998) and has been corroborated by extensive nuclear DNA studies recently (Eizirik *et al.* 2001; Madsen *et al.* 2001; Murphy *et al.* 2001a). However, any morphological support for an Afrotherian clade is missing so far. The recent aardvark–elephant–human Zoo-FISH study (Yang *et al.* 2003) also did not define common derived chromosomal characteristics for aardvark and elephant. However, the comparison of our more detailed elephant Zoo-FISH data with the aardvark data of Yang *et al.* delineates the syntenic association of HSA 5 and 21 in both genomes (aardvark chromosome 2 and elephant chromosome 3). As the previously undescribed HSA 21 signal is located near the centromere of LAF 3, it could have potentially been caused by cross hybridization of repetitive DNA sequences. However, sequence

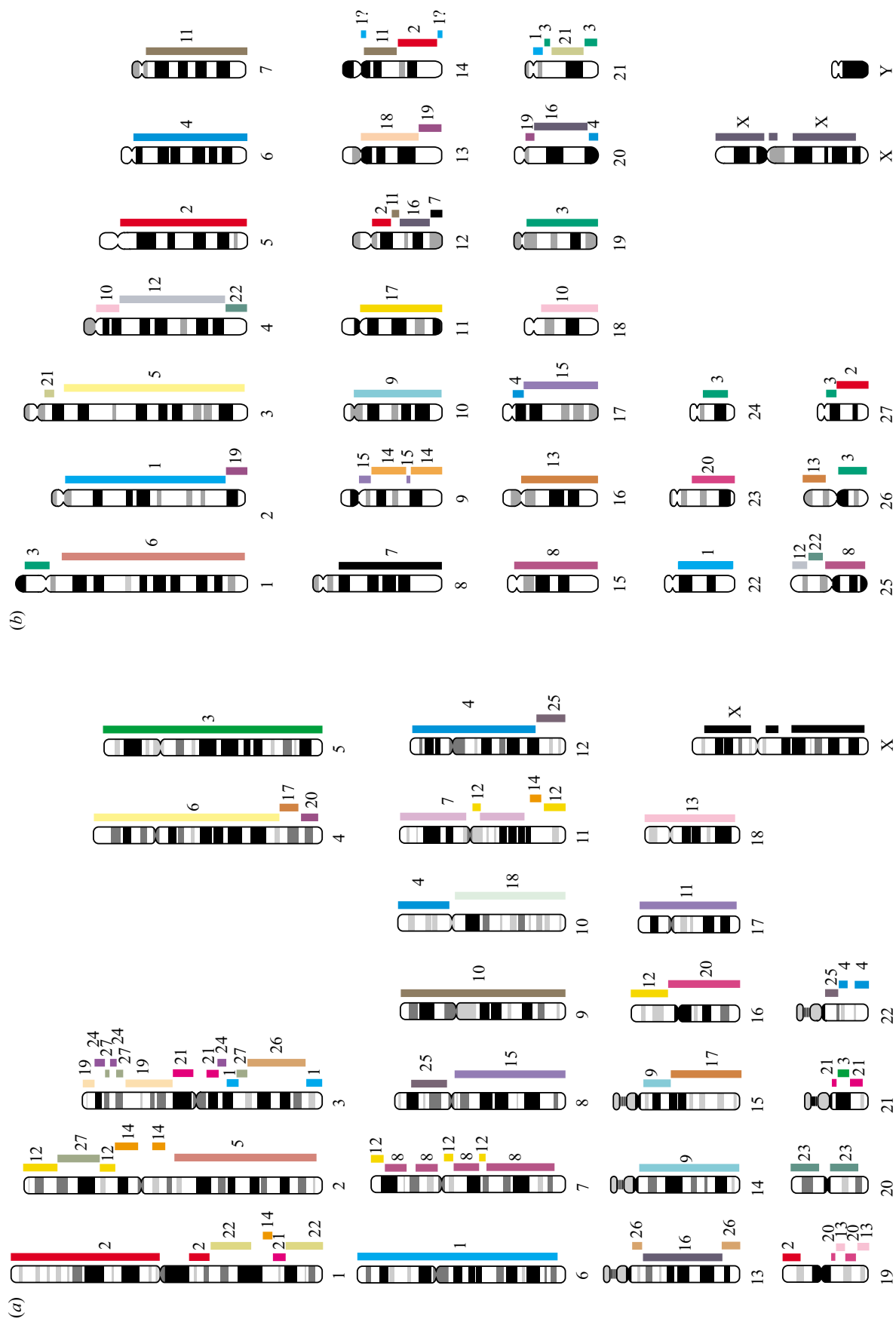


Figure 3. Comparative human and African elephant genome maps summarizing the Zoo-FISH results. (a) The chromosomal homologies delineated by elephant paint probes in the human karyotype are indicated to the right of human chromosome ideograms by coloured and numbered bars. (b) Distribution of 53 conserved segments on an ideogram of the elephant karyotype based on the GTG banded karyotype presented by Houck *et al.* (2001).

homologies in repetitive DNA are highly unlikely between such distantly related mammals. A comparison with the C-banded karyotype (Houck *et al.* 2001) shows that the HSA 21 signal is located in a euchromatic region between two small C-band positive blocks and therefore is unlikely to be due to repetitive sequences. This association has not been reported for other species and therefore constitutes chromosomal rearrangement linking the two Afrotherian species.

An association between HSA 1 and 19 is probably a second linking trait. Yang *et al.* hypothesize that an association of HSA 1 and 19 is part of the ancestral placental karyotype, because it is also found outside the Afrotheria in a primate, the galago (Stanyon *et al.* 2002). However, this is the only occurrence of this association in a species which is not a well suited model for ancestral karyotype forms, because of its highly rearranged karyotype (Stanyon *et al.* 2002). A comparison of G-banding patterns of the galago and the aardvark and elephant makes it seem very likely that the HSA 19 homologous segment is fused to different parts of HSA 1 in the galago and in the Afrotheria. Whereas the HSA 19 segment seems to be joined to HSA 1q homologous material in the former, it seems to be connected to HSA 1p material in the latter species. For these reasons it is most likely that the HSA 1/19 association in the galago has an independent origin from the one found in Afrotheria.

The Zoo-FISH data therefore provide, to our knowledge, the first non-DNA-sequence data which support the grouping of Proboscidea and Tubulidentata into an Afrotherian clade. The chromosome painting studies delineate two chromosomal rearrangements (the associations HSA 1/19 and 5/21) which phylogenetically link the two Afrotherian species. But, because of the special phylogenetic position of the Afrotheria at the root of the placental evolutionary tree and because of the lack of suitable outgroup data, the question remains open whether these cytogenetic traits constitute Afrotherian synapomorphies or eventually ancestral Placentalia features.

### (c) Homologies to other Placentalia karyotypes

The elephant karyotype features the five well-known ancestral syntenic associations of human chromosome homologues that have been observed in various different placental orders (HSA 3/21, 7/16, 12/22 (2×), 14/15, 16/19; Chowdhary *et al.* 1998; Wienberg *et al.* 2000). Reciprocal Zoo-FISH studies identified the 16/19 association as a conserved segment consisting of human chromosome 16q and 19q homologues (Goureau *et al.* 1996; Wienberg *et al.* 1997; Breen *et al.* 1999; Korstanje *et al.* 1999). The elephant data agree with these findings but LAF 20 hybridizes also to the proximal part of the HSA 16 short arm in addition to the chromosome 16 and 19 long arms. This signal might be caused by cross-hybridizations to repetitive sequences present on both sides of the HSA 16 centromere (Dauwerse *et al.* 1992) or it might reflect the special evolutionary plasticity of this region (Haig 1999). For example, it has been shown that this region harbours the integration sites for duplicated material from at least four other human chromosomes (Eichler 1998).

In addition to the known ancestral associations, the elephant shares a chromosome homologous to

HSA10/12/22 (LAF 4) with the aardvark and two carnivores with highly conserved karyotypes, the American mink (Hameister *et al.* 1997) and the harbour seal (Frönicke *et al.* 1997). These findings provide good arguments that the ancestral chromosome not only showed homologies to HSA 12pter-q23 and 22q (Chowdhary *et al.* 1998; Wienberg *et al.* 2000; Murphy *et al.* 2001b) but also to HSA 10p.

Besides the already discussed associations, 20 further syntenic associations are present in the elephant genome map. Eight of these have been described for other species (HSA 1/2, 1/11, 2/11, 3/6, 4/15, 4/16, 7/10). They most probably represent convergent events because they have been reported for only a single species with a highly rearranged genome (dog, horse or zebra (Raudsepp *et al.* 1996; Breen *et al.* 1999; Sargan *et al.* 2000; Richard *et al.* 2001)). Others show associations at the centromeres (giant panda (Nash *et al.* 1998)). As Robertsonian rearrangements (whole arm translocations) seem to be by far the most common type of evolutionary translocation they are prone to homoplasies (Wienberg *et al.* 2000). Out of the aforementioned associations only HSA 1/2 is more common and found in three different mammalian orders (Rettenberger *et al.* 1995; Chowdhary *et al.* 1996; Wienberg *et al.* 1997; Breen *et al.* 1999; Iannuzzi *et al.* 1999). However, it can be shown for those three species for which reciprocal Zoo-FISH data are available (the cat, the dog and the elephant), that this association involves different segments in each case and therefore has been generated by independent events three times.

It has also been proposed that human chromosome 4 and 8 homologous segments were syntenic in the ancestral Placentalia genome (Richard *et al.* 2001). However, because it is missing in several species (the primates, the tree shrew, the dolphin and the horse) its evolutionary history is difficult to determine (Richard *et al.* 2001). The same problem persists in the new Zoo-FISH data. In the Afrotheria this association is present in the aardvark (Yang *et al.* 2003) but not in the elephant (Yang *et al.* 2003; the present data).

The published Zoo-FISH data combined with our unpublished data indicate the presence of the 4/8 association in representatives of all analysed eutherian orders except Primates, Scandentia (Müller *et al.* 1999) and Insectivora (Dixkens *et al.* 1998); however, the 4/8 association might have been missed as discussed by Volleth *et al.* (2002). Reciprocal Zoo-FISH identifies an association of the entire HSA 8p arm with the complete human chromosome 4 (cat) or with the telomeric end of the HSA 4q arm (4q34-qter) (the dog, the rabbit, the squirrel and the aardvark). The present comparative human and elephant maps and additional experiments with cat probes show that the 4q telomeric region is not associated with HSA 8 but instead with HSA 16 in the elephant. The HSA 8 p-arm homologous region is found associated with HSA 12 and 22.

The most parsimonious explanation in congruence with the phylogenetic data would suggest an ancestral chromosome homologous to HSA 4 and 8p in which the two conserved segments have been separated by a centromere. Such a chromosome form has not been observed by Zoo-FISH yet, but would explain the convergent fissions of the



two homologous segments blocks by the most common Robertsonian rearrangements.

The absence of the 4/8 association (and the conservation of the HSA 8 synteny) in all primates as well as in tree shrews might, however, indicate a fission of HSA 4 and 8 and a fusion of 8p and 8q homologous segments in a common ancestor, providing a common derived trait for both orders. This idea would be in agreement with classical hypotheses based on morphological comparisons, which identified them as sister-groups or even as a single order (Martin 1990).

#### (d) *Reconstructions of ancestral eutherian karyotypes*

Comparative chromosome painting is a powerful tool for establishing global chromosomal homologies between distantly related species. By this approach large ECSs have now been delineated in species from 10 mammalian orders. These data allowed the suggestion of ancestral karyotypes at a cytogenetic level (Chowdhary *et al.* 1998; Wienberg *et al.* 2000; Murphy *et al.* 2001b).

Up until recently the taxon at the root of the eutherian evolutionary tree (Afrotheria) had not been analysed by Zoo-FISH. The mentioned reconstructed karyotypes cannot be attributed to the common ancestor of all Eutheria, but should instead apply to the ancestor of the Boreoeutheria (Eutheria excluding the older clades Xenarthra and Afrotheria). The comparative human and elephant genome maps show that the elephant shares many of the features of the reconstructed proto-boreoeutherian karyotype. These features are the conservation of homologues to entire human chromosomes 2q, 9, 10q, 17, 20, conserved synteny of homologues to human chromosomes 5, 6, 8p, 8q, 10p, 18, and the above mentioned syntenic associations of HSA 3/21, 7/16, 12/22 (2×), 14/15, 16/19.

The new comparative data from the elephant karyotype and the highly conserved aardvark complement (Yang *et al.* 2003) provide first insights into the genome organization of the ancestor of extant placental mammals, the proto-afrotherian. They add the following characteristics to previous reconstructions. As discussed above and also suggested by Yang *et al.* (2003), the new data indicate the presence of an ancestral chromosome homologous to HSA 10p/12pter-q23/22q and corroborate the presence of a chromosome homologous to HSA 4 and 8p in ancestral placental karyotypes. Yang *et al.* (2003) furthermore suggest an ancestral chromosome homologous to the complete human chromosome 1 and 19p. However, as described previously, the fusion of chromosome 1p and 19 is most probably restricted to the two afrotherian species and therefore can be rejected for the proto-boreoeutherian karyotype. Because no suitable outgroup for placental mammals has yet been analysed by Zoo-FISH, it is impossible to determine whether this rearrangement is ancestral for all placental mammals or a derived trait of the oldest placental clade, the Afrotheria. The conservation of the entire human chromosome 1 synteny however, which has been observed in the bottlenosed dolphin and the aardvark is also corroborated by new gene mapping and Zoo-FISH data (Murphy *et al.* 2003).

The available Zoo-FISH data therefore suggest ancestral proto-afrotherian and proto-boreoeutherian

karyotypes of  $2n = 46$  chromosomes consisting of chromosomes homologous to human chromosomes: 1, 2pter-q13, 2q13-qter, 3/21, 4/8p, 5, 6, 7a (7a: 7p21-cen, 7q11.3-21.1), 7b/16p (7b: 7pter-p22, 7q11.1-11.2, 7q21.2-21.3), 8q, 9, 10q, 10p/12pter-q23/22q, 11, 12q23-qter/22q11-q12.3, 13, 14/15, 16q-19q, 17, 18, 19p, 20, X, Y. Whereas the reconstruction of the proto-boreoeutherian karyotype is now well founded, our suggestion has to be preliminary regarding the proto-afrotherian karyotype because of missing outgroup data.

It can be expected that further studies of Xenarthran and Afrotherian genomes will provide additional insights into the proto-afrotherian karyotype. Future comparative genome studies in the Afrotheria should be greatly assisted by the complete set of paint probes of the African elephant, which is now available. To allow for a more confident interpretation of the Zoo-FISH data an outgroup comparison to marsupial genome data would be very desirable. However, the new Zoo-FISH studies covering an evolutionary distance of ca. 95–105 Myr between Afrotheria and humans (Eizirik *et al.* 2001) might have reached the limit for detailed Zoo-FISH analyses. Attempts to study marsupial genomes by chromosome painting with eutherian probes as well as the reciprocal experiments have been limited to signals of X-chromosome probes (Glas *et al.* 1999). Therefore, it will require either significant technical advances in the Zoo-FISH protocol or a comprehensive gene mapping effort in marsupials like the announced Kangaroo genome project (Graves & Westerman 2002) to enable tests of ancestral eutherian genome hypotheses and to attempt a reconstruction of the earliest mammalian genome evolution.

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