



A chromosome painting test of the basal Eutherian karyotype

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Abstract

We studied the chromosomes of an Afrotherian species, the short-eared elephant shrew *Macroscelides proboscideus* with traditional banding techniques and mapped the homology to human chromosomes by *in-situ* hybridization of human chromosome paints. Here we present for the first time the karyotype of this species, including banding patterns. The chromosome painting allowed us to test various hypotheses of the ancestral Eutherian karyotype, the validity of the radical taxonomic assemblage known as Afrotheria and the phylogenetic position of the elephant shrew within the Afrotheria. Current hypotheses concerning the Eutherian ancestral karyotype include diploid numbers ranging from $2n=44$ to 50 while molecular studies have proposed a new superordinal grouping of extant Eutherians. In particular, the Afrotheria is hotly debated, as it appears to be an odd mixture of species from Ungulata, Tubulidentata, Macroscelidea and Lipotyphla, which have no apparent morphological traits to unite them. The hybridization pattern delimited a total of 37 segments in the elephant shrew genome and revealed 21 different associations of human chromosome segments. Associations 1/19 and 5/21 link all Afrotheria so far studied and support the Afrotheria assemblage. Associations 2/8, 3/20, and 10/17 strongly link aardvarks and elephant shrews after the divergence of the line leading to elephants. The most likely ancestral Eutherian karyotype would be $2n=48$ chromosomes. However, the lack of comparative chromosome painting data between Eutherians and an appropriate outgroup is a severe limitation on attempts to delineate the ancestral genome of Eutherians. Current attempts lack legitimacy until this situation is corrected.

Introduction

Establishing the ancestral Eutherian karyotype has been one of the aims of interspecies chromosome painting studies. Most authors appeal to the concept of parsimony and consider that likely ancestral chromosomes are commonly present in species of a number of divergent Eutherian orders. Various authors have presented hypotheses on the ancestral Eutherian karyotype (see Table I). They include diploid numbers ranging from a high of $2n=50$ to a low of $2n=44$ (Chowdhary *et al.* 1998, Wienberg *et al.* 2000,

Murphy *et al.* 2001c, Froenicke *et al.* 2003, Murphy *et al.* 2003, Richard *et al.* 2003, Yang *et al.* 2003). Sixteen chromosomes are common to all hypotheses and correspond to human chromosomes, 2p+q, 2q, 3/21, 5, 6, 9, 11, 12/22, 13, 14/15, 16q/19q, 17, 18, 20, X and Y (Table I). Various disruptions and syntenic associations of the remaining chromosomes account for the discrepancies of the proposed ancestral Eutherian karyotype.

Chowdhary *et al.* (1998), in one of the first attempts to reconstruct a putative ancestral Eutherian karyotype, proposed a diploid number of $2n=48$.

Table I. Comparison of suggested ancestral Eutherian karyotypes

	Chromosome syntenies that differ between proposals																			
References	1	1p	1q	1/19p	4	4/8p	8	8q	7	7a	7b/16p	12/22	22a	10p/12p/ 22b	16p	19p	10	10p	10q	Diploid number
Chowdhary <i>et al.</i> 1998		X	X		X		X		X			X	X		X	X	X			48
Wienberg <i>et al.</i> 2000; Murphy <i>et al.</i> 2001c; Richard <i>et al.</i> 2003		X	X			X		X		X	X	2X				X		X	X	50
Murphy <i>et al.</i> 2003	X					X		X		X	X	2X				X		X	X	48
Frönicke <i>et al.</i> 2003	X					X		X		X	X	X		X		X			X	46
Yang <i>et al.</i> 2003				X		X		X		X	X	X		X					X	44

Chromosomes common to all proposed ancestral Eutherian karyotypes: Human chromosomes completely conserved as synteny: 2p + q, 2q, 5, 6, 9, 11, 13, 17, 18, 20, X, Y and Associations of human chromosome found as synteny: 3/21, 14/15, 16q/19q.

These authors hypothesized that human 4, 7, 8, 10, 16p were present as single chromosomes and that the association 12/22a with an additional chromosome 22b was also likely present in the Eutherian ancestral karyotype. However, with the inclusion of improved chromosome painting data from further taxa, it was deemed likely that chromosomes 4/8p, 7p, 7q/16p, 8q, 10p, 10q, 12q/22a, 12p/22b were present in the ancestral Eutherian karyotype and that $2n=50$ was the ancestral diploid number (Wienberg *et al.* 2000, Murphy *et al.* 2001c, Richard *et al.* 2003).

More recently, Murphy *et al.* (2003) performed an elegant analysis of comparative gene mapping data and reciprocal chromosome painting with homologs to human 1 in seven species from six Eutherian orders of mammals, demonstrating that the segments previously considered equivalents of human 1p and 1q in these species were not identical. These data, combined with the presence of a single human chromosome 1 homolog in three highly divergent Eutherian orders, strongly indicated that a single large chromosome, corresponding to human 1, was present in the ancestral Eutherian karyotype (Murphy *et al.* 2003). Different disruptions and rearrangements of

chromosome 1 would explain the present-day painting pattern and gene mapping data among mammalian taxa. Based on these findings, a karyotype with $2n=48$, differing from the $2n=50$ only in the presence of an intact chromosome 1 was proposed. Frönicke *et al.* (2003) later considered an additional combination HSA10p/12p/22q as present in the ancestral Eutherian karyotype, leading to a $2n=46$. Yang *et al.* (2003) proposed the lowest diploid number for the ancestral Eutherian karyotype, $2n=44$. As in Frönicke *et al.* (2003), they included a chromosome composed of human 10q/12p/22q but hypothesized that human 1 and 19p were fused into a single syntenic unit.

Over the last few years, molecular data from sequencing analysis of not only mtDNA but also most recently nuclear DNA, have provided radical grouping of primordial mammalian divergence (Murphy *et al.* 2001a, 2001b). These interpretations, which have shaken the phylogenetic tree to its very roots, deal with the surviving 18 Eutherian mammalian orders. The phylogenetic analyses of these DNA sequences, consisting mostly of nuclear exons, led investigators to identify four primary superordinal clades: (1) Afrotheria (elephants, manatees, hyraxes, tenrecs, aardvark

and elephant shrews); (2) Xenarthra (sloths, anteaters and armadillos); (3) Euarchontoglires (rodents and lagomorphs as a sister taxon to primates, flying lemurs and tree shrews); and (4) Laurasiatheria (cetaceans, artiodactyls, perissodactyls, carnivores, pangolins, bats and insectivores).

Initially, it was not completely clear whether Afrotheria or Xenarthra were basal (Liu *et al.* 2001, Murphy *et al.* 2001a). Murphy *et al.* (2001a) placed Afrotheria as the most basal Eutherian clade even though they could not statistically reject Xenarthra as the earliest and most divergent clade. Subsequent analysis with an enlarged data set, more confidently placed Afrotheria at the base of Eutherian phylogeny (Murphy 2001b). These authors proposed that the basal split between Afrotheria and other placentals, driven by the separation of South America and Africa in the Cretaceous, was probably in excess of 100 million years (Eizirik *et al.* 2001). Afrotheria and Xenarthra, the two oldest Eutherian clades are both from the Southern hemisphere and one conclusion is that crown-group Eutheria may have their most recent common ancestry in the Southern hemisphere (Gondwana; Eizirik *et al.* 2001). The other two clades (Laurasiatheria and Euarchontoglires) can be grouped as Boreoeutheria (Springer *et al.* 2003).

Paleontologists and morphologists have hotly contested the conclusions from molecular biology. Fossil and morphological analyses provide very different trees. Afrotheria, in particular, appears to be a hodgepodge composed of species from Ungulata, Tubulidentata, Macroscelidea and Lipotyphla, which have no apparent morphological traits to unite them. Further, traditional analyses suggest a divergence of Eutherian mammals at around 60 million years ago, almost half of what is derived from molecular data (Easteal 1999, Benton & Ayala 2003). However, some recent paleontological finds have extended the fossil dating for Eutherian origins to about 125 million years (Ji *et al.* 2002).

There is no consensus for the exact branching order within Afrotheria. Some authors have viewed the Macroscelidea, elephant shrews, as the most basal and early divergent order within the Afrotheria (Liu *et al.* 2001, Murphy *et al.* 2001a). However, Murphy *et al.* (2001b) placed the triumvirate of sirenians, hyrax and elephant (Paenungulata) as basal, a position since supported by a number of

other authors (Arnason *et al.* 2002, Delsuc *et al.* 2002, Murata *et al.* 2003, Springer *et al.* 2003).

In addition to the temporal and direct testimony of fossil remains, the tracks of evolutionary history are left on all levels of the genomic hierarchy, from the genotype to the phenotype. Precious complementary data can be gleaned from living species, ranging from DNA sequences to morphologic characteristics.

In this paper, specific human chromosome probes were used to paint chromosome spreads of an Afrotherian, the short-eared elephant shrew. Two previous publications deal with chromosome painting in elephants and the aardvark, two other taxa united under Afrotheria (Froenicke *et al.* 2003, Yang *et al.* 2003). Comparisons through chromosome painting provide an independent test of contrasting hypotheses of mammalian evolution and phylogeny. This research has three complementary goals: (1) To test various hypotheses of the ancestral Eutherian karyotype; (2) To test the validity of the radical taxonomic assemblage known as Afrotheria; and (3) To test various phylogenies of the elephant shrew within the Afrotheria. In this report, we also present the first cytogenetic data on the elephant shrew and include G- and C-banding patterns, as well as the Ag-NOR distribution.

Materials and methods

Chromosome preparations of a male short-eared elephant shrew *Macroscelides proboscideus* ($2n=26$; $NF=48$) were obtained from cultured fibroblasts kindly supplied by the Center for Reproduction of Endangered Species (CRES) of the San Diego Zoo, CA, in January 2000. Cells were cultivated in alpha-DMEM supplemented with 10% fetal bovine serum. Routine procedures were used for chromosome preparations. G- and C-banding patterns were obtained according to Seabright (1971) and Sumner (1972), respectively, with modifications. Silver staining of the nucleolus organizer regions (Ag-NORs) was performed according to Howell & Black (1980).

Chromosome suspensions of a human lymphoblastoid cell line were flow sorted with a dual laser sorter (FACS Vantage SE; Becton Dickinson) directly into PCR tubes containing 30 μ l of distilled water. The DOP-PCR amplification and labeling with the 6MW primer of each chromosome

probe was performed as previously described (Telenius *et al.* 1992, Stanyon *et al.* 1999), and either biotin-dUTP or digoxigenin-dUTP (Roche) were used for labeling.

Interspecific *in-situ* hybridization of human probes onto elephant shrew chromosomes were performed with 300 to 500 ng of each biotin or digoxigenin labeled probe, 10 µg of human Cot-1 DNA, 5 µg of sonicated elephant shrew genomic DNA and 5 µg of ssDNA. The mixture was precipitated and dissolved in 13–15 µl of hybridization mix (formamide 50%, dextran sulfate 10%, in $2 \times$ SSC), denatured for 10 min at 80°C and then reannealed at 37°C for 90 min before being applied to the hybridization areas. Chromosomal DNA was denatured in 70% formamide/ $2 \times$ SSC, at 65°C for 90–120 s, followed by dehydration in ice-cold ethanol. Hybridization was carried out in a wet chamber at 37°C for five days. Post-hybridization washes followed standard procedures at 40°C. Detection was performed with avidin conjugated with FITC (Vector) or anti-digoxigenin conjugated with rhodamine (Roche) for 45 min at 37°C. Counterstaining was performed with DAPI (0.8 ng/µl) for 10 min and the slides were mounted with antifade (PPD).

Analyses were performed under a Zeiss Axiophot 2 fluorescence microscope coupled with a CCD camera (Photometrics) and images were captured with the Smart Capture software (Digital Scientific Inc.).

Results

The karyotype of the short-eared elephant shrew (*Macroscelides proboscideus*), presented here for the first time, has 26 chromosomes (Figure 1). All autosomes are biarmed, ranging from metacentric to subtelocentric, resulting in a fundamental number of 48. The X chromosome is a small submetacentric and the Y, the smallest chromosome in this karyotype, is acrocentric. C-banding shows that large pericentromeric heterochromatin blocks are present in pairs 2, 3 and 4 (Figure 2a). These blocks are lightly stained with G-banding. Chromosome pairs 8 and 9 have large blocks of heterochromatin in their long arms and most of the Y chromosome is heterochromatic (Figure 2a). Silver staining revealed the presence of a single nucleolus organizer region on the long arm of pair 8 (Figure 2b).

Hybridization with human chromosome paints

Figure 3 shows examples of human chromosome paints hybridized to short-eared elephant shrew metaphases. Thirteen out of the 22 human autosomes (human 1, 4, 5, 6, 9, 11, 13, 14, 15, 17, 18, 20, and 21) and the X chromosome provided single signals. Five human chromosomes (7, 8, 10, 12, and 22) gave two hybridization signals. Three chromosome paints to human 2, 16 and 19 gave three signals while the paint to human 3 provided four signals. Chromosomes 16 and 19 each painted two chromosomes, but, on elephant shrew chromosome 8, each paint provided two alternating signals, indicative of a pericentric inversion. The Y chromosome was the only human probe that failed to provide a signal in the elephant shrew. Thus, the human chromosome paints delimited a total of 37 segments in the elephant shrew genome.

The hybridization pattern revealed that 21 different associations of human chromosome segments are found in the elephant shrew genome: 1/19, 1/2, 2/8, 2/9, 2/10, 3/21, 3/13, 3/15, 3/18, 3/20, 4/8, 5/21, 6/7, 7/16, 8/11, 10/12, 10/17, 12/22 ($2 \times$), 16/19 ($2 \times$), 14/15, 16/22. Associations separated by centromeres or heterochromatic regions were counted.

Discussion

Chromosome painting has proved an invaluable tool for dissecting the genomes of mammals in the quest for their common ancestral karyotype. Although some questions remain, the general picture that has emerged is one of conservation, reflected by the uniformity of proposals for the ancestral Eutherian karyotype as depicted in Table I.

The chromosome painting pattern of the elephant shrew was compared with results recently published on two other Afrotheria orders (Proboscidea and Tubulidentata); (Froenicke *et al.* 2003, Yang *et al.* 2003). Our results show that the elephant shrew, aardvark and elephant all have eight associations in common (1/19, 3/21, 5/21, 7/16, 10/12, 12/22, 14/15, 16/19).

From the eight associations found in all the Afrotheria analyzed, five are considered ancestral to all Eutherians by almost all proposals (3/21, 7/16, 12/22 twice, 14/15, and 16/19). All these

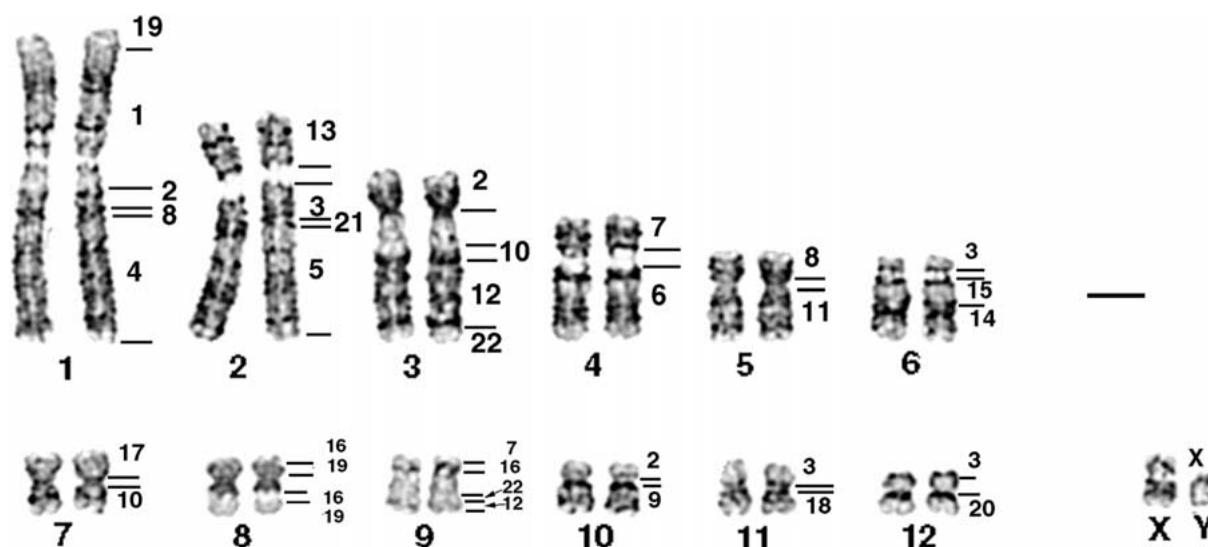


Figure 1. G-banded karyotype of a male short-eared elephant shrew *Macroselides proboscideus* ($2n=26$; $NF=48$). The correspondence to human chromosome segments as revealed by chromosome painting is shown on the right of each chromosome.

associations occur in most Eutherian orders and their presence in all Afrotheria so far studied certainly strengthens the conclusion that they are ancestral.

Three associations (1/19, 5/21, and 10/12) are found in all Afrotheria. The two contrasting hypotheses are whether they should be considered part of the ancestral Eutherian karyotype

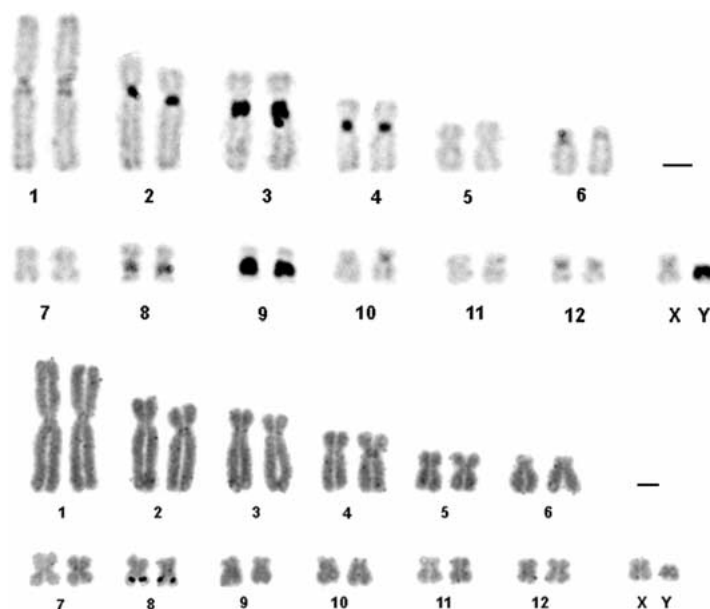


Figure 2. C-banded (top) and Ag-NOR stained (bottom) karyotypes of a male short-eared elephant shrew *Macroselides proboscideus* ($2n=26$; $NF=48$).

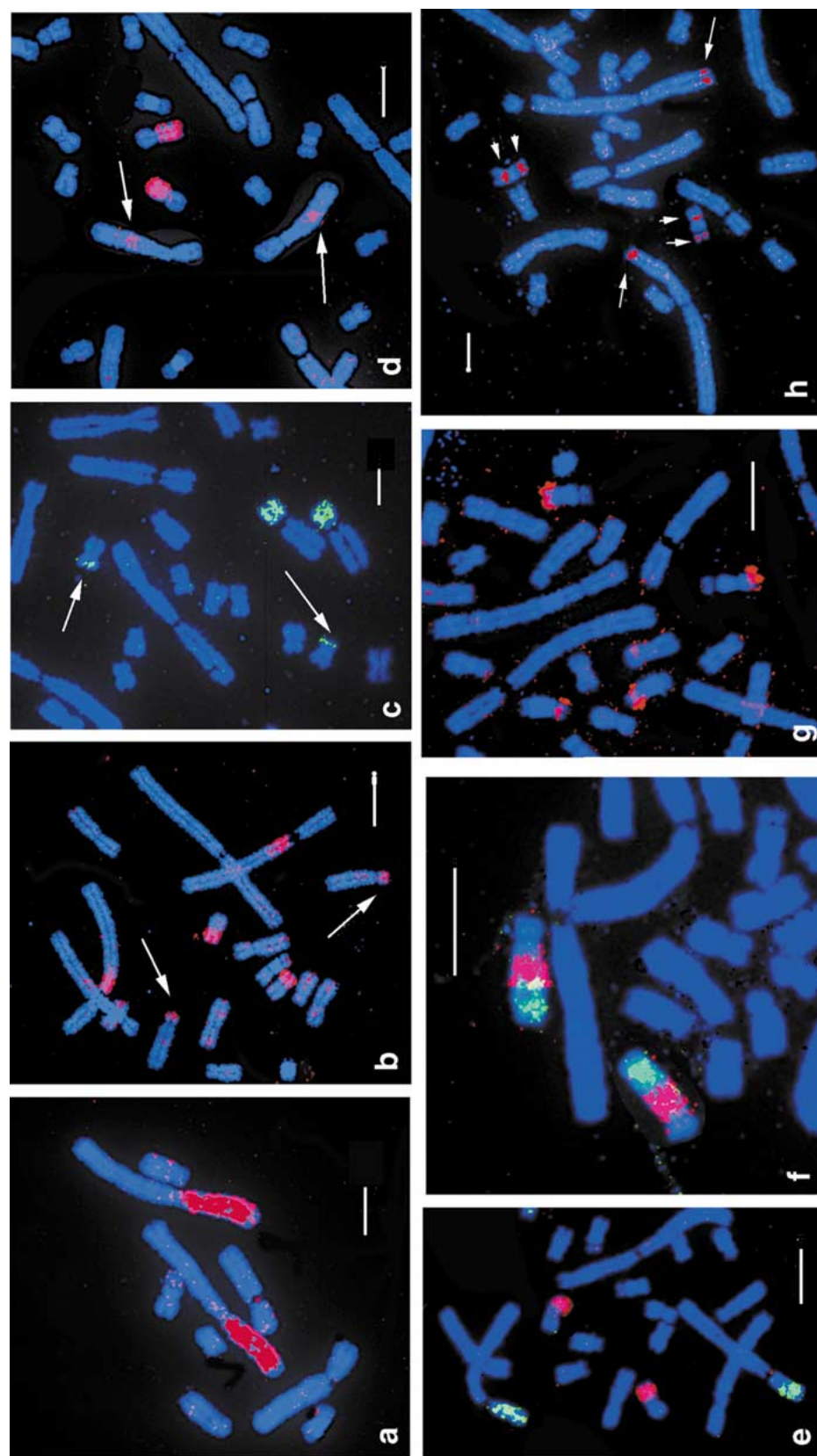


Figure 3. Partial metaphases of the short-eared elephant shrew (MPR) after chromosome painting with human (HSA) chromosome-specific probes: (a) HSA1 on MPR1; (b) HSA3 painted segments of MPR2, MPR6, MPR11 and MPR12; (c) HSA7 on MPR4 and MPR9; (d) HSA10 on MPR3 and MPR7; (e) HSA13, in green, on MPR2 and HSA17, in red, on MPR7; (f) HSA14, in green, and HSA15, in red, on MPR6; (g) HSA16 on MPR8 and MPR9; (h) HSA19 on MPR1 and MPR8. The chromosomes were counterstained with DAPI and the hybridizations were detected with avidin-FITC (green) or antidigoxigenin-rhodamine (red).

or, instead, represent derived associations linking Afrotheria taxa.

The association 5/21 is not found in any species outside of the Afrotheria and therefore cannot be considered for inclusion in the ancestral Eutherian karyotype.

The presence of human chromosome 1 as a single block in the elephant shrew, aardvark and elephant reinforces the conclusion of Murphy *et al.* (2003) that HSA1 was conserved as a syntenic block in the ancestral Eutherian karyotype (Figures 1 & 3a). A conserved synteny for this chromosome has now been found in each of the superordinal taxa: Afrotheria (aardvark and elephant shrews), Xenarthra (sloths), Euarchontoglires (primates), and Laurasiatheria (cetaceans).

The combination human 1/19p, found in the four studied species of Afrotheria (Froenicke *et al.* 2003, Yang *et al.* 2003), was considered as common to the ancestral Eutherian karyotype by Yang *et al.* (2003), who proposed a $2n=44$ (Table I). The presence of this combination in the aardvark and elephant, as well as in the galago (Stanyon *et al.* 2002), led these authors to assume that it was ancestral. Nevertheless, the galago karyotype is highly rearranged in relation to other primates and the segment of human 19 combined to the counterpart of human chromosome 1 in the galago may not be the same as the HSA19 region combined to HSA1 in the Afrotheria. Further, in primates, that association is probably not homologous because it is probably formed by different segments of chromosome 1 (Froenicke *et al.* 2003). In conclusion, associations 1/19 have been found in only one order outside of Afrotheria (primates). This limited frequency is not sufficient for inclusion in the ancestral Eutherian karyotype as it could result from convergence.

The status of the third association, 10/12, is more difficult to determine. A combination of human 10p/12p/22a and a single human 10q was previously found in Afrotheria, the aardvark and elephant karyotypes. This finding led to the suggestion that 10p/12q/22a was the arrangement in the ancestral Eutherian karyotype (Froenicke *et al.* 2003, Yang *et al.* 2003). We found an apparently identical sequence of segments in the elephant shrew. The elephant shrew data support the conclusion that these associations are widespread in Afrotheria and appears by reciprocal painting to

be homologous to that found in Carnivores. However, up to now, the association 10/12/22 has not been found in any further Eutherian order and therefore it is not possible to rule out convergence for this trait between Afrotheria and Carnivores. Multiple reciprocal painting between Afrotheria and Carnivore (such as done for chromosome 1 homologs between diverse mammalian orders; Murphy *et al.* 2003) could help establish whether the 10/12/22 chromosome is truly homologous.

It appears that the associations 5/21 and 1/19 can safely be considered as derived associations, i.e. cytogenetic landmarks, linking Afrotheria species, thus providing cytogenetic evidence that Afrotheria is a natural clade and supporting the conclusions of molecular studies.

Three additional associations (2/8, 3/20, and 10/17) are found only between elephant shrew and aardvark. Both publications on elephant painting found a 3/13 association and one publication found a 1/2 association (Froenicke *et al.* 2003, Yang *et al.* 2003). Apparently, these two associations could phylogenetically link elephants and elephant shrew (1/2, 3/13). However, Froenicke *et al.* (2003) indicate that the presence of a signal to human chromosome 1 associated with a segment of chromosome 2 was considered only a vague possibility. In the elephant shrew, the probe to human 3 gave signals on three different chromosomes and was found in four associations: 3/21, 3/13, 3/15, and 3/18. From banding and the reciprocal hybridization pattern, it is clear that 3/13 associations are not homologous as they do not involve the same segments of chromosome 3 (Muller *et al.* 2000, Froenicke *et al.* 2003, Yang *et al.* 2003). In conclusion, the common derived association pattern of 2/8, 3/20 and 10/17 indicates a strong phylogenetic link between elephant shrew and aardvark after the divergence of the elephant. Thus, the chromosome data help resolve some uncertainty as to the placement of the Macroscelidae within the Afrotheria (Liu *et al.* 2001, Murphy *et al.* 2001a) and in concordance with more recent molecular phylogenetic results (Murphy *et al.* 2001b, Arnason *et al.* 2002, Murata *et al.* 2003, Springer *et al.* 2003).

The association of human 4/8p as part of the ancestral Eutherian karyotype gains support from this report as this association was also found in the aardvark (Yang *et al.* 2003) and in

the elephant shrew (Figure 1). Both publications (Fronicke *et al.* 2003, Yang *et al.* 2003) on the elephant indicate that this association is lacking. It was either not detected or, if lost, it probably represents a derived trait of the lineage leading to the elephant. The widespread occurrence of the 4/8 association in all mammalian orders outside of elephants and primates lends credence to its inclusion in the ancestral mammalian karyotype. Further painting in Proboscidea and Sirenia would be informative as to when this association was lost. For instance, our unpublished data of *in-situ* hybridizations in additional Scadentia species shows that the 4/8 association was probably missed in earlier work (Muller *et al.* 1999).

The addition of chromosome painting data in the elephant shrew led us to conclude that, based on the current available data, the most likely ancestral Eutherian karyotype would be the $2n=48$, basically the same as proposed by Murphy *et al.* (2003). In this karyotype, human chromosome 1 is represented as a single chromosome and all the other features are the same as detailed in Murphy *et al.* (2001c).

Finally, an essential point that has not been strongly noted is that all reconstructions of the ancestral Eutherian karyotype are preliminary until an appropriate outgroup is studied with chromosome painting. The lack of comparative chromosome painting data between Eutherians and other mammals, monotremes and marsupials, is a severe limitation on attempts to delineate the ancestral genome of Eutherians. Current attempts lack legitimacy until this situation is corrected.

Recent molecular data suggest that marsupials (Metatheria) are the most appropriate outgroup because they may share a period of common descent with Eutherians after the divergence of the platypus and echidna (Prototheria) (Killian *et al.* 2001). Further data on Afrotheria and especially Xenarthans, groups considered basal in the Eutherian tree, are also scarce. Only partial and sketchy data is available on Xenarthans (Murphy *et al.* 2003, Richard *et al.* 2003). The need for a taxonomically rich array of species supported by appropriate outgroups in the reconstruction of mammalian genome evolution cannot be sufficiently stressed. The analysis of marsupials as

well as other Afrotherians and Xenarthrans may shed light on the genome evolution, phylogeny and origin of Eutherians.

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