

Defining the ancestral karyotype of all primates by multidirectional chromosome painting between tree shrews, lemurs and humans

S. Müller^{1,2}, R. Stanyon³, P.C.M. O'Brien², M.A. Ferguson-Smith², R. Plesker⁴, J. Wienberg^{1,3}

¹ Institut für Anthropologie und Humangenetik, Ludwig-Maximilians Universität München, München Germany

² Department of Pathology, University of Cambridge, Cambridge, UK

³ Laboratory of Genomic Diversity, National Cancer Institute, Building 560, Room 11-75, Frederick, MD 21702-1201, USA

⁴ Paul-Ehrlich Institut, Frankfurt, Germany

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Abstract. We used multidirectional chromosome painting with probes derived by bivariate fluorescence-activated flow sorting of chromosomes from human, black lemur (*Eulemur macaco macaco*) and tree shrew (*Tupaia belangeri*, order Scandentia) to better define the karyological relationship of tree shrews and primates. An assumed close relationship between tree shrews and primates also assists in the reconstruction of the ancestral primate karyotype taking the tree shrew as an “out-group” species. The results indicate that *T. belangeri* has a highly derived karyotype. Tandem fusions or fissions of chromosomal segments seem to be the predominant mechanism in the evolution of this tree shrew karyotype. The 22 human autosomal painting probes delineated 40 different segments, which is in the range found in most mammals analyzed by chromosome painting up to now. There were no reciprocal translocations that would distinguish the karyotype of the tree shrew from an assumed primitive primate karyotype. This karyotype would have included the chromosomal forms 1a, 1b, 2a, 2b, 3/21, 4–11, 12a/22a, 12b/22b, 13, 14/15, 16a, 16b, 17, 18, 19a, 19b, 20 and X and Y and had a diploid chromosome number of $2n=50$. Of these forms, chromosomes 1a, 1b, 4, 8, 12a/22a, and 12b/22b may be common derived characters that would link the tree shrew with primates. To define the exact phylogenetic relationships of the tree shrews and the genomic rearrangements that gave rise to the primates and eventually to humans further chromosome painting in Rodentia, Lagomorpha, Dermoptera and Chiroptera is needed, but many of the landmarks of genomic evolution are now known.

Introduction

It has been demonstrated that fluorescence in situ hybridization (FISH) allows karyotypes to be compared with more accuracy and with higher resolution than classical banding analysis (Wienberg et al. 1990, 1992; Jauch et al. 1992; Wienberg and Stanyon 1997, 1998). The increased quality of chromosome-specific paints generated by flow sorting and the degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) (Telenius et al. 1992) has made FISH ever more attractive for comparison of the human karyotype with those of distantly related mammalian species (Scherthan et al. 1994; Wienberg and Stanyon 1998). Chromosome-specific paints for any mammalian species can be established by fluorescence-activated chromosome flow sorting (FACS) (Ferguson-Smith 1997). These probes can be used for reciprocal painting experiments (Arnold et al. 1996; Goureau et al. 1996; Müller et al. 1997; Wienberg et al. 1997).

The present work was initiated to gather more information on the origin of primate chromosomes and the karyological and phylogenetic relationship of tupaia or tree shrews (family Tupaiidae). Tree shrews exhibit a relatively uniform body plan typical of unspecialized placental mammals. Their taxonomic classification is controversial and discussions mostly concern whether or not tree shrews should be considered primates. Various authors have described morphological traits that supposedly link tupaia with primates, with insectivores, or with rodents while others rank them as a separate mammalian order (Scandentia) (Butler 1972; Thenius 1979; Martin 1990; Cartmill 1992; De Jong 1998). Certainly, tree shrews are considered to be closely related to primates and are an appropriate outgroup to analyze by chromosome painting to determine better the ancestral karyotype of all primates.

There are very few reports on tree shrew chromosomes. Early classical non-banding studies showed that diploid numbers for species belonging to the genus *Tupaia* are relatively high ($2n=60, 62, 68$) (Chu and Bender 1962; Klinger 1963; Egozcue et al. 1968;

Edited by: T. Hassold

Correspondence to: J. Wienberg
e-mail: wienbergj@ncifcrf.gov

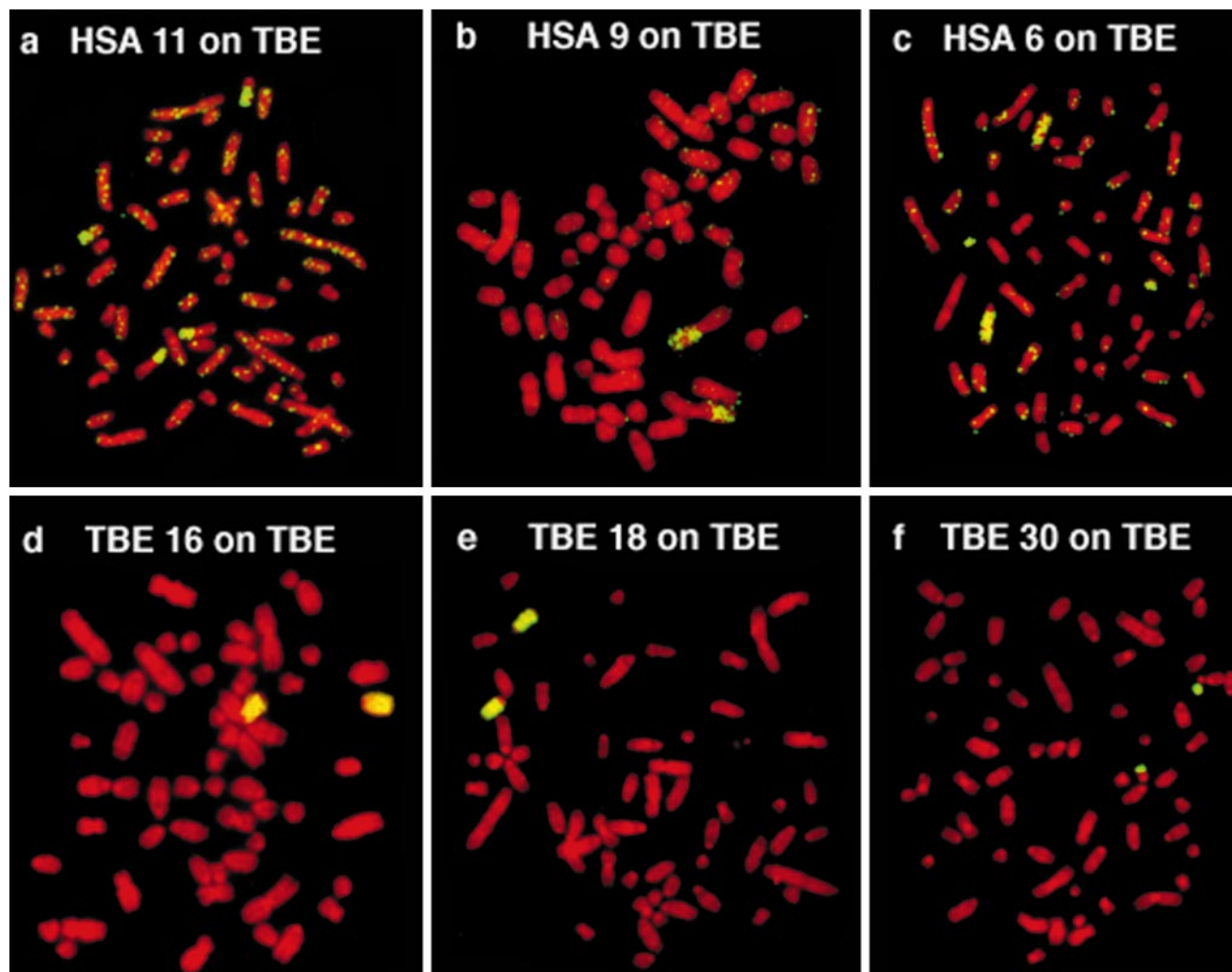


Fig. 1. Examples of in situ hybridization of human (*HSA*) chromosome-specific paints to *Tupaia belangeri* (*TBE*) metaphases (**a–c**), and *T. belangeri* chromosome-specific paints to metaphase spreads of the same species (**d–f**)

Arrighi et al. 1969; Elliot et al. 1969). Even chromosome banding has not contributed much useful information for the comparison of tree shrews with primates and chromosomal homologies have been proposed only within the order Scandentia (Toder et al. 1992).

We established painting probes from a tree shrew (*Tupaia belangeri*) by FACS. The tupaia chromosome probe set allowed reciprocal painting between humans and tree shrews. In order to extend and confirm these results tupaia chromosome paints were also hybridized to black lemur (*Eulemur macaco macaco*) metaphases. The chromosomal homology between black lemurs and humans was established previously by reciprocal chromosome painting (Müller et al. 1997).

Materials and methods

Cell samples, tissue culture and chromosome preparation

Primary skin fibroblast cell lines were established from one female individual of *T. belangeri* ($2n=62$) according to standard

protocols. In general, *T. belangeri* chromosomal nomenclature followed Toder et al. (1992). However, for the many acrocentric *T. belangeri* chromosomes, matching our G-banded chromosomes with the previously published R-banding karyotype was difficult, therefore we independently ordered acrocentric chromosomes according to size. A lymphoblastoid cell line of a normal human male and fibroblasts of a male *E. m. macaco* ($2n=44$), described in Müller et al. (1997), provided the metaphase spreads for reciprocal hybridization experiments.

Generation and labeling of chromosome-specific paints

Chromosome flow sorting was performed on a dual-laser cell sorter (FACStar Plus, Becton Dickinson Immuno-Cytometry Systems) (Rabbitts et al. 1995; Ferguson-Smith 1997). Chromosome preparation for flow sorting of human and *T. belangeri* chromosomes, subsequent primary DOP-PCR and secondary labeling DOP-PCR in the presence of biotin-dUTP were performed as described earlier (Telenius et al. 1992; Yang et al. 1997).

In situ hybridization and probe detection

Hybridization in situ and probe detection were carried out using previously described protocols (Wienberg et al. 1997; Yang et al.

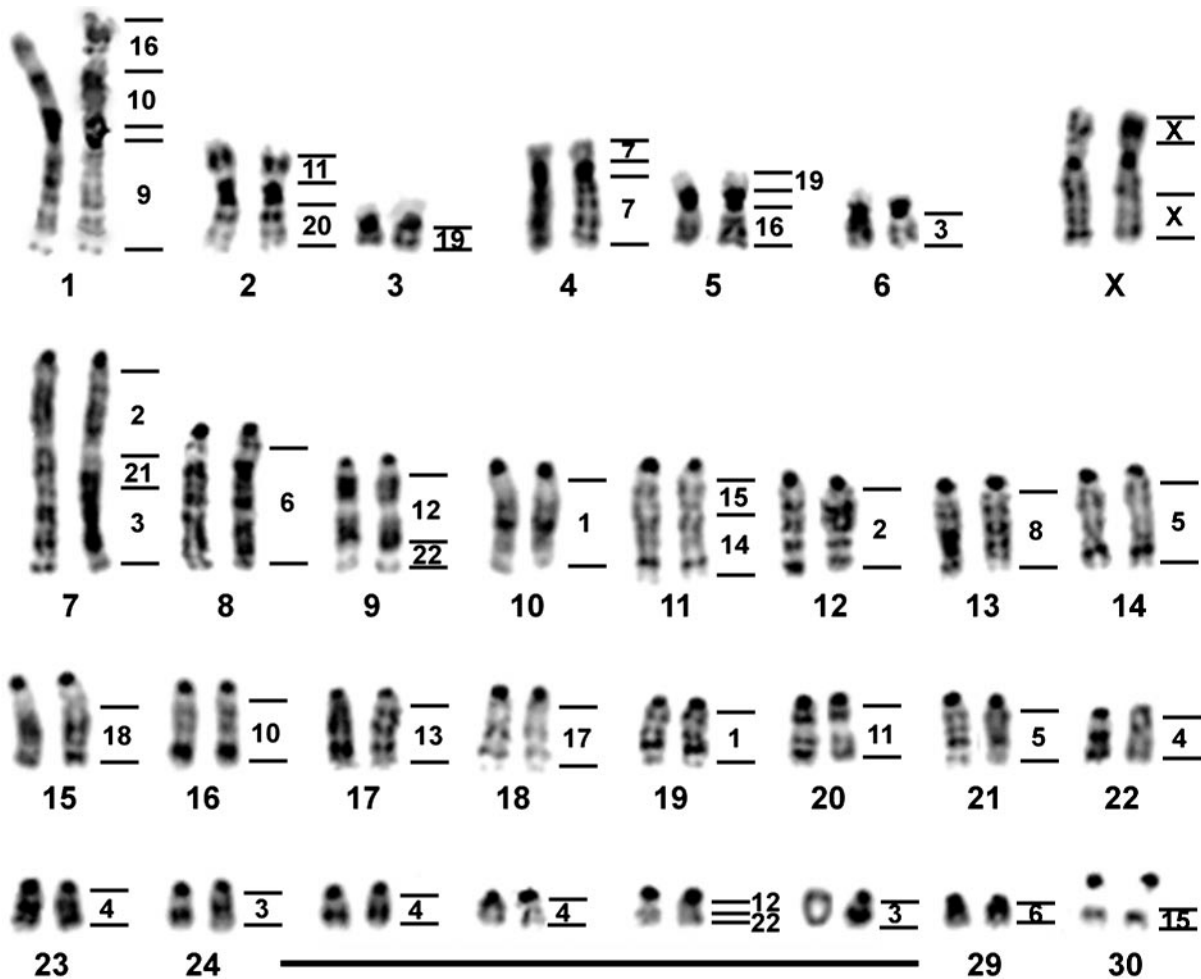


Fig. 2. G-banded karyotype of a female *T. belangeri* with the location of chromosome painting for all single human chromosome probes. The tree shrew chromosomes are numbered below and the human chromosome probes are numbered to the right of the ho-

mologous *T. belangeri* chromosomal segments. The small chromosomes 24–29 were difficult to distinguish by banding but defined through their position in the flow karyotype (see Fig. 3)

1997). 100 ng of biotinylated secondary DOP-PCR product was used in 15 µl hybridization buffer (50% deionized formamide, 10% dextran sulfate, 2×SSC). (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.) DNA probes were denatured at 68°C for 7 min and preannealed by incubation at 37°C for 30 min. In experiments where *T. belangeri* chromosome-specific paints were hybridized to chromosomes of *T. belangeri* or *E. m. macaco*, DNA probes were hybridized overnight. Post-hybridization washes included twice for 5 min in 50% formamide, 1×SSC at 45°C and twice for 5 min in 2×SSC at 45°C. When human chromosome-specific paints were applied to *T. belangeri* chromosomes and vice versa, probe concentration was increased to 300 ng and a less stringent hybridization protocol was followed: DNA probes were hybridized for up to 96 h, post-hybridization washes included twice for 5 min in 50% formamide, 1×SSC at 37°C and twice for 5 min in 2×SSC at 37°C. Biotinylated DNA probes were detected by avidin-fluorescein isothiocyanate and visualized without further amplification of the signal.

Microscopy and image analysis

Metaphases were analyzed with a cooled CCD camera (Photometrics NU200 equipped with a KAF1400 chip) coupled to a Zeiss Axiophot microscope. Camera control and digital image acquisi-

tion were as described by Ried et al. (1992). Merging of chromosomal images was performed by using Adobe Photoshop 3.0 software. Chromosomes were identified by computer-enhanced 4',6-diamidino-2-phenylindole (DAPI) banding (SmartCapture, Digital Scientific, Cambridge, UK).

Results

Hybridization of human chromosome-specific paints to *T. belangeri* metaphases

All human chromosome-specific DNA probes with the exception of the Y yielded hybridization signals on *T. belangeri* metaphase spreads. The hybridization quality, however, was significantly lower than that seen on other distantly related species investigated under comparable standard internal laboratory conditions (Wienberg et al. 1997; Yang et al. 1997). Additionally, because of their small size and similar banding pattern, DAPI chromosome banding did not always permit easy identification of some *T. belangeri* acrocentric chromosomes. Figure 1

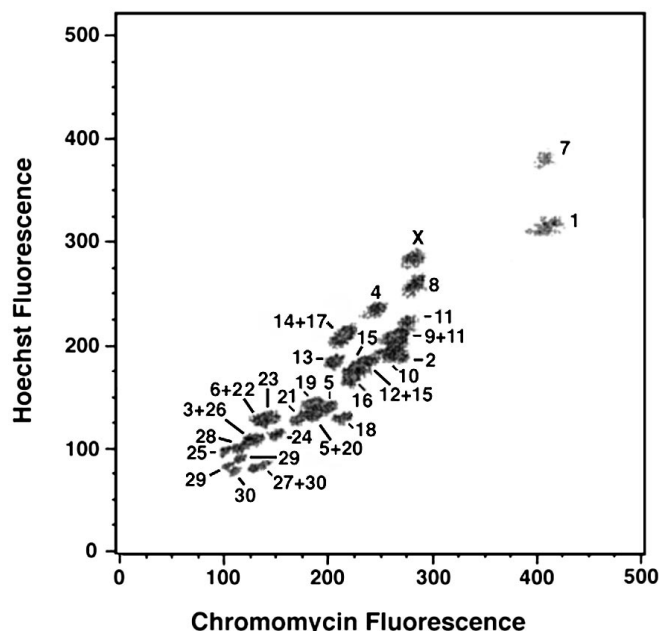


Fig. 3. Flow karyotype of *T. belangeri* with chromosomal assignments: 28 peaks were identified. From all peaks chromosomal material could be amplified by the degenerate oligonucleotide-primed polymerase chain reaction. Mapping these probes to chromosomes of *T. belangeri* revealed that 21 chromosome-specific paints could be generated, whereas seven peaks contained two chromosomes (9+11, 2+15, 5+20, 27+30, 14+17, 6+22 and 3+26). Probes for chromosomes 29 and 30 were represented in two peaks

shows representative examples of FISH, and Fig. 2 shows the karyotype of *T. belangeri* together with a summary of regions homologous to human chromosomal regions.

Probes specific for human chromosomes 7, 8, 9, 13, 14, 17, 18, 20, 21 and X identified homologous chromosomal regions on single *T. belangeri* chromosomes. Paints derived from human chromosomes 7, 8, 13, 17, 18 and X painted entire *T. belangeri* chromosomes. All other DNA probes of human origin showed homology to more than one and up to four *T. belangeri* chromosomes (Fig. 2).

Hybridization of T. belangeri chromosome-specific paints to human metaphases

Reciprocal hybridizations of *T. belangeri* probes to human chromosomes independently confirmed the results obtained with human probes on *T. belangeri* chromosomes. Bivariate flow sorting and two subsequent rounds of DOP-PCR established chromosome-specific paints of *T. belangeri*. In the flow karyotype 28 distinct peaks were identified (Fig. 3). To assign the content of each peak to particular chromosomes the paints were hybridized to metaphase preparations of *T. belangeri*. Single chromosomes were represented in 21 peaks, whereas seven peaks contained two chromosomes (*T. belangeri* chromosomes 9+11, 12+15, 5+20, 27+30, 14+17, 6+22

and 3+26). Chromosomes 29 and 30 of *T. belangeri* were represented in two peaks. Examples of hybridizations are given in Fig. 1.

Reciprocal painting of tree shrew paints to human chromosomes also gave a high background. However, in this case chromosome identification was straightforward from the DAPI banding. In cases where the "forward painting" with human probes were not satisfactory the "reverse painting" of tree shrew paints on human chromosomes provided further data for the identification of *T. belangeri* chromosomes. Examples of these hybridization experiments are given in Fig. 4, and a summary of the results is provided in Fig. 5. Human chromosomes 7, 8, 9, 13, 14, 17, 18, 20, 21 and X were painted entirely by one *T. belangeri* probe. Human chromosomes 1–6, 10–12, 15, 19, and 22 were painted by two to four *T. belangeri* probes. Most tree shrew paints hybridized to one human chromosome or chromosomal segment but paints 1, 2, 5, 7, 9, 11, and 27 showed homology to more than one human chromosome.

Hybridization of T. belangeri chromosome-specific paints to metaphases of E. m. macaco

In order to confirm the reciprocal painting results between humans and tree shrews and to identify chromosomal homologies between tree shrew and lemurs, *T. belangeri* chromosome-specific paints were also hybridized to *E. m. macaco* metaphases. The chromosomal homology between this lemur and humans was already known from our previous work (Müller et al. 1997). Compared with studies of reciprocal painting between humans and tree shrew, these experiments yielded reproducible hybridization efficiencies with all *T. belangeri* chromosome-specific paints. Only *E. m. macaco* chromosome 21, which had previously not hybridized with human chromosome paints, was not hybridized by any tree shrew chromosome probe (Müller et al. 1997). A *T. belangeri* Y chromosome-specific paint was not available for the experiments.

The *T. belangeri* probes mainly painted entire chromosomes, or chromosomal arms, indicating Robertsonian transformations or tandem fusion and fission events as the most likely mechanism of karyological evolution. No reciprocal translocations were observed. Examples of these hybridizations are shown in Fig. 4, and Fig. 6 summarizes the results.

Discussion

With the rapidly accumulating database of comparative chromosome painting it is possible to identify the direction of chromosomal changes in particular phylogenetic lines with increased accuracy (reviewed in Wienberg and Stanyon 1997, 1998; Chaudhary et al. 1998; Glas et al. 1998). The aim of the present work was to establish the ancestral karyotype for all primates and help clarify the phylogenetic and taxonomic position of the tree shrews.

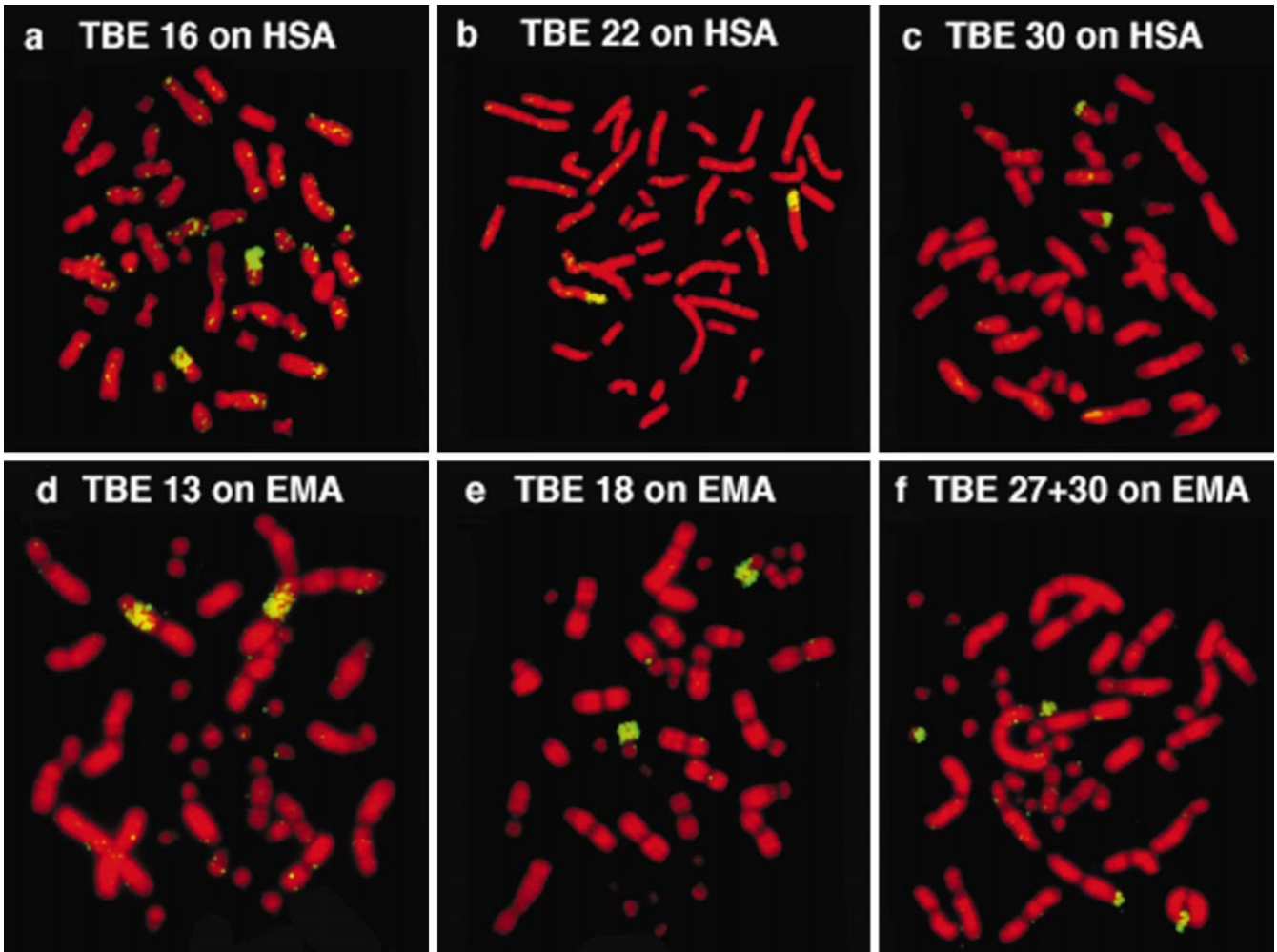


Fig. 4. Examples of in situ hybridization of *T. belangeri* (TBE) chromosome-specific painting probes on human (HSA) chromosomes (a–c) and *T. belangeri* paints on black lemur chromosomes (*Eulemur macaco macaco*, EMA) (d–f)

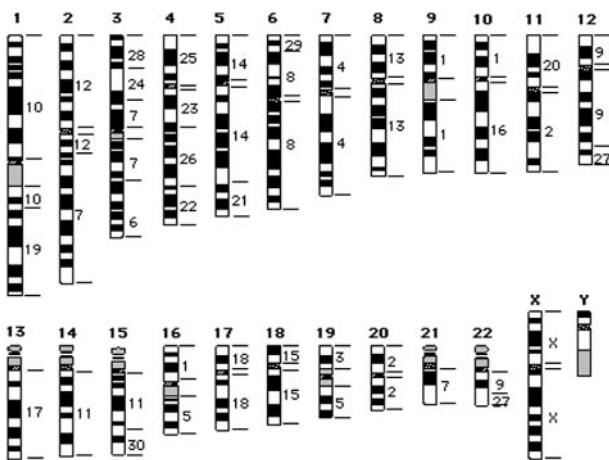


Fig. 5. Idiogram of the G-banded human karyotype with the assignment of the chromosome-painting results for all tree shrew chromosome-specific probes. The human chromosomes are *numbered* above and the tree shrew chromosome-specific probes are *numbered to the right* of the homologous human chromosomal segments

We used multidirectional painting between three species: humans, lemurs and tree shrews. Multidirectional painting allows more complete and satisfactory results when hybridization efficiency is low or signals difficult to analyze between two of the species. Chromosomal homologies between human and *T. belangeri* were established with confidence by direct comparison with a third, already characterized species, *E. m. macaco* (Müller et al. 1997), because hybridization of all tree shrew chromosome-specific paints to lemur metaphases gave strong, consistent signals. It is not clear why tree shrew paints should give better hybridization signals on lemurs than on humans. However, this difference cannot be due to differences in phylogenetic distance because all tree shrews should be equidistant from all primate species.

Our painting results indicate that *T. belangeri* has a highly derived karyotype. Tandem fusions or fissions of chromosomal segments seem to be the predominant mechanism in the evolution of the tree shrew karyotype. There were no reciprocal translocations that would dis-

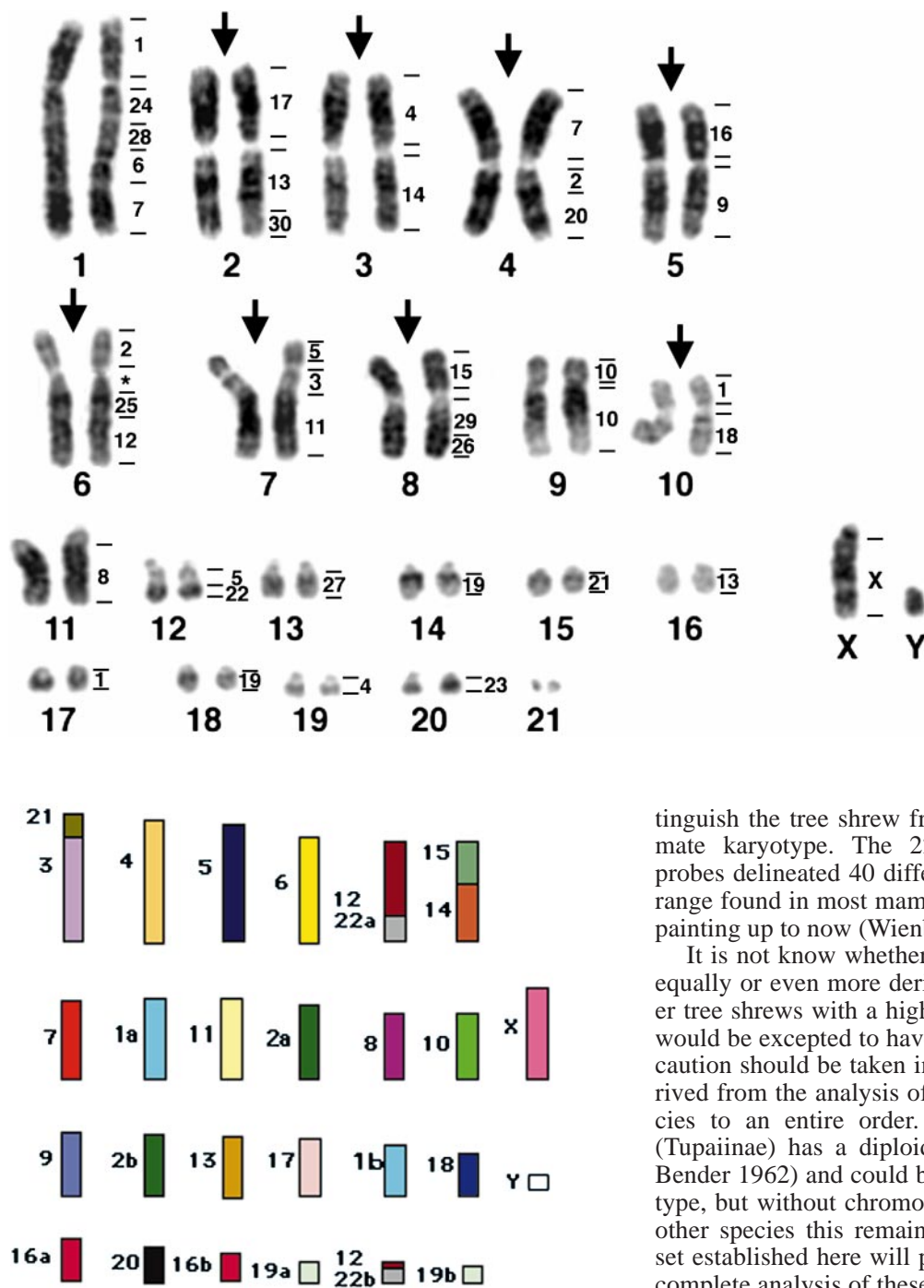


Fig. 6. Computer-enhanced 4',6-diamidino-2-phenylindole-banded karyotype of the black lemur (*E. m. macaco*) together with the location of chromosome painting for tree shrew chromosome 1–30 and X probes. The lemur chromosomes are numbered below and the tree shrew chromosome-specific probes are numbered to the right of the homologous lemur chromosomal segments. No signal could be observed on lemur chromosome 21 or at the G-negative band on chromosome 6 indicated by an asterisk. Arrows highlight *E. m. macaco* chromosomes that can be derived from their *Eulemur fulvus mayottensis* homologs by Robertsonian fusions (Müller et al. 1997)

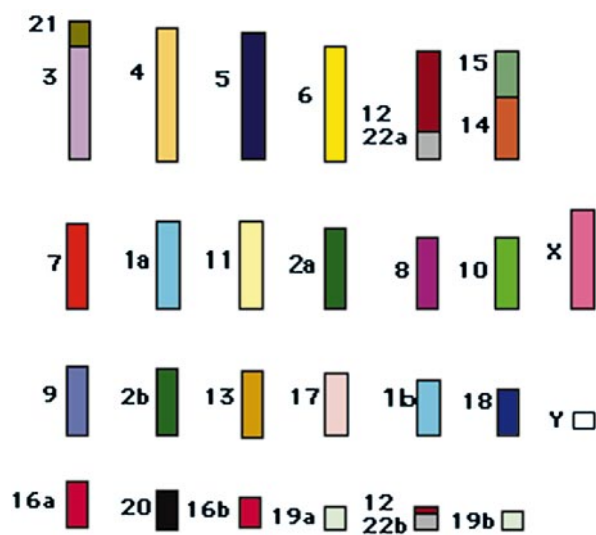


Fig. 7. A proposed ancestral karyotype for primates with $2n=50$ based on comparisons of chromosome-painting data both within primates and between primates and other mammals that can serve as outgroups for both primates and tree shrews. Chromosomes are ordered according to approximate length. The numbers on the left indicate the human homolog. Each human homolog has been assigned a different color. The position of the centromeres is not shown since this cannot be deduced from the chromosome-painting data. The Y chromosome is not colored since it was not hybridizing with the probes used

tinguish the tree shrew from the assumed primitive primate karyotype. The 22 human autosomal painting probes delineated 40 different segments, which is in the range found in most mammals analyzed by chromosome painting up to now (Wienberg and Stanyon 1997).

It is not known whether all Tupaiiformes species have equally or even more derived karyotypes. Certainly other tree shrews with a high diploid chromosomal number would be expected to have derived karyotypes; however, caution should be taken in extrapolating conclusions derived from the analysis of the karyotype of a single species to an entire order. (Scadentia). *Urogale everetti* (Tupaiainae) has a diploid number of $2n=44$ (Chu and Bender 1962) and could be closer to the ancestral karyotype, but without chromosome-painting data on this and other species this remains only speculation. The probe set established here will provide the material for a more complete analysis of these taxa.

Recently, a working hypothesis for the ancestral karyotype of all primates was proposed by making a few basic assumptions (Wienberg and Stanyon 1998). The hybridization map of primates and various "outgroup" mammals was analyzed by the principle of parsimony. Parsimony suggests that the same syntenic group may often be disrupted *independently* by chromosomal rearrangements. However, it is much less likely that the same syntenic group would be brought together independently in different lineages. When chromosomal synteny is found intact between various species this condition is likely to be ancestral. Associations of various syntenic groups seen in different species may indicate common derived traits (synapomorphies) that phylogenetically

link those species. For example, human chromosome 1 and 2 homologous segments are found associated in various carnivores and artiodactyls (Wienberg and Stanyon 1997, 1998; Chaudhary et al. 1998; Glas et al. 1998). Gene-mapping data for mouse and rat indicate the presence of this association in rodents. We can therefore hypothesize that this chromosomal form was ancestral for many mammalian orders.

According to these assumptions ancestral chromosomal synteny for primates would probably include the following homologous human chromosomes: 1a, 1b, 2a, 2b, 3/21, 5, 6, 7, 9, 10, 11, 12a/22a, 12b/22b, 13, 14/15, 17, 18, 20 and X and Y (Wienberg and Stanyon 1998). The results obtained in the present study suggest that chromosomes 4, 8, 16a, 16b, 19a, and 19b could be included in the primitive primate karyotype. The 16/19 association that is present in various mammals is still found in the tree shrew but is broken up in the lemur with the formation of two independent chromosomes (Müller et al. 1997). Therefore, the hypothetical karyotype would have had a diploid chromosome number of $2n=50$ (Fig. 7), which is one pair more than previously suggested (Wienberg and Stanyon 1998).

When comparing the tree shrew and the proposed ancestral primate karyotype we can identify various intact ancestral chromosomal forms (chromosomes 1a, 1b, 2a, 7, 8, 9, 13, 12a/22a, 12b/22b, 17, 18, 19b and X) that are conserved as single chromosomes. Other chromosomes were involved in derived fissions (chromosomes 3/21, 4, 5, 6, 10, 11, 14/15a, and 15b) or derived fusions (entire chromosomes or segments of 2b, 3/21, 9, 10, 11, 16a and 20).

The assumed primitive primate karyotype and the karyotype of the tree shrew share several common derived (synapomorphic) rearrangements. (i) In both primates and the tree shrew human chromosome 1 homologs form two separate chromosomes, which are not associated with any other homolog. Except for the dolphin (*Tursiops truncatus*, Bielec et al. 1998), complete synteny of chromosome 1 is seen only in primates. (ii) Various outgroups show a small insertion of a chromosome 8 segment in the homolog to chromosome 4. Reciprocal painting with cat and human probes shows that the small insertion comes from 8p (Wienberg et al. 1997). Gene mapping shows that the 8p/4 association is also present in rodents. However, these segments are separated both in the tree shrew and primates and may represent another common derived character. (iii) Both the tree shrew and the assumed primitive primate karyotype share two individual chromosomes consisting of fragments homologous to human chromosomes 12 and 22 (12a/22a, 12b/22b). However, 12/22 is associated with different chromosomal segments in various species. There is a 4/12/22 association in the cow (and a 4/12 association in the muntjak). A 12/22/18 association is seen in an apparently wide array of species, including cat, mink, horse, and dolphins (22/18 is found in the harbor seal). Two hypotheses can be proposed to account for these association patterns. (i) The 12/22/18 may be ancestral for many mammalian orders including primates. Then fission of the 18 segments would be a synapomorphy link-

ing primates and tree shrews. (ii) The alternate hypothesis is that the 12/22 is ancestral for placental mammals and the 12/22/18 association is a synapomorphy of ferungulates (carnivores, perissodactyls, artiodactyls, cetaceans). To allow a choice to be made with confidence between these competing hypotheses more hybridization maps are needed from a richer array of mammalian species distributed in a greater number of mammalian orders.

In the near future more complete data will allow more accurate reconstruction of the landmarks of mammalian chromosomal evolution. However, the technical difficulties in using FISH between distantly related species are illustrated by the 4/8 translocation found in many mammals. The chromosome 8p fragment associated with chromosome 4 is small. Its apparent absence in cattle, pig and horse may only be the result of low hybridization efficiencies when painting across great phylogenetic distances. However, with multidirectional painting as introduced here chromosome painting can be done in a stepping stone fashion from more closely to more distantly related species. Multidirectional painting promises to improve the paint map database and allow better interpretations of genomic evolution.

Tree shrew and lemur chromosome-specific paints promise to be useful in analyzing chromosomal phylogeny and evolution in prosimian as well as non-primate mammalian species. Although the tree shrew certainly has a derived karyotype there are some chromosomes that may link it to primates. Further studies are needed especially in Lagomorpha, Rodentia, Dermoptera and Chiroptera (De Jong 1998) to define the exact genomic rearrangement that gave rise to the primates and eventually to humans.

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