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RESEARCH ARTICLES

Fluorescence In Situ Hybridization (FISH) Maps Chromosomal Homologies Between the Dusky Titi and Squirrel Monkey

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The Platyrrhini are one of the most karyologically derived groups of primates and the evolution of their karyotypes is far from understood. The identification of the origin and direction of chromosome rearrangements will contribute to a better understanding of New World monkey phylogeny, taxonomy, and evolution. We mapped homology and identified translocations in the chromosomes of the dusky titi monkey (*Callicebus moloch*, 2n = 50) and the squirrel monkey (*Saimiri sciureus,* 2n = 44) by fluorescence in situ hybridization (FISH) of human chromosome paints. The hybridization results established chromosomal homologies between these New World primates, humans, other primates, and more distantly related mammalian species and show that both species have highly rearranged karyotypes. The total number of hybridization signals was 37 in *C. moloch* and 40 in *S. sciureus*, which is in the range of most comparisons of human chromosomes with phylogenetically more distant species outside of the primate order. Parsimony analyses of outgroup painting patterns allowed us to propose an ancestral karyotype for New World monkeys consisting of 2n = 56 with homologs to the following human chromosomes or chromosome segments: 1b; 1c; 2a; 2b; 3a; 3b; 3/21; 4; 5; 6; 7; 8a; 8/18; 9; 10a; 10/16; 11; 12; 13; 14/15; 15a; 16a; 17; 19; 20; 22; X; Y. Associations 8/18 and 10/16 are derived ancestral associations for all Platyrrhini. A 2/16 association found in *S. sciureus* and *C. moloch* was also seen in *Ateles geoffroyi* and *Cebus capucinus*; a 5/7 association in *S. sciureus* was present in *A. geoffroyi, C. capucinus*, and *Alouatta belzebul*. Other associations seen in the dusky titi monkey or the squirrel monkey are probably aupomorphisms. Comparison with chromosome phylogenies based on R-banding [Dutrillaux et al., 1986] showed that there were many

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errors in assigning homology with human chromosomes. The chromosomal phylogeny of New World monkeys based on banding patterns is in need of revision using modern molecular methods. Am. J. Primatol. 50:00–00, 1999. Am. J. Primatol. 50:95–107, 2000.

Key words: New World monkeys; Cebidae; cytogenetics; chromosome painting; phylogeny

INTRODUCTION

Over the last decade, molecular techniques have become valuable taxonomic and phylogenetic tools in mammalian cytogenetics, especially when integrated with other data, including classical morphological comparisons. As a result of the human genome project, DNA probes of various complexities are now available to the scientific community and can be readily used for comparative genome analysis. Currently, the most attractive probes are chromosome paints for fluorescence in situ hybridization (FISH). A chromosome paint is a labelled mixture of DNA sequences usually derived from flow sorted or microdissected chromosomes. Each probe is specific for a single chromosome and hybridizes to the entire chromosome or chromosome segments in a target metaphase [Wienberg & Stanyon, 1997, 1998; Ried et al., 1998]. This method allows a simple and reliable analysis of chromosomal translocations that separate the karyotypes of two species. These data are especially useful for taxonomy in taxa, such as New World monkeys, that show high evolutionary rates of chromosome evolution.

Cytogenetic analyses have shown that New World monkey biodiversity is not yet well known. These primates are karyologically variable and highly derived [Chu & Bender, 1961; Bender & Chu, 1963; Egozcue & Egozcue, 1966; Koiffmann & Saldanha, 1974, 1981; Armada et al., 1987]. The number of species might be underestimated by traditional taxonomy [Stanyon et al., 1995; Consigliere et al., 1996, 1998]. Here we report on the hybridization of human chromosome specific paints on metaphase of the dusky titi monkey (*Callicebus moloch*) and the squirrel monkey (*Saimiri sciureus*). These two species were chosen because they represent two major taxonomic divisions of cebids not yet studied by chromosome painting. Further, samples where available for the present study as already established cell lines.

The results from chromosome painting were then compared with the hybridization maps published for other primates and more distantly related mammal species. This comparison lead to the identification of various ancestral and derived chromosome forms that in the near future will allow an improved cladistic interpretation of New World monkey evolution.

Saimiri sciureus

The genus *Saimiri* is the only representative of the subfamily Saimirinae. Traditionally, only one species, *S. sciureus*, was recognized, but the genus is now commonly divided into four species [Herschkovitz, 1984]: *S. sciureus, S. boliviensis, S. oerstedii*, and *S. ustus. S. sciureus* is the most widely used New World monkey in biomedical research [Abee, 1985], and many reports are available in the literature on the cytogenetics of this species [Bender & Mettler, 1958; Jones et al., 1973; Jones & Ma, 1975; Ma et al., 1974; Ma & Jones, 1975; Lau et al., 1977; García et al., 1979; Fogel, 1984; Herschkovitz, 1984; Assis & Barros, 1987; Moore et al., 1990]. The diploid number is unusually constant for a South American monkey, with 2n = 44, but there have been reports that pericentric inversions produce three geographically defined karyotypes [Jones & Ma, 1975; Ma et al., 1974].

Callicebus moloch

Callicebus is the only genus of the subfamily Callicebinae and may be closely related both to the genus *Aotus* and to the recent ancestors of the subfamily Pithecinae [Martin, 1990]. It is traditionally divided into three species: *C. moloch, C. personatus*, and *C. torquatus* [Herschkovitz, 1963; Kinzey, 1983], but Herschkovitz [1988, 1990] described 13 species of *Callicebus*. Although cytogenetic studies of *C. moloch* are limited they are sufficient to show that variability is high. Diploid numbers between subspecies vary from 46 to 50 [Egozcue et al., 1969; Benirschke & Bogart, 1976; Pieczarka & Nagamachi, 1988; Minezawa et al., 1989; de Boer, 1974; Minezawa & Valdivia, 1984]. According to Minezawa et al. [1989] the distribution of the karyotypes shows a clear geographical cline, with 2n = 46 in the north to 2n = 50 in the south. It has been suggested that five pericentric inversions and two Robertsonian transformations separate the 2n = 46 from the 2n = 50 karyotypes. Such differences may be sufficient to ensure reproductive isolation [de Boer, 1974; Koiffmann, 1982; Pieczarka & Nagamachi, 1988; Minezawa et al., 1989].

MATERIALS AND METHODS

Both species samples consisted of male fibroblast cell lines (repository numbers: AG06115 for *Callicebus molloch* and AG05311 for *Saimiri sciureus*, Coriell Institute for Medical Research) kindly provided by W. Schempp of the Institute for Anthropology and Human Genetics of the University of Freiburg, Germany. Various multi-color hybridizations were done on metaphase preparations from the lymphoblastoid cell line 533B from a female *S. sciureus* kindly provided by C. Roos, Munich, Germany.

Standard tissue culture and chromosome preparation techniques were followed. Sequential G-banding and in situ hybridization were as previously described [Wienberg et al., 1990, 1992; Consigliere et al., 1996]. Chromosome numbering followed Jones and Ma [1975].

Chromosome painting probes were labeled with biotin-dUTP, digoxigenin-dUTP (Boehringer-Mannheim, Indianapolis, IN), or Cy3-dUTP (Amersham, Arlington Heights, IL) by DOP-PCR according to previously published protocols [Telenius et al., 1992]. After hybridization and washing of the slides biotinylated DNA probes were detected with avidin coupled with Cy5 (Amersham, Arlington Heights, IL) and digoxigenin labelled probes with fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA).

G-banded metaphases were photographed on Agfa-Ortho 25 or Kodak Technical Pan film. Photographs of hybridized metaphases were taken with Kodak (ASA 1600) color slide film or Kodak T-max (ASA 400) black-and-white film. Digital images were taken using a cooled CCD camera (Photometrics NU200 equipped with a Kodak KAF1400 CCD chip) coupled to the microscope. Imaging software was SmartCapture (Digital Scientific, Cambridge, UK).

RESULTS

Karyotype and Hybridization Pattern of *Callicebus moloch*

Callicebus moloch had a diploid number of 2n = 50 (Fig. 1), with 24 meta/submetacentrics and 24 acrocentrics. The individual we studied had a karyotype similar to that of *C. moloch donacophilus* [Benirshke & Bogart, 1976]. The Y-chromosome is an extremely small acrocentric and the sex-chromosome system is XX/XY. The fundamental number is FN = 75 for males and FN = 76 for females.

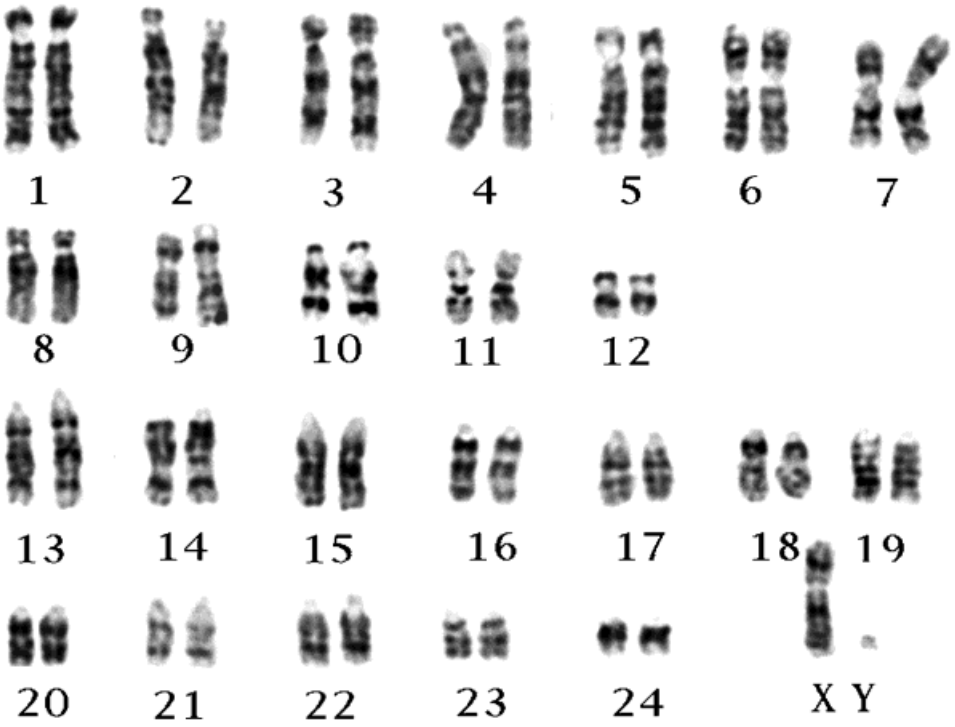

Fig. 1. G-banded karyotype of *Callicebus moloch*.

The idiogram shown in Figure 2 summarizes the hybridization results of human chromosome specific paints on *C. moloch* chromosomes. With the exception of the human Y-chromosome probe all human paints gave bright hybridization signals (Fig. 3). The total number of hybridization signals obtained was 37 excluding the Y. Every *C. moloch* chromosome was hybridized by at least one chromosome paint. Paints from thirteen human chromosomes hybridized one chromosome pair each: seven human chromosomes (paints 4, 6, 9, 13, 17, 20, and X) each completely hybridized only one *C. moloch* homolog, while six human autosomes (paints 7, 11, 14, 18, 19, and 21) hybridized a *C. moloch* chromosome along with other human paints. The remaining ten chromosomal probes gave multiple signals on a number of different *C. moloch* chromosomes, showing that they have been fragmented: six human chromosome paints (5, 8, 10, 12, 15, and 22) gave signals on two chromosomes per haploid set, while four human paints (1, 2, 3, and 16) labeled three chromosomes segments on two or more chromosomes per haploid set.

When a single chromosome is hybridized by multiple paints associations are formed between contiguous segments that are homologous to the reference species chromosomes. The *C. moloch* chromosomes painted by multiple human paints produced nine associations of segments homologous to human chromosomes or chromosome segments: 2/22, 2/16, 3/21, 7/15, 8/18, 10/11, 10/16, 12/19, and 14/15.

Karyotype and Hybridization Pattern of *Saimiri sciureus*

Our sample had a diploid number of 2n = 44 with sixteen meta/submetacentric and five acrocentric pairs (Fig. 4) and corresponds to that of Peruvian mon-

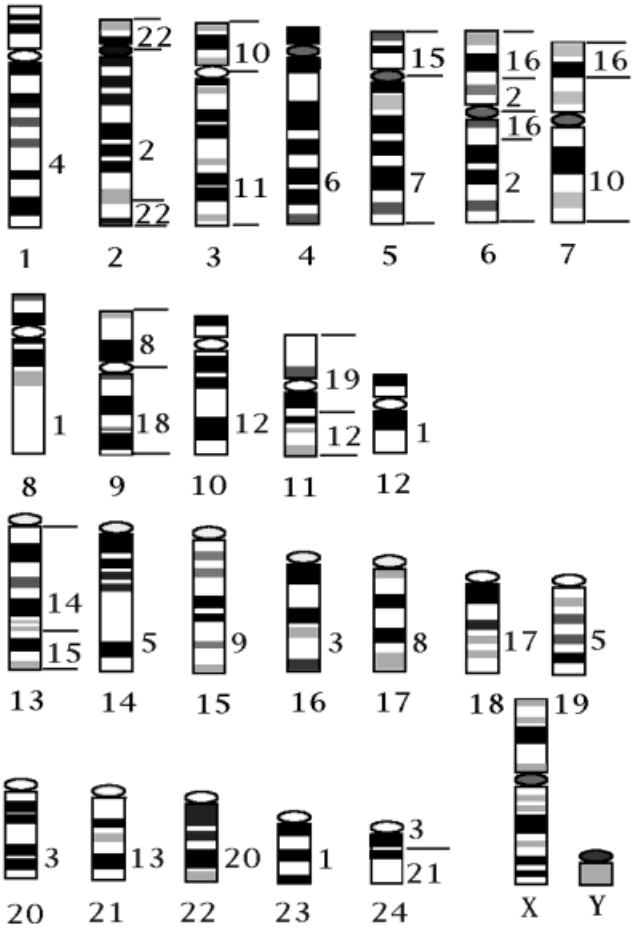

Fig. 2. Idiogram and chromosome painting map of *C. moloch*. The hybridization sites and the numbers of the human painting probes are given on the right of each chromosome.

keys [Jones & Ma, 1975]). The Y-chromosome is a small acrocentric and the sex-chromosome system is the normal XX/XY. The fundamental number of FN = 77 for males and FN = 78 for females.

The idiogram shown in Figure 5 summarizes the painting results on *S. sciureus* chromosomes. With the exception of the Y-chromosome probe all paints gave bright hybridization signals. The total number of hybridization signals obtained was 40 (excluding the Y). DNA paints from thirteen chromosomes each hybridized a single monkey chromosome pair: eight chromosome paints (4, 6, 11, 12, 13, 17, 22, and X) each completely hybridized only one *S. sciureus* homolog, while five other paints (9, 18, 19, 20, and 21) hybridized a *S. sciureus* chromosome along with other human paints. The remaining ten chromosomal probes gave multiple signals on a number of different *S. sciureus* chromosomes, showing that they have been fragmented: Four human paints (5, 7, 8, and 10) gave signals on two chromosomes per haploid set; five human paints (1, 2, 3, 14, and 16) labeled three chromosomes segments per haploid set; and human paint 15 labeled four chromosomes segments per haploid set.

The *S. sciureus* chromosomes hybridized by multiple human paints produced

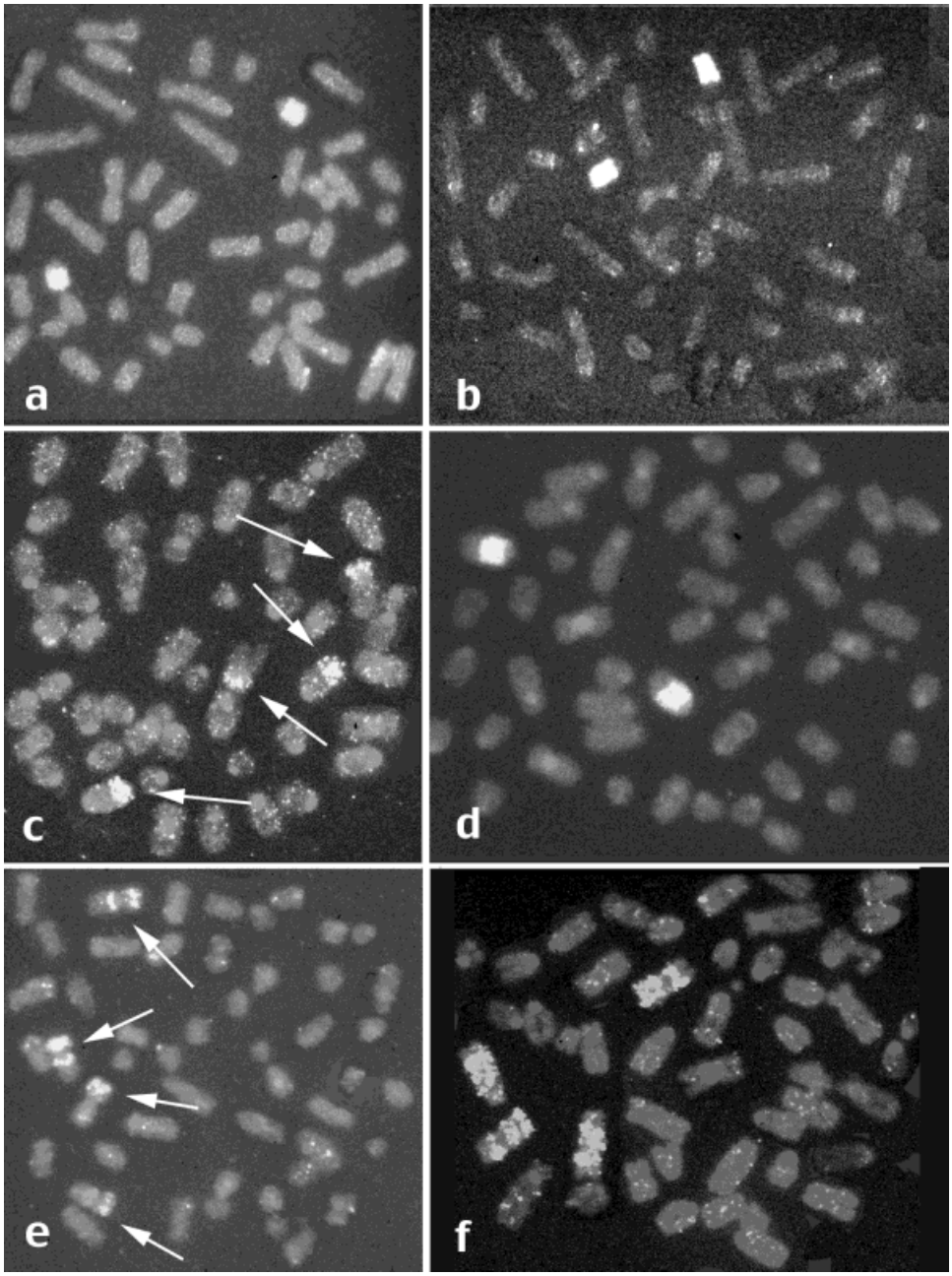

Fig. 3. Examples of in situ hybridizations of human painting probes hybridized on *C. moloch* chromosomes. All images were taken by conventional microscopy on photographic film. Chromosome identification was made by analyzing the same metaphases by G-banding before hybridization. **a,b:** Human chromosome 20 and 17 paints, respectively, paint a single *C. moloch* homolog each, showing that they have been conserved in the evolution of human and this New World monkey. **c:** Human chromosome 15 paint hybridized to two chromosome pairs (arrows), one chromosome pair is also painted by chromosome 14 painting probe (**d**). In **e** hybridization sites of human chromosome 16 paint label three segments on two *C. moloch* chromosomes (arrows). The chromosome with two signals is also labeled by human chromosome 2 (**f**).

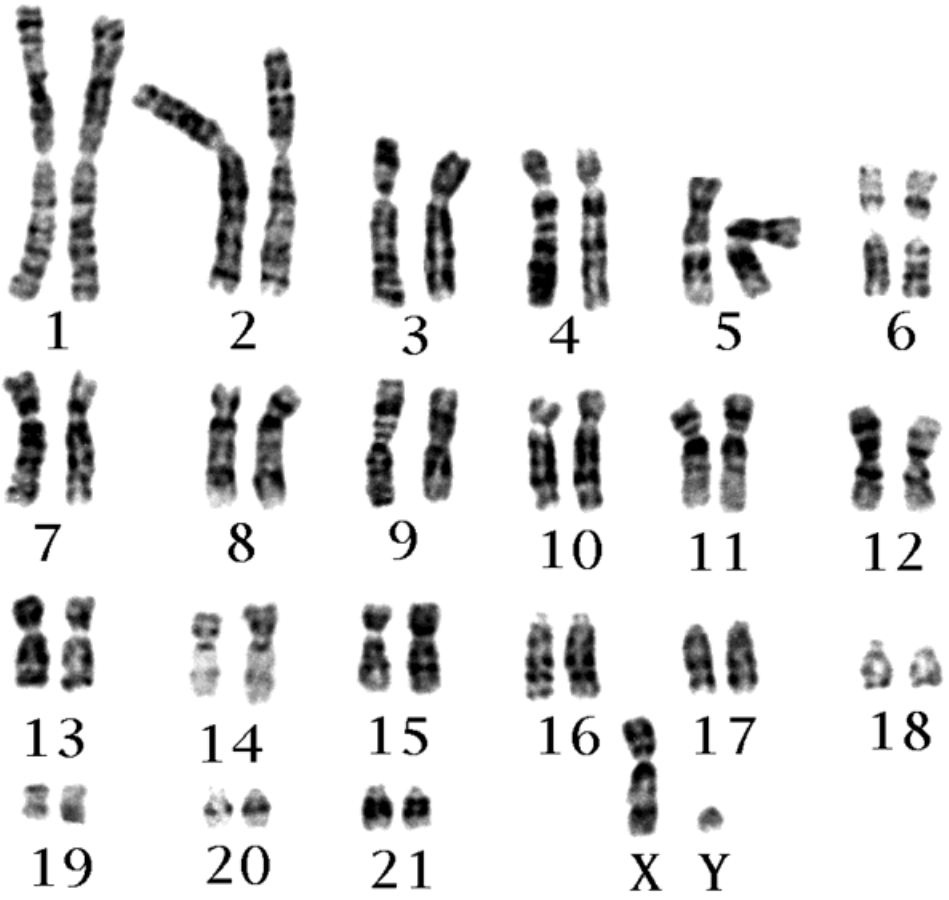

Fig. 4. G-banded karyotype of *Saimiri sciureus*.

twelve associations of segments homologous to human chromosomes or chromosome segments: 1/19, 2/15, 2/16, 3/10, 3/20, 3/21, 5/7, 5/16, 8/18, 9/14, 10/16, and 14/15. Squirrel monkey associations were independently controlled by multi-color hybridizations using up to three different probes in single experiments (Fig. 6).

DISCUSSION

The aim of the present study was to analyze whether molecular cytogenetic data from the dusky titi monkey and the squirrel monkey could contribute to our understanding of New World monkey phylogeny and taxonomy. Indeed, a number of species of New World primates have now been studied with chromosome painting including *Callicebus moloch* and *Saimiri sciureus* (this report), *Alouatta sara* and *A. seniculus arctoidea* [Consigliere et al., 1996], *A. belzebul* [Consigliere et al., 1998], *Ateles geoffroyi* [Morescalchi et al., 1997], *Cebus capucinus* [Richard et al., 1996], and *Callithrix jacchus* [Scherlock et al., 1996]. These data are beginning to provide insight into platyrhinne chromosome evolution. However, a number of other species belonging to diverse taxonomic divisions of New World monkeys need to be studied with molecular cytogenetic techniques before any

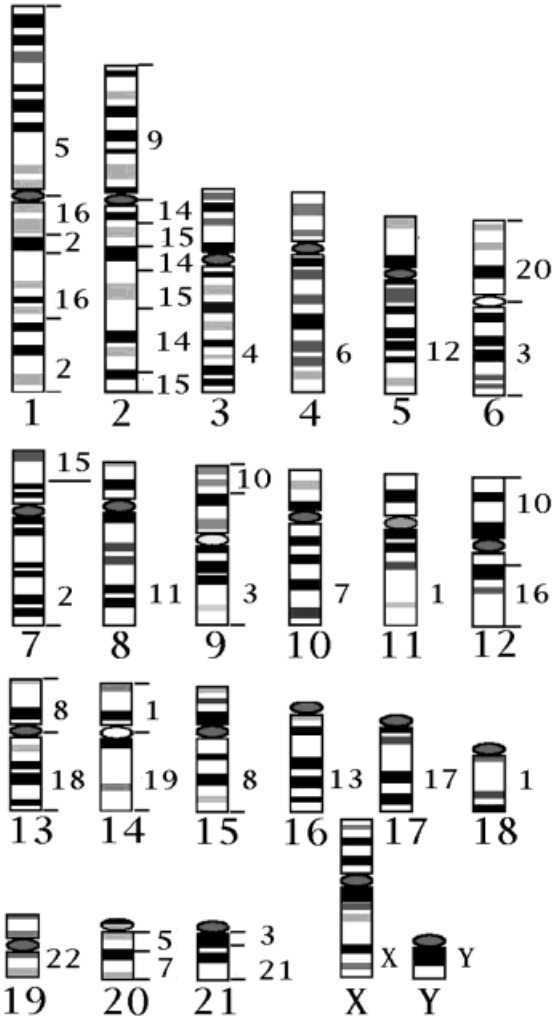

Fig. 5. Idiogram and hybridization map of *S. sciureus*. The hybridization sites and the number of the human painting probes are given on the right of each chromosome.

final phylogenetic conclusions are drawn: especially Pitheciini, Aotus, Callimico, and various Callitrichini.

The hybridization results show that both species have highly rearranged karyotypes and confirms that the Platyrrhini, along with the Hylobatidae, are one of the most karyologically derived groups of primates. The high rate of karyotypic evolution in Platyrrhini holds promise that that cytogenetic data can contribute to resolving phylogenetic problems in New World monkeys. However, the data also confirm that there is certainly no "molecular clock" for chromosome translocations in mammalian evolution. Various more distantly related primates and even non-primate mammals show a much more conserved karyotype than the two New World monkeys analyzed in the present work [Wienberg & Stanyon, 1997, 1998].

We can compare the hybridization pattern of New World monkeys with those

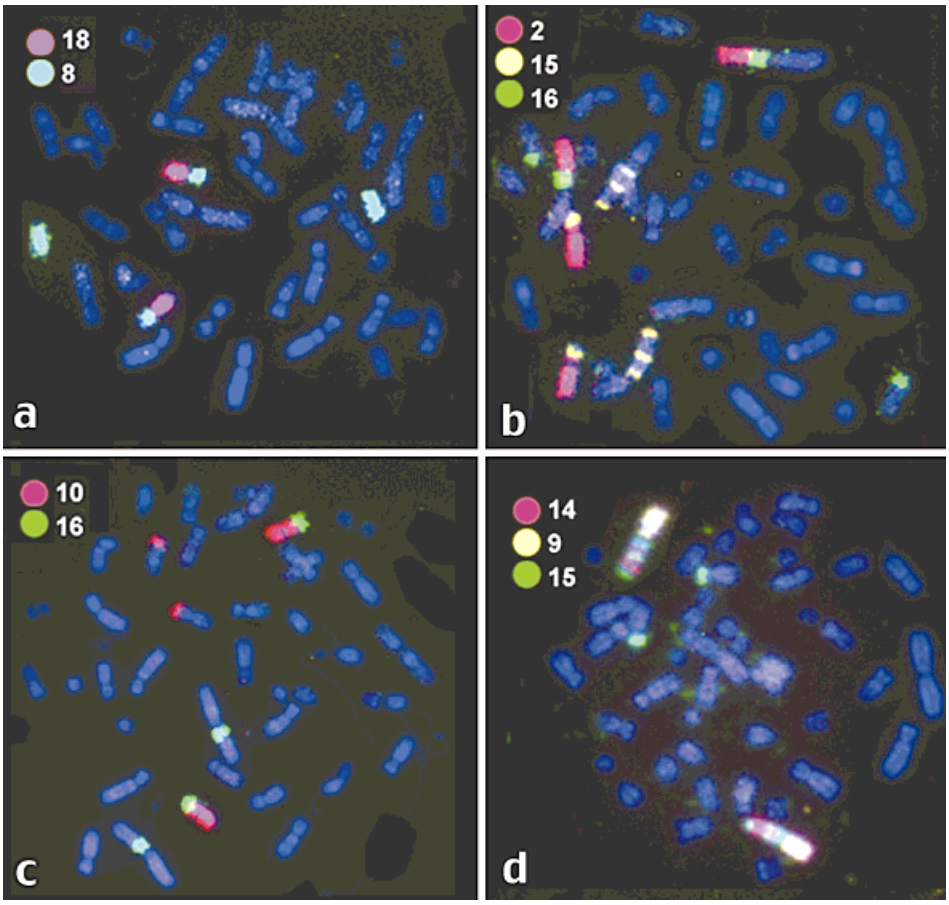

Fig. 6. Examples of multi-color hybridizations of human painting probes to chromosomes of *S. sciureus*. This approach allowed us to independently control results obtained by single chromosome paints for associations of hybridization signals on individual chromosomes. **a:** Human chromosome 8 and 18 both hybridize *S. sciureus* chromosome 13. Human chromosome 8 probe gave another signal entirely covering *S. sciureus* chromosome 15. **b:** Human chromosome 2 and 16 paints show associations on *S. sciureus* chromosome 1 (arrow). A small pericentric of chromosome 2 material within the chromosome 16 homologous block is not resolved by multi-color painting. On *S. sciureus* chromosome 7, the human chromosome 2 paint also identifies an association with the chromosome 15 specific paint. **c:** Human chromosome probes 16 and 10 both label *S. sciureus* chromosome 12. **d:** *S. sciureus* chromosome 2 (arrows) is hybridized by three human paints (probe 9, 14, and 15). The three hybridization signals obtained with the chromosome 15 probe are also evident in (b).

found in other primates and in outgroup mammals by following a few basic assumptions. Pasimony indicates that the same syntenic group may often be disrupted *independently* by chromosome rearrangements. However, it is much less likely that the same syntenic group can be brought together independently in different lineages. When chromosomal synteny is found intact between various species this condition may be ancestral. Associations of various syntenic groups seen in different species may indicate common derived traits (synapomorphies) that phylogenetically link those species. When integrated with other data, including classical morphological comparisons, these synapomorphies will become valuable cladistic landmarks for the taxonomy of Platyrrhines. Furthermore, these characters can be distinguished from ancestral chromosome

forms with high confidence. For example, the associations of segments homologous to human chromosomes 3/21 and 14/15 found in both the dusky titi monkey and the squirrel monkey are typical not only of Platyrrhines and other primate taxa, but many other mammals. Given their wide distribution in primates and other mammalian orders, associations 3/21 and 14/15 are certainly ancestral for many mammalian orders, and derived chromosome forms can be found in Old World monkeys and hominoids, respectively [for review, see Wienberg & Stanyon, 1997, 1998].

Associations of segments homologous to human chromosomes 8/18 and 10/16 are derived ancestral associations for all Platyrrhini analyzed up to now, while other associations variously link *S. sciureus* and *C. moloch* with different New World primate species. A 2/16 association found in *S. sciureus* and *C. moloch* is also seen in *Ateles geoffroyi*, and *Cebus*; a 5/7 association in *S. sciureus* is present in *Ateles geoffroyi, Cebus capucinus*, and *Alouatta belzebul*. Other associations seen in either the dusky titi monkey or the squirrel monkey are most likely derived apomorphisms.

Hypothesis for the Inferred Ancestral Karyotype of New World Monkeys

A parsimony analysis of chromosome painting results in other primates and more distantly related mammals supports the hypothesis that the synteny of 13 chromosomes (including the X) homologous to entire human chromosomes was conserved in the ancestral karyotypes of New World monkeys. Other human syntenies were probably fragmented in the ancestral platyrrhine karyotypes.

From the hybridization maps now available from the various New World monkeys studied [see species list above and Wienberg & Stanyon, 1998, for a more complete discussion of outgroup species analyzed], we propose that the ancestral karyotype of New World monkeys may have had a diploid number of 2n = 56 and consisted of the following homologs to human chromosomes or chromosome segments: 1a; 1b; 1c; 2a; 2b; 3a; 3b; 3/21; 4; 5; 6; 7; 8a; 8/18; 9; 10a; 10/16; 11; 12; 13; 14/15; 15a; 16a; 17; 19; 20; 22; X; Y.

If found in a wide range of platyrrhine the association 5/7 might also be eventually included in the ancestral karyotype.

Derivation of the *Callicebus moloch* and *Saimiri sciureus* Karyotypes From the Ancestral Platyrrhine Karyotype

Nine *Callicebus moloch* autosomes differ for inter-chromosomal rearrangements from the proposed ancestral karyotype for the Platyrrhini, for a total of two fissions, five fusions, and three inversions. Six *Saimiri sciureus* autosomes differ for inter-chromosomal rearrangements from the ancestral karyotype for the Platyrrhine, for a total of two fissions, six fusions, and six inversions. Inversions account for the alternating pattern of segments homologous to human 2 and 16 on *S. sciureus* chromosome 1 and to human chromosomes 14 and 15 on *S. sciureus* chromosome 2. There is no particular karyological association linking *S. sciureus* and *C. moloch*. This result apparently reflects the possibility that *Callicebus* may be closely related both to the genus *Aotus* and to the recent ancestors of the subfamily Pithecinae. However, representative species from these taxa have yet to be studied with chromosome painting.

Tenability of Chromosome Banding Phylogenies of New World Monkeys

A previous attempt to reconstruct the ancestral karyotype for platyrrhines on the basis of R-banding proposed a similar diploid number to our hypothesis

(58 vs. 56) [Dutrillaux et al., 1986]. However, the homologies proposed by banding with the human are in agreement with our proposal for only 15 out of 26 autosomes. Furthermore, Dutrillaux et al. [1986] attributed the syntenic association 8/18 to the ancestral karyotype but not 10/16, 14/15, and 3/21 and included the association 14/20, which cannot be found in any New World monkey with chromosome painting. The Dutrillaux et al. reconstruction of the ancestral karyotype can be tested against the chromosome homologies between humans and New World monkeys established by FISH.

Indeed, the reconstruction of the ancestral karyotype by Dutrillaux et al. [1986] leads to the identification of homologies with the human karyotype in various species, which are far off the mark. For example, in the squirrel monkey they correctly identified the syntenic association 5/2/16 and 5/7 but incorrectly proposed 5/3, 10/14/1q, 19/3, and missed associations 1/10, 2/15, 3/10, 3/20, 10/16, and 14/15. In *Callithrix jacchus* FISH finds nine syntenic associations [Sherlock et al., 1996]. Dutrillaux et al. [1986] show only four associations: but only the association of a region homologous to human chromosome 1 with an unidentified segment is correct. The eight other associations found with in situ techniques were not seen with R-banding. Clearly the chromosomal phylogeny of New World monkeys based on banding pattern comparisons is in need of revision. In particular, further molecular cytogenetic data is needed on a large number of species. More refined methods such as reciprocal chromosome painting, and in situ hybridization with subregional and band specific probes will better define break points and add information on interchromosomal rearrangements.

Currently, chromosome painting due to the labor intensive and high cost of FISH experiments is restricted to the analysis of only one or a few individuals for a given species. It would be preferable to analyze good sample sizes for each species especially when taxa are known to be chromosomally variable. Recent progress in molecular cytogenetic techniques, however, promises to overcome these limitations. Entire karyotypes can now be analyzed with a single hybridization experiments [Schröck et al., 1996; Speicher et al., 1996]. These methods when applied to non-human primate species will eventually permit the study of New World monkey species at the population level.

CONCLUSIONS

1. Both the dusky titi monkey and the squirrel monkey, like many New World primates, have highly rearranged karyotypes.

2. An ancestral karyotype for Platyrrhini is proposed with a diploid number of 2n = 56.

3. The short chromosomal distance separating the Callithrichidae and the Cebidae supports a monophyletic origin of New World monkeys, but it is difficult without additional painting data from an array of species to assess the phylogenetic groupings within the Cebidae.

4. Comparison with previous chromosome phylogenies based on R-banding showed that there were many errors in assigning homology with human chromosomes. Clearly the chromosomal phylogeny of New World monkeys based on banding pattern comparisons is in need of revision using modern molecular methods.

5. More refined molecular techniques such as reciprocal and multi-color FISH and in situ hybridization with subregional probes could better define ancestral states and break points and will better identify chromosome rearrangements.

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