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Mapping genomic rearrangements in titi monkeys by chromosome flow sorting and multidirectional *in-situ* hybridization

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Abstract

We developed chromosome painting probes for *Callicebus pallescens* from flow-sorted chromosomes and used multidirectional chromosome painting to investigate the genomic rearrangements in *C. cupreus* and *C. pallescens*. Multidirectional painting provides information about chromosomal homologies at the subchromosomal level and rearrangement break points, allowing chromosomes to be used as cladistic markers. Chromosome paints of *C. pallescens* were hybridized to human metaphases and 43 signals were detected. Then, both human and *C. pallescens* probes were hybridized to the chromosomes of another titi monkey, *C. cupreus*. The human chromosome paints detected 45 segments in the haploid karyotype of *C. cupreus*. We found that all the syntenic associations proposed for the ancestral platyrrhine karyotype are present in *C. cupreus* and in *C. pallescens*. The rearrangements differentiating *C. pallescens* from *C. cupreus* are one inversion, one fission and three fusions (two tandem and one Robertsonian) that occurred on the *C. cupreus* lineage. Our results support the hypothesis that karyological evolution in titi monkeys has resulted in a reduction in diploid number and that species with higher diploid numbers (with less derived, more ancestral karyotypes) are localized in the centre of the geographic range of the genera, while more derived species appear to occupy the periphery.

Introduction

The diversity of living Platyrrhini (New World monkeys) is the result of an impressive adaptive radiation. This group displays a wide range of morphological and behavioural modifications, so that it is sometimes difficult to determine homologous morphological characteristics (Fleagle 1988). Sister species with almost identical morphology, but with marked genetic divergence, are not uncommon. Previously, marked differences in karyotypes have revealed sister species hidden in a single taxon such as *Alouatta seniculus* (Stanyon *et al.* 1995). The discovery of multiple species within single taxa is not surprising because the biogeography of Amazonian primates is far from well known (van Roosmalen *et al.* 2002). Many studies and classifications used morphological data collected from museum skins that are often without skulls and skeletons, so that even potentially discriminating descriptions of head and extremities are missing. These

interpretative difficulties are reflected in the number of different phylogenetic trees based on different sets of morphological characteristics by different authors (Rosenberger 1981, Rosenberger & Coimbra-Filho 1984, Ford 1986, Kay 1990, Ford & Davis, 1992).

The continual revision of the number of species within the genus *Callicebus* and the relationships of titi monkeys with other New World primates is illustrative of these problems. *Callicebus* has been linked with *Aotus* for morphological behavioural characteristics and chromosomal data (Dutrillaux *et al*. 1986, Ford 1986, Kinzey 1992). *Callicebus* has been also considered as the most basal platyrrhine genus (Kay 1990). A different conclusion was reached by studies based on molecular data (Horovitz *et al*. 1998, Porter *et al*. 1999, von Dornum & Ruvolo 1999, Schneider 2000, Schneider *et al*. 2001) that included *Callicebus* in the family Pitheciidae, together with the tribe Pithecini (*Pithecia*, *Cacajao* and *Chiropotes*). If the phylogenetic position of *Callicebus* is far from clear, the internal taxonomy of the genus is not less controversial. Hershkovitz (1963) listed only two species (*Callicebus moloch* and *C. torquatus*), each with a number of subspecies. According to this classification, *Callicebus cupreus* and *C. donacophilus* were considered subspecies of *C. moloch*. In 1990, Hershkovitz revised the taxonomy of the genus *Callicebus*. He listed 13 species organized in four species groups: *C. modestus* group (only one species), *C. donacophilus* group (*C. donacophilus*, *C. olallae*, *C. oenanthe*), *C. moloch* group (*C. cinerascens*, *C. moloch*, *C. hoffmannsi*, *C. brunneus*, *C. cupreus*, *C. caligatus*, *C. dubius*, *C. personatus*) and *C. torquatus* group (only one species).

In 1995, Kobayashi published a new phylogeny of the genus *Callicebus*, based on morphometric data, where titi monkey species were arranged into five groups: *C. torquatus*, *C. personatus*, *C. moloch*, *C. cupreus* and *C. donacophilus*. The genus *Callicebus* was also divided into two branches, one including *torquatus* and *personatus* and another splitting in *moloch* and *cupreus*/*donacophilus*. Kobayashi & Langguth (1999) added a new species, *C. coimbrai*, to the *C. personatus* group. Groves (2001) agreed with Hershkovitz regarding the arrangement into four groups but changed the status of some taxa and included a new species, *C. coimbrai*, to the *moloch* group.

Van Roosmalen *et al*. (2002), in the latest revision of this genus, recognized 28 species (and no subspecies) included in five species groups or clades: *C. donacophilus* group (including *C. pallescens*), *C. moloch*, *C. cupreus*, *C. torquatus* (including *C. lugens* that in previous classifications was always considered a *C. torquatus* subspecies) and *C. personatus*. Two new species, *C. bernahardi* and *C. stephennashi*, were added respectively to the *moloch* and to the *cupreus* groups.

As can be well appreciated, comparing data from different publications can be difficult due to the rapid increase in the number of recognized species and relative changes in nomenclature, but what remains noteworthy is the wide difference in chromosomal number and morphology. An interesting contrast arises from studies investigating divergence between biochemical and cytogenetic differences in the *Callicebus moloch* group (*C. moloch*, *C. brunneus*, *C. cupreus*) showing the presence of low values of genetic distance (Schneider *et al*. 1993). These authors also suggest that recent karyological rearrangements are possibly the major evolutionary mechanism at the origin of the speciation process in this group of Primates.

The karyological variability of genus *Callicebus* is clearly noteworthy, despite the fact that less than half of the species have been studied with chromosome banding (Table 1). Recent studies showed that the species *Callicebus lugens* has a diploid number $2n = 16$, the lowest found in primates (Bonvicino *et al*. 2003, Stanyon *et al*. 2003) while the highest diploid number found in the genus *Callicebus* is $2n = 50$ (de Boer 1974, Minezawa & Borda 1984, Stanyon *et al*. 2000, Rodrigues *et al*. 2001, Barros *et al*. 2003).

Platyrrhini are karyologically one of the most derived groups of Primates (Consigliere *et al*. 1996, Barros *et al*. 2000, de Oliveira *et al*. 2002, Garcia *et al*. 2002, Barros *et al*. 2003, Stanyon *et al*. 2003). We know that the rate of karyological evolution is high but the mechanisms that have facilitated such rapid genome evolution are still poorly understood. Nevertheless, molecular cytogenetic techniques are potentially an important tool in the understanding of Platyrrhini evolution since systems with high evolutionary rates are expected to provide high phylogenetic resolution. Yet there are only three

Table 1. Cytogenetic studies of titi monkeys.

Species	*2n*	*Method*	*References*
C. pallescens I	50	M, B, F	Stanyon *et al.* 2000; this report
C. donacophilus I	50	M, B,	Minezawa & Borda 1984, de Boer 1974,
		M, B, F	Barros *et al.* 2003
C. hoffmannsi II	50	M, B	Rodrigues *et al.* 2001
C. brunneus II	48	M, B	Minezawa *et al.* 1989
C. moloch II	48	M, B	Pieczarka & Nagamachi 1988
C. ornatus III	46	M, B	de Boer 1974
C. cupreus III	46	M, B	Benirschke & Bogart 1976, de Boer 1974,
		M, B, F	this report
C. discolor III	46	M, B	Schneider *et al.* 1993
C. personatus IV	44	M, B	Rodrigues *et al.* 2004
C. nigrifrons IV	42	M, B, F	Nagamachi *et al.* 2003
C. torquatus ssp. V	22	M, B	Barros *et al.* 2000
C. torquatus V	20	M, B, M	Egozcue *et al.* 1969, Benirschke & Bogart 1976
C. lugens V	16	M, B, F	Bonvicino *et al.* 2000, Stanyon *et al.* 2003

On the left, the species with the Roman numeral which refers to the species group: I = *donacophilus* group, II = *moloch* group, III = *cupreus* group, IV = *personatus* group, V = *torquatus* group. The cytogenetic methods used: M = morphology from classical staining, B = chromosome banding, F = Fluorescence *in-situ* hybridization (FISH). *C. pallescens* was previously reported in Stanyon *et al.* 2000 to be *C. moloch* (see text).

reports in titi monkeys using molecular cytogenetic methods (Stanyon *et al.* 2000, Barros *et al.* 2003, Stanyon *et al.* 2003). Here we used multidirectional chromosome painting to study the chromosomal variability in *Callicebus cupreus* and *C. pallescens* by developing a set of chromosomal probes for *C. pallescens*.

Multidirectional painting provides information about chromosome homologies at the subchromosomal level and rearrangement break points that allow chromosomes to be used as cladistic markers. A more precise knowledge of chromosome evolution in these primates will contribute to a better understanding of New World monkey phylogeny, taxonomy and conservation. These monkeys are also considered important bio/medical models for many diseases. The considerable chromosomal variability in New World monkeys also provides an opportunity to better understand the mechanisms and causes of genome evolution and eventually its relationship with cytogenetic changes seen in disease (Bailey *et al.* 2002, 2004).

Materials and methods

Sodium citrate/dextrose-treated blood samples of 79 *Callicebus cupreus* individuals (49 males, 30 females) were kindly provided by California Regional Primate Research Center, University of California, Davis. The founders of the colony were obtained in the early 1970s from Iquitos (Peru). Other animals from the same location were added in the early 1990s.

Chromosome spreads from Concavalin-A (CON-A)-stimulated peripheral blood lymphocytes were prepared according to standard methods (Small *et al.* 1985). Briefly, 0.3 ml of blood was added to 10 ml of RPMI 1640 enriched with 15% FBS and 50 μg/ml CON-A. Cultures were harvested for 72–96 h.

A fibroblast cell line from a single male, was obtained from the Coriell Institute for Medical Research and described in the catalogue as *Callicebus moloch pallescens* (paraguayan titi), repository number AG06115. This cell line was studied previously in Stanyon *et al.* 2000 and reported as *Callicebus moloch*. Further information on this cell line was obtained from Coriell and, according to the taxonomic revisions of Groves 2001 and van Roosmalen *et al.* 2002, this cell line belongs to the species *Callicebus pallescens*. For this and other titi monkeys, we followed the nomenclature of van Roosmalen *et al.* (2002) for genus and species names (the classification of van Roosmalen does not include any splitting into subspecies)

regardless of the designations in the original publications.

Standard tissue culture and chromosome preparation techniques were followed. To facilitate chromosome identification, sequential G-banding before *in-situ* hybridization on most chromosome preparations was performed as previously described (Klever *et al.* 1991).

Flow sorting and in-situ *hybridization*

Both human and *C. pallescens* chromosome-specific probes were made by degenerate oligonucleotide primed PCR (DOP-PCR) from flow-sorted chromosomes using PCR primers amplification and labelling conditions as previously described (Telenius *et al.* 1992, Wienberg & Stanyon 1997). Chromosome sorting was performed using a dual laser cell sorter (FACSDiVa). This system allowed a bivariate analysis of the chromosomes by size and base-pair composition. About five hundred chromosomes were sorted from each peak in the flow karyotype. Chromosomes were sorted directly into PCR tubes containing 30 µl of distilled water. The same 6MW primer (5′-ccgactcgagnnnnnnatgtgg-3′) was used in the primary reaction and to label the chromosomal DNA with biotin-dUTP or digoxigenin-dUTP in a secondary PCR for indirect detection. Direct labelling was with Rodamine 110-dUTP (Perkin-Elmer) for green, Spectrum Orange (Vysis) for red and Cy5-dUTP (Amersham) for infrared as previously described (Muller *et al.* 1999). *In-situ* hybridization and probe detection were carried out following common FISH procedures. About 300–400 ng of each PCR product per probe, together with 10 µg of human Cot-1 (Gibco BRL) were precipitated and then dissolved in 14 µl hybridization buffer. After hybridization and washing of the slides, biotinylated DNA probes were detected with avidin coupled with fluorescein isothiocyanate (FITC, Vector, Burlingame, CA). Digoxigenin-labelled probes were detected with antidigoxigenin antibodies conjugated with Rhodamine (Roche, Eugene, Oregon).

Digital images were taken using a cooled Photometrics CCD camera coupled to the microscope. Imaging software was SmartCapture (Digital Scientific, Cambridge, UK).

Results

Flow sorting of C. pallescens

The bivariate flow karyotype of *C. pallescens* was resolved into 24 peaks (Figure 1). The flow karyotype is numbered according to Stanyon *et al.* (2000). The karyotype was organized in two groups: chromosomes 1–12 are submeta- or metacentric , while chromosomes 13–24 are acrocentric. Within each group, chromosomes were then ordered by size. Flow sorting and DOP-PCR provided chromosome paints from each peak. These paints were then hybridized to *C. pallescens* metaphases to identify the chromosome content of each peak of the flow karyotype. All but two peaks contained single chromosomes. All peaks provided exceptional chromosome paints.

Chromosomes 2 and 3 were sorted in a single peak and chromosomes 14 and 15 were also contained in one peak. In reciprocal hybridizations, this fact does not usually represent a problem. It is

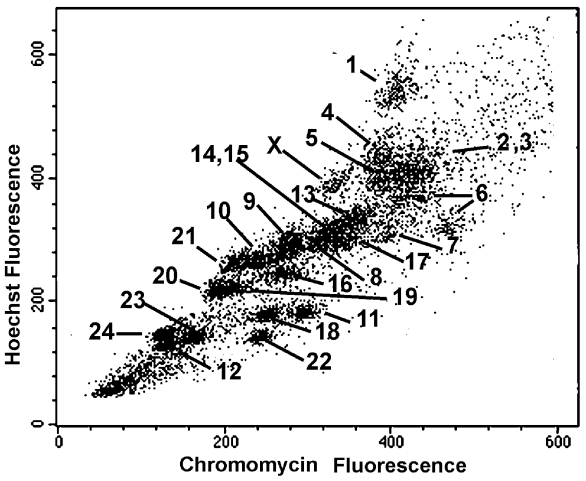

Figure 1. The density plot of the bivariate flow karyotype of *Callicebus pallescens* is shown. The bivariate flow karyotype of *C. pallescens* was resolved into 24 peaks. All but two peaks contained single chromosomes. Chromosomes 2 and 3 sorted together in a single peak. Also chromosomes 14 and 15 sorted together in one peak. Chromosome 6 was present in two different peaks. Numbers of chromosomes are according to G-banded karyotype of *Callicebus pallescens* by Stanyon *et al.* 2000. Chromosomes 1–12 are biarmed chromosomes while chromosomes 13–24 are acrocentrics. Therefore, the numbering in the flow karyotype does not reflect the size of the chromosomes contained in different peaks.

possible to integrate the new data with those already available from hybridizations of human probes on the same monkey species (Stanyon *et al.* 2000) and assign the hybridization signals to specific monkey chromosome.

Chromosome 6 was present in two different peaks. The most likely explanation is that these two chromosomes differ in the amount of repetitive DNA, but we have no further data. It is interesting to note that these two chromosomes 6 also differ for an inversion, but inversions do not cause a change in genome size and therefore it does not explain the presence of chromosome 6 in two different peaks.

Hybridization of C. pallescens *paints on human metaphases*

Paints of *C. pallescens* chromosomes were used to hybridize human metaphases (Figure 2d–h) and 43 clear signals were detected on the human karyotype (Figure 3). Eleven human chromosomes (HSA 4, 6, 9, 11, 14, 17, 18, 20, 21, 22 and X) were entirely painted by titi monkey chromosome paints. However, the titi chromosome paints that hybridized to human chromosomes 11, 14, 17, 18, 21 and 22 hybridized also to other human chromosomes. Ten human chromosomes (HSA 2, 5, 7, 8, 10, 12, 13, 15, 16, 19) were each hybridized by two titi monkey chromosome paints and two human chromosomes (HSA 1 and 3) were each hybridized by three titi monkey paints.

Hybridization of human chromosome paints on C. pallescens *metaphases*

In a previous publication (Stanyon *et al.* 2000), we mapped the chromosomal homology of *C. pallescens* by hybridizing human chromosome paints on *C. pallescens* metaphases. The vast majority of previous results were confirmed by the reciprocal painting of *C. pallescens* paints on human metaphases. Additionally, we determined the subchromosomal homology of human chromosomes, which are found fragmented in the *C. pallescens* genome.

By hybridization of human chromosomes probes on the *C. pallescens*, we confirmed the previous results, but we could also add on to the map other small segments that were not detected in the previous publication where the same titi monkey cell line was used for FISH analysis (Stanyon *et al.* 2000). One small segment of human paint 19 was detected on the bottom of *C. pallescens* chromosome 10 in association with human paint 12. Two small segments of human paint 7 were found in association with human paint 5 on *C. pallescens* chromosome 14. The titi chromosome 7 was split into four segments by human paints 10 and 16. Finally, on the titi chromosome 18, we found a proximal band painted by human probe 13 in association with human probe 17 and a small proximal segment of human paint 17 in association with human paint 20 on titi chromosome 18. The number of hybridization signals on titi monkey autosomes changed, therefore, from 36 (Stanyon *et al.* 2000) to a total of 44. We also found a polymorphism in *C. pallescens* chromosome 6 due to an inversion.

Karyotype of Callicebus cupreus

The karyotype has a diploid number of $2n = 46$ and autosomal $FN = 33$ confirming previous results (de Boer 1974, Benirschke & Bogart 1976). We found eleven pairs of meta/submetacentric autosomes and eleven pairs of acrocentric ones (Figure 4). The sex-chromosome system is XX/XY. The X chromosome is a submetacentric, typical for most mammals and Y is a very small acrocentric. No differences in G-banding pattern were noted in the 79 individuals studied; therefore the two groups of monkeys that formed the colony between the early 1970s and the 1990s must have had the same karyotype.

Hybridization of human chromosome probes on C. cupreus

With the exception of the Y-chromosome probe, every human probe gave a bright hybridization signal on the *C. cupreus* chromosomes (Figure 2 a–c). The human chromosome paints detected 44 segments on the *C. cupreus* haploid set of autosomes. Figure 4 summarizes the hybridization results of human chromosome-specific paints on *Callicebus cupreus* banded chromosomes.

The synteny of only one human autosome (HSA 6) was found intact as this human probe

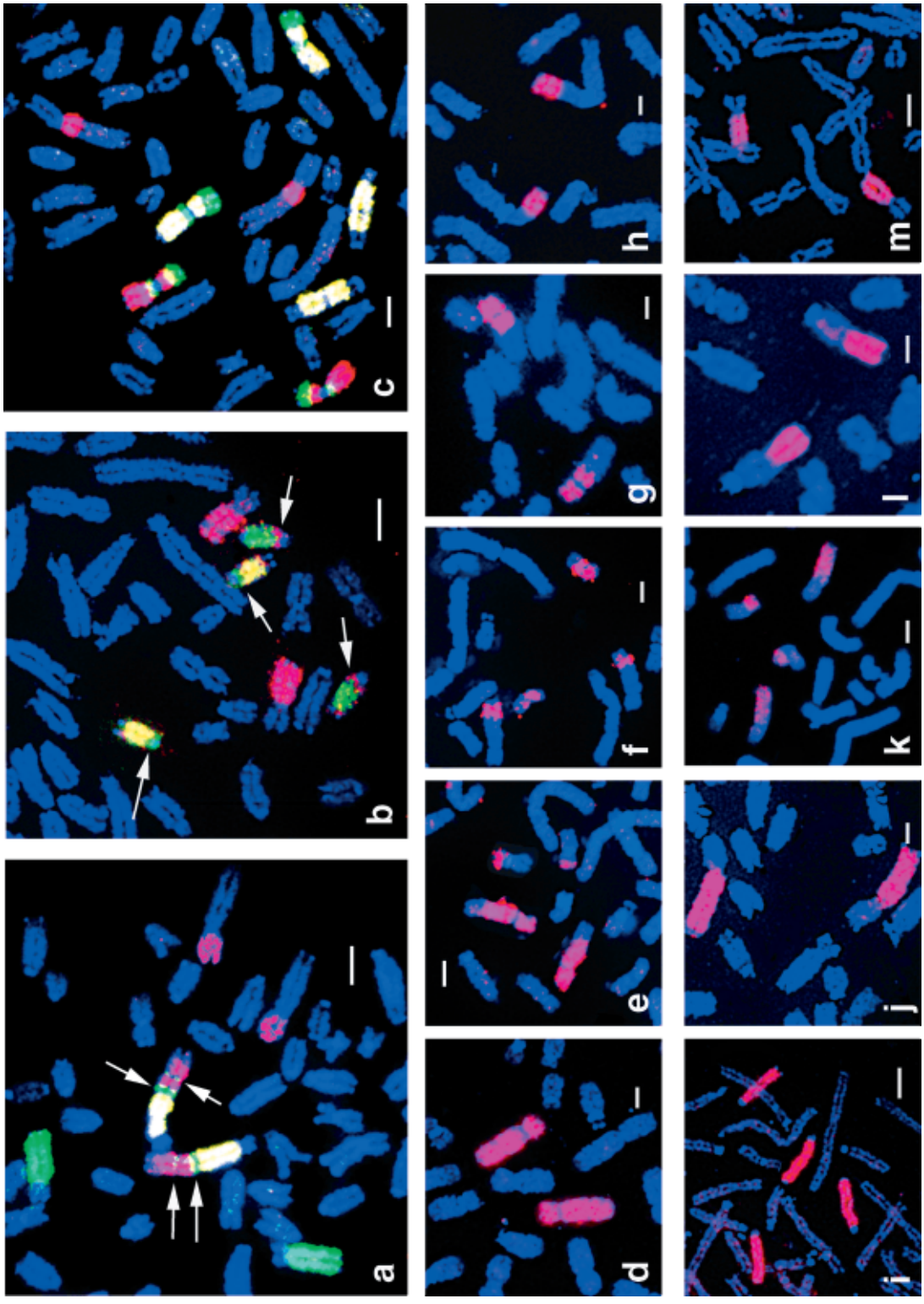

Figure 2. Examples of hybridization: (**a**–**c**) show human chromosome-specific probes hybridized to metaphases of *Callicebus cupreus*, (**a**) HSA7 in green, 5 red, 9 yellow, (**b**) HSA17 in green, 13 red, 20 yellow, (**c**) HSA16 in green, 10 red, 2 yellow; (**d**–**h**) show *C. pallescens* chromosome-specific paints hybridized to human metaphases, (**d**) CPA1; (**e**) CPA10; (**f**) CPA11, (**g**) CPA20, (**h**) CPA23; (**i**–**m**) show *C. pallescens* probes hybridized to metaphases of *C. cupreus*, (**i**) CPA1, (**j**) CPA3, (**k**) CPA7, (**l**) CPA14, (**m**) CPA21. Bars = 2 μm.

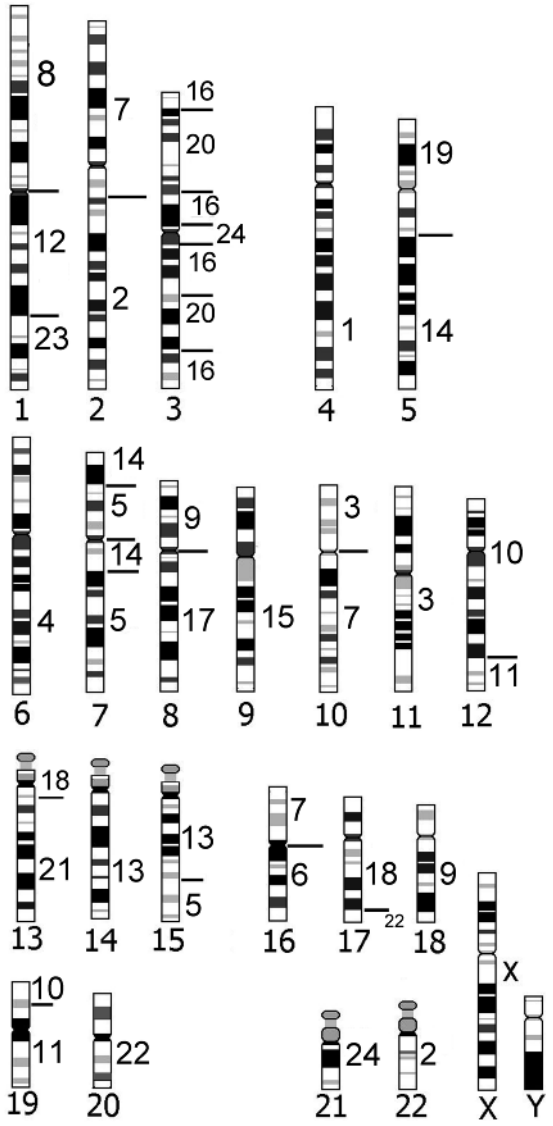

Figure 3. The idiogram of human chromosome numbered below with the hybridization pattern of *C. pallescens* chromosome paints to the right.

hybridized completely one pair of *C. cupreus* chromosomes. All the other human paints were found fragmented on the *C. cupreus* karyotype. Paints specific for human chromosomes 2, 4, 5, 7, 8, 10, 12, 13, 15, 16 and 17 gave signals on two different couples of *C. cupreus* chromosomes. Among these, the probe for human chromosome 4 hybridized completely *C. cupreus* chromosomes 18 and 20. The paint for human chromosomes 1 hybridized completely *C. cupreus* chromosomes 11, 13 and 22. Human probes 2, 3, 7 and 10 were found split into three segments and the human probe 16 into four segments. The paint for human chromosome 3 covered completely *C. cupreus* chromosomes 15 and 21 and was found also on *C. cupreus* chromosome 7 in association with human probes 21 and 13. The paint for human chromosome 17 was found on *C. cupreus* chromosome 17 in association with a segment of probe for human chromosome 13 and on chromosome 19 in association with probe 20. The following 15 associations of human chromosome paints were found on *C. cupreus* chromosomes: 2/16, 2/22, 3/21, 5/7, 5/10, 7/9, 7/15, 8/18, 10/11, 10/16, 12/19, 13/17, 13/21, 14/15 and 17/20.

Hybridization of C. pallescens *chromosome probes on* C. cupreus

All *C. pallescens* probes gave bright signals on *C. cupreus* chromosomes (Figure 2i–m). *C. pallescens* chromosome 1 paint was split in two segments that covered *C. cupreus* chromosomes 18 and 20. The probes for *C. pallescens* chromosomes 3 and 19 were present on the same *C. cupreus* chromosome 1. The probes for *C. pallescens* chromosomes 21, 24 and the probes for *C. pallescens* chromosomes 14, 15, painted segments on the same *C. cupreus* chromosomes, respectively *C. cupreus* 7 and 12.

Discussion

The most recent hypothesis about the ancestral New World monkey karyotype proposed a diploid number of $2n = 54$ (Stanyon *et al.* 2003). According to this hypothesis, ten human syntenic groups are still conserved (4, 6, 9, 11, 12, 13, 17, 19, 20, 22). Human syntenic groups 1 and 3 are fragmented into three segments and six human syntenies (2, 7, 8, 10, 15 and 16) are found fragmented in two segments. The hypothetical karyotype shows the following six associations: 2/16, 3/21, 5/7, 8/18, 10/16 and 14/15. We found that all the associations proposed for the ancestral New World monkey karyotype are present in *Callicebus cupreus* and in *C. pallescens*. In *C. cupreus*, we detected an additional

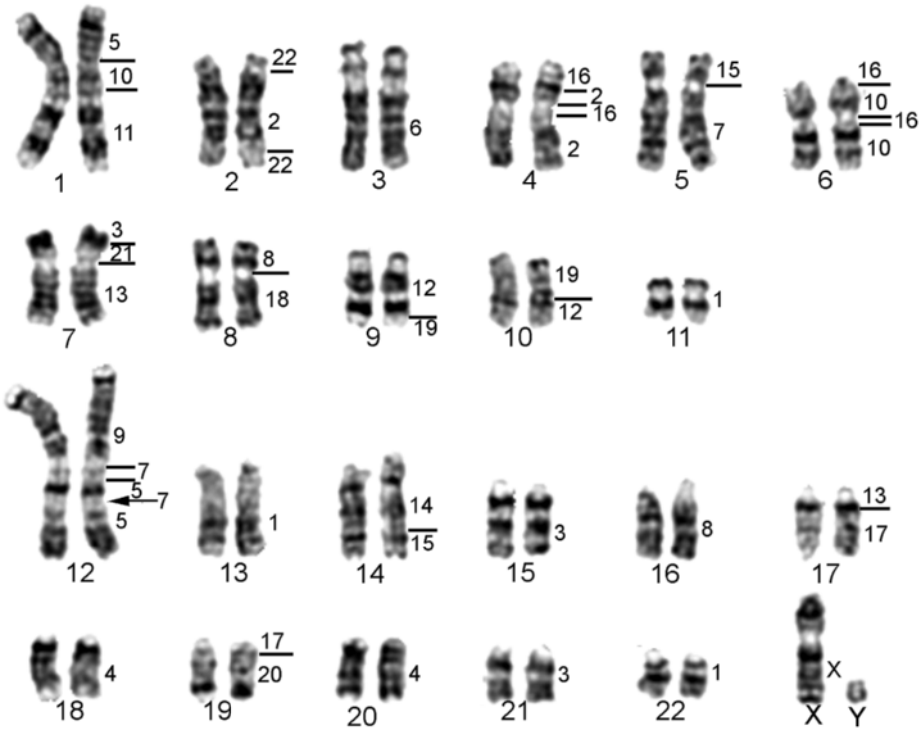

Figure 4. The G-banded karyotype of *Callicebus cupreus* (male). The chromosomes are numbered below. Homology with the human chromosomes is shown on the right.

eight associations: 2/22, 5/10, 7/9, 7/15, 10/11, 12/19, 13/17 and 13/21. All *C. cupreus* associations, with the exception of 5/10, 7/9 and 13/21, are also found in *C. pallescens*. We did not detect any other new association of human chromosomes in *C. pallescens* that is not present in *C. cupreus*. The differences in associations can be linked to the differences in diploid number, *C. cupreus* $2n = 46$ and *C. pallescens* $2n = 50$. The rearrangements differentiating *C. pallescens* from *C. cupreus* are the result of one inversion, one fission and three fusions (two tandem and one Robertsonian) that occurred on the *C. cupreus* lineage. However, it is possible that other small inversions are present but went undetected. The one inversion identified explains the different morphology of the homologue C. *cupreus* chromosome 13 (acrocentric) and *C. pallescens* chromosome 8 (submetacentric), both hybridized by a segment of HSA1. The hybridization of human probe 4 on *C. cupreus* chromosomes 2 and 18 can be explained as a result of the one fission event we found. Finally, three fusions created *C. cupreus* chromosome 1 (association of human probes 5/10/11), chromosome 7 (association 3/21/13) and chromosome 12 (association 5/7/9).

Comparison with C. donacophilus

Despite the nomenclature, the sample of *C. pallescens* studied here and those studied by Barros *et al.* (2003) do not have the same karyotype. On the contrary, the *C. pallescens* karyotype by Barros *et al.* seems closer to *C. cupreus.* The comparison between hybridization data on our two species of *Callicebus* shows that *C. cupreus* is more derived, while *C. pallescens* is relatively conserved. The karyotype of *C.* (*donacophilus*) *pallescens* by Barros *et al.* (2003) seems to represent an intermediate stage, sharing the fusion between human segments 9 with 7/5/7/5 and the fission of human chromosome 4 with *C. cupreus*, but lacking the fusion of human segments 13 with 21/3. We found two associations, 13/17 and 17/20, in *C. cupreus* that

previously went undetected in *C. pallescens*, thanks to reciprocal hybridization, superior chromosome paints and improved imaging now available. Barros *et al.* (2003) did not report these associations in *C. donacophilus*. It is probable that these associations are present but went undetected. Surprisingly, the associations 13/17 and 17/20 (both found in *cupreus* and *pallescens*) have been previously found in the Callithricidae species *S. oedipus*, *C. pygmaea, C. argentata* and *C. jacchus* (Neusser *et al.* 2001) and therefore, according to most studies, in taxa not closely related to *Callicebus*.

Comparison with C. lugens *the primate with the lowest diploid number*

We already underlined the wide range of diploid numbers present in the karyotypes of *Callicebus* species and in particular the recent report on *C. lugens*, the primate species with the lowest diploid number known ($2n = 16$). Due to multiple fusions, there are 34 associations not found in humans. Despite dramatic differences in the diploid number, many elements forming the *C. pallescens* and *C. cupreus* karyotypes are also present in *C. lugens* chromosomes. For example, a number of derived associations present in *C. pallescens* and *C. cupreus* are also present in *C. lugens* (10/11, 2/22/, 15/7, 17/20). We can propose that the associations 10/11, 2/22 and 15/7 were present in the ancestor of all three species. The hybridization pattern shows that three derived inversions are also held in common (16/2/16/2, 16/10/16/10, 22/2/22).

Chromosome fissions

Another possible phylogenetic link between species is the fission of homologues to various human chromosomes (i.e. the number of signals for each human probe on different *Callicebus* species). Comparing the number of signals obtained with the hypothetical New World monkey karyotype, human probes 2 (split in three segments on *Callicebus* species), 10 (three or more segments), 16 (four segments), 19 (two segments) and 22 (two segments) are a common feature for the studied species of *Callicebus* (Table 2). The species group internally on this basis: *C. cupreus*, *C. pallescens* and *C. donacophilus* share the same number of segments revealed by human probes 5 (three segments), 7 (three segments) and 12 (two segments). Finally, *C. cupreus* and *C. donacophilus* may be phylogenetically linked by a fission of the homologue to human chromosome 4.

As already indicated, the problem of the different number of segments regarding human probe 13 and 17 in *Callicebus* species and others can be explained with convergence or as an ancestral trait that was lost or finally and more probably, just with the very small size of the segments involved and subsequent difficulties in the detection process.

Comparison with chromosome painting data in other New World monkeys

To assess the phylogenetic weight of the number of segments present in each species, we need to know if the fission breakpoints involved in producing the fragments are the same (i.e. homologous). Reciprocal painting, when chromosome paints produced from another species are painted back onto human metaphases, provides a good estimate of breakpoint location. Counting the present paper, there are now four reports on reciprocal painting between humans and New World monkeys: *Lagothrix lagotricha* (Stanyon *et al.* 2001), *Saguinus oedipus* (Muller *et al.* 2001), *Aotus nancymae* (Stanyon *et al.* 2004) and *Callicebus pallescens* (this report). The great majority of breakpoints in these three species is apparently equivalent (Table 3) and shows that the fragmentation of these chromosomal syntenies occurred in a common ancestor.

The breakpoint analysis shows that the additional fissions of homologues to human chromosome 5 and 7 are different in the titi and the woolly monkey and provides no phylogenetic link between these species as proposed in some molecular studies (Horovitz *et al.* 1998, Porter *et al.* 1999). A fission of chromosome 13 is found in both the titi monkey and the tamarin. Apparently, also, these breakpoints are different and provide no link between these species. However, since the breakpoints are very close (q12.3 vs. q13) and near the chromosome painting limits of assignment, they merit further investigation. Indeed, since the associations 13/17 and 17/20 found in *Callicebus*

Table 2. Number of segments of homologues to each human chromosome found in four species of titi monkeys.

	Species			
Chromosome	*CDO*	*CPA*	*CCU*	*CLU*
1	3	3	3	3
2	3	3	3	3
3	3	3	3	3
4	2	1	2	1
5	3	3	3	1
6	1	1	1	2
7	3	3	3	2
8	2	2	2	2
9	1	1	1	1
10	3	3	3	4
11	1	1	1	2
12	2	2	2	1
13	1	2	2	1
14	1	1	1	1
15	2	2	2	2
16	4	4	4	4
17	1	2	2	1
18	1	1	1	1
19	2	2	2	2
20	1	1	1	1
21	1	1	1	1
22	2	2	2	2

CDO = *Callicebus donacophilus*, CPA = *C. pallescens*, CCU = *C. cupreus*, CLU = *C. lugens*. Nomenclature follows, as in the article, van Roosmalen *et al.* 2002.

Table 3. Location of breakpoints determined by hybridization to human metaphase of chromosome paints from three species of New World monkeys.

	Species		
Chromosome	*Callicebus pallescens*	*Saguinus oedipus*	*Lagothrix lagotricha*
1	cent, q32	cent, q32	cent, q32, p21
2	q13	q13	q13
3	p24, p14, p12 q13.1, q22, q26.3	p24, p14, p12 q13.1, q22, q26.3	p24, p14, p12 q13.1, q22, q26.3
4			q24, q31.2
5	q13.3		q31
7	p21, cent, q21	p21, cent, q21, q22	p21, cent, q21, q34
8	cent	cent	cent
10	cent	cent	cent
12	q23		
13	q12.3	q13	
15	q24	q24	q13, q21.2, q24
16	cent	cent	cent
17	q25		
19	p13.2		

pallescens are also found in *Callitrichidae* (Neusser *et al.* 2001), it would be of value to have further data on the breakpoints in these and other New World monkeys to determine if they are homologous or not. Intriguingly, Kay (1990) proposes that *Callicebus* is basal to all New World monkeys.

A number of recent studies link *Callicebus* with the *Pitheciini*. Groves (2001) goes so far as to say that this conclusion is one of the few consistent simplifications to emerge from recent revisions of platyrrhine phylogeny.

Recent molecular cytogenetic data (Stanyon *et al.* in press) revealed karyological features which could be interpreted to provide support for diverse phylogenetic arrangements of *Callicebus*. An association of human 10 and 11 suggests a link between *Callicebus* and *Aotus* while an inversion between homologues to segments of human chromosome 10 and 16 in both *Callicebus* and *Chiropotes* suggests a link between these two genera.

Early karyological studies on *Callicebus* species already hypothesized that the direction of karyotypic evolution followed a reduction in number of chromosomes from 2n = 50 to lower diploid numbers (de Boer 1974, Benirschke & Bogart 1976) and that Robertsonian translocation mechanisms played an important role. Recent results seem to support the hypothesis of reduction in diploid number because the ancestral platyrrhine karyotype is thought to have had 2n = 54. Our data indicate that Robertsonian fissions are an important mechanism in transforming the karyotype, but that fissions, inversions and non-Robertsonian translocations are also important mechanisms of genome rearrangement in *Callicebus* and other New World primates. Many breakpoints seem to be located at the G-/R-band boundary. The resolution of whole chromosome paints is not sufficient to test this hypothesis. Other smaller sized probes, such as BACs, are more suitable for more precisely mapping breakpoints and would be a first step toward testing hypotheses about the location of rearrangement breakpoints.

It is probable, given what we now know about titi monkey cytogenetics, that we can expect to find other intermediate diploid numbers as more

species are studied. Another important aspect to investigate is the correlation between karyotypes and geographical distribution. It is now clear that rivers form the borders between many titi monkey species. There is some indication that species with a higher diploid number (with less-derived more ancestral karyotypes) are localized in the centre of the geographical range of the genera, while more derived species appear to occupy the periphery. This hypothesis needs to be tested and awaits fuller explanation. However, molecular cytogenetics has the potential to test these and other hypotheses of New World phylogeny and evolution given that a sufficient number of samples of known geographical origin are studied with ever more refined molecular methods.

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