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RESEARCH ARTICLE

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Chromosome painting in *Callicebus lugens*, the species with the lowest diploid number (2*n*=16) known in primates

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Abstract Cytogenetic studies have shown that New World primates are karyologically diverse and highly derived. The genus *Callicebus* is the best example of this karyological diversity, with diploid numbers ranging from 2*n*=50 to 2*n*=16. We report on *Callicebus lugens*, which has the lowest diploid number (2*n*=16) yet found in the primate order and represents a striking example of extreme karyotypic shuffling. To better understand the genomic rearrangements that have resulted in this extremely low diploid number, we mapped chromosome homologies between *C. lugens* and humans by in situ hybridization. The total number of hybridization signals was 42, excluding the Y chromosome, with a total of 34 syntenic associations not found in humans. This species has one of the most derived karyotypes among the Platyrrhini. Fusion has been the predominant mode of karyological evolution, although fissions and inversions have also transformed the *C. lugens* karyotype. Remarkably in such a highly rearranged karyotype, the synteny of 11 human chromosomes (4, 5, 9, 12, 13, 14, 17, 18, 20, 21, and X) was maintained intact, even if most of these human-homologous gene clusters were translocated. Other human syntenies, such as homologues to human chromosomes 10 and 16, were highly fragmented. Comparisons of the *C. lugens*-human homology map with those of other New World primates have not yet helped establish a phylogenic arrangement between congeneric species or link *Callicebus* with any other genus.

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Introduction

The genus *Callicebus* provides a good example of how our understanding of New World monkeys is contentious with many outstanding phylogenetic and evolutionary questions. Morphological analyses in conflicting topologies placed *Callicebus* either as the most basal branch of the main platyrrhine stock (Kay 1990) or as a sister lineage of *Aotus*. Rosenberger and Coimbra-Filho (1984) and Rosenberger et al. (1990) proposed that *Callicebus* and *Aotus* were derived, sister lineages closely related to the pithecine clade (*Pithecia*, *Chiropotes*, and *Cacajao*) while Ford (1986) placed *Callicebus* and *Aotus* as very basal sister lineages, second only to *Cebus* and *Saimiri* and very distantly related to the pithecines.

Molecular phylogenetic analysis has provided a different picture of platyrrhine phylogeny. For example, a recent analysis based on four different DNA datasets, representative of all extant neotropical primate genera, comprising some 6,763 base pairs with 2,086 variable characters, and 674 informative sites placed *Callicebus* as basal to *Pithecia* and to the more derived sister groups (*Cacajao* and *Chiropotes*) (Schneider et al. 2001) (Fig. 1).

Dutrillaux et al. (1986), on the basis of chromosome banding, proposed an association between *Callicebus* and the Atelinae. Conversely, extensive analyses of molecular data clearly established the phylogenetic relationships of all platyrrhine genera, separating *Callicebus* from *Aotus* and grouping *Callicebus* as a basal offshoot of a derived clade that included the pithecines (Schneider et al. 2001).

Callicebus is the only recognized genus of the tribe Callicebini (sensu Schneider et al. 2001) but the number of extant species is controversial. Traditional taxonomy until a decade or so ago divided the genus into only three

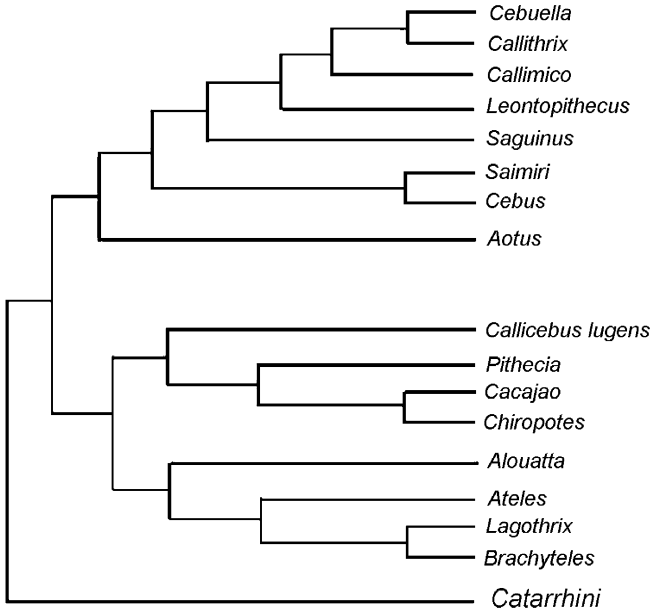

Fig. 1 A recent phylogeny of New World primates based on DNA sequence analysis from all extant neotropical primate genera (modified from Schneider et al. 2001). Note that the phylogenetic line leading to *Callicebus lugens* is basal to *Pithecia*, *Cacajao*, and *Chiropotes*. Catarrhini comprise Old World monkeys, apes, and humans

species: *C. moloch*, *C. personatus*, and *C. torquatus* (Hershkovitz 1963), although 13 species were described later (Hershkovitz 1988; Hershkovitz 1990). More recently, 28 species and their respective geographic range were reported, although the phylogenetic relationship between them remains unclear and speculative (van Roosmalen et al. 2002).

Cytogenetic studies have shown that New World primates are karyologically diverse, and some species are highly derived with respect to the chromosome complement of humans or a presumed platyrrhine ancestor (Chu and Bender 1962; Koiffmann and Saldanha 1981; Consigliere et al. 1996, 1998; Stanyon et al. 2000; Bonvicino et al. 2003). The genus *Callicebus* is one of the best examples of this diversity because diploid numbers among species range from 2*n*=50 (Rodrigues et al. 2001) to 2*n*=16 (Bonvicino et al. 2003). This latter karyotype, reported in *Callicebus lugens*, shows the lowest diploid number yet found in the primate order and represents a striking example of extreme genomic shuffling.

This extensive karyological diversity within a specious genus provides reason and stimulus to investigate more fully the cytogenetics of *Callicebus* species. The high evolutionary rate of chromosome evolution within this genus provides confidence that molecular cytogenetic data may eventually help to clarify the phylogeny and evolutionary relationships, both between *Callicebus* species and between *Callicebus* and other New World primate taxa.

To better understand the genomic rearrangements that resulted in the lowest diploid number known in the primates, we identified and mapped chromosome homologies between *C. lugens* and humans by in situ hybridization. We subsequently used this homology map to compare *C. lugens* with other congeneric species and platyrrhine taxa and discuss the presumed evolutionary rearrangements that took place in the chromosome complement of titi monkeys.

Materials and methods

Collecting sites, morphological traits, and karyotypic description of the *C. lugens* samples used here for chromosome painting were recently described (Bonvicino et al. 2003). Briefly, metaphase preparations were obtained from bone marrow cultures from four wild, caught *C. lugens* after a brief culture.

Human chromosome paints were made by flow-activated chromosome sorting followed by DOP-PCR as previously described (Telenius et al. 1992). For indirect detection, biotin-dUTP and digoxigenin-dUTP (Roche) were incorporated by secondary DOP-PCR. Direct labeling was done with rhodamine 110-dUTP for green, Tamra-dUTP (both from Perkin-Elmer) 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 probe-PCR product was precipitated together with 10 µg of Cot-1 DNA (Gibco BRL) and then dissolved in 14 µl of hybridization buffer.

Slides were G-banded, re-fixed in formaldehyde, and denatured for 30–40 s in 70% formamide/2× SSC at 55°C. Unbanded slides were denatured for 2 min at 65°C. DNA probes were denatured at 80°C for 5 min, pre-annealed at 37°C for 90 min, and hybridized for 48 h at 37°C. All post-hybridization washes were at 42°C and included 2×6 min in 50% formamide/2× SSC, 2×6 min in 2× SSC, 3 min in 4× SSC plus Tween. Biotinylated DNA probes were detected with avidin-fluorescein isothiocyanate (FITC) and digoxigenin was detected with mouse anti-digoxigenin coupled with rhodamine (Vector Laboratories). For chromosome identification, slides were counterstained with DAPI (4′, 6-diamidino-2-phenylindol, Sigma).

The telomeric probe was purchased from Cytocell (Cambridge, UK). Hybridization and detection was done according to the protocol supplied with the kit.

Digital images were taken using a cooled CCD camera (Photometrics) and SmartCapture (Digital Scientific, Cambridge, UK). Alternatively, slides were observed with an Olympus B-60 fluorescence microscope, and image capture was carried out with a Sensys Photometrics camera and processed with PathVision (VYSIS).

Results

Callicebus lugens specimens showed 2*n*=16; autosomal *FN*=22. With the exception of the human Y-chromosome probe, all human paints gave bright hybridization signals (Fig. 2a–g). The total number of hybridization signals obtained was 42, excluding the Y chromosome. The smallest *C. lugens* chromosomes (5, 6, and 7) were hybridized by single human chromosome probes, while the largest four chromosomes showed 6–13 hybridization signals. Figure 3 summarizes the hybridization results of human chromosome-specific paints on *C. lugens* G-banded chromosomes.

Even in such a highly rearranged karyotype, the synteny of 11 human chromosomes (4, 5, 9, 12, 13, 14,

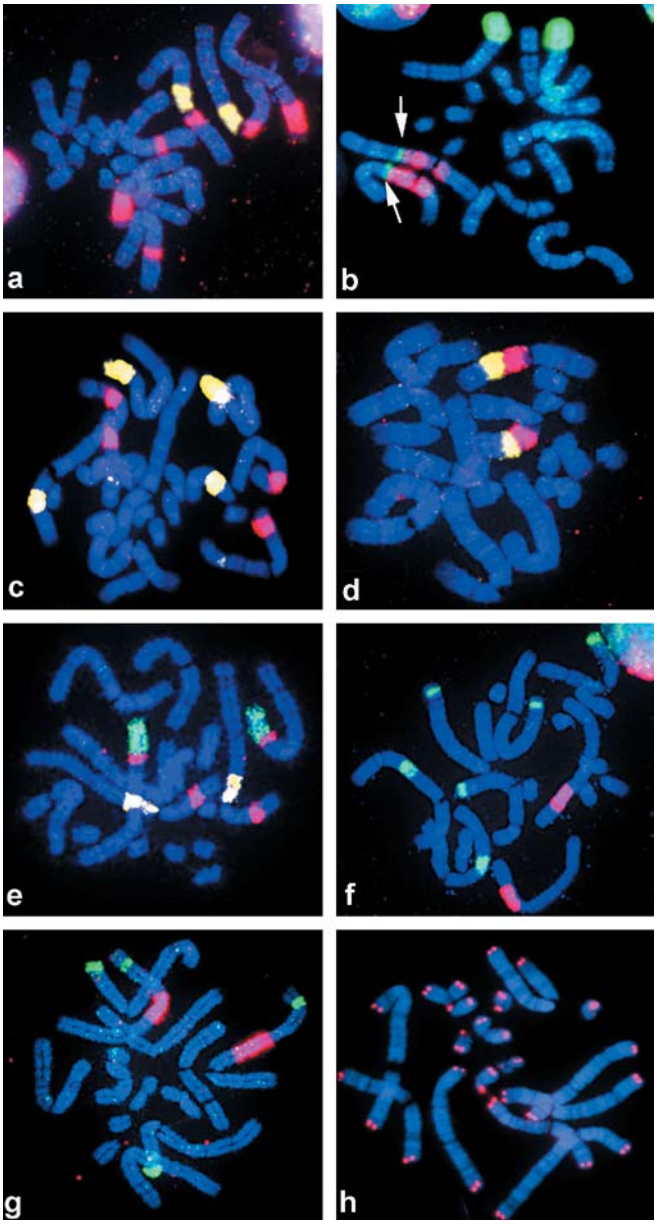

Fig. 2 Examples of hybridization of human chromosome specific probes (**a**–**g**) to metaphases of *Callicebus lugens*: **a** 1 in red and 13 in yellow; **b** 7 in green and 5 in red, the *arrows* point to a small segment of 7 associated with 5; **c** 11 in red and 3 in yellow; **d** 17 in red and 20 in yellow; **e** 15 in red, 7 in green and 13 in yellow; **f** 9 in red and 10 in green; **g** 19 in green and 12 in red; and **h** a telomeric probe

17, 18, 20, 21, and X) was maintained without disruption. However, they were, with the exception of the syntenic homologues to human chromosomes 18 and X, associated with other human chromosomes or chromosome fragments. The remaining 12 human chromosome paints provided multiple signals showing clear fragmentation in the *C. lugens* chromosome complement. Chromosomes 10 and 16 were the most highly disrupted and showed four signals. Chromosomes 1, 2, and 3 each showed three

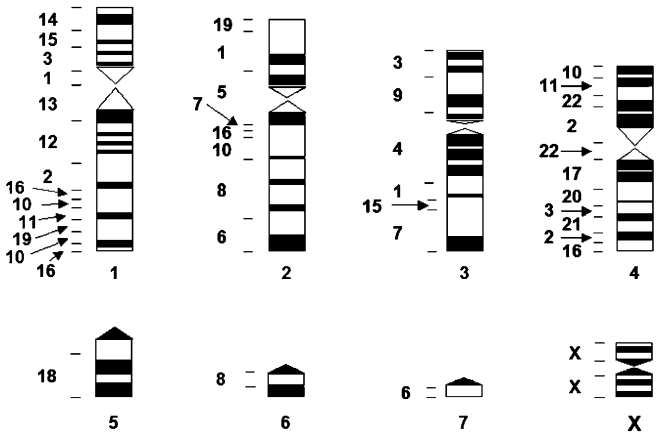

Fig. 3 Idiogram of *Callicebus lugens* (2*n*=16). The *C. lugens* chromosomes are numbered *below* and the hybridization with human chromosome paints is indicated to the *left*

signals, while chromosomes 6, 7, 8, 11, 15, 19, and 22 each showed two signals.

When a single chromosome is hybridized by multiple paints, contiguous segments represent associations of homologous syntenic clusters of the reference species. As anticipated from a species with an extremely low diploid number, the *C. lugens* chromosomes painted by multiple human chromosome probes produced a high number of associations (34) of human-homologous chromosomes or chromosome segments: 1/3, 1/4, 1/5, 1/13, 1/15, 1/19, 2/12, 2/16 (twice), 2/21, 2/22 (twice), 3/9, 3/15, 3/20, 3/21, 4/9, 5/7, 6/8, 7/15, 7/16, 8/10, 10/11 (twice), 10/16 (thrice), 10/19, 11/19, 11/22, 12/13, 14/15, 17/20, and 17/22.

The telomeric probe produced bright signals at the telomeres of most chromosomes (Fig. 2h), but no interstitial signals were observed using the standard protocol.

Discussion

Hybridization patterns show, as might be expected from the extremely low diploid number (2*n*=16), that *Callicebus lugens* has one of the most derived karyotypes among the Platyrrhini. There are 29 different associations that are not found in the human genome (totaling 34, due to multiple occurrences of four associations). It is clear from the low diploid number of *C. lugens* that fusion has been the predominant type of rearrangement in the karyological evolution of this species. Indeed, we used a telomeric probe in anticipation that it might reveal interstitial sites of telomeric repeats left from tandem fusions. However, telomeric signals were restricted to the end of *C. lugens* chromosomes. It may be that interstitial telomere sequences, if they exist, are below the resolution of the probe and the protocol used.

The mode of chromosome evolution becomes clearer if we compare the *Callicebus* hybridization patterns with

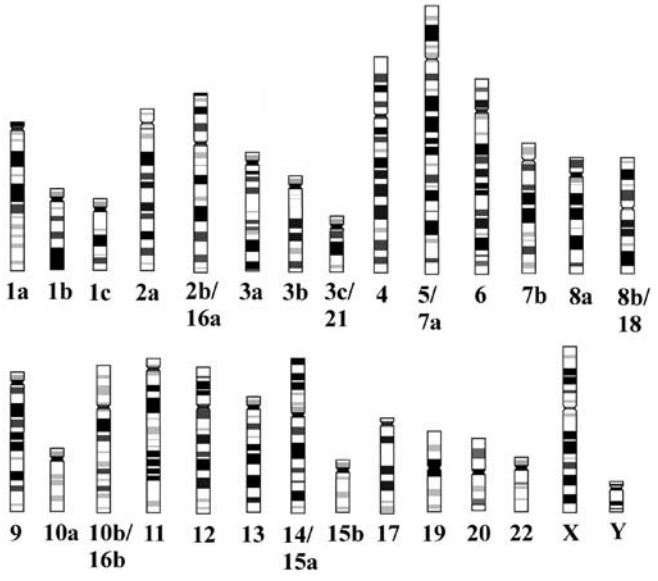

Fig. 4 Idiogram of the ancestral platyrrhine karyotype. This hypothetical karyotype has a diploid number of 2*n*=54. The exact morphology and banding pattern of chromosomes are speculative

that of the proposed karyotype of ancestral New World monkeys (Stanyon et al. 2000; Neusser et al. 2001; Garcia et al. 2002). The most recent of these hypotheses proposed that the ancestral New World monkey karyotype had a diploid number of 2*n*=54, similar to the karyotype of *Cebus capucinus* and *C. apella*, and that seven human syntenies (chromosomes 1, 2, 7, 8, 10, 15, and 16) were fragmented into two segments, while chromosome 3 was split in three segments (Fig. 4). The ancestral platyrrhine karyotype would have contained six associations: 2/16, 3/21, 5/7, 8/18, 10/16, 14/15 (Neusser et al. 2001); two associations (3/21 and 14/15) are presumably very old because they are hypothesized to have been present in the ancestral karyotype of placental mammals (Muller et al. 1999; O'Brien and Stanyon 1999; Murphy et al. 2001), while the 2/16, 5/7, 8/18, and 10/16 would be derived associations common to all New World monkeys.

Eleven human syntenic clusters (4, 5, 9, 12, 13, 14, 17, 18, 20, 21, and X) are conserved without disruption in *Callicebus lugens*, as in the presumed ancestral karyotype of all platyrrhines. Moreover, *C. lugens* shows five presumably ancestral associations; three of them are present only once (3/21, 5/7 and 14/15), but the other two (2/16 and 10/16) are present twice and thrice, respectively. A survey of associations present in other primates indicates that the 7/16 association found in *C. lugens* is not the same association proposed to be present in the ancestral mammalian karyotype (Muller et al. 1999).

It is thus evident that fissions are responsible for the multiplicity of syntenic disruptions and these additional associations, and for the finding that several human syntenic clusters, conserved in the ancestral platyrrhine karyotype (homologues to human 6, 11, 19, and 22), were fragmented in two segments. Additional fissions and inversions further fragmented some human clusters already disrupted in the platyrrhine ancestor (10 and 16 in four segments vs two ancestral segments each, and 1 and 2 in three segments vs two ancestral segments each). Furthermore, the ancestral platyrrhine 8/18 association is not present in *C. lugens*, and its disruption is clearly indicative of another fission event.

The fissions observed in *C. lugens* indicate that several associations must not necessarily represent identical syntenic assemblages with the ones found in the ancestral platyrrhine karyotype. This is the case in *C. lugens* associations involving human homologous syntenies 1, 2, 10, and 16, because these clusters were far more fragmented than they were in the proposed ancestral platyrrhine genome. In general, the higher the fragmentation, the lower the possibility of conserving the same syntenic clusters across disparate taxa, and the higher the possibility that novel syntenic associations may be produced by subsequent fusions. Conversely, a given association might still be conserved despite synteny disruption. For example, the association between the 14/15 human homologues is an ancestral mammalian association found conserved in all primates except the hominoidea (Murphy et al. 2001). In all platyrrhines, chromosome 15 is fissioned, but the 14/15 association is still found.

Common inversions have most likely produced the alternating signals between 2 and 22 as well as between 10 and 16. The *Callicebus* ancestral genome also seems to have experienced further fragmentation of the homologues to human chromosomes 1, 2, 10, 16, and 22. A fission event, accounting for the apparent loss of the 8/18 association, resulted in an apparently derived characteristic of the *C. lugens* karyotype. A comparison with *C. moloch* shows that two other human homologues have been fissioned in *C. lugens*: chromosome 6 (2 vs 1 signal) and chromosome 10 (4 vs 2 signals). In conclusion, although there is a preponderance of fusions, also fissions and inversions have transformed the *C. lugens* karyotype.

Two other species of the genus *Callicebus* were previously studied using chromosome painting. A specimen identified as *C. moloch* (2*n*=50) showed 37 signals and 12 associations (Stanyon et al. 2000), while two specimens of *C. donacophilus pallescens* (2*n*=50) produced 44 signals and 17 associations (Barros et al. 2003). *C. lugens* showed a similar number of signals (42), but the total number of associations (34) was clearly higher as a consequence of fusions leading to a drastic reduction in diploid number.

Associations 2/22, 7/15, and 10/11 would represent derived associations present in all three *Callicebus* species and most probably in the genome of their common ancestor. One association, 5/7, was found in *C. d. pallescens* and *C. lugens* but not in *C. moloch*. This association may have simply been missed in *C. moloch* because in *C. lugens*, the region is small and close to the limit of resolution of FISH (Fig. 2b, e). In fact, the 5/7 association is found in a wide range of neotropical primates and was hypothesized to be present in the ancestral platyrrhine genome. Another association, 12/19,

was found in *C. moloch* and *C. d. pallescens* but not in *C. lugens*, despite multiple hybridization attempts, a finding that suggests a closer relationship between *C. moloch* and *C. d. pallescens*. Regardless of these findings, however, hybridization data do not at this time provide conclusive evidence for determining phylogenetic relationships between these *Callicebus* species.

Similarly, hybridization data do not link *Callicebus* with any other platyrrhine previously analyzed by chromosome painting. The 2/22, 7/15, and 10/11 associations that are presumably ancestral in *Callicebus* are neither present in the inferred ancestral karyotype of the Atelinae nor in the presumed ancestral karyotype of *Alouatta*, the most basal Ateline lineage (Consigliere et al. 1996; de Oliveira et al. 2002). Although some associations, like 3/15, clearly absent in the presumed platyrrhine ancestor, are present in *C. lugens*, some species of *Alouatta* and two species of *Ateles*, *A. geoffroyi* and *A. paniscus chamek* (Morescalchi et al. 1997; Seuanez et al. 2001), a phylogenetic link between these taxa cannot be established. In *Alouatta*, for example, the 3/15 association was reported in the X_2 chromosome of species with an $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex chromosome system (Consigliere et al. 1996; Murphy et al. 2001; de Oliveira et al. 2002), while in *C. lugens* and in both *Ateles* species, the 3/15 association is autosomal. Further, if 3/15 were a phylogenetic link between *Callicebus* and *Ateles*, we would expect to find this association present in all *Callicebus* species, not just one.

Although the 2/16 association is present twice in *C. lugens*, *A. geoffroyi*, and the presumed ancestral karyotype of *Alouatta*, each association is located in two different chromosomes of *C. lugens*, unlike the others in which both 2/16 associations are present in a single chromosome. Separation of 2/16 associations was shown to occur as a derived characteristic of a clade of several *Alouatta* species: *A. sara arctoidea*, *A. s. maconnelli*, and *A. fusca fusca* (de Oliveira et al. 2002), indicating that this characteristic is the result of independent inversions and fissions in each phylogenetic lineage.

Unfortunately, there is no hybridization data on any Pithecinae species to test the proposed phylogenetic link of *Callicebus* with these species as supported by most molecular data (Schneider et al. 2001). Further work within the genus *Callicebus*, especially on samples of known geographic origin from a wide array of species, will be necessary to resolve the phylogenetic relationship within this genus.

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