Original Article

Cytogenetic and Genome Research

Cytogenet Genome Res 2009;125:46–53
DOI: 10.1159/000207518

Accepted after revision: December 22, 2008
by M. Schmid

The Y Chromosome of the Atelidae Family (Platyrrhini): Study by Chromosome Microdissection

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Key Words
Atelidae · Chromosomal evolution · Homologies · Platyrrhini · Y chromosome

Abstract
In order to study the intergeneric variability of the Y chromosome, we describe the hybridization of the Y chromosome of *Brachyteles arachnoides*, obtained by microdissection, to metaphases of *Ateles belzebuth marginatus*, *Lagothrix lagothricha*, and *Alouatta* male specimens. *Brachyteles arachnoides* (Atelinae) has 62 chromosomes and a very small Y chromosome. Our results showed that the *Brachyteles arachnoides* Y chromosome probe hybridized to *Lagothrix lagothricha* metaphases yielding one hybridization signal on only the tiny Y chromosome, and when hybridized with *Ateles belzebuth marginatus* metaphases it yielded one hybridization signal on two thirds of the small acrocentric Y chromosome. However, no hybridization signal was observed in *Alouatta* metaphases (subfamily Alouattinae), a closely related genus in the Atelidae family. Furthermore, our data support a close phylogenetic relationship among *Brachyteles*, *Ateles*, and *Lagothrix* and their placement in the Atelinae subfamily, but exclude *Alouatta* from this group indicating its placement as basal to this group.

All placental mammals have an XX-female XY-male sex determining system or some variant of this, with the exception of *Ellobius lutescens* [Vogel et al., 1998] and *Tokudaia osimensis* [Honda et al., 1977] species without a Y chromosome. Gene mapping and chromosome painting show that the X chromosome is almost identical, even between the most distantly related species. However, the Y chromosome shows remarkable differences both morphologically and genetically between species [Waters et al., 2007] and shows considerable variation in heterochromatin content [Houck et al., 2001]. Repetitive sequences are very poorly conserved, and a Y chromosome paint prepared from one species usually will not hybridize to the Y of even a quite closely related species [Waters et al., 2007]. The human Yq has a large terminal heterochromatin block that appears to be evolutionarily derived. The ancestral Y chromosome was probably considerably smaller in all ancestral karyotypes and has been subject to numerous rearrangements during the evolution of the hominoids [Wimmer et al., 2005].

The mammalian Y chromosome shows a broad spectrum of species-specific rearrangements. As long as some of these rearrangements do not interfere with male fertility they are transmitted obligatorily to male offspring and thus evolutionary fixation is possible. This has led to the assumption that Y chromosomes should offer a large number of evolutionary breakpoints [Wimmer et al., 2005].

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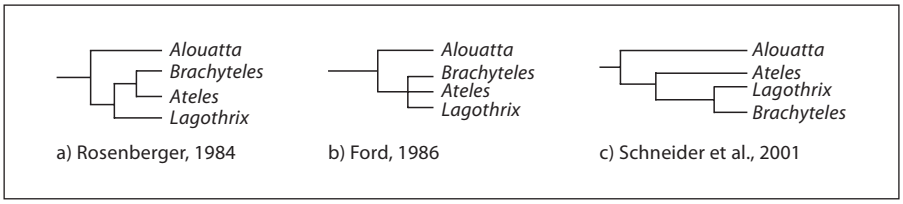


Fig. 1. Three phylogenies for Atelidae family modified from: a) Rosenberger [1984], b) Ford [1986], and c) Schneider et al. [2001].

The Infraorder Platyrrhini (Neotropical primates or New World monkeys) is most commonly divided into 3 families: Cebidae, Pitheciidae, and Atelidae [Goodman et al., 1998; Schneider et al., 2001; Steiper and Ruvolo, 2002; Seuánez et al., 2005]. The phylogenetic relationships of genera within the Atelidae family are not clearly established. Morphological studies have shown conflicting results: Ford [1986] found *Brachyteles*, *Lagothrix*, and *Ateles* to form an unresolved trichotomy; Rosenberger [1981, 1984] and Rosenberger et al. [1990] placed *Brachyteles* and *Ateles* in one clade followed by *Lagothrix* and then *Alouatta* (fig. 1a, b); and Kay [1990] separated this family into 2 sister clades, one grouping *Alouatta* and *Brachyteles* and another clade grouping *Lagothrix* and *Ateles*. Chromosomal studies have indicated that *Brachyteles*, *Lagothrix*, and *Ateles* form a separate and distinct evolutionary branch, and that *Lagothrix* is more closely related to *Ateles* than to *Brachyteles* [Dutrillaux et al., 1986]. Molecular data showed congruent branching inside the atelid clade, placing *Alouatta* as the most basal lineage followed by *Ateles* and a more derived branch including *Brachyteles* and *Lagothrix* as sister groups [Schneider et al., 1993, 2001; Meireles et al., 1999] (Fig. 1c). On the other hand, Ruiz-García and Alvarez [2003] and Nieves et al. [2005] showed a close phylogenetic relationship among atelids *(Ateles*, *Lagothrix*, and *Brachyteles)*, while the placement of *Alouatta* was basal within the Atelidae.

Our analysis of the Y chromosome in platyrrhines includes all 4 genera within the Atelidae: the Woolly Spider monkey *(Brachyteles)*, the Woolly monkey *(Lagothrix)*, the Spider monkey *(Ateles)*, and the Howler monkey *(Alouatta)*. Previous chromosomal analyses on these genera are summarized as follows.

The Woolly spider monkey (genus *Brachyteles*) is endemic to the Atlantic coastal forests of Brazil [Pope, 1998]. The karyotype of a few specimens of *B. arachnoides* have been analyzed and the diploid number is 2n = 62 [Koiffmann and Saldanha, 1978; Viegas-Péquignot et al., 1985; De Oliveira et al., 2005]. The Y chromosome was found to be very small and similar to the Y chromosome described for *Lagothrix* [De Oliveira et al., 2005].

The Woolly monkey (genus *Lagothrix*) is distributed in the regions of the Brazilian Amazon rainforest, Bolivia, Colombia, Ecuador, Peru, and Venezuela [Fooden, 1963]. This genus shares with *Brachyteles* the highest diploid chromosome number found among New World monkeys (2n = 62) and the Y chromosome has been described as acrocentric, metacentric, or a tiny chromosome [Egozcue and Perkins, 1970; De Boer, 1974; Koiffmann and Saldanha, 1974; García et al., 1980; Stanyon et al., 2001].

The Spider monkey (genus *Ateles*) is distributed in the regions of Mexico, Costa Rica, Panama, Colombia, and Brazil [Kellogg and Goldman, 1944; Silva-López et al., 1996]. The diploid number is 2n = 34 in all species and subspecies except in *A. paniscus paniscus* (2n = 32), and the Y chromosome has been reported as an acrocentric or a metacentric [De Boer, 1974; Kunkel et al., 1980; Koiffmann and Saldanha, 1981a, b, 1982; De Boer and De Bruijn, 1990; Medeiros et al., 1997; Morescalchi et al., 1997].

The Howler monkey *(Alouatta)* shows the largest area of geographical distribution among Platyrrhini (between southern Mexico and northern Argentina) and one of the greatest diversity of species [Stanyon et al., 1995; De Oliveira et al., 2002]. *Alouatta* gained special interest because of the presence of high diploid number variability (2n = 42 to 56), the presence of microchromosomes and complex sex chromosome systems due to the Y-autosome translocations found within this genus [De Boer, 1974; Koiffmann and Saldanha, 1974, 1981a; Armada et al., 1987; Lima and Seuánez, 1991; De Oliveira et al., 1995, 2002; Stanyon et al., 1995; Consigliere et al., 1996, 1998; Vassart et al., 1996; Mudry et al., 1998; Torres and Ramírez, 2003].

In order to study the intergeneric variability of the Y chromosome, we describe the hybridization of the Y chromosome of *Brachyteles arachnoides* obtained by microdissection to metaphases of *Ateles belzebuth marginatus*, *Lagothrix lagothricha*, *Alouatta caraya*, and *A. guariba clamitans*.

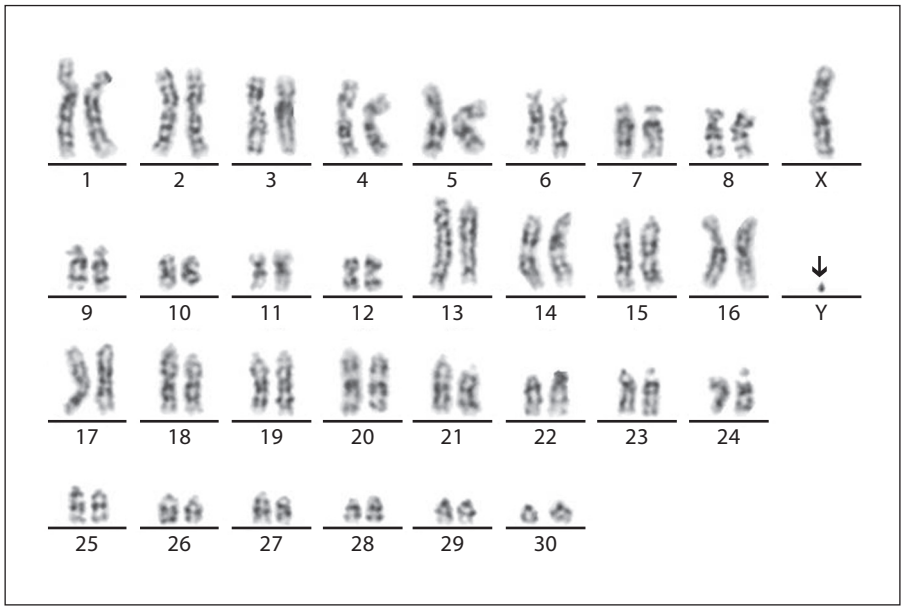


Fig. 2. G-banded karyotype of *Brachyteles arachnoides*. Arrow indicates the small Y chromosome.

Materials and Methods

Peripheral blood samples of *Brachyteles arachnoides*, *Lagothrix lagothricha*, and *Ateles belzebuth marginatus* males [Zoológico Municipal Quinzinho de Barros (Sorocaba, Brazil)], a male of *Alouatta caraya* [Porto Primavera (Presidente Epitácio, Brazil)] and a male of *Alouatta guariba clamitans* [Centro de Estudo e Manejo de Animais Silvestres – Fundação Florestal (São Paulo, Brazil)] were collected with a heparinized (Liquemine, Roche, Switzerland) disposable syringe. Lymphocyte cultures were done according to Moorhead et al. [1960]. GTG banding [Seabright, 1971] and conventional Giemsa (Merck, Germany) metaphases were analyzed on an Axiophot 2 microscope (Zeiss, Germany) using digital images acquired via CCD video camera and karyotyped using the IKAROS 3 software (MetaSystems, Germany).

A chromosome microdissection protocol with slight modification [Weimer et al., 1999; Henning et al., 2008] was done to obtain the *Brachyteles arachnoides* Y chromosome probe. The lymphocyte culture derived cells were stored as fixed cells in 3:1 methanol:acetic acid. Suspensions were dropped onto coverslips and subjected to regular Giemsa staining. The *Brachyteles arachnoides* Y chromosome was identified with an inverted microscope (Axiovert S100; Zeiss, Germany), microdissected [TransferMan NK 2 (Eppendorf, Germany)] with needles, and transferred by inserting the tip of the needle into the siliconized pipette containing a collection solution added by capillarity (30% glycerol, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM EDTA pH 7.5–8.0, 0.1% SDS, 0.1% Triton X-100, and 500 μM proteinase K). Only 3 chromosomes were microdissected. Needles [borosilicate rods (Harvard Apparatus, USA)] and pipettes were prepared using a two-step pipette puller (Narishige, Japan). All equipment was exposed to UV light prior to use.

The pipette containing the chromosomes was incubated in a humid chamber at 60°C for 1 h. The chromosomes were transferred to the pre-amplification mixture by breaking the tip of the pipette inside the microtube. The pre-amplification PCR mix consisted of 24 mM Tris-HCl pH 7.5, 5 μM 6-MW primer (5′ CCG ACT CGA GNN NNN NAT GTG G 3′, 10 mM), 30 mM NaCl (0.6× reaction buffer), 0.2 mM dNTP's and 12 mM $MgCl_2$, in a 5 μl final volume. The reaction profile was 90°C for 1 min, 25°C for 2 min, and 4°C for 2 min; 0.3 U of T7 DNA polymerase – Sequenase Version 2.0 (USB, USA) was added at each of the 8 cycles during annealing. A first step of denaturation (5 min at 90°C) was necessary to inactivate the proteinase activity in the collection solution. For the standard DOP amplification (30 cycles), after the pre-amplification step, 50 μl of the following mix was added to the previous PCR product: 10 μl of 10× NH_4 reaction buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20], 5 μM 6-MW primer (10 mM), 0.2 mM dNTPs and 0.1 U Taq polymerase. The high temperature reaction profile was 92°C for 1 min, 56°C for 2 min and 70°C for 2 min. A final 5 min extension step at 72°C was added. DOP-PCR generates on an agarose gel a smear of DNA fragments between 200 and 500 bp.

The probe was PCR labeled with biotin-16-dUTP (Roche, USA) in 28 DOP-PCR cycles using the same template and primer of the previous high stringency cycles but with the substitution of 0.1 mM dTTP to 0.1 mM biotin-16-dUTP, or labeled by nick translation with the DIG-Nick translation mix (Roche, USA) carried out according to the manufacturer's protocol. In addition we used Human COT-1 DNA (Invitrogen, USA) to prevent non-specific signals [Rubtsov et al., 1996].

Slides with metaphase spreads of *Brachyteles arachnoides*, *Lagothrix lagothricha*, *Ateles belzebuth marginatus*, *Alouatta caraya*, and *A. guariba clamitans* males were subjected to RNase (100 μg/ml, 1 h at 37°C), pepsin (0.005% pepsin in 10 mM HCl), and PBS pretreatments with 50 mM $MgCl_2$ for 5 min and with 50 M $MgCl_2$ and 1% formaldehyde for 10 min. The labeled DNA probe in hybridization mixture (10% dextran sulfate, 50% formamide and 2× SSC) was denatured and pre-hybridized. The slides were denatured, followed by dehydration and the hybridization was left for

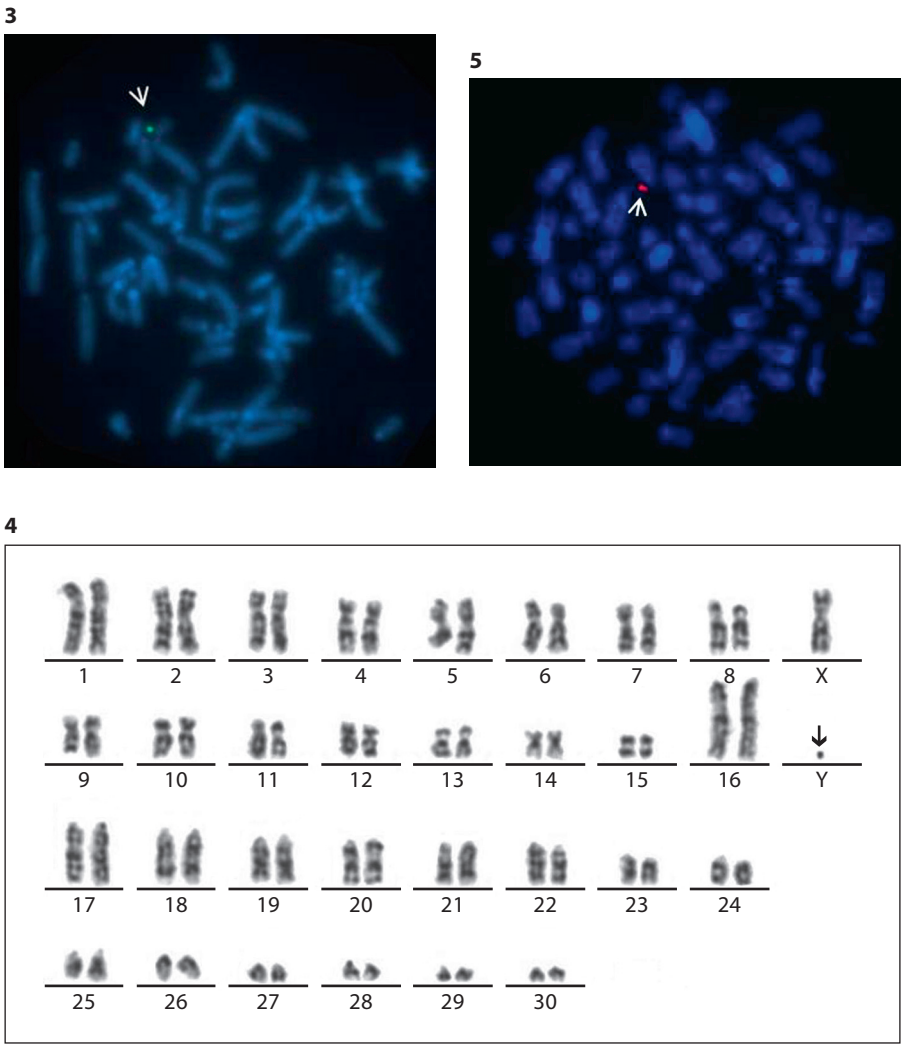


Fig. 3. In situ hybridization of *Brachyteles arachnoides* Y chromosome probe on *Brachyteles arachnoides* metaphase. Arrow indicates the Y chromosome.
Fig. 4. G-banded karyotype of *Lagothrix lagothricha*. Arrow indicates the small Y chromosome.
Fig. 5. In situ hybridization of *Brachyteles arachnoides* Y chromosome probe on *Lagothrix lagothricha* metaphase. Arrow indicates the Y chromosome.

6 days at 37°C. The signal detection for biotin was performed with Fluorescein Avidin (Vector, USA) and for digoxigenin with Anti-Digoxigenin-Rhodamine (Roche, USA). Chromosomes were counterstained in blue with DAPI (4′,6-diamidino-2-phenylindole) and Vectashield (Switzerland). An Axiophot 2 motorized microscope equipped with a CCD video camera (Zeiss, Germany) was used. Digital images were captured and further processed using the ISIS software (MetaSystems, Germany).

Results

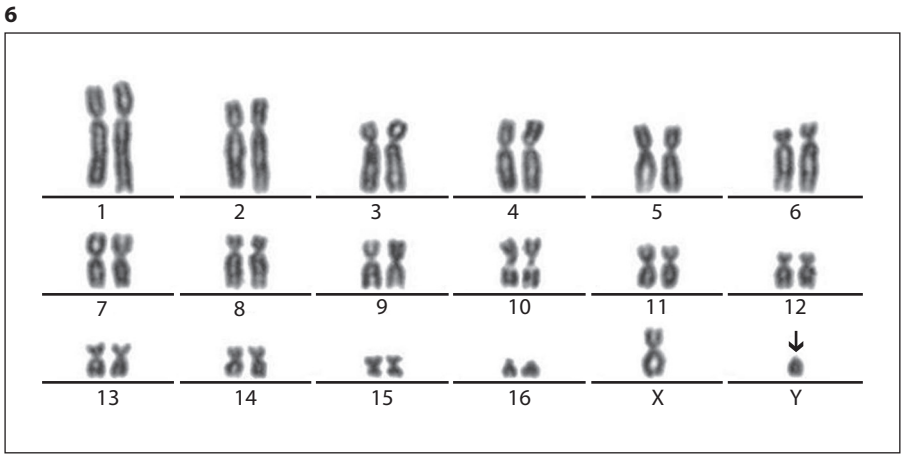
The *Brachyteles arachnoides* male specimen showed 2n = 62 with 24 biarmed and 36 acrocentric autosomes, a submetacentric X chromosome and a very small Y chromosome (fig. 2).

The *Brachyteles arachnoides* Y chromosome probe, generated by chromosome microdissection, was hybridized to metaphases of the same specimen to confirm the efficiency of the chromosome microdissection technique. A bright hybridization signal was observed only on the tiny chromosome (fig. 3).

The *Brachyteles arachnoides* Y chromosome probe was also hybridized to metaphases of *Lagothrix lagothricha*, *Ateles belzebuth marginatus*, *Alouatta caraya*, and *A. guariba clamitans* male specimens.

Lagothrix lagothricha presented a diploid number of 62 with 30 biarmed and 30 acrocentric autosomes, a submetacentric X chromosome and a very small Y chromosome (fig. 4).

The *Brachyteles arachnoides* Y chromosome probe hybridized to *Lagothrix lagothricha* metaphases showed one hybridization signal only on the tiny chromosome (fig. 5).



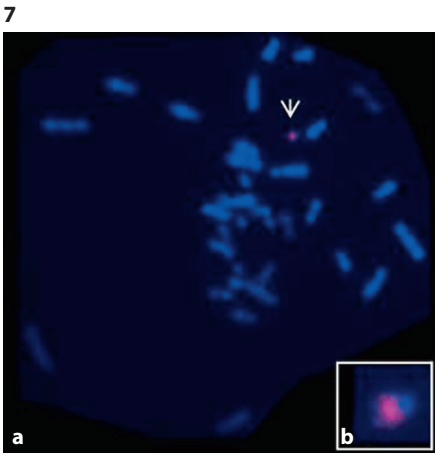


Fig. 6. Conventional karyotype of *Ateles belzebuth marginatus*. Arrow indicates the Y chromosome.
Fig. 7. *Brachyteles arachnoides* Y chromosome probe hybridization on *A. belzebuth marginatus* metaphase. In situ hybridization (**a**) and the *Ateles belzebuth marginatus* Y chromosome in detail (**b**). Arrow indicates the Y chromosome.

The *Ateles belzebuth marginatus* male specimen showed 2n = 34 with 30 biarmed and 2 acrocentric autosomes, a submetacentric X chromosome and a small acrocentric Y chromosome (fig. 6).

The *Brachyteles arachnoides* Y chromosome probe hybridized to *Ateles belzebuth marginatus* metaphases showed one hybridization signal on two thirds of one small acrocentric chromosome (fig. 7).

The *Alouatta caraya* male specimen presented a diploid number of 52 with 19 biarmed and 31 acrocentric autosomes, a submetacentric X chromosome and an acrocentric Y chromosome (fig. 8).

The *Alouatta guariba clamitans* male specimen showed 2n = 49 with 19 biarmed and 29 acrocentric autosomes, a submetacentric X chromosome and an apparent absence of the Y chromosome due to a Y-autosome translocation (fig. 9).

Despite several attempts, no hybridization signals were observed in *Alouatta caraya* and *A. guariba clamitans* metaphases with the *Brachyteles arachnoides* Y chromosome probe (data not shown).

Discussion

In general, the Y chromosome in Platyrrhini is highly variable in size and morphology and the diploid chromosome numbers in males of some species decrease due to a Y-autosome translocation, originating the multiple sex chromosome systems [Mudry et al., 2001]. There is also a considerable variation of Y gene content between species, although they overlap and all contain *SRY* plus several genes known to be required for spermatogenesis [Waters et al., 2007]. The very small Y chromosome observed in *Brachyteles arachnoides* and *Lagothrix lagothricha* must have a high degree of specialization in sexual differentiation because it would need to contain all genetic information required for male sexual differentiation.

Recent modifications from the original protocols have made the microdissection procedure applicable for molecular cytogenetics and comparative genomic research. The possibility of developing paints from few or even single microdissected chromosomes is promising and this method is very effective to generate probes that are difficult to sort due to the small size of chromosomes. Although few probes developed using microdissection have revealed homologies between species from distantly related taxa, they appear to be more suited to compare closely related species groups.

Our *Brachyteles arachnoides* Y chromosome probe hybridized to the Y chromosome in *Ateles* and *Lagothrix*. However, a hybridization signal was not observed in *Alouatta* metaphases (subfamily Alouattinae) that along with the Atelinae genera comprise the family *Atelidae*.

As described above, there is no consensus concerning phylogenetic relationships between Atelidae genera (fig. 1). Our data support the phylogenetic relationships proposed by Ruiz-García and Álvarez [2003] and Nieves et al. [2005] on the bases of DNA analysis evidence. The

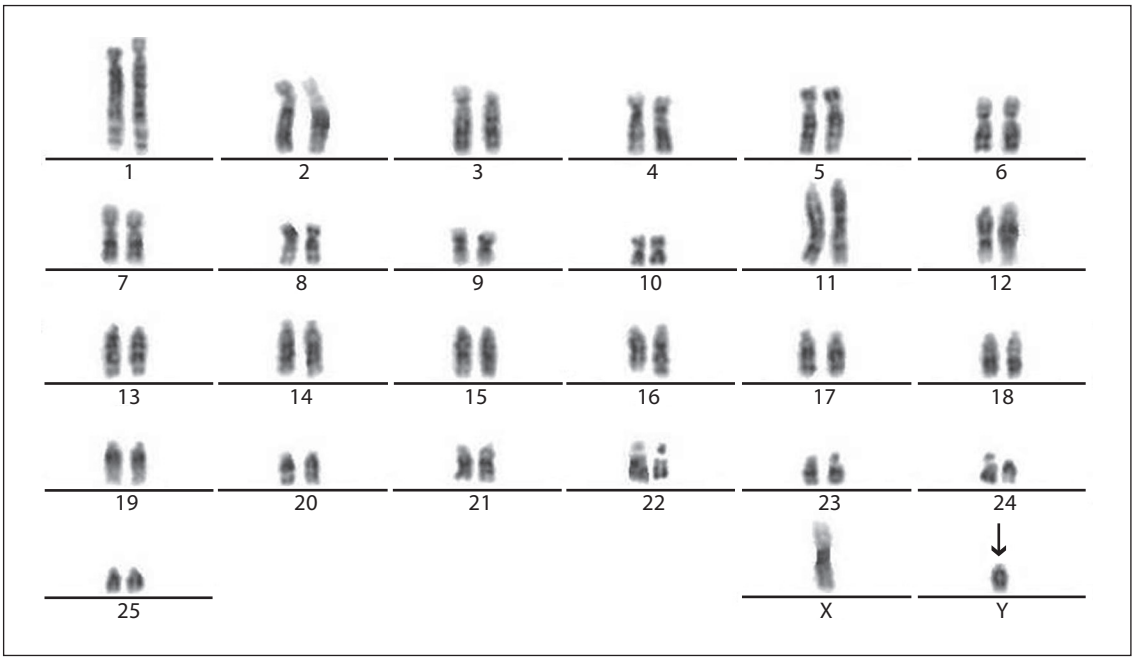


Fig. 8. G-banded karyotype of *Alouatta caraya*. Arrow indicates the Y chromosome.

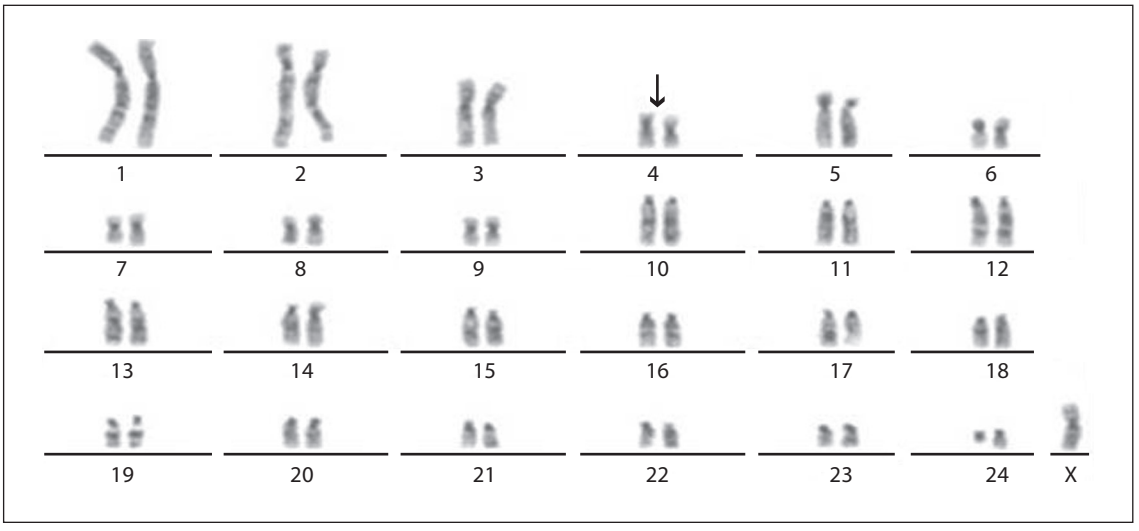


Fig. 9. G-banded karyotype of *Alouatta guariba clamitans*. The arrow points to a heteromorphic pair that is the carrier of the Y-autosome translocation.

Y chromosomes in *Ateles*, *Lagothrix* and *Brachyteles* share a common history. The *Ateles* Y chromosome appears to contain additional genes as a consequence of the extensive chromosome rearrangements experienced by this genus leading to the chromosome number reduction to 34. Furthermore, our data support a close phylogenetic relationship among these 3 genera and their placement in the subfamily Atelinae, and the placement of *Alouatta* outside the Atelinae group indicating its placement as basal to this group.

Acknowledgements

We would like to thank Eppendorf do Brasil application specialist Dr. Ana Paula de Andrade Aukar, Dr. Eduardo Gorab (Universidade de São Paulo) for the permission to use facilities and equipment, Frederico Henning and Nathália Fernandes de Azevedo (Universidade de São Paulo) for general help and advice, Carlos Yamashita, Dr. Daniel Antônio Salati Marcondes and Dr. Sílvio Marsaru Ichihara (*Alouatta caraya*), Zoológico Municipal Quinzinho de Barros (Sorocaba, Brazil) and CEMA – Centro de Estudo e Manejo de Animais Silvestres, Fundação Florestal (São Paulo, Brazil) for providing blood sample, and we really appreciate the critical review of Dr. Eugene E. Harris.

This research was supported by grants from FAPESP, CEPID-FAPESP and CNPq.

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