

# Chapter 4

## Cytogenetics of Howler Monkeys

Marta D. Mudry, Mariela Nieves, and Eliana R. Steinberg

**Abstract** Cytogenetic studies of howler monkeys show diploid numbers ranging from  $2N=43$  in *Alouatta seniculus* to  $2N=58$  in *A. pigra* with several interspecific chromosomal rearrangements such as translocations and inversions. Other remarkable genetic features are the multiple sex chromosome systems and the presence of microchromosomes. Multiple sexual systems are originated by Y-autosome translocations, resulting in the formation of trivalents  $X_1X_2Y$  in males of *A. belzebul* and *A. palliata* and quadrivalents  $X_1X_2Y_1Y_2$  in males of *A. seniculus*, *A. pigra*, *A. macconnelli*, and *A. caraya*. Fluorescence *in situ* hybridization (FISH) analyses in the South American species have revealed that segments with homeology to human chromosomes #3 and #15 (synteny 3/15) are involved in these sexual systems. Different authors agreed with the assumption that these diverse sex chromosome systems share the same autosomal pair and the rearrangement may have occurred once. Recent cytogenetic characterization of *A. pigra* and *A. palliata* has shown that the autosomes involved in the translocation that formed the sex chromosome systems in the Mesoamerican and South American species are different. Two independent events of Y-autosome translocations might have led to different sexual systems. Together with the multiple autosomal rearrangements found in the genus, the howler monkey's sex chromosome systems constitute an illustrative example of the possible chromosomal evolutionary mechanisms in Platyrrhini.

**Resumen** Los estudios citogenéticos realizados en monos aulladores muestran números diploides que van de  $2N=43$  para *Alouatta seniculus* a  $2N=58$  para *A. pigra*, con reordenamientos cromosómicos interespecíficos de tipo translocaciones e inversiones. Otras características genéticas notables son los sistemas sexuales múltiples y la presencia de microcromosomas. Los sistemas sexuales múltiples son resultado de translocaciones Y-autosoma, originando trivalentes  $X_1X_2Y$  en los machos de *A. belzebul* y *A. palliata* y cuadrivalentes  $X_1X_2Y_1Y_2$  en los machos de

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M.D. Mudry (✉) • M. Nieves • E.R. Steinberg

Grupo de Investigación en Biología Evolutiva (GIBE), Labs 46/43, 4° piso, Depto. de Ecología, Genética y Evolución, IEGEBA, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pab II. Ciudad Universitaria, Ciudad Autónoma de Buenos Aires, Argentina

Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET),  
Ciudad Autónoma de Buenos Aires, Argentina

e-mail: [martamudry@yahoo.com.ar](mailto:martamudry@yahoo.com.ar); [mnieves@ege.fcen.uba.ar](mailto:mnieves@ege.fcen.uba.ar); [steinberg@ege.fcen.uba.ar](mailto:steinberg@ege.fcen.uba.ar)

*A. seniculus*, *A. pigra*, *A. macconnelli* y *A. caraya*. La Hibridación in situ Fluorescente (FISH) ha revelado que en la formación de los sistemas sexuales de las especies sudamericanas están involucrados ciertos segmentos homeólogos de los cromosomas humanos #3 y #15 (sintenia 3/15). Diferentes autores están de acuerdo con la hipótesis de que estos sistemas de cromosomas sexuales comparten un mismo par autosómico y que el reordenamiento que les dio lugar habría ocurrido una única vez. Una caracterización citogenética reciente de *A. pigra* y *A. palliata* puso en evidencia que diferentes cromosomas están involucrados en los sistemas sexuales de las especies mesoamericanas y sudamericanas. Dos eventos independientes de translocaciones Y-autosoma parecerían haber originado los diferentes sistemas sexuales. Junto con los múltiples reordenamientos autosómicos que se observan en el género, los sistemas de cromosomas sexuales presentes en aulladores son un ejemplo ilustrativo de los posibles mecanismos de evolución cromosómica en Platyrrhini.

**Keywords** Karyosystematics • Multiple sex chromosome systems • Cytogenetics • Chromosomal synteny

## Abbreviations

FISH	Fluorescence in situ hybridization
G-banding	Giemsa banding
NWP	New World Primates
PAR	Pseudoautosomal region
R-banding	Reverse banding
REs banding	Restriction enzymes banding
SC	Synaptonemal complex

## 4.1 Introduction

Karyosystematics, or the study of the natural relationships of species using the information provided by chromosomes, can provide valuable information for taxonomic classifications and evolutionary analyses. In primates, during the last three decades, researchers have proposed chromosomal speciation as a probable evolutionary mechanism to interpret diversity of living primates (De Grouchy et al. 1972; Dutrillaux et al. 1975, 1980; Seuánez 1979; Dutrillaux and Couturier 1981; De Grouchy 1987; Clemente et al. 1990). More recently, chromosomal data began to be used as phylogenetic markers, since they are inherited as Mendelian characters and are conserved within species (Sankoff 2003; Dobigny et al. 2004; Stanyon et al. 2008). Following the maximum parsimony criterion, karyological comparisons allow the identification of chromosomal forms shared by common ancestry (Dobigny et al. 2004).

In order to obtain better taxonomic inferences, a wide battery of variables including genetic, morphological, and ecological should be employed into the

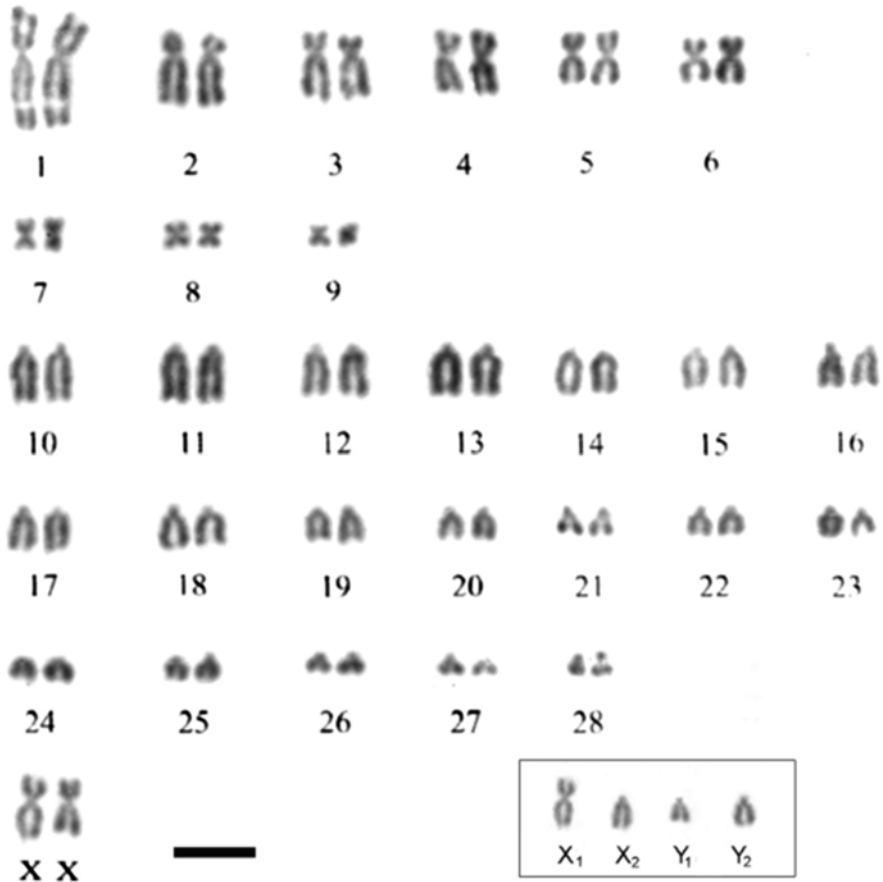
theoretical framework referred to as “Total Evidence” (Kluge 1989). The systematics of New World Primates (NWP) is still under discussion (Rylands 2000; Groves 2001, 2005; Schneider et al. 2001; Rylands and Mittermeier 2009; Perelman et al. 2011; Rylands et al. 2012), and the genus *Alouatta* is not the exception (Cabrera 1957; Hill 1962; Groves 2001; Gregorin 2006; Rylands and Mittermeier 2009; Rylands et al. 2012; Cortés-Ortiz et al. 2014). In this context, cytogenetic studies have become an important tool complementing molecular and morphological data traditionally used in systematic studies.

## 4.2 Karyological Features of the Genus *Alouatta*

### 4.2.1 Classical Cytogenetic Analysis

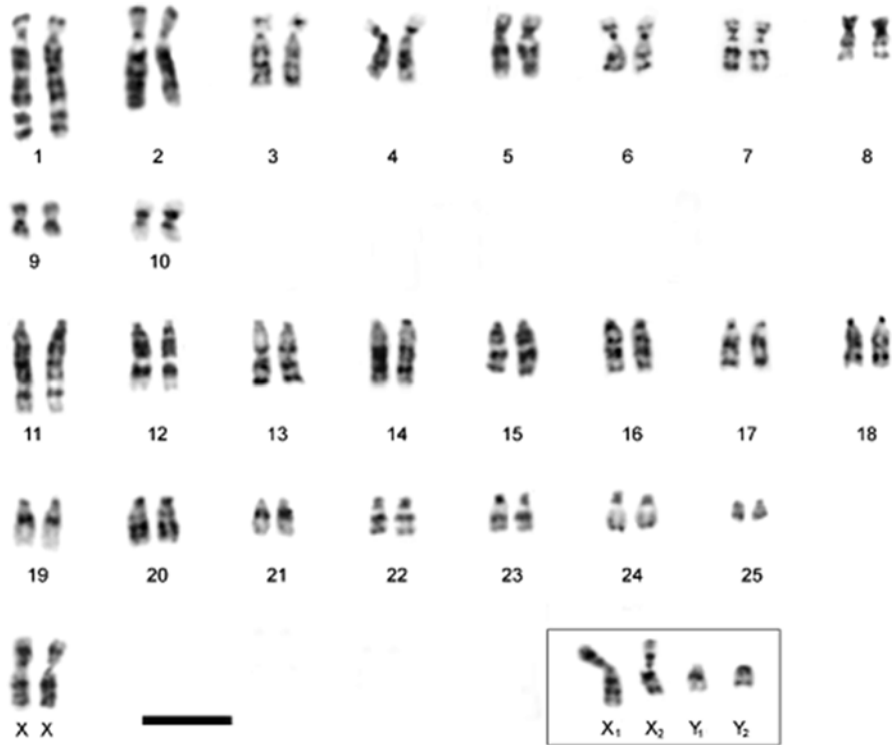
Since the first cytogenetic characterization of the red howler monkey, *Alouatta seniculus* (Bender and Chu 1963), the karyological features of the genus have attracted the scientists’ attention, due to the multiple interspecific autosomal rearrangements and, particularly, for the multiple sex chromosome systems observed. The first cytogenetic studies employed standard staining techniques (Fig. 4.1), established the diploid numbers, and allowed to arrange the chromosomes by size and morphology (*A. seniculus*: Bender and Chu 1963; *A. palliata*: Hsu and Benirschke 1970; *A. caraya*: Egozcue and de Egozcue 1965, 1966). The advent of the chromosome banding techniques in the 1970s (Giemsa banding or G-banding, reverse banding or R-banding, and restriction enzyme banding or REs banding) made it possible to reveal a characteristic pattern of specific dark and light bands along each chromosome (Fig. 4.2).

Using these differential banding techniques, homologous chromosomes, chromosome segments, and chromosomal rearrangements could be identified with precision in each karyotype, thus allowing the first interspecific comparisons (Koiffmann and Saldanha 1974; Yunis et al. 1976; Mudry et al. 1981, 1984, 1994; Minezawa et al. 1985; Armada et al. 1987; Lima and Seuánez 1991; Stanyon et al. 1995; Rahn et al. 1996; Vassart et al. 1996; Steinberg et al. 2008). Cytogenetic analyses using these staining techniques in somatic cells showed high chromosomal variability with drastic differences in the chromosome number among species (Table 4.1). Howler monkeys exhibit diploid numbers (2N) ranging from 2N=43 in *A. seniculus* to 2N=58 in *A. pigra*. Several interspecific chromosomal rearrangements such as translocations and inversions have been described (De Boer 1974; Mudry et al. 1994; Consigliere et al. 1996, 1998; de Oliveira et al. 2002). This interspecific chromosomal variation coincides with the one observed in the night monkey, genus *Aotus* (Ma 1981; Torres et al. 1998; Ruiz Herrera et al. 2005; Defler and Bueno 2007), but contrasts with other platyrrhine genera such as the squirrel monkeys, genus *Saimiri* (Hershkovitz 1984; Moore et al. 1990), or the capuchin monkeys, genus *Cebus* (Matayoshi et al. 1987; Mudry et al. 1987; Ponsà et al. 1995), in which interspecific variation in 2N is not observed (species show constant diploid numbers).



**Fig. 4.1** Female standard karyotype of *Alouatta pigra*. Chromosome pairs were numbered consecutively following decreasing size within each type of morphology. *Inset*: sex chromosome system observed in the male *Alouatta pigra*. Bar = 10  $\mu$ m

*Alouatta* chromosomal variability is not restricted to differences among species. A few intraspecific polymorphisms have been described in *A. caraya*, but other species, such as *A. guariba*, show multiple intraspecific rearrangements involving differences in diploid number and sexual systems (see Table 4.1). This high intraspecific variability suggests that these may be species complexes rather than single species (Stanyon et al. 1995; Consigliere et al. 1996, 1998; de Oliveira et al. 2000). Within *Alouatta*, karyological studies have contributed to the taxonomic reassessment of several taxa previously considered as subspecies, elevating them to the species level: *A. nigerrima*, previously *A. seniculus nigerrima*; *A. macconnelli*, previously *A. seniculus macconnelli*; *A. stramineus*, previously *A. seniculus stramineus* (here considered within *A. macconnelli*); and *A. belzebul*, previously *A. seniculus belzebul* (Lima and Seuánez 1991; de Oliveira 1996).



**Fig. 4.2** G-banded karyotype of *Alouatta caraya* female. *Inset*: sex chromosome system observed *Alouatta caraya* males. Bar = 10  $\mu$ m

#### 4.2.1.1 Heterochromatin

Traditionally, the term heterochromatin was used to denote chromosomal regions of the karyotype showing increased condensation (Heitz 1928), revealed as regions of intense staining (denoted as C+ bands) (Fig. 4.3) by C-banding (Sumner 1971). Later, cytomolecular techniques showed that these regions are constituted by distinct medium and highly repetitive DNA sequences (Copenhaver et al. 1999; Fransz et al. 2000; Avramova 2002). In Platyrrhini, heterochromatic regions are highly variable in quantity, quality, and location. C-banding techniques and restriction enzymes digestion have demonstrated that there are different kinds of heterochromatin, and particularly among platyrrhine species, a great variability was described, not only in sequence types but also in their location (centromeric, interstitial, and telomeric blocks) (Matayoshi et al. 1987; Mudry de Pargament and Labal de Vinuesa 1988; Pieczarka et al. 2001; García et al. 2003). Compared with other platyrrhine genera, like *Saimiri*, *Cebus*, and *Aotus*, that show big C+ blocks of extracentromeric heterochromatin,

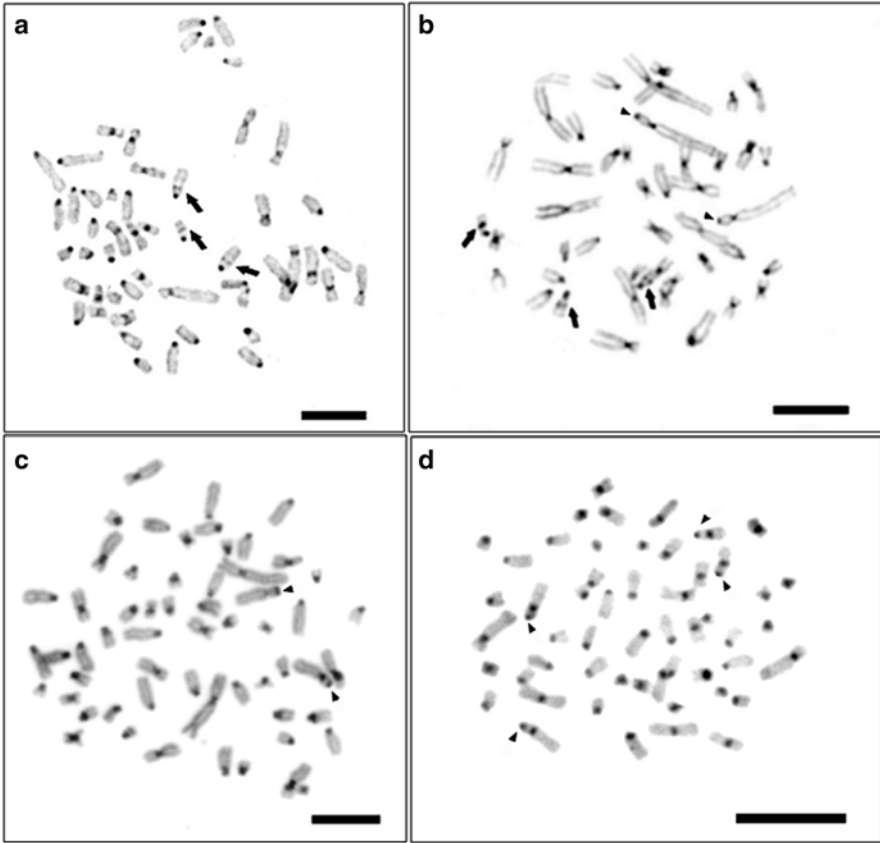
Table 4.1 Cytogenetic data published for howler monkeys

Species	2N		Autosomal complement in males		Sexual system (female/male)	References
	♀	♂	#NA	#A		
<i>A. caraya</i>	52	52	20	30	XX/XY	Egozcue and de Egozcue (1965) (specimens from Argentina)
<i>A. caraya</i>	–	52	20	30	XX/XY	Egozcue and de Egozcue (1966) (specimens from Argentina)
<i>A. caraya</i>	52	52	20	30	XX/XY	Mudry et al. (1981, 1990), Mudry de Pargament et al. (1984) (specimens from Argentina)
<i>A. caraya</i>	52	52	18	30	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub> / X <sub>1</sub> X <sub>2</sub> Y <sub>1</sub> Y <sub>2</sub>	Rahn et al. (1996), Mudry et al. (1998, 2001) (specimens from Argentina)
<i>A. guariba clamitans</i>	50	50	20	28	XX/XY	Koiffmann and Saldanha (1974) (São Paulo, Brazil)
<i>A. guariba clamitans</i>	50	49	20	27	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub> /X <sub>1</sub> X <sub>2</sub> Y	Koiffmann and Saldanha (1981) (São Paulo, Brazil)
<i>A. guariba clamitans</i>	46	45	22	20	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub> /X <sub>1</sub> X <sub>2</sub> Y	de Oliveira et al. (1995) (specimens from Parana and Santa Catarina, south of Brazil)
<i>A. guariba clamitans</i>	–	52	22	28	XY	de Oliveira et al. (1995, 2000) (one male from Espírito Santo, Brazil)
<i>A. guariba guariba</i>	–	49	14	32	X <sub>1</sub> X <sub>2</sub> Y	de Oliveira et al. (1998) (Rio de Janeiro, Brazil)
<i>A. guariba clamitans</i>	–	45	22	18	X <sub>1</sub> X <sub>2</sub> X <sub>3</sub> Y <sub>1</sub> Y <sub>2</sub>	de Oliveira et al. (2002) (in captivity in Parana, Brazil)
<i>A. guariba guariba</i>	50	49	20	24	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub> X <sub>3</sub> X <sub>3</sub> / X <sub>1</sub> X <sub>2</sub> X <sub>3</sub> Y <sub>1</sub> Y <sub>2</sub>	de Oliveira et al. (2002) (in captivity in Minas Gerais, Brazil)
<i>A. nigerrima</i>	50	–	18	30	XX	Armada et al. (1987) (geographic origin unknown)
<i>A. villosa</i> (= <i>A. palliata</i> )	54	–	22	30	XX	Hsu and Benirschke (1970) (one female from Guatemala)
<i>A. palliata</i>	54	53	20	30	X <sub>1</sub> X <sub>1</sub> X <sub>2</sub> X <sub>2</sub> /X <sub>1</sub> X <sub>2</sub> Y	Ma et al. (1975) (specimens from Panama)
<i>A. palliata</i>	56	56	20	34	XX/XY	Torres and Ramirez (2003) (specimens from Colombia)
<i>A. palliata</i>	–	53	22	28	X <sub>1</sub> X <sub>2</sub> Y	Solari and Rahn (2005) (specimens from Mexico)

<i>A. pigra</i>	58	58	18	36	$X_1X_1X_2X_2/$ $X_1X_2Y_1Y_2$	Steinberg et al. (2008) (specimens from Mexico)
<i>A. seniculus</i>	44	44	12	30	XX/XY	Bender and Chu (1963) (specimens from Colombia)
<i>A. seniculus</i> (previously considered <i>A. s. seniculus</i> by the author)	43–45 <sup>a</sup>	43–45 <sup>a</sup>	12	26	XX/XY	Yumis et al. (1976) (specimens from Colombia)
<i>A. macconnelli</i> (previously considered <i>A. seniculus</i> by the author)	47–49 <sup>a</sup>	47–49 <sup>a</sup>	18	24	$X_1X_1X_2X_2/$ $X_1X_2Y_1Y_2$	Vassart et al. (1996) (specimens from French Guiana)
<i>A. seniculus</i>	43–45 <sup>a</sup>	43–45 <sup>a</sup>	12	26	XX/XY	Torres and Leibovici (2001) (specimens from Colombia)
<i>A. macconnelli</i> (previously considered <i>A. s. stramineus</i> by the author)	47–49 <sup>a</sup>	47–49 <sup>a</sup>	20	22	$X_1X_1X_2X_2/$ $X_1X_2Y_1Y_2$	Lima and Seuánez (1991) (specimens from Brazil)
<i>A. arctoidea</i> (previously considered <i>A. s. arctoidea</i> by the author)	44	45	10	30	$X_1X_1X_2X_2/$ $X_1X_2Y_1Y_2$	Stanyon et al. (1995) (specimens from Venezuela; variation in diploid number between sexes is due to microchromosome number)
<i>A. sara</i> (previously considered <i>A. s. sara</i> by the author)	48–51 <sup>a</sup>	48–51 <sup>a</sup>	16	24	$X_1X_1X_2X_2/X_1X_2Y$	Minezawa et al. (1985) (specimens from Bolivia)
<i>A. sara</i> (previously considered <i>A. s. sara</i> by the author)	–	46+4m	14	28	$X_1X_2Y_1Y_2$	Stanyon et al. (1995) (specimens from Bolivia)
<i>A. macconnelli</i> (previously considered <i>A. seniculus</i> by the author)	47–49 <sup>a</sup>	47–49 <sup>a</sup>	20	22	$X_1X_1X_2X_2/$ $X_1X_2Y_1Y_2$	Lima et al. (1990) (specimens from Brazil)
<i>A. belzebul</i>	50	49	22	24	$X_1X_1X_2X_2/X_2Y$	Armada et al. (1987) (specimens from Brazil)

NA non-acrocentric chromosomes, A acrocentric chromosomes, *m* microchromosomes

<sup>a</sup>Differences are due to the presence of microchromosomes



**Fig. 4.3** C-banded metaphases of male howler monkeys. The *arrows* indicate the interstitial C+ bands. *Single arrowheads* indicate telomeric C+ bands. Bar = 10  $\mu\text{m}$ . (a) *Alouatta caraya* 2N=52, X<sub>1</sub>X<sub>2</sub>Y<sub>1</sub>Y<sub>2</sub>, (b) *A. guariba* 2N=45, X<sub>1</sub>X<sub>2</sub>X<sub>3</sub>Y<sub>1</sub>Y<sub>2</sub>, (c) *A. pigra* 2N=58, X<sub>1</sub>X<sub>2</sub>Y<sub>1</sub>Y<sub>2</sub>, and (d) *A. palliata* 2N=53, X<sub>1</sub>X<sub>2</sub>Y

*Alouatta* is the genus with the lowest heterochromatin proportion (Ma et al. 1976; García et al. 1979; 1983; Matayoshi et al. 1987; Mudry de Pargament and Slavutsky 1987; Mudry de Pargament et al. 1984; Mudry 1990; Mudry et al. 1990; 1991; Ponsà et al. 1995; Rahn et al. 1996; Nieves et al. 2005a; Nieves 2007). Within howler monkeys, *A. seniculus* group shows the lowest C+ heterochromatin proportion, with centromeric location only (Lima et al. 1990); meanwhile *A. guariba clamitans* (de Oliveira et al. 1998) and *A. palliata* (Ma et al. 1975) show the highest heterochromatin content within the genus, with centromeric, interstitial, and telomeric C+ blocks (Table 4.2).



**Table 4.2** Heterochromatin location in somatic cells of howler monkeys revealed by C-banding technique

Species	C-bands location	References
<i>A. caraya</i>	C+ Cent in all chromosomal pairs C+ Int in pairs #16 and #21	Mudry et al. (1984, 1994)
<i>A. guariba clamitans</i>	C+ Cent in all pairs; C+ Int in pair #17 C+ Tel in pair #2; 5p and 6p heterochromatic	de Oliveira et al. (1998)
<i>A. guariba clamitans</i>	C+ Cent in all pairs; C+ Int in pairs #16, #17 C+ Tel in pair #2	Steinberg (2011)
<i>A. nigerrima</i>	C+ Cent in all pairs	Armada et al. (1987)
<i>A. pigra</i>	C+ Cent in all pairs C+ Tel in pair #2	Steinberg et al. (2008)
<i>A. palliata</i>	C+ Cent in all pairs C+ Tel in pairs #3 and #5	Steinberg (2011)
<i>A. palliata</i>	C+ Cent in all pairs; C+ Int in pairs A1, A5, B19, B25 and C+ Tel in pairs A3, A6	Ma et al. (1975)
<i>A. macconnelli</i> (previously considered <i>A. s. stramineus</i> by the author)	C+ Cent in all pairs Microchromosomes C-negative	Yunis et al. (1976)
<i>A. macconnelli</i> (previously considered <i>A. s. stramineus</i> by the author)	C+ Cent in all pairs, except in 2 biarmed ones; C+ band polymorphism in #19; Microchromosomes C+ positive	Lima and Seuáñez (1991)
<i>A. macconnelli</i> (previously considered <i>A. seniculus</i> by the author)	C+ Cent in all pairs, except in #4 and #8 9q heterochromatic Microchromosomes C+ positive	Vassart et al. (1996)
<i>A. sara</i> (previously considered <i>A. s. sara</i> by the author)	C+ Cent in all pairs Microchromosomes C+ positive and C-negative	Minezawa et al. (1985)
<i>A. seniculus</i>	C+ Cent in all pairs Microchromosomes C+ positive and C-negative	Torres and Leibovici (2001)
<i>A. macconnelli</i> (previously considered <i>A. seniculus</i> by the author)	C+ Cent in all pairs, except in #4 and #8 Microchromosomes C+ positive	Lima et al. (1990)
<i>A. belzebul belzebul</i>	C+ Cent in all pairs; C+ Int in 2 pairs; C+ Tel in 2 pairs	Armada et al. (1987)

C+ Cent=C+ Centromeric bands, C+ Tel=C+ Telomeric bands, C+ Int=C+ Interstitial bands

#### 4.2.1.2 Microchromosomes

Microchromosomes are supernumerary chromosomes and their presence is unusual among Primates. Some authors denominate these chromosomes as “B chromosomes” in contrast to the chromosome complement, generally denominated as “A chromosomes”. Their name is derived from the fact that many of these

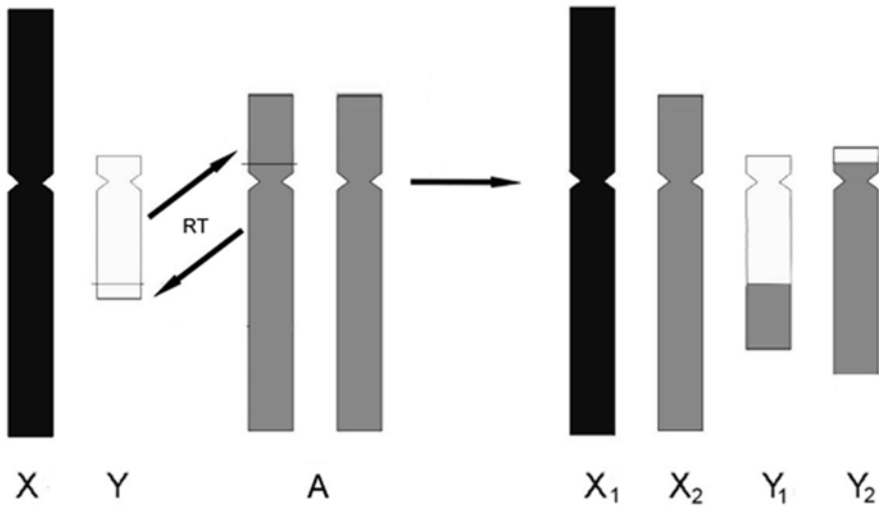
supernumerary chromosomes are smaller than the smallest A chromosomes (reviewed in Vujošević and Blagojević 2004). In Platyrrhini, *A. seniculus* (Yunis et al. 1976; Torres and Leibovici 2001), *A. sara* (Minezawa et al. 1985), and *A. macconnelli* (Lima et al. 1990; Vassart et al. 1996) exhibit this kind of accessory chromosomes. The structure of microchromosomes has mostly been characterized by C-banding technique (Arrighi and Hsu 1971; Sumner 1971). These minute chromosomes have been reported as small segments of heterochromatin (Patton 1977). However, in howler monkeys, there are reports of microchromosomes being either C-band negative (C-) such as in *A. seniculus* (Yunis et al. 1976) or C-band positive (C+) like in *A. macconnelli* (previously *A. stramineus*, Lima and Seuáñez 1991; Lima et al. 1990; Vassart et al. 1996). In *A. sara*, some microchromosomes are C-positive and others are C-negative, and there is no formal hypothesis about this particularity (Minezawa et al. 1985; Torres and Leibovici 2001).

In some howler monkey species, there is variation in microchromosome number between sexes and between individuals (Yunis et al. 1976; Minezawa et al. 1985; Lima et al. 1990; Lima and Seuáñez 1991; Vassart et al. 1996). Battaglia (1964) suggested that variation in microchromosome number in mammals could affect the frequency of chiasmata as well as growth, viability, and fertility. Little is known about the meiotic behavior of these microchromosomes, since few analyses in germ cells have been performed. More studies need to be developed in order to elucidate the nature and transmission of these supernumerary chromosomes in howler monkeys.

#### 4.2.1.3 Sex Chromosomes

In mammals, and particularly in Primates, the most frequently described sexual system is the XX/XY (reviewed in Solari 1993). As variants of the ancestral male XY sexual system, Y-autosome translocations that generate multiple sex chromosome systems in males (Fig. 4.4) have been described in Platyrrhini (Hsu and Hampton 1970; Ma et al. 1975; Dutrillaux et al. 1981; Armada et al. 1987; Lima and Seuáñez 1991; Rahn et al. 1996; Mudry et al. 1998, 2001; Solari and Rahn 2005; Steinberg et al. 2008). Only the karyological study of germ cells (meiotic analysis) allows the identification and confirmation of these sexual systems; however, meiotic studies are remarkably scarce.

Meiotic karyotypes of platyrrhines have only been described for a small number of species, confirming the sex determination XX/XY in *Cebus libidinosus* (formerly *C. apella paraguayanus*; Seuáñez et al. 1983; Mudry et al. 2001), *Ateles geoffroyi* and *Ateles paniscus* (Mudry et al. 2001; Nieves et al. 2005b), and *Saimiri boliviensis boliviensis* (Egozcue 1969; Steinberg et al. 2007) and multiple sex chromosome systems in *Aotus azarae* (Ma et al. 1976), *Callimico* sp. (Hsu and Hampton 1970), *Cacajao* sp. (Dutrillaux et al. 1981), and five species of *Alouatta* (Armada et al. 1987; Lima and Seuáñez 1991; Rahn et al. 1996; Mudry et al. 1998, 2001; Solari and Rahn 2005; Steinberg et al. 2008). All meiotic studies of howler monkeys were performed using testes biopsies and confirmed two types of multiple sex chromosome systems (Table 4.3): (1)  $X_1X_2Y_1Y_2$  (which forms a chain of four elements or quadrivalent at Metaphase I) in *A. macconnelli* (Lima and Seuáñez 1991), *A. caraya*



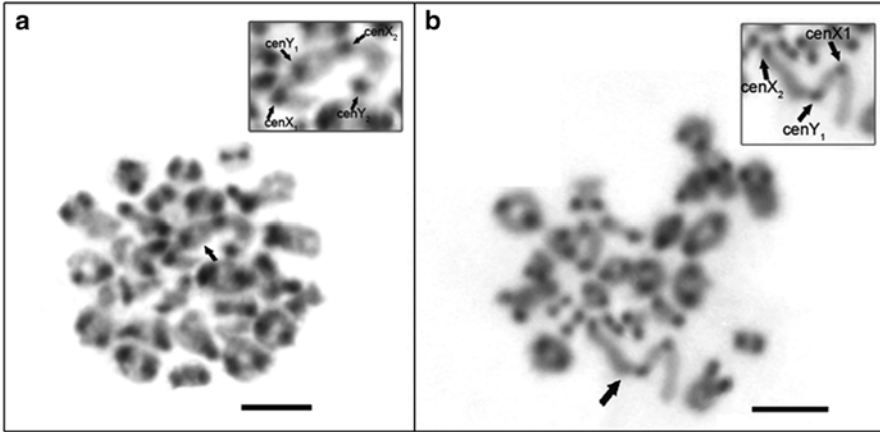
**Fig. 4.4** Hypothesis on the origin of the sexual system X<sub>1</sub>X<sub>2</sub>Y<sub>1</sub>Y<sub>2</sub> in mammalian males. The ancestral X is shown in *black*, the ancestral Y in *white*, and the autosomal pair (A) involved in the translocation in *gray*. From an XY sexual system, two simultaneous breaks on the proximal region of the short arm (A<sub>p<sub>prox</sub></sub>) and the terminal region of the long arm (Y<sub>q<sub>ter</sub></sub>), followed by a reciprocal translocation (RT), give origin to the chromosomes Y<sub>1</sub> and Y<sub>2</sub>. The homologous chromosome of the autosomal pair not involved in the translocation became known as X<sub>2</sub>, and the ancestral X is now denominated X<sub>1</sub>

**Table 4.3** Confirmation of howler monkey sexual systems through meiotic studies in males

Species	2N (♂)	Autosomal complement in males		Confirmed sexual system	References
		#NA	#A		
<i>A. belzebul</i>	49	22	24	X <sub>1</sub> X <sub>2</sub> Y	Armada et al. (1987)
<i>A. macconnelli</i> (previously considered <i>A. s. stramineus</i> by the author)	47–49 <sup>a</sup>	20	22	X <sub>1</sub> X <sub>2</sub> Y <sub>1</sub> Y <sub>2</sub>	Lima and Seuánz (1991)
<i>A. caraya</i>	52	18	30	X <sub>1</sub> X <sub>2</sub> Y <sub>1</sub> Y <sub>2</sub>	Rahn et al. (1996), Mudry et al. (1998, 2001)
<i>A. palliata</i>	53	22	28	X <sub>1</sub> X <sub>2</sub> Y	Solari and Rahn (2005)
<i>A. pigra</i>	58	18	36	X <sub>1</sub> X <sub>2</sub> Y <sub>1</sub> Y <sub>2</sub>	Steinberg et al. (2008)

NA number of non-acrocentric chromosomes, A number of acrocentric chromosomes

<sup>a</sup>These differences are due to presence of microchromosomes



**Fig. 4.5** Howler monkey C-banded spermatocytes in Metaphase I showing the location of the C+ heterochromatic regions. This staining technique allows the identification of C+ centromeres, thus revealing the structure of the multivalents. Bar=5  $\mu\text{m}$ . **(a)** *A. caraya* spermatocyte. The arrow indicates the sexual  $X_1X_2Y_1Y_2$  (four centromeres C+). Inset: detail of the sexual quadrivalent. The arrows indicate the centromeres of each of the four chromosomal components. **(b)** *A. palliata* spermatocyte. The arrow indicates the sexual  $X_1X_2Y_1$  (three centromeres C+). Inset: detail of the sexual trivalent. The arrows indicate the centromeres of each of the three chromosomal components

(Fig. 4.5a) (Mudry et al. 1998, 2001), and *A. pigra* (Steinberg et al. 2008) and (2)  $X_1X_2Y$  (which forms a chain of three elements or trivalent at Metaphase I) in *A. belzebul* (Armada et al. 1987) and *A. palliata* (Fig. 4.5b) (Solari and Rahn 2005). The multivalent configurations observed in Metaphase I of howler monkey spermatocytes (Fig. 4.5) allow an alternate segregation in Anaphase I, ensuring a balanced gamete production and maintaining the fertility of the individual carriers.

Some mitotic studies of *Alouatta* described the presence of a typical XY male sexual system in *A. seniculus* (Yunis et al. 1976), *A. guariba clamitans* (Koiffmann and Saldanha 1974; de Oliveira et al. 1995, 2000), and *A. palliata* (Torres and Ramírez 2003), and other studies suggested the presence of a  $X_1X_2X_3Y_1Y_2$  sexual system (which would form a chain of five elements or pentavalent in Metaphase I) in *A. guariba guariba* and *A. g. clamitans* (de Oliveira et al. 2002), a  $X_1X_2Y$  system in *A. guariba guariba* (de Oliveira et al. 1998) and *A. sara* (Minezawa et al. 1985), and a  $X_1X_2Y_1Y_2$  system in *A. sara* (Consigliere et al. 1998). However, the occurrence of all these sex chromosome systems in *Alouatta* still awaits confirmation by meiotic analysis.

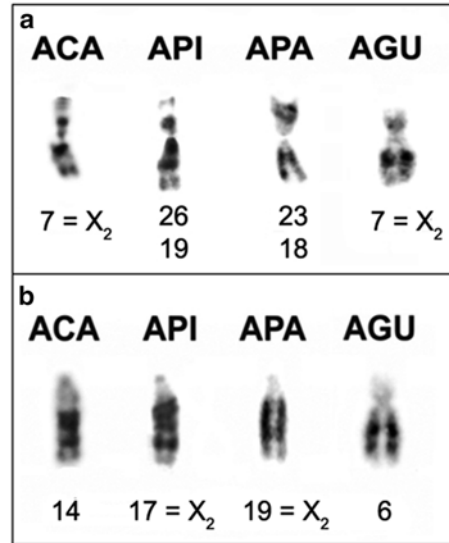
The meiotic behavior in early meiotic stages of the howler monkey sexual multivalents has been studied in just a few cases (Rahn et al. 1996; Mudry et al. 1998, 2001; Solari and Rahn 2005). The analysis of the sexual quadrivalent of *A. caraya*

at pachytene showed that the maximum extent of synapsis in  $Y_2$  is 51 % of the length of  $X_2$ , whereas the maximum extent of synapsis of  $Y_1$  with  $X_2$  is 42.9 %. The synaptonemal complex (SC) between the  $X_1$  axis and  $Y_1$  is the smallest pairing segment in the whole quadrivalent (Mudry et al. 1998). In the sexual trivalent of *A. palliata*, however, the long arms of  $X_2$  and  $Y_1$  are paired in almost all their length, and the short arm of  $Y_1$  forms the pseudoautosomal region (PAR) with  $X_{1p}$  (Solari and Rahn 2005). The end-to-end joining between the  $X_1$  and  $Y_1$  chromosomes is similar in both multivalents, although the  $Y_1$  is a much longer acrocentric in *A. palliata*. In both *A. caraya* and *A. palliata*, the  $X_1$  axis has the typical characteristics (branchings, tangling) that are common in spermatocytes at pachytene stages described for other mammalian X axis (Solari 1993), including other Neotropical primates with XX/XY sexual systems (Mudry et al. 2001).

To understand the mechanisms underlying chromosomal evolution and speciation in mammalian species, experimental descriptions of recombination maps are needed. Few studies using “in situ” immunolocalization of recombination proteins have been applied in nonhuman primates (Garcia-Cruz et al. 2009, 2011; Hassold et al. 2009). Only one of such studies has been carried out in howler monkeys (on spermatocytes of *A. caraya* by Garcia-Cruz et al. 2011), analyzing MLH1 foci, which correspond to recombination spots and are equivalent to the chiasmata observed in Metaphase I. The mean MLH1 foci number per autosomal set was  $40.6 \pm 4.3$  (standard deviation), with a range of 31–50 MHL1 foci per cell. This value is lower than the one observed for human males ( $49.8 \pm 4.3$ , Sun et al. 2004) but similar to those observed in *Cebus libidinosus* ( $41.3 \pm 4.8$ ), *C. nigrinus* ( $39.2 \pm 3.3$ ) (Garcia-Cruz et al. 2011), and *Macaca mulatta* ( $39.0 \pm 3.0$ ) (Hassold et al. 2009). The sexual quadrivalent formed a convoluted sex body, which folded back onto itself, not allowing for a correct visualization of the MLH1 foci. More studies are needed in order to understand the meiotic process in howler monkeys.

Classical cytogenetic analysis showed that the chromosomal pair involved in the sexual systems in Mesoamerican howler monkeys, *A. pigra* (API) and *A. palliata* (APA), share no homeology (see Sect. 4.2.2) with the pair involved in the South American species (Steinberg et al. 2008). Chromosomal pair API17 (denominated APIX<sub>2</sub> in males) is involved in *A. pigra*'s multiple sexual system, and APA19 (APA19 in females, APAX<sub>2</sub> in males) is involved in the multiple sexual system of *A. palliata*. These chromosomal pairs share homeology with *A. caraya* autosome 14 (ACA14) (Fig. 4.6). Therefore, the autosomal pair that is involved in the formation of the sexual systems in *A. pigra* and *A. palliata* is not homeologous with the one involved in the sexual system in *A. caraya* (known as ACA7 in females and ACAX<sub>2</sub> in males). ACA7 shares homeology with the autosomal pairs involved in the sexual systems of all South American howler monkey species studied so far (Rahn et al. 1996; Mudry et al. 1998, 2001): *A. seniculus* (Lima and Seuánez 1991), *A. belzebul* (Armada et al. 1987), *A. sara* (Minezawa et al. 1985), *A. guariba* (de Oliveira et al. 2002), and *A. macconnelli* (Lima et al. 1990). This suggests that the multiple sexual systems originated independently in South American and Mesoamerican howler monkeys.

**Fig. 4.6** Chromosome homeologies among *A. caraya* (ACA), *A. pigra* (API), *A. palliata* (APA), and *A. guariba* (AGU). **(a)** ACA7 ( $X_2$ ) shares homeology with API26, API19, APA23, APA18, and AGU7 ( $X_2$ ). **(b)** ACA14 shares homeology with API17 ( $X_2$ ), APA19 ( $X_2$ ), and AGU6



## 4.2.2 Cytomolecular Analysis

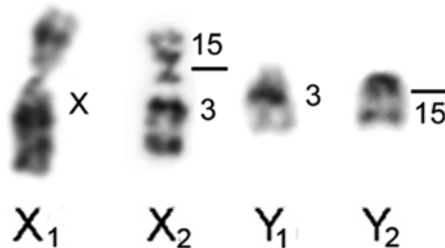
Homeologies at chromosomal level refer to the recognition of chromosome pairs carrying the same information among different organisms. Homologous chromosomes are defined as chromosome pairs of approximately the same length, centromere position, and staining pattern, with genes for the same characteristics at corresponding *loci*. One homologous chromosome is inherited from the organism's mother and the other from the organism's father. When we consider genetic information in different karyotypes of different organisms, we apply the term "homeolog" (Andersson et al. 1996), which becomes useful for phylogenetic analysis. The homeologies identified by G-banding pattern and employed for karyological comparisons are often not informative enough. This is the case when the homeologies involve complex chromosomal rearrangements, small translocated chromosomal fragments or highly rearranged karyotypes, such as *Alouatta*'s (Dobigny et al. 2004; Stanyon et al. 2008).

In the past 20 years, the Fluorescence in situ Hybridization (FISH) technique has proven to be a fast and reliable method to establish chromosomal homeologies between taxa. In this technique, labeled DNA probes specific for entire chromosomes or chromosome regions of a given species are used to hybridize to entire chromosome or chromosome segments in target metaphases (John et al. 1969; Pardue and Gall 1969; Pinkel 1986; Wienberg and Stanyon 1997). Several authors applied this technique to characterize genome conservation in primates (Wienberg et al. 1990; Morescalchi et al. 1997; Consigliere et al. 1998; Stanyon et al. 2004, 2011; Dumas et al. 2007; Amaral et al. 2008). FISH technique provides an unequivocal confirmation of the homeologies previously described by G-banding, giving a higher definition at the cytomolecular level (Wienberg et al. 1990; Wienberg 2005;

Müller 2006; Stanyon et al. 2008). Conserved chromosomal synteny (regions of chromosomes that can be located together side by side on the same chromosome arm) may be used as markers to investigate possible common evolutionary origin. Cytogeneticists refer to a broken chromosomal synteny when a region in a single chromosome of one taxon is found located in different chromosomes of another taxon. Therefore, it is said that the synteny is broken in the later taxon. Chromosomal synteny can be broken by fissions or translocations. The analysis of these synteny throughout the phylogeny of a group, such as Primates, allows the identification of the chromosomal rearrangements that might be involved in the speciation process. In the last two decades, cross-hybridization using probes of human chromosomes and/or other primate species (Wienberg and Stanyon 1997; Wienberg 2005; Stanyon et al. 2008) has been especially helpful to analyze genomic conservation.

Only five species of *Alouatta* have been analyzed using FISH: *A. caraya* (Mudry et al. 2001; Stanyon et al. 2011), *A. guariba* (both *A. g. clamitans* and *A. g. guariba*, de Oliveira et al. 2002; Stanyon et al. 2011), *A. sara* and *A. arctoidea* (Consigliere et al. 1996), and *A. belzebul* (Consigliere et al. 1998). FISH analyses in *Alouatta* were concordant with the G-banding studies, showing high levels of interspecific chromosomal variability, with interchromosomal rearrangements (Consigliere et al. 1996, 1998; Mudry et al. 2001; de Oliveira et al. 2002; Stanyon et al. 2011). Two syntenic associations (4/15 and 10/16) and the loss of the ancestral association 2/16 were proposed as synapomorphies of *Alouatta*. All howler monkeys share the synteny 14/15 from the ancestral mammalian karyotype and 8/18 from the ancestral Platyrrhini (Consigliere et al. 1996, 1998; de Oliveira et al. 2002; Stanyon et al. 2011).

The synteny 3/15 was found in all South American *Alouatta* species with the exception of *A. belzebul* (Fig. 4.7), and it is involved in their multiple sexual systems (Consigliere et al. 1996, 1998; Mudry et al. 2001; de Oliveira et al. 2002; Stanyon et al. 2011) (see Sect. 4.2.1.3). The 3/15 synteny was also observed in other atelids, such as *Ateles geoffroyi* and *A. belzebuth hybridus*, but is not involved in their XY “human-like” sexual systems (Morescalchi et al. 1997; García Haro 2001; de Oliveira et al. 2005). This synteny was not found in other platyrrhine genera



**Fig. 4.7** Fluorescence in situ Hybridization with human chromosome probes X, #3, and #15 performed in South American species of *Alouatta* with male sexual system X<sub>1</sub>X<sub>2</sub>Y<sub>1</sub>Y<sub>2</sub>. The numbers to the right of the chromosome indicate the hybridization signal of each human chromosome on its corresponding *Alouatta* homeolog chromosome

such as *Cebus libidinosus* or *Saimiri boliviensis boliviensis* (Mudry et al. 2001). It was then proposed that this synteny could be ancestral for the Atelidae, and an association with multiple sex chromosomes would have only occurred in South American howler monkeys (de Oliveira et al. 2012). However, this 3/15 synteny is not involved in the sexual systems of *A. pigra* and *A. palliata* (see Fig. 4.6), and it is not conserved in the autosomes of these species (Steinberg et al. 2014). Considering that in other species of atelids, such as *Lagothrix* and *Brachyteles*, the 3/15 synteny has also not been found (Stanyon et al. 2001; de Oliveira et al. 2005), the hypothesis of the ancestrality of this association is not supported. The 3/15 association may have arisen independently in both *Ateles* and the South American howler monkeys. More cytogenetic studies in both genera are needed in order to confirm this last hypothesis.

### 4.3 Concluding Remarks

*Alouatta* is a genus with high chromosomal variability, showing multiple interspecific chromosomal rearrangements. C+ heterochromatin is scarce, suggesting that it might not play a prominent role in *Alouatta*'s chromosomal speciation, which contrasts with observations in other platyrrhines (such as *Cebus* sp.). Instead, structural rearrangements might be the main factor promoting the karyological evolution of the genus.

The high inter- and intraspecific karyological variability in the genus needs to be considered when assessing the taxonomy of *Alouatta*. Several species still lack a cytogenetic characterization (either requiring mitotic or meiotic studies, or both). Considering that karyology contributed to the reassessment of several taxa in the past, it seems plausible that the number of species and subspecies could be underestimated (or overestimated) if genetic data are not considered. The characterization of meiotic behavior in *A. sara*, *A. guariba*, and *A. seniculus*, as well as studies in somatic and germ cells in *A. p. coibensis* and *A. nigerrima*, would contribute to *Alouatta* taxonomy and allow testing hypotheses on the chromosomal evolution in the genus.

As stated in Sect. 4.2.1.3, *Alouatta* is one of the NWP genera that present multiple sex chromosome systems, together with *Aotus*, *Callimico*, and *Cacajao*. In Old World Primates, multiple sexual systems have only been suggested by mitotic studies for one species, the silvered leaf monkey *Presbytis cristata* (Bigoni et al. 1997). The involvement of the NWP Y-chromosome in multiple sex chromosome systems, together with the absence of homeology with the human Y-chromosome observed by FISH analysis (Consigliere et al. 1996, 1998; Mudry et al. 2001; de Oliveira et al. 2002), highlights the highly different genomic composition and behavior of Y-chromosomes in platyrrhines compared to those of catarrhines.

Finally, the complex multiple sex chromosome systems observed in *Alouatta* constitute an interesting case study to understand the evolution of sex chromosomes, not only for the diversity of sexual systems but also because it is the only reported case of an independent origin of multiple sex chromosome systems in Primates.



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