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Chromatin diminution in Copepoda (Crustacea): pattern, biological role and evolutionary aspects

Andrey Grishanin

1 *Papanin Institute for Biology of Inland Waters Russian Academy of Sciences 152742, Borok Yaroslavl Prov., Russia* **2** *Dubna International University for Nature, Society and Man, 141980, Dubna, Moscow Prov., Russia*

Corresponding author: *Andrey Grishanin* (andreygrishanin@mail.ru)

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Abstract

This article provides an overview of research on chromatin diminution (CD) in copepods. The phenomenology, mechanisms and biological role of CD are discussed. A model of CD as an alternative means of regulating cell differentiation is presented. While the vast majority of eukaryotes inactivate genes that are no longer needed in development by heterochromatinization, copepods probably use CD for the same purpose. It is assumed that the copepods have exploited CD as a tool for adaptation to changing environmental conditions and as a mechanism for regulating the rate of evolutionary processes.

Keywords

Chromatin diminution, evolution, Cyclopoida, Copepoda, Crustacea

The pattern of chromatin diminution in Cyclopoida (Copepoda, Crustacea)

Chromatin diminution (CD) in Cyclopoida is the removal of part ofthe chromosomal material from cells of the somatic cells line in one or two sequential cleavage divisions, while germ-line cells retain their nuclear DNA unchanged throughout ontogeny (Beermann 1977, Grishanin et al. 1996, 2006b, Akifyev and Grishanin 2005). CD in freshwater copepods was initially treated as a pathological event (Amma 1911) or as a manifestation of extranuclear DNA synthesis (Stich 1954, 1962). Later study of the marine copepods *Pseudocalanus* Boeck, 1872 revealed a large number of Feulgen-positive mate-

rial (Robins and McLaren 1982). This material is concentrated by the division spindle prior to the first cleavage division and becomes dispersed soon afterwards. During the second cleavage division about one tenth of Feulgen-positive material is reduced. Robins and McLaren (1982) noted that the phenomenon of loss of nuclear DNA for marine copepods is not like the CD in freshwater copepods, because, despite the elimination of chromatin, during the first two maturation divisions of the embryo, reduction of the genome is not observed in the somatic cells line. They also suggested that the main cause of fluctuations in the nuclear DNA content in marine copepods is to maintain the ratio of nuclear DNA to the size of the nucleus, which is probably related to body size and the speed of development. Later study found a correlation between the size of the somatic genome and the rate of development for the marine copepods (McLaren et al. 1989).

The phenomenon of chromatin diminution (CD) has been discovered in 23 species of freshwater copepods (Table 1). The timing of CD is species-specific and occurs during one or two cell cycles in early embryogenesis. Numerous studies of CD have shown that during early embryonic cells divisions, somatic cells lose from 45% to 94% of DNA whereas germ line cells preserve the initial amount of DNA *Cyclops furcifer* Claus, 1857*, Cyclops strenuus divulsus* Lindberg, 1957*, Cyclops strenuus strenuus* Fisher, 1851 and *Mesocyclops edax* Forbes, 1891 during prophase of their first meiotic division (Beermann 1966, 1977, Chinnappa 1980, Wyngaard and Chinnappa 1982). The number of dense segments changes from 20 to 40 in anaphase chromosomes of embryonic presomatic cells of *Cyclops kolensis* Lilljeborg 1901 (Grishanin 1995). These observations suggest that the condensation pattern of prediminution chromosomes in some way contributes to its specification for excision and elimination. The heterochromatin localization is strongly species-specific. Heterochromatin is localized in the telomeric area of *C. divulsus* and in or near the centromeres and telomeres of *C. furcifer* and *M. edax*, but is evenly distributed throughout the chomosomes in *C. strenuus strenuus* (Germany population) and *C. kolensis* (Beermann 1977, Wyngaard and Chinnappa 1982, Grishanin 1995, Grishanin et al. 1996). Standiford (1989) compared C-banding patterns of *Acanthocyclops vernalis* Fischer, 1853 chromosomes before and after chromatin diminution to identify the heterochromatin regions eliminated during CD, and found that as a result of CD part of the heterochromatin of *A. vernalis* chromosomes cut out. Embryonic cells of German populations of *C. strenuus strenuus* until fourth cleavage division showed clear separation of paternal and maternal chromosomes (Beermann 1977). Heterozygous females have two type of pronuclei: one with a set of heterochromatin-rich chromosomes and the other with a low heterochromatin set. CD totally eliminates a significant difference in the size between homologous chromosomes. In other words, the length of euchromatin part of chromosome is constant , while the length of the heterochromatic regions varies. Lecher et al. (1995) proposed that the heterochromatin segments that are excised in CD consist not only from highly repetitive DNA and might be considered as facultative heterochromatin. Subsequent studies have shownthat the eliminated DNA of *C. kolensis* is composed ofmany direct and inverted repeats with a complex internal structure present within the same fragment (Degtyarev et al. 2004). The repetitive sequences (motifs) of *C. ko-*

Table 1. Cytogenetic characteristics of Cyclopoida species with chromatin diminution (CD). **1** Akif'ev 1974 **2** Beermann 1959 **3** Beermann 1977 **4** Chinnappa 1980 **5** Dorward and Wyngaard 1997 **6** Einsle 1964 **7** Einsle 1975 **8** Einsle 1993 **9** Einsle 1994 **10** Einsle 1996 **11** Grishanin et al. 1996 **12** Grishanin et al. 2004 **13** Ivankina et al. 2013 **14** Kochina and Monchenko 1986 **15** Rasch and Wyngaard 2006 **16** Rasch et al. 2008 **17** Semeshin et al. 2011 **18** Standiford 1989 **19** Wyngaard et al. 2011 **20** Zagoskin et al. 2010, nd= no data, PD/SC = DNA ratio of prediminuted germ cell nuclei and somatic cell nuclei.

Species	PD/ SC	n	CD time (cleavage division)	References
Acanthocyclops incolotaenia Mazepova, 1950	nd	nd	nd	13
A. robustus Sars G.O., 1863	nd	4	6	5,18
A. vernalis Fischer, 1853	nd	nd	5	1
Apocyclops paramensis Marsh, 1913	nd	nd	7	5
Cyclops abyssorum Sars GO, 1863	nd	nd	5	8
C. bohater Kozminski, 1933	nd	nd	5	8
C. insignis Claus, 1857	nd	nd	5	8
C. furcifer Claus, 1857	2	11	6,7	3
C. heberti Einsle, 1996	nd	nd	5	10
C. kikuchi Smirnov, 1932	nd	11	nd	9,14
C. kolensis Lilljeborg 1901	15.6–16.4	11	4	11,20
//------//-------//	11.2–12.4	11	4	17
//------//-------//	31–40	11	4	19
C. singularis Einsle, 1996	nd	nd	4	10
C. strenuus divulsus Lindberg, 1957	1.7	11	5	3
C. strenuus strenuus Fisher, 1851	2.4	11	4	3
//------//------//	4	12	5,6	11
//------//------//	5.7	nd	nd	15
C. vicinus Ulyanin, 1875	nd	11	nd	7,9
Diacyclops galbinus Mazepova 1950	11.9–13.2	nd	nd	13
D. navus Herrick, 1882	nd	nd	5	5
Mesocyclops edax Forbes, 1891	5.2–10.5	7	4	4,15,16
M. longisetus Thiébaud, 1912	9.5	nd	6	5,15
M. longisetus curvatus Dussart, 1987	14.6	nd	nd	15
Metacyclops mendocinus Wierzeiski, 1892	10	nd	nd	15
Microcyclops varicans Sars G.O., 1863	nd	nd	nd	2
Paracyclops affinis Sars G.O., 1863	1.75	nd	nd	12

lensis eliminated DNA have a mosaic structure consisting of submotifs (short repeats) and are distributed throughout the eliminated genome (Degtyarev et al. 2004). The study of inter simple sequence repeats (ISSR) of *C. kolensis* showed that most of them are stored after CD (Zagoskin et al. 2008) Some sequences of eliminated DNA are selectively reduced during CD (Grishanin et al. 2006a, Zagoskin et al. 2008). The investigation of *C. kolensis* rDNA before and after CD demonstrated a reduction of three hundred times of rRNA genes in the somatic cells line (Zagoskin et al. 2010).

Comparative analysis of eliminated DNA of Moscow and Baikal populations of *C. kolensis* showed a high level homology of repeats (97–98%) (Grishanin et al. 2006a). This means that, despite the huge number of generations that have passed since the divergence of Moscow and Baikal *C. kolensis* population (at least 25 million), the sequence data has been under strong selection to not change.

Of special interest is the research on genome endoreduplication in cyclops with CD (Rash et al. 2008, Wyngaard et al. 2011). The mechanism of endoreduplication makes it possible to reverse the process of CD.

The mechanism of chromatin diminution in freshwater copepods

Beermann (1977) proposed that CD in cyclops involved the synthesis or activation of enzymes that initiate CD in presomatic cells during the prediminution interphase. In her opinion, the absence, inactivation or repression of diminution enzymes in germ line cells is sufficient to explain their failure to undergo CD. Beermann (1977) assumed that ectosomes in germ line cells contain either a non-specific repressor with such functions or a factor that induces the formation of such repressor. According to Beermann (1977), the mechanism for eliminating chromatin from the chromosomes of somatic cells is analogous to the mechanism of excising bacteriophage DNA from *Escherichia coli* Escherich, 1885 chromosomes. It involves looping of the eliminated region of chromosomes, recombination of homologous sites in the loop basement and joining of the chromosome fragments. This hypothesis is supported by the fact that the diploid chromosomes number before and after CD remains unchanged in the studied species of *Cyclops* Muller, 1776. It is also supported by the presence in embryonic cells of *C. divulsus* and *C. furcifer* of numerous chromatin rings 25–30 nm in diameter and 0.6–100 microns in length immediately after the beginning of diminution events irrespective of the localization of the eliminated chromosome regions (Beermann and Meyer 1980, Beermann 1984). Beermann explains the evolutionary changes affecting the localization and size of eliminated regions of chromosomes as a result of chromosome rearrangements, primarily deletions and duplications. The model of organization of the higher order chromatin loop (Mirkovitch et al. 1984) explains the data of Beermann and Meyer (Beermann and Meyer 1980, Beermann 1984). The domain-loops excised from cyclops chromosomes could be cut out in the base of loops at the site of Matrix Associated Regions (MAR). With this theory in mind it is now possible to complete the explanation of CD mechanism for *C. kolensis* which was presented earlier (Akif'ev et al. 1998, 2002) and propose the following CD stages that occurs in the presomatic cells of cyclops:

1) Preparation for the reduction of a major part of the genome, which involves lengthening of the prediminution interphase and the appearance of G-bands in chromosomes prior to diminution. The G-bands might be involved in reprogramming the functionally active part of the genome through the mechanisms of DNA methylation and histone modifications of somatic cells chromosomes before CD;

2) Activation of the sites of chromosomes breaks during interphase of the cell cycle when CD occurs. It is probable that the chromosomes breakage sites are localized in regions associated with the nuclear matrix;
3) Cutting at chromosomes breakage sites. Immediately after cutting, the chromosome DNA strand is restored;
4) Compacting of the excised DNA and formation of a pore-free membrane around it to produce granules;
5) Degradation of the granules of excised DNA during 2–3 subsequent divisions. Thus, the CD process, presumably, involve many genes.

Biological role and evolutionary significance of CD

CD is unique in producing a dramatic reorganization of the entire nuclear genome during a relatively short period of ontogeny. During CD large regions of heterochromatin are removed from chromosomes of the somatic cells line. Prior to CD, presomatic cells of cyclops in interphase have a nuclear structure that is highly ordered in terms of the spatial distribution of eu- and heterochromatin. There is a strong opinion based on numerous facts that silent genes are localized in the heterochromatin compartments at the nuclear periphery, whereas active genes are located in the central part (Dillon 2004, Meaburn and Misteli 2007, Schofer and Weipoltshammer 2008). Hollick et al. (1997) showed that rapid genome reorganization is associated with repetitive DNA, its methylation and insertions of a transposable element. Therefore, the excision of heterochromatin from chromosomes during CD can remove genes, change their position, and through the mechanisms of DNA methylation and histone modifications modify their regulatory status. Moreover, presumably, the excision of heterochromatin segments by CD will decrease the distance between many previously distantly located genes. This is expected to increase the amount ofinterference from crossover exchanges and decrease the number of possible recombinations, which in turn is expected to reduce adaptiveness. There is an alternative, albeit radical evolutionary solution of reducing the number of recombinations during meiosis – an absence of chiasmata. It's usual for Cyclopoidae species to have achiasmatic meiosis (Chinnappa 1980, Grishanin et al. 2005). However some cryptic species have meiosis with well-defined chiasmata, variable genome and maybe capable of rapid evolutionary changes (Grishanin et al. 2005, 2006b).

Monchenko (2003) emphasizes that macromorphological traits are of little importance in the speciation of cyclops, which has many cryptic species. Cryptic speciation is apparently common in the Cyclopoida. The study *A. vernalis* revealed a complex population structure of this species, where some populations not only have different cytogenetic characteristics, but also show a partial or complete reproductive isolation from other populations (Grishanin et al. 2005, 2006c). These data suggest that these populations can be considered as cryptic species (Dodson et al. 2003). Comparative study of the Cyclopoida species (*A. vernalis, C. insignis, C. kolensis, C. strenuus strenuus*) has revealed that a large-scale rearrangement of the genome has arisen in this suborder with-

out any visible morphological changes. Evolutionary events that have involved changes in genome size but not changes in chromosome number are evidenced by multiple genome size differences within the genera *Mesocyclops* Sars, 1913 and between populations of *Thermocyclops crassus* Fischer, 1853 (Table 2) (it's probably by mechanisms of endoreduplication); the chromosome polymorphism observed in *C. strenuous strenuus* like gonomery (Beermann 1977),the cytogenetics differences observed between Russian and Germany populations of *C. strenuous strenuus*; and the presence and absence of CD for Russian and German populations of *C. insignis* (Grishanin and Akifiev 2000).The molecular data complete the cytogenetics pattern. The high level of conservation of the *C. kolensis* genome, the complex structure of its eliminated DNA, and the selective removal of some sequences, (Degtyarev et al. 2004, Grishanin et al. 2006a, Zagoskin et al. 2010) suggests a special role of eliminated DNA in its development and evolution. So, CD process should be considered as an evolutionary innovation that leads directly to the appearance of cryptic Cyclopoida species that are distinguished by the occurrence or lack of CD, by peculiarities of the CD process and by other cytogenetic characteristics. The mechanism of cryptic speciation is not known yet, but it is likely that hybrids of cyclops with CD and without CD will fail as a result of a compromised ability to regulate CD. Disruption of the coordinated network of genes that control each of the above-proposed stages of CD will inevitably lead to the disturbance of the CD process and, as a result, to errors in the processes of development and differentiation of the organism that will most likely cause the death for the given organism. Consequently, the occurrence of CD in *Cyclops* evolution should automatically lead to the emergence of a new species. To confirm this hypothesis, it would be of interest to cross the German population of *C. insignis*, which have CD and the Russian population of *C. insignis*, in which CD absent.

The detection of polyploid cells in some *Cyclops* species raises another side of the biological significance of CD (Grishanin et al. 1996). With the conventional notion of polypoidy arising during or before conception, the functional advantage of having multiple copies of some

DNA sequences is offset by the necessity of replicating and maintaining multiple copies of much additional genetic material that will never be required in differentiated somatic cells. An alternative but more economical path to the same end is to eliminate unused DNA from the somatic cells line genome during CD and then to repeatedly amplify the genome of somatic cells.

Based on the above reasoning we can make the following assumptions about biological role of CD:

1. Chromatin diminution is an alternative form of regulation of cell differentiation during which there is a total loss of mostly redundant DNA, while in the vast majority of eukaryotes part of the genome is inactivated through heterochromatinization;
2. CD coevolves with polyploidy to regulate gene dosage in somatic tissues;
3. CD is a tool for adaptation to changing environmental conditions;
4. CD is a mechanism for regulating the rate of evolutionary processes.

Table 2. Cytogenetic characteristics of *Thermocyclops crassus* Fischer, 1853 and some species of *Mesocyclops* Sars, 1913. **1** Wyngaard and Rasch 2000 **2** Rasch and Wyngaard 2006 **3** Grishanin et al. 2004 **4** Grishanin 2008, SC = somatic cell nuclei.

Species	1C(pg) SC	n	CD	References
Mesocyclops edax Forbes, 1891	1.47	7	present	1,2
M. ruttneri Kiefer, 1981	0.72	7	no CD	1
M. leuckarti Claus, 1857	0.38	7	no CD	1
M. woutersi van de Velde, 1987	0.38	no data	no CD	1
Thermocyclops crassus (russian population)	1.2	7	no CD	3,4
Th. crassus (vietnamese population)	0.42	no data	no CD	1

Thus, even a brief acquaintance with the facts established recently permits a conclusion that the phenomenon of CD is a unique tool to study the eukaryotic nucleus organization and some questions of evolution.

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RESEARCH ARTICLE

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New karyologycal data and cytotaxonomic considerations on small mammals from Santa Virgínia (Parque Estadual da Serra do Mar, Atlantic Forest, Brazil)

Camilla Bruno Di-Nizo[1], Carolina Lima Neves[2], Júlio Fernando Vilela[3], Maria José de J. Silva[1]

1 *Laboratório de Ecologia e Evolução, Instituto Butantan, Avenida Vital Brazil, 1500, CEP 05503-900, São Paulo, SP, Brazil* **2** *Laboratório de Biologia da Conservação, Departamento de Ecologia, Universidade Estadual Paulista (UNESP), Avenida 24 A,1515. CEP 13506-900, Rio Claro, SP, Brazil* **3** *Laboratório de Biologia Evolutiva Teórica e Aplicada, Universidade Federal do Rio do Janeiro (UFRJ), Avenida Professor Rodolpho Paulo Rocco, s/n – CCS, bl. A, sala A2-92. CEP 21941-617, Rio de Janeiro, RJ, Brazil*

Corresponding author: *Maria José de J. Silva* (mariajo@butantan.gov.br)

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Abstract

Atlantic Forest, in the eastern coast of Brazil, is a hotspot of biodiversity of mammals, and Parque Estadual da Serra do Mar (PESM) is the largest continuous area of this biome. Here, we characterized the karyotype composition of the small mammals from Santa Virgínia, a region in the northern part of PESM. Specimens were collected from July 2008 to September 2009. We identified 17 species (13 rodents and 4 marsupials) from which 7 exhibited species-specific karyotypes, illustrating the importance of karyotype information in cytotaxonomy. We report for first time the karyotype of *Monodelphis scalops* (Thomas, 1888) and two new records for PESM: *Akodon montensis* Thomas, 1913 and *Brucepattersonius soricinus* Hershkovitz, 1998. Cytogenetic polymorphisms were detected for some species trapped in the area. Our results show the importance of Santa Virgínia / PESM in addressing studies for the conservation of small mammal wildlife in the Atlantic Forest.

Keywords
Atlantic Forest, conservation, cytotaxonomy, *Monodelphis scalops*

Introduction

The Atlantic Forest is the fourth biodiversity hotspot in the world (Myers et al. 2000, Ceballos and Ehrlich 2006, Carnaval et al. 2009). Geographical aspects combined with the large altitudinal and longitudinal ranges have favored the emergence of high endemism and species richness in this biome (Leal and De Gusmão Câmara 2003, Ribeiro et al. 2009). Nevertheless, the remaining forest represents only approximately 11% of the original extent, which highlights the biome as a priority for biodiversity conservation (Ribeiro et al. 2009).

The Parque Estadual da Serra do Mar (PESM), located in the state of São Paulo, Brazil was created in 1977, and is considered the largest remaining block of Atlantic Forest with 315.390 hectares (Instituto Florestal 2006).

Studies the mammal fauna of this park are scarce and the majority of the reports were presented in undergraduate theses and master's dissertations, focusing on large mammals (Wang 2002, Norris et al. 2012). The most comprehensive article about small mammals from PESM was performed in Picinguaba (Northern of PESM) and reported morphology and karyotype information of 27 species belonging to the orders Didelphimorphia, Carnivora, and Rodentia (Pinheiro and Geise 2008).

According to Paglia et al. (2012), small mammals of the orders Rodentia and Didelphimorphia are important components of the Atlantic Forest mammal fauna, representing approximately 40% of the species. Morphological studies combined with cytogenetics and geographical distribution information allow the proper identification of taxa, particularly in cases of cryptic or morphologically similar species. Moreover, cytogenetic study can reveal genetic variability within and among individuals.

This study aims to characterize the karyotype composition and contribute to the identification of small rodents and marsupials from Santa Virgínia, since there is only one published study focusing on small mammals of this area. Data about geographical distribution of trapped species are also given.

Material and methods

Study area

Santa Virgínia (lat. 23°24.00'S to 23°17.00'S, long. 45°03.00'W to 45°11.00'W) is located in the Northern of PESM (Fig. 1) covering an area of 17,000 hectares (Instituto Florestal 2006), and altitudes ranging from 870 to 1,100 meters (Tabarelli and Mantovani 1999). The vegetation is defined as a dense montane humid forest ('Floresta Ombrófila Densa Montana') (Veloso et al. 1991) and the annual precipitation is about 2200 mm. The annual mean temperature varies from 18°C to 22°C.

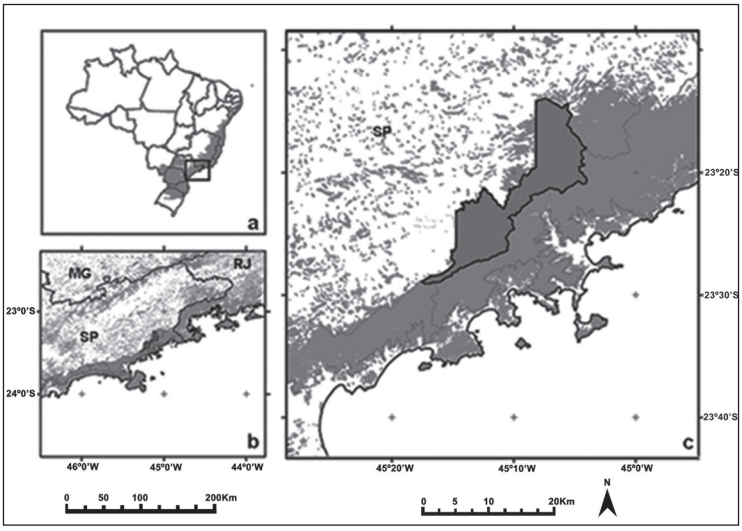

Figure 1. a Map of Brazil with original Atlantic Forest cover in grey and the region of Parque Estadual da Serra do Mar (PESM) indicated (square) **b** Parque Estadual da Serra do Mar (PESM) in grey **c** Santa Virgínia is highlighted (extracted and modified from Instituto Florestal 2006).

Field work

Small mammals were sampled by commercial live-traps (Sherman and Tomahawk-like traps) and pitfall-traps. In July 2008, a pilot experiment was performed from one to three nights, with a total sampling effort of 300 live-traps/night. From September 2008 to September 2009, field survey was carried out bimonthly during five consecutive nights. During this period, we set up six grids with 30 live-traps per grid and 12 transects of pitfall-traps. Live-traps were arranged in a 0.6 ha grids (60 × 100 m each) with 24 trap stations spaced every 20 meters. Each trap station received one Sherman of different size, randomly set (small, 25 × 7.5 × 9.5 cm; medium, 30 × 7.5 × 9.5 cm; large, 37.5 × 10 × 12 cm; H.B. Sherman Trap®, Inc., Tallahassee, Florida, USA). We also set randomly a Tomahawk-like trap (45 × 16 × 16 cm; Rosaminas Serviço Engenharia e Comércio Ltda. Piraúba, Minas Gerais, Brazil) at six trapping stations. Overall, we had 6300 live-trap/night.

The 12 transects of pitfall-traps were pairwise 30 meters apart, from November 2008 to September 2009. Each transect received four plastic buckets (60L, 40 cm top diameter, 35 cm bottom diameter, and 56 cm depth) buried with the rim at ground level, spaced every 10 meters each. The buckets on each line were connected with a 0.5 meters tall plastic drift fence that extended an additional 10 meters at each end, totaling 50 meters of fence. In total, we used 48 buckets, resulting in 1,440 pitfall-traps/night.

Different sizes and models of traps were used to optimize the sampling, aiming to reduce the selectivity based on body size and/or habits of the animals. Attractive baits (mashed bananas, peanut butter, bacon and corn meal) were placed in both kinds of traps. All traps were checked daily, preferably on the first hours in the morning.

Trapping and handling were carried out under ICMBio licence (number 14428-2) of Instituto Chico Mendes de Conservação da Biodiversidade.

Animals were euthanized according to the protocol of the "Animal experimentation ethics" (Carpenter et al. 1996) and under permission of Instituto Butantan Ethics Committee (242/05). The skins, skulls and partial skeletons were deposited in the Museu de Zoologia da Universidade de São Paulo (MZUSP) (still without MZUSP number), Museu Nacional da Universidade Federal do Rio de Janeiro (MN) and Coleção de Mamíferos da Universidade Federal do Espírito Santo (UFES) (Table 1).

The nomenclature used in this work follows Gardner (2005), Musser and Carleton (2005), Weksler et al. (2006) and Percequillo et al. (2011). External morphologic traits of marsupials were compared with voucher specimens preserved at MZUSP.

Chromosome preparation

Metaphases were obtained from bone marrow and spleen after *in vivo* injection of a 0.1% colchicine solution (1mL/100g of weight). Cells were suspended in 0.075M KCl solution for 20 minutes at 37°C and fixed in three washes of methanol: acetic acid (3:1). GTG and CBG-banding were performed according to Seabright (1971) and Sumner (1972), respectively. At least 20 metaphases per individual were analyzed to define the diploid number (2n) and fundamental number of autosome arms (FNa). Chromosomes were measured using the program ImageJ version 1.46 (Rasband 2011) to establish the fundamental number, according to Levan et al. (1964). Karyotypes were set up according to the literature, when available.

Specimen identification was carried out through a comparison of our data with previous cytogenetic information, external morphological characteristics, and geographic distribution (see Table 1 references).

Results

A total of 706 small mammal specimens were captured (600 rodents and 106 marsupials) and 54 specimens were selected for chromosome preparations (46 rodents and 8 marsupials, Table 1).

On the whole, 13 species of rodents belonging to two families were cytogenetically analyzed (Table 1): *Akodon montensis* Thomas, 1913; *Blarinomys breviceps* (Winge, 1887); *Brucepattersonius soricinus* Hershkovitz, 1998; *Thaptomys nigrita* (Lichtenstein, 1829); *Drymoreomys albimaculatus* Percequillo, Weksler & Costa, 2011; *Euryoryzomys russatus* (Wagner, 1848); *Nectomys squamipes* (Brants, 1827); *Oligoryzomys nigripes* (Olfers, 1818); *Sooretamys angouya* (Fischer, 1814); *Calomys tener* (Winge, 1887); *Rhipidomys itoan* Costa, Geise, Pereira and Costa, 2011; *Juliomys pictipes* (Osgood, 1933) of family Cricetidae, and *Trinomys iheringi* (Thomas, 1911) of family Echimyidae.

Four marsupial species (Didelphimorphia) were karyotyped: *Marmosops incanus* (Lund, 1840); *Micoureus paraguayanus* (Tate, 1931); *Monodelphis scalops* (Thomas, 1888) and *Philander frenatus* (Olfers, 1818) (Table 1).

First cytogenetic information for *Monodelphis scalops*

Eight individuals were collected, although only one male had been cytogenetically studied. Morphological data and geographic distribution comparisons allow us to identify all as *Monodelphis scalops*. The morphological traits of these individuals are similar to voucher specimens of *M. scalops* preserved at MZUSP under catalogue numbers1528, 30702, 30712 and 30757. This species has also been reported in São Paulo state, Brazil (Gardner 2005), agreeing to our collecting site (Fig. 1).

Here we present, for the first time, the karyotype of *Monodelphis scalops.* The karyotype of a male showed 2n=18, FNa=30. Pair 1 is a large submetacentric, pair 2 is a medium metacentric, pairs 3, 4 and 6 are medium subtelocentric, pair 5 is a medium acrocentric and pairs 7 and 8 are medium submetacentric. X chromosome is a small subtelocentric, and the Y is a minute acrocentric (Fig. 2). The short arm of pairs 4 and 6 are difficult to see depending on the condensation of the chromosome and so it was necessary to analyze and measure more than 30 metaphases to define their morphology.

New records for PESM

Cytogenetic data helped us to report for first time the presence of *Akodon montensis*, and *Brucepattersonius soricinus* in PESM. Cytogenetic information of these species are shown in Fig. 3, Table 1. Briefly, *Akodon montensis* showed 2n=24, 25 (24+1B), FNa=42 and one individual showed a heteromorphic X chromosome with an enlarged short arm. We also detected one small supernumerary submetacentric (B) in three out of nine individuals analyzed (Fig. 3a).

B. soricinus had 2n=52, FNa=52 (Fig. 3b) and this is the first time that banding-pattern is presented in this species. The CBG-banding pattern in the female specimen showed rather pronounced amount of pericentromeric heterochromatin in all chromosomes (Fig. 3c). GTG-banding allowed the identification of all autosomic pairs and X chromosomes (Fig. 3d).

Chromosomal variability and species-specific karyotypes

The remaining species studied in this work have already been recorded in PESM and their karyotypes are in accordance to the literature. Karyotype information of all species analyzed and the chromosomal variability found in this work is shown inTable 1 and Figs 4–7.

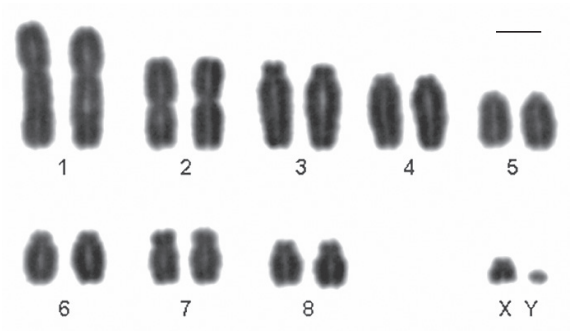

Figure 2. Conventional stained karyotype of *Monodelphis scalops* (2n=18, FNa=30, male). Bar = 10μm.

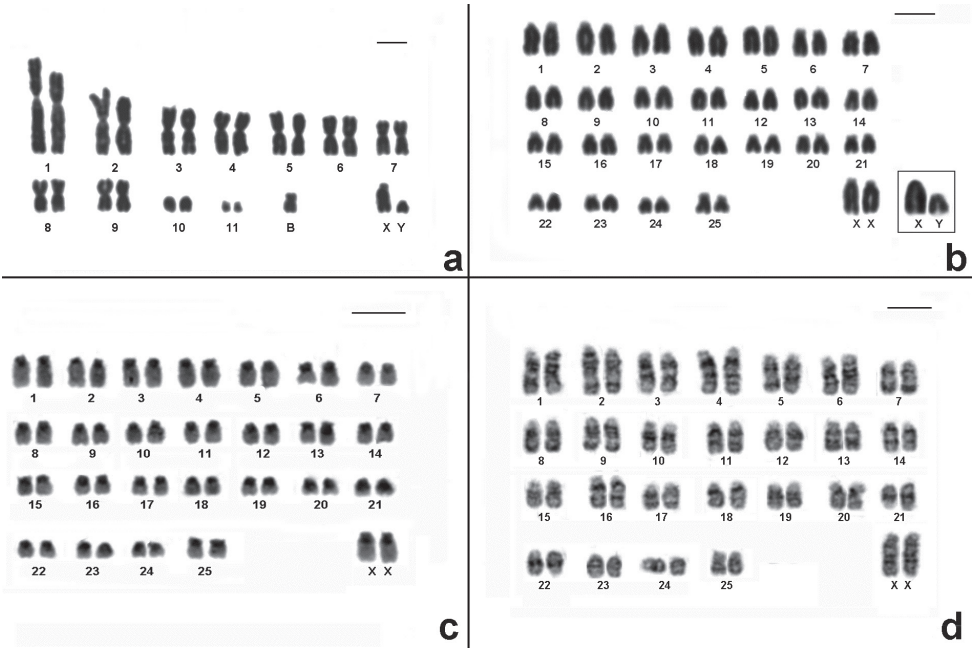

Figure 3. Karyotypes of the new records for PESM. **a** Conventional stained karyotype of *Akodon montensis* (2n=24+1B, FNa=42, male) **b** Conventional stained karyotype of *Brucepattersonius soricinus* (2n=52, FNa=52, female). Inset: sex chromosomes of a male **c** CBG-banding pattern of *B. soricinus* (2n=52, FNa=52, female) **d** GTG-banding pattern of *B. soricinus* (2n=52, FNa=52, female). Bar = 10μm.

Seven out of the 13 rodent species showed species-specific karyotypes: *Akodon montensis*, *Drymoreomys albimaculatus*, *Oligoryzomys nigripes*, *Sooretamys angouya*, *Calomys tener*, *Juliomys pictipes* and *Trinomys iheringi* (grey cells in Table 1). The identification of the remaining species (*Blarinomys breviceps*, *Brucepattersonius soricinus, Thaptomys nigrita*, *Euryoryzomys russatus*, *Nectomys squamipes*, and *Rhipidomys itoan*) required additional morphological and molecular investigation and geographic distribution information (Table 1).

Marsupials presented conserved diploid numbers of 14, 18 and 22 and were identified here by external morphological comparisons.

Table 1. A list of cytogenetically studied small mammals from Santa Virgínia, Parque Estadual da Serra do Mar, state of São Paulo, Brazil. N: number of individuals analyzed. Specimens voucher/Museum Field number: ROD and MARS - Laboratório de Ecologia e Evolução Instituto Butantan, Brazil; MN - Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; UFES - Coleção de Mamíferos da Universidade Federal do Espírito Santo, Brazil. 2n: diploid number, and FNa: number of autosomes arms. **Morphologies: A=acrocentric; M=metacentric; SM=submetacentric; ST=subtelocentric. Grey cells correspond to species-specific karyotypes**.

ORDER **Family** Tribe *Species*	**N**	**Specimens voucher/ museum field number**	**Distribution**	**2n**	**FNa**	**Autosome pairs**[a]	**Sex chromosomes**	**Variable cytogenetic characteristics**	**Karyotype reference**	**Figure No.**
ORDER RODENTIA **Family Cricetidae** Tribe Akodontini *Akodon montensis*	3♀6♂	ROD 3*, 6*, 11*, 28*, 29* UFES 2235-2237, 2239	From Rio de Janeiro to Rio Grande do Sul and Minas Gerais, Brazil [1, 2]	24, 24 (+ 1B)	42	9 large to medium M/SM; 1 A; 1 small M	X: medium A Y: small A	X chromosome polymorphism (enlarged short arm), 1 SM B-chromosome	Kasahara and Yonenaga-Yassuda (1982)	3a
Blarinomys breviceps	1♀	UFES 2263	Endemic of Atlantic Forest, Brazil[1, 2]	29 (+2B)	50	11 medium M/SM 1 A Heteromorphic pair:1 M + 2 A	X: large A	Heteromorphic pair, 2 M B-chromosomes	Ventura et al. (2012)	See Ventura et al. (2012)
Brucepattersonius soricinus	1♀, 1♂	MN 78955, 78956	Southeastern Brazil, exclusively in Atlantic Forest[1, 2, 3]	52	52	24 medium to small A; 1 small SM	X: large ST Y: small A	-	Bonvicino et al. (1998)	3b–d
Thaptomys nigrita	2♂	ROD 2*, 4*	South Bahia to the north of Rio Grande do Sul, Brazil[1, 2]	52	52	24 medium to small A; 1 small SM	X: large A Y: small SM	-	Kasahara and Yonenaga-Yassuda (1984)	5a
Tribe Oryzomyini *Drymoreomys albimaculatus*	1♀, 1♂	UFES 2271, 2272	Endemic of Atlantic Forest, Brazil [4]	62	62	29 medium to small A; 1 small M	X: large SM Y large SM, smaller than the X	-	Suárez-Villota et al. (2013)	See Suárez-Villota et al. (2013)
Euryoryzomys russatus	1♀, 7♂	ROD 5*, 12*, 30* UFES 2242-2244, 2265-2266	Coastal region of Brazil from Bahia to Rio Grande do Sul[1, 2]	80	86	35 A decreasing in size; 4 small M	X: large SM Y: small A or small ST	Sex chromosomes polymorphisms	Andrades-Miranda et al. (2000)	5b

ORDER **Family** Tribe *Species*	**N**	**Specimens voucher/ museum field number**	**Distribution**	**2n**	**FNa**	**Autosome pairs**[a]	**Sex chromosomes**	**Variable cytogenetic characteristics**	**Karyotype reference**	**Figure No.**
Nectomys squamipes	1♀	UFES 2270	Eastern Brazil [2]	56 (+2B)	56	26 A decreasing in size; 1 small M	X: large SM	2 small SM B-chromosomes	Silva and Yonenaga-Yassuda (1998)	4a
Oligoryzomys nigripes	4♀, 4♂	ROD 34*, UFES 2274-2280	From South Bahia to Rio Grande do Sul, Brazil[1, 2]	62	80–82	11 M/SM decreasing in size; 19 A decreasing in size	X: large SM or large M Y: medium M or medium SM	Pericentric inversions in pair 3, sex chromosomes polymorphisms	Paresque et al. (2007)	4b
Sooretamys angouya	1♀, 4♂	UFES 2262, 2282-2285	From Espírito Santo to Santa Catarina, Brazil[2]	58	60	26 A decreasing in size; 2 small M	X: large A Y: medium A	-	Andrades-Miranda et al. (2000)	5c
Tribe Phyllotini *Calomys tener*	1♂	UFES 2264	Widespread in the state of São Paulo, Brazil[1, 2]	66	66	31 medium to small A; 1 M	X: large SM Y: medium A	-	Mattevi et al. (2005)	6a
Tribe Thomasomyini *Rhipidomys itoan*	1♀	UFES 2281	PESM[5, 6]	44	50	17 A decreasing in size; 1 medium SM; 3 small M	X: large SM	-	Pinheiro and Geise (2008); Costa et al. (2011)	4c
Incertae sedis *Juliomys pictipes*	3♂	UFES 2267-2269	Minas Gerais to Rio Grande do Sul, Brazil[1, 2]	36	34	17 A decreasing in size	X: medium A Y: small A	-	Bonvicino and Otazu (1999)	6b–c
Family Echimyidae *Trinomys iheringi*	2♀, 1♂	ROD 7*, 10*, UFES 2286	West of Rio de Janeiro, São Paulo to north of Paraná, Brazil[2, 7]	60+1B, 60+4B	116	29 M or SM decreasing in size	X: large SM Y: small SM	1 or 4 dot-like B-chromosomes; Secondary constriction on pair 7	Yonenaga-Yassuda et al. (1985)	4d
ORDER DIDELPHIMORPHIA **Family Didelphidae** *Marmosops incanus*	2♀, 1♂	MARS 1*, 5*, 6*	Eastern Brazil[8]	14	24	6 SM decreasing in size	X: small SM Y: small A	-	Carvalho et al. (2002)	7a

ORDER **Family** Tribe *Species*	**N**	**Specimens voucher/ museum field number**	**Distribution**	**2n**	**FNa**	**Autosome pairs[a]**	**Sex chromosomes**	**Variable cytogenetic characteristics**	**Karyotype reference**	**Figure No.**
Micoureus paraguayanus	1♀, 1♂	MARS 3*, 4*	Atlantic Forest; Eastern Brazil, until Rio Grande do Sul state[8]	14	20	4 M or SM 2 A	X: medium A Y: medium A, smaller than X	-	Pereira et al. (2008)	7b
Monodelphis scalops	1♂	MN 78961	Espírito Santo, Rio de Janeiro and São Paulo, Brazil[8]	18	30	4 SM 3 ST 1 A	X: small ST Y: minute A	-	Present study	**2**
Philander frenatus	1♀, 1♂	UFES 2287-2288	From Bahia to Santa Catarina, Brazil[8]	22	20	10 A	X: medium A Y: small A		Pereira et al. (2008)	7c

Geographic distribution according to: 1. Musser and Carleton (2005); 2. Bonvicino et al. (2008); 3. Bonvicino et al. (1998); 4. Percequillo et al. (2011); 5. De Vivo et al. (2011); 6. Pinheiro and Geise (2008); 7. Woods and Kilpatrick (2005); 8. Gardner (2005).

*Specimens voucher deposited in Museu de Zoologia da Universidade de São Paulo (MZUSP) without catalog number yet. [a] Autosomal morphologies do not include Bs.

Discussion

Importance of cytogenetic study for Neotropical rodents

We proved the cytogenetic analyses as a taxonomic tool, since 7 out of 13 rodent species present species-specific karyotypes (53.8%). Besides, we identified 94% of all species, when cytogenetic data were combined with information of external morphology and geographical distribution (Table 1).

Cryptic species are relatively common in some Neotropical rodent groups and cytogenetic information was indispensable for identifying such species. For instance, *A. montensis* is morphologically indistinguishable from *A. cursor* (Winge, 1887) and both species occur in sympatry in the Atlantic Forest (Christoff et al. 2000). In addition, the occurrence of *A. cursor* previously recorded in Santa Virgínia/PESM (Instituto Florestal 2006) was doubtful till this study, as we proved the occurrence of *A. montensis* by karyotypic analysis.

Another cryptic species case occurs in the genus *Thaptomys*. *Thaptomys* sp. (2n=50) and *T. nigrita* (2n=52) are morphologically identical, so the karyotypes are the diagnostic information to distinguish both species (Ventura et al. 2004, 2010).

By contrast, *T. nigrita* and *B. soricinus* present very similar karyotypes (2n=52, FNa=52) however their identification can be safely done at the level of genera by external morphological characters. An accurate observation on the karyotypes of *B. soricinus* and *T. nigrita* showed that the pair 1 of *T. nigrita* is the largest of the chromosome set (Fig. 5a) meanwhile *B. soricinus* has the pair 1 similar in size to the others of the set (Figs 3b–d). We also noticed differences regarding sex chromosome morphologies of both species (Table 1). This feature could be a diagnostic tool to differentiate each karyotype, but additional cytogenetic studies (including comparative and molecular cytogenetic data) are needed to support these first observations.

Blarinomys breviceps presents a peculiar karyotype and it could not be considered species-specific due to the great variability in 2n and FNa (Geise et al. 2008, Ventura et al. 2012). Moreover, Ventura et al. (2012) suggested the existence of more species for the monotypic genus *Blarinomys* in Atlantic Forest since molecular phylogenetic analyses showed two geographically distinct lineages.

Euryoryzomys russatus does not have species-specific karyotype also. *E. emmonsae* Musser, Carleton, Brothers and Gardner, 1998, and *E. nitidus* (Thomas, 1884) share the same 2n=80, NFa=86 (Bonvicino and Geise 2006). However, when cytogenetic information is combined with morphologic and geographic distribution data, *E. russatus* can be confirmed.

Concerning *Nectomys squamipes*, it is not possible to affirm that this species possess species-specific karyotype with classical cytogenetic data because, when compared to *Holochilus brasiliensis* (Desmarest 1819), both karyotypes are identical (Yonenaga-Yassuda et al. 1987). Nevertheless, the association of cytogenetic, geographic distribution and external morphological characters allows the recognition of *N. squamipes* as occurring at PESM (Bonvicino et al. 2008). *Nectomys squamipes* was considered for

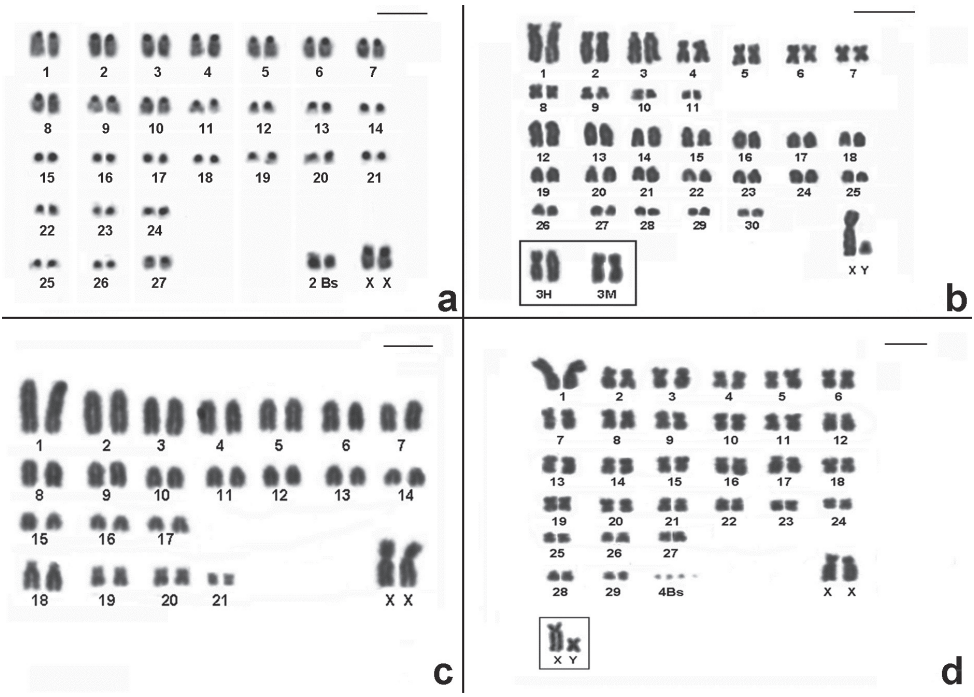

Figure 4. CBG-banding pattern of *Nectomys squamipes* (2n=56 + 2B, FNa=56, female) **b** Conventional stained karyotype of *Oligoryzomys nigripes* (2n=62, FNa=80, male). Inset: different forms of pair 3: heteromorphic (3H) and homomorphic metacentric (3M) **c** Conventional stained karyotype of *Rhipidomys itoan* (2n=44, FNa=50, female) **d** Conventional stained karyotype of *Trinomys iheringi* (2n=60+4Bs, FNa=116, female). Inset: sex chromosomes of a male. Bar = 10μm.

years as a carrier of two basic distinct karyotypes: 2n=56 (1 to 3Bs) and 2n=52 (1 to 3Bs), and only after crossings in laboratory, Bonvicino et al. (1996) noticed that two different species could be diagnosed - *N. squamipes* (2n=56) and *N. rattus* (Pelzeln, 1883), (2n=52).

The karyotype of *Rhipidomys itoan* presented here (2n=44, FNa=50 Fig. 4c) is the same one as described by Zanchin et al. (1992) and Silva and Yonenaga-Yassuda (1999). Pinheiro and Geise (2008) also found an identical karyotype for a species referred as *Rhipidomys* sp., trapped in Picinguaba (PESM), and De Vivo et al. (2011) reported an undescribed species of *Rhipidomys* that occurs at the Parque Estadual da Serra do Mar. Recently, two new species from Atlantic Forest were described: *R. tribei* Costa, Geise, Pereira and Costa, 2011 and *R. itoan*; and the latter presented 2n=44, FNa=48, 49, 50 (Costa et al. 2011). Santa Virgínia is embedded in the geographical distribution described for this species and molecular analyzes confirmed that this sample belongs to *R. itoan* species. Nevertheless, we do not consider this karyotype species-specific.

Finally, cytogenetic analysis was useful in identifying *T. iheringi* as two species – *T. iheringi* and *T. dimidiatus* (Günther, 1876) - occur in Atlantic Forest. Despite the

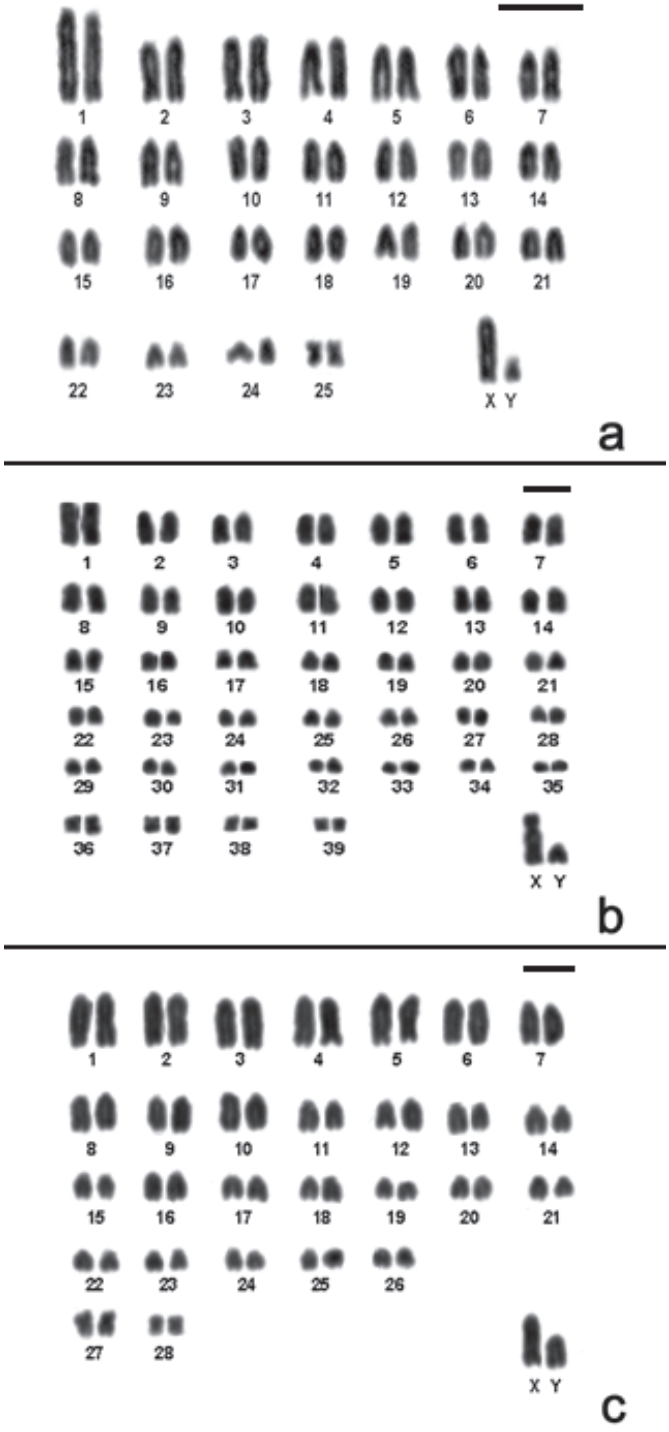

Figure 5. Conventional stained karyotypes: **a** *Thaptomys nigrita* (2n=52, FNa=52, male) **b** *Euryoryzomys russatus* (2n=80, FNa=86, male) **c** *Sooretamys angouya* (2n=58, FNa=60, male). Bar = 10μm.

regular chromosome set of *T. iheringi* (not considering B chromosomes) is identical to the one described for the species *T. dimidiatus* (2n=60, FNa=116) by Pessoa et al. (2005), the presence of at least one B and the morphology of Y chromosome in *T. iheringi* represent good characters to diagnose the species.

Chromosome variations

Mammals have remarkable diversity in species karyotypes, and rodents exhibit noteworthy variability of diploid chromosome number (O'Brien et al. 2006, Romanenko et al. 2012). For instance, in this work, diploid numbers of rodents ranged from 24 in *A. montensis* to 80 in *E. russatus*.

The chromosome variation observed here is due to the presence of supernumerary chromosomes (B chromosomes), sex chromosome heteromorphism and/or polymorphism, as well as autosomal polymorphisms. This chromosome variability does not cause a problem in characterizing the species, except in the case of *T. iheringi*, in which the presence of at least one B chromosome is sufficient to confirm its identity.

Structural rearrangements may explain much of the observed karyotype diversity in rodents. In this regard, Robertsonian fusions/fissions (whole-arm translocations) and pericentric inversions, have long been considered the predominant rearrangements in natural populations of rodents (Patton and Sherwood 1983). Nevertheless, studies with more refined techniques such as fluorescent *in situ* hybridization and chromosome painting demonstrate that tandem fusions, reciprocal translocations, and paracentric inversions are much more common than previously thought (Hass et al. 2008, Ventura et al. 2009, Romanenko et al. 2012).

Our data showed two species with pericentric inversion rearrangements, *O. nigripes* and *R. itoan*. *O. nigripes* showed variation in autosomal pair 3 (Fig. 4b) but this rearrangement had also been reported in pairs 2, 4 and 8, which places this species as one of the most polymorphic within Neotropical rodents (Paresque et al. 2007). The genus *Rhipidomys* frequently shows 2n=44, except for the 2n=50 reported by Silva and Yonenaga-Yassuda (1999) from Amazonas, in contrast with differences in the FNa (Zanchin et al. 1992, Costa et al. 2011). The variation of FNa, which represents the commonest chromosome change observed for the genus, may be a consequence of pericentric inversion events.

Karyotype diversity is also enhanced in mammals due to the presence of B chromosomes. B chromosomes are extra elements found in the karyotypes of many eukaryotic species. Their functions and molecular composition remain obscure but, apparently in mammals, these chromosomes neither promote phenotypic alterations nor affect fitness of individuals (Jones and Rees 1982, Trifonov et al. 2010). B chromosomes are known in nine Brazilian rodent species (Silva and Yonenaga-Yassuda 2004, Ventura et al. 2012). Herein, we found B chromosomes in four out of 13 species of rodents (30,76%, i.e. almost a third of the total): *A. montensis*, *B. breviceps, N. squamipes* and *T. iheringi*. Silva and Yonenaga-Yassuda (2004) found B chromosomes in *S. angouya*

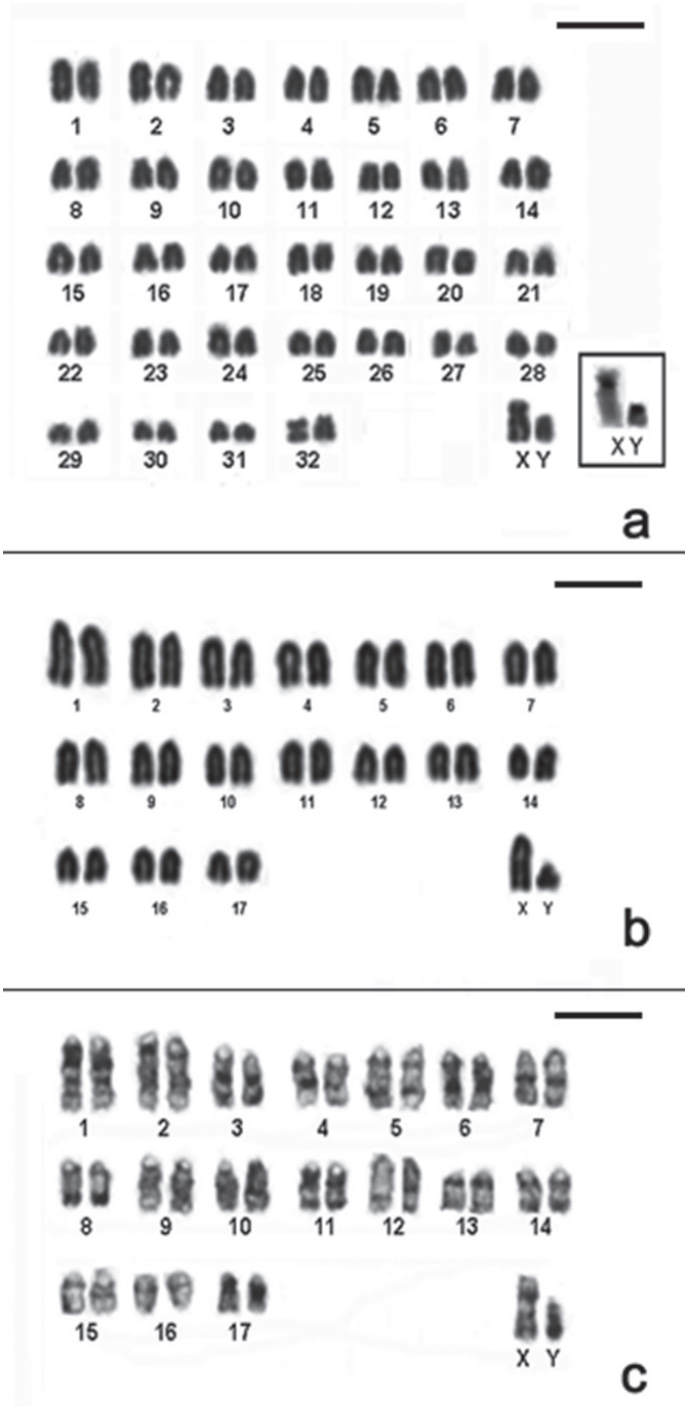

Figure 6. a Conventional stained karyotype of *Calomys tener* (2n=66, FNa=66, male). Inset: Sex chromosomes CBG-banded **b** Conventional stained karyotype of *Juliomys pictipes* (2n=36, FNa=36, male) **c** GTG-banding pattern of *Juliomys pictipes* (2n=36, FNa=36, male). Bar = 10μm.

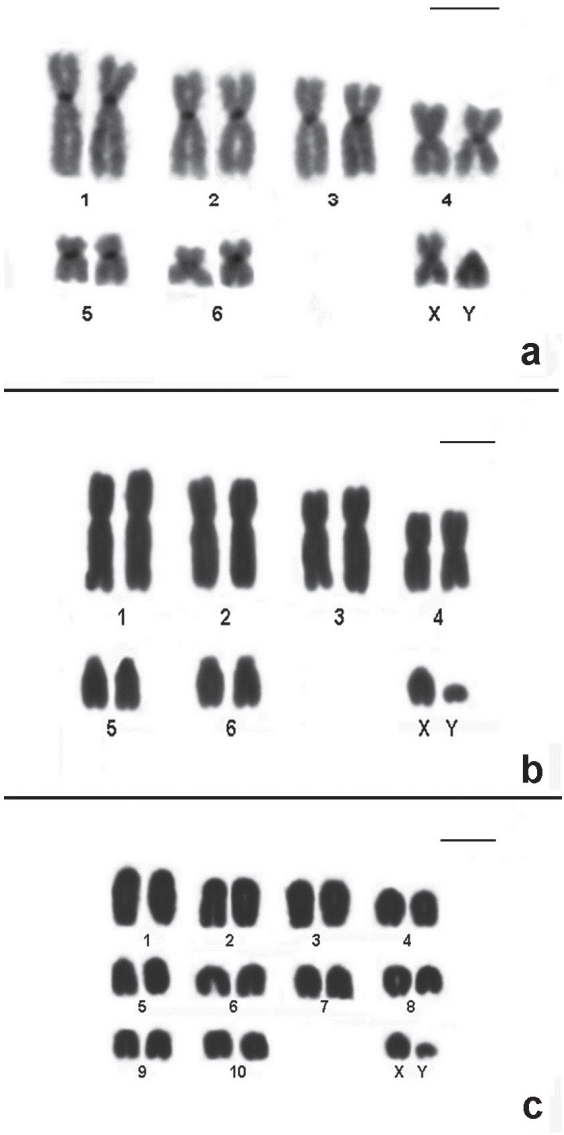

Figure 7. a CBG-banding pattern of *Marmosops incanus* (2n=14, FNa=24, male) **b** Conventional stained karyotype of *Micoureus paraguayanus* (2n=14, FNa=20, male) **c** Conventional stained karyotype of *Philander frenatus* (2n=22, FNa=20, male). Bar = 10μm.

(referred at that time as *Oryzomys angouya*), however, in our sample, B chromosomes were not observed for this species (Fig. 5c).

Sex chromosome heteromorphisms/polymorphisms were found in *A. montensis* and *O. nigripes*, and the variation is due to addition/deletion of constitutive heterochromatin, as described by Kasahara and Yonenaga-Yassuda (1982) and Paresque et al. (2007), respectively.

Marsupials

Cytogenetic data exposed three diploid numbers for the family Didelphidae: 2n=14, 18 and 22 (Reig et al. 1977, Carvalho et al. 2002). As the karyotypes of American marsupials are conserved, cytogenetic analyses cannot be considered as a diagnostic tool to identify species. However, differences in banding patterns could help in the characterization of some taxa, for instance, *Marmosops incanus* (Svartman 2009).

In the present paper we report for the first time the karyotype of *Monodelphis scalops* which is similar to the one described for *M. kunsi* Pine, 1975 and *M. brevicauda* (Erxleben, 1777) by Carvalho et al. (2002), except for the morphology of the sex chromosomes (Fig. 2). Besides, *M. scalops* karyotype differs from *M. rubida* (Thomas, 1899) (2n=18, FNa=32) (Pereira et al. 2008) due to the presence of one acrocentric pair (#5) instead of a biarmed pair (Fig. 2).

Final considerations

Our species list is an evidence of the limited knowledge of small mammals in PESM since the karyotype of *Monodelphis scalops* is reported for the first time and *A. montensis* and *B. soricinus* are new records for the park. According to De Vivo et al. (2011), it is important to increase samples in areas of dense humid forest since these areas are poorly surveyed. The number of species collected during the period of 14 months in Santa Virgínia should be considered highly representative, and this effort brought to light new findings. This includes the specimen of *Blarinomys breviceps* herein mentioned which was added to a larger sample with animals collected from different localities of Brazil and the diploid numbers ranged from 28 to 52 (Ventura et al. 2012), as well as *Drymoreomys albimaculatus* which was studied by Suárez-Villota et al. (2013).

The improvements to the list of mammals of PESM could be attributed to different methods of capture (live and pitfall traps) to enhance the success of trapping in different habitats. The multidisciplinary approach employed is also evidently important in some cases as presented above. Additionally, data on diversity and geographical distribution of species are essential to reach conservation strategies, and the significance of Santa Virgínia / PESM in the preservation of the Neotropical fauna becomes more clear.

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RESEARCH ARTICLE

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Karyotypes of parasitic wasps of the family Eulophidae (Hymenoptera) attacking leaf-mining Lepidoptera (Gracillariidae, Gelechiidae)

Vladimir E. Gokhman[1], Zoya A. Yefremova[2], Ekaterina N. Yegorenkova[3]

1 *Botanical Garden, Moscow State University, Moscow 119234, Russia* **2** *Department of Zoology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel* **3** *Department of Geography, Ulyanovsk State Pedagogical University, Ulyanovsk 432700, Russia*

Corresponding author: *Vladimir E. Gokhman* (vegokhman@hotmail.com)

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Abstract

Karyotypes of eleven parasitoid species of the family Eulophidae were examined, namely, *Chrysocharis laomedon* (Walker, 1839) (2n = 10), *Chrysocharis* sp. aff. *laomedon* (n = 5, 2n = 10), *Chrysocharis* sp. aff. *albipes* (Ashmead, 1904) (2n = 12), *Mischotetrastichus petiolatus* (Erdös, 1961) (n = 6, 2n = 12), *Minotetrastichus frontalis* (Nees, 1834) (n = 5, 2n = 10), *Cirrospilus pictus* (Nees, 1834) (2n = 12), *Hyssopus geniculatus* (Hartig, 1838) (2n = 16), *Sympiesis gordius* (Walker, 1839) (2n = 12), *S. sericeicornis* (Nees, 1834) (2n = 12), *Pnigalio agraules* (Walker, 1839) (2n = 12 + 0–2B) and *Pnigalio gyamiensis* Myartseva & Kurashev, 1990 (2n = 12 + 0–6B) reared from *Phyllonorycter acerifoliella* (Zeller, 1839), *Ph. apparella* (Herrich-Schäffer, 1855), *Ph. issikii* (Kumata, 1963) (Gracillariidae) and *Chrysoesthia sexguttella* (Thunberg, 1794) (Gelechiidae). Chromosome sets of all species except *P. agraules* and *P. gyamiensis* were studied for the first time. B chromosomes were detected in the two latter species; in *P. gyamiensis*, the maximum number of B chromosomes represents the highest value known for parasitic wasps to date.

Keywords

Hymenoptera, Chalcidoidea, Eulophidae, Lepidoptera, Gracillariidae, Gelechiidae, chromosomes, karyotypes, B chromosomes

Introduction

The Eulophidae are one of the largest and most diverse families of the hymenopteran superfamily Chalcidoidea. This group currently contains about 300 genera and 4500 described species (Noyes 2013). Chromosomal study of these parasitoids is becoming a rapidly developing research field due to its implications for taxonomy and evolutionary history of this morphologically challenging group, with about 60 species of the family Eulophidae (i.e. more than 1% of described species) karyotyped up to now. Specifically, two reviews of Eulophidae karyology were published in the 2000s (Gokhman 2002, 2004), and a number of other papers on this subject appeared since that time (e.g. Gebiola et al. 2012a, Gokhman and Gumovsky 2013, and references therein) including a monograph on karyology of hymenopteran parasitoids (Gokhman 2009). Furthermore, certain members of the family have become objects of an advanced cytogenetic study (Bolsheva et al. 2012). We have recently examined chromosome sets of a number of Eulophidae species associated with leaf-mining Lepidoptera of the families Gracillariidae and Gelechiidae. This group of the family Eulophidae has been chosen for the present study because its members are well known as the most abundant parasitoids of leaf-mining Lepidoptera (see e.g. Yefremova and Mishchenko 2008, Yefremova et al. 2009, 2011). At the same time, many species of these parasitic wasps belong to the subfamily Eulophinae that is, in turn, the most karyotypically diverse group of the Eulophidae (Gokhman 2002, 2009).

Material and methods

The material used in this study was collected by V.E. Gokhman and E.N. Yegorenkova in the Moscow (Ozhigovo, 60 km SW Moscow; 55°27'N; 36°52'E) and Ulyanovsk Provinces (Ulyanovsk; 54°16'N; 48°20'E) of Russia in 2012–2013 respectively (Table 1). All parasitoids were reared from *Phyllonorycter acerifoliella* (Zeller, 1839) on *Acer platanoides* Linnaeus, *Ph. apparella* (Herrich-Schäffer, 1855) on *Populus tremula* Linnaeus, *Ph. issikii* (Kumata, 1963) (Gracillariidae) on *Tilia cordata* Miller and *Chrysoesthia sexguttella* (Thunberg, 1794) (Gelechiidae) on *Chenopodium album* Linnaeus. Preparations of mitotic chromosomes (as well as meiotic chromosomes where available) of all species except *Minotetrastichus frontalis* were obtained from ovaries of adult females according to the protocol developed by Gokhman (2009) with minor modifications. Specifically, the extracted ovaries were incubated in 0.5% hypotonic colchicine-added sodium citrate solution for 30 min (the original technique implies incubation in 1% hypotonic solution for 20 min). Alternatively, chromosomes of gregarious *M. frontalis* were studied on preparations of cerebral ganglia of prepupae according to the slightly modified technique developed by Imai et al. (1988). Again, above-specified incubation parameters were used; a few individuals from some broods were reared to the adult stage and then identified. Numbers of examined specimens as well as mitotic and meiotic divisions for each species are given in

Table 1. Cell divisions were studied and photographed using an optic microscope Zeiss Axioskop 40 FL fitted with a digital camera AxioCam MRc. To obtain karyograms, the resulting images were processed with image analysis programs Zeiss AxioVision version 3.1 and Adobe Photoshop version 8.0. Mitotic chromosomes were subdivided into four groups: metacentrics (M), submetacentrics (SM), subtelocentrics (ST) and acrocentrics (A) following guidelines provided by Levan et al. (1964) and Insua et al. (2006). For species with already known karyotypes, morphometric analysis of chromosomal morphology was performed. Selected metaphase plates with the clearly visible centromeric position of every chromosome were measured using Adobe Photoshop; relative lengths and centromeric indices of all chromosomes were calculated and given in Table 2. Meiotic chromosomes were classified according

Table 1. The main results of karyotypic study of the Eulophidae (Hymenoptera) attacking leaf-mining Lepidoptera.

Species	Origin		Number		(n), 2n	Chromosomal formula: (n), 2n
	Locality	Host	(Males), females	(Meiotic), mitotic divisions		
Chrysocharis laomedon	Ozhigovo	*Phyllonorycter issikii*	1	11	10	10M
Chrysocharis sp. aff. *laomedon*	Ozhigovo, Ulyanovsk	*Phyllonorycter acerifoliella, Ph. issikii*	7	(3), 13	(5), 10	10M
Chrysocharis sp. aff. *albipes*	Ozhigovo	*Phyllonorycter apparella*	1	10	12	10M + 2ST
Mischotetrastichus petiolatus	Ditto	*Ph. issikii*	2	(4), 2	(6), 12	10M + 2A
Minotetrastichus frontalis	Ulyanovsk	Ditto	(1), 7	15	(5), 10	(5M), 10M
Cirrospilus pictus	Ozhigovo	*Ph. apparella*	2	6	12	6M + 2SM + 4ST
Hyssopus geniculatus	Ditto	*Ph. issikii*	3	14	16	6M + 2SM + 4ST + 4A
Sympiesis gordius	Ditto	Ditto	2	2	12	12M
S. sericeicornis	Ditto	*Ph. apparella*	2	10	12	10M + 2A
Pnigalio agraules	Ditto	*Ph. apparella, Ph. issikii*	3	13	2n = 12 + 0–2B	10M + 2M/SM
P. gyamiensis	Ulyanovsk	*Chrysoesthia sexguttella*	1	17	2n = 12 + 0–6B	6M + 4M/SM + 2ST

Table 2. Relative lengths (RL) and centromeric indices (CI) of *Pnigalio agraules* and *P. gyamiensis* chromosomes (mean ± SD; B chromosomes not included). For each species, numbers of analyzed metaphase plates are given in brackets.

Chromosome no.	P. agraules (6)		P. gyamiensis (8)	
	RL	CI	RL	CI
1	21.09 ± 1.09	44.74 ± 3.50	20.44 ± 0.75	45.43 ± 3.37
2	18.89 ± 0.84	43.32 ± 3.56	19.06 ± 0.85	40.87 ± 5.13
3	17.31 ± 0.70	41.82 ± 3.69	18.04 ± 0.54	41.40 ± 4.59
4	16.06 ± 0.61	44.40 ± 4.63	16.69 ± 0.76	44.82 ± 3.79
5	14.37 ± 0.83	45.18 ± 3.92	14.18 ± 0.93	45.50 ± 4.61
6	12.28 ± 1.42	38.39 ± 5.36	11.59 ± 1.24	18.82 ± 5.34

to Darlington (1965). Parasitoids were identified by Z.A. Yefremova (Eulophinae and Entedoninae) and E.N. Yegorenkova (Tetrastichinae) using keys provided by Trjapitzin (1978) and Storozheva et al. (1995), Hansson (1985) as well as by Graham (1987) respectively; however, most individuals belonging to the taxonomically complicated genus *Chrysocharis* Förster, 1856 could not be reliably assigned to any named species. Voucher specimens are deposited in the Zoological Museum, Moscow State University, Moscow, Russia.

Results

The principal results of the present study are listed in Table 1; some additional details are given below.

Subfamily Entedoninae

Chrysocharis laomedon (Walker, 1839) (Fig. 1a). All chromosomes are obviously metacentric; chromosomes of the first, second and third pair, and those of the fourth and fifth pair, form three size groups.

Chrysocharis sp. aff. *laomedon* (Fig. 1b–c). Karyotype structure of the mitotic chromosome set as in *Ch. laomedon* (Fig. 1b). The meiotic karyotype contains five bivalents; each of them apparently bears two chiasmata in diplotene (Fig. 1c).

Chrysocharis sp. aff. *albipes* (Ashmead, 1904) (Fig. 1d). As in the two previous species, chromosomes of the five largest pairs are metacentric, but a pair of small subtelocentrics is present in the karyotype as well. The metacentrics also form three size groups; however, apart from previous species, these groups include chromosomes of the first and second, third and fourth, and fifth pair respectively.

Subfamily Tetrastichinae

Mischotetrastichus petiolatus (Erdös, 1961) (Fig. 1e–f). The karyotype contains five pairs of metacentric chromosomes; the first and the last pair are visibly longer/shorter respectively than the remaining ones. In addition, a pair of small acrocentrics is present in the chromosome set (Fig. 1e). Six bivalents are found in the meiotic karyotype of this species; in diplotene, almost all of them bear two chiasmata except for the last one with a single chiasma (Fig. 1f).

Minotetrastichus frontalis (Nees, 1834) (Fig. 1g–h). Both haploid (Fig. 1g) and diploid karyotypes (Fig. 1h) were studied. All chromosomes are obviously metacentric; those of the fifth pair are substantially smaller than chromosomes of the preceding ones.

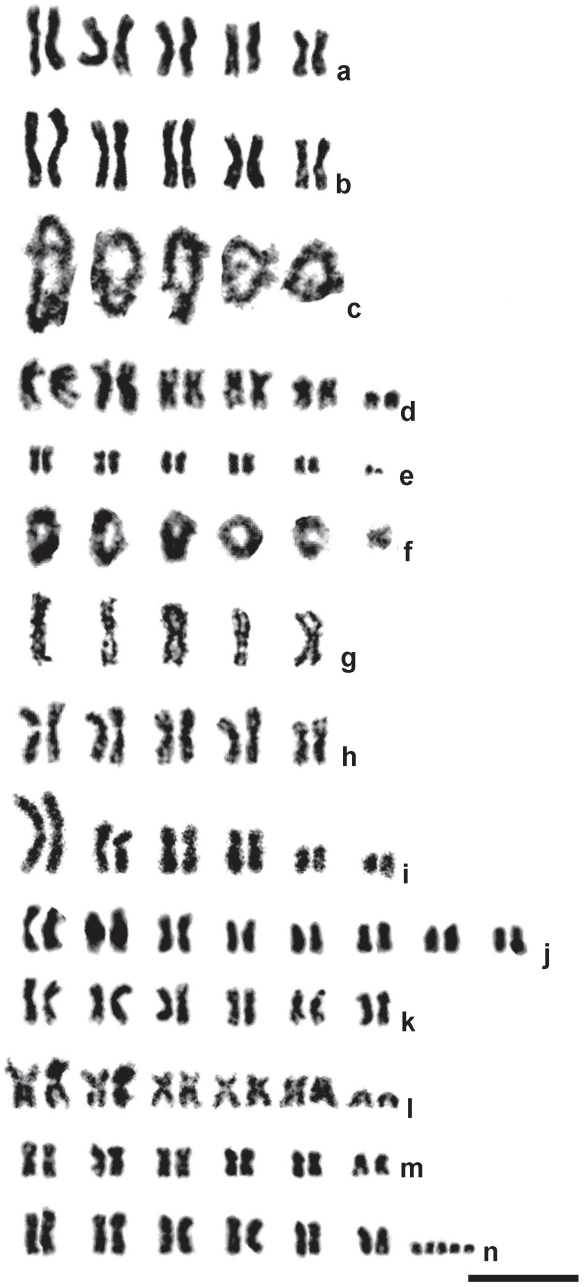

Figure 1. Mitotic (**a–b, d–e, g–n**) and meiotic (diplotene; **c, f**) karyograms of male (**g**) and female (**a–f, h–n**) parasitic wasps of the family Eulophidae. **a** *Chrysocharis laomedon* **b–c** *Chrysocharis* sp. aff. *laomedon* **d** *Chrysocharis* sp. aff. *albipes* **e–f** *Mischotetrastichus petiolatus* **g–h** *Minotetrastichus frontalis* **i** *Cirrospilus pictus* **j** *Hyssopus geniculatus* **k** *Sympiesis gordius* **l** *S. sericeicornis* **m** *Pnigalio agraules* **n** *P. gyamiensis*, karyotype with five B chromosomes. Bar = 10 μm (6.7 μm for **c**).

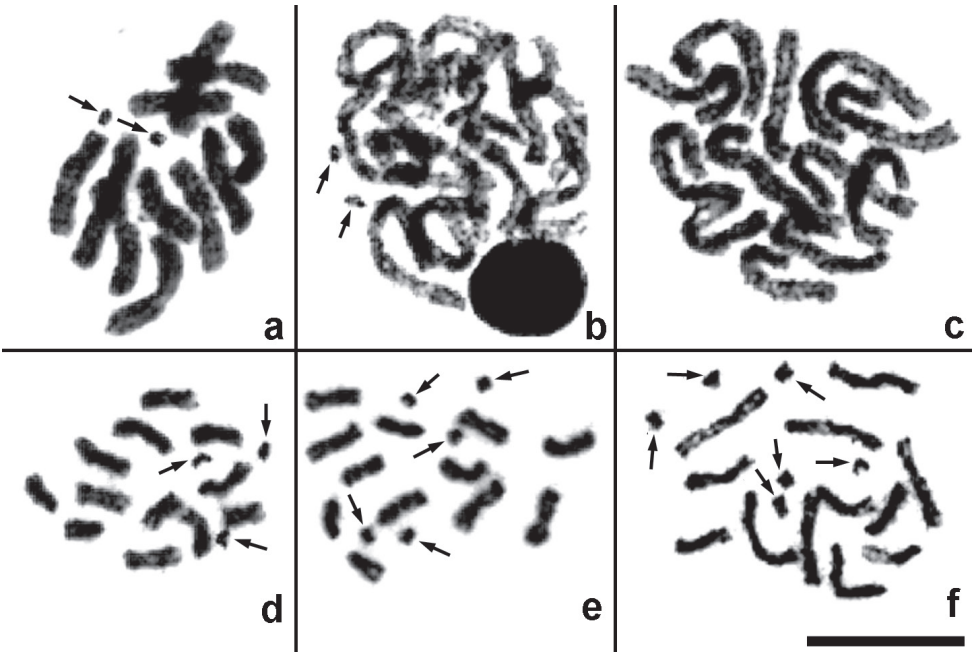

Figure 2. Mitotic divisions in *Pnigalio agraules* (**a–c**) and *P. gyamiensis* (**d–f**). **a** Fragment of a metaphase plate with two B chromosomes **b** Same individual, late prophase with two B chromosomes **c** Same individual, prometaphase without B chromosomes **d–f** Metaphase plates with three, five and six B chromosomes respectively. Arrows indicate B chromosomes. Bar = 10 μm.

Subfamily Eulophinae

Cirrospilus pictus (Nees, 1834) (Fig. 1i). Metacentrics of the first pair very large, at least more than 1.5 times longer than the remaining chromosomes. Metacentrics of the second and third pair as well as submetacentrics of the fourth pair more or less gradually decrease in length. Subtelocentric chromosomes of the fifth and sixth pair substantially differ in size and visibly smaller than the preceding ones.

Hyssopus geniculatus (Hartig, 1838) (Fig. 1j). The diploid chromosome number in this species is substantially higher than in many other members of the family. First three chromosome pairs obviously differ in size and are somewhat longer than the remaining ones. The karyotype contains metacentric (the first, third and eighth pair), submetacentric (the second pair), subtelocentric (the fourth and sixth pair) and acrocentric chromosomes (the fifth and seventh pair).

Sympiesis gordius (Walker, 1839) (Fig. 1k). All chromosomes are metacentric; metacentrics of the first pair substantially differ in size from the remaining ones. Chromosomes of the fifth pair bear distinct secondary constrictions.

S. sericeicornis (Nees, 1834) (Fig. 1l). The karyotype contains five pairs of large metacentric chromosomes and a small pair of acrocentrics; chromosomes of the first pair are visibly longer than the other metacentrics.

Pnigalio agraules (Walker, 1839) (Figs 1m, 2a–c). The first and the last chromosome pair are obviously longer/shorter respectively than the remaining ones that form a continuous gradation in length (Table 2). Most chromosomes are metacentric except for the sixth pair that can be either metacentric or submetacentric (Fig. 1m). In addition, a single specimen carrying B chromosomes was found. Although we were unable to obtain full metaphase plates in the former individual, fragments of these plates (Fig. 2a) as well as certain mitotic divisions in late prophase or early metaphase (Fig. 2b) clearly demonstrate presence of the two B chromosomes. Nevertheless, other cell divisions from the same individual show no trace of the chromosomes of that kind (Fig. 2c).

P. gyamiensis Myartseva & Kurashev, 1990 (Figs 1n, 2d–f). The overall karyotype structure as in the preceding species (Table 2), but the second and third chromosome pair can be either metacentric or submetacentric, and the last chromosome pair is subtelocentric (Fig. 1n). In addition, most metaphase plates carry a few very small apparently subtelocentric or acrocentric B chromosomes (usually two to five, but sometimes one or six; Figs 1n, 2d–f).

Discussion

The family Eulophidae is the most karyotypically studied group of parasitoids of its taxonomic rank in terms of the relative number of studied species. The results presented here provide new information on chromosome number and morphology in certain groups of the family. All species listed in the present paper (except for *Pnigalio agraules* and *P. gyamiensis*; see below) as well as the genera *Mischotetrastichus* Graham, 1987, *Minotetrastichus* Kostjukov, 1977 and *Chrysocharis* Förster, 1856 were studied for the first time. In addition, new karyotypic information was obtained for the genus *Cirrospilus* Westwood, 1832. Within this genus, only the chromosome number was studied earlier for *Cirrospilus diallus* Walker, 1838 (n = 6; Gokhman and Quicke 1995). Specifically, the haploid chromosome number in the species studied varies from n = 5 to n = 8. Interestingly, this variation range is also observed in the family Eulophidae in general (Gokhman 2002). The new data confirm our previous conclusion that the haploid set of five long bi-armed chromosomes and a short acro- or subtelocentric represents the ancestral feature of the Eulophidae (Gokhman 2002, 2004), possibly a synapomorphy for the family (Gokhman 2009). Since many Torymidae (together with a few Ormyridae) as well as certain Agaonidae also have similar karyotypes, this feature is likely to have been independently acquired by various groups of the superfamily Chalcidoidea (Gokhman 2013).

Nevertheless, a few deviations from the above mentioned pattern have been recorded up to now, including "*Elachertus* sp." with n = 8 (Gokhman 2002, 2009) and *Cirrospilus pictus* with n = 6 in the present study. As for the former, this aberrant chromosome number together with a characteristic karyotype structure (see Fig. B.207 in Gokhman 2009) was detected in a single specimen identified by V.V. Kostjukov. Since the same n value was found during the present study in *Hyssopus geniculatus*, we have re-examined the former specimen that is also deposited in the Zoological Museum of Moscow State University. In fact, this time it was identified by Z.A. Yefremova as a member of the genus *Hyssopus* Girault, 1916, namely *H. nigritulus* (Zetterstedt, 1838). *Hyssopus* was actually placed within *Elachertus* Spinola, 1811 as its subgenus or a separate species group for a certain period (e.g. Bouček 1965). The aberrant karyotype structure found in both studied *Hyssopus* species can therefore be considered as a autapomorphy of this genus. On the other hand, the chromosome set of *Cirrospilus pictus* (n = 6) represents a particular karyotype structure that was previously unknown in the Eulophidae. In addition, chromosomes of the last pair in *Pnigalio agraules* and *P. gyamensis* with the same haploid number are substantially longer than those characteristic of the common karyotype structure in the family (see also Gebiola et al. 2012a).

Chromosomal rearrangements involved in the karyotype evolution of the Eulophidae possibly include chromosomal fusions in a few species (e.g. *Minotetrastichus frontalis* and *Chrysocharis laomedon*). In these cases, the smallest chromosome fused to one of the larger elements to form a karyotype with n = 5. On the other hand, an increase in chromosome number in certain groups (e.g. in the genus *Hyssopus*) could take place by aneuploidy and the subsequent restoration of even chromosome numbers (Gokhman 2009). An alternative explanation, i.e. chromosomal fissions followed by inversions (see e.g. Imai et al. 1988), seems less likely, mainly due to the lack of smaller chromosomes that could arise from these rearrangements in *Hyssopus* and a few other Eulophidae.

Karyotype structure found in *Mischotetrastichus* and *Minotetrastichus* showed certain resemblance to that of *Tetrastichus* Haliday, 1844 s.str. Specifically, metacentrics of the last pair are substantially shorter than the preceding ones (Gokhman 2004).

The meiotic figures obtained in *Chrysocharis* sp. aff. *laomedon* and *Mischotetrastichus petiolatus* generally correspond to mitotic karyotypes of these parasitoids. Specifically, bivalents apparently formed by metacentric chromosomes are relatively large and gradually decrease in size. These ring-like bivalents bear two terminal/subterminal chiasmata in diplotene. Alternatively, the only acrocentric pair found in *M. petiolatus* forms a small open bivalent with a single chiasma.

We have also detected B chromosomes in *Pnigalio agraules* and *P. gyamiensis*. Interestingly, chromosome sets of both members of this genus have been recently examined by Gebiola et al. (2012a), with the latter species listed there as *Pnigalio soemius* (Walker, 1839) (i.e. *P. soemius*_CS; see Gebiola et al. 2012b). Overall karyotype structure of the two *Pnigalio* Schrank, 1802 species studied by Gebiola et al. (2012a) generally coincides with our results except for the last chromosome pair of *P. gyamiensis* which appeared to be subtelocentric according to the present study. However,

Gebiola et al. (2012a) could define this pair as acrocentric arbitrarily, since no quantitative data on its centromeric position are given in the cited paper. Moreover, no B chromosomes were previously detected in both members of the genus *Pnigalio*. Up to now, chromosomes of that kind were found only in a few species of chalcid wasps belonging to the families Pteromalidae, Trichogrammatidae and possibly also Aphelinidae (see Gokhman 2009 for review), and Eulophidae (Gebiola et al. 2012a). In all those cases, only one chromosome per karyotype was detected. In addition, chromosomes of a particular pair found in *Aphidius ervi* Haliday, 1834 (Braconidae) with 2n = 12 (Gokhman and Westendorff 2003) can also be considered, with certain reservations, as B chromosomes. Gebiola et al. (2012a) found B chromosomes in the genus *Pnigalio*, i.e. in *P. mediterraneus* Ferrière & Delucchi, 1957. Again, karyotypes of a few individuals of this species carried the only B chromosome. We found two B chromosomes in the karyotype of a certain female of *P. agraules*. However, identity of the latter species poses a separate problem. Indeed, *P. mediterraneus* and *P. agraules* cannot be reliably separated on the basis of adult external morphology alone (Gebiola et al. 2009). Nevertheless, karyotype of the former species contains strictly metacentric chromosomes, whereas certain chromosomes of *P. agraules* can be submetacentric (Gebiola et al. 2012a), and this is characteristic of our specimens as well. On the other hand, borders between closely related taxa (perhaps even between different genera) are probably not impermeable for B chromosomes, as it was suggested for an analogous chromosome found in the pteromalid *Nasonia vitripennis* (Walker, 1836) (McAllister and Werren 1997). We also found one to six B chromosomes in *P. gyamiensis*. The latter value therefore represents the highest number of B chromosomes per diploid karyotype known for parasitic wasps to date. As far as other members of the order Hymenoptera are concerned (see Gokhman 2009 for a brief review), up to 12 chromosomes per haploid set were found in the ant, *Leptothorax spinosior* Forel, 1901 (Imai 1974), which is one of the highest records in the animal world (Camacho et al. 2000). Although B chromosomes often carry sex-ratio distorters in parasitoid Hymenoptera, as opposed to the aculeate members of the order (Gokhman 2009), this is probably not the case in *P. gyamiensis*, thus providing a possible explanation for accumulation of these chromosomes in the latter species.

The present research also revealed differences between karyotypes of closely related taxa (e.g. within the genus *Chrysocharis*), thus confirming that chromosomal studies can be used for identifying cryptic species in the Eulophidae, as in many other parasitoid families (Gokhman 2009, Gebiola et al. 2012a). Furthermore, data provided in the present paper have some important implications for parasitoid karyology in general. For example, the number of B chromosomes found in *P. gyamiensis* appeared to be the highest among all other parasitic wasps, although the previous study (Gebiola et al. 2012a) did not reveal B chromosomes in this particular species as well as in *P. agraules*. This also suggests the importance of karyotypic study of every available population of parasitoid Hymenoptera, even if it apparently belongs to an already examined species (see also Gokhman 2009).

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Multiple sex chromosome systems in howler monkeys (Platyrrhini, Alouatta)

Eliana Ruth Steinberg[1], Mariela Nieves[1], Marta Dolores Mudry[1]

1 *Grupo de Investigación en Biología Evolutiva (GIBE) – Departamento de Ecología, Genética y Evolución – Facultad de Ciencias Exactas y Naturales – Universidad de Buenos Aires – IEGEBA (CONICET-UBA) – Ciudad Universitaria – Pab. II –4º piso - Labs 43-46 - (C1428EGA) – Buenos Aires – Argentina*

Corresponding author: *Eliana Ruth Steinberg* (steinberg@ege.fcen.uba.ar)

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Abstract

In light of the multiple sex chromosome systems observed in howler monkeys (*Alouatta* Lacépède, 1799) a combined cladistic analysis using chromosomal and molecular characters was applied to discuss the possible origin of these systems. Mesoamerican and South American howlers were karyologically compared. FISH analysis using the chromosome painting probes for the #3 and #15 human chromosomes was applied to corroborate the homeology of the sexual systems. We found that the HSA3/15 syntenic association, present in the sex chromosome systems of South American Howlers, is not present in those of Mesoamerican ones. The autosomes involved in the translocation that formed the sexual systems in the Mesoamerican and South American species are different, thus suggesting an independent origin. Parsimony analysis resolved the phylogenetic relationships among howler species, demonstrating utility of the combined approach. A hypothesis for the origin of the multiple sex chromosome systems for the genus is proposed.

Keywords

Multiple sex chromosome systems, combined phylogenetic analysis, FISH, Neotropical Primates, cytochrome b

Introduction

Howler monkeys (genus *Alouatta* Lacépède, 1799 of the family Atelidae) exhibit one of the widest geographic distributions recorded to date for Neotropical Primates. Their distribution extends from southern Mexico to northern Argentina (Crockett and Eisenberg 1987, Rylands 2000). They inhabit a diverse range of environments, including tropical rain forests, flood forests, gallery forests, patches of forest and deciduous and semideciduous seasonal environments (Crockett and Eisenberg 1987, Zunino et al. 2001). There remains a lack of consensus regarding both the number of species within the genus, which, depending on the author, ranges from 9 to 14 species (Rylands 2000, Groves 2001, 2005, Gregorin 2006, Rylands and Mittermeier 2009), and the phylogenetic relationships among them. This shows the complexity of the taxonomy of *Alouatta* and highlights the importance of including a larger number of variables for a more accurate characterization of the species in the genus. We adhere to the classification proposed by Groves (2001, 2005) in recognizing 10 species (*Alouatta belzebul* Linnaeus, 1766, *A. seniculus* Linnaeus, 1766, *A. sara* Elliot, 1910, *A. macconnelli* Linnaeus, 1766, *A. caraya* Humboldt, 1812, *A. palliata* Gray, 1849, *A. pigra* Lawrence, 1933, *A. guariba* Humboldt, 1812, *A. nigerrima* Lönnberg, 1941, *A. coibensis* Thomas, 1912), since it considers both morphological and genetic information.

In this context, and to contribute to the description of the phylogenetic relationships in the genus, several authors have proposed that chromosomal data can also 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 ancestrality.

In primates, different researchers in the last three decades have proposed chromosomal speciation as a probable evolutionary mechanism to explain the diversity observed in living species (de Grouchy et al. 1972, Seuánez 1979, Dutrillaux and Couturier 1981, Clemente et al. 1990, Stanyon et al. 2008, de Oliveira et al. 2012). In howler monkeys, species exhibit diploid numbers (2N) ranging from 44 in *Alouatta seniculus* to 58 in *Alouatta pigra*, and in a large number of species, multiple sex chromosome systems in males originated from Y-autosome translocations have been described (Table 1). The chromosomes involved in the Y-autosome translocations in *Alouatta caraya*, *Alouatta macconnelli, Alouatta guariba guariba* Humboldt, 1812, *Alouatta guariba clamitans* Cabrera, 1940, *Alouatta sara* and *Alouatta seniculus arctoidea* Cabrera, 1940, are homeologous to the same regions of human chromosomes #3 and #15 (Consigliere et al. 1996, 1998, Mudry et al. 2001, de Oliveira et al. 2002).

The phylogenies proposed so far for *Alouatta* have used either molecular markers (γ^1-globin (Meireles et al. 1999), Mt ATP synt 8 and 6, Mt cyt b, CAL and RAG1 (Cortés-Ortiz et al. 2003)) or chromosomal characters (de Oliveira et al. 2002). However, the combination of different variables can improve the phylogenetic sig-

Table 1. Cytogenetic characteristics of howler monkeys (*Alouatta*).

Species	2N	Sex Chromosome Systems	References
A. belzebul	♀50 ♂49	$X_1X_1X_2X_2/X_1X_2Y$	Armada et al. 1987§
A. s. seniculus	♀♂47 to 49†	XY	Yunis et al. 1976
A. s. stramineus	♀♂47 to 49†	$X_1X_1X_2X_2/X_1X_2Y_1Y_2$	Lima and Seuánez 1991§
A. s. arctoidea	♀44 ♂45‡	$X_1X_1X_2X_2/X_1X_2Y_1Y_2$	Stanyon et al. 1995
A. sara	♀♂ 48 to 51†	$X_1X_1X_2X_2$ / X_1X_2Y	Minezawa et al. 1985
	♀♂50	$X_1X_1X_2X_2$ / $X_1X_2Y_1Y_2$	Stanyon et al. 1995
A. macconnelli	♀♂ 47 to 49†	$X_1X_1X_2X_2/X_1X_2Y_1Y_2$	Lima et al. 1990
A. caraya	♀♂52	XX/XY	Egozcue and De Egozcue 1966, Mudry et al. 1984, 1994
		$X_1X_1X_2X_2/X_1X_2Y_1Y_2$	Rahn et al. 1996§, Mudry et al. 1998§, 2001§
A. palliata	♀♂56	XX/XY	Torres and Ramírez 2003
	♀54 ♂53	$X_1X_1X_2X_2/X_1X_2Y$	Ma et al. 1976 Solari and Rahn 2005§
A. pigra	♀♂58	$X_1X_1X_2X_2/X_1X_2Y_1Y_2$	Steinberg et al. 2008§
A. guariba guariba	♀50 ♂49	XX/XY	Koiffmann and Saldanha 1974
	♂49	X_1X_2Y	de Oliveira et al. 1995
	♀50♂49	$X_1X_1X_2X_2X_3X_3$ / $X_1X_2X_3Y_1Y_2$	de Oliveira et al. 2002
A. guariba clamitans	♀46 ♂45	XX/XY	de Oliveira et al. 1995
		$X_1X_1X_2X_2/X_1X_2Y$	de Oliveira et al. 1998
		$X_1X_1X_2X_2X_3X_3$ / $X_1X_2X_3Y_1Y_2$	de Oliveira et al. 2002
A. nigerrima	♀50	XX	Armada et al. 1987
A. coibensis	ND	ND	---

†These differences are due to the presence of microchromosomes (1 to 3 per nuclei); ‡Differences due to a variation in microchromosome number between sexes. ND: not yet cytogenetically characterized. §Meiotic studies performed to corroborate the sex chromosome system.

nal due to the possible common shared history of different datasets. At the same time, this combination can increase the support of a tree, since different characters evolve at particular rates and will support different parts of the tree (Kluge 1989, Whittaker et al. 2007).

In the present contribution, howler species were karyologically compared and FISH analyses were carried out to corroborate the homeology of the sex chromosome systems among them. Using these data and molecular data obtained from the literature, a phylogenetic analysis combining them in a single matrix was performed.

Methods

Sampled specimens: A total of 29 adult specimens of both sexes of four species of howlers, both from captivity as well as from the wild within their natural geographical distribution, were analyzed: *Alouatta caraya* (9 ♂ and 6 ♀), *A. guariba clamitans* (1 ♂), *A. pigra* (6 ♂ and 5 ♀) and *A. palliata* (2 ♂).

The origin of the animals was as follows:

Argentina

A. caraya, 1 ♂ from Corrientes Zoo, Corrientes; 1 ♂ from Ecological Park "El Puma", Misiones; 1 ♂ and 2♀ from Mendoza Zoo, Mendoza; 6 ♂ and 4 ♀ from the Black Howler Monkey Reeducational Center, La Cumbre, Córdoba.
A. g. clamitans, 1 ♂ from "Güira-Oga", Misiones.

Mexico

A. pigra: 4 ♂ and 4 ♀ were sampled in the wild in Campeche, Yucatán Península; 2 ♂ and 1 ♀ from San Juan de Aragón Zoo, Mexico City.
A. palliata: 1 ♂ from San Juan de Aragón Zoo, Mexico City; 1 ♂ from Chapultepec Zoo, Mexico City.

Classical cytogenetic analysis

Chromosome preparation: Peripheral blood samples were collected from all animals with previously heparinized disposable syringes. Lymphocytes were cultured for 72 h at 37 °C following Mudry (1990). At least 50 metaphases were analyzed to determine the diploid number (2N) at 1000×. Metaphase spreads were treated with G-Wright banding (Steinberg et al. 2007). At least 10 G-banded metaphases with the species diploid number (2N) were photographed with a Leica DFC 340 FX camera. Chromosomes were arranged according to previously described karyotypes using Photoshop CS (Adobe) and the species assignation of each specimen was corroborated.

Analysis of homeologies: For *A. caraya* and *A. g. clamitans*, the homeologies with human chromosomes and the homeologies with the other South American howlers are well known (Consigliere et al. 1998, Mudry et al. 2001, de Oliveira et al. 2002, Stanyon et al. 2011). The G-banded chromosomes of *A. pigra* and *A. palliata* were first compared with those of *A. caraya* and *A. g. clamitans*. We took *A. caraya*'s karyotype as the reference for the comparisons with Mesoamerican howlers (Mudry et al. 2001, Szapkievich and Mudry 2003). To compare homeologies, the G-banded metaphases obtained for *A. caraya*, *A. g. clamitans*, *A. pigra* and *A. palliata* were also compared with those published for *A. g. guariba* (de Oliveira et al. 2002, Stanyon et al. 2011), *A. macconnelli* (de Oliveira et al. 2002), *A. s. arctoidea* (Consigliere et al. 1996), *A. belzebul* (Armada et al. 1987, Consigliere et al. 1998) and *A. sara* (Consigliere et al. 1996).

Cytomolecular study

FISH analysis with human chromosome painting probes #3 and #15 was used as a tool to confirm the identity of the sex chromosome systems in howlers. Whole chromosome painting probes for human chromosomes #3 (red), #15 (green), #21 (green), X (green) and Y (red) (PCT3 Cy3, PCT15 FITC, PCT21 FITC, PCTX FITC, PCTY

Cy3, LEXEL S.R.L., Buenos Aires, Argentina) were used for FISH analysis on the metaphases of *A. pigra*, *A. caraya*, *A. g. clamitans* and *A. palliata*. *Homo sapiens* (HSA) metaphases were used as a positive control of hybridization. The HSA3/21 syntenic association, considered ancestral in mammals and conserved in most primate species (Müller et al. 2000), was analyzed simultaneously as a control of synteny conservation. Human X and Y chromosomes were also tested.

FISH was performed according to the supplier's instructions (LEXEL S.R.L., Buenos Aires, Argentina). Slides were counterstained with DAPI (Sigma) and analyzed with a Leica DMLB fluorescence microscope. Chromosome images were obtained with a Leica DFC 340 FX camera. Images were processed with Image Pro-Plus 4.5 (Media Cybernetics Inc.).

Our results were compared with those previously described (Consigliere et al. 1996, 1998, Mudry et al. 2001, de Oliveira et al. 2002, Stanyon et al. 2011).

Phylogenetic analysis

Chromosomal dataset: We used data obtained from the comparisons of G-banding patterns and the analysis of chromosomal syntenic associations, both from the present study and from previous reports (Consigliere et al. 1996, 1998, García et al. 2001, 2002, Mudry et al. 2001, de Oliveira et al. 2002, Amaral et al. 2008, Stanyon et al. 2001, 2011). We considered the structural changes as characters. The pattern observed before and after their occurrence, i.e. their presence or absence, was considered as the character states. The matrix (see Appendix 1) was produced taking into consideration the characters proposed by Neusser et al. (2001) and modified for howlers by de Oliveira et al. (2002). These authors used an abbreviated nomenclature for ancestral Platyrrhini chromosome forms with a correspondence in the human karyotype. In the present contribution, for the character nomenclature, we refer directly to the human G band ideogram (Table 2). New characters were obtained from our karyological comparisons and introduced in the chromosomal dataset.

Molecular dataset: The sequences available in GenBank for the same species used in the G-banding pattern and FISH comparisons were taken into to choose the molecular marker. The only molecular marker that fullfiled all the requirements was cyt b. The sequences used were (Genbank Accession Numbers): *A. belzebul* (AY374348.2), *A. caraya* (AY374359.2), *A. s. arctoidea* (AY065886.1), *A. sara* (AY065887.1), *A. macconnelli* (AY065888.1), *A. g. guariba* (AY065899.1), *A. g. clamitans* (DQ679782.1), *A. pigra* (AY065884.1), *A. palliata* (AY065879.1) (Cortés-Ortiz et al. 2003, Harris et al. 2005, Lorenz et al. 2005, Nascimento et al. 2005, Casado et al. 2010). *Cebus apella* Linnaeus, 1758 (FJ529102.1) and *Lagothrix lagotricha* Humboldt, 1812 (AY671799.1) were used as outgroups. *C. apella*, from the Cebidae family, was taken as an outgroup species, since it is accepted that this species presents the most ancestral karyotype within Platyrrhini (Clemente et al. 1990, García et al. 2000). *Lagothrix lagotricha*, also a member of the Atelidae family, was chosen as the second outgroup to test the monophyly of the group. All sequences were aligned using CLUSTALW (Thompson et al. 1994).

Table 2. Human chromosome syntenic association considered as characters and used to construct the binary matrix of chromosomal homeologies among howler monkeys (modified from Neusser et al. 2001, de Oliveira et al. 2002).

1. 1p21-pter/1p12-21
2. 5q31.3-qter/ 7p22; q11 q21
3. 5pter-q31.2/5q31.3-qter
4. 2pter q12/16q
5. 4q31.3-qter/4q23-q31.2
6. 4q23-q31.2/4pter-q22
7. $(10q/16p)_3$
8. 6
9. 8p/18
10. 15q21.3-q24/15q13-q21.2
11. 15q11 q13; q25 qter
12. 7p21 p11; q11 q21; q22 qter
13. 8q
14. 12
15. 11
16. 13
17. 9
18. 3pter p24; p21 p12; q12 q13; q27 qter
19. 3p24 q21; q13 q26
20. 1q32 qter
21. 1q21 q31
22. 3p12/21
23. 10p
24. 22
25. X
26. Y
27. 5pter-q31.2
28. $(5q31.3\text{-}qter/7p22; q11\ q21)_2$
29. 1p21-pter
30. 1p12-21
31. 4q31.3-qter
32. 4q23-q31.2/15q13-q212
33. 4pter-q22
34. 14/15q21.3-q24
35. $(10q/16p)_2/(10q/16p)_1$
36. 15q11 q13; q25 qter/Y
37. 2pter q12
38. 16q
39. 3p24 q21; q13 q26/ 15q11 q13; q25 qter
40. 11/5pter-q31.2
41. 5pter-q31.2/7p22; q11; q21
42. 12/9
43. 1p21-pter/2pter q12
44. 16q/4pter-q22
45. 22/14
46. 2q13 qter/20
47. 2q13 qter/4q23-q31.2
48. 8q/2q13 qter
49. 7p22; q11; q21/8q
50. 7p22; q11; q21/8q
51. 17/2pter q12/12
52. 2pter q12/12
53. 1q32 qter/11
54. 1q32 qter/$(11/5pter\text{-}q31.2)_2$
55. $(11/5pter\text{-}q31.2)_2$
56. 18/14
57. 3pter p24; p21 p12; q12 q13; q27 qter/15q21.3-q24
58. 3pter p24; p21 p12; q12 q13; q27 qter/15q21.3-q24/16q
59. 15q21.3-q24/16q
60. 17/10p
61. 17/10p/19
62. 10p/19
63. 22/20
64. 22/20/1q21 q31
65. 20/1q21 q31
66. $(11/5pter\text{-}q31.2)_3$
67. 1p12-21/8p
68. 7p21 p11; q11 q21; q22 qter /14/15q21.3-q24
69. $(10q/16p)_2$
70. $11/(10q/16p)_2$
71. 10q/16p
72. 10q/16p/4pter-q22
73. 10p/10q/16p/4pter-q22
74. 10p/10q/16p
75. 19/13
76. 19/22
77. 22/1p21-pter
78. 19/22/1p21-pter
79. 2q13 qter/4q23-q31.2
80. 6/1p12-21
81. Y/15q11 q13; q25 qter/ 3p24 q21; q13 q26
82. 1p12-21/5pter-q31.2/7p22; q11; q21/5q31.3-qter/ 7p22; q11 q21
83. 9/22
84. 17/11
85. 3pter p24; p21 p12; q12 q13; q27 qter/8p
86. 15q21.3-q24/1q32 qter
87. 16q/15q21.3-q24/1q32 qter
88. 4pter-q22/1p12-21
89. 14
90. 2pter q12/4pter-q22
91. 15q13-q21.2/7p22; q11; q21/5q31.3-qter/ 7p22; q11; q21
92. 6/15q21.3-q24
93. 14/1p12-21
94. 6/15q21.3-q24/14/1p12-21
95. 17/8p/18
96. 22/10q/16p
97. 2q13 qter/11
98. $(10q/16p)_2$/1q21 q31/20
99. Y/7

/: separates the chromosomal segments that constitute an association. $(\ )_n$: n= number of repeats in the segment

Phylogeny: A Maximum Parsimony phylogeny using the exhaustive search option was obtained with PAUP 4.0 software (Phylogenetic Analysis Using Maximum Parsimony, (Swofford 2002)), for each separate partition and the combination of both the chromosomal and molecular datasets. All characters had the same weight, based on the premise that chromosome rearrangements occur by equal chance (de Oliveira et al. 2002, Dobigny et al. 2004). The relative stability of nodes was assessed by bootstrap estimates (Felsenstein 1985) based on 200 iterations. Each bootstrap replicate involved a heuristic parsimony search with 10 random taxon additions and tree-bisection reconnection (TBR) branch swapping.

Results

Classical cytogenetic analysis

Karyological analysis: The cytogenetic characterization of the *Alouatta* specimens showed diploid numbers, sex chromosome systems and G-bandings patterns in agreement with the ones previously described for each species. Figures 1a and 1b show all the comparisons performed.

Chromosomal homeologies between A. caraya and A. palliata: The chromosomal rearrangements that could explain the homeologies were grouped in two categories: 1) *A. palliata* chromosomes with no rearrangements with respect to *A. caraya* chromosomes: 3, 5, 6, 7, 8, 9, 10, 13, 14, 16, 17, 19, 20, 21, 22, 24, 25 and X_1; 2) *A. palliata* chromosomes with more than one rearrangement with respect to *A. caraya* chromosomes: 1, 2, 4, 11, 12, 15, 18 and 23. No homeologies were allocated for *A. palliata* chromosome 26 and chromosome arms 4q and 2p using the level of resolution of the classical cytogenetic techniques applied. The rearrangements detected between the *A. caraya* and *A. palliata* karyotypes included at least seven fissions/fusions, two paracentric inversions and one deletion. *A. caraya* chromosome 7 (X_2 in males) shares homeology with two *A. palliata* chromosome pairs, 23 and 18, which are not the ones involved in the sex chromosome system in *A. palliata*. The *A. palliata* chromosomal pair 19 (X_2 in males) shares homeology with chromosome 14 of *A. caraya*.

Chromosomal homeologies between A. caraya and A. pigra: The chromosomal rearrangements that could explain the homeologies were grouped in two categories: 1) *A. pigra* chromosomes with no rearrangements with respect to *A. caraya* chromosomes: 2, 5, 6, 7, 8, 10, 15, 16, 17, 20, 22, 23, 25, 28 and X_1; 2) *A. pigra* chromosomes with more than one rearrangement with respect to *A. caraya* chromosomes: 1, 3, 4, 9, 11, 13, 18, 19, 24, 26 and 27. No homeologies were allocated for *A. pigra* chromosomes $4p_{prox}$, 12, 14 and 21 using the level of resolution of the classical cytogenetic techniques applied. The rearrangements detected between the *A. caraya* and *A. pigra* karyotypes included at least 12 fissions/fusions, two paracentric inversions, two translocations and one deletion. *A. caraya* chromosome 7 (X_2 in males) shares homeology with two *A. pigra* chromosome pairs, 26 and 19, which are not the ones involved in the sex chromosome system in *A. pigra*. *A. pigra* chromosome 17 (X_2 in males) shares homeology with chromosome 14 of *A. caraya* (which in turn has homeology with HSA7).

Chromosomal homeologies among all howlers: The chromosomal homeologies found among all howlers are shown in Table 3 and Figures 1a and 1b. Results show that Mesoamerican howlers share several human chromosomal syntenic associations with South American ones: HSA15q13-q21.2/4q23-q31.2 and HSA16p/10q, shared with all howlers; HSA15q21.3-q24/14, shared with all howlers except *A. s. arctoidea* and *A. macconnelli*, and HSA8p/18, shared with all howlers except *A. s. arctoidea*. Two new chromosomal syntenic associations, HSA$4p_{ter}$-q22/9/11 and HSA15q21.3-q24/14/21q, were found for *A. pigra* in chromosomes 1 and 4q, respectively.

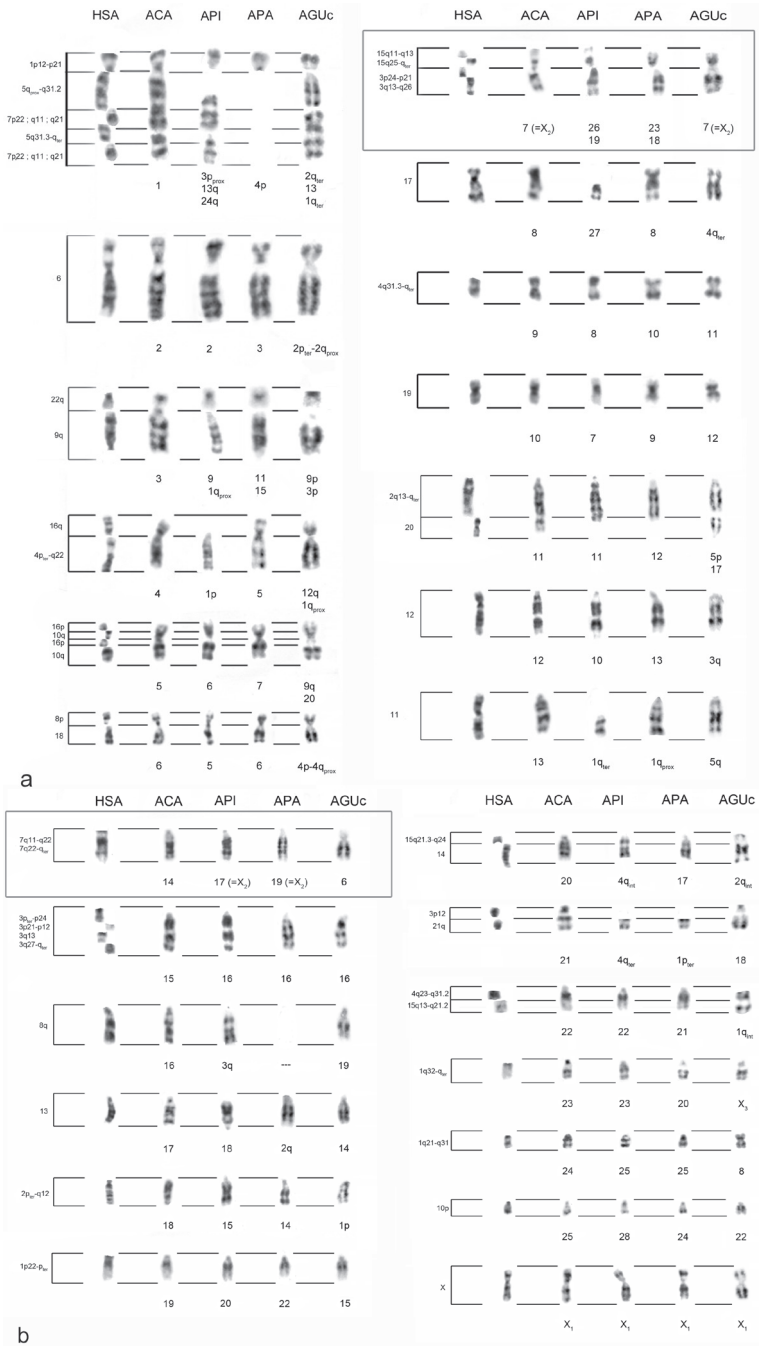

Figure 1. Comparison of *Homo sapiens* (HSA), *Alouatta caraya* (ACA), *A. pigra* (API), *A. palliata* (APA) and *A. guariba clamitans* (AGUc) G-banded chromosomes, taking *A. caraya*'s karyotype as reference. On the left, human chromosomal bands with homeology for its corresponding ACA chromosome segment are indicated. The boxes highlight the homeologies of the autosomes involved in the sex chromosome systems in these species **a** Comparison for ACA chromosomes #1 to #13 **b** Comparison for ACA chromosomes #14 to X_1

Table 3. Chromosomal homeologies between howlers, obtained from data both from this contribution and from previous reports. ACA: *A. caraya*; API: *A. pigra*; APA: *A. palliata*; AGU: *A. guariba*; ASEa: *A. s. arctoidea*; AMA: *A. macconnelli*; ASA: *A. sara*; ABE: *A. belzebul*.

Human Chromosomal associations†	ACA	API	APA	AGU	ASEa	AMA	ASA	ABE
1p12-p21	1	$3p_{prox}$	4p	$2q_{ter}$	$9q_{ter}$	$18q_{ter}$	$16q_{ter}$	23
$5q_{prox}$-q31.2				13	$3q_{ter}$	13	$1q_{ter}$	1q
7p22; q11; q21		13q		$1q_{ter}$	$12q_{prox}$	$12q_{ter}$	$13q_{ter}$	
5q31.3-q_{ter}					$8q_{ter}$		$7q_{ter}$	
7p22; q11; q21		24q			$1q_{ter}$		$4q_{ter}$	
6	2	2	3	$2p_{ter}$-q_{prox}	4	$18p_{ter}$-q_{prox}	5	4
						8		
22q	3	9	11	9p	$9q_{prox}$	$5p_{prox}$	$8q_{prox}$	$6p_{ter}$
9q		$1q_{prox}$	15	3p	13	15	11	2q
16q	4	1p	5	12q	$6q_{ter}$	3q	$9p_{ter}$	5
$4p_{ter}$-q22				$1q_{prox}$	11		14	
16p	5	6	7	9q	10	$3p_{prox}$	21	7
10q				20		2p	19	
16p								
10q								
8p	6	5	6	$4p_{ter}$-q_{prox}	$15q_{ter}$	6	$2q_{ter}$	8
18					$5q_{prox}$			
15q11-q13	7 (X_2)	26q	23	7 (X_2)	X_2	X_2	X_2	24
15q25-q_{ter}								
3p24-p21		19q	18					17 (X_2)
3q13-q26								
17	8	27q	8	$4q_{ter}$	$7q_{prox}$	7	$1p$-$1q_{prox}$	9
					2p			
4q31.3-q_{ter}	9	8	10	11	18	10	16p-q_{prox}	11
19	10	7	9	12	$7q_{ter}$	$5p_{ter}$	15p-q_{prox}	10
						4p		
2q13-q_{ter}	11	11	12	5p	$2p_{prox}$	$11q_{prox}$	$3q_{prox}$	12
20				17	$9q_{int}$	$16q_{prox}$	$8q_{int}$	
12	12	10	13	3q	2	14	6	2p
11	13	$1q_{ter}$	$1q_{prox}$	5q	$3q_{prox}$	2q	$1q_{int}$	1p
					$12q_{ter}$		$13q_{prox}$	
7 q11-q21	14	17 (X_2)	19 (X_2)	6	8	1q	7	13
7q22-q_{ter}								
$3p_{ter}$-p24	15	16	16	16	$6q_{prox}$	19	$2q_{prox}$	15
3p21-p12								
3q13								
3q27-q_{ter}								
8q	16	3q	--	19	$1q_{prox}$	$12q_{prox}$	$4q_{prox}$	16
13	17	18	2q	14	16	4q	12	14
$2p_{ter}$- q12	18	15	14	1p	$2q_{prox}$	17	10	3q
1p21-p_{ter}	19	20	22	15	14	5q	17	3p

Human Chromosomal associations†	ACA	API	APA	AGU	ASEa	AMA	ASA	ABE
15q21.3-q24	20	$4q_{int}$	17	$2q_{int}$	$6q_{int}$	1p	$9q_{prox}$	$6p_{prox}$-q_{ter}
14					$5q_{ter}$		18	
3p12	21	$4p_{ter}$	$1p_{ter}$	18	17	9	20	$18q_{ter}$
21q								
4q23-q31.2	22	22	21	$1q_{int}$	$1p_{ter}$	$1q_{ter}$	$3q_{ter}$	20
15q13-q21.2								
1q32-q_{ter}	23	23	20	X_3	$15q_{prox}$	21	$8q_{ter}$	19
1q21-q31	24	25	25	8	3p	$16q_{ter}$	$9q_{ter}$	21
10p	25	28	24	22	$7q_{int}$	$3p_{ter}$	$15q_{ter}$	22
X	X_1	X_1	X_1	X_1	X_1	X_1	X_1	X_1

†from pter to qter.

Cytomolecular study

In the *Homo sapiens* metaphases, the hybridization signals on chromosomes HSA3, HSA21, HSA15, HSAX and HSAY for chromosome painting probes #3 (red), #21 (green), #15 (green) (Figure 2a), X and Y (data not shown) were corroborated.

In *A. g. clamitans*, the signal for HSA21 was observed in $18q_{ter}$, the signal for HSA3 was observed in $18q_{prox}$ (thus corroborating the HSA3/21 synteny in *A. g. clamitans*), 16q, 7q (X_2 in males) and Y_2, and the signal for HSA15 was observed in 1_{int}, 2_{int}, 7p (X_2) and Y_1. This corroborates the HSA3/15 syntenic association to the multiple sex chromosome system $X_1X_1X_2X_2X_3X_3/X_1X_2X_3Y_1Y_2$ of this species (Figures 2b and 3b).

In *A. pigra*, the signal for HSA3 was observed in 16q and 19q, while that for HSA21 hybridized in $4p_{ter}$, thus indicating that the HSA3/21 synteny is not present in *A. pigra* (Figures 2c and 3c). The probe for HSA15 hybridized in *A. pigra* metaphases in $4q_{ter}$, $22q_{ter}$ and 26q, showing that the HSA3/15 syntenic association is also absent. None of these *A. pigra* chromosomes is involved in the sex chromosome system of this species (Figures 2d and 3c).

In *A. caraya,* the signal for HSA21 was observed in $21q_{ter}$, whereas that for HSA3 was observed in $21q_{prox}$, thus confirming the conservation of the HSA3/21 synteny. HSA15 hybridized in 7p (X_2 in males) and Y_{1ter}, and HSA3 in 7q and Y_{1prox}, exhibiting the HSA3/15 syntenic association in the sex chromosome system $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ (Figure 3a).

A. palliata showed a pattern similar to that of *A. pigra* (therefore Figure 2 illustrates only the latter). HSA3 hybridized in 16q and 18q, HSA21 hybridized in $1p_{ter}$ and HSA15 in $2q_{ter}$, $21q_{ter}$ and 23q (Figure 3d). Both the HSA3/21 and HSA3/15 syntenic associations are absent in *A. palliata* and chromosomes with homeology to HSA3 and HSA15 are also not involved in the sex chromosome system of this species.

The probe for the human X chromosome showed positive hybridization signal in X_1 of all the species analyzed. The probe for the human Y chromosome did not hybridize in any of the howler species (data not shown).

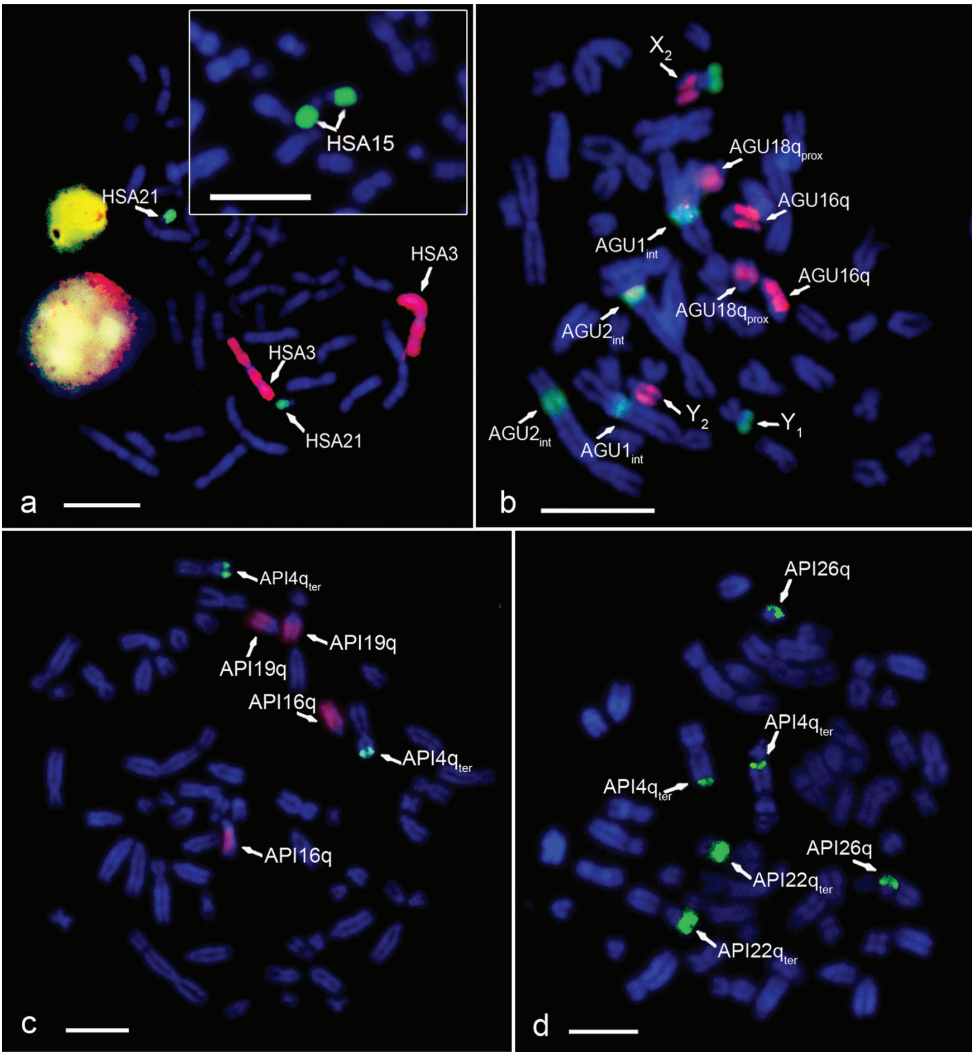

Figure 2. Analysis of the conservation of the HSA3/21 and HSA3/15 syntenic chromosomal associations in howlers (bar=10 μm). The arrows indicate the chromosomes with positive FISH signal **a** *Homo sapiens* partial metaphase hybridized with probes HSA21 (green) and HSA3 (red) (control of the hybridization). Inset: *Homo sapiens* partial metaphase hybridized with HSA15 (green) **b** AGUc metaphase hybridized with HSA15 (green) and HSA3 (red) **c** API metaphase hybridized with HSA3 (red) and HSA21 (green) **d** API metaphase hybridized with HSA15 (green).

Phylogenetic analysis

The data obtained from the G-banding pattern and FISH homeologies, together with cyt b sequences obtained from previous reports, were used as the basis to perform a cladistic analysis. The HSAY/7 association, corresponding to the Y-autosome translocation that gave rise to the multivalents observed in *A. pigra* and *A. palliata*, was added

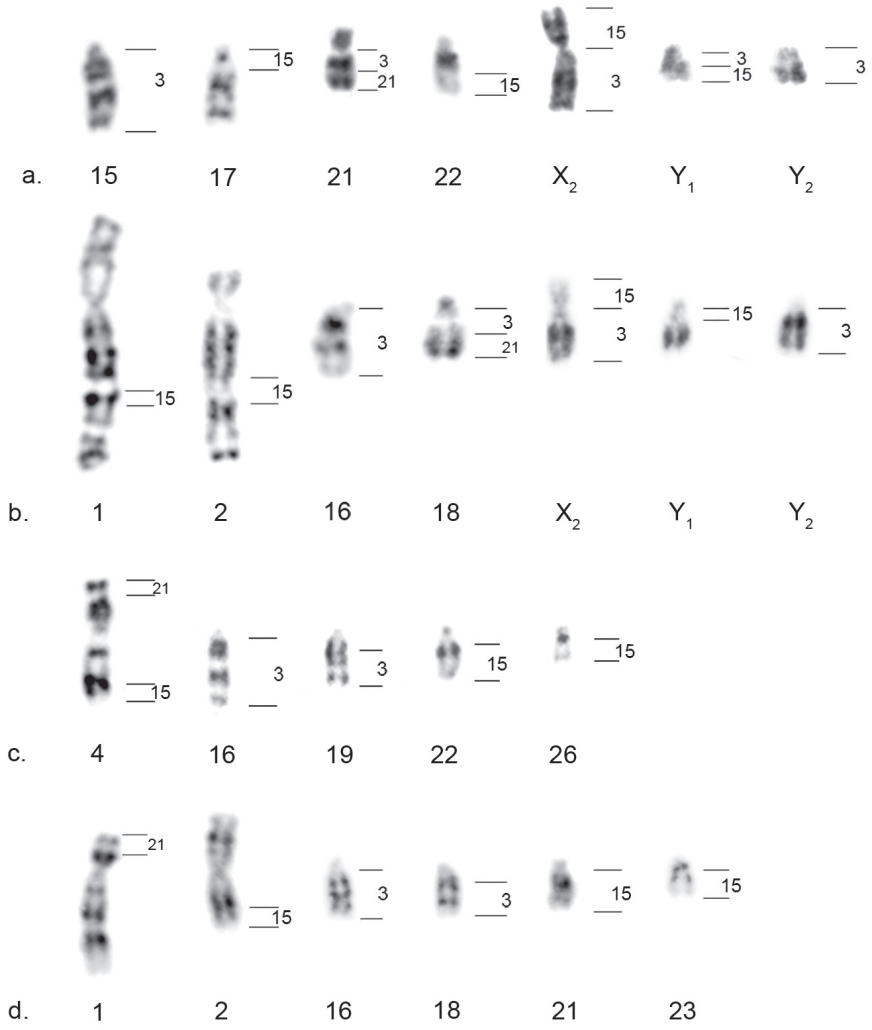

Figure 3. Howler monkeys G-banded chromosomes with positive signal for the human chromosome painting probes analyzed. On the right, the hybridization pattern of human chromosomes #3, #21 and #15. **a** *A. caraya* **b** *A. guariba clamitans* **c** *A. pigra* **d** *A. palliata*.

as an extra character to the original list (de Oliveira et al. 2002). The syntenic associations HSA4p$_{ter}$-q22/9/11 observed in chromosome 1 of *A. pigra* and HSA15q21.3-q24/14/21q observed in chromosome arm 4q were not included in the analysis, because, as autopomorphies for *A. pigra*, they are considered non-informative.

Three data matrices were obtained: one including only chromosomal data, another including only molecular data and the last one including both types of characters (chromosomal and molecular) in a single matrix (see Appendix 1).

Chromosomal partition: The analysis of chromosomal data resulted in 36 informative characters, 23 constant characters and 40 non-informative characters. After ana-

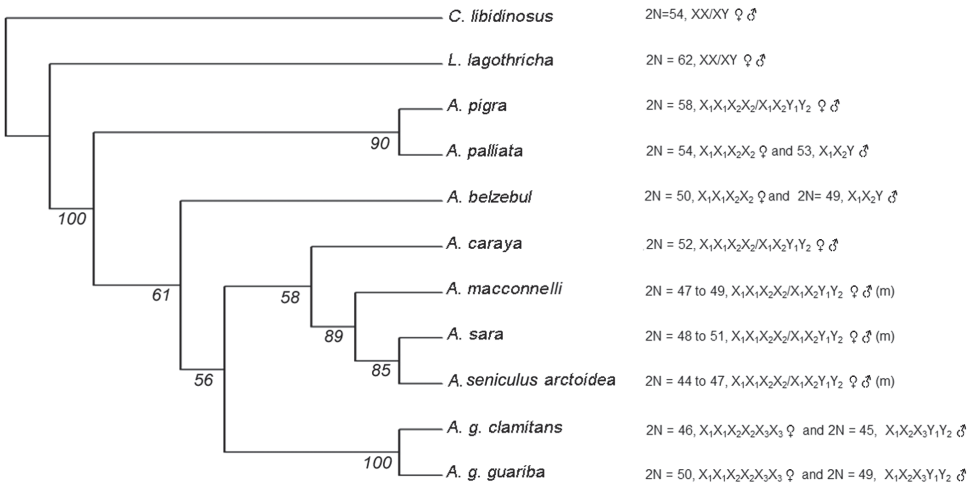

Figure 4. 50% majority consensus tree obtained by "bootstrap" for the combined analysis. Next to the name of each species, the diploid number (2N) and sex chromosome system is described. (m)=microchromosomes.

lyzing 704 trees, PAUP retained the two most parsimonious trees (Appendix 2: Figures Sa and Sb), both with a length of 87 (L = 87). The analysis using only the partition of chromosomal data did not resolve the node ((*A. palliata*, *A. pigra*), (*A. caraya*, *A. belzebul*), ((*A. g. clamitans*, *A. g. guariba*), (*A. macconnelli* (*A. sara*, *A. s. arctoidea*))), since it was established in a polytomy (Appendix 2: Figure Sc).

Molecular partition: Heuristic analysis of cyt b gene sequences, made from a total of 800 characters, produced 109 informative characters, 551 constant characters and 140 non-informative characters. After analyzing 916 trees, PAUP retained a single most parsimonious tree (Appendix 2: Figure Sd), with a length of L = 366. The analysis using only molecular data did not resolve the node (*A. sara*, *A. macconnelli*, *A. s. arctoidea*, *A. caraya*), which was established as a polytomy different from that described from chromosomal data.

Combined analysis: The heuristic analysis of the combined data showed a total of 899 characters, 145 of which were informative, 180 non-informative and 574 constant. After analyzing 684 trees, PAUP retained only one, with a length of L=460 (Figure 4). This type of analysis allowed us to solve all the nodes, resulting in a fully resolved tree.

Discussion

We present the first phylogenetic study using a combined analysis of chromosomal and molecular characters in Ceboidea to contribute to the characterization of the speciogenic processes in howler monkeys. The homoplasy distribution is likely to be different

in each dataset because these are subject to different constraints. Therefore, when different datasets are analyzed simultaneously, the signal common to all of them is more likely to overwhelm the homoplasy signal on the data (Kluge 1989).

In primates, few studies have compared and taken into account more than one type of character. Bonvicino et al. (2001) superimposed chromosomal information on the phylogeny obtained from molecular characters. Villalobos et al. (2004) used numerical and metric values that describe the karyotype, such as diploid number (2N) and fundamental number (FN), in a combined phylogenetic analysis with morphological characters. However, these values (2N, FN, etc) can be identical simply by chance and, if interpreted in a phylogenetic context, may be spurious indicators of relatedness (Dobigny et al. 2004). Our encoding strategy (using the rearrangements as characters) is quite similar to that used for morphological data but in cytogenetics one can retrieve information on the mutational event itself, something that is clearly not available to morphologists. As such, chromosomal mutations that accumulate along the tree are comparable to transitions, transversions, and insertions/deletions in molecular phylogenies (Dobigny et al. 2004). Our combined phylogeny evidences the accuracy of this encoding strategy.

In all the above-mentioned contributions, the $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex chromosome system was proposed as the ancestral condition for the genus. However, as discussed by Solari and Rahn (2005), the $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system is simpler and is present in other genera of Neotropical Primates, such as *Aotus* Illiger, 1811, *Callimico* Miranda Ribeiro, 1912, and *Cacajao* Lesson, 1840 (Ma et al. 1976, Seuánez et al. 1989, Moura-Pensin et al. 2001). The $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system as an ancestral state appears to be a more parsimonious hypothesis. Moreover, since Mesoamerican howlers (*A. pigra* and *A. palliata*) were poorly karyologically characterized at the time, data on these howlers are missing in all previous contributions.

Homeology analysis

The karyotypes of *A. pigra* and *A. palliata* share more syntenic associations with those of *A. caraya* and *A. belzebul* than with those of the "*A. seniculus* group" (*A. s. arctoidea*, *A. sara*, *A. macconnelli*, denominated as such because they were once all subspecies of *A. seniculus* together with *A. s. seniculus* Linnaeus, 1766, and *A. s. stramineus* Hill, 1962). This supports the basal grouping of the *A. pigra-A. palliata* Mesoamerican clade and the basal grouping of *A. belzebul* among South American howlers.

The chromosomal comparisons showed that *A. pigra* and *A. palliata* conserved the HSA8/18 and HSA14/15 syntenies, considered ancestral for Platyrrhini (Stanyon et al. 2008), as well as the HSA10/16/10/16 syntenic association, ancestral for Atelidae (de Oliveira et al. 2002), but lost the HSA3/21 synteny, ancestral for mammals (Müller et al. 2000, Müller 2006).

According to our combined phylogeny, the HSA2/20 and HSA5/7/5/7 syntenic associations, previously considered as synapomorphies of the *A. caraya-A. belzebul* group (de Oliveira et al. 2002), would be homoplasies (parallelism). The HSA16/4

syntenyc association would be ancestral for the genus and might either be absent in *A. pigra*, *A. guariba* and the *A. seniculus* group or might constitute a parallelism among *A. palliata*, *A. caraya* and *A. belzebul*.

Like the HSA3/21 synteny, the HSA3/15 syntenic association, involved in the sex chromosome systems in South American howlers, is not present in Mesoamerican ones. This syntenic association of human 3/15 chromosomal segments has been described in other Atelidae species such as *Ateles geoffroyi* Kuhl, 1820 and *Ateles belzebul hibridus* Geoffroy, 1829, although not associated with the sex chromosome system (Morescalchi et al. 1997), but not observed in *Lagothrix* Geoffroy, 1812, and *Brachyteles* Spix, 1823 (Stanyon et al. 2001, de Oliveira et al. 2005). This association has not been observed in other genera of Neotropical Primates such as *Cebus libidinosus* Spix, 1823, or *Saimiri boliviensis boliviensis* Geoffroy & Blainville, 1834 (Mudry et al. 2001). Therefore, the HSA3/15 syntenic association either could be interpreted as the ancestral condition for the family Atelidae, where the association with multiple sex chromosomes would be an evolutionary novelty (apomorphy) in howlers and the loss of the association a apomorphy for the *Lagothrix* and *Brachyteles* group, or could have appeared independently in *Alouatta,* involved in the Y-autosome translocation, and in *Ateles*, not involved in the sex chromosome system (de Oliveira et al. 2005). However, our results suggest that the HSA3/15 syntenic association is not an ancestral condition for *Alouatta,* since the most basal species (see Figure 4) *A. pigra* and *A. palliata* (this contribution) and *A. belzebul* (Consigliere et al. 1996) do not possess this association.

Possible origin of the multivalents

Taking into consideration the data obtained, a hypothesis can be proposed regarding the origin of the sex chromosome systems in the genus. Within the family Atelidae, with the exception of *Alouatta*, all genera have an XX/XY sex chromosome system. Therefore, it can be considered that the *Alouatta* ancestor possessed a chromosomal sex determination XX/XY, prior to the biogeographic separation of Mesoamerican and South American groups (see below). After this separation, both groups independently acquired the multiple sex chromosome systems currently observed through independent Y-autosome translocations.

The sex chromosome system $X_1X_1X_2X_2/X_1X_2Y$ would have arisen independently in the lineages of Meso and South American howlers by a Y-autosome translocation (Figure 5a). In males, two fissions, one in Yp_{ter} and another in q_{prox} of the autosomal pair involved (Aq_{prox}), followed by translocation of Aq_{prox} to Yq-p_{prox}, formed the new chromosome Y_1. The Yp_{ter} segment is lost and the proximal region of the fissioned autosome either is lost or, in certain howler species, could have given rise to microchromosomes (e.g.: *A. seniculus* (Yunis et al. 1976, Lima and Seuánez 1991, Torres and Leibovici 2001), *A. sara* (Minezawa et al. 1985) and *A. macconnelli* (Lima et al. 1990)). The homologous autosomal pair involved in the translocation is the one now denominated X_2. In the case of South American howlers, the autosomal pair involved

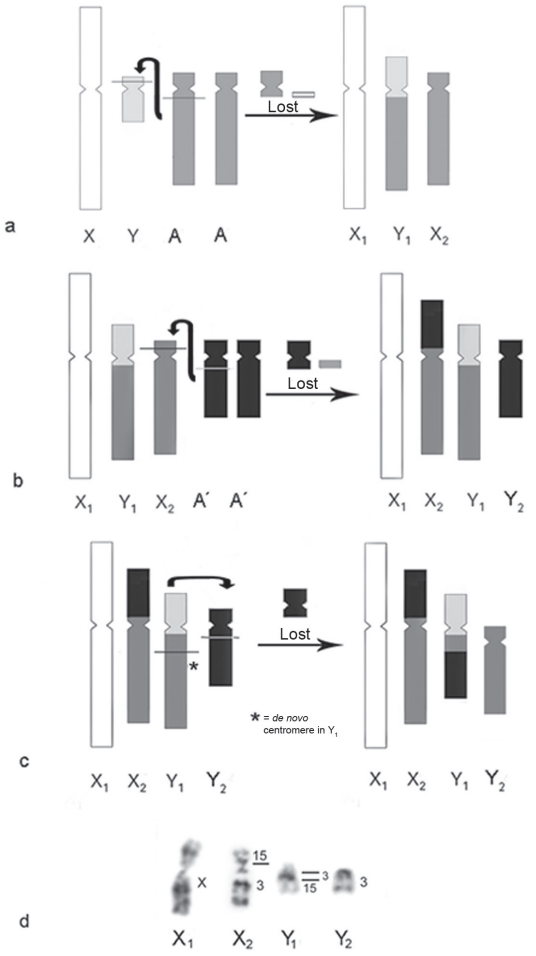

Figure 5. a Possible origin for $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system in the genus *Alouatta*. The ancestral X chromosome is shown in white, the ancestral Y chromosome in light gray and the autosomal pair (A) in dark gray. Two fissions occurs, one in Yp_{ter} and another in q_{prox} of the autosomal pair involved (Aq_{prox}). The translocation of Yq-p_{prox} to the Aq formed the new Y_1 chromosome and the homolog of the autosomal pair involved in the translocation is now denominated X_2. The Yp_{ter} acentric fragment is lost and the rest of the autosome (Ap and Aq_{prox}) could either be lost or remain as a microchromosome in some howlers **b** Possible origin for the $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex chromosome systems from a $X_1X_1X_2X_2/X_1X_2Y$ system. The ancestral X is shown in white, the ancestral Y in light gray, the autosomal pair involved in the first translocation (A) in dark gray and the autosomal pair (A´) involved in the formation of this new sex chromosome system in black. Simultaneous breaks in X_2p_{prox} and A`$q_{prox}$ followed by the translocation of the rest of the A`q to X_2p_{prox} give origin to the new X_2 chromosome. The X_2p_{ter} acentric fragment could be lost and the rest of the autosome (A´) could either be lost or remain as a microchromosome in some howlers. The homolog to the autosomal chromosome in question is now identified as Y_2 **c** Simultaneous breaks in Y_1q and Y_2q and a translocation between Y_1 and Y_2 further explain the hybridization pattern observed in the sex chromosome systems of South American howlers. A *de novo* centromere arises in the remains of the old Y_1 (now Y_2). The remains of the old Y_2 could either be lost or remain as a microchromosome in some howlers **d** Hybridization pattern in South American howlers.

would share homeology with HSA3, whereas in the Mesoamerican species it would share homeology with HSA7.

From this $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system, an $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ system could have arisen from a new translocation (Figure 5b). Under this hypothesis, simultaneous breaks in X_2p_{prox} and q_{prox} of another autosome ($A'q_{prox}$), followed by the translocation of most of the A´q arm to X_2p_{prox}, gave rise to the new X_2 chromosome. The X_2p_{prox} acentric fragment is lost and the rest of the autosome (A´) either is lost or could have remained as a microchromosome in some howler species (see above). The chromosome homologous to the autosome in question (A`) became Y_2. In the case of South American howlers, the new autosomal pair involved in the sex chromosome system would share homeology with HSA15. A further translocation between Y_1 and Y_2 (Figure 5c) would explain the hybridization pattern of the segments with homeology to human chromosomes 3 and 15 observed in the sex chromosome systems $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ in South American howlers (Figure 5d).

On the other hand, in the Mesoamerican species, the $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex chromosome system could have arisen either as described in Figure 5b (with the autosomal pair involved sharing homeology with a human chromosome not yet identified by G-banding pattern) or by a fission in Y_1 that would have given rise to two chromosomes, the new Y_1 (containing the segment corresponding to the ancestral Y chromosome) and Y_2 (containing a portion of the autosomal pair with homeology to HSA7). This last hypothesis would require a centromeric activation in Y_2.

However, considering the observation of the independent origin of the multiple sex chromosome systems in these two groups of howlers, the possibility of an independent origin of the $X_1X_1X_2X_2/X_1X_2Y$ and $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex chromosome systems within the Meso and South American groups cannot be ruled out until further studies.

It can be considered that multiple sex chromosome systems would be an extremely rare phenomenon due to complication in meiosis. Extreme cases are platypus and echidna, with a large number of sex chromosomes (Bick and Jackson 1967, Renz et al. 2007). In primates, multiple sex chromosome systems are even more infrequent. Moreover, *Alouatta* would be the first case where an independent origin of multiple sex chromosome systems is described. In other taxa, such as *Drosophila* (Flores et al. 2008), Erythrinidae fishes (Cioffi et al. 2013) and mole-rats (Deuve et al. 2006), a few cases have been observed, but these descriptions are still scarce.

Phylogeny of *Alouatta*

The chromosomal homeologies and FISH analysis were used to construct a data matrix for the phylogenetic analysis. For comparison purposes, independent phylogenetic reconstructions were performed with each type of partition (Appendix 2: Figure Sa, b, c and d), along with the combined analysis of the two datasets (Figure 4). The chromosome partition grouped *A. caraya* and *A. belzebul* as sister taxa, in agreement with that reported by de Oliveira et al. (2002), a relationship that was not observed in the other

two analyses, which grouped *A. caraya* with *A. sara*, *A. s. arctoidea* and *A. macconnelli* (although in the case of the molecular partition this relationship constituted a polytomy). This last species arrangement was also proposed by Nascimento et al. (2005) and Cortés-Ortiz et al. (2003) using molecular characters. In our molecular data partition (Figure Sd), *A. belzebul* was grouped with the clade of *A. guariba*, in agreement with that reported by Bonvicino et al. (2001) and Cortés-Ortiz et al. (2003). The three types of analyses agreed to place *A. sara*, *A. s. arctoidea* and *A. macconnelli* into a single group, although the molecular data partition did not resolve the relationships between them, as they formed a polytomy. The grouping of all those species ("*A. seniculus* group") was observed in all phylogenetic studies performed so far (see above). Another coincidence was that the Mesoamerican species were placed as a separate clade from other South American species and, as expected, the two subspecies of *A. guariba* in one group. All sets of taxa analyzed in this new approach were solved without polytomies only with the combined analysis, demonstrating the usefulness of incorporating more than one source of data for a more accurate elucidation of the relationships among current taxa.

The grouping of South American species as a separate group of the Mesoamerican group coincides with previous phylogenetic analyses using only molecular characters (Cortés-Ortiz et al. 2003, Ellsworth and Hoelzer 2006) and with the hypothesis of monophyletic origin of the Mesoamerican howlers previously proposed by Smith (1970). Smith's hypothesis holds that Mesoamerican howlers originated by an expansion of the geographic distribution of South American howlers after the formation of the Isthmus of Panama, estimated to be completed about 3 million years ago (Coates et al. 2004). However, other studies indicate that the rise of the isthmus was a process rather than an event (Knowlton and Weigt 1998), resulting in intermittent periods with connected and divided lands during the past 18 million years. Given this last fact, another hypothesis was postulated to explain the current geographic distribution of the species of the genus. Instead of a single colonization event, various founder events, either across the Isthmus of Panama during one of the periods in which the two Americas were connected or across islands in the Caribbean archipielago, could have occurred (Cortés-Ortiz et al. 2003, Ellsworth and Hoelzer 2006). Primate fossils have been found in Cuba and Jamaica, but the origin and relationships of these specimens with modern Platyrrhini are still under debate (Fleagle 1999, Gutiérrez Calvache and Jaimez Salgado 2007, Rosenberger et al. 2009, Cooke et al. 2011).

Independently of the biogeographic scenario under consideration, it is clear that the evolutionary history of Mesoamerican howlers is different from that of South American howlers, an assertion that would be supported by the evidence provided by our new data.

This contribution provides new useful information for the systematics of the genus *Alouatta*, while supporting the hypothesis of chromosomal evolution in primates as a speciogenic strategy. The combined analysis resolved the phylogenetic relationships between howler species of both American origins, as a first approach to the "Total Evidence" concept and towards clarifying the controversies related to the Taxonomy and Evolution of Ceboidea.

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All research reported in this manuscript has met the appropriate national and institutional guidelines for the legal acquisition and use of laboratory animals and authorized study of wild animals. The authors also adhered to the Guide for Care and Use of Experimental Animals, as promulgated by the Canadian Council of Animal Care, and to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of non-human Primates. The work which took place both in Argentina as in Mexico was done in accordance with the laws of these countries.

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Appendix 1

Data matrix. (doi: 10.3945/CompCytogen.v8i1.6716.app1) File format: Microsoft Word file (doc).

Explanation note: Data matrix contains: Chromosomal data matrix, Molecular data matrix and Combined data matrix.

Citation: Steinberg ER, Nieves M, Mudry MD (2014) Multiple sex chromosome systems in howler monkeys (Mammalia, Platyrrhini). Comparative Cytogenetics 8(1): 43–69. doi: 10.3897/CompCytogen.v8i1.6716 Data matrix. doi: 10.3897/CompCytogen.v8i1.6716.app1

Appendix 2

Supplementary Figure S. (doi: 10.3945/CompCytogen.v8i1.6716.app2) File format: Microsoft Word file (doc).

Explanation note: Figure S: a) and b) Most parsimonious trees obtained for the chromosomal partition c) 50% majority consensus tree obtained by "bootstrap" d) 50% majority consensus tree obtained by "bootstrap" for the molecular partition.

Citation: Steinberg ER, Nieves M, Mudry MD (2014) Multiple sex chromosome systems in howler monkeys (Mammalia, Platyrrhini). Comparative Cytogenetics 8(1): 43–69. doi: 10.3897/CompCytogen.v8i1.6716 Supplementary Figure S. doi: 10.3897/CompCytogen.v8i1.6716.app2

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Karyomorphometry on three polyploid species of *Arum* L. (Araceae, Aroideae)

Alessio Turco[1], Pietro Medagli[1], Antonella Albano[1], Saverio D'Emerico[2]

1 *Dept. of Biological and Environmental Sciences and Technologies, University of the Salento, Via prov.le Lecce-Monteroni 6, Lecce, Italy* **2** *Dept. of Plant Biology, University of Bari "Aldo Moro", Via Orabona 4, Bari, Italy*

Corresponding author: *Alessio Turco* (alessio.turco@unisalento.it)

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Abstract

In this study three polyploid *Arum* Linnaeus, 1753 species from Southern Italy were chromosomally investigated. *Arum italicum* Miller, 1768 was found to have 2n = 84 chromosomes and a karyotype composed of numerous asymmetric chromosomes. *Arum maculatum* Linnaeus, 1753 and *A. apulum* (Carano) P. C. Boyce, 1993 were found to have 2n = 56 chromosomes. In the examined taxa some chromosome pairs were characterized by the presence of weakly coloured Feulgen-stained segments. The karyotype morphology of *A. italicum* was found to be similar to that of *A. maculatum*, but the more asymmetrical karyotype and numerous weakly coloured Feulgen-stained segments observed in the former suggest the existence of more extensive rearrangements. In contrast, *A. apulum* was observed to have a symmetrical karyotype. The A_1, A_2 and SYi karyotype asymmetry indices are presented. The relationships between these taxa in terms of karyotype morphology and evolution are discussed.

Keywords

Allocyclic segments, karyotype asymmetry, karyotype evolution, *Arum apulum*, *Arum italicum*, *Arum maculatum*

Introduction

The high biodiversity of *Araceae* Jussieu, 1789, with ca. 109 genera and over 3700 species (Mayo et al. 1997), reflects their ability to occupy a wide range of environments. This family also displays a large variety of life forms, from epiphytic to aquatic, attesting to extensive adaptive radiation during the Cretaceous period (Chase et al. 2006, Anderson

and Janssen 2009). Some *Araceae* genera exhibit heat production (Minorsky 2003). Indeed, Lamark first noticed that the inflorescences of *Arum italicum* Miller, 1768, produced heat in 1778 (Meeuse 1973). It was subsequently shown that several *Araceae* taxa can produce heating up to 22°C above the environmental temperature (Meeuse 1959). This is related to the group's biology, as heat increases the volatilization rate of its odour, facilitating pollination (Dafni 1984). Chromosome counts have been conducted for 862 *Araceae* taxa, with the number varying from 2n = 10 for *Typhonium jinpingense* Z. L. Wang, H. Li & F. H. Bian, 2002 to 2n = 168 for *Arisaema heterophyllum* Blume, 1835 and *Typhonium eliosurum* (Bentham) O. D. Evans, 1961 (Cusimano et al. 2012 and references therein).

In this study we conducted a karyomorphometric survey of *Arum* Linnaeus, 1753, a small herbaceous genus containing about 28 species (Lobin et al. 2007), five of which are found among Italian vascular flora (Abbate et al. 2005, Conti et al. 2007). *Arum maculatum* Linnaeus, 1753 and *A. italicum* have rhizomatous tubers while *A. apulum* (Carano) P. C. Boyce, 1993 has a discoid tuber (Bedalov and Küpfer 2005). Bedalov and Küpfer (2005) suggested that the discoid tuber shape may represent the ancestral state of *Arum* with respect to the rhizomatous form, and this was confirmed by molecular studies conducted by Espìndola et al. (2010).

From a karyological point of view, the basic number for the *Arum* genus is x = 14 (Petersen 1993) with most of the species diploid rather than polyploid (Prime 1980). *Arum maculatum* and *A. apulum* are tetraploid (2n = 56), while *A. italicum* is hexaploid (2n = 84) (Marchi 1971, Beuret 1971, Bedalov et al. 2002, Lendel et al. 2006, Bedini et al. 2012). Most of the polyploid *Arum* taxa have been reported to occupy broader geographic ranges than their diploid counterparts (Bedalov 1981). The distribution of *Arum italicum* extends from the Caucasus through the Mediterranean region to the Atlantic coast (Bonnier 1931, Meusel et al. 1965, Dihoru 1970, Bedalov 1975). According to Meusel et al. (1965), Terpò (1973) and Bedalov (1981), *A. maculatum* is distributed across Central and Western Europe. The broader geographical range of *A. italicum* and *A. maculatum* with respect to diploids such as *A. pictum* Linnaeus filius, 1782 or *A. orientale* M. Bieberstein, 1808 (Prime 1980) may be therefore explained by their capacity to colonize new areas. However, the diploid *A. alpinum* Schott & Kotschy, 1851 has a very wide distribution (Bedalov and Fischer 1995) and the tetraploid *A. apulum* has a very limited distribution, restricted to Southern Italy (Puglia) (Carano 1934, Gori 1958, Bianco et al. 1994).

Cytological investigations of *Arum* chromosome numbers have sought to clarify its taxonomy (Gori 1958, Marchi et al. 1964, Beuret 1971, 1972, Marchi 1971, Bedalov 1975, 1981). D'Emerico et al. (1993) and Bianco et al. (1994) also described the karyotypes of six species for the genus, and found that the studied taxa all had a "basic karyotype" characterized by the presence of marker-chromosome pairs. Specifically, they noticed that the diploids' 14th pair is characterized by chromosomes with one satellite on the short arm and another on the long arm; this feature was also shown in pair 27 for *A. maculatum* and *A. apulum* (Bedalov et al. 1992, D'Emerico et al. 1993).

The purpose of this study is to acquire detailed new information on the karyomorphometry and chromosome structure of *A. italicum*, *A. maculatum*, and *A. apulum* from Southern Italy.

Materials and methods

Samples of *Arum italicum* were collected from various sites in Puglia and Lucania, while samples of *A. maculatum* were collected near Muro Lucano - Potenza (Lucania) and *A. apulum* near Quasano, Sammichele, Turi - Bari (Puglia) (Table 1). Only *A. apulum* and *A. italicum* are cultured in the Museo Orto Botanico di Bari (Bari). The nomenclature used for classification follows Boyce (1989).

Root-tips were pretreated in 0.3% aqueous colchicine at 20°C for two hours, and subsequently fixed for five min in a 5:1:1:1 (volume ratio) mixture of absolute ethanol, chloroform, glacial acetic acid and formalin. Hydrolysis was carried out at 20°C in 5.5 N HCl for 20 min (Battaglia 1957 a, b), then stained with Schiff's reagent. Root tips were squashed in a drop of 45% acetic acid.

The nomenclature used for describing karyotype composition followed Levan et al. (1964). The karyotype parameters were composed following D'Emerico et al. (1996) and evaluated by calculating haploid complement lengths, the SYi index introduced by Greilhuber and Speta (1976) and the A_1 and A_2 indices proposed by Romero Zarco (1986). The SYi index describes the average symmetry of the karyotype, A_1 is the intrachromosomal asymmetry index (i.e. the average position of the centromere in a karyotype) and A_2 is the interchromosomal asymmetry index (i.e. variation in chromosome length). As a standard procedure, chromosome metaphase plates from at least five cells were measured.

For Giemsa C-banding, a modification of Schwarzacher et al. (1980) was used, but unfortunately in these taxa C-Banding staining was unable to differentiate chromosomal or nuclear structures.

Results and discussion

This study provides new cytological information on three polyploid *Arum* taxa. The present analysis is in agreement with the sectional segregation based on tuber structure in the classification of the *Arum* genus suggested by Boyce (1989).

Table 1. *Arum taxa* investigated and origin of samples.

Taxon	Locality	Collector
Arum apulum	Apulia: Quasano (Bari)	Medagli and D'Emerico 13.IV.2010
	Apulia: Sammichele (Bari)	Medagli and D'Emerico 15.IV.2010
	Apulia: Turi (Bari)	Medagli and D'Emerico 15.IV.2010
A. italicum	Apulia: Quasano (Bari)	Medagli and D'Emerico 13.IV.2010
	Apulia: Sammichele (Bari)	Medagli and D'Emerico 15.IV.2010
	Apulia: Turi (Bari)	Medagli and D'Emerico 15.IV.2010
	Lucania: Matera	Medagli and D'Emerico 22.IV.2010
	Lucania: Grottole (Matera)	Medagli and D'Emerico 23.IV.2010
	Lucania: Pomarico (Matera)	Medagli and D'Emerico 23.IV.2010
A. maculatum	Lucania: Muro Lucano (Potenza)	Medagli and D'Emerico 27.V.2010

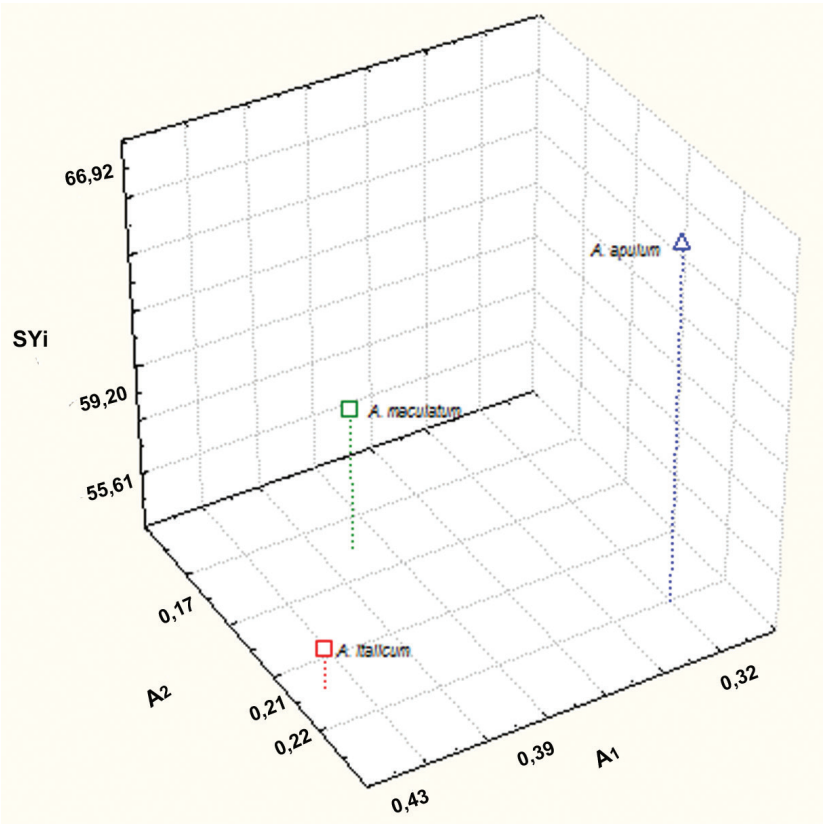

Figure 1. Scatter diagram of A1, A2 and SYi values of *Arum* taxa examined.

In *A. italicum* the chromosome number 2n = 84 (Fig. 2a) was observed in all the investigated populations, which is consistent with previous reports (Marchi 1971, Bedalov 1981). However, one individual from the Gargano Peninsula was found to have the chromosome number 2n = 85, as previously reported by Marchi (1971). The detailed karyotype morphology of this species consists of 38m+30sm+14st+2t chromosomes. Pairs 5, 7, 28, 33, 35 and 42 show weakly coloured segments with Feulgen-staining on the long arm, while pairs 9, 11 and 21 show these on the short arm, and pair 15 has a slightly Feulgen-stained segment on both arms. Pair 39 has a microsatellite on the short arm, while pairs 37 and 41 have a microsatellite on the short arm and a secondary constriction on the long arm (Figs 3, 4a).

Arum maculatum was found to have 2n = 56 somatic chromosomes (Fig. 2b), confirming earlier counts for this species on samples from the Balkan Peninsula (Bedalov 1981, D'Emerico et al. 1993). Our analyses show that the karyotype is similar to the previous reports and that it is characterized by the presence of 26m+24sm+6st chromosomes. However, individuals from central Puglia showed some differences in terms of the number and position of secondary constrictions. Pairs 1, 6, 19 and 28 have weakly coloured segments with Feulgen-staining on the long arm, while pairs 5, 18, 20

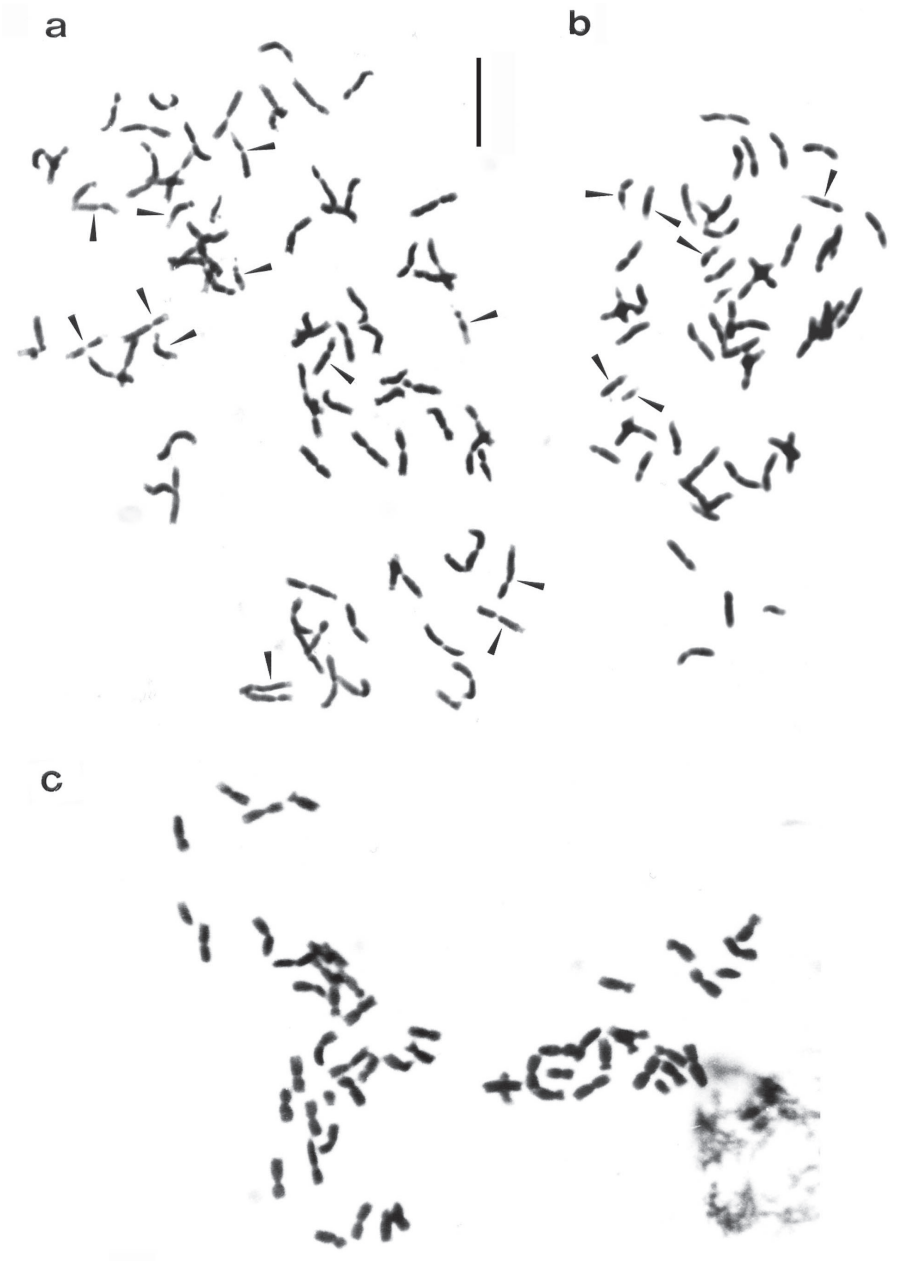

Figure 2. Somatic chromosomes of *Arum* species: **a** *Arum italicum* (2n = 84) **b** *Arum maculatum* (2n = 56) **c** *A. apulum* (2n = 56). (Arrows show chromosomes with weakly coloured Feulgen-stained segments) Bar = 5μm.

and 24 have these on the short arm and pair 27 has a microsatellite on the short arm and a secondary constriction on the long arm (Fig. 4b).

The samples of *A. apulum* from Quasano, Sammichele and Turi (Bari) showed 2n = 56 chromosomes (Fig. 2c), in agreement with previous reports (Bianco et al. 1994).

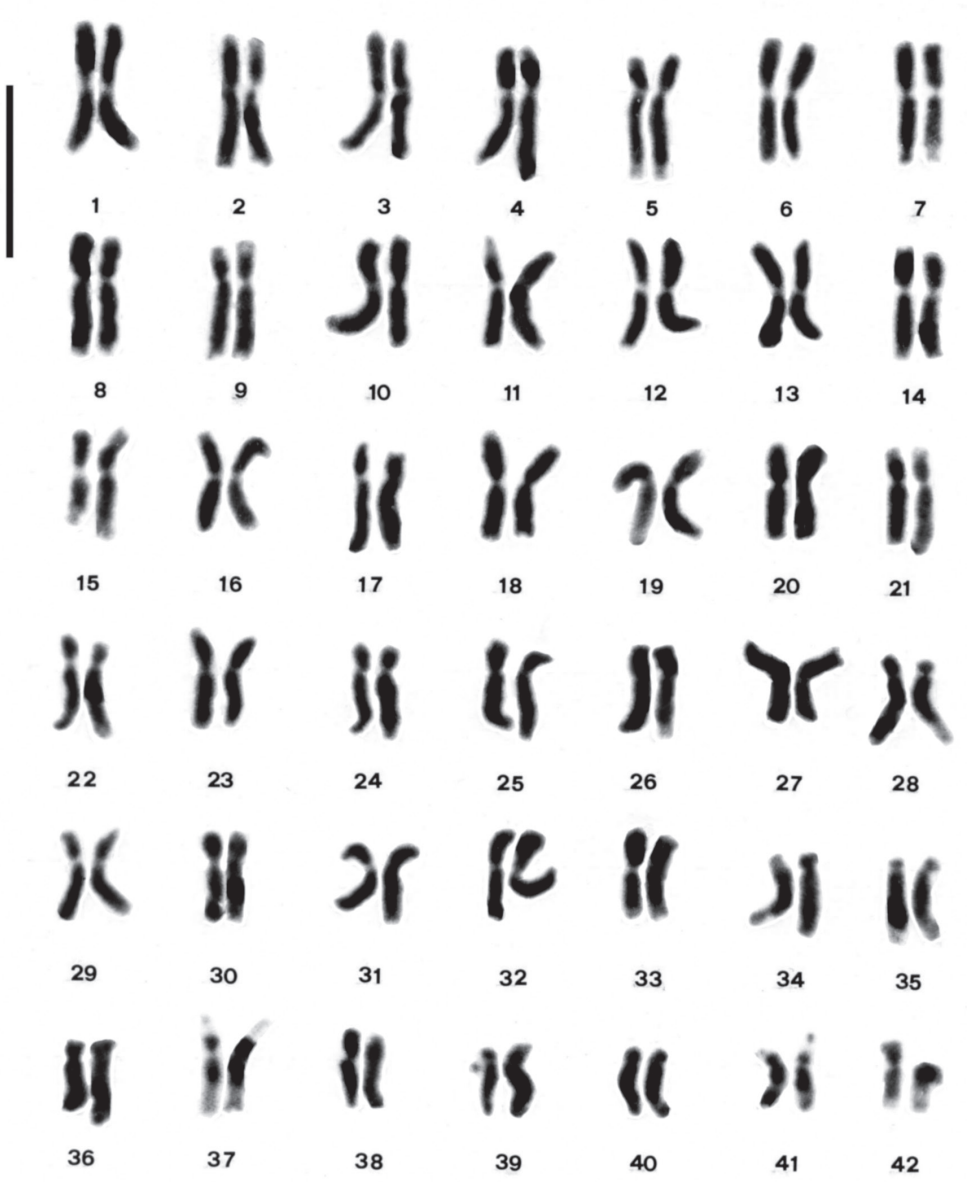

Figure 3. Karyotype of *Arum italicum.* Bar = 5μm.

This species is characterized by a rather symmetrical karyotype, comprising mainly metacentric chromosomes. The karyotype morphology consists of 40m+16sm chromosomes. Pairs 1, 6 and 18 have weakly coloured segments with Feulgen-staining on the long arm; pair 16 has these on the short arm and pair 27 has a secondary constriction on the short arm and a microsatellite on the long arm (Fig. 4c).

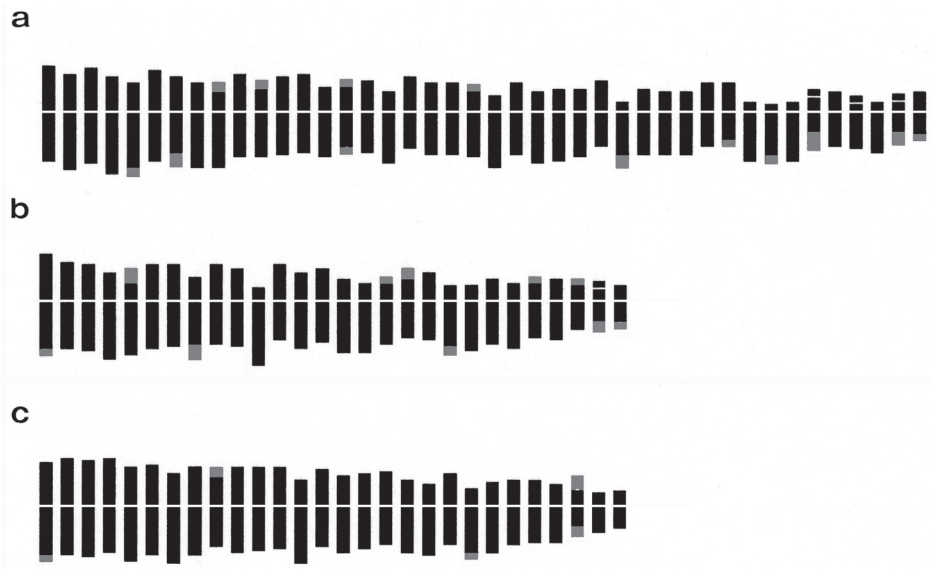

Figure 4. Haploid idiograms of *Arum* species: **a** *Arum italicum* **b** *Arum maculatum* **c** *A. apulum*. (Telomeres shaded in gray show chromosomes with allocyclic segments).

The karyotype morphology of *A. italicum* is similar to that of *A. maculatum*. *Arum italicum* shows a more asymmetrical karyotype, with a higher intrachromosomal asymmetry index (A1 = 0.43) than *A. maculatum* (A1 = 0.39). By contrast, *Arum apulum* possesses the most symmetrical karyotype of the three (A1 = 0.32) (Fig. 1, Table 2), being composed of mainly metacentric chromosomes and having few allocyclic segments. According to Stebbins (1971) the presence of metacentric chromosomes in the karyotype could be considered indicative of early divergence by a species. On the other hand, geographical isolation accompanied by ecological variation seems to support the current karyotype structure of *A. apulum*.

In all the examined taxa some chromosome pairs are characterized by the presence of weakly stained segments, formerly described as secondary constrictions (D'Emerico et al. 1993). Dyer (1963) and Vosa and Colasante (1995) reported that similar segments have been found in several groups of plants (e.g. *Gasteria* Duval, 1809, *Iris* Linnaeus, 1753, *Aloe* Linnaeus, 1753). Moreover, they suggest that in somatic metaphase some chromosomes can exhibit non-contracted telomeric segments called "allocyclic segments". Vosa and Bennett (1990) and Bennett and Grimshaw (1991) suggested that the presence of this type of segment could be used to distinguish species with similar karyotypes. In our study, *A. italicum* showed numerous chromosomes with these segments, in contrast to *A. maculatum* and *A. apulum*. Polyploidy associated with structural changes in chromosomes is involved in bringing about further diversifications of karyotype morphology (Stebbins 1971). Therefore, on this basis we suggest *A. italicum* is characterised by more rearrangement in its chromosome complement than the other two species.

Table 2. Morphometric parameters (mean ± S. E.) of the karyotypes of three *Arum* taxa studied. Haploid complement length; Chromosome number; A_1, A_2 (Romero Zarco 1986) and Syi (Greilhuber and Speta 1976).

Taxa	Haploid complement (µm)	Chromosome number 2n	A_1	A_2	SYi
A. apulum	90.58 (± 3.12)	56	0.32 (± 0.01)	0.22 (± 0.01)	66.92 (± 1.61)
A. maculatum	96.63 (± 2.46)	56	0.39 (± 0.01)	0.17 (± 0.01)	59.20 (± 0.27)
A. italicum	169.22 (± 16.36)	84	0.43 (± 0.02)	0.21 (± 0.02)	55.61 (± 1.90)

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