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Authors: Barros, Luísa Antônia Campos, Teixeira, Gisele Amaro, Aguiar, Hilton Jeferson Alves Cardoso De, Mariano, Cléa Dos Santos Ferreira, Delabie, Jacques Hubert Charles, et al.

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BANDING PATTERNS OF THREE LEAFCUTTER ANT SPECIES OF THE GENUS *ATTA* (FORMICIDAE: MYRMICINAE) AND CHROMOSOMAL INFERENCES

LUÍSA ANTÔNIA CAMPOS BARROS^{(1,2)*}, GISELE AMARO TEIXEIRA⁽¹⁾, HILTON JEFERSON ALVES CARDOSO DE AGUIAR^(1,2,3), CLÉA DOS SANTOS FERREIRA MARIANO^(4,5), JACQUES HUBERT CHARLES DELABIE^(4,6) AND SILVIA DAS GRAÇAS POMPOLO⁽¹⁾

¹Laboratório de Citogenética de Insetos, Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa - MG, 36570-000, Brazil

²Programa de Pós-graduação em Genética e Melhoramento, Universidade Federal de Viçosa, Viçosa - MG, 36570-000, Brazil

³Universidade Federal do Amapá; Campus Binacional, Oiapoque - AP, 68980-000, Brazil

⁴Laboratório de Mirmecologia, CEPEC/CEPLAC; Itabuna - BA, CP 7, 45600-000, Brazil

⁵Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz; Ilhéus - BA, 45650-000, Brazil

⁶Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz, Ilhéus - BA, 45650-000, Brazil

*Corresponding author; E-mail: luufv@yahoo.com.br

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ABSTRACT

Among Neotropical ants, the genera *Acromyrmex* and *Atta* are of great importance because they include the major agricultural pest species. Regarding the genus *Atta*, considered one of the most derived of the tribe Attini, cytogenetic data exists for 5 taxa that present $2n = 22$ chromosomes; however, information regarding banding patterns is only available for *Atta colombica*. Cytogenetics has contributed to several aspects related to the family Formicidae, in particular to the understanding of its evolution. Therefore, the aim of this study was to increase the cytogenetic data on *Atta sexdens rubropilosa*, *Atta laevigata* and *Atta bisphaerica* belonging to populations in the state of Minas Gerais, Brazil. The 3 species were characterized as having $2n = 22$ chromosomes with a karyotypic formula of $2n = 18m + 2sm + 2st$. Using the C-banding technique, small heterochromatic markings were observed in the centromeres of most of the chromosomes. The presence of GC-rich blocks in the interstitial region on the long arm of the fourth pair of metacentric chromosomes was indicated by CMA₃ fluorochrome. The data from this study enabled a comparison with *Acromyrmex striatus*, a phylogenetically close species possessing the same chromosome number as the *Atta* species studied in this work, although clear differences were evident in the morphologies of 2 chromosome pairs and in the distribution and composition of heterochromatin. These results show the constancy of the chromosome number, morphology and banding pattern for the species studied, highlighting the importance of measuring the chromosomes to enable accurate comparison and using banding techniques to compare species of genera with a conserved chromosome number.

Key Words: Attini, chromosome evolution, fluorochrome, heterochromatin

RESUMO

Os gêneros *Acromyrmex* e *Atta* são de grande importância entre as formigas neotropicais devido a sua posição entre as pragas agrícolas. O gênero *Atta* é considerado um dos mais derivados da tribo Attini e dados citogenéticos estão disponíveis para cinco táxons que apresentam $2n = 22$ cromossomos, enquanto que informações sobre padrões de bandamentos estão disponíveis apenas para *Atta colombica*. A citogenética tem contribuído em muitos aspectos relacionados à família Formicidae, em particular referentes aos estudos evolutivos,

e assim o objetivo do presente trabalho foi ampliar os dados citogenéticos de *Atta sexdens rubropilosa*, *Atta laevigata* e *Atta bisphaerica* de populações do estado de Minas Gerais, Brasil. As três espécies foram caracterizadas com $2n = 22$ cromossomos e fórmula cariotípica $2n = 18m + 2sm + 2st$. Pequenas marcações heterocromáticas foram observadas no centrômero dos cromossomos de acordo com a técnica de banda C. A presença de blocos ricos em GC na região intersticial do braço longo do quarto par de cromossomos metacêntricos foi indicada pelo fluorocromo CMA₃. Os dados deste estudo permitiram a comparação com *Acromyrmex striatus*, espécie filogeneticamente próxima que tem o mesmo número cromossômico das espécies de *Atta* embora exista uma nítida diferença na morfologia de dois pares de cromossomos e na distribuição e composição de heterocromatina. Esses resultados mostram a constância do número, morfologia e padrão de bandamentos dos cromossomos das espécies estudadas, ressaltando a importância da medição dos cromossomos e do uso de técnicas de bandamentos para comparação entre espécies de gêneros com número cromossômico conservado.

Palavras Chave: Attini, evolução cromossômica, fluorocromo, heterocromatina

Leafcutter ants are considered the most derived of the tribe Attini and their origin is considered recent (Hölldobler & Wilson 1990; Schultz & Meier 1995; Villesen et al. 2002; Fernández-Marín et al. 2004; Schultz & Brady 2008; Mehdiabadi & Schultz 2010). These ants practice what Mehdiabadi & Schultz (2010) termed “leafcutter agriculture”. Leafcutter ants of the genera *Atta* and *Acromyrmex* have been referred to as the dominant herbivores of the Neotropics (Hölldobler & Wilson 1990), because they use plant, flower and seed fragments to cultivate a mutualistic fungus garden on which they feed (Della Lucia & Fowler 1993). These ants are significant agricultural pests of the Neotropical region because they cause great damage to pastures and cultivated agricultural crops (Della Lucia & Fowler 1993; Della Lucia & Souza 2011). In the natural ecosystems, leafcutter ants play an important role in seed dispersal (Teixeira et al. 2008), in modifying the structure of the vegetation surrounding the colonies (Della Lucia & Souza 2011), and in soil aeration which facilitates plant root penetration (Della Lucia 2003).

Bacci et al. (2009) conducted a molecular phylogenetic investigation of the genus *Atta* for 13 of the 15 described species, using DNA sequence information from the fragments of 4 genes (1 nuclear and 3 mitochondrial genes). The data supported monophyly of the genus and indicated its subdivision into 4 monophyletic groups: *Atta sensu stricto*, *Archeatta*, *Neoatta* and *Epiatta*, which was a major redefinition in the face of the various classifications of the genus proposed by different authors using morphological data.

Species are typically identified based on external morphological traits. However, morphological traits may or may not become altered over time by the selective pressure exerted by the environment to which each species is subjected, with consequences to the genotype. Thus, cytogenetic analysis is a useful tool

which enables the identification and comparison of species (or sometimes populations) because chromosomal rearrangements can result in speciation (Kasahara 2009). Cytogenetics is, therefore, a suitable tool for phylogenetic, taxonomic and evolutionary studies and inferences (MacGregor 1993).

The chromosome numbers observed within the tribe Attini range from $2n = 8$ in *Mycocepurus goeldii* (Barros et al. 2010) and *Mycocepurus* sp. (Murakami et al. 1998) up to $2n = 54$ in *Mycetarotes parallelus* (Barros et al. 2011). Cytogenetic information can be used to compare populations, sympatric species and closely related ant groups (Mariano et al. 2012).

Atta and *Acromyrmex* are characterized by a conserved chromosome number within each genus of $2n = 22$ chromosomes and $2n = 38$ chromosomes, respectively, and by similar chromosome morphology within each genera (revised in Barros et al. 2011; Cristiano et al. 2013). Recent cytogenetic studies on *Acromyrmex* have reported some differences in the chromosome morphology and banding patterns in 6 species from Minas Gerais, Brazil and Barro Colorado, Panamá (Barros 2010), indicating that further cytogenetic studies in leafcutter ants are warranted. In the genus *Acromyrmex*, *Acromyrmex striatus* ($2n = 22$) differs the most from the other already known species with respect to both morphological traits and chromosome number. Its karyotype bears closer similarity to that of the species of the genus *Atta* ($2n = 22$) studied earlier, and associated with the molecular data it provided clear insights into a distinct phylogenetic position of this species among the leafcutter ants (Cristiano et al. 2013).

Cytogenetic data is now available for 5 taxa of *Atta*, all of which have $2n = 22$ chromosomes (Table 1). Information on chromosome banding is available for a single *Atta* species, i.e., *A. colombica*, (Table 1) with heterochromatin in the interstitial region of 4 chromosomes added to the centromeric heterochromatic blocks on all the chromosomes (Murakami et al. 1998).

TABLE 1. *ATTA* SPECIES STUDIED TO DATE, LOCALITIES, DIPLOID CHROMOSOME NUMBER (2N) AND CHROMOSOME BANDING DATA (FLUOROCHROMES – FL AND C-BANDING – CB) STUDIED IN BRAZIL: MINAS GERAIS (MG), RIO GRANDE DO SUL (RS); PANAMA: BARRO COLORADO AND THE RESPECTIVE SUBGENERA ACCORDING TO THE MOLECULAR PHYLOGENY BY BACCI ET AL. (2009)

<i>Atta</i> spp.	Locality, Coordinates	2n	Chromosome banding	Subgenera	Reference
<i>A. bisphaerica</i>	Lavras – MG ; 21° 14' S 44° 59' W	22	CB, FL	<i>Epiatta</i>	This study
<i>A. bisphaerica</i>	Teixeiras – MG ; 20° 38' S 42° 51' W	22	CB, FL	<i>Epiatta</i>	This study
<i>A. bisphaerica</i>	Viçosa – MG ; 20° 45' S 42° 51' W	22	—	<i>Epiatta</i>	Fadini & Pompolo (1996)
<i>A. colombica</i>	Barro Colorado – Panamá ; 9° 09' N 79° 50' W	22	CB	<i>Atta sensu stricto</i>	Murakami et al. (1998)
<i>A. laevigata</i>	Viçosa – MG ; 20° 45' S 42° 51' W	22	—	<i>Epiatta</i>	Fadini & Pompolo (1996)
<i>A. laevigata</i>	Viçosa – MG ; 20° 45' S 42° 51' W	22	CB, FL	<i>Epiatta</i>	This study
<i>A. sexdens rubropilosa</i>	Ponte Nova – MG ; 20° 45' S 42° 52' W	22	CB, FL	<i>Neotia</i>	This study
<i>A. sexdens rubropilosa</i>	Teixeiras – MG ; 20° 38' S 42° 51' W	22	CB, FL	<i>Neotia</i>	This study
<i>A. sexdens rubropilosa</i>	Viçosa – MG ; 20° 45' S 42° 51' W	22	—	<i>Neotia</i>	Fadini & Pompolo (1996)
<i>A. sexdens piriventris</i>	Pelotas – RS ; 31° 44' S 52° 21' W	22	—	<i>Neotia</i>	Santos-Colares et al. (1997)

Due to the lack of cytogenetic data on leafcutter ants, the aim of the present work was to broaden the studies, adding information on 3 species of *Atta* as well as to enable a better understanding of the karyotype evolution of this group and further comparative studies with other phylogenetically close groups, such as the genus *Acromyrmex*, including *Ac. striatus* or studies on *Trachymyrmex*, considered the sister group of the leafcutter ants. This is the first time that chromosome morphology of the genus *Atta* was classified by measurement, allowing an accurate comparison among similar karyotypes. The classification proposed by Imai (1991) is based on heterochromatin location and details in chromosome size mainly among similar karyotypes are not highlighted, especially when the species have low amount of heterochromatin.

MATERIAL AND METHODS

Cytogenetic studies were conducted on 3 species of the *Atta* genus, i.e., *Atta sexdens rubropilosa* (Forel, 1908), *Atta laevigata* (Smith, 1858) and *Atta bisphaerica* Forel, 1908, all collected in the state of Minas Gerais, Brazil (Table 1). A total of 26 individuals were analyzed for *A. bisphaerica*, 10 for *Atta laevigata* and 43 for *A. sexdens rubropilosa*. Mitotic metaphases were obtained according to Imai et al. (1988) by dissecting the cerebral ganglia of larvae after *meconium* elimination. Some slides were stained with 4% Giemsa and the metaphases were observed and photographed by a BX 60 microscope with a 100X lens attached to the Q-Color3 Olympus® image capture system. At least 6 individuals per species were subjected to the C-banding and fluorochrome techniques. The chromosomes were paired and ranked in decreasing order of size to determine the karyotype.

A total of 10 metaphases per species with similar degree of condensation, without overlappings and evident centromeres were measured and classified according to the specifications of Levan et al. (1964), based on the chromosome arm ratio (r) using the following features: long arm length (L), short arm length (S) and arm ratio between the long and short arms ($r = L/S$). The chromosomes were classified as: m = metacentric ($r = 1-1.7$), sm = submetacentric ($r = 1.7-3$), st = subtelocentric ($r = 3-7$) and a = acrocentric ($r > 7$). Karyotypes were organized using the Corel Photopaint X3® and Image Pro Plus® programs. The C-banding technique was performed according to Sumner (1972) with modifications proposed by Barros et al. (2013). The GC and AT rich regions were detected using the fluorochromes Chromomycin A₃ (CMA₃) and 4'6-diamidin-2-phenylindole (DAPI) according to Schweizer (1980).

Adult ant specimens were deposited as vouchers in the reference collection of the Laboratório

de Mirmecologia, Centro de Pesquisas do Cacau (CPDC/Brazil) under the following numbers: #5712 (*A. sexdens rubropilosa*), #5715 (*A. laevigata*) and #5713 (*A. bisphaerica*).

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RESULTS

The 3 species of the *Atta* studied presented $2n = 22$ chromosomes with a karyotypic formula was $2n = 18m + 2sm + 2st$ (Fig. 1). The morphometric data of the chromosome pairs 10 and 11 of *Atta* spp. are shown in Table 2.

Small and weak heterochromatic bands were observed in the centromeric region of the chromosomes in all the 3 species (Fig. 2). Besides the centromeric blocks, the presence of a small interstitial band was observed on the long arm of the fourth pair of metacentric chromosomes for *A. sexdens rubropilosa* (Fig. 3a – inbox).

The CMA₃ fluorochrome staining showed interstitial marking on the long arm of the fourth pair of metacentric chromosomes for the 3 species, indicating that this region is rich in GC base pairs (Fig. 3; color version in Suppl. Fig. 3). CMA₃ positive regions in *A. sexdens rubropilosa* corresponded to a heterochromatic region.

Regions with differential staining with DAPI, indicative of regions rich in AT base pairs, were not observed.

DISCUSSION

The diploid number of $2n = 22$ chromosomes reported for the *Atta* species in the present study was also observed in other studies (Table 1). The chromosome morphology was shown to be constant in the different *Atta* species, unlike the observations made for the species of *Acromyrmex* (Barros 2010; Cristiano et al. 2013). The genera *Atta* and *Acromyrmex* are considered phylogenetically close and recognized for the constancy of their karyotype, with respect to both chromosome number and similarity in morphology in most of the species studied (revised in Barros et al. 2011; Cristiano et al. 2013). This characteristic has already been observed in the different ant groups, like those of the subgenera *Myrmothrix* and *Myrmamblys* in genus *Camponotus* (Formicinae) (Mariano et al. 2003) and genus *Pheidole* (Myrmicinae) (revised in Lorite & Palomeque 2010). However, recent cytogenetic studies on the genus *Acromyrmex* have revealed differences in the morphology and chromosome banding patterns in 7 species studied in Brazil and Panamá (Barros 2010; Cristiano et al. 2013).

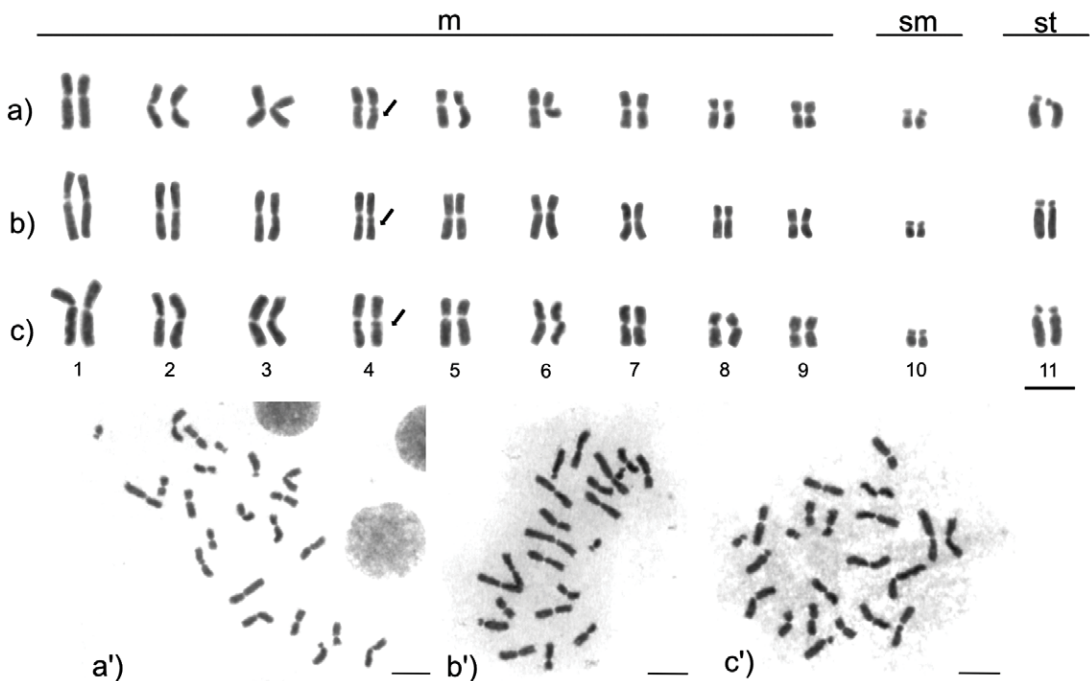


Fig. 1. Karyotypes of *Atta* spp.: (a) *A. sexdens rubropilosa*, (b) *A. laevigata* and (c) *A. bisphaerica* with $2n = 22$ chromosomes and karyotypic formula $2n = 18m + 2sm + 2st$. Arrows indicate the presence of a secondary constriction on the fourth pair of chromosomes. Bar: 5µm.

TABLE 2. MORPHOLOGICAL ANALYSES OF TWO CHROMOSOME PAIRS OF *ATTA* SPP. AND *ACROMYRMEX STRIATUS*

Species	Chromosome	S (μm)*	L (μm)*	r	Chromosome classification	Reference
<i>A. bisphaerica</i>	10	0.612	1.26	2.083	Submetacentric	This study
		0.601	1.282	2.155		
<i>A. laevigata</i>		0.69	1.384	2.015		
		0.679	1.379	2.03		
<i>A. sexdens rubropilosa</i>		0.636	1.266	2.02		
		0.606	1.227	2.055		
<i>Acromyrmex striatus</i>		0.85	1.03	1.22	Metacentric	Cristiano et al. (2013)
		0.79	0.99	1.26		
<i>A. bisphaerica</i>	11	0.707	2.834	4.051	Subtelocentric	This study
		0.745	2.84	3.833		
<i>A. laevigata</i>		0.855	3.443	4.038		
		0.812	3.312	4.071		
<i>A. sexdens rubropilosa</i>		0.736	3.01	4.139		
		0.694	2.929	4.267		
<i>Acromyrmex striatus</i>		0.94	2.29	2.51	Submetacentric	Cristiano et al. (2013)
		0.95	2.45	2.62		

L: long arm length; S: short arm length; RL: relative length; r: arm ratio ($r = L/S$)
* short and long arms mean length obtained from 10 metaphases

In the present study, the C-banding technique indicated the existence of a small amount of heterochromatin on the chromosomes of the *Atta* species, with a distribution pattern in the centromeric region. Similar results regarding the heterochromatin distribution were reported by Murakami et al. (1998) for *A. colombica*, which has centromeric heterochromatic bands on all the chromosomes and additional interstitial bands on 2 pairs of chromosomes. However, metaphase was not illustrated in the study of Murakami et al. (1998). The genome of *Atta cephalotes*, included in the *sensu stricto* group has been sequenced and reveals that there is a low percentage of satellite DNA (Suen et al. 2011), the principal com-

ponent of the constitutive heterochromatin. The C-banding technique confirmed the low amount of heterochromatin when using modern genome sequencing techniques.

The interstitial heterochromatic band located on the fourth pair of chromosomes of *A. sexdens rubropilosa* corresponded to a region positively stained with CMA₃ fluorochromes, indicating that this heterochromatic block was rich in GC base pairs (Fig. 3; color version in Suppl. Fig. 3). This correspondence to the CMA₃ bands, however, was not observed in the centromeric heterochromatic bands of the chromosomes revealed by the C-banding technique. The interstitial heterochromatic band of the fourth pair of chromosomes was

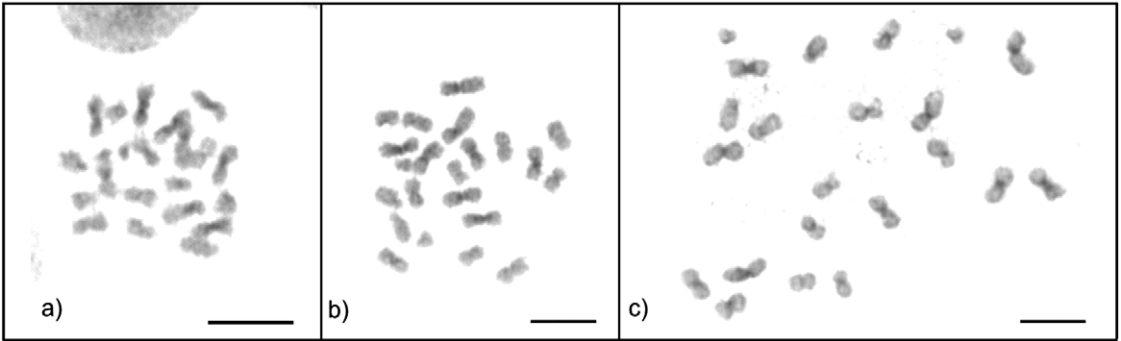


Fig. 2. Metaphases of *Atta* spp. submitted to the C-banding technique: (a) *A. sexdens rubropilosa*, (b) *A. laevigata* and (c) *A. bisphaerica*. The darker centromeric regions reveal the presence of heterochromatin. Bar: 5μm.

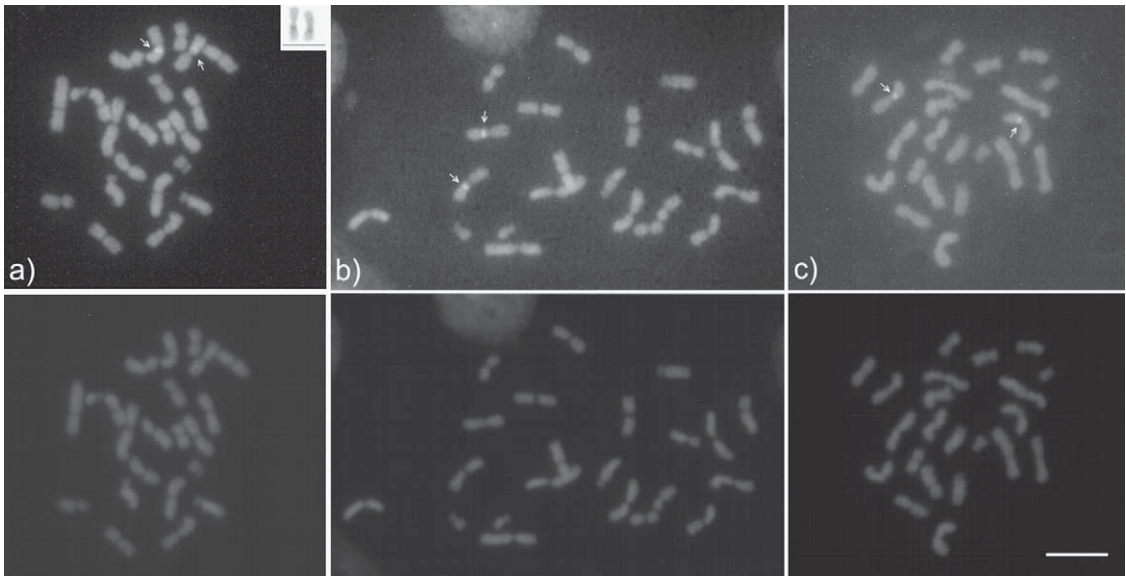


Fig. 3. Metaphases of *Atta* stained with the fluorochromes CMA₃ (metaphases on the top) and DAPI fluorochromes (metaphases on the bottom) of: (a) *Atta sexdens rubropilosa*, (b) *Atta laevigata* and (c) *Atta bisphaerica*. The arrows indicate GC-rich blocks and complementary negative AT regions, respectively. The fourth metacentric chromosome pair is highlighted in Fig. 3a (in box) with GC-rich interstitial heterochromatin. Bar: 5µm.

observed only for *A. sexdens rubropilosa*, which was certainly due to the small band size in other species.

The interstitial region positively stained with CMA₃ fluorochrome on the fourth pair of chromosomes was observed in all 3 *Atta* species; a secondary constriction was also visible, indicating that this GC rich heterochromatic region probably corresponds to the nucleolus organizer region (NOR). To date no published reports are available on the location of secondary constrictions in this genus of leafcutter ants. The GC-rich regions generally correspond to the NOR, which are well conserved during evolution. In the *Tapinoma nigerrimum* (Lorite et al. 1997) and *Dinoponera lucida* (Mariano et al. 2008) species the CMA₃ fluorochrome pattern coincided with the NOR site verified by the fluorescence in situ hybridization (FISH) technique, similar to most eukaryotes (Reed & Phillips 1995).

Recent cytogenetic studies on *Ac. striatus* revealed $2n = 22$ chromosomes (Cristiano et al. 2013), the same chromosome number found in all the *Atta* species studied to date. However, the karyotype of this species did not have the same morphological traits of the chromosomes found in *Atta* species and morphological analyses of chromosome pairs 10 and 11 that differed between species are shown in Table 2. In *Ac. striatus*, the submetacentric pair is probably homeologous to the subtelocentric pair of the *Atta* species investigated in the present study. This difference suggests chromosome rearrangements, as for

example, duplication and heterochromatin inversion during the evolution of this species, such that the positioning of a heterochromatic block on the telomere of the submetacentric pair of *Ac. striatus* would be possible. However, the opposite is also possible, in which the heterochromatic block could have been lost in an ancestor of the modern *Atta* species. Another pair of chromosomes that was also observed to be different in *Ac. striatus* was the smallest chromosome pair with their characteristic morphology. This small pair clearly has the 2 arms (long and short arm) which differ in size in all the *Atta* species already studied, although these 2 chromosome arms are similar in size in *Ac. striatus*. Both chromosomes have heterochromatin on the centromere of this homologous chromosome pair, while duplication of a portion of the centromeric heterochromatin may have modified the chromosome morphology. However, variations in the euchromatic regions cannot be disregarded.

The heterochromatin distribution pattern seen in *Atta* species is similar to that of *Ac. striatus* (Cristiano et al. 2013) and is characterized by the presence of small and weak heterochromatic bands in the centromeric region of the chromosomes. However, *Ac. striatus* can be distinguished by the presence of additional pericentromeric bands and a telomeric marking on the short arm of the submetacentric pair. The differences noted in chromosome morphology and heterochromatin distribution patterns revealed by a comparison of the *Atta* species assessed in the present study

with *Ac. striatus* show a divergence between these ant groups.

Karyotypes of the 3 leafcutter ant species of genus *Atta*, added to those of previously described karyotypes, were shown to be conserved in chromosome number, morphology and banding pattern, and constancy is inferred for chromosome number within the genus for the species studied to date. It included species in 3 of the 4 groups of the *Atta* genus based on the molecular data (Bacci et al. 2009) pertaining to *A. laevigata* and *A. bisphaerica* in the *Epiatta* group; *A. colombica* in the *Atta sensu stricto* group; *A. sexdens rubropilosa* and *A. sexdens privertris* in the *Neoatta* group.

Data obtained from the chromosome banding techniques are scarce not only for the species of the genus *Atta* but for other species of the tribe Attini as well, (Barros et al. 2010; Barros et al. 2014) even though they could be very informative for our understanding of the evolution of the tribe.

Data in the present study revealed the importance of measuring the chromosomes to enable accurate interspecific comparison among the *Atta* spp. as well as the published information available on *Ac. striatus* which revealed interesting differences. The chromosome banding data indicated the importance of the techniques mentioned above for comparison among the species within the genera with a conserved chromosome number.

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