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Abstract

Leaf-cutting ants are among the New World's most conspicuous and studied ant species due to their notable ecological and economic roles. Cytogenetic studies carried out in *Atta* show remarkable karyotype conservation among the species. We performed classical cytogenetics and physical mapping of repetitive sequences in the leaf-cutting ant *Atta cephalotes* (Linnaeus, 1758), the type species of the genus. Our goal was to test the karyotype conservation in *Atta* and to understand the genomic organization and diversity regarding repetitive sequences in leaf-cutting ants. *Atta cephalotes* showed $2n = 22$ (18m + 2sm + 2st) chromosomes. The heterochromatin followed a centromeric pattern, and the GC-rich regions and 18S rDNA clusters were co-located interstitially in the 4th metacentric pair. These cytogenetic characteristics were observed in other *Atta* species that had previously been studied, confirming the karyotype conservation in *Atta*. Evolutionary implications regarding the conservation of the chromosome number in leaf-cutting ants are discussed. Telomeric motif $(TTAGG)_n$ was detected in *A. cephalotes* as observed in other ants. Five out of the 11 microsatellites showed a scattered distribution exclusively on euchromatic areas of the chromosomes. Repetitive sequences mapped on the chromosomes of *A. cephalotes* are the first insights into genomic organization and diversity in leaf-cutting ants, useful in further comparative studies.

Key words: Attina, cytogenetic markers, Amazon, euchromatin, chromosomal evolution, FISH

Résumé

Les fourmis coupe-feuille sont parmi les espèces les plus répandues et les plus étudiées dans le Nouveau Monde en raison de leurs rôles écologiques et économiques notables. Des études cytogénétiques réalisées chez le genre *Atta* ont montré une conservation remarquable du caryotype chez ces espèces. Les auteurs ont réalisé des analyses cytogénétiques classiques ainsi que la cartographie physique des séquences répétées chez la fourmi coupe-feuille *Atta cephalotes* (Linnaeus, 1758), l'espèce-type au sein de ce genre. Le but était de vérifier la conservation du carytoype au sein du genre *Atta* et de comprendre l'organisation génomique et la diversité des séquences répétées chez les fourmis coupe-feuille. L'*Atta cephalotes* compte $2n = 22$ chromosomes (18m + 2sm + 2st). L'hétérochromatine présentait une distribution centromérique tandis que les régions riches en GC et les amas d'ADNr 18S colocalisaient dans les régions interstitielles de la quatrième paire de chromosomes métacentriques. Ces caractéristiques cytogénétiques ont été observées chez d'autres espèces du genre *Atta* qui ont été étudiées précédemment, ce qui confirme la conservation caryotypique au sein de ce genre. Les auteurs discutent des implications évolutives de la conservation du nombre de chromosomes chez les fourmis coupe-feuille. Le motif télomérique $(TTAGG)_n$ détecté chez l'*A. cephalotes* est le même que celui observé chez les autres fourmis. Cinq des 11 microsatellites étaient largement distribués au sein des régions euchromatiques des chromosomes. Cette cartographie des séquences répétées sur les chromosomes de l'*A.*

cephalotes apporte les premières informations sur l'organisation génomique et la diversité chez les fourmis coupe-feuille, une information qui sera utile pour des études comparées chez d'autres espèces. [Traduit par la Rédaction]

Mots-clés : Attina, marqueurs cytogénétiques, Amazonie, euchromatine, évolution chromosomique, FISH

Introduction

Leaf-cutting ants are considered major herbivores in the Neotropics (Hölldobler and Wilson 1990). They exert a substantial influence on ecosystem processes (Swanson et al. 2019) and are among the most conspicuous and studied ant species in the New World (Brandão et al. 2011). These ants are regarded as pests in landscapes deeply impacted by urbanization or extensive agriculture. However, in natural environments, they contribute with several environmental benefits by modifying soil properties, contributing to seed germination and seed dispersal; accordingly, they are regarded as ecosystem's engineers (reviewed by Della Lucia et al. 2014; Swanson et al. 2019).

Currently, leaf-cutting ants include 54 valid species distributed in four genera: *Atta* Fabricius, 1804; *Acromyrmex* Mayr, 1865; *Amoimyrmex* Cristiano, Cardoso & Sandoval, 2020, and *Pseudoatta* Gallardo, 1916 (reviewed by Bolton 2021). Due to their notable characteristics mentioned above, these ants have been the focus of several research lines, including cytogenetics (Table 1; reviewed by Barros et al. 2021; Micolino et al. 2022). Particularly for *Atta*, cytogenetic data are available for five species (Table 1), with representatives in three of the four monophyletic groups defined by molecular phylogenies previously proposed (Bacci et al. 2009; Barrera et al. 2022). *Atta sexdens* (Linnaeus, 1758) and *Atta robusta* Borgmeier, 1939 are included in the *Neoatta* group, *Atta bisphaerica* Forel, 1908 in the *Epiatta* group, and *Atta colombica* Guérin-Méneville, 1844 in the *Atta sensu stricto* group. No cytogenetic data are available for the ancestral *Archeatta* group, which is restricted to the Caribbean and North and Central America (Barrera et al. 2022).

Thus far, all of the *Atta* species submitted to chromosomal measurements had a diploid chromosome number of $2n = 22$ and the karyotype formula 18m + 2sm + 2st (Table 1); therefore, they are considered as having a conserved karyotype (Barros et al. 2014, 2015; Aguiar et al. 2020). In addition, distinctive GC-rich chromatin was detected in the 4th metacentric chromosome pair, which co-localized in the secondary constriction of *A. bisphaerica*, *A. laevigata*, *A. sexdens rubropilosa*, and *A. robusta* (Barros et al. 2014, 2015). This distinct GC-rich chromatin corresponds to the 18S ribosomal sites (Barros et al. 2015; Teixeira et al. 2017). *Atta cephalotes* also belongs to the *Atta sensu stricto* group (Bacci et al. 2009; Barrera et al. 2022) and is the type species of the genus; it is widely distributed in the Neotropical region (Janicki et al. 2016) and has high environmental importance since it is a deep forest species and, therefore, not a major agricultural pest (Jaffe and Vilela 1989; Urbas et al. 2007; Forti et al. 2020). Despite having its genomic data available at DDBJ/EMBL/GenBank (Suen et al. 2011), no information regarding the karyotype configuration of *A. cephalotes* has been published thus far. Within the *Atta sensu stricto* group, *A. colombica* is the only species that has been karyotyped (Murakami et al. 1998); however, information concerning the physical mapping of its chromosomes is lacking. Despite the importance of *A. colombica* karyological data, it is challenging to compare the karyotype to that of other *Atta* species since it was published over 20 years ago and image quality is highly limited.

Molecular cytogenetic studies in ants have been focused predominantly on rDNA clusters (reviewed by Teixeira et al. 2021). In addition to ribosomal DNA, other repetitive sequences, such as microsatellites, make up the genome of eukaryotes; they comprise short repeats of 1–6 bp, also denominated simple sequence repeats (SSRs), which are common and widely distributed in the genomes of organisms (reviewed by Martins et al. 2011; López-Flores and Garrido-Ramos 2012). SSRs have been widely used as landmarks in different organisms, including insects, appearing as a nonrandom distribution on the chromosomes (Cuadrado et al. 2008; Ruiz-Ruano et al. 2015; Travenzoli et al. 2019*a*, 2019*b*; Marchioro et al. 2020). In some grasshoppers' karyotypes, microsatellites have shown scattered or clustered distribution only in euchromatin (Ruiz-Ruano et al. 2015). Among Hymenoptera, in bees of the genera *Melipona* Illiger, 1806 and *Partamona* Schwarz, 1939, microsatellites including di- and trinucleotides are observed in euchromatic regions (Piccoli et al. 2018; Travenzoli et al. 2019*a*, 2019*b*; Lopes et al. 2020). An exception is $(CA)_{15}$, which shows a scattered distribution only in the heterochromatin of *Melipona seminigra merrilae* Cockerell, 1919 (Barbosa et al. 2021). Besides, *Friesella* Moure, 1946 species bees have a predominantly euchromatic $(GA)_{15}$ pattern with some marks in heterochromatin (Elizeu et al. 2021). Contrastingly, *Frieseomelitta* Ihering, 1912 species stingless bees show a prevalence of marks coincident with heterochromatic regions, in addition to euchromatic regions in some species (Santos et al. 2018). In the wasp *Polybia fastidiosuscula* Saussure, 1854, most SSRs are located predominantly in heterochromatic regions, and only $(GA)_{15}$ was observed in euchromatin (Marchioro et al. 2020).

In ants, reports of SSRs are scarce and, so far, are available for some fungus-farming ants (Barros et al. 2018; Micolino et al. 2019, 2022; Teixeira et al. 2022). Scattered distribution of the $(GA)_{15}$ microsatellite was observed exclusively in the euchromatin of the chromosomes (Barros et al. 2018; Teixeira et al. 2022), although specific blocks with differences among some populations were also reported for the same species (Micolino et al. 2019, 2022; Teixeira et al. 2022). In contrast, the $(GAG)_{10}$, $(CAA)_{10}$, and $(CGG)_{10}$ microsatellites did not show specific bands in *Mycetomoellerius holmgreni* (Wheeler, 1925) (Micolino et al. 2019). Additionally, other repetitive sequences, such as telomeric sequences, have been mapped on ant chromosomes, including leaf-cutting ants; they show that the TTAGG motif constitutes their telomeres (Meyne et al. 1995; Lorite et al. 2002; Pereira et al. 2018; Castro et al. 2020; Teixeira et al. 2022).

In the present study, we performed classical cytogenetics and physical mapping of repetitive sequences (18S

Table 1. Summary of cytogenetic data available, including this study, for leaf-cutting ants of the genus *Atta*, organized in monophyletic groups according to Bacci et al. (2009) and Barrera et al. (2022).

Group	*Atta* species	2*n*	Karyotypic formula	Heterochromatic pattern	18S rDNA location	Reference
Atta s. str.	*A. colombica*	22	18M + 4A	Centromeric	—	Murakami et al. 1998
	A. cephalotes	22	18m + 2sm + 2st*	Centromeric	4th m	Present study
Epiatta	*A. bisphaerica*	22	18M + 4A	—	—	Fadini and Pompolo 1996
		22	18m + 2sm + 2st*	Centromeric	4th m	Barros et al. 2014
	A. laevigata	22	18M + 4 A	—	—	Fadini and Pompolo 1996
		22	18m + 2sm + 2st*	Centromeric	4th m	Barros et al. 2014
Neoatta	*A. robusta*	22	18m + 2sm + 2st*	Centromeric	4th m	Barros et al. 2015
	A. sexdens	22	18M + 4A	—	—	Fadini and Pompolo 1996
		22	18m + 2sm + 2st*	Centromeric	—	Barros et al. 2014
		22	18m + 2sm + 2st*	—	4th m	Teixeira et al. 2017
		22	—	—	—	Santos-Colares et al. 1997
		22	18m + 2sm + 2st*	—	—	Aguiar et al. 2020

Note: Diploid chromosome number (2*n*), diploid karyotypic formula, heterochromatin distribution, 18S rDNA chromosome location, and references. Chromosome types: M/m, metacentric; sm, submetacentric; st, subtelocentric; A, acrocentric. *Karyotypic formula using chromosome measurements according to the classification provided by Levan et al. (1964).

rDNA, telomeric motifs, and microsatellites mono-, di-, and trinucleotides) on the chromosomes of the leaf-cutting *A. cephalotes*. Our goal was to verify the karyotype conservation of this species compared to other *Atta* species and start to understand the genomic organization and diversity of repetitive sequences in leaf-cutting ants.

Materials and methods

Three colonies of *A. cephalotes* were collected in the Amazonian Guiana Shield, two in Kourou, French Guiana (5.17312, −52.65480; 5.102658, −52.705830), and one in Oiapoque, State of Amapá, Brazil (3.84151, −51.84112). Sampling authorizations were given by ICMBio/SISBIO in Brazil (accession number: 62598) and by ABSCH-CNA-FR-240495-2 in French Guiana (accession number: TREL1820249A/61). Ant vouchers (workers) were deposited in the reference collection at the Laboratório de Mirmecologia, Centro de Pesquisas do Cacau (CPDC/Brazil).

The metaphases were obtained from cerebral ganglia of the larvae after meconium elimination, according to Imai et al. (1988). To determine the morphology of the chromosomes, a total of 10 metaphases with evident centromeres were analyzed. The measurements were obtained in micromeres for short arm lengths (p), long arm lengths (q), total lengths ($TL = p + q$), and relative lengths ($RL = TL \times 100/\Sigma TL$) in percentage. Chromosomes were arranged in order of decreasing size and based on the ratio of the chromosome arm lengths (r = long arm/short arm), according to the classification proposed by Levan et al. (1964). The chromosomes were classified as m, metacentric (r = 1–1.7); sm, submetacentric (r = 1.7–3); st, subtelocentric (r = 3–7); a, acrocentric ($r > 7$). Adobe Photoshop 2021 and Image Pro Plus® were used for mounting the chromosomal karyotype and measurements, respectively. A total of 49 individuals from three colonies were analyzed.

The heterochromatin distribution pattern was obtained using the C-banding technique according to Sumner (1972), with time adaptations (Barros et al. 2013). Specific GC- and AT-rich regions were detected using sequential staining with the fluorochrome chromomycin A_3 (CMA_3) and 4′6-diamidino-2-phenylindole (DAPI) following Schweizer (1980).

Different repetitive sequences were physically mapped on the chromosomes of *A. cephalotes*. Ribosomal 18S probe was obtained by amplification via polymerase chain reaction (PCR) employing the primers rDNA 18SF1 (5′-GTCATAGCTTTGTCTCAAAGA-3′) and 18SR1.1 (5′-CGCAAATGAAACTTTTTTAATCT-3′) designed for the bee *Melipona quinquefasciata* Lepeletier, 1836 (Pereira 2006) and isolated from total DNA of the ant *Camponotus rufipes* (Fabricius, 1775). 18S rDNA probes were labeled maintaining the conditions for PCR amplification (Pereira 2006) by the indirect method using digoxigenin-11-dUTP (Roche, Mannheim, Germany), and the fluorescence *in situ* hybridization (FISH) signals were indirectly detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol. Also, different microsatellites and telomeric sequences were used as probes for physical mapping (Table 2) directly labeled with cyanine-3 (Cy3) in the 5′ terminal during synthesis by Sigma (St. Louis, MO, USA).

The mapping of the repetitive sequences was performed by FISH following the protocol of Pinkel et al. (1986) with modifications: metaphase chromosomes were denatured in 70% formamide/2 × SSC at 75 °C for 3 min; the probes were hybridized with chromosomes in 20 μL of hybridization mix (200 ng of labeled probe, 2 × SSC, 50% formamide, and 10% dextrane sulfate). The hybridization mix was heated for 10 min at 85 °C, and the slides were kept in a moist chamber at 37 °C overnight. Afterward, the slides with rDNA 18S probe were incubated in a detection solution containing anti-digoxygenin-rhodamine for 1 h in a humid chamber at 37 °C. In the case of microsatellite and telomeric probes, this incubation step with the antibody on the second day was omitted. Then, the slides were washed in 4 × SSC/Tween (4 × SSC, 0.05% [*v*/*v*] Tween 20) and dehydrated in an alcohol series 50%,

Table 2. Summary of the chromosomal hybridization signals of microsatellites and telomeric sequences detected in chromosomes of *Atta cephalotes* using FISH technique.

Motif length class	Repetitive motif	Hybridization pattern
Microsatellites		
Mononucleotides	$(A)_{30}$	SP- euchromatin
	$(C)_{30}$	—
Dinucleotides	$(TA)_{15}$	—
	$(GA)_{15}$	SP- euchromatin
	$(CA)_{15}$	SP- euchromatin
Trinucleotides	$(GAG)_{10}$	SP- euchromatin
	$(CAA)_{10}$	SP- euchromatin
	$(CAT)_{10}$	—
	$(CGG)_{10}$	—
	$(CAG)_{10}$	—
	$(TAT)_{10}$	—
Telomeric sequences		
Pentanucleotides	$(TTAGG)_6$	T
	$(TCAGG)_6$	—
Hexanucleotides	$(TTAGGG)_n$	—

Note: SP, scattered pattern of the FISH signal; T, telomeric region; –, no hybridization signal.

70%, and 100%. Lastly, the chromosomes were counterstained with DAPI (DAPI Fluorshield, Sigma–Aldrich).

The metaphases were observed and documented using a fluorescence microscope Olympus BX 53F coupled with an Olympus MX10 camera and the image software CellSens® with the filter WG (510–550 nm) for the probe rich in Cy3 and rhodamine, WB (450–480 nm) for the CMA_3, and WU (330–385 nm) for the DAPI. At least 30 metaphases were analyzed for fluorochrome staining and FISH technique with different probes. Due to the abundance of chromosomes in metaphase and good quality slides, it was not necessary to use the same slide for different probing procedures, avoiding unwanted markings and background.

The phylogenetic relationship among *Atta* spp. was determined according to previously published molecular phylogeny from Bacci et al. (2009) and Barrera et al. (2022).

Results

Atta cephalotes showed $2n = 22$ chromosomes and the karyotype formula of 18m + 2sm + 2st based on the karyomorphometric analyses performed (Fig. 1; Table 3). This is the first cytogenetic report concerning this species. Small and subtle heterochromatic blocks on centromeric regions were detected on all chromosomes (Fig. 2*a*). The 18S rDNA genes and GC-rich regions (CMA_3^+) were co-located interstitially in the long arm of the 4th metacentric pair in the secondary constriction region (Figs. 2*b* and 3*a*) and negative-DAPI regions (Fig. 2*c*). The telomeric motif $(TTAGG)_6$ was present on the telomeres of all the chromosomes (Fig. 3*b*). Five out of the 11 microsatellite motifs analyzed resulted in signals on *A. cephalotes* chromosomes following the FISH technique, including $(A)_{30}$, $(GA)_{15}$, $(CA)_{15}$, $(GAG)_{10}$, and $(CAA)_{10}$ (Table 2). They all showed a scattered distribution exclusively on euchromatic areas of the chromosomes, covering almost the entire chromosome lengths except for the small heterochromatic centromeric regions (Figs. 3*c*–3*g*). No signals were observed in the chromosomes of *A. cephalotes* with the other telomeric motifs $(TCAGG)_6$ and $(TTAGGG)_n$ or the microsatellites $(C)_{30}$, $(TA)_{15}$, $(TAT)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, and $(CAG)_{10}$ (Table 2). Figure 4 shows the ideogram of the *A. cephalotes* haploid complement with all the chromosomal markers that were analyzed in the present study.

Discussion

The leaf-cutting ant *A. cephalotes* showed the same chromosome number ($2n = 22$) and karyotypic formula as the other *Atta* species that had been studied previously using measurements in the Brazilian savannah, Atlantic, and Amazon rainforests (Table 1). Such karyomorphometric analyses enable more reliable comparisons among closely related species (Gokhman 2009; Barros et al. 2016; Aguiar et al. 2020). Our data enhanced the cytogenetic knowledge surrounding the *Atta* genus as well as the *Atta sensu stricto* phylogenetic group.

In addition, *A. cephalotes* showed other karyotype characteristics that were similar to the previously studied *Atta* species, such as heterochromatin distribution restricted to the centromeric region (Table 1). In general, *Atta* species do not have large heterochromatic clusters and serve as a good example of the challenge involved in detecting heterochromatic patterns through the basic C-banding technique on leaf-cutting ants (Barros et al. 2014, 2015). The heterochromatin distribution observed in the karyotypes of *Atta* species may be related to their functions in the chromosomal structure. Centromeric heterochromatin is part of the centromere structure; it plays a crucial role in the structure's functionality since it helps in the assembly of kinetochores and in the recruitment of large amounts of cohesins, which are proteins that maintain cohesion between sister chromatids (Pidoux and Allshire 2005; reviewed by Allshire and Madhani 2018). Consequently, such heterochromatin has a great level of influence over the correct segregation of chromosomes during cell division (Allshire and Madhani 2018).

Also, secondary constrictions co-localized in GC-rich regions and 18S rDNA clusters were observed interstitially in the 4th metacentric pair of *A. cephalotes*, like other *Atta* species (Barros et al. 2014, 2015; Teixeira et al. 2017). The intrachromosomal location of GC-rich 18S rDNA sites is the most common pattern in ants and influences the restriction of rDNA genes to a single chromosome pair, as observed in *Atta* species (Teixeira et al. 2021). Regions with differential staining with DAPI were not detected, as usually observed in different ant groups (Barros et al. 2014; Velasco et al. 2014; Correia et al. 2016; Teixeira et al. 2021).

Considering that insects are highly diverse with regard to their telomere structures (Kuznetsova et al. 2020; Lukhtanov 2022), the presence of such different repetitive sequences associated or not with the telomeres should be investigated in fungus-farming ant species. Traces of the $(TTAGGG)_n$ mo-

Fig. 1. Diploid karyotype (*a*) and metaphase (*b*) of *Atta cephalotes* female ($2n = 22$; $2n = 18m + 2sm + 2st$). Arrowhead indicates the presence of a secondary constriction in the fourth metacentric pair. Bars = 5 μm.

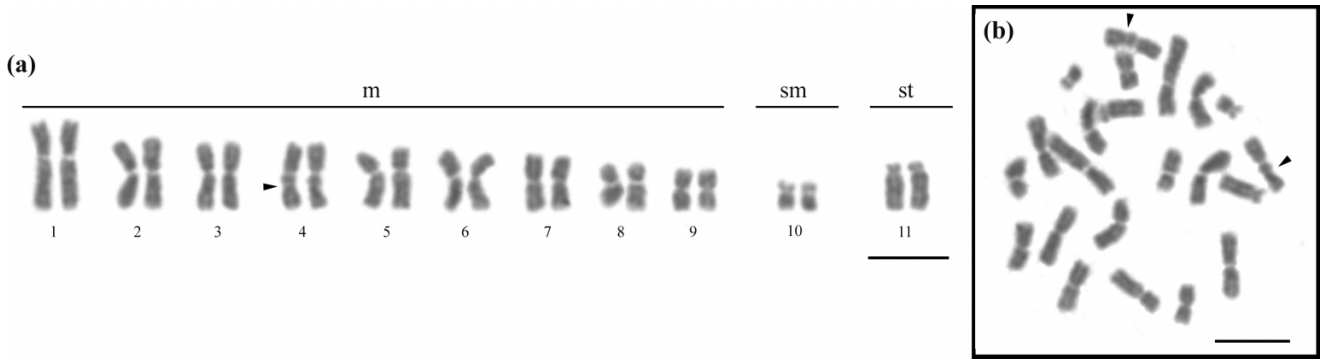


Table 3. Karyomorphometrical analyses of *Atta cephalotes* female.

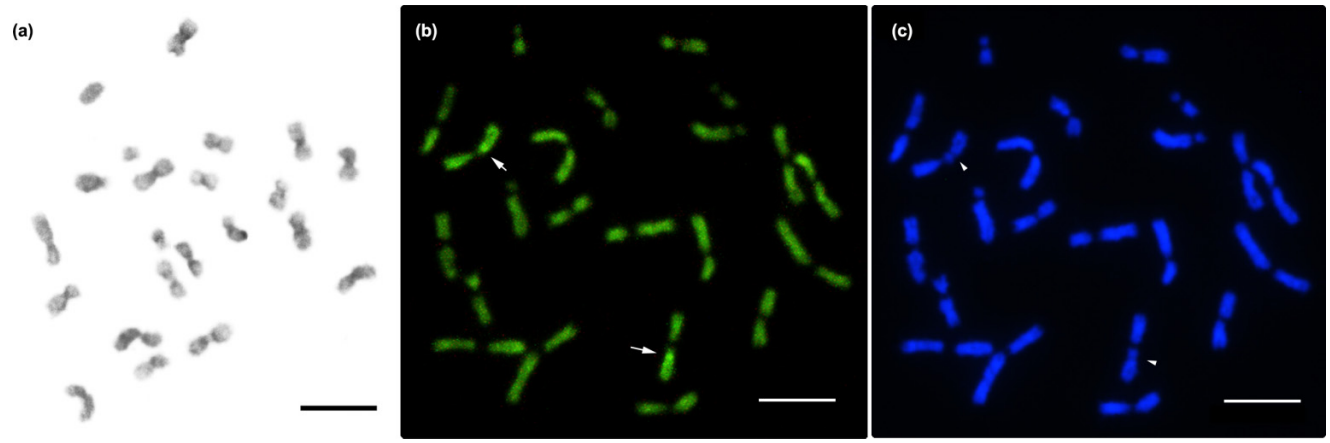
Chromosomes	*p*	*q*	TL	RL	*r*	Classification
1	2.21 ± 0.29	3.19 ± 0.44	5.39 ± 0.70	6.73%	1.45 ± 0.11	Metacentric
1	2.17 ± 0.24	3.22 ± 0.42	5.40 ± 0.65	6.75%	1.48 ± 0.09	Metacentric
2	2.08 ± 0.36	2.47 ± 0.33	4.55 ± 0.66	5.68%	1.20 ± 0.13	Metacentric
2	2.06 ± 0.29	2.43 ± 0.32	4.49 ± 0.59	5.61%	1.19 ± 0.08	Metacentric
3	1.86 ± 0.23	2.40 ± 0.27	4.26 ± 0.49	5.32%	1.30 ± 0.10	Metacentric
3	1.90 ± 0.27	2.38 ± 0.25	4.27 ± 0.49	5.33%	1.27 ± 0.13	Metacentric
4	1.76 ± 0.26	2.32 ± 0.29	4.08 ± 0.54	5.10%	1.32 ± 0.06	Metacentric
4	1.68 ± 0.21	2.35 ± 0.31	4.04 ± 0.49	5.05%	1.40 ± 0.14	Metacentric
5	1.57 ± 0.13	2.30 ± 0.35	3.87 ± 0.45	4.83%	1.47 ± 0.16	Metacentric
5	1.53 ± 0.16	2.29 ± 0.33	3.82 ± 0.47	4.77%	1.50 ± 0.13	Metacentric
6	1.65 ± 0.19	2.21 ± 0.31	3.85 ± 0.48	4.85%	1.32 ± 0.10	Metacentric
6	1.59 ± 0.18	2.09 ± 0.25	3.75 ± 0.41	4.68%	1.26 ± 0.10	Metacentric
7	1.55 ± 0.19	1.91 ± 0.27	3.46 ± 0.43	4.32%	1.23 ± 0.12	Metacentric
7	1.66 ± 0.19	1.85 ± 0.28	3.41 ± 0.44	4.26%	1.19 ± 0.11	Metacentric
8	1.55 ± 0.15	1.73 ± 0.18	2.95 ± 0.31	3.68%	1.43 ± 0.13	Metacentric
8	1.56 ± 0.14	1.75 ± 0.17	2.89 ± 0.28	3.61%	1.50 ± 0.13	Metacentric
9	1.22 ± 0.13	1.63 ± 0.19	2.72 ± 0.32	3.40%	1.22 ± 0.06	Metacentric
9	1.16 ± 0.14	1.49 ± 0.23	2.66 ± 0.36	3.32%	1.28 ± 0.08	Metacentric
10	0.58 ± 0.08	1.18 ± 0.14	1.75 ± 0.20	2.19%	2.07 ± 0.29	Submetacentric
10	0.57 ± 0.10	1.19 ± 0.14	1.76 ± 0.20	2.20%	2.13 ± 0.39	Submetacentric
11	0.64 ± 0.11	2.72 ± 0.33	3.36 ± 0.40	4.20%	4.30 ± 0.63	Subtelocentric
11	0.70 ± 0.16	2.64 ± 0.30	3.34 ± 0.44	4.17%	3.87 ± 0.52	Subtelocentric
			ΣTL = 80.01			

Note: The values (μm) represent an average obtained from 10 metaphases. Chromosomal classification according to Levan et al. (1964). *p*, short arm length; *q*, long arm length; TL, total length ($p + q$); RL, relative length ($RL = TL \times 100/\Sigma TL$); *r*, arm ratio ($q/p$).

tif, the canonical telomeric sequence in vertebrates, have been previously observed in some chromosomes of bulldog ants through FISH (Meyne et al. 1995) and even in the fire ant *Solenopsis invicta* Buren, 1972 (Myrmicinae) through sequencing analysis (Wurm et al. 2011). The $(TCAGG)_n$ motif has been detected in telomeres of some clades of the order Coleoptera (reviewed by Kuznetsova et al. 2020) and has recently been characterized as a microsatellite located in the centromeric heterochromatin of the social wasp *P. fastidiosuscula* (Marchioro et al. 2020). Our physical mapping data showed that such telomeric sequence variations are absent in any portion of the *A. cephalotes* chromosomes. However, we cannot discard the possibility that these motifs may be present at low levels that might be detectable only through genome sequencing analyses. The presence of different mechanisms of telomeric maintenance may also lead to variations in the ancestral $(TTAGG)_n$ motif along with insect evolution (Kuznetsova et al. 2020).

By contrast, the telomeric canonical motif $(TTAGG)_n$ of insects showed strong signals at the end of all the chromosomes of *A. cephalotes*, as in other ant species (Meyne et al. 1995; Lorite et al. 2002), including leaf-cutting ants (Pereira et al. 2018; Castro et al. 2020; Barros et al. 2021; Micolino et al. 2022; Teixeira et al. 2022). Interstitial telomeric sequences can be considered relicts of the fusions of ancestral chromosomes (Lin and Yan 2008; Olsson et al. 2018). However, no in-

Fig. 2. Diploid metaphases of *Atta cephalotes* female ($2n = 22$) submitted to (*a*) C-banding showing small centromeric heterochromatic blocks (dark regions), staining with fluorochromes chromomycin A_3 (CMA_3) and 4′6-diamidino-2-phenylindole (DAPI), respectively. Arrows in (*b*) indicate GC-rich regions, and arrowheads in (*c*) show negative regions for AT base pairs in the secondary constrictions. Bars = 5 μm.



terstitial telomere signal was detected on the chromosomes of *A. cephalotes* as expected since it has the same chromosome number proposed for the ancestor of leaf-cutting ants ($2n = 22$) (Pereira et al. 2018).

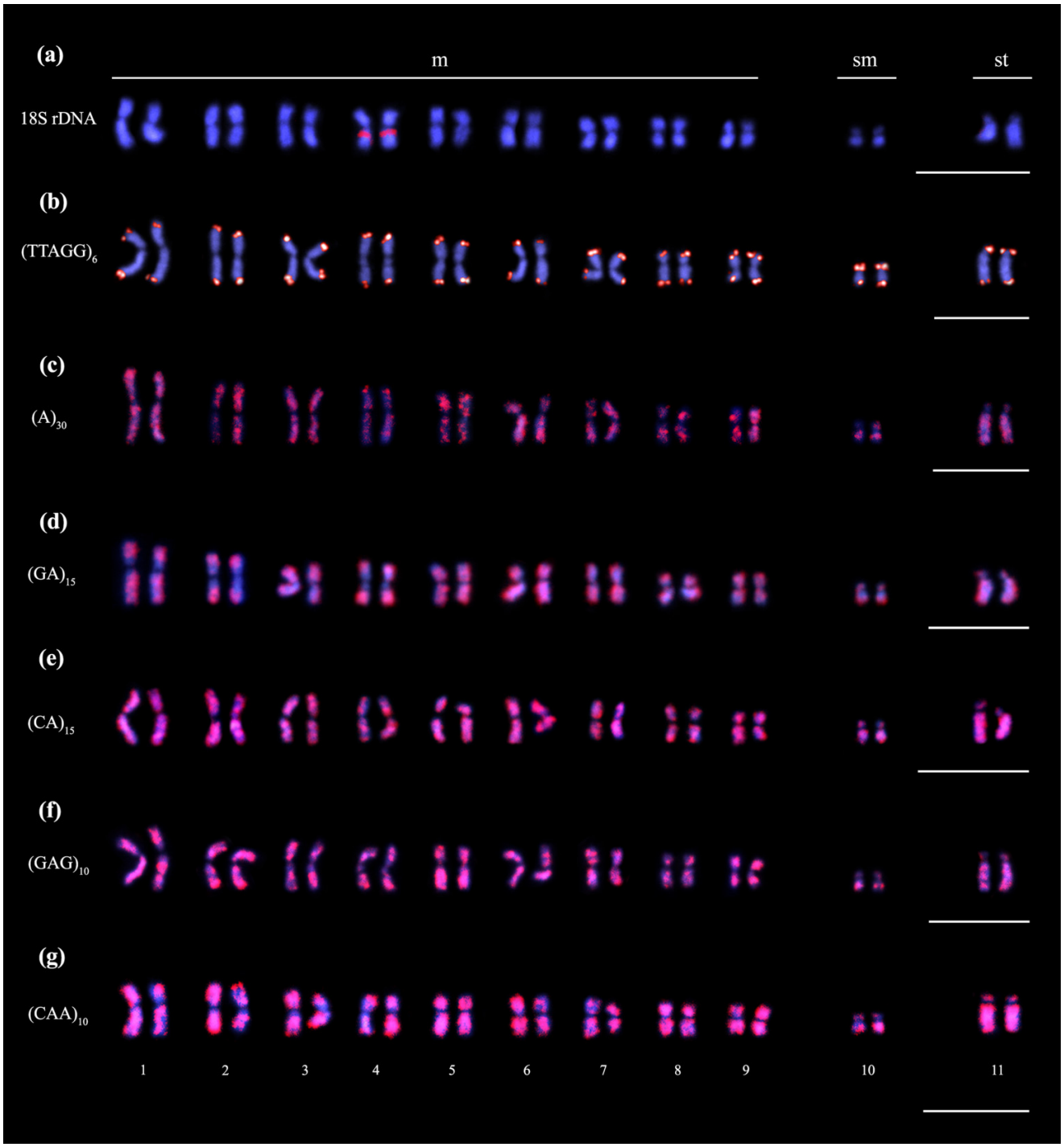
The genomic organization of all microsatellites showed scattered but nonrandom distribution, exclusively on the euchromatic regions of *A. cephalotes*, which corresponds to most chromosome lengths with the exception of the centromeric heterochromatin region. This pattern was also observed in other *Atta* species (Teixeira et al. 2022). The euchromatic location of microsatellites is common in insects (Palacios-Gimenez and Cabral-de-Mello 2015; Ruiz-Ruano et al. 2015; Travenzoli et al. 2019*a*, 2019*b*; Lopes et al. 2020). Associations between microsatellites or satellite DNA and transposable elements have been previously observed in different organisms, indicating that mobile elements may also be involved in both the origin and the genomic propagation of microsatellites (Ellegren 2004; Zhang 2004; Palomeque et al. 2006; Ruiz-Ruano et al. 2015). The repetitive portion of the *A. cephalotes* genome includes many transposable elements (Suen et al. 2011) that may be responsible for this intense spread of microsatellites in the euchromatin of this particular species. Future studies involving the mapping of this species' transposable elements will aid in investigating this hypothesis.

In the present study, the heterochromatin of *A. cephalotes* had no trace of several microsatellites, such as $(GA)_{15}$, $(CA)_{15}$, $(TAT)_{10}$, $(CGG)_{10}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(TCAGG)_6$. However, these microsatellites are present in the heterochromatic regions of other Hymenoptera species, like the bees' *Frieseomelitta* species and *M. seminigra merrilae*, and the wasp *P. fastidiosuscula* (Santos et al. 2018; Marchioro et al. 2020; Barbosa et al. 2021). Either these microsatellites were located in euchromatin, or there were no signals produced on the *A. cephalotes* chromosomes. These results suggest that the heterochromatin in *A. cephalotes* comprises other types of repetitive sequences, such as satellite DNA, or that it has low levels or other microsatellites undetectable by FISH and therefore warrant further investigations.

The microsatellites do not cluster in any specific region of the *A. cephalotes* chromosomes, similar to the pattern observed for a population of *M. holmgreni* (Barros et al. 2018) and bees (Piccoli et al. 2018; Travenzoli et al. 2019*a*, 2019*b*; Lopes et al. 2020; Barbosa et al. 2021), mainly the *Melipona* species of Group I (with a low proportion of heterochromatin restricted to pericentromeric regions). It contrasts to some populations of *M. holmgreni*, in which the $(GA)_{15}$ microsatellite is clustered in one or two chromosomal pairs (Micolino et al. 2019) besides *Mycocepurus goeldii* (Forel, 1893) and *Sericomyrmex* sp. that showed $(GA)_{15}$ bands in some metacentric chromosomes (Teixeira et al. 2022). Other insects like the grasshoppers *Abracris flavolineata* (De Geer, 1773) (Milani and Cabral-de-Mello 2014), *Locusta migratoria* (Linnaeus, 1758), and *Eyprepocnemis plorans* (Charpentier, 1825) (Ruiz-Ruano et al. 2015), and the cricket *Eneoptera surinamensis* (De Geer, 1773) (Palacios-Gimenez et al. 2015) showed clustered microsatellite patterns, in addition to scattered distribution. These studies show a diversity of distribution patterns of microsatellites on chromosomes of different organisms, highlighting the importance of these sequences' chromosome mapping to understand their organization and evolutionary patterns in the eukaryotic genome.

Conserved chromosome numbers have been observed in two other genera of leaf-cutting ants. *Acromyrmex* species show predominantly $2n = 38$ chromosomes (reviewed by Barros et al. 2021), with the single exception of *Acromyrmex ameliae* De Souza et al. 2007. This social parasite displays $2n = 36$ chromosomes, probably due to the Robertsonian chromosome translocation, representing a derivation from the genus. Chromosomal data on *Amoimyrmex* showed that *Amoimyrmex bruchi*, *Amoimyrmex silvestrii*, and *Amoimyrmex striatus* (Roger, 1863) have the same chromosome number, $2n = 22$ (Cristiano et al. 2013; Micolino et al. 2022). Distinct ant groups like *Odontomachus* (*haematodus* group), with

Fig. 3. Diploid karyotypes of *Atta cephalotes* female ($2n = 22$) submitted to fluorescence *in situ* hybridization (FISH) with different repetitive probes (red regions): (*a*) 18S rDNA genes, (*b*) $(TTAGG)_6$, (*c*) $(A)_{30}$, (*d*) $(GA)_{15}$, (*e*) $(CA)_{15}$, (*f*) $(GAG)_{10}$, and (*g*) $(CAA)_{10}$. Bars = 5 μm.



$2n = 44$, and *Camponotus* (*Myrmothrix*) (reviewed by Mariano et al. 2019), with $2n = 40$, also show a conserved chromosome number. However, in all these genera, other cytogenetic characteristics vary between species. For example, variations in chromosome morphology were observed between *Acromyrmex* species (reviewed Barros et al. 2021) and *Odontomachus* species (Aguiar et al. 2020). *Camponotus* species (*Myrmothrix*) have different 18S rDNA site numbers (Aguiar et al. 2017), and *Amoimyrmex* species show distinct microsatellite $(GA)_{15}$ patterns (Micolino et al. 2022). Furthermore, according to our perceptions based on both the karyotype figure and the chromosome measurements of *Amoimyrmex bruchi* and *Amoimyrmex silvestrii,* as presented in Micolino et al. (2022), other differences also seem to exist. The total chromosome sizes of the 9th and 10th metacentric pairs seem to be smaller than those of the 8th chromosome pair in *Amoimyrmex sil-*

Fig. 4. Ideogram showing the *Atta cephalotes* haploid complement with all the chromosomal markers analyzed in the present study.

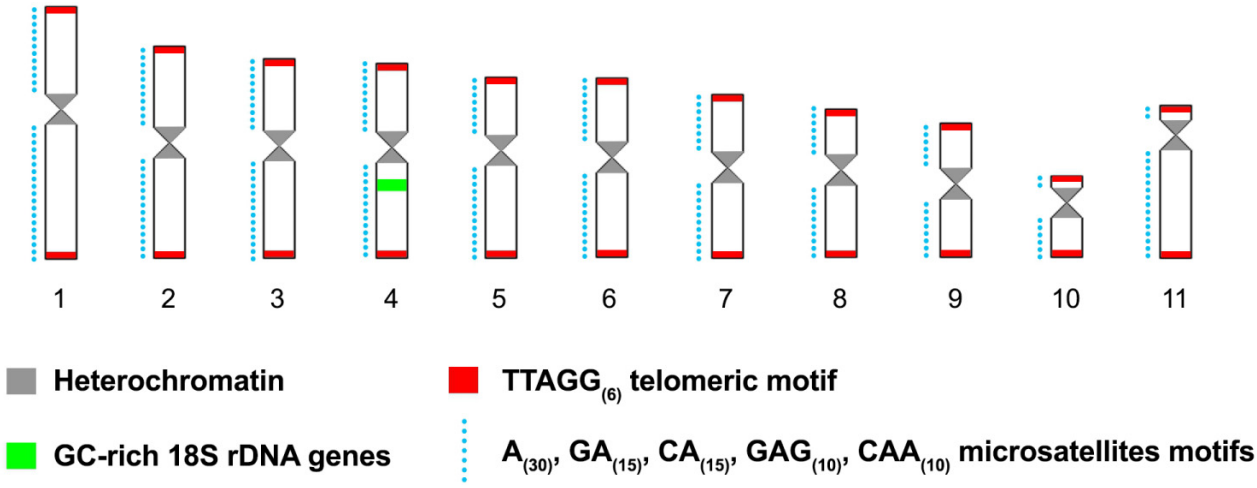
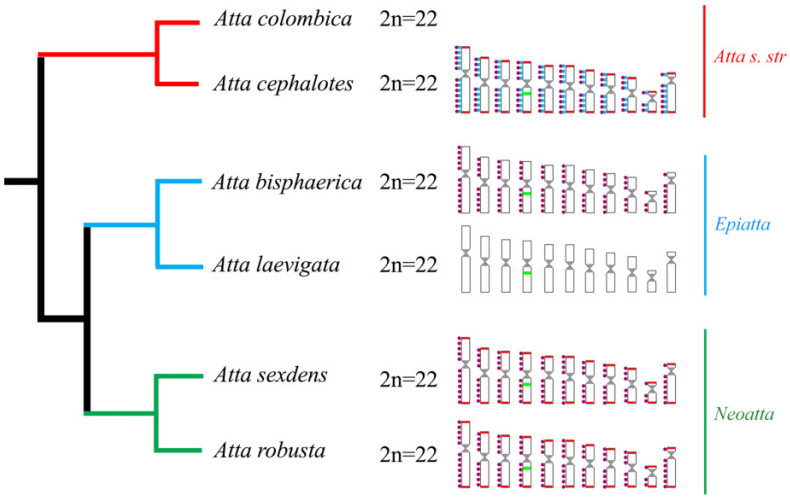


Fig. 5. Summary of available cytogenetic data (this study and literature) from *Atta* species included in three of the four monophyletic groups, namely *Neoatta*, *Epiatta*, and *Atta sensu stricto*, defined in the molecular phylogeny proposed by Bacci et al. (2009) and Barrera et al. (2022). Ideograms show chromosome morphology, heterochromatin (gray), 18S rDNA clusters (green), telomeres (red), and microsatellites $(A)_{30}$, $(CA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$ (blue dotted line), and $(GA)_{15}$ (purple dotted line) in the haploid complement. Phylogenetic relationships among the species are based on Bacci et al. (2009) and Barrera et al. (2022).



vestrii. However, the 8th, 9th, and 10th metacentric pairs seem to have the same size in *Amoimyrmex bruchi*. Measurement data provided by Micolino et al. (2022) confirm these differences. Our results corroborate the karyotype conservation in three of the four *Atta* groups, including chromosome number and morphology, heterochromatin pattern, location of GC-rich region, 18S rDNA clusters, and the $(GA)_{15}$ microsatellite, contributing with data on another *Atta* phylogenetic group (*Atta sensu stricto*) (Fig. 5). Karyotype conservation as observed in *Atta*, which includes different chromosome information and distinct species and groups (Fig. 5), has not yet been described in other genera. We also highlight that cytogenetic data for *A. sexdens* are available for the subspecies *A. sexdens rubropilosa* and *A. sexdens piriventris* that were previously synonymized by Borgmeier (1959).

It has been suggested that heterochromatin plays an essential role in ant karyotype evolution by dispersing in the karyotype and increasing the blocks in the chromosomes (Imai 1991). According to the recent model proposed by Hirai (2020), the physical associations of distal heterochromatic blocks in the meiotic prophase favor its dispersion in karyotype. However, intrachromosomal heterochromatic blocks do not form associations. *Acromyrmex* species show a higher heterochromatin distribution by the C-banding technique, which is located in some intrachromosomal regions but predominantly in small, short chromosome arms (Barros et al. 2016), denominated by Imai (1991) as pseudo-acrocentric. The location of these heterochromatic blocks in *Acromyrmex* seems to follow Hirai's model, leading to heterochromatin dispersion and variations in the chromosome morphology

among the species. Heterochromatin variations display an important role in karyotype evolution in *Acromyrmex* (Barros et al. 2016). In contrast, *Atta* species show smaller heterochromatin blocks restricted to intrachromosomal regions (Barros et al. 2014, 2015), which may influence the karyotype conservation among species.

Cytogenetic data indicate a wide increase and diversification in the chromosome number and morphology in ant genera of the ancient subfamilies, such as Ponerinae and the Australian Myrmeciinae. In contrast, smaller variations are observed within genera of the derived subfamilies Dolichoderinae, Formicinae, and Myrmicinae (Mariano et al. 2019). According to these authors, geographic barriers or recent diversification time did not drastically influence the karyotypes, especially with regard to the chromosome number found in these ants. Thus, another explanation for the conserved chromosome number would be the recent radiation and restricted geographic distribution of the leaf-cutting ants (Barrera et al. 2022).

In the present study, we confirmed the karyotype conservation in *Atta* using classical and molecular cytogenetic techniques. Other types of markers may bring new information regarding karyotype stability, such as transposable elements and satellite DNA; the latter, when located in centromeric regions, has high variation rates. In addition, we brought the use of several microsatellites, including mono-, di-, and trinucleotides, and different telomeric sequences in ant chromosomes that can be useful in further comparative studies. We contributed to the beginning of the karyotypic organization, understanding, and repetitive sequence diversity of leaf-cutting ants and Formicidae in general.

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Competing interests

The authors declare there are no competing interests.

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