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Chromosome Mapping of Ribosomal DNA Clusters in Four Leaf-cutting Ant Species of the Genus *Acromyrmex*, with Description of a Triploid Individual in *A. laticeps*

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Abstract

An increase in cytogenetic data in the leaf-cutting ant genus *Acromyrmex* has shown exciting contributions to evolutionary and taxonomic approaches, including the creation of a new genus and the description of chromosomal patterns, allowing comparative studies within *Acromyrmex* as well as between this genus and other similar taxa. The conserved chromosome number in *Acromyrmex* is a notable feature, with some distinguishable chromosomes among species. In this study, we described the diploid karyotype of *Acromyrmex laticeps* in addition to an individual with all the metaphases triploids, suggesting a triploid individual. To the best of our knowledge, this is the first report of polyploidy in a fungus-growing ant. Furthermore, we mapped 18S ribosomal DNA on the chromosomes of *A. laticeps* and three other *Acromyrmex* species. Among them, three species showed the ribosomal DNA cluster pattern observed in other previously studied members of this genus, with a terminal location in the largest subtelocentric pair. Meanwhile, *Acromyrmex balzani* had these ribosomal DNA clusters in its entire short arm. The role of triploidy in ant evolution has also been discussed. The increase in chromosomal data of leaf-cutting ants may provide significant insights into the evolution of this peculiar ant group.

Introduction

Leaf-cutting ants are the most conspicuous members of an important group that cultivates fungi for nourishment: fungus-growing ants (Schultz, 2021). They are mostly Neotropical (Mayhé-Nunes & Jaffé, 1998) and constitute a monophyletic group of >250 described species (Schultz, 2021). Among the leaf-cutting ants, the *Acromyrmex* Mayr, 1865 genus underwent remarkable restructuring based on recent integrative taxonomic findings from molecular biology, cytogenetics, and morphological data, culminating in the creation of the new genus *Amoimyrmex* Cristiano et al., 2020 (Cristiano et al., 2013, 2020). In addition, using morphological and molecular data, Forti et al. (2022) elevated three subspecies of the *Acromyrmex subterraneus* group to the species level: *A. molestans* Santschi, 1925, *A. brunneus* (Forel, 1912), and *A. subterraneus* (Forel, 1893).

Cytogenetic data on leaf-cutting ants, which include the genera *Acromyrmex*, *Atta* Fabricius, 1804, and the new genus *Amoimyrmex,* have increased in the last decade (Cristiano et al., 2013, 2020; Barros et al., 2014, 2016, 2021; Teixeira et al., 2017, 2022), but many gaps remain to fully comprehend the karyological evolution of these peculiar ants. Cytogenetic data are available for *Acromyrmex* spp. collected in Brazil, French Guiana, Panama, and Uruguay (reviewed by Barros et al., 2021), and to date, all of them have been shown to contain 2n = 38 chromosomes, with a single exception: *Acromyrmex ameliae* De Souza et al. (2007), which has 2n = 36 chromosomes, as a result of a Robertsonian translocation, which shaped the karyotype of the species (Barros et al., 2021).

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Despite conserved chromosome numbers in *Acromyrmex* spp., differences in chromosomal morphology, banding patterns, and molecular cytogenetics have been reported. Therefore, a different pattern observed in the genus *Atta,* which shows stability in chromosome number and other chromosomal traits, has already been analyzed (see discussion in Teixeira et al., 2022).

Molecular cytogenetic techniques, such as Fluorescent *in situ* Hybridization (FISH), for the physical mapping of major 45S rDNA genes (18S, 5.8S, and 28S) have provided important insights into ant cytogenetics (Teixeira et al., 2021a), contributing to our understanding of chromosomal evolution and phylogenetic relationships. Physical mapping of 45S rDNA has been performed for seven *Acromyrmex* (Table 1; reviewed by Teixeira et al., 2021a; Barros et al., 2021), five *Atta* (reviewed by Teixeira et al., 2022), and three *Amoimyrmex* species (Teixeira et al., 2017; Micolino et al., 2022). Similar to most other Formicidae, a single chromosome pair bearing ribosomal genes has been observed in leaf-cutting ants (reviewed in Teixeira et al., 2021a; Barros et al., 2021; Micolino et al., 2022). Generally, GC-rich regions contain ribosomal genes in different organisms (Symonová, 2019), including ants (Teixeira et al., 2021a).

The pattern of major rDNA clusters in *Acromyrmex* spp. is GC-rich and is considered conserved and restricted to the terminal region of the shorter arm of the highest subtelocentric pair (designated by Barros et al. (2016) as ST1). To date, *Acromyrmex echinatior* (Forel, 1899) has been considered an exception, with rDNA clusters located in the interstitial region of the short arm, a derived condition resulting from a paracentric inversion (Barros et al., 2016; Teixeira et al., 2021a).

Of the 34 described species of *Acromyrmex* (Bolton, 2023), the karyotypes of 17 species are known and a conserved chromosome number in the genus has been observed (reviewed in Barros et al., 2021). In this study, we described the karyotype of *Acromyrmex laticeps* (Emery, 1905) for the first time. Furthermore, we mapped the 18S rDNA clusters on the chromosomes of this species, as well as those of three other *Acromyrmex* species, to investigate whether they followed the pattern observed in this genus.

Material and Methods

The colony of *A. laticeps* was collected in Petrópolis, state of Rio de Janeiro, Brazil (22.50723° S, 43.18047° W), on 14 February 2021. This region is located in the Atlantic Rainforest and, therefore, is highly endangered due to historical anthropogenic interventions. Colonies of *Acromyrmex subterraneus* (Forel, 1893) and *Acromyrmex brunneus* (Forel, 1912) collected in Paraopeba, and *Acromyrmex balzani* (Emery, 1890) in Viçosa, both localities in the state of Minas Gerais, Brazil, were previously sampled for other studies of our research group (Barros et al., 2016, 2021).

Sampling permission was given by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (SISBIO accession number 32459). The metaphases were obtained from the cerebral ganglia of workers' larvae after meconium elimination, according to Imai et al. (1988). Chromosomes were measured, arranged in order of decreasing size, and classified according to Levan et al. (1964). The software Corel Photopaint X3 and Image-Pro Plus were used to assemble and measure the chromosomes, respectively.

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), with the treatment with distamycin omitted.

The sequences of 18S rDNA were mapped by FISH, following the protocol of Pinkel et al. (1986) with modifications described by Teixeira et al. (2021a). The 18S rDNA probe was obtained by amplification via polymerase chain reaction employing the primers rDNA 18SF1 (5'-GTC ATA GCT TTG TCT CAA AGA-3') and 18SR1.1 (5'-CGC AAA TGA AAC TTT TTT AAT CT-3') designed for the bee *Melipona quinquefasciata* Lepeletier, 1836, (Pereira, 2006) and isolated from total DNA of the ant *Camponotus rufipes* (Fabricius, 1775). The 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 FISH signals were indirectly detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol.

Results and Discussion

A set of 11 individuals from the colony of *A. laticeps* showed 2n = 38 chromosomes and a karyotype formula of 2n = 12m + 12sm + 12st + 2a (Figure 1a). This species showed the chromosome number conserved within the genus. The single exception thus far is *A. ameliae*, with 2n = 36. The karyotype is similar to that observed in other *Acromyrmex* spp., with some chromosomes present in different species, such as the longest metacentric, two longest subtelocentric, and even the acrocentric pair (reviewed in Barros et al., 2021).

Within this colony, one individual had an uncommon karyotype with 57 chromosomes. Detailed karyotype analysis indicated a triploid configuration with 3n = 18m + 18sm + 18st + 3a chromosomes (Figure 1b). We analyzed 74 metaphasic cells of this individual, all of which showed triploidy.

Polyploidy is considered a special class of mutation. It can result from distinct processes, such as genome doubling, gametic non-reduction, and polyspermy, which play significant roles in plant evolution (Otto & Whitton, 2000). A triploid individual within a natural diploid population is an exception because the development and retention of a triploid lineage is unlikely in a sexually reproducing diploid population among animals. Triploid individuals can usually emerge within diploid populations via three main mechanisms: fertilization

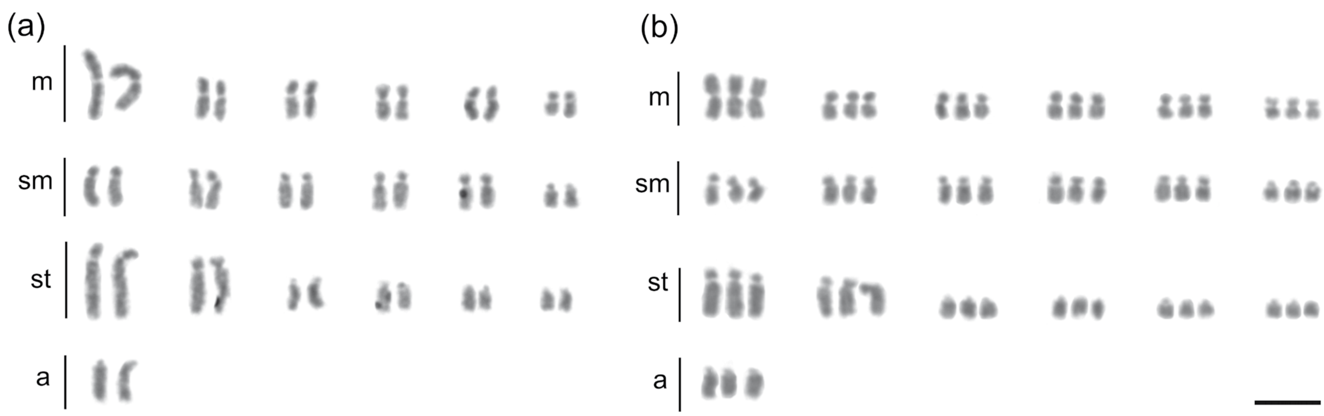

Fig 1. a) Karyotypes of a diploid female (2n = 38, 2n = 12m + 12sm + 12st + 2a) and b) triploid female (3n = 57, 3n = 18m + 18sm + 18st + 3a) of the leaf-cutting ant *Acromyrmex laticeps*. Bar = 5µm.

of a diploid egg by a haploid sperm owing to unreduced eggs; fertilization of a haploid egg by a diploid sperm, which is considered very rare compared to a female gamete; or polyspermy, which is the fertilization of a haploid egg by two haploid sperms (reviewed in Toda & Okamoto, 2016).

Hymenopterans are characterized by a haplodiploid sexual system, in which males are generally haploid, and females are diploid (reviewed in Gokhman & Kuznetsova, 2017; Gokhman, 2023). Despite the significance of the number of chromosome sets for the fertility of the natural population of hymenopterans, triploid individuals are considered unusual and unprecedented in fungus-growing ants (reviewed by Mariano et al., 2019; Cardoso & Cristiano, 2021), including an intensive analysis on leaf-cutting ants (Barros et al., 2014, 2016, 2021; Micolino et al., 2022), and polyploidy is not seen as a relevant mechanism in the evolution of ant genomes (Imai et al., 1977; Lorite & Palomeque, 2010).

Polyploid individuals of species belonging to different ant groups have been reported. In ants, tetraploid individuals have been reported in *Camponotus* sp. (Imai et al., 1977) and triploid individuals in *Crematogaster* sp. (Imai et al., 1977), *Vollenhovia* sp. (Crozier, 1975, apud Mariano et al., 2015), and *Solenopsis invicta* Buren, 1972 (Krieger et al., 1999). Triploidy in workers in *S. invicta* has been associated with the high frequency of diploid males in the analyzed polygyne population (Krieger et al., 1999). In addition, a wide variation in the ploidy level of cells in *S. invicta* has been reported among individuals (immature phase) within colonies, including variation in the same individual (Murakami et al., 2021; De Andrade et al., 2023). Silva (2016) detected diploid and polyploid cells in *Solenopsis saevissima* (Smith, 1855) using the same tissue (cerebral ganglia) but in distinct proportions according to the analyzed development (larvae and pupae). A decrease in the proportion of tetraploid cells was observed during the development of the individuals, suggesting that polyploidy is not permanent in adult worker tissues. Murakami et al. (2021) associated ploidy variation in the species with the effect of hybridization with other fire ants and even using insecticides to control these ants. In contrast, De Andrade et al. (2023) suggested that polyploid cells might be advantageous during the immature phase, as reversion was detected from larvae to pupae and adults by Silva (2016). Considering that cell variation was not detected in the individual with 57 chromosomes of *A. laticeps* in the present study using the cerebral ganglia, this strongly suggests a triploid individual.

The rDNA clusters were physically mapped on the terminal region of the ST1 pair in *A. laticeps*, *A. brunneus*, and *A. subterraneus* (Figures 2b, 2e, 2f). This pattern has also been reported in other *Acromyrmex* species (Table 1).

Table 1. Summary of physical mapping of 45S or 18S rDNA clusters in leaf-cutting ants of the genus *Acromyrmex*. Diploid chromosome number (2n).

Species	2n	Ideogram	Reference
Acromyrmex ameliae	36		Barros et al. (2021)
Acromyrmex aspersus	38		Teixeira et al. (2017)
Acromyrmex balzani	38		**This study**
Acromyrmex brunneus	38		**This study**
Acromyrmex coronatus	38		Barros et al. (2016)
Acromyrmex disciger	38		Barros et al. (2016)
Acromyrmex echinatior	38		Teixeira et al. (2021a)
Acromyrmex laticeps	38		**This study**
Acromyrmex niger	38		Barros et al. (2016)
Acromyrmex molestans	38		Teixeira et al. (2017)
Acromyrmex subterraneus	38		**This study**

The triploid individual of *A. laticeps* showed terminal markings on the three ST1 chromosomes (Figure 2c), and GC-rich regions were detected in the ST1 pair of diploid individuals (Figure 2a), with the same marking pattern as that observed for other *Acromyrmex* spp. (Barros et al., 2021). With a different pattern, the rDNA clusters in *A. balzani* were mapped on the entire short arm of the ST1 pair (Figure 2d), corresponding to the CMA_3^+ regions previously observed in this species (Barros et al., 2016). The karyotype of *A. balzani* was slightly different from those of other *Acromyrmex* species, with the largest metacentric and ST1 pairs of the same size. In contrast, in *A. balzani*, the largest metacentric pair was approximately twice as long as the ST1 pair. Species of the new genus *Amoimyrmex*, as well as *A. balzani*, were previously placed in the subgenus *Moellerius*, highlighting the importance of analyzing the species of this non monophyletic group. The distinct pattern of rDNA locations in *A. balzani* corroborated the differences in the size ratios of the chromosomes mentioned above. This may have been involved in the rearrangement that resulted in the distinct pattern observed in the species.

In contrast to the terminal location of rDNA in *Acromyrmex*, the intrachromosomal location observed in other leaf-cutting ant species, such as *Atta, Amoimyrmex*, and the close phylogenetic clade *Mycetomoellerius* (reviewed by Teixeira et al., 2021a, b; Micolino et al., 2022), suggests that this pattern corresponds to a plesiomorphic trait among leaf-cutting ants (Barros et al., 2021).

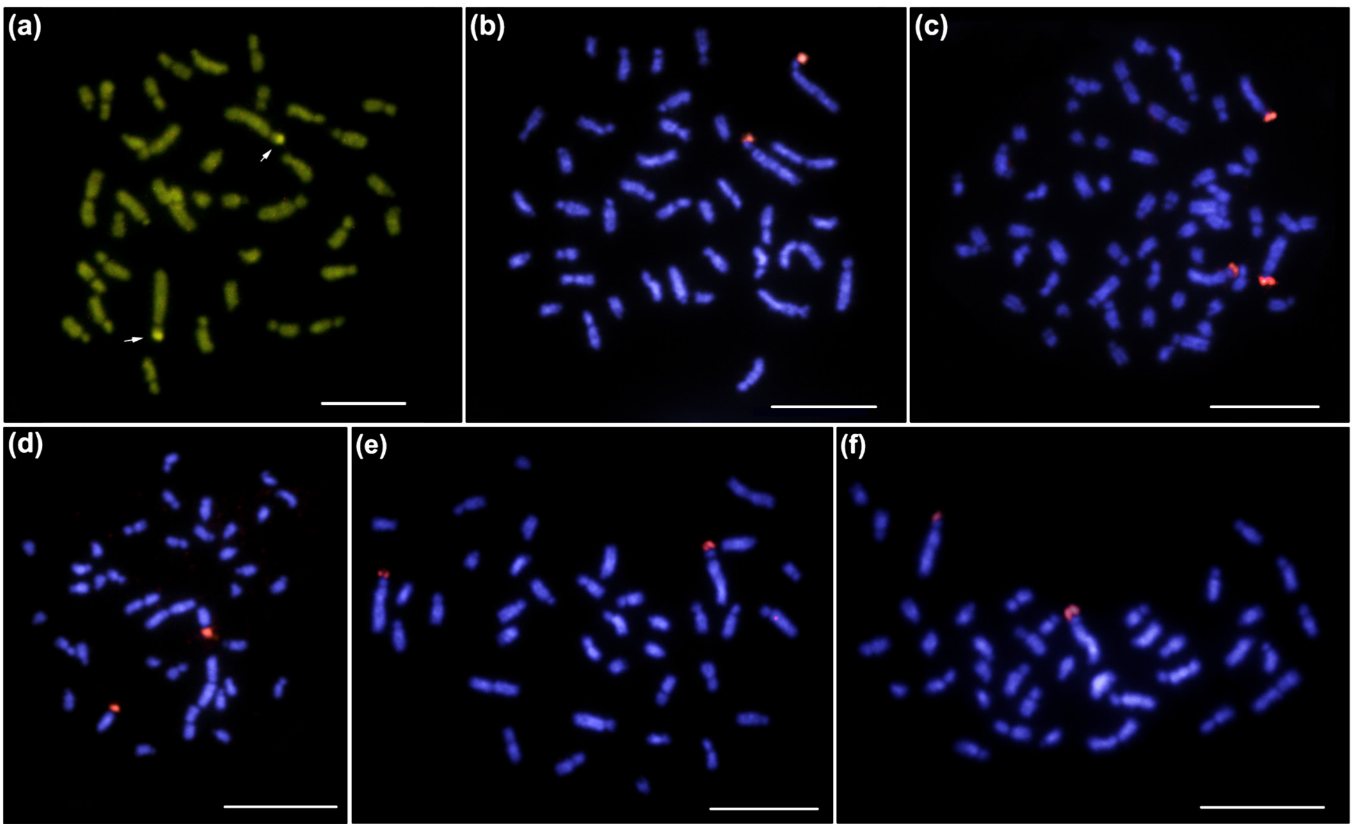

Fig 2. Metaphases of *Acromyrmex* leaf-cutting ant species submitted to different cytogenetic techniques. Metaphase of *Acromyrmex laticeps* (2n = 38) submitted to the fluorochrome chromomycin A_3 (CMA_3) with arrows indicating GC-rich regions (a). Metaphases of *Acromyrmex* species submitted to the Fluorescent *in situ* Hybridization with 18S rDNA probes (red signals): (b) and (c) *Acromyrmex laticeps* (2n = 38 and 3n = 57, respectively), (d) *Acromyrmex balzani* (2n = 38), (e) *Acromyrmex brunneus* (2n = 38) and (f) *Acromyrmex subterraneus* (2n = 38). Bars = 5µm.

In addition to the conserved chromosome number in *Acromyrmex*, karyological differences involving distinct chromosomal rearrangements have also been reported. Some notable rearrangements include a paracentric inversion in *A. echinatior*, a Roberstsonian translocation in *A. ameliae*, differential heterochromatin growth contributing to chromosome morphology differences in *Acromyrmex* spp., and complex chromosome rearrangements involving the karyotype of *A. balzani* (Barros et al., 2016, 2021; Teixeira et al., 2021a). The observation of a triploid individual in this study highlights the important role of cytogenetics in understanding the evolution of leaf-cutting ants, particularly Attina (Mariano et al., 2019; Cardoso & Cristiano, 2021). Future studies focusing on this group, including studies on the heterochromatin composition of these ants, will provide new insights into the evolution of the genus *Acromyrmex*.

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Authors' Contributions

LACB: Conceptualization, investigation, resources, writing-original draft, writing-eview & editing, visualization, supervision, project administration, funding acquisition
GAT: Investigation, iesources, writing-original draft, writing-review & editing, visualization.
RBL: Investigation.
AA: Data curation, writing-original draft.
HJACA: Investigation, resources, writing-original draft, writing-review & editing, visualization, project administration, funding acquisition.

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