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Cytogenetic studies in *Trachymyrmex holmgreni* Wheeler, 1925 (Formicidae: Myrmicinae) by conventional and molecular methods

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Abstract

Over the past several decades, ant cytogenetic studies have focused on chromosome number and morphology; however, recently, additional information concerning heterochromatin composition and 45S rDNA location has become accessible. The fungus-growing ants are a peculiar ant group that cultivates fungus for food, and *Trachymyrmex* is suspected to be the sister group of leafcutter ants. Cytogenetic data are so far available for sixn *Trachymyrmex* species. The present study aimed to increase the knowledge about *Trachymyrmex* cytogenetics by the chromosomal characterization of *Trachymyrmex holmgreni* including the karyotyping, fluorochromes staining, 18S rDNA, and microsatellite $(GA)_{15}$ fluorescence *in situ* hybridization (FISH). Karyotyped samples from four ant colonies showed 2n = 20 metacentric chromosomes. Centromeric heterochromatin rich in GC base pairs was detected in all chromosomes. FISH revealed the presence of rDNA clusters on the fourth chromosome pair, and an intense spreading of the microsatellite $(GA)_{15}$ including exclusively euchromatic areas of the chromosomes. The GC-rich heterochromatin observed in different ant species may have a common origin and, thus, phylogenetic implication that needs to be further investigated. To the best of our knowledge, this study is the first report of the use of chromosomal physical location of repetitive DNA sequences by means of microsatellite probes in Formicidae.

Introduction

Fungus-growing ants are found exclusively in the New World, primarily in the Neotropical region (Mayhé-Nunes & Jaffé, 1998; Schultz & Meier, 1995), and are suggested to have originated around 50 Mya (Chapela et al., 1994; Schultz & Brady, 2008). These ants comprise nearly 300 described species divided into 15 genera (Brandão et al., 2011).

Trachymyrmex Forel, 1983, currently includes 47 species (Bolton, 2017), and is suspected to be the sister group of the leafcutter ants, *Atta* and *Acromyrmex* genera. It is therefore a key group for understanding the relationship among "higher attine" (Brandão et al., 2011). The *Trachymyrmex septentrionalis* group, a clade of North American species, is considered by Schultz and Brady (2008) to be closely related to the leafcutter ants. *Trachymyrmex* is possibly a paraphyletic group (Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010) and taxonomic uncertainties remain.

Ant cytogenetics has drawn the attention of myrmecologists (Delabie et al., 2012) and is useful in phylogenetic, taxonomic, evolutionary and conservation applications (e.g., Cristiano et al., 2013; Barros et al., 2015; Santos et al., 2016; Aguiar et al., 2017). Cytogenetic studies in fungus-growing ants are scarce, especially for the so called "lower attine" group (reviewed in Barros et al., 2011). Cytogenetic data in *Trachymyrmex* are available for six species (Table 1), and the chromosome numbers range from 2n = 12 to 2n = 22 chromosomes. *Trachymyrmex septentrionalis* (McCook 1881),

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included in the *T. septentrionalis* group, is closely related to leafcutter ants (Schultz & Brady 2008), and presents 2n=20 chromosomes (Murakami et al., 1998). This chromosome number is similar to that observed in *Atta* spp. already studied, and also *Ac. striatus*, with 2n = 22, although minor chromosome morphology differences are observed between these two group species. The other leafcutter clade, with the other *Acromyrmex*, present 2n = 38, meaning, a derived karyotype was originated by centric fissions, according to Barros et al. (2016).

Studies concerning heterochromatin information are valuable in cytogenetic analysis of ants owing to the central role of heterochromatin in the chromosome evolution of the group (Imai et al., 1994). The cytogenetic data available for fungus-growing ants show heterochromatin distribution mainly at the centromeric and/or pericentromeric regions for species with low chromosome numbers n ≤ 12 (sensu Imai et al., 1988), such as *Mycocepurus goeldii* (Forel, 1893) (Barros et al., 2010), *Trachymyrmex relictus* Borgmeier, 1934 (Barros et al., 2013), *Trachymyrmex fuscus* Emery, 1934 (Barros et al., 2014a), and *Atta* spp. (Barros et al., 2014b, 2015). This pattern is expected in species with a low chromosome number, according to the minimum interaction theory (Imai et al., 1994).

Other cytogenetic techniques including fluorescent *in situ* hybridization (FISH) using rDNA probes have been used to detect differences among fungus-growing ants (Barros et al., 2016; Teixeira et al., 2017). The detection of heterochromatic GC-rich regions using the fluorochrome CMA_3 pointed to single bands for most species of the Neotropical region, including the leafcutter ants of the genus *Atta* (Barros et al., 2014b, 2015) and *Acromyrmex* (Barros et al., 2016). However, some fungus-growing ants showed multiple GC-rich regions, which correspond to heterochromatic bands such as those observed in *M. goeldii* (Barros et al., 2010), *T. fuscus* (Barros et al., 2014a), and *Acromyrmex striatus* (Roger, 1863) (Cristiano et al., 2013).

In ants from the Neotropical region, the reports of 18S or 45S physical location are available for 25 species (Mariano et al., 2008; Aguiar et al., 2011; Santos et al., 2010, 2016; Barros et al., 2012, 2015, 2016; Teixeira et al., 2017; Aguiar et al., 2017). The majority of these species present the GC-rich regions coinciding with the nucleolus organizer regions (NORs). The exceptions are *M. goeldii* (Barros et al., 2010, 2012), *Acromyrmex niger* (Smith, 1858) (Barros et al., 2016), *Dolichoderus lutosus* (Smith, 1858), *Dolichoderus voraginosus* MacKay, 1993, *Dolichoderus bidens*(Linnaeus, 1758) (Santos et al., 2016), and *Ac. striatus* (Cristiano et al., 2013; Teixeira et al., 2017), which presented multiple GC-rich heterochromatic bands and a single pair of NORs.

Recently, the detection of specific microsatellites as landmarks has been used in different organisms including insects such as orthopterans (Milani & Cabral-de-Mello, 2014, Palacios-Gimenez et al. 2015, Palacios-Gimenez & Cabral-de-Mello, 2015).These microsatellites can be useful markers in evolutionary studies. Microsatellites distribution is highly variable in the species genomes, and can be found in specific regions or dispersed throughout the chromosomes (Sumner, 2003). Many of the studied species present a scattered distribution pattern of microsatellites along the chromosomes.

Regarding the phylogenetic position of *Trachymyrmex* within the "higher attine" group, and the absence of previous physical mapping of rDNA genes data in this genus, this study aimed to describe the fungus-growing ant *T. holmgreni*, Wheeler 1925, included in the *Iheringi* group (reviewed in Mayhé-Nunes & Brandão, 2005) by means of classical and molecular cytogenetics.

Material and Methods

Four colonies of *T. holmgreni* were collected in Itutinga, State of Minas Gerais, Brazil (21º 17' S; 44º 39' W), on July 29th, 2012. Sample collection was done under the authorization of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for the collection of biological material issued to Luísa Antônia Campos Barros (SISBIO accession number 32459). Ant vouchers (workers) were identified by Jacques Hubert Charles Delabie and deposited in the reference collection at the Laboratório de Mirmecologia, Centro de Pesquisas do Cacau (CPDC/Brazil) under the record #5725.

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 were measured and chromosomes were classified according to Levan et al. (1964). Corel Photopaint X3® and Image Pro Plus® were the softwares used for mounting the chromosomal karyotype and measurements, respectively. For subsequent techniques, at least 15 metaphases were analyzed. At least five individuals per colony were analyzed.

C-banding was performed according to Sumner (1972) with minor adaptations as suggested by Barros et al. (2013). Specific GC- and AT-rich regions were detected using sequential staining with the fluorochrome Cromomicin A_3 (CMA_3) and 4'6- diamidino-2-phenylindole (DAPI) following Schweizer (1980).

Ribosomal 18S genes were detected by FISH, following Pinkel et al. (1986). The 18S rDNA probe was obtained via PCR (polymerase chain reaction) amplification 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* (Pereira, 2006). Total DNA of the ant *Camponotus rufipes* was used as template in the PCR reactions. 18S rDNA probes were labeled maintaining the conditions for PCR amplification (Pereira, 2006) by an indirect method using digoxigenin-11-dUTP (Roche, Mannheim, Germany), and the FISH signals were detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol.

Microsatellite $(GA)_{15}$ was used as probe in the physical location of repetitive DNA. The sequence of this probe was directly labeled with Cyanine-3 (Cy3) in the 5' terminal during synthesis by Sigma (St. Louis, MO, USA). The microsatellite hybridization procedures were performed according to Kubat et al. (2008), with the modifications of Cioffi et al. (2010).

The metaphases were observed and documented using a fluorescence microscope Olympus BX 60, coupled with capture system Q-Color3 Olympus® images, using the software Q capture® with the filters WB (450-480 nm), WU (330-385 nm) and WG (510-550 nm) for analyzing CMA_3, DAPI and rhodamine, respectively. The metaphases labeled with the microsatellite $(GA)_{15}$ probe were observed using a 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 WU forthe chromosomes (330-385 nm).

Results

T. holmgreni presented 2n = 20 chromosomes, all of them metacentric (Fig 1a; Table 1). Centromeric heterochromatin (Fig 1d) rich in GC-base pairs (Fig 1b) was observed in all chromosomes. Differentially, AT base pairs rich regions were not found (Fig 1c), but only negative regions complementing the fluorochrome CMA_3 (Fig 1d).

The detection of ribosomal 18S genes by FISH analysis showed bands in the centromeric region of the fourth pair of metacentric chromosomes (Fig 1e). The results indicated an intense spreading of the dinucleotide microsatellite $(GA)_{15}$ in the *T. holmgreni* genome including only euchromatic areas of the chromosomes (Fig 1f).

Discussion

The karyotype presented by *T. holmgreni* is similar in number and morphology to that of *T. septentrionalis* (Murakami et al., 1998), a species closely related to leafcutter ants (Schultz & Brady 2008), and *T. relictus* (Barros et al., 2013). The predominance of metacentric chromosomes is a karyotypic characteristic of the species of *Trachymyrmex* studied so far (Murakami et al., 1988; Barros et al., 2013, 2014a). The heterochromatin pattern observed in *T. holmgreni* is similar to that previously described in *T. fuscus* (2n = 18) (Barros et al., 2014a), with centromeric and pericentromeric bands that coincided with GC-rich regions (CMA_3^+).

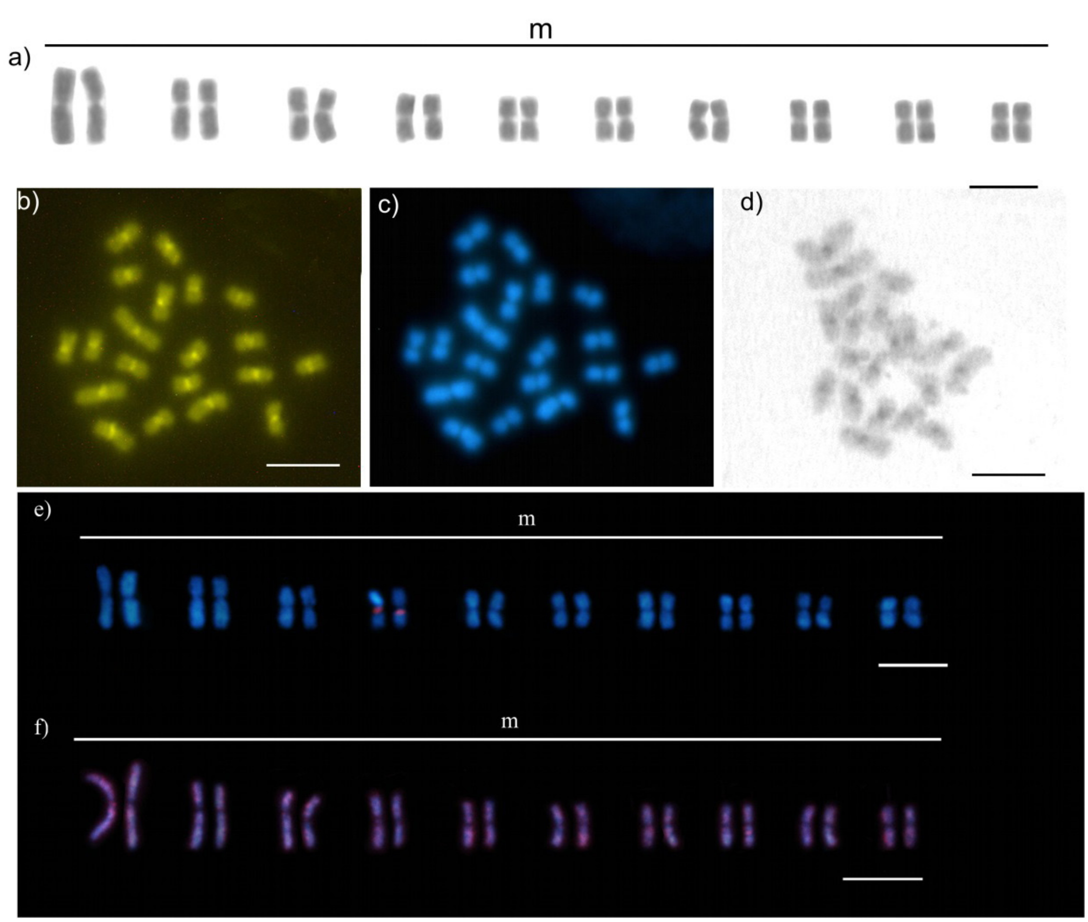

Fig 1. Conventional and molecular cytogenetics of mitotic cells of *Trachymyrmex holmgreni*. a) Diploid karyotype arranged in descending order of size (2n = 20), b) CMA_3 and c) DAPI staining for the detection of GC and AT rich blocks, respectively, d) C-banding for heterochromatin detection, e) FISH analysis for 18S rDNA, and f) FISH analysis for dinucleotide microsatellites repeats (GA)15.

Chromosomal fusion hypothesis was suggested for the taxa with 2n = 12 owing to the low chromosome number and the presence of interstitial heterochromatic blocks (Murakami et al., 1998). Considering the chromosome number available for *Trachymyrmex* spp. (Table 1), associated with the location and composition of heterochromatin for the studied species (Murakami et al., 1998; Barros et al., 2013, 2014, present study), centric fusion rearrangements seems to have occurred during the chromosomal evolution of this genus. Further cytogenetic studies will enable more robust inferences.

Although *T. holmgreni* had presented multiple GC-rich bands, only a single pair of NOR-bearing chromosomes was observed, demonstrating that most of the GC-rich heterochromatin bands in this species do not correspond to the 18S ribosomal genes. This was also observed in other fungus-growing ants such as *M. goeldii* (Barros et al., 2010, 2012), *A. niger* (Barros et al., 2016) and *Ac. striatus* (Cristiano et al., 2013, Teixeira et al. 2017).

Cytogenetic data of *T. holmgreni* in the present study showed predominance of metacentric chromosomes, multiple GC-rich heterochromatic bands and a single 18S rDNA pair: similar chromosomal traits that are observed in *Ac. striatus* (Cristiano et al., 2013). However, most *Acromyrmex* (2n = 38) and all *Atta* species already studied have a single pair of 18S rDNA rich in GC (Barros et al., 2014b, 2015, 2016; Teixeira et al., 2017). It is suggested that *Ac. striatus* is the sister group of the remaining leafcutter ants (Cristiano et al., 2013), and the GC-rich patterns observed in *Ac. striatus* which are also found in *T. holmgreni* and *T. fuscus* may have a common origin and, thus, a phylogenetic implication. This must be further investigated in other *Trachymyrmex* and other fungus-growing ant groups.

Trachymyrmex holmgreni (2n = 20), as well as *Ac. striatus* (2n = 22) and *Atta* spp. (2n = 22), presented 18S rDNA located in metacentric chromosomes (Barros et al., 2015; Teixeira et al. 2017), thus differing from *Acromyrmex* spp. which presented these genes in the terminal region of the larger subtelocentric chromosome pair (Barros et al., 2016; Teixeira et al., 2017). Considering the phylogenetic relationships of these species (Schultz & Brady, 2008; Cristiano et al., 2013), the 18S rDNA locations in metacentric chromosomes seem to be the ancestral condition of the group.

Usually NORs are rich in GC base pairs (Sumner, 2003). Another peculiar observation was described in *D. voraginosus* in which GC-rich regions did not correspond with rDNA 45S clusters (Santos et al. 2016), indicating the importance of the extension of rDNA mapping in Formicidae. Regions with differential staining with DAPI, indicative of regions rich in AT base pairs, were not observed in *T. holmgreni*, a similar pattern to that observed in other ants such as *T. fuscus* Emery, 1934 (Barros et al. 2014a), *Atta* spp. (Barros et al. 2014b, 2015), *Dolichoderus* (Santos et al., 2016), and *Pseudoponera* (Correia et al., 2016).

The repetitive probe $(GA)_{15}$ presented dispersed distribution in the euchromatic regions of the chromosomes. The dispersed pattern of the $(GA)_{15}$ microsatellite differs from that observed in the orthopteran *Abracris flavolineata* (De Geer, 1773),which presented specific euchromatic and heterochromatic bands (Milani & Cabral de Mello, 2014). In Formicidae, there are no data of chromosomal physical location using repetitive DNA sequences for comparisons. However, initial descriptive data can generate new insights into the comprehension of the ant genome. These data open new possibilities for population and evolutionary studies and the use of additional probes.

Table 1 – *Trachymyrmex* species studied cytogenetically. Chromosome number (2n). Chromosome morphology. Sampling sites (MG = Minas Gerais state). References of the data

Species	2n	Morphology	Locality	Reference
Trachymyrmex fuscus	18	16m + 2a	Paraopeba - MG - Brazil	Barros et al., 2014a
Trachymyrmex holmgreni	20	20m	Itutinga - MG - Brazil	Present study
Trachymyrmex relictus	20	20m	Viçosa - MG - Brazil	Barros et al., 2013
Trachymyrmex septentrionalis	20	20m	Barro Colorado - Panama	Murakami et al., 1998
Trachymyrmex sp. 1	12	12m	Barro Colorado - Panama	Murakami et al., 1998
Trachymyrmex sp. 2	18	18m	Barro Colorado - Panama	Murakami et al., 1998
Trachymyrmex sp.	22	18m + 4sm	Viçosa -MG - Brazil	Barros et al., 2013

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Compliance with Ethical Standards:

All the authors declare that they have no conflict of interest.

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