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# Population-Based Cytogenetic Banding Analysis and Phylogenetic Relationships of the Neotropical Fungus-Farming Ant *Trachymyrmex holmgreni* Wheeler, 1925

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**Keywords**
Fungus-farming ants · Phylogeny · Polymorphisms · Speciation

**Abstract**
*Trachymyrmex* is one of the most species-rich genera within fungus-farming ants and presents intraspecific cytogenetic polymorphisms as well as possible cryptic species. This ant genus is currently paraphyletic. Therefore, to unravel systematic and taxonomic misunderstandings, it is necessary to incorporate new information. We aimed to cytogenetically and genetically examine *Trachymyrmex holmgreni* populations from southern and northern Brazil to identify intraspecific chromosomal variations that support incipient speciation and reveal the species' position in a molecular phylogeny. Our cytogenetic approach did not show population variation in the mapping of both 18S rDNA and the $TTAGG_{(6)}$ motif, presenting instead a pattern characteristic of correlated species. However, the clustered pattern of the microsatellite $GA_{(15)}$ showed significant differences among populations: a well-defined block in each homologue, distinctly irregular signs between homologues, and blocks in 2 pairs of homologues. Our phylogenetic reconstruction yielded unexpected results, grouping representatives of 3 former morphological groups into 1 clade, namely *T. urichii*, *T. papulatus*, and *T. holmgreni*. Previously, it was suggested that northern and southern populations of *T. holmgreni* may be undergoing incipient speciation, but we can only indicate that the southernmost population differs prominently from the others in its distribution pattern of the microsatellite $GA_{(15)}$. Our study also supports the uniformity of karyotypes and repetitive DNA from both telomeric sequences and ribosomal DNA in *Trachymyrmex* studied here. In addition, we clarify some phylogenetic uncertainties within the genus and suggest further relevant systematic changes. Finally, additional studies utilizing other probes and additional populations may allow the detection of hidden genetic variation.



The eukaryotic genome is organized into a set of chromosomes that contain functional genes but consist, in large part, of repetitive sequences. Such sequences may have some functionality and could be involved in gene repair and expression as well as in the formation of heterochromatin [Shapiro and von Sternberg, 2005; Biémont and Vieira, 2006]. Repetitive DNA elements, located mainly in specific regions of chromosomes, such as centromere and telomeres, may induce chromosomal re-



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arrangements and thus cause significant evolutionary events in populations or species [Cazaux et al., 2011; Farré et al., 2015]. Karyotypes are usually well conserved in a panmictic population or species, whereas uneven intraspecific karyotypes may be related to meiotic incompatibilities that may have long-term effects on the emergence of incipient species [White, 1973; Guerra, 2008]. Moreover, intraspecific structural differences involving variations in shape, size, and DNA sequences, which do not alter the number of chromosomes, can also lead to prominent genetic changes and ultimately could promote speciation by restricting gene flow [Zolan, 1995; Hoffmann and Rieseberg, 2008].

The remarkable karyotypic inconstancy of ants is reflected in their high species diversity and terrestrial geographic spread, and it has been proposed that chromosomal rearrangements, such as Robertsonian fissions and fusions, may have directed speciation and diversification in ants [Lorite and Palomeque, 2010]. The controversial minimum interaction theory states that fission events, by increasing chromosome number, were primarily responsible for ant chromosome evolution [Imai et al., 1988; 2001]. Though these authors do not disregard the possibility of other chromosomal rearrangements in ant karyotype evolution, fusions have also been important in the diversification of *Mycetophylax* fungus-farming ants [Cardoso et al., 2014]. Therefore, taking into account parsimony and precision, both Robertsonian rearrangements must play equally important roles [Micolino et al., unpublished]. Natural populations constantly exhibit chromosomal polymorphisms [White, 1973], and these have been reported among ant populations [Imai et al., 1977; Lorite et al., 1996; Cardoso et al., 2014; 2018a]. Intraspecific cytogenetic divergences may be involved in the maintenance of different karyotypes in the same population, and their high frequency may be explained by recurrent mutation [Seifert, 2009; Lorite and Palomeque, 2010].

*Trachymyrmex* (Formicidae: Myrmicinae) is one of the most species-rich genera within fungus-farming ants and comprises species with a range of morphological variations, leading to complications in species identification [Mayhé-Nunes and Brandão, 2002; 2005; 2007; Brandão and Mayhé-Nunes, 2007; Rabeling et al., 2007]. The chromosomal number in *Trachymyrmex* ranges from 2n = 12 to 2n = 22 and appears to have structural stability with a predominance of metacentric chromosomes [Cardoso et al., 2018b]. According to morphological similarity, the genus *Trachymyrmex* was divided into groups, e.g., the Iheringi group includes those species endemic to South America [Mayhé-Nunes and Brandão, 2005]. *Trachymyrmex holmgreni* Wheeler, 1925 is a member of the Iheringi species group and is a widely distributed species occupying savannas, forests, and coastal environments [Mayhé-Nunes and Brandão 2005; Vasconcelos et al. 2008; Cardoso and Cristiano, 2010]. As the distribution of the Iheringi group does not cross the 10°S parallel [Brandão and Mayhé-Nunes, 2007], *T. holmgreni* can be separated geographically into northern and southern populations, with the boundary between them more or less at the 20°S parallel. The karyotype of this species consists of 2n = 20 chromosomes, all of them metacentric [Barros et al., 2018]. In addition, previous studies considering geographically distant populations of *T. holmgreni* have suggested a potential barrier to gene flow between northern and southern populations due to significant differences in genome size and karyotype length [Cardoso et al., 2018a].

Considering phylogenetic relationships, *Trachymyrmex*, together with *Sericomyrmex* and *Xerolitor*, form paraphyletic branches comprising a succession of 3 nested clades that fall as sister groups to the remainder of *Trachymyrmex* and with leafcutter ants [Schultz and Brady, 2008; Mehdiabadi and Schultz, 2009; Sosa-Calvo et al., 2018] thus making their phylogenetic relationship controversial. For instance, the monophyletic clade of *Sericomyrmex* plus the monotypic genus *Xerolitor* form a sister group of the Opulentus and Iheringi species groups [Schultz and Brady, 2008; Sosa-Calvo et al., 2018]. However, the phylogenetic position of *T. holmgreni* and their relatives is not yet known exactly, i.e., which lineages have descended from a common ancestor and their patterns of divergence.

Since chromosomal changes may be related to speciation [Rieseberg, 2001; Faria and Navarro, 2010], an effective way to test hypotheses about species delimitation can be to search for cytogenetic markers using FISH, which is a banding technique that allows for physical mapping of specific DNA sequences on chromosomes using 2 basic elements, a DNA probe and a target sequence [Speicher and Carter, 2005]. Cytogenetic data can be incorporated into phylogenetic trees and may give more precise estimates of the possible evolutionary paths of a group in question. Taking into account previous karyomorphometric differences across *T. holmgreni* populations [Cardoso et al., 2018a] as well as its slight morphological polymorphism in color, tubercles, spines, and pilosity [see figure 1 from Cardoso et al., 2018a], we aimed to analyze *T. holmgreni* cytogenetically and evaluate the phylogenetic positions of populations from southern and northern

Brazil. We sought FISH markers to determine if differential signals could reveal changes in telomeric and rDNA sequence length that could represent variation in genome size. Such highly repeated sequences are used with increasing frequency in studies based on insect biodiversity [e.g., Vershinina et al., 2015; Maryańska-Nadachowska et al., 2016; Micolino et al., unpublished], producing results that often lead to species delimitation. Indeed, the pattern of repetitive DNA distribution, such as that of microsatellite repeats, can vary considerably among natural populations [Cioffi et al., 2011], and there is evidence of preferential and nonrandom accumulation of some microsatellites on chromosomes [Ruiz-Ruano et al., 2015]. The distribution pattern of tandem DNA repeats could provide cytogenetic markers that would allow additional clues to whether these populations are undergoing incipient speciation and further contribute data for future systematic analyses in *Trachymyrmex*.

## Material and Methods

*Sampled Colonies*

Colonies of *T. holmgreni* were sampled in 6 different localities: Cidreira, RS (CI: 30°08′39′S; 50°12′19′W), Torres, RS (TO: 29°24′01′S; 49°46′33′W), Balneário Gaivota, SC (BG: 29°11′42′S; 49°36′31′W), Araranguá, SC (MC: 28°56′08′S; 49°21′28′W), Laguna, SC (IT: 28°21′04′S; 48°43′05′W), and Cachoeira do Campo, MG (CC: 20°20′56′S; 43°40′20′W). The number of colonies was variable, ranging from 5 to 20 colonies per locality. After sample collection, colonies of workers, queens, and broods (pupae and larvae) were taken to the Laboratório de Genética Evolutiva e de Populações at Universidade Federal de Ouro Preto, Minas Gerais, Brazil and kept under laboratory conditions according to Cardoso et al. [2011] until broods became available. Additional details such as year of collection, habitat type, and distances between populations can be found in Cardoso et al. [2018a] and Cristiano et al. [2019].

*Chromosome Preparation*

Metaphase chromosomes were obtained from brain ganglia of pre-pupal larvae according to Imai et al. [1988], with modifications described by Cardoso et al. [2012]. The selected brain ganglion was dissected in hypotonic colchicine solution (0.005%) under a stereoscopic microscope. Quality metaphase chromosomes were stained with 4% Giemsa solution diluted in Sørensen's buffer, pH 6.8 and were classified by centromere position and arm ratio, following the standard nomenclature proposed by Levan et al. [1964] with modifications by Crozier [1970]. At least 10 good non-overlapping metaphases from each colony were selected for subsequent analysis.

*FISH Analysis*

We performed FISH to physically locate the 45S rDNA cluster by means of a sequenced 18S rDNA probe, while using the $TTAGG_{(6)}$ motif to confirm the position of the telomere sequence and the oligonucleotides $GA_{(15)}$, $GAG_{(10)}$, $CAA_{(10)}$, and $CGG_{(10)}$ as microsatellite probes. Microsatellites and the TTAGG(6) motif were directly labeled with Cy3 at the 5′ terminal end during synthesis (Sigma, St. Louis, MO, USA), while the 18S rDNA sequence was obtained from the ant *Mycetophylax morschi* and amplified by PCR using the primers 18SF (5′-GTCATATGCTTGTCTCAAAGA-3′) and 18SR (3′-TCTAATTTTTTCAAAGTAAACGC-5′) [Pereira, 2006], which corresponded to a ~750-bp segment. The PCR reaction consisted of an initial denaturation for 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and final extension for 5 min at 72°C. FISH experiments were performed as previously described by Kubat et al. [2008] with detailed modifications as follows: the chromosomal preparations were incubated in RNAse (10 μg/mL) for 1 h at 37°C, then washed for 5 min in 2× SSC, pH 7.0 (0.03 M sodium citrate and 0.3 M sodium chloride). Then, 0.005% pepsin was added for 10 min at room temperature (RT), washed in 1× PBS (1.36 M sodium chloride and 0.07 M disodium phosphate) for 5 min followed by a time-out in 10% formaldehyde for 10 min at RT, and again washed in 1× PBS for 5 min. The chromosomal preparations on the slides were dehydrated in a series of alcoholic solutions for 2 min each (50, 70, and 100%, respectively) prior to denaturation by 70% formamide/2× SSC at 75°C on a heating plate for 5 min. Next, the spreads were dehydrated again in an alcoholic series of which the thermal shock in cold alcohol was emphasized, and finally, the chromosomes were hybridized with the mixture (200 ng of labeled probe, 50% formamide, 2× SSC, and 10% dextran sulfate 20× SSC) previously amplified at 75°C for 10 min and then kept in a moist chamber at 37°C overnight (i.e., the probe was hybridizing for at least 18 h). The next day, the chromosomal preparations were washed in 2× SSC and 1× SSC for 5 min each, plus a further wash in 4× SSC/Tween for 5 min. For the $TTAGG_{(6)}$ and microsatellite probes, a final alcohol dehydration was performed under the same conditions mentioned above, and chromosomal metaphases were assembled in antifade solution with DAPI (4′,6-diamidino-2-phenylindole) (Sigma-Aldrich). For the 18S rDNA probe, after the initial saline washes, the slides were incubated in 3% NFDM 4× SSC (i.e., a mix of distilled water, 20× SSC, and powdered milk). A detection solution (3% NFDM 4× SSC and anti-digoxigenin-rhodamine) was then added to each slide and kept in a moist chamber at 37°C for 1 h. The final steps were the same as described for the other probe used here.

*Phylogenetic Analyses*

Genomic DNA extraction was performed according to the standard CTAB/chloroform technique [Sambrook and Russell, 2001] from an individual representative of each population, not from the sampled colony. A total set of collected colonies were characterized as belonging to the same population if they were sampled a short geographic distance apart from each other (<20 km). We used 5 primer pairs previously described in the literature to sequence fragments of nuclear protein-coding genes (Table 1). The amplifications were carried out via PCR based on methodologies outlined in previous ant studies (references shown in Table 1). We checked the quality of sequencing by using Geneious R7 software [Kearse et al., 2012], from which nucleotides with a Phred score <20 were trimmed (at both sequence tips). The nucleotide sequences (GenBank accession numbers: MN562056-MN562085) were aligned using the Muscle algorithm implemented in MEGA7 software [Kumar et al., 2016] and manually concatenated. We

**Table 1.** Primers used to sequence fragments of nuclear protein-coding genes

| Gene region | Primer | Sequence 5′-3′ | Fragment size, bp | Annealing temperature, ° C | Source |
|---|---|---|---|---|---|
| *EF1a-F1* | 1424F<br>1829R | GCGCCKGCGGCTCTCACCACCGAGG<br>GGAAGGCCTCGACGCACATMGG | 278 | 60 | Brady et al. [2006] |
| *EF1a-F2* | 557F<br>1118R | GAACGTGAACGTGGTATYACSAT<br>TTACCTGAAGGGGAAGACGRAG | 484 | 56 | Brady et al. [2006] |
| *LW Rh* | 143F<br>639ER | GACAAAGTKCCACCRGARATGCT<br>YTTACCGRTTCCATCCRAACA | 348 | 56 | Ward and Downie [2005] |
| *Wg* | 578F<br>1032R | TGCACNGTGAARACYTGCTGGATGCG<br>ACYTCGCAGCACCARTGGAA | 373 | 55 | Ward and Downie [2005]<br>Abouheif and Wray [2002] |
| *Top1* | 1339F<br>2192R | GARCAYAARGGACCKGTRTTYGCACC<br>GARCARCARCCYACDGTRTCHGCYTG | 748 | 58 | Ward and Sumnicht [2012] |

placed *Cyphomyrmex* and *Mycetagroicus* species as outgroups based on their previous phylogenetic positions as well as differences in their morphology. A total of 45 fungus-farming ant sequences were taken from GenBank (accession numbers available at doi: 10.13140/RG.2.2.27634.66246) for the same gene fragments and added to our data matrix. Selection of the best evolutionary model for the concatenated dataset was performed by MEGA7 based on the Bayesian Information Criterion (BIC). This analysis indicated that GTR + I + G was the most suitable model. For Bayesian phylogenetic analysis, 2 independent simultaneous Monte Carlo Markov chain (MCMC) runs were carried out in MrBayes 3.2 software [Ronquist et al., 2012] with 10 million generations, sampling every 1,000 generations. Runs were evaluated using Tracer 1.7 [Rambaut et al., 2018] with effective sample size values for all parameters >200, and the first 10% of the sampled tree topologies were discarded as burn-in. The tree-generated and posterior probabilities were visualized in FigTree 1.4 software [Rambaut, 2009].

## Results

All analyzed colonies presented female individuals with 2n = 20 metacentric chromosomes (male ants have a haploid chromosome set, i.e., n = 10). Our comparative molecular cytogenetic analyses on the physical localization on the chromosomes of both ribosomal and telomere markers revealed no differences among *T. holmgreni* populations. The 18S rDNA probe was labeled in the pericentromeric region of the 4th chromosomal pair in all populations (Fig. 1). Likewise, the $TTAGG_{(6)}$ probe appeared restricted to the ends of all chromosomes, as there was no signal at interstitial telomeric sites (Fig. 2). In contrast, we found variation in size or brightness across signals that could represent variation in length (Fig. 1, 2). Similarly, the $GAG_{(10)}$, $CAA_{(10)}$, and $CGG_{(10)}$ microsatellite probes indicated a random distribution across the chromosomes that did not allow population differentiation (data not shown, sample results are available at doi: 10.13140/RG.2.2.27634.66246). On the other hand, the $GA_{(15)}$ microsatellite marker has been shown to be polymorphic across populations. All karyotypes showed scattered uniform signals across all chromosomes, except for the centromeric region, but $GA_{(15)}$ repeat accumulation was observed in some chromosomal regions forming clusters as follows: in the south-central (TO, BG, and IT) and northernmost (CC) populations, there were markings on a pair of homologues in the pericentromeric region; surprisingly, the southernmost (CI) population showed markings on 2 homologous pairs also in the pericentromeric regions, while the MC population differed in signal brightness in one homologous pair (Fig. 3).

We reconstructed a molecular phylogeny by Bayesian inference to unravel the phylogenetic relationships of *T. holmgreni* populations with the congeneric species. Our phylogenetic analyses indicated a monophyletic group (defined herein as the Iheringi group) composed of *T. urichii*, which diverged prior (Bayesian posterior probability, PP = 0.86), plus the sister species *T. papulatus* and *T. holmgreni* (PP = 1.0). We also recovered 3 phylogenetically separated clades: the first one comprises the Iheringi group lineages together with representatives of the Opulentus and Jamaicensis species group (PP = 1.0), the second encompasses the so-called Intermedius (or Cornetzi) group and the third, consisting of mostly North American lineages (also recognized as a sister group of leafcutter ants), the Septentrionalis

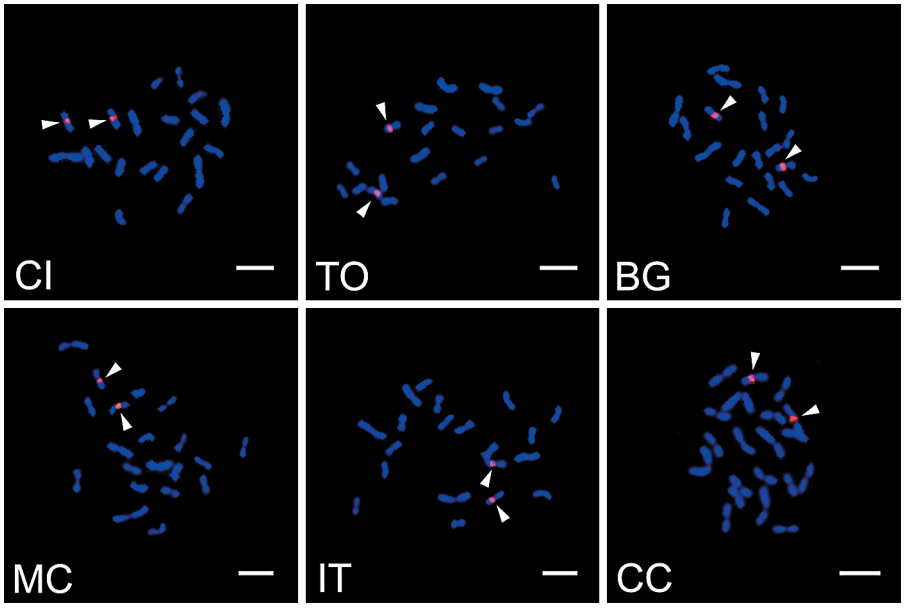



**Fig. 1.** Chromosomal metaphases stained with DAPI showing the 18S rDNA clusters (in red) in the 4th chromosome pair in different populations of *T. holmgreni*. CI: Cidreira; TO: Torres; BG: Balneário Gaivota; MC: Araranguá; IT: Laguna; CC: Cachoeira do Campo. Scale bars, 5 μm.

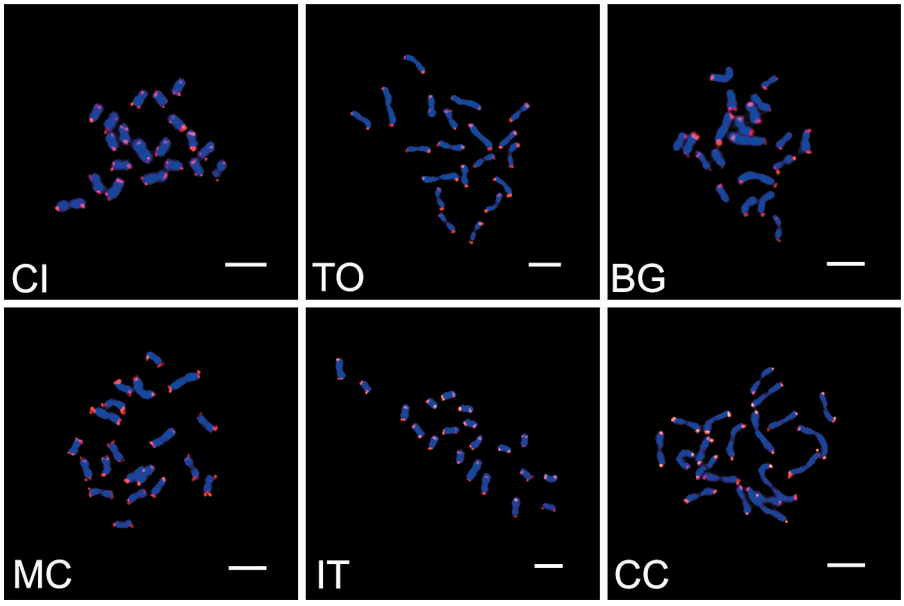
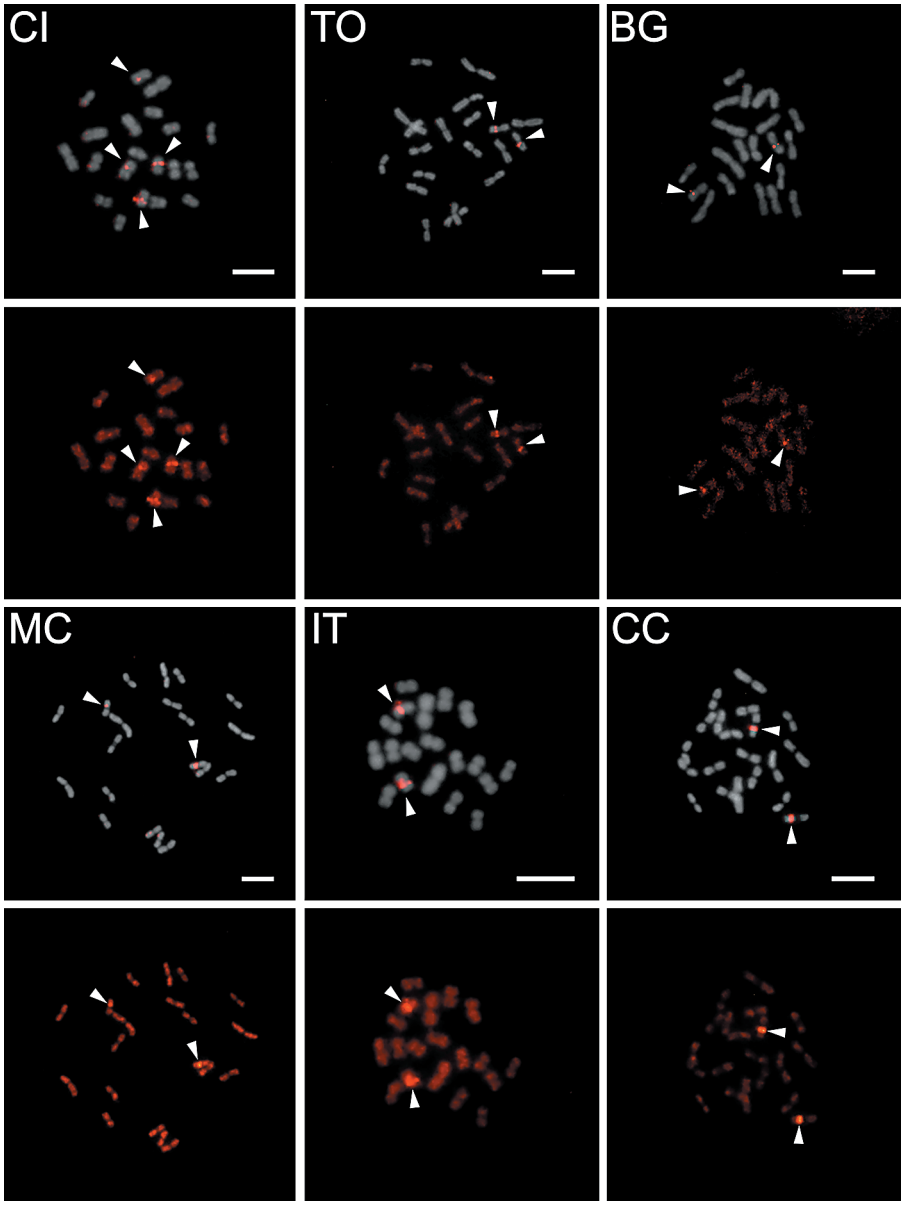



**Fig. 2.** Chromosomal metaphases stained with DAPI showing the $TTAGG_{(6)}$ telomeric sequences (in red) at the ends of all chromosomes in different populations of *T. holmgreni*. CI: Cidreira; TO: Torres; BG: Balneário Gaivota; MC: Araranguá; IT: Laguna; CC: Cachoeira do Campo. Scale bars, 5 μm.

group (PP = 1.0) (Fig. 4). Furthermore, despite the cytogenetic variations indicated for *T. holmgreni* populations, we did not obtain striking phylogenetic differences among them.

## Discussion

Our results did not show cytogenetic banding differences with the 18S rDNA and TTAGG telomere motif probes among the *T. holmgreni* populations analyzed here despite the considerable geographical distance, which is also related to nest habitats, as well as to the previously designated chromosomal length polymorphism [Cardoso et al., 2018a]. Such cytogenetic markers are widely conserved intraspecifically, although these markers have been increasingly used for species delimitation and cryptic identification [e.g., Grozeva et al., 2011; Chirino et al., 2017; Štundlová et al., 2019; Micolino et al., unpublished]. Mapping of the 18S rDNA cluster to the pericentromeric region of the 4th chromosomal pair corresponds to the previous finding in a *T. holmgreni* population




Color version available online

**Fig. 3.** Chromosomal metaphases stained with DAPI showing the $GA_{(15)}$ repeat sequences (in red in the merged up image; only probe in the bottom image) in different populations of *T. holmgreni*. CI: Cidreira; TO: Torres; BG: Balneário Gaivota; MC: Araranguá; IT: Laguna; CC: Cachoeira do Campo. Scale bars, 5 μm.

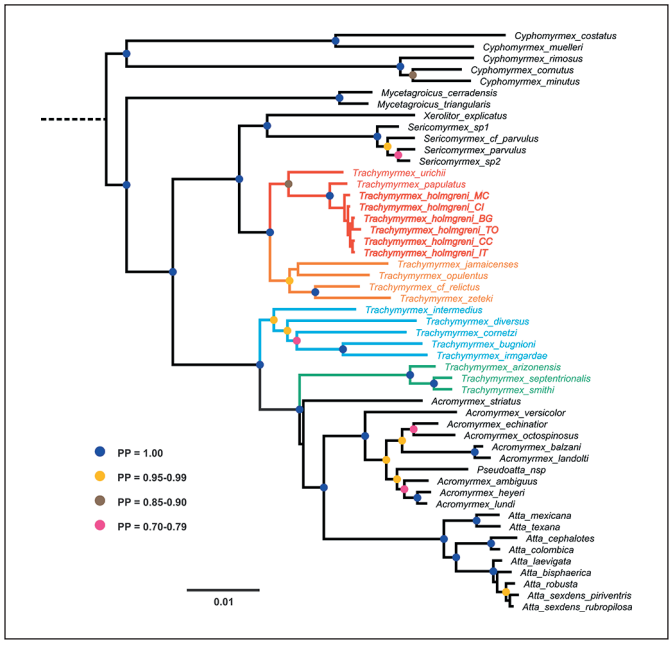
occurring north of its distribution [Barros et al., 2018], near to our CC population (our population closest to the registered border of its geographical distribution). The rDNA is extremely conserved within a particular species or natural population, and any intraspecific variation in these regions may be related to speciation processes [Raskina et al., 2004; 2008]. However, as mentioned before, both northern and southern populations showed no difference in this chromosomal feature, which implies stability at least in the 45S rDNA cluster.

Additionally, for the first time, we have mapped the telomeric regions within the genus *Trachymyrmex*. The TTAGG probe has been characterized for a range of organisms and is supposed to be the ancestral DNA motif of telomeres in insects, although it has been repeatedly lost within some insect orders [Sahara et al., 1999; Frydrychová et al., 2004]. Also, it is suggested that the TTAGG sequence was putatively lost in the Apocrita ancestor with at least 2 subsequent independent recoveries (in Formicidae and Apidae) [Lorite et al., 2002; Menezes et al., 2017]. Moreover, the ancestral nature of this repetition in the order Hymenoptera has been shown [Gokhman and Kuznetsova, 2018]. However, until then, the TTAGG motif had been confirmed in the chromosomes of very few

species of fungus-farming ants, namely *Acromyrmex striatus* [Pereira et al., 2018] and *Mycetophylax* spp. [Micolino et al., unpublished]. Here, we add another representative of fungus-farming ants that has the TTAGG sequence on its telomeres. It has been hypothesized that centric fusions play a relevant role in speciation, in that they can be fixed in isolated populations with negligible meiotic problems when combined in heterozygosis [Baker and Bickham, 1986]. Since others have pointed out that centric fusion rearrangements seem to have occurred during the chromosomal evolution of the genus *Trachymyrmex* [Barros et al., 2018], the use of a telomeric probe could unveil such rearrangements [Ruiz-Herrera et al., 2008]. However, we did not find any evidence for telomere-related rearrangements in *T. holmgreni* due to the absence of interstitial telomeric sites usually associated with tandem fusion rearrangements.

On the other hand, of the microsatellites tested, only the $GA_{(15)}$ repeat represented a polymorphic cytogenetic marker for *T. holmgreni* populations, and geographically close populations (such as MC and BG) showed slight differences in signal brightness intensity for the accumulated $GA_{(15)}$ regions. Such variations may represent differential accumulation between homologs during replication of this microsatellite region or unequal crossing over [Eckert and Hile, 2009]. Similarly, we highlight another interesting finding, where the southernmost population of Brazil (CI) exhibited 4 markings for the same microsatellite repeat, rather than 2 as in the other populations. Genomic studies indicate that microsatellites are often found in close association with transposable elements (TEs), including in insects [Wilder and Hollocher, 2001; Zhang, 2004]. Therefore, once a microsatellite arises, it can multiply and disperse in the genome via transposition of mobile elements [Zhang, 2004]. Thus, we can deduce that there might well have been a transposition of the GA-rich region associated with the centromere repeats [Hartley and O'Neill, 2019]. Perhaps an analysis comprising cytogenetic markers from transposons could highlight such an idea.

The accumulation of this microsatellite in blocks in the pericentromeric region of 1 or 2 chromosomal pairs appears to be a peculiar feature. Indeed, evident $GA_{(15)}$ microsatellite blocks, such as those presented here, are generally disseminated by chromosomes of the species characterized with this probe. For example, the grasshopper *Abracris flavolineata* had most of its chromosomes labeled with prominent interstitial, terminal, and proximal blocks [Milani and Cabral-de-Mello, 2014]. Similarly, stingless bee species of the genus *Melipona* had markings




Color version available online

**Fig. 4.** Phylogenetic tree emphasizing the position of *T. holmgreni* and the different clades of *Trachymyrmex* from Bayesian analysis of 5 nuclear protein-coding genes. In red the (Urichii + Iheringi) group, in orange the (Opulentus + Jamaicensis) group, in light blue the Intermedius (or Cornetzi) group, and in dark blue the Septentrionalis group is shown.

confined to euchromatin regions on all chromosomes, particularly in subterminal regions [Travenzoli et al., 2019]. As specified by Barros et al. [2018] from C-band analysis, the centromeres of *T. holmgreni* chromosomes are composed of heterochromatin while the remainder is predominantly euchromatic. Nevertheless, our results were unexpectedly different from those obtained with the $GA_{(15)}$ probe by Barros et al. [2018], who found no evident cluster. We emphasize that we performed several replications and obtained the same results for all populations.

Overall, ribosomal and telomeric sequences are indeed well-conserved cytogenetic markers, so that even in ant populations with incongruences that may be related to centromere length [Cardoso et al., 2018a] they have been preserved entirely on the chromosomes of these same populations. Microsatellite cytogenetic markers have also proved advantageous for possible delimitation of cryptic species. This suggests that modes of chromosomal speciation are still quite intriguing, and, since repetitive sequences change rapidly within properly isolated populations, could lead to genome remodeling and hence dif-

ferentiation [Hughes and Hawley, 2009]. Further cytogenetic information, including FISH mapping, from other closely related species of *Trachymyrmex* may be indispensable for characterizing the role of chromosomal changes in the evolution of these lineages and the particular importance of chromosomal rearrangements in genomic repatterning.

The combination of cytogenetic and phylogenetic data can indicate precisely the evolutionary relationships among lineages. As *Trachymyrmex* has been assembled into morphologically similar groups, additional phylogenetic information within them could be used to further refine their phylogenetic relationships. Our phylogenetic reconstruction indicates that *T. holmgreni* is more closely related to *T. papulatus*, which is not included in the Iheringi group because it lacks lobated antennal scapes, although there were clues to its phylogenetic closeness [Brandão and Mayhé-Nunes, 2007]. Eventually, we reconstructed the *T. urichii* species as belonging to the Iheringi group, being the most basal species phylogenetically known for this clade. Since no further species from Urichii group were used in our analyses, this group may indeed be the sister clade of the Iheringi group. Yet, the last proposition made by Brandão and Mayhé-Nunes [2007], where they join the former groups Opulentus and Jamaicensis [Mayhé-Nunes and Brandão, 2002; 2007] within a so-called Urichii group, must be dubious and systematically unassertive from a molecular phylogenetic point of view. Our results indicate that the Urichii group is closely related to the Iheringi group, whereas the Opulentus and Jamaicensis groups appear to be more closely related. Furthermore, it is worth noting that nomenclature problems are found within *T. urichii* species due to the indiscriminate use of one of its synonyms, known as *T. fuscus* [e.g., Vieira et al., 2012; Barros et al., 2013a; Araújo et al., 2016]. According to Bolton [1995], *T. urichii* has 4 junior synonyms, among them *T. urichii fusca,* which would be the former *T. fuscus*. We therefore reiterate that the invalid name *T. fuscus* should no longer be used. Essentially, our phylogenetic reconstruction also suggests that clustering based on morphological features may not delineate natural groups, which denotes the key role of multilocus analyses, assisting and often re-arranging phylogenetic trees based exclusively on morphological data. In summary, *Trachymyrmex* appears to be a paraphyletic genus, but 3 clades are evidently well defined: the Urichii and Iheringi plus Opulentus and Jamaicensis clade, the Intermedius (or Cornetzi) clade, and the Septentrionalis clade [Mehdiabadi and Schultz, 2009; Adams et al., 2012; Sánchez-Peña et al., 2017; present study]. Given the cladistic discrimination among these monophyletic branches, it can be suggested that each one belongs to a distinct genus.

Our comparative approach also provides clues about karyotype evolution in fungus-farming ants. The predominance of metacentric chromosomes in *Trachymyrmex* lineages seems to be a synapomorphic feature, as opposed to only being a karyotypic characteristic of the clade encompassing the Iheringi group. Such implications come from the following circumstances: *T. holmgreni* has 2n = 20 chromosomes, all metacentric, and *T. urichii* has 2n = 18, of which 16 are metacentric and 2 are submetacentric. Likewise, *T. relictus* from the Opulentus group has 2n = 20, all metacentric, and *T. septentrionalis* from North America clade has 2n = 20, also all metacentric [Murakami et al., 1998; Barros et al., 2013a, b; 2018]. Further, leafcutter ants of the *Atta* genus have a well-conserved number of chromosomes in their karyotypes with 2n = 22, as does their likely ancestor, *A. striatus*, which presents 2n = 22 with 20 metacentric plus 2 submetacentric chromosomes [Cristiano et al., 2013]. Obviously, the lack of *Trachymyrmex* species that have been characterized cytogenetically hinders an accurate reconstruction of its evolutionary history and the role of chromosomes on their lineage diversification besides implying taxonomic misunderstandings.

Our comparative study contributes to a better understanding of *Trachymyrmex* lineages by fusing cytogenetic and molecular data. The characterization of highly repeated sequences on chromosomes, mainly microsatellites, has led to distinctions rarely encountered previously. The output to test processes on putatively incipient species comes from limited analyses and may be needed for a complete understanding to place the role of gradual changes on chromosomes in the speciation process. Further studies taking into account other probes, as well as a larger number of species to reconstruct their phylogenetic relationships, may allow more precise detection of genetic variation and help in the identification of cryptic species within this genus, which is one of the most diverse of the fungus-farming ants.

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## Statement of Ethics

The samples were collected by the authorization of ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade) by permission number 49332–1 and 49336–1. We thank the editor and 3 reviewers for their helpful comments on the manuscript.

## Disclosure Statement

The authors declare no conflicts of interest.

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## Author Contribution

R.M., M.P.C., and D.C.C. conceived the study; M.P.C. and D.C.C. collected the samples; M.P.C. and D.C.C. contributed with reagents/materials/and analysis tools; R.M. and D.C.C. conducted the molecular cytogenetic experiments; R.M. conducted and analyzed the molecular data; R.M. and D.C.C. wrote the manuscript. All authors revised and approved the final version of the manuscript.

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