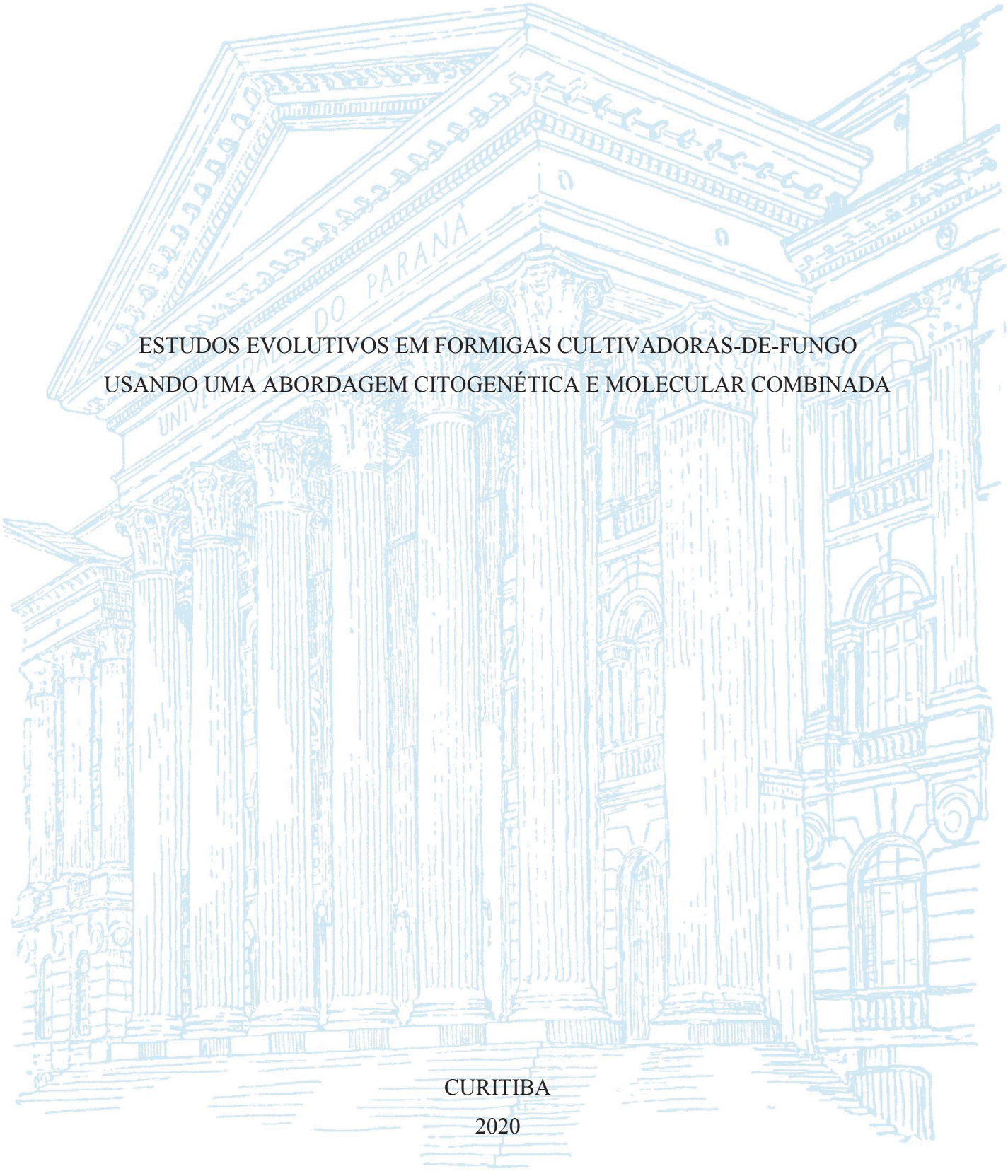
UNIVERSIDADE FEDERAL DO PARANÁ

RICARDO MICOLINO



ESTUDOS EVOLUTIVOS EM FORMIGAS CULTIVADORAS-DE-FUNGO USANDO UMA ABORDAGEM CITOGENÉTICA E MOLECULAR COMBINADA

CURITIBA

2020

RICARDO MICOLINO

ESTUDOS EVOLUTIVOS EM FORMIGAS CULTIVADORAS-DE-FUNGO USANDO UMA ABORDAGEM CITOGENÉTICA E MOLECULAR COMBINADA

Tese apresentada ao curso de Pós-Graduação em Genética, Setor de Ciências Biológicas, Universidade Federal do Paraná (UFPR), como requisito parcial à obtenção do título de Doutor em Genética.

Orientador: Prof. Dr. Danon Clemes Cardoso
Coorientator: Prof. Dr. Maykon Passos Cristiano

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em GENÉTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **RICARDO MICOLINO** intitulada: **Estudos evolutivos em formigas cultivadoras-de-fungo usando uma abordagem citogenética e molecular combinada**, sob orientação do Prof. Dr. DANON CLEMES CARDOSO, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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Dedico este trabalho a todos aqueles que
acreditam e apoiam a ciência brasileira.

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RESUMO

Estudos evolutivos conferem uma gama de oportunidades para se testar hipóteses sobre relações taxonômicas, filogenéticas, comportamentais, biogeográficas, citogenéticas, entre outras. As formigas cultivadoras-de-fungo são um grupo natural fascinante devido ao fato de terem estabelecido uma relação simbiótica com fungos e, portanto, praticam a agricultura há muito mais tempo que os seres humanos. Na presente tese de doutorado, o objetivo foi obter maior compreensão sobre a história evolutiva de alguns grupos de formigas cultivadoras-de-fungo focando em análises citogenéticas e filogenéticas. No primeiro capítulo, discutiu-se a história evolutiva de cinco linhagens de *Mycetophylax*, todas endêmicas em dunas de areia. As descobertas suportam o papel fundamental das mudanças cromossômicas correlacionadas com o aumento do estresse ecológico durante as transições climáticas passadas na diversificação dessas linhagens, bem como a provável rota de colonização da costa atlântica brasileira. No segundo capítulo, examinou-se citogeneticamente populações de "*Trachymyrmex holmgreni*" (nome válido atual *Mycetomoellerius holmgreni*) com o intuito de procurar por variações cromossômicas intraespecíficas que poderiam suportar uma especiação incipiente. Além disso, discutiu-se a posição filogenética desta espécie e sua relação com espécies intimamente relacionadas. No terceiro capítulo, descreveu-se o cariótipo de *Mycetomoellerius iheringi* e, com a posse de valiosas informações disponíveis sobre características citogenéticas de espécies congêneres, sugeriram-se os possíveis modos de especiação cromossômica entre elas, tomados à luz de suas relações filogenéticas. No quarto capítulo, analisaram-se os cariótipos de populações de *Acromyrmex striatus* provenientes do Chaco argentino e de *Acromyrmex silvestrii*. Destacou-se o alto nível de conservação cariotípica e morfológica dessas linhagens e discutiram-se possíveis modos de especiação sem alterações cromossômicas identificáveis. No quinto capítulo, usou-se uma abordagem combinada de dados morfológicos e moleculares que estavam disponíveis na literatura para desvendar profundamente as relações evolutivas de "*Trachymyrmex*" latu sensu (atuais gêneros *Mycetomoellerius*, *Paratrachymyrmex* e *Trachymyrmex* stricto sensu) e as possíveis rotas biogeográficas adotadas por seus principais clados que poderiam explicar suas distribuições geográficas atuais.

Palavras-chave: cromossomos, especiação, evolução, filogenia, formigas.

ABSTRACT

Evolutionary studies provide a range of opportunities to test hypotheses about taxonomic, phylogenetic, behavioral, biogeographic, cytogenetic relationships. Fungus-farming ants are a fascinating natural group, due to the fact that they have established a symbiotic relationship with fungi and, therefore, have been practicing agriculture for much longer than humans. In the present doctoral thesis, the objective was to get a better understanding of the evolutionary history of some groups of fungus-farming ants, focusing on cytogenetic and phylogenetic analyzes. In the first chapter, the evolutionary history of five lineages of *Mycetophylax*, all endemic in sand dunes, was discussed. The findings support the fundamental role of chromosomal changes correlated with the increase of ecological stress during past climatic transitions in the diversification of those lineages, as well the likely colonization route of the Brazilian Atlantic coast. In the second chapter, populations of "*Trachymyrmex holmgreni*" (current valid name *Mycetomoellerius holmgreni*) were cytogenetically examined in order to look for intraspecific chromosomal variations that could support incipient speciation. Moreover, the phylogenetic position of this species and its relationship with closely related species were discussed. In the third chapter, the *Mycetomoellerius iheringi* karyotype was described and, with valuable information available on cytogenetic features of similar species, the likely modes of chromosomal speciation among them were suggested, taken in light of their phylogenetic relationships. In the fourth chapter, the karyotypes of *Acromyrmex striatus* populations from the Argentine Chaco and *Acromyrmex silvestrii* were analyzed. The high level of karyotypic and morphological conservation of these lineages was highlighted and likely modes of speciation without identifiable chromosomal changes were discussed. In the fifth chapter, a combined approach of available morphological and molecular data was used to deeply unravel the evolutionary relationships of the former "*Trachymyrmex*" (current genera *Mycetomoellerius*, *Paratrachymyrmex* and *Trachymyrmex* stricto sensu) and the likely biogeographical routes traced by their major clades that could explain their current geographic distributions.

Keywords: ants, chromosomes, evolution, phylogeny, speciation.

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INTRODUÇÃO

Considerações sobre o objeto de estudo

A classe Insecta (Arthropoda: Hexapoda) é o grupo animal predominante nos ecossistemas terrestres. Estimativas sugerem que os insetos surgiram há cerca de 450 milhões de anos atrás, praticamente no mesmo período de surgimento das plantas terrestres e sofreram diversas radiações adaptativas (Misof *et al*. 2014). Os insetos desempenham um papel ecológico extremamente relevante para os ambientes, atuando como responsáveis por grande parte da ciclagem de nutrientes e da polinização de plantas, além de serem fundamentais em diversas redes alimentares (Grimaldi & Engel 2005). As formigas (Hymenoptera: Formicidae) são um dos grupos mais bem sucedidos de insetos. Estimativas propõem que as formigas surgiram há mais de 100 milhões de anos atrás e se diversificaram para mais de 16.000 espécies e subespécies descritas atualmente (revisado por Borowiec *et al*. 2019; Bolton, 2020). O grande sucesso ecológico das formigas tem sido atribuído à sua organização social – algumas colônias são descritas como um superorganismo, tamanha a complexidade cooperativa dos indivíduos que a compõem – e às complexas relações que estabelecem com o ambiente físico e nas relações biológicas com os demais organismos da comunidade (Hölldobler & Wilson 2008). Uma das associações biológicas mais fascinantes é a agricultura de fungos (fungicultura). As formigas cultivadoras-de-fungo (Myrmicinae: Attini) representam um exemplo clássico de mutualismo, estabelecido há cerca de 60 milhões de anos atrás na região Neotropical (Mueller *et al*. 2001; Branstetter *et al*. 2017). Essas espécies dependem obrigatoriamente do cultivo de jardins de fungos como alimento e, em troca, fornecem ao fungo proteção contra patógenos e competidores, além do substrato para seu alimento (Weber 1972). Atualmente, cerca de 260 espécies são reconhecidas, distribuídas em 19 gêneros (Bolton 2020), das quais ocorrem do sul dos Estados Unidos (latitude 40ºN) à Argentina (latitude 44ºS) (Weber 1972).

Considerações filogenéticas

A árvore da vida das formigas, bem como do clado das formigas cultivadoras-de-fungo está relativamente bem estabelecida (Brady *et al*. 2006; Moreau *et al*. 2006; Borowiec *et al*. 2019), esta última mostrando dois clados bem definidos denominados Paleoattini e Neoattini (Schultz & Brady 2008; Branstetter *et al*. 2017). Análises filogenéticas

baseadas em dados genômicos estão permitindo a reconstrução da história evolutiva das formigas de uma maneira mais robusta; no entanto, dados moleculares provenientes de fragmentos de genes nucleares e/ou mitocondriais resolvem bem árvores pouco compreendidas, como as parafiléticas ou polifiléticas (*e.g.*, Solomon *et al*. 2019). A datação dos tempos de divergência entre linhagens, como as citadas acima, são estimadas conjuntamente com análises filogenéticas acopladas a dados de registro fóssil, assim, permitindo inferir a história evolutiva de um determinado táxon de um modo mais concreto. Os resultados são geralmente associados aos eventos climáticos e geológicos passados e relacionados aos processos de especiação (*e.g.*, Moreau & Bell 2013; Branstetter *et al*. 2017). Além disso, a incorporação da distribuição geográfica *a priori* das linhagens nas mesmas árvores filogenéticas pode gerar informações biogeográficas adicionais que permitem traçar a rota de dispersão e/ou colonização de um grupo (clado, gênero, populações, etc.) para um determinado local (*e.g.*, Clouse *et al*. 2015; Economo *et al*. 2015).

A combinação de técnicas genéticas/moleculares e morfológicas, por exemplo, possibilitou várias descobertas importantes dentro das formigas cultivadoras-de-fungo, como o entendimento da evolução da fungicultura em um contexto filogenético e a descrição de muitas novas espécies e gêneros. Por exemplo, o gênero *Mycetosoritis* foi reformulado, dando origem ao gênero *Xerolitor* – grupo irmão das *Sericomyrmex* –, além de outras espécies transferidas para o gênero *Mycetophylax*, que por sua vez abrange um clado com as espécies do antigo grupo *Cyphomyrmex strigatus* e hoje contém pelo menos 21 espécies (Sosa-Calvo *et al*. 2017, 2018). Os gêneros monotípicos *Kalathomyrmex* e *Cyatta* foram identificados morfologicamente e filogeneticamente como o grupo irmão de todas as Neoattini (Klingenberg & Brandão 2009; Sosa-Calvo *et al*. 2013). Outro caso semelhante foi encontrado no gênero *Mycetagroicus*, do qual mais tarde foi identificado como o grupo irmão das chamadas "*higher-attine*", que incluem as bem conhecidas formigas cortadeiras (gêneros *Atta* e *Acromyrmex*), e caracterizam-se por terem seus cultivares de fungos poliploides e simbiontes obrigatórios (Brandão & Mayhé-Nunes 2001; Schultz & Brady 2008). Considerando o caráter parafilético do gênero *Trachymyrmex*, Solomon *et al*. (2019) usaram uma amostragem significativa de espécies e genes e reconheceram dois novos gêneros dentro deste grupo, *Mycetomoellerius* e *Paratrachymyrmex*, além de no mínimo 11 novas espécies. Ainda, Rabeling *et al*. (2011) relataram indiretamente cinco novas espécies de *Mycocepurus* – um dos três gêneros do clado das Paleoattini, enquanto Mehdiabadi *et al*. (2012)

sugeriram que todos os supostos casos de especiação no grupo *Cyphomyrmex wheeleri* poderiam estar correlacionados com uma mudança ancestral na associação de seus cultivares de fungos. Vale destacar que a maioria massiva desta nova biodiversidade descoberta pela ciência se encontra na América do Sul, predominantemente no Brasil, ressaltando inferências prévias de que metade da fauna mirmecológica global ainda precisa ser descoberta, necessariamente nos Trópicos (Lach *et al*. 2010).

Considerações citogenéticas

Outra característica biológica notável das formigas é sua impressionante variação citogenética, tanto no número quanto na estrutura de seus cromossomos (revisado por Lorite & Palomeque 2010). O menor número de cromossomos foi observado em *Myrmecia croslandi*, com n=1 e o maior número em *Dinoponera lucida*, com n=60 (Crosland & Crozier 1986; Mariano *et al*. 2008). De fato, observações sobre essa alta diversidade cariotípica das formigas foram determinantes para a proposição da Teoria da Interação Mínima, que sugere que rearranjos cromossômicos, tais como as fissões Robertsonianas, são a principal força motriz da evolução cromossômica em longo prazo e são eficazes para minimizar os riscos genéticos devido a interação dos cromossomos no núcleo (Imai *et al*. 1988, 1994, 2001). Uma quantidade considerável de evidências indiretas levou à ideia clássica de que rearranjos cromossômicos podem desempenhar um papel na especiação (*e.g.*, White 1978; King 1993; Rieseberg 2001; Ayala & Coluzzi 2005; Faria & Navarro 2010). Como o número de cromossomos tende a ser estável dentro de uma espécie, um potencial polimorfismo nessa característica (dado por fissões e fusões Robertsonianas ou poliploidia) em uma população ou linhagem se torna um passo importante para a delimitação intraespecífica (White 1978; Guerra 2008). Além disso, inversões cromossômicas foram identificadas para ter um papel central para a evolução de muitas espécies, mas muito mais dados e novos modelos são necessários para entender os mecanismos complexos envolvidos (Faria *et al*. 2019). Dessa forma, esforços para caracterizar cariótipos são extremamente importantes para estudos evolutivos, de modo que podem fornecer indicações sobre possíveis trajetórias cario-evolutivas envolvendo rearranjos cromossômicos e seu papel na divergência de linhagens.

As formigas cultivadoras-de-fungo também apresentam variação equivalente nos seus cariótipos. A espécie filogeneticamente basal *Mycocepurus goeldii* tem n=4, enquanto uma das formigas "*higher-attine*" *Sericomyrmex amabilis* tem n=25 (revisado

por Cardoso *et al*. 2018). Estudos anteriores identificaram variação cariotípica intraespecífica em populações de *Mycetophylax morschi*, das quais mostraram dois cariótipos distintos: n=13 e n=15 (Cardoso *et al*. 2014a). Este estudo também integrou dados citogenéticos à árvore filogenética de *Mycetophylax* buscando compreender parcialmente a evolução cromossômica do gênero, até então composto de três espécies endêmicas de dunas arenosas (ver Klingenberg & Brandão 2009 e Cardoso *et al*. 2014b). Dessa forma, sugeriu-se que fusões cromossômicas desempenharam um papel igualmente importante para sua evolução deste pequeno grupo de formigas psamófilas (Cardoso *et al*. 2014a). Por outro lado, o número de cromossomos nas formigas cortadeiras parece ser bastante conservado, *Atta* com n=11 e *Acromyrmex* com n=19, exceto por *Acromyrmex striatus*, que tem n=11 e características morfológicas compartilhadas por ambos os gêneros (Cristiano *et al*. 2013). Estudos filogenéticos constataram que *A. striatus* é parafilético em relação às outras espécies de *Acromyrmex*, sendo então, o grupo irmão de todas as formigas cortadeiras remanescentes (Cristiano *et al*. 2013; Branstetter *et al*. 2017). O gênero *Acromyrmex* possui um elevado número de espécies cariotipadas em relação a outros gêneros de formigas cultivadoras-de-fungo (revisado por Cardoso *et al*. 2018), o que poderia proporcionar certa vantagem na análise de mudanças cromossômicas entre suas linhagens. Adicionalmente, a citogenética molecular envolve a combinação de biologia molecular com citogenética e pode resultar em achados mais específicos e atraentes, dado o problema a ser analisado. Uma de suas técnicas mais usadas é a hibridização *in situ* fluorescente (FISH), que permite o mapeamento físico de sequências específicas de DNA nos cromossomos usando dois elementos básicos: uma sonda de DNA e uma sequência alvo (Speicher & Carter 2005). A técnica de FISH pode fornecer caracteres úteis para diagnóstico de espécies morfologicamente indistinguíveis (*i.e.*, espécies crípticas) e tornou-se uma ferramenta importante para descrever e delimitar novas espécies, especialmente insetos (*e.g.*, Lukhtanov *et al*. 2015; Panzera *et al*. 2015).

OBJETIVOS E JUSTIFICATIVA

O principal objetivo da presente tese de doutorado visou analisar as relações filogenéticas de populações e/ou espécies de formigas cultivadoras-de-fungo, das quais ainda não havia registros, e relacioná-las com dados citogenéticos a fim de revelar as trajetórias evolutivas até a divergência dessas linhagens. Os supostos polimorfismos encontrados a partir dessa abordagem integrativa levaram a descobertas que, juntas, podem ser uma fonte excelente de informações para estudos taxonômicos e evolutivos. O objeto de estudo provém de espécies de três gêneros de formigas cultivadoras-de-fungo: *Mycetophylax*, *Mycetomoellerius* e *Acromyrmex*. Cada população e/ou linhagem estudada forneceu contribuições evolutivas significativas que foram discutidas primariamente à luz do papel das mudanças cromossômicas na especiação, bem como de suas relações filogenéticas.

CAPÍTULO 1:

CHROMOSOMAL DYNAMICS IN SPACE AND TIME: EVOLUTIONARY HISTORY OF *Mycetophylax* ANTS ACROSS PAST CLIMATIC CHANGES IN THE BRAZILIAN ATLANTIC COAST

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Chromosomal dynamics in space and time: evolutionary history of *Mycetophylax* ants across past climatic changes in the Brazilian Atlantic coast

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Fungus-farming ants of the genus *Mycetophylax* exhibit intra and interspecific chromosome variability, which makes them suitable for testing hypotheses about possible chromosomal rearrangements that endure lineage diversification. We combined cytogenetic and molecular data from *Mycetophylax* populations from coastal environments to trace the evolutionary history of the clade in light of chromosomal changes under a historical and geographic context. Our cytogenetic analyses revealed chromosomal differences within and among species. *M. morschi* exhibited three distinct karyotypes and considerable variability in the localization of 45S rDNA clusters. The molecular phylogeny was congruent with our cytogenetic findings. Biogeographical and divergence time dating analyses estimated that the most recent common ancestor of *Mycetophylax* would have originated at about 30 Ma in an area including the Amazon and Southern Grasslands, and several dispersion and vicariance events may have occurred before the colonization of the Brazilian Atlantic coast. Diversification of the psammophilous *Mycetophylax* first took place in the Middle Miocene (*ca.* 18–10 Ma) in the South Atlantic coast, while "*M. morschi*" lineages diversified during the Pliocene-Pleistocene transition (*ca.* 3–2 Ma) through founder-event dispersal for the Northern coastal regions. Psammophilous *Mycetophylax* diversification fits into the major global climatic events that have had a direct impact on the changes in sea level as well as deep ecological impact throughout South America. We assume therefore that putative chromosomal rearrangements correlated with increased ecological stress during the past climatic transitions could have intensified and/or accompanied the divergence of the psammophilous *Mycetophylax*. We further reiterate that "*M. morschi*" comprises a complex of at least three well-defined lineages, and we emphasize the role of this integrative approach for the identification and delimitation of evolutionary lineages.

Ants (Hymenoptera: Formicidae) exhibit astonishing ecological success that has been attributed to their social organization as well as the ability to associate with other organisms, *e.g.*, fungi[1,2]. Another remarkable feature of the ants is their impressive karyotype variation that ranges from $2n = 2$ to $2n = 120$[3,4]. Such exceptional variations in the chromosomal number may indicate that ants underwent speciation processes concomitantly with chromosomal rearrangements (CRs). Chromosome numbers tend to be stable within a species, so a potential polymorphism of this trait into a population or lineage becomes an important step toward intraspecific delimitation[5,6]. It has been proposed that CRs may promote speciation through reproductive isolation[7–10], although it remains a source of debate, increasingly studies agree[11–13]. In fact, observations of ant karyotypic diversity were determinant for the proposition of the Minimum Interaction Theory, which suggests that Robertsonian (Rb) fissions are the

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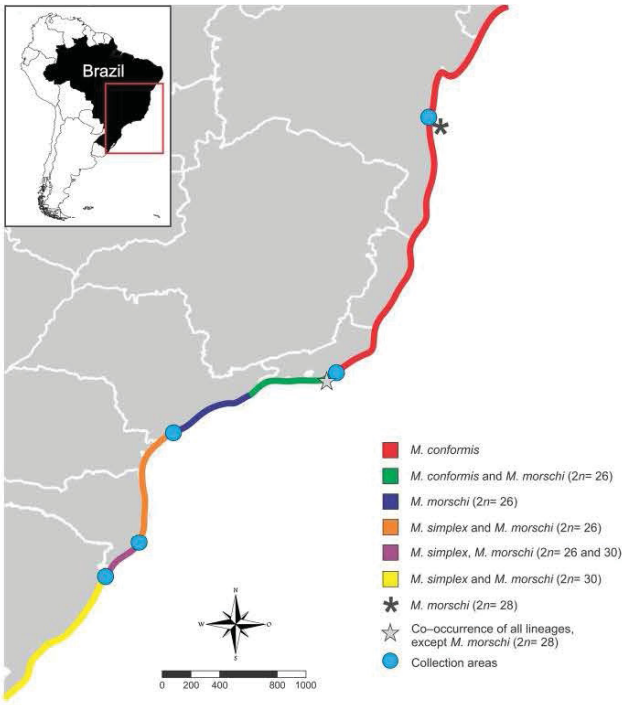

Figure 1. Map of geographic distribution and sampling localities of *Mycetophylax* populations along Brazilian Atlantic coast. The legends of the colors and symbols are embedded in the figure. The scale bar is represented in kilometers. Adapted from Cardoso *et al.*[60].

main driving force in long-term chromosome evolution and are effective at minimizing genetic risks and increasing the potential for genetic divergence[14–16].

In contrast, in a population-based cytogenetic study with fungus-farming ants of the genus *Mycetophylax*, the role of centric fusions in the karyotypic diversification of those ant lineages was proposed[17]. *Mycetophylax* was formerly composed of three endemic species of coastal sand dune environments, known as Restinga[18,19]: *M. conformis* Mayr, 1884; *M. morschi* Emery, 1888; and *M. simplex* Emery, 1888 (see Fig. 1 for details of occurrence areas). Differences in both the number and structure of their chromosomes were found, including intraspecific variation within *M. morschi*, showing populations with two distinct karyotypes ($2n = 26$ and $2n = 30$). It has therefore been suggested that *M. morschi* comprises a complex of cryptic species or even different lineages that have been subjected to consecutive CRs[17]. It is noteworthy that through phylogenetic analyses, members of the former *Cyphomyrmex strigatus* group, including *Mycetosoritis asper* and *Mycetosoritis clorindae*, were shown to form a well-defined clade with strong statistical support along with *Mycetophylax sensu stricto*. Thus, these species were transferred to the genus *Mycetophylax*, which currently comprises 21 species[20].

The integration of cytogenetic data, phylogenetic trees, and molecular dating considerably improved the findings on evolutionary history within lineages as well as providing implications that perhaps could not be found independently. However, few studies have combined such approaches, but what has emerged from this integration are interesting and even unexpected results[21–24]. Divergence times and phylogenetic relationships can be estimated simultaneously under a Bayesian inference. A method for divergence time estimation and fossil calibration using a stochastic branching process and relaxed-clock model in a Bayesian framework was recently developed[25,26]. The Fossilized Birth-Death (FBD) process has the advantage of assuming that fossils and molecular sequences from extant species comprise the same diversification process[25–27]. On the other hand, under a molecular cytogenetic approach, such as Fluorescence *in situ* Hybridization (FISH) chromosome mapping, useful diagnostic characters for morphologically conservative species may be provided as variations in the number and/or position of ribosomal DNA (rDNA) clusters or CR indications given by changes in chromosome number. Hence, the use of FISH data has become an important tool for describing and delimiting new taxa, especially

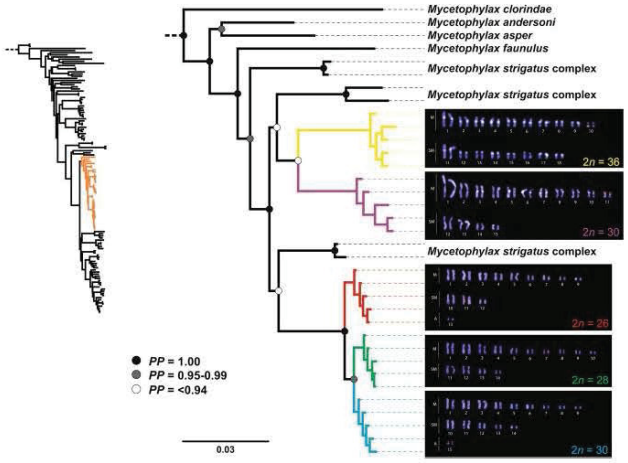

Figure 2. Pruned phylogenetic tree from *Mycetophylax* fungus-farming ants based on a Bayesian analysis of five nuclear protein-coding genes and their DAPI-stained karyotypes showing the FISH mapping of the 18S rDNA probe (in red). *M. simplex* shown in yellow branches and karyotype $2n = 36$. *M. conformis* shown in purple branches and karyotype $2n = 30$. *M. morschi* (lineage A) shown in red branches and karyotype $2n = 26$. *M. morschi* (lineage B) shown in green branches and karyotype $2n = 28$. *M. morschi* (lineage C) shown in blue branches and karyotype $2n = 30$. In the karyotypes images: (M) metacentric, (SM) submetacentric, and (A) acrocentric chromosomes.

for insects[28–30]. In this way, we can accurately test the assumptions made previously on the likely chromosomal evolution within the *Mycetophylax* lineages.

Additionally, the endemism of the three former *Mycetophylax* species to the Atlantic coastal environments remains a peculiar and attractive feature since the correlation between chromosomal and genetic variability needs to be examined in the context of geographic distributions of the species[31,32]. The Brazilian Atlantic coastal area (often referred to as the Restinga ecosystem) can be physically and biologically defined as coastal plains of marine sedimentary origin[33] that were subjected to ecological and geological stress during the major climatic transitions associated eustatic change[34,35]. Global past climate changes have certainly had biogeographic impacts due to the effects of alterations in temperature and moisture on species[36,37]. Ancestral range estimation contributes to a better understanding of species distributions over evolutionary time scales, and it is possible to infer dispersion and/or vicariance events from previous climatic and/or geological events in a given geographic area[38]. The Restinga ecosystem is a coastal environment within the Atlantic Forest biome that is floristically and geomorphologically heterogeneous. It is composed of a range of organisms that have intrinsically adapted to adverse conditions[39]. These habitats have been deeply influenced by the long-term effects of climate change that may have contributed to species diversification due to dynamism. Therefore, we hypothesized that *Mycetophylax* populations may have diversified through chromosomal changes triggered by the climatic events that have continuously affected their habitat.

In order to test the assumptions raised above, we correlated cytogenetic, molecular, and biogeographic data from the additional *Mycetophylax* species with the phylogenetic tree of the fungus-farming ants, and under a historical and geographical context, we aimed to describe the evolutionary relationships and chromosomal changes that raised the karyotype observed today. In addition, with this integrative approach, we aimed to decipher the taxonomic status of *M. morschi*, which is currently considered a single species, but which certainly represents a complex of independent evolutionary lineages.

Results

Karyotype determination and FISH mapping. The detailed cytogenetic analysis with *Mycetophylax* populations revealed the presence of intra- and inter-specific karyotype variations related to the number of diploid chromosomes ($2n$), the karyotypic formula (KF), and the fundamental number (FN). FISH signals with the 18S rDNA probe showed a stable number of rDNA clusters, denoting only one pair of chromosomes, but its localization and position along the chromosome differed between karyotypes (Fig. 2). The distribution pattern of the $TTAGG_{(6)}$ telomeric repeat was restricted to the terminals of both chromosome arms in all karyotypes and intercalated stronger and weaker signals at random (Fig. S1). No signals for interstitial telomeric sites (ITS) were detected.

Mycetophylax morschi surprisingly exhibited three distinct cytotypes (*i.e.*, organisms considered to be from the same species but differing in both number and morphology of the chromosomes) instead of two, and thus, it was divided into three lineages, as follows:

Lineage A. Colonies from the southern (Torres/RS) and southeast (Cabo Frio/RJ and Ilha Comprida/SP) Brazilian coast presented $2n = 26$ chromosomes. This karyotype is composed of nine metacentric, three submetacentric, and one acrocentric chromosome pairs; the karyotype formula is KF = 9 M + 3SM + 1 A; and the fundamental number (FN) is 50. The rDNA clusters are located in the pericentromeric region of the long arms of submetacentric chromosome pair 11 (Fig. 2).

Lineage B. Colonies from the northeast Brazilian coast (Ilhéus/BA) presented $2n = 28$ chromosomes. This unexpected karyotype is composed of ten metacentric and four submetacentric chromosome pairs with a karyotype formula of KF = 9 M + 5SM and an FN of 56. The rDNA clusters are located in the pericentromeric region of the short arms of submetacentric chromosome pair 7 (Fig. 2). Note that, unlike the others, this lineage does not present acrocentric chromosomes.

Lineage C. Colonies of both localities of the southern Brazilian coast (Torres/RS and Araranguá/SC) presented $2n = 30$ chromosomes. This karyotype is composed of nine metacentric, five submetacentric, and one acrocentric chromosome pairs with a karyotype formula of KF = 9 M + 5SM + 1 A and an FN of 58. The rDNA clusters are located in the terminal region of the short arms of the acrocentric chromosome pair (Fig. 2). For the first time, the presence of two cytotypes of "*M. morschi*" was recorded at the same geographic area. It is therefore conclusive that they live sympatrically.

Mycetophylax conformis. All colonies of *M. conformis* presented $2n = 30$ chromosomes. This karyotype is composed of eleven metacentric and four submetacentric chromosome pairs with a karyotype formula of KF = 11 M + 4SM and an FN of 60. The rDNA clusters are located in the terminal region of the short arms of metacentric chromosome pair 11 (Fig. 2).

Mycetophylax simplex. All colonies of *M. simplex* presented $2n = 36$ chromosomes. This karyotype is composed of ten metacentric and eight submetacentric chromosome pairs with a karyotype formula of KF = 10 M + 8SM and an FN of 72. The rDNA clusters are located in the pericentromeric region of the long arms of metacentric chromosome pair 10 (Fig. 2).

Molecular phylogeny. Our phylogenetic analyses were based on Bayesian Inference (BI), which exhibited strong statistical support for most nodes (Figs. 2 and S2). We use the aligned dataset provided by Sosa-Calvo *et al.*[20] and inserted additional *Mycetophylax* sequences obtained in this work. The phylogenetic tree generated showed three well-supported clades of "*M. morschi*": the first one corresponded to the cytotype $2n = 26$ (lineage A) (Bayesian posterior probability (PP) = 1.0), and the others diversifying from it formed a sister group of cytotypes $2n = 28$ (lineage B) and $2n = 30$ (lineage C) (PP = 0.96) (Fig. 2). These three lineages were grouped with unknown species of the former *Cyphomyrmex strigatus* complex, composing a clade with intriguing weak support. *M. simplex* and *M. conformis* fell in a sister group (PP = 0.75), which, together with another unknown species of the former *C. strigatus* complex, formed a major clade with "*M. morschi*" lineages and the unknown species from the *C. strigatus* complex (PP = 1.0) (Fig. 2). This major clade will henceforth be called "psammophilous *Mycetophylax*".

FBD-based divergence dating. The FBD-based divergence dating analysis recovered the stem-group age (*i.e.*, the earliest possible origin) of fungus-farming ants as 64.6 million years ago (Ma) and the crown-group age (*i.e.*, the latest possible origin) as 61.9 Ma (95% highest posterior density interval, HPD = 76–49 Ma) (Fig. S3). The stem- and crown-group ages for the *Mycetophylax* clade were reconstructed as 37.4 and 29.9 Ma, respectively (HPD = 39–22 Ma), and psammophilous *Mycetophylax* emerged at about 14 Ma (HPD = 18–10 Ma) (Fig. 3). Divergence age estimates of ((*M. conformis* + *M. simplex*) + (*strigatus* complex "01")) and (("*M. morschi*" lineages) + (*strigatus* complex "02")) indicated that they diverged almost simultaneously at 12.5 Ma (HPD = 17.2–8.6 Ma) and 11.4 Ma (HPD = 15.9–7.6 Ma), respectively. While *M. conformis* and *M. simplex* split at about 10 Ma (HPD = 14.3–5.6 Ma), the "*M. morschi*" lineages diversified more recently: lineage A diverged from lineages B + C at ~3 Ma (HPD = 4.75–1.65 Ma), while lineages B + C diverged at ~2 Ma (HPD = 3.2–0.95 Ma) (Fig. 3).

Historical biogeography. The three independent estimates of the ancestral range showed similar results. In the BGB analysis, the BAYAREALIKE + j model yielded the best statistical fit for the data (AIC = 101.9; $p = 0.06$), although it had no significant difference to the model without the founder-event speciation parameter (Table S1). All models pointed out that the most recent common ancestor (MRCA) of *Mycetophylax* would have originated in an area including the Amazon and Southern Grasslands (BGB: $p = 0.4$, BayArea: $p = 0.43$, and BBM: $p = 0.5$) (Fig. 3). Most of the nodes estimated dispersal while vicariance was recovered in a few nodes, including the split of the "*M. morschi*" lineages.

The colonization of the coastal regions probably involved major dispersal events that would have directed the *Mycetophylax* ancestors to the Atlantic Forest from the Amazon and subsequently spread to the South Coast region at about 17 Ma (BGB: $p = 0.37$, BayArea: $p = 0.59$, and BBM: $p = 0.62$). All models strongly estimated the ancestral range of all psammophilous *Mycetophylax*, including the remaining ants from the "*strigatus* complex", which apparently lived on the Atlantic coast of Southern Brazil and later scattered farther north in both coastal and rainforest regions, including the Amazon (BGB: $p = 0.98$, BayArea: $p = 0.89$, and BBM: $p = 0.89$) (Fig. 3). No dispersion events were recovered, primarily indicating diversification within the area or sympatric range inheritance.

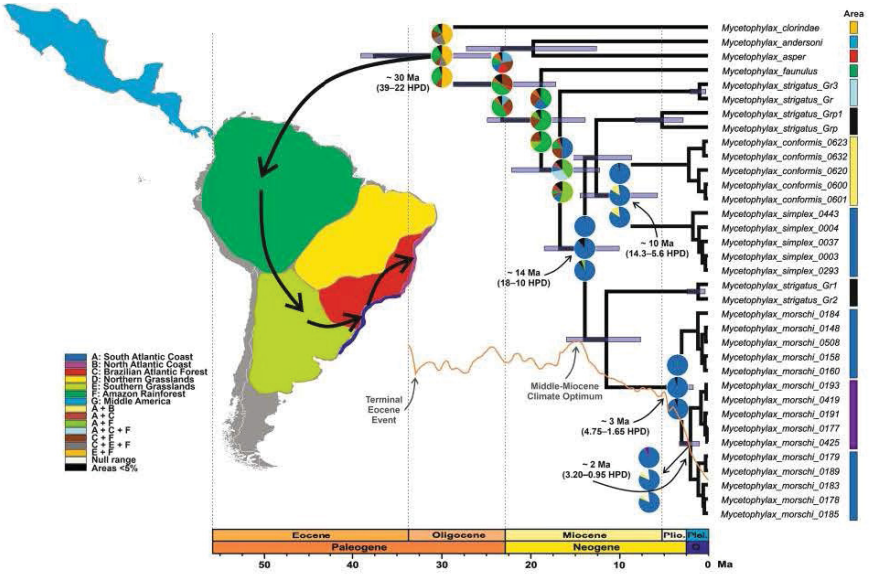

Figure 3. Phylogenetic tree based on Fossilized Birth-Death process representing the divergence time estimates along with ancestral range estimates based on three alternative biogeographic model. The arrows on the map represent a possible route of the *Mycetophylax* ants along the colonization of the Brazilian Atlantic coast. Pie charts showing (**a**) "BioGeoBEARS" analyses (model selected: BAYAREALIKE + j) on the top, (**b**) the BayArea model on the middle, and (**c**) the Bayesian Binary MCMC model on the bottom. The pie charts at the nodes represent 95% confidence intervals of the relative frequencies of the ancestral range optimizations across the restricted tree of the genus *Mycetophylax*. The horizontal blue bars at the nodes represent the 95% highest posterior density (HPD) intervals of the estimated node ages. The numbers on the main nodes represent the mean ages of lineage divergence as well as the confidence intervals. The scale axis bar represents million years ago (Ma). Q.: Quaternary, Plio.: Pliocene, and Plei.: Pleistocene. The orange curve represents temperature fluctuations during the past ~35 Ma, as depicted by Zachos *et al.*[34].

The ancestral range of *M. conformis* and *M. simplex* was estimated as the South Atlantic Coast for all models (BGB: $p = 0.97$, BayArea: $p = 0.84$, and BBM: $p = 0.84$). Thus, *M. simplex* inherited the ancestral range, whereas *M. conformis* colonized the North Atlantic coast via founder-event dispersal. Likewise, the "*M. morschi*" lineages would have inhabited the South Atlantic Coast (BGB: $p = 0.99$, BayArea: $p = 0.95$, and BBM: $p = 0.95$), and their diversification may have been sympatrical, as no dispersal event was estimated. Finally, lineages B and C of "*M. morschi*" would have diverged through two dispersion events and one of vicariance. Lineage C could have colonized the North Atlantic Coast via founder-event dispersal (BGB: $p = 0.93$, BayArea: $p = 0.81$ and BBM: $p = 0.81$) (Fig. 3).

Chromosome evolution. Our ancestral chromosome numbers reconstruction suggests that the best model to support chromosome evolution is linear gain, loss, and duplication. This model infers that the rates of gain (fission) and loss (fusion) of a chromosome are constant and depend linearly on the current number of chromosomes. The estimated rate parameters in the best model are shown in Table S2. The number of haploid chromosomes estimated in the MRCA of the genus *Mycetophylax* ranged from $n = 15$ to $n = 17$ between the ML and BI analyses (Figs. 4 and S4). In the psammophilous *Mycetophylax* clade, the most likely haploid numbers were $n = 15$ (ML) and $n = 16$ (BI; PP = 0.20). The ancestral karyotype number of the sister clades *M. conformis* and *M. simplex* was $n = 16$ (ML) and $n = 17$ (BI; PP = 0.25). Likewise, the likely chromosomal ancestry of the "*M. morschi*" lineages was $n = 14$ for both approaches (PP = 0.50 for BI). On the other hand, the ancestral karyotype number of the clade containing "*M. morschi*" lineages B and C was $n = 14$ (ML) and $n = 15$ (BI; PP = 0.51) (Figs. 4 and S4).

Discussion

Chromosome evolution and diversification of psammophilous *Mycetophylax*. Our study involved extensive sampling of psammophilous *Mycetophylax* populations in order to evaluate the level of diversification of these lineages on their geographic distribution along the Brazilian Atlantic coast and to deeply investigate the mechanisms of chromosome evolution in a phylogenetic context. Previous analyses with psammophilous

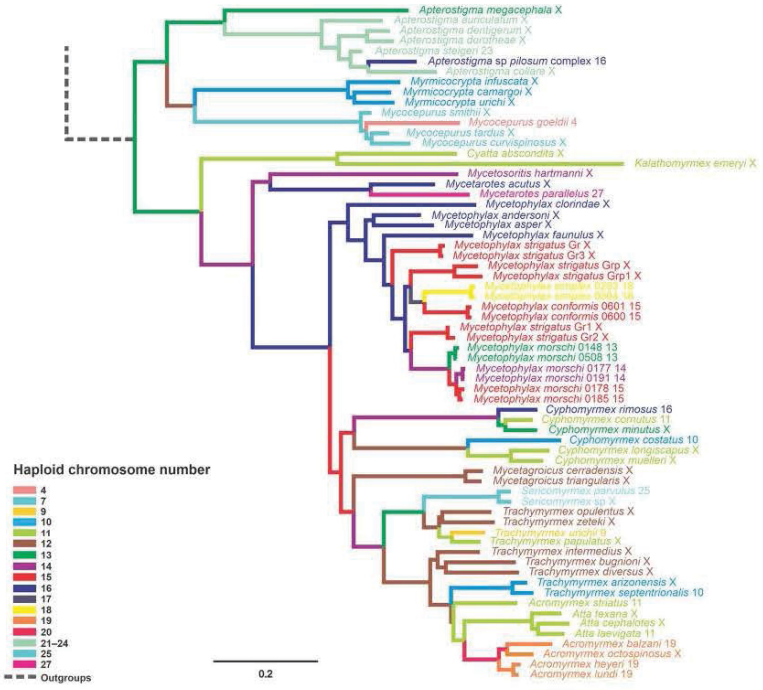

Figure 4. Chromosome number evolution and inferred ancestral chromosome state in the phylogenetic tree of fungus-farming ants from ChromEvol results based on Bayesian inference, chosen because it provides posterior probabilities as a statistical parameter. The numbers at the tips are the known haploid chromosome numbers of species, while "X" represents unknown number. The various colors on the branches of the tree represent the base haploid chromosome number for each node, given in the legend of the figure.

Mycetophylax showed remarkable chromosomal polymorphisms among populations[17], which provoked us to seek additional knowledge about their evolutionary relationships. Our data corroborate the previous karyotypic findings[17] on the species *M. conformis* and *M. simplex*, both in terms of the number and structure of their chromosomes. Likewise, our data support the findings of dissimilar karyotypes within *M. morschi* populations with the unexpected discovery of a new karyotype ($2n = 28$) in a geographic area previously unknown to this species, the northeast Brazilian Atlantic coast. No universal rule seems to govern karyotype evolution, but a chromosomal variation between closely related lineages, such as psammophilous *Mycetophylax*, strongly suggests the role of CRs in the lineage diversification. In fungus-farming ants, few species have had their karyotypes described so far (45 out of 245), but available data show extensive variation among species ($2n = 8$ to $2n = 50$)[4]. This aspect, which appears to be typical of Formicidae, also corresponds to the clade of the fungus-farming ants and may be the outcome of diverse CRs, such as Rb fusion/fission.

In an attempt to partially understand the speciation history of the psammophilous *Mycetophylax*, we integrated cytogenetic and phylogenetic data. Our cytogenetic information has provided some evidence that psammophilous *Mycetophylax* comprises distinct and new evolutionary lineages, since different chromosomal numbers eventually do not match during meiosis, acting as post-zygotic barriers. Besides, as rDNA is considered one of the most conservative fraction from the eukaryotic genome[40,41], variations in the location and chromosome bearer of the rDNA clusters, as observed in the karyotypes analyzed here, may be related to the differentiation and divergence of natural populations[42]. In fact, rDNA clusters are even considered hotspots of chromosomal changes and lineage diversification in mice[43]. Here, we found that all of the *Mycetophylax* karyotypes and cytotypes showed only two signals of 45S rDNA but at distinct locations and homologue pairs suggesting that changes in ribossomal cluster appears to be related to the diversification of these ants. Some chromosomal mechanisms have been suggested to explain such mobility of rDNA clusters within sister lineages, including ectopic recombination[44], transposition[45], and centric fission[46]. The fact that there is no variation in the number of these clusters can be attributed to homologous recombination between repetitive sequences dispersed throughout genome (*i.e.*,

ectopic recombination) or more likely to transposition of these sequences into arbitrary position and chromosomes. However, we cannot state any further inferences, only that the intraspecific chromosomal diversity in *M. morschi* gives us further indications that these lineages may indeed be considered distinct species.

The application of molecular phylogenetics on the study of karyotype evolution has enabled us to deduce the direction of changes that give rise to chromosome variation in the most objective way[47,48]. For example, just because two species of a genus share the same chromosome number does not mean that they are phylogenetically closer to each other than two species with different chromosome numbers[5]. However, the base chromosome number (*i.e.*, the haploid number present in the initial population of a monophyletic clade) may be directly related to the chromosomal variability inside that clade and its sister group[5]. Our phylogenetic reconstruction is congruent with our cytogenetic findings showing that the *Mycetophylax* cytotypes are phylogenetically close. The lineage A diverged prior to the other "*M. morschi*" lineages, which would imply that $2n = 26$ ($n = 13$) was probably the ancestral karyotype. However, our results on chromosome evolution reconstructed its ancestral karyotype as $2n = 28$ ($n = 14$), whereas it was $2n = 32$ ($n = 16$) for the clade *M. conformis* + *M. simplex*. Previous estimates have shown slightly contrasting results, with an ancestral diploid number for "*M. morschi*" lineages ($n = 15$) and the clade *M. conformis* + *M. simplex* ($n = 17$), leading the authors to propose fusion as the main mechanism of chromosome evolution in *Mycetophylax*[17].

Our FISH-based comparative approach indicates that is more likely that both Rb fissions and fusions contributed to the karyotypic diversification in psammophilous *Mycetophylax* and similarly in Formicidae rather than a larger role for Rb fissions, as has been proposed[14–16]. In fact, specifying which chromosomes are involved in such rearrangements is a difficult task, especially since large-scale CRs can modify karyotypes in a striking way. For example, a karyotypic reduction observed in *Cucumis* species (from $2n = 24$ to $2n = 14$) involved at least 59 CRs, including five fusions, four translocations, and 50 inversions[49]. Not least, the presence of only a single pair of chromosomes bearing the 45S rDNA clusters in fungus-farming ants appears to be uniform. Taking into account the phylogenetic relationships of fungus-farming ants from the basal species *Mycocepurus goeldii*[50] and cross referencing our data with the most derived species of leafcutter ants from *Acromyrmex* and *Atta* genera[51,52], we noticed that there are only a pair of chromosomes bearing the 45S rDNA cluster, which would imply that this chromosomal feature may be plesiomorphic.

Fusions can be directly observed using telomeric probes. After *in tandem* fusions, the canonical telomeric sequences may remain in the interstitial chromosome regions, serving as clues of fusion and inversions involving the telomeres. Telomeric sequences can either be eliminated or not be eliminated by chromosome breakage, and when complete elimination does not occur, interstitial telomeric sites (ITS) can be observed[53]. Considering the fusion hypothesis proposed by Cardoso *et al.*[17] as the mechanism shaping *Mycetophylax* karyotypes, signals for ITS would be expected, but we did not find any evidence for ITS, suggesting that if tandem fusion did take place the telomeric regions would have been lost. Lastly, we added more Formicidae species containing the TTAGG as the ending motif on their chromosomes, and thus we reinforce the hypothesis that this sequence could have been the ancestral motif of the ant telomeres[54,55].

Evolutionary history of psammophilous *Mycetophylax* under a cytogenetic perspective. Since the beginning of the Cenozoic (*ca.* 65 Ma), the Earth's biota has experienced both rapid and gradual climate changes[34,35] that have had profound impacts on species diversification and composition[56]. Indeed, the major climate transitions has triggered higher rates of species turnover, and the Paleocene-Eocene Thermal Maximum "PETM" (*ca.* 55 Ma), the Terminal Eocene Event "TEE" (*ca.* 34 Ma), Middle Miocene Climatic Optimum "MMCO" (*ca.* 16–14 Ma), and Pliocene-Pleistocene intervals (from *ca.* 3 Ma) have been reported as striking events for mammals[56]. Coincidentally, our time-calibrated phylogenetic reconstruction suggested that the basal divergence of the genus *Mycetophylax* took place in the Eocene-Oligocene boundary (*ca.* 38–22 Ma), while psammophilous *Mycetophylax* diverged into the Middle Miocene (*ca.* 18–10 Ma) and thereafter diversified into the existing lineages. Both inferred ages fit with one of these major events mentioned above, which makes them relevant to other taxa than mammals, including here invertebrates.

The fungus-farming ants originated about 60 Ma in South America[57], likely during the post-extinction event recovery period and shortly before the PETM. After that, an abrupt drop in temperature began at around 52 Ma and continued to the end of the Eocene (*i.e.*, TEE)[34] that had left hallmarks characterized by cooler and more seasonal climates, increase topographic heterogeneity, and persistent phylogenetic and ecologic diversification of lineages[58]. It was suggested therefore that this global cooling event spurred both fungus-farming ants' lineages diversification and ant-fungus coevolution[57]. Likewise, this assumption agrees with our estimated age for diversification of the genus *Mycetophylax* in the Miocene (*ca.* 23–5.3 Ma). The Miocene was a climatically dynamic interval from Cenozoic, when long-term global climatic cooling was punctuated by extreme climatic optima (*i.e.*, MMCO)[59]. After this period of global warming — the warmest one in the last 24 Ma[34] — a climate transition associated with major Antarctic ice sheet expansion and global cooling began, the so-called Middle Miocene Glaciation "MMG"[59], which drastically extended to the Pleistocene glacial intervals. As these global climate oscillations were directly associated with marine transgressions and regressions[35], the evolutionary history of psammophilous *Mycetophylax* on sand dunes could plausibly be related to the heterogeneity of the Brazilian Atlantic coast as well to their topographic and climatic gradients.

The basal split between *M. conformis* and *M. simplex* took place in the Middle to Late Miocene, *ca.* 10 Ma (HPD = 14.3–5.6 Ma), corresponding to the initial cooling period (*i.e.*, MMG) and a consequent drop in sea levels reaching near to the current levels[35]. These species are morphologically distinguishable[18] and are not found to inhabit the same coastline area, except in Cabo Frio, Rio de Janeiro[60]. *M. conformis* is further distributed in the north of the Atlantic coast with the southern limit of its occurrence at about 24°S, while *M. simplex* occurs from the southern coast of São Paulo (~25°S) to the border of southern Brazil (~34°S)[60]. Our ancestral range estimates place the species ancestor as having inhabited the South Atlantic Coast, and we identified a major dispersal event.

The putative biogeographic scenario would be that *M. simplex* inherited the ancestral range, while *M. conformis* would have colonized the North Atlantic Coast via founder-event dispersal. Founder-event speciation refers to changes in the lineage distribution during split (cladogenesis), where one daughter lineage inherits the ancestral range and the other lineage "jumps" to a new area[61,62]. Considering the current contrasting geographic range and respective karyotypes of *M. simplex* and *M. conformis*, the fixation of different CRs either by genetic drift or geographic isolation could have been triggered by the recurrent climatic events that continuously changed the environment, leading CRs to play some role in lineage diversification. Moreover, relict populations of *M. simplex* living sympatrically with *M. conformis* could further indicate that species were sympatric in the past and that during successive Pleistocene climatic transitions they could have become allopatric majority. Phylogeographic analyses with *M. simplex* populations corroborate this, as it was identified that the species maintained a stable population size until about 75,000 years ago when it underwent a gradual demographic expansion[63].

Continuous climate changes have been more abrupt since the Early Pliocene leading to extreme periods of glaciation (most temperate regions) and aridification (tropical regions) later in the Pleistocene[58]. Particularly in South America, these fluctuations were responsible for chorological changes that expanded and contracted the distribution areas of taxa, communities, and biomes[36]. Indeed, increasing climatic oscillations during the past 3 Ma have driven many range changes for a range of taxa, and higher speciation rates were identified during the Pliocene and Pleistocene[64,65], which may well be associated with chromosomal changes promoting lineage diversification and potential reproductive isolation[32,66]. The chromosomal diversity observed in psammophilous *Mycetophylax* is in agreement with this scenario, especially considering "*M. morschi*" lineages. The divergence of these lineages was recovered at the Pliocene-Pleistocene boundary (*ca.* 3.6–1.8 Ma). Historical biogeography analyses have estimated the ancestral range as the South Atlantic Coast with no dispersion events, suggesting that at least the initial diversification of this group may have occurred sympatrically. Interestingly, it has been argued that sympatric sister taxa have a higher average karyotypic diversity than allopatric sister taxa[67]. Populations that have multiple accumulated CRs can persist more easily in secondary sympatry than populations whose karyotypes are insufficiently divergent, thus leading sympatric populations to reproductive divergence[7,8,67]. On the other hand, the split of "*M. morschi*" lineages B and C would have involved both dispersion and vicariance events, allowing a possible colonization route of diversification to be traced.

The Pliocene-Pleistocene intervals had large-scale effects on habitat change, including the Brazilian coast, which was remarkably influenced by the sea level[35,58]. Sea level fluctuations related to climate change and geological disturbances would have dramatically increased over these periods to −100 m from current sea level[35,58], thus allowed the opening of new previously unexploited regions by psammophilous taxa, including *Mycetophylax* ants. A direct consequence of this severe drop in sea level during the glacial periods would be the emergence of the Brazilian continental shelf[68]. This displacement of the southeastern South American coastline would have reached hundreds of kilometers to the east, enabling suitable climatic conditions for forest-adapted species[68] and presumably extending the geographic range of psammophilous taxa to areas previously flooded by the sea. Populations of "*M. morschi*" subjected to putative CRs would have been confronted with adaptively adverse conditions as a result of climatic and ecological stress during colonization, favoring CRs that would increase adaptation to these conditions whenever such conditions arose[10,11]. This pattern of chromosomal changes and local adaptation has been reported for *Anopheles gambiae*[11] and supports the diversity and karyotypic evolution in the psammophilous *Mycetophylax* clade. Additionally, the perspective of CRs fixation in isolated and/or peripheral founder populations through genetic drift and inbreeding is raised by many of the chromosomal speciation models[9–11]. In this way, the northernmost dispersion of the Brazilian Atlantic coast by the psammophilous *Mycetophylax* where molded by several historical events coupled with chromosomal changes, likely Rb fissions and fusions that shaped the karyotypes and lineage differentiation.

In conclusion, our integrative approach combining cytogenetic and molecular data has once again proved to be effective in supporting systematics and taxonomic research. We emphasize that the lack of cytogenetic data is still a hindrance to better application of these approaches. Further, we shed light in the cryptic diversity within "*M. morschi*" and, as previously suggested[17], should be treated as phylogenetically isolated lineages or even as distinct species as they form well-resolved clades. The entire genus *Mycetophylax* has undergone systematic and taxonomic changes over the years, reinforcing the need for taxonomic revision. Psammophilous *Mycetophylax* represents a cytogenetically diverse group, showing great variation in terms of the number and morphology of the chromosomes and the localization of highly conserved sequences, such as the 45S rDNA cluster. Fossil-calibrated molecular dating suggests that the genus *Mycetophylax* probably originated at the Eocene-Oligocene boundary in the Amazon and/or Southern Grasslands, while psammophilous *Mycetophylax* emerged in the Middle Miocene in the South Atlantic coast, and most diversification occurred during subsequent major climatic events in the sand dune areas of the Brazilian Atlantic Forest biome. Putative CRs in the context of global climatic events and subsequent ecological stress could have somehow triggered and accompanied the diversification of the lineages in this highly dynamic environment.

Materials and Methods

Biological material. Populations of *Mycetophylax* were previously collected throughout their area of geographic distribution along the Brazilian Atlantic coast (Fig. 1). Colonies of *M. morschi* were collected in coastal regions belonging to the following Brazilian cities: Torres/RS (29°22′00″S; 49°44′45″W), Araranguá/SC (28°54′37″S; 49°22′00″W), Ilha Comprida/SP (24°44′29″S; 47°32′12″W), Cabo Frio/RJ (22°54′37″S; 42°02′40″W), and Ilhéus/BA (14°29′60″S; 39°02′07″W). Colonies of *M. conformis* were collected from Cabo Frio/RJ (22°54′29″S; 42°02′15″W), while *M. simplex* colonies were collected from Araranguá/SC (28°56′57″S; 49°22′15″W) (Fig. 1). Each population collected consisted of at least five colonies. Subsequent to collection, the colonies were transported to and conditioned *in vivo* at the Laboratório de Genética Evolutiva e de Populações from Universidade Federal de Ouro Preto, Minas Gerais, Brazil to obtain pre-pupae larvae, as reported by Cardoso *et al.*[69].

Chromosome preparation and FISH. Mitotic metaphases were obtained by the method described by Imai *et al.*[14] using brain ganglia of pre-pupae larvae through dissection in colchicine-hypotonic solution, following the adjusts described by Cardoso *et al.*[70]. Conventional giemsa staining was used to determine the chromosome number and morphology. We classified the chromosomes following the standard nomenclature for centromere position proposed by Levan *et al.*[71] with the small modification on the term acrocentric referring to all the chromosomes with an arm ratio greater than 7.0. The arm ratio (r) is the ratio of the length of the long arm of a chromosome to that of the short arm. Thus, metacentric chromosomes (M) are those with $r = 1.0–1.7$, submetacentrics (SM) have $r = 1.7–3.0$, subtelocentrics (ST) have $r = 3.0–7.0$, and acrocentrics have values from 7.0 to above.

FISH experiments were performed according to previous descriptions[72] with detailed modifications by Micolino *et al.*[73]. Highly conserved DNA repeat sequences (18S rDNA and TTAGG-telomeric motif) were used as probes. The $TTAGG_{(6)}$ motif was directly labeled with Cy3 at the 5′ terminal during synthesis (SIGMA, ST. LOUIS, MO, USA). The 18S rDNA sequence was isolated from bee *Melipona quinquefasciata* and amplified by PCR using the primers 18SF (5′-GTCATATGCTTGTCTCAAAGA-3′) and 18SR (3′-TCTAATTTTTTCAAAGTAAACGC-5′)[74], which corresponded to a 750 base pair (bp) segment. The PCR reaction consisted of 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. Metaphase chromosomes were incubated with RNAse (40 μg/mL) for 1 h at 37 °C. Thereafter, chromosomes were washed several times with saline solutions and then denatured in 70% formamide/2xSSC. The probes were hybridized with the chromosomes in 20 μL of the hybridization mix (200 ng of labeled probe, 50% formamide, 2xSSC, and 10% dextran sulfate 20xSSC) overnight. After this, metaphases were washed in 4xSSC/Tween, dehydrated in a series of alcoholic solutions (50%, 70%, and 100%, respectively) and assembled in antifade solution with DAPI (4′,6-diamidino-2-phenylindole) (DAPI FLUOROSHIELD, SIGMA-ALDRICH). Metaphase chromosomes were analyzed under an OLYMPUS BX53 epifluorescence microscope with OLYMPUS CELLSENS IMAGING software (OLYMPUS AMERICAN, INC., CENTER VALLEY, PA, USA), using WU (330–385 nm) and WG (510–550 nm) filters for DAPI and rhodamine, respectively. From 10 to 20 metaphases were analyzed per colony and the images were edited with ADOBE PHOTOSHOP CC software.

DNA extraction, amplification, and sequencing. Genomic DNA extraction was performed from one ant worker per colony according to the standard CTAB/chloroform techniques[75] with adaptations for ant extraction. We sequenced fragments of five nuclear protein-coding genes: *elongation factor 1-alpha-F1* (EF1α-F1), *elongation factor 1-alpha-F2* (EF1α-F2), *wingless* (Wg), *long-wavelength rhodopsin* (LW Rh), and *topoisomerase 1* (Top1) (GenBank accession numbers: MN745212-MN745286). The primers used to generate the sequence data are listed in Table S3. PCR was performed in a final volume of 25 μL which included 12.5 μL of Go*Taq* G2 Hot Start Colorless MasterMix (*Taq* DNA Polymerase, dNTPs, $MgCl_2$, and buffer), 9.5 μL of Nuclease-Free Water, 1 μL of each pair of primers, and 1 μL of DNA, according to the manufacturer's instructions (PROMEGA, MADISON, WI, USA). Amplifications reaction conditions and sequencing were based on the methodology outlined in previous studies[20,76,77]. The chromatogram quality was verified by a Phred quality score higher than 30, and then the sequences were manually trimmed in GENEIOUS R7[78].

Bayesian phylogenetic analysis. The concatenated dataset consisting of ~2.2 kbp (279 bp for EF1α-F1, 333 bp for Wg, 387 bp for LW Rh, 459 bp for EF1α-F2, and 669 bp for Top1) was added to the aligned dataset of Sosa-Calvo *et al.*[20] and aligned by using the MEGA7[79]. Data were partitioned and modeled using PARTITIONFINDER 2[80] under the Bayesian Information Criterion (BIC), greedy search scheme and with 15 input data blocks consisting of the first, second, and third codon positions of each of the five gene fragments. Thereafter, nine partitions were employed in independent Bayesian analyses using MRBAYES v3.2[81]. The standard nucleotide model was used for all partitions, and the nucleotide substitution models were variable for each partition (Table S4). Two simultaneous independent Monte Carlo Markov chain (MCMC) runs were performed with seven heated chains and one cold; the temperature was set at 0.05; and 10 million generations were performed with sampling every 1000 generations. The convergence among runs was verified by the average standard deviation of split frequencies that had to reach <0.01, and the appropriated burn-in was determined to be 10%. The tree-generated and posterior probabilities were visualized in FIGTREE v1.4[82].

Divergence time estimates. For divergence time analyses, we used BEAST v2.5[83] under the Fossilized Birth-Death (FBD) model[26] using an uncorrelated, log normal relaxed clock model to describe the branch-specific substitution rates[84]. The FBD model has become the most appropriate way to calibrate divergence time estimates when the calibration dates represented fossil occurrence times[26]. Accordingly, we integrated the occurrence times of 13 sets of Myrmicinae ant species fossils[85] into the tree prior to impose a time structure on the tree and to calibrate the analysis to absolute time. We calibrated internal nodes with minimum-age constraints using Myrmicinae amber fossils (Table S5). The root age was set at 98.6 Ma based on the estimated age for the subfamily Myrmicinae[77]. We set the nucleotide substitution model as GTR + I + G for all genes. Independent MCMC analyses were run for 60 million generations, sampling every 1000 generations. Runs were evaluated using TRACER v1.7[86] with effective sample size (ESS) values for all parameters over 200. The first 20% of the sampled tree topologies from the analyses were discarded as burn-in, and the remaining trees were summarized in TREEANNOTATOR v2.5. Before this, all fossils were removed from the tree using the FullToExtantTreeConverter tool (implemented in BEAUTI v2.5). The generated trees and credible intervals were visualized in both FIGTREE v1.4[82] and ICYTREE[87].

Biogeographical analysis and ancestral range estimation. Biogeographical reconstructions were performed using a phylogenetic tree constructed only with *Mycetophylax* species plus their basal relatives:

Mycetarotes, *Mycetosoritis*, *Kalathomyrmex* and *Cyatta* species. It should be stressed that some specimens of the "*strigatus* group" were pruned from the tree as they are unknown lineages with unknown geographic ranges as well. We ran the phylogenetic tree using BEAST v2.5[83] with 25 million generations, sampling every 1000 generations, and a final burn-in of 10%. For this, we used a Yule speciation process, an uncorrelated, log normal relaxed clock model, and GTR + I + G for all genes. We characterized seven geographic ranges by the Neotropics that corresponded to the species distributions used: South Atlantic Coast (A); North Atlantic Coast (B); Brazilian Atlantic Forest (C); Northern Grasslands, including the Caatinga and Cerrado biomes (D); Southern Grasslands, including Chaco and Pampas biomes (E); Amazon Rainforest (F); and Middle America, covering all of Central America and Southern USA (G). The species distributions were taken from www.antmaps.org [88].

We used the RASP v4.1[89] software package to estimate the likely ancestral ranges of *Mycetophylax*. RASP implements the major model-based approaches currently used, which enables comparisons to be made. Three approaches were employed for such estimates. The first one was carried out with the BioGeoBEARS (BGB)[90] to compare alternative biogeographic models. BGB can implement six specific models: the dispersal-extinction-cladogenesis model (DEC)[91], a likelihood version of the dispersal-vicariance analysis (DIVALIKE), and the Bayesian inference of historical biogeography for discrete areas (BAYAREALIKE); and all three models can also be run with the founder-event speciation parameter (+j)[38,61]. The model also implements the rate of "dispersal" (range expansion) and "extinction" (range contraction) along the internal branches of the phylogeny[38,61]. In order to analyze the connectivity between the areas, we set up dispersion multipliers so that adjacent areas received a dispersion multiplier of 1 and non-adjacent areas received a value of 0.5. AIC results were used to assess the statistical significance of data-to-model fit.

The second approach used was BayArea[92] as it re-calculates the ancestral state probabilities of each unit area with alternative burn-in values[89]. BayArea also incorporates geographic coordinate data, making it an attractive feature for biogeographic investigations. However, BayArea may need to be repeated several times to get a stable result[89], so we carried out independent runs with 10 million generations each, setting different burn-in values. Lastly, the third approach used was the Bayesian Binary MCMC (BBM)[93]. BBM analysis was performed using an estimated model, F81 + G, with a null root distribution, and the MCMC was run for 10 million generations with sampling every 1000 generations.

Ancestral chromosome number reconstruction. The chromosome evolution model and the reconstruction of ancestral chromosome numbers was recovered with CHROMEVOL v2.0[94,95] using both the maximum likelihood (ML) and Bayesian approaches. The chromosome evolution models evaluated were implemented as specified by Cardoso *et al.*[17]. To avoid over-representing the OTUs, and as several karyotype data on fungus-farming ants are unavailable, we used a restricted phylogeny, comprising data with cytogenetic information and only two species per genus per clade, since some genera were paraphyletic. The phylogeny was generated under the same specifications cited above for the MRBAYES analyses. The chromosome models over the phylogeny estimated in Bayesian inference and its null hypotheses were analyzed with 10,000 simulations, and the model that best fit the data set was selected under the Akaike Information Criterion (AIC).

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Author contributions
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., D.M.L. and D.C.C. contributed with reagents/materials/and analysis tools; R.M., N.M.T. and D.C.C. conducted cytogenetic experiments; R.M. and D.C.C. conducted and analyzed the molecular data; R.M. and D.C.C. wrote the manuscript; all authors read, discussed and approved the final version of the paper.

Competing interests
The authors declare no competing interests.

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Supplementary material

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Table S1. Comparisons of likelihood values (LnL), dispersal (d) and extinction (e) rates, *P*-value compared to the models with and without event-founder dispersal (j), and the Akaike Information Criterion (AIC) scores from each of the analyses in "BioGeoBEARS".

Model	LnL	d	e	j	AIC
DEC	–63.79	5	2.13	–	132.5
DEC+j	–59.23	5	3.8	0.077	126.5
DIVALIKE	–66.5	5	3.90E–07	–	137.9
DIVALIKE+j	–63.2	5	1.05	0.072	134.4
BAYAREALIKE	–64.33	4.51	5	–	133.6
BAYAREALIKE+j	–46.95	1.89	4.86	0.06	101.9

Table S2. The estimated rate parameters for the chromosome evolution model that best fit the data – the linear gain, loss and duplication model. Loss refers to chromosomal fusions, gain refers to chromosomal fissions and duplication refers to complete duplication of the genome (polyploidy). The total of events inferred throughout the karyotype evolution along the phylogenetic tree.

Rate parameters			Total events
Loss	Constant (δ)	11.58	
	Linear (δ_1)	1.88	
			487.93
Gain	Constant (λ)	31.52	
	Linear (λ_1)	–0.74	
			276.06
Duplication	Constant (ρ)	1.12	
			12.33

Table S3. List of primers used for amplification of the nuclear genes *EF1α-F1*, *EF1α-F2*, *Wg*, *LW Rh* and *Top1* in fungus-farming ants of the genus *Mycetophylax*.

Gene region	Primer	Sequence 5′ to 3′	Source
EF1a-F1	1424F	GCGCCKGCGGCTCTCACCACCGAGG	Brady *et al.*[1]
	1829R	GGAAGGCCTCGACGCACATMGG	Brady *et al.*[1]
EF1a-F2	557F	GAACGTGAACGTGGTATYACSAT	Brady *et al.*[1]
	1118R	TTACCTGAAGGGGAAGACGRAG	Brady *et al.*[1]
LW Rh	LR143F	GACAAAGTKCCACCRGARATGCT	Ward & Downie[2]
	LR639ER	YTTACCGRTTCCATCCRAACA	Ward & Downie[2]
Wg	wg578F	TGCACNGTGAARACYTGCTGGATGCG	Ward & Downie[2]
	wg1032R	ACYTCGCAGCACCARTGGAA	Abouheif & Wray[3]
Top1	TP1339F	GARCAYAARGGACCKGTRTTYGCACC	Ward & Sumnicht[4]
	TP2192R	GARCARCARCCYACDGTRTCHGCYTG	Ward & Sumnicht[4]

Table S4. The 15 partitions and models identified by PartitionFinder 2 and used in the Bayesian analyses of the concatenated dataset.

Partition	Data blocks	Best model
p1	EF1aF1 pos1	TRN+I
p2	EF1aF1 pos2, EF1aF2 pos2	F81+I
p3	Wg pos3, EF1aF1 pos3	GTR+I+G
p4	Wg pos1, Wg pos2	K80+I+G
p5	Top1 pos2, LWRh pos1	HKY+I+G
p6	LWRh pos2	GTR+I+G
p7	LWRh pos3	K80+I+G
p8	Top1 pos1, EF1aF2 pos1	GTR+I+G
p9	Top1 pos3, EF1aF2 pos3	TRNEF+I+G

Table S5. List of a priori age distributions applied to 13 fossil calibration points for BEAST divergence dating analysis.

Taxon (stem-group)	Offset (in Ma)	Location	Source
Myrmica spp.	42	Baltic and Saxonian ambers	Radchenko *et al.*[5]
Tetramorium spp.	42	Baltic and Saxonian ambers	Dlussky & Rasnitsyn[6]
Pristomyrmex spp.	42	Late Eocene amber	Dlussky & Radchenko[7]
Pogonomyrmex fossilis	34	Florissant Formation	Carpenter[8]
Pheidole spp.	34	Florissant Formation	Carpenter[8]
Cataulacus spp.	30	Sicilian amber	Emery[9]
Cephalotes atratus	15	Dominican amber	De Andrade & Baroni Urbani[10]
Crematogaster acuta	15	Dominican amber	Blaimer[11]
Strumigenys ambatrix	15	Dominican amber	Baroni Urbani & De Andrade[11]
Apterostigma electropilosum	15	Dominican amber	Schultz[12]
Cyphomyrmex maya	15	Dominican amber	De Andrade[13]
Cyphomyrmex taino	15	Dominican amber	De Andrade[13]
Trachymyrmex primaevus	15	Dominican amber	Baroni Urbani[14]

Figure S1. FISH mapping of $TTAGG_{(6)}$ telomeric probe (in red) in DAPI-stained karyotypes from psammophilous *Mycetophylax*. a) *M. morschi* (2*n* = 26); b) *M. morschi* (2*n* = 28); c) *M. morschi* (2*n* = 30); d) *M. conformis* (2*n* = 30); and e) *M. simplex* (2*n* = 36). Scale bar = 5 μm.

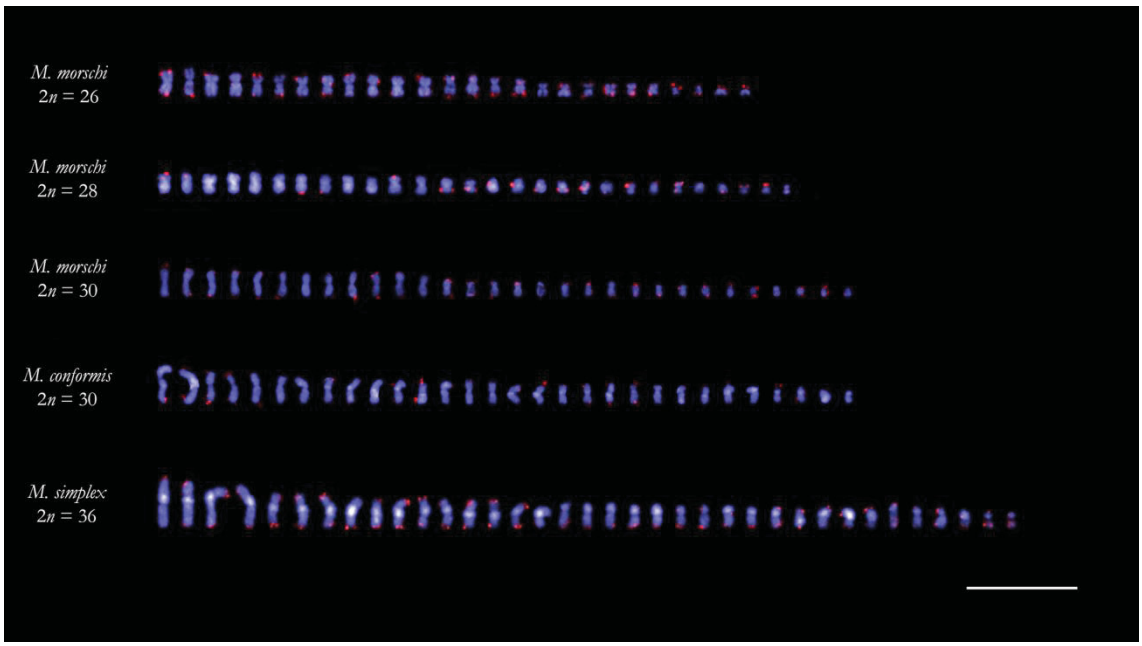


Figure S2. Phylogeny of fungus-farming ants based on a Bayesian analysis of five nuclear protein-coding genes plus *Mycetophylax* lineages analyzed here, as follows: *M. simplex* (yellow); *M. conformis* (purple); "*M. morschi*" lineage A (red); "*M. morschi*" lineage B (green); and "*M. morschi*" lineage C (blue). Black dots on branches represent a Bayesian posterior probability of 100, while gray dots represent a Bayesian posterior probability of 95–99. Outgroups are represented by the dotted gray line.

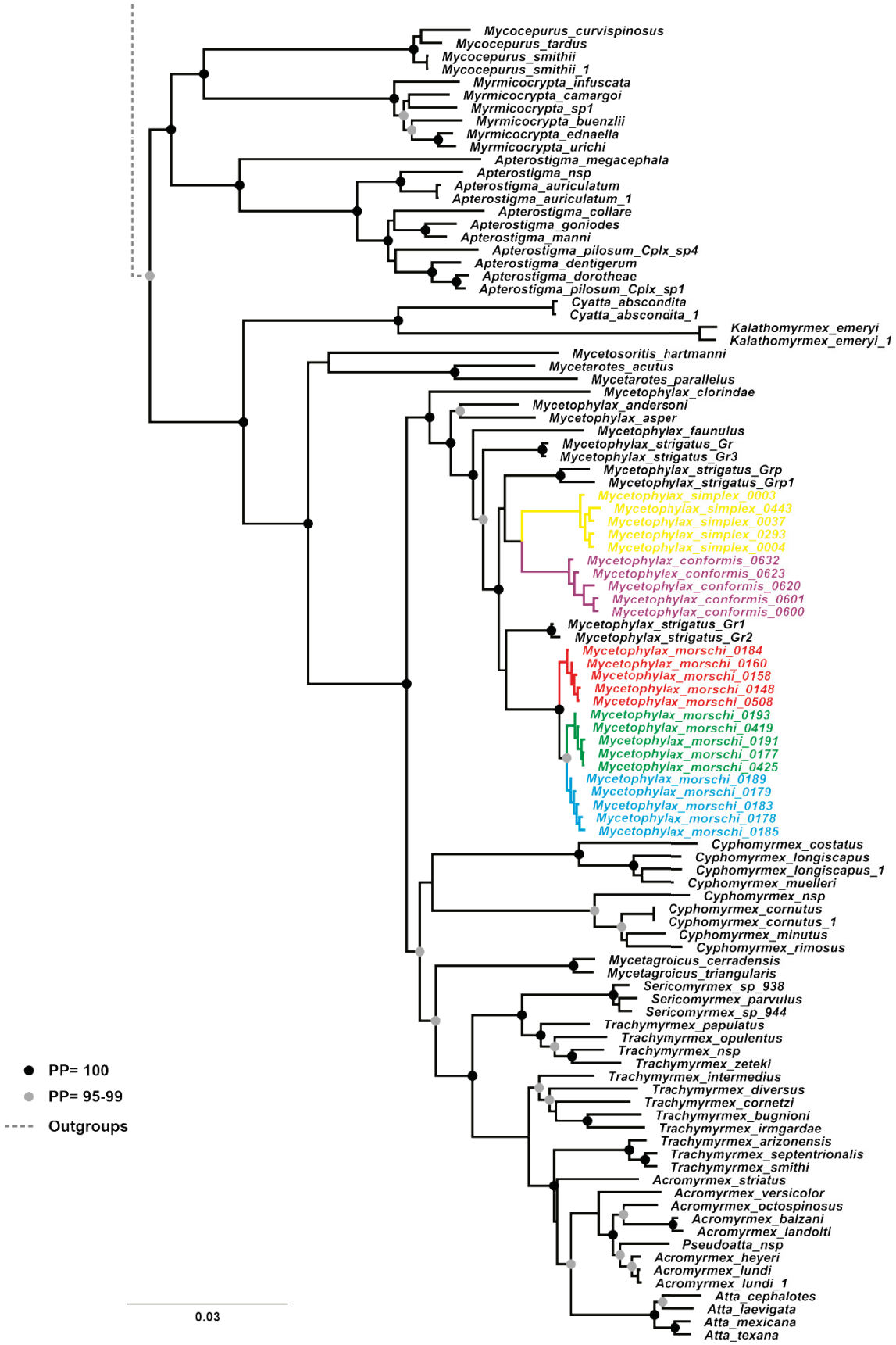

Mycocepurus_curvispinosus
Mycocepurus_tardus
Mycocepurus_smithii
Mycocepurus_smithii_1
Myrmicocrypta_infuscata
Myrmicocrypta_camargoi
Myrmicocrypta_sp1
Myrmicocrypta_buenzlii
Myrmicocrypta_ednaella
Myrmicocrypta_urichi
Apterostigma_megacephala
Apterostigma_nsp
Apterostigma_auriculatum
Apterostigma_auriculatum_1
Apterostigma_collare
Apterostigma_goniodes
Apterostigma_manni
Apterostigma_pilosum_Cplx_sp4
Apterostigma_dentigerum
Apterostigma_dorotheae
Apterostigma_pilosum_Cplx_sp1
Cyatta_abscondita
Cyatta_abscondita_1
Kalathomyrmex_emeryi
Kalathomyrmex_emeryi_1
Mycetosoritis_hartmanni
Mycetarotes_acutus
Mycetarotes_parallelus
Mycetophylax_clorindae
Mycetophylax_andersoni
Mycetophylax_asper
Mycetophylax_faunulus
Mycetophylax_strigatus_Gr
Mycetophylax_strigatus_Gr3
Mycetophylax_strigatus_Grp
Mycetophylax_strigatus_Grp1
Mycetophylax_simplex_0003
Mycetophylax_simplex_0443
Mycetophylax_simplex_0037
Mycetophylax_simplex_0293
Mycetophylax_simplex_0004
Mycetophylax_conformis_0632
Mycetophylax_conformis_0623
Mycetophylax_conformis_0620
Mycetophylax_conformis_0601
Mycetophylax_conformis_0600
Mycetophylax_strigatus_Gr1
Mycetophylax_strigatus_Gr2
Mycetophylax_morschi_0184
Mycetophylax_morschi_0160
Mycetophylax_morschi_0158
Mycetophylax_morschi_0148
Mycetophylax_morschi_0508
Mycetophylax_morschi_0193
Mycetophylax_morschi_0419
Mycetophylax_morschi_0191
Mycetophylax_morschi_0177
Mycetophylax_morschi_0425
Mycetophylax_morschi_0189
Mycetophylax_morschi_0179
Mycetophylax_morschi_0183
Mycetophylax_morschi_0178
Mycetophylax_morschi_0185
Cyphomyrmex_costatus
Cyphomyrmex_longiscapus
Cyphomyrmex_longiscapus_1
Cyphomyrmex_muelleri
Cyphomyrmex_nsp
Cyphomyrmex_cornutus
Cyphomyrmex_cornutus_1
Cyphomyrmex_minutus
Cyphomyrmex_rimosus
Mycetagroicus_cerradensis
Mycetagroicus_triangularis
Sericomyrmex_sp_938
Sericomyrmex_parvulus
Sericomyrmex_sp_944
Trachymyrmex_papulatus
Trachymyrmex_opulentus
Trachymyrmex_nsp
Trachymyrmex_zeteki
Trachymyrmex_intermedius
Trachymyrmex_diversus
Trachymyrmex_cornetzi
Trachymyrmex_bugnioni
Trachymyrmex_irmgardae
Trachymyrmex_arizonensis
Trachymyrmex_septentrionalis
Trachymyrmex_smithi
Acromyrmex_striatus
Acromyrmex_versicolor
Acromyrmex_octospinosus
Acromyrmex_balzani
Acromyrmex_landolti
Pseudoatta_nsp
Acromyrmex_heyeri
Acromyrmex_lundi
Acromyrmex_lundi_1
Atta_cephalotes
Atta_laevigata
Atta_mexicana
Atta_texana
PP= 100
PP= 95-99
Outgroups
0.03

Figure S3. FBD-based phylogenetic tree showing divergence time estimates of the fungus-farming ants. The horizontal blue bars at the nodes represent the 95% highest posterior density (HPD) intervals of the estimated node ages. The scale axis bar represents million years ago (Ma).

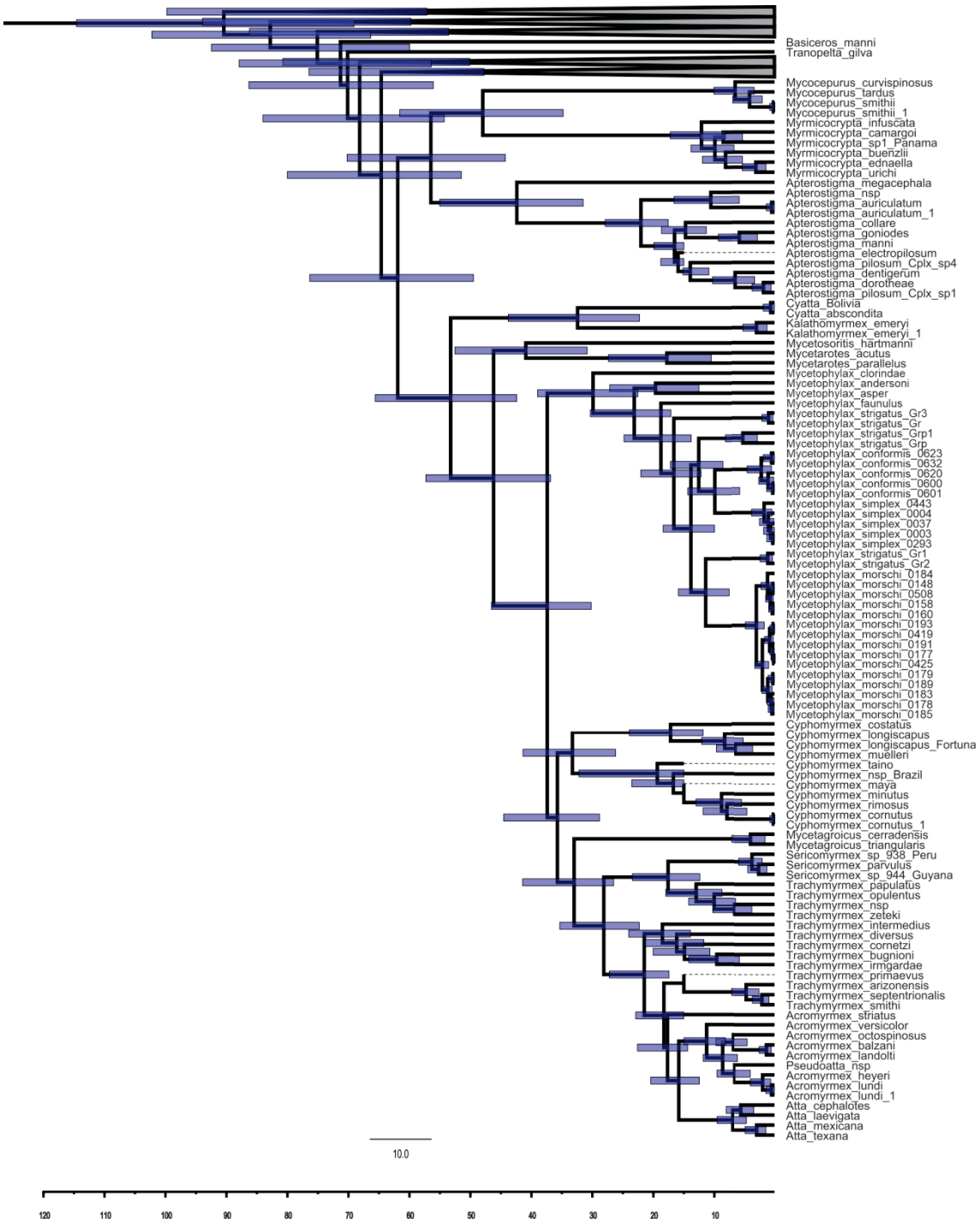
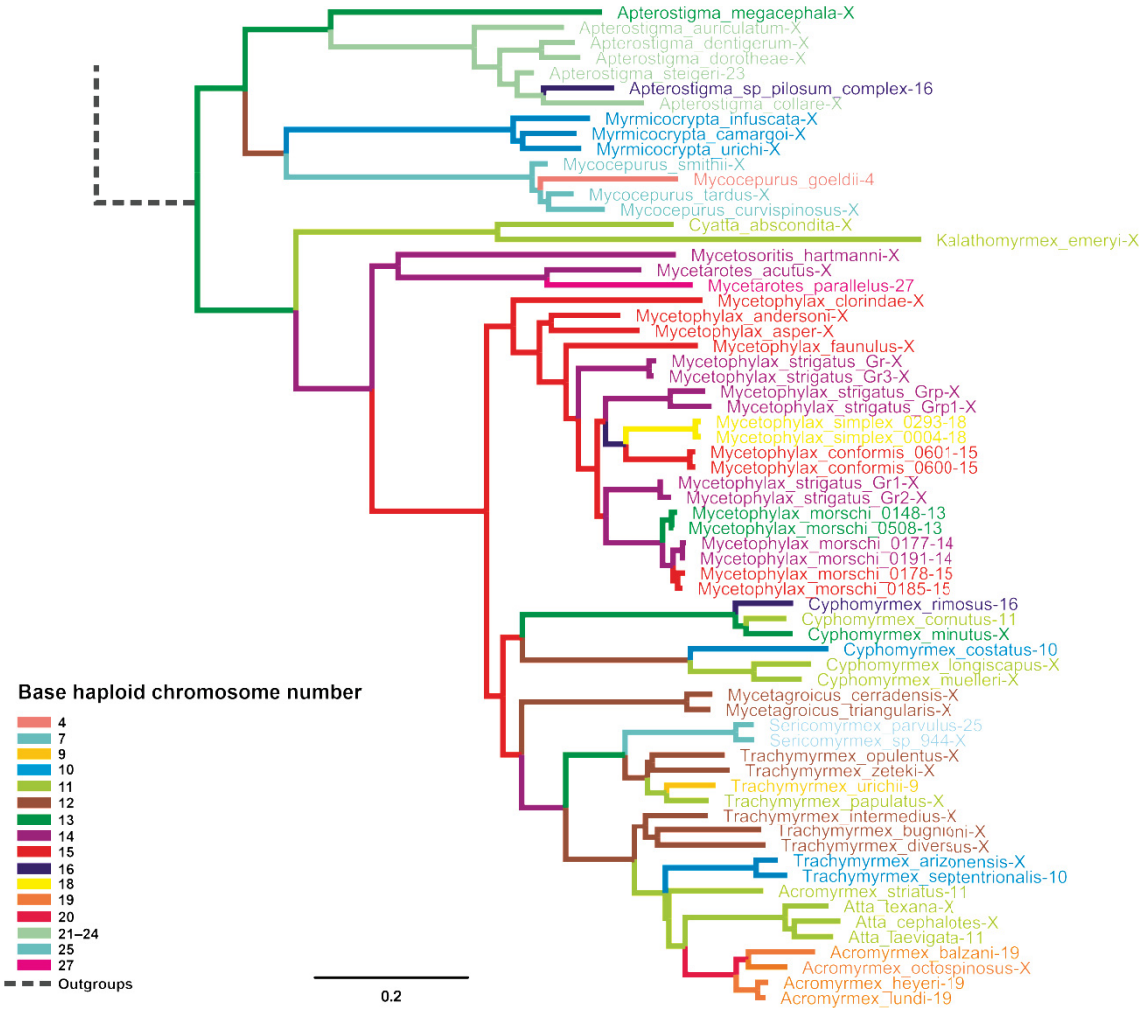


Figure S4. Chromosome number evolution and inferred ancestral chromosome state in the fungus-farming ants from ChromEvol results based on maximum likelihood (ML). The numbers at the tips are the known haploid chromosome numbers of species, while "X" represents unknown numbers. The various colors on the branches of the tree represent the base haploid chromosome number for each node, given in the legend of the figure.



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CAPÍTULO 2:

POPULATION-BASED CYTOGENETIC BANDING ANALYSIS AND PHYLOGENETIC RELATIONSHIPS OF THE NEOTROPICAL FUNGUS-FARMING ANT *Trachymyrmex holmgreni* WHEELER, 1925

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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 1829R	GCGCCKGCGGCTCTCACCACCGAGG GGAAGGCCTCGACGCACATMGG	278	60	Brady et al. [2006]
EF1a-F2	557F 1118R	GAACGTGAACGTGGTATYACSAT TTACCTGAAGGGGAAGACGRAG	484	56	Brady et al. [2006]
LW Rh	143F 639ER	GACAAAGTKCCACCRGARATGCT YTTACCGRTTCCATCCRAACA	348	56	Ward and Downie [2005]
Wg	578F 1032R	TGCACNGTGAARACYTGCTGGATGCG ACYTCGCAGCACCARTGGAA	373	55	Ward and Downie [2005] Abouheif and Wray [2002]
Top1	1339F 2192R	GARCAYAARGGACCKGTRTTYGCACC 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

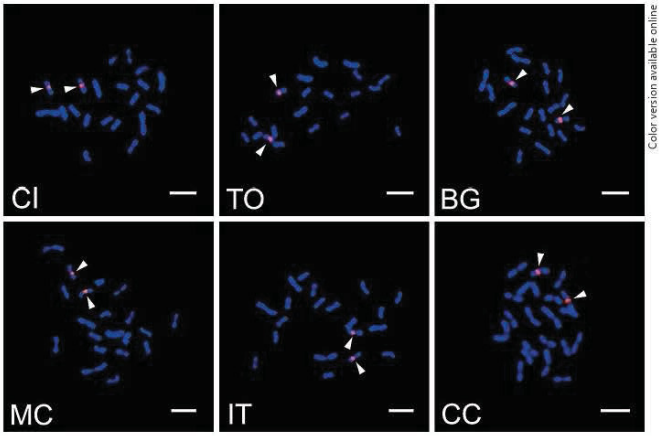


Fig. 1. Chromosomal metaphases stained with DAPI showing the 18S rDNA clusters (in red) in the 4th chromosome pair in different populations of *T. holmgreni*. CI: Cidreira; TO: Torres; BG: Balneário Gaivota; MC: Araranguá; IT: Laguna; CC: Cachoeira do Campo. Scale bars, 5 μm.

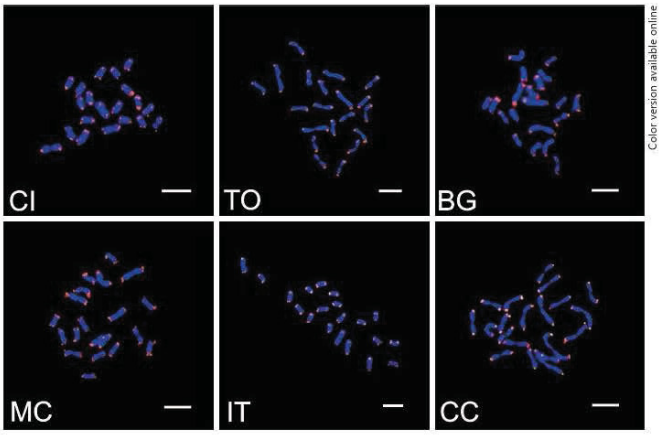
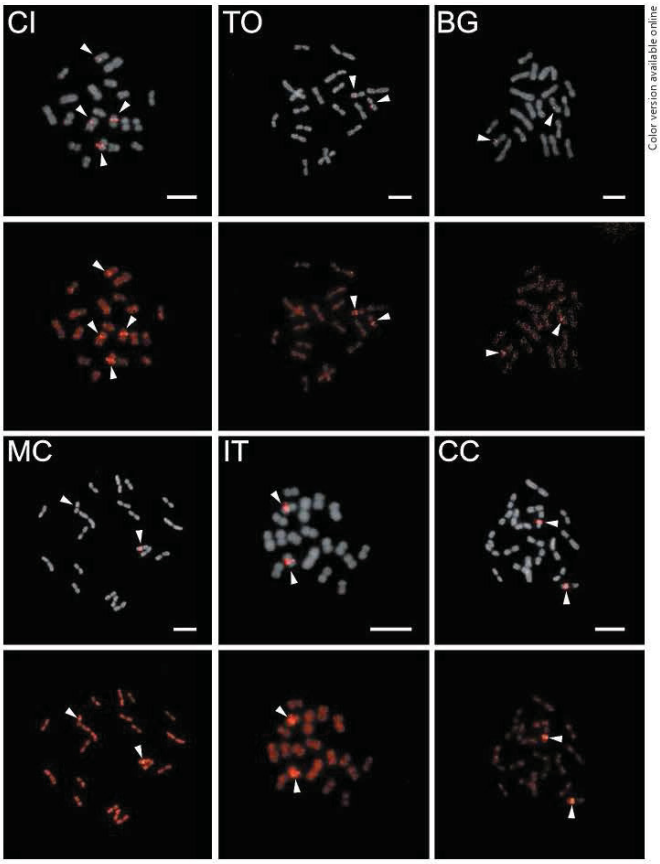


Fig. 2. Chromosomal metaphases stained with DAPI showing the $TTAGG_{(6)}$ telomeric sequences (in red) at the ends of all chromosomes in different populations of *T. holmgreni*. CI: Cidreira; TO: Torres; BG: Balneário Gaivota; MC: Araranguá; IT: Laguna; CC: Cachoeira do Campo. Scale bars, 5 μm.

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

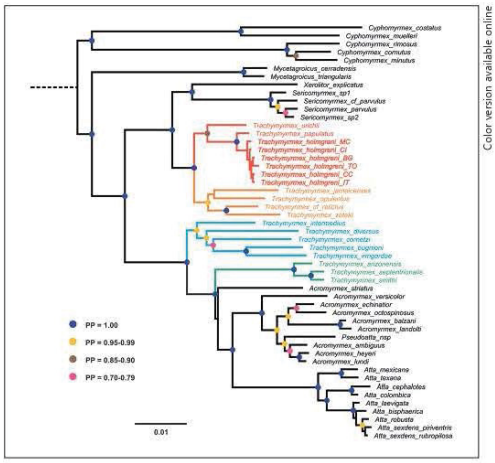


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Fig. 4. Phylogenetic tree emphasizing the position of *T. holmgreni* and the different clades of *Trachymyrmex* from Bayesian analysis of 5 nuclear protein-coding genes. In red the (Urichii + Iheringi) group, in orange the (Opulentus + Jamaicensis) 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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Supplementary material

(Available in:
https://www.researchgate.net/publication/336749947_Population_Banding_and_Phylogenetic_Relationships_of_Trachymyrmex_homlgreni_-_Supplementary_Data)

Table S1. Accession numbers by molecular marker of the specimens retrived from GenBank used for the phylogenetic inference.

Species	**EF1aF1**	**EF1aF2**	**WG**	**LW RH**	**TOP1**
Acromyrmex ambiguus	KC478127	KC478127	JX198231	JX198244	-
Acromyrmex balzani	EU204323	EU204565	EU204170	EU204490	KY828557
Acromyrmex echinatior	KC478113	KC478130	KC478095	KC478104	-
Acromyrmex heyeri	KC478114	KC478131	JX198235	JX198247	KY828555
Acromyrmex landolti	EU204454	EU204605	EU204211	EU204530	KY828558
Acromyrmex lundi	EU204422	EU204573	EU204178	EU204497	KY828553
Acromyrmex octospinosus	EU204389	EU204541	EU204145	EU204465	KY828554
Acromyrmex striatus	KC478119	KC478136	KC478096	KC478107	KY828501
Acromyrmex versicolor	EF013211	EF013373	EF013662	EF013534	KJ861521
Atta bisphaerica	KC478121	KC478138	KC478098	KC478102	-
Atta cephalotes	EU204441	EU204591	EU204197	EU204516	KY828559
Atta colombica	KC478122	KC478139	KC478099	KC478103	-
Atta laevigata	EU204405	EU204556	EU204161	EU204481	KY828561
Atta mexicana	EU204415	EU204566	EU204171	EU204491	KY828560
Atta robusta	KC478123	KC478140	JX198242	JX198254	-
Atta sexdens piriventris	KC478124	KC478141	KC478100	KC478105	-
Atta sexdens rubropilosa	KC478125	KC478142	KC478101	KC478106	-
Atta texana	EU204449	EU204600	EU204206	EU204525	KY828562
Cyphomyrmex cornutus	EU204456	EU204607	EU204213	EU204532	KY828533
Cyphomyrmex costatus	EU204412	EU204563	EU204168	EU204488	KY828535
Cyphomyrmex minutus	EU204433	EU204583	EU204189	EU204508	KY828537
Cyphomyrmex muelleri	EU204459	EU204610	EU204216	EU204535	KY828542
Cyphomyrmex rimosus	EU204299	EU204390	EU204146	EU204466	KY828540
Mycetagroicus cerradensis	-	-	-	-	KY828500

Mycetagroicus triangularis	EU204612	EU204461	EU204218	EU204537	-
Pseudoatta sp.	EU204569	EU204418	EU204174	EU204493	KY828556
Sericomyrmex cf. parvulus	EU204300	EU204542	EU204147	EU204467	KY828531
Sericomyrmex parvulus	-	-	KC964656	KC964633	-
Sericomyrmex sp1	KY828580	KY828486	KY828496	KY828590	KY828569
Sericomyrmex sp2	KY828581	KY828487	KY828497	KY828591	KY828570
Trachymyrmex arizonensis	EU204388	EF013526	EF013783	EF013655	KJ861741
Trachymyrmex bugnioni	EU204303	EU204545	EU204150	EU204470	KY828550
Trachymyrmex cf relictus	EU204333	EU204575	EU204180	EU204499	-
Trachymyrmex cornetzi	EU204301	EU204543	EU204148	EU204468	KY828548
Trachymyrmex diversus	EU204302	EU204544	EU204149	EU204469	KY828549
Trachymyrmex intermedius	EU204336	-	EU204183	EU204502	-
Trachymyrmex irmgardae	EU204322	EU204564	EU204169	EU204489	KY828546
Trachymyrmex jamaicenses	-	-	DQ353036	DQ353224	--
Trachymyrmex opulentus	EU204332	EU204574	EU204179	EU204498	KY828547
Trachymyrmex papulatus	EU204429	EU204579	EU204185	EU204504	KY828545
Trachymyrmex septentrionalis	EU204337	EU204578	EU204184	EU204503	KY828551
Trachymyrmex smithi	EU204613	EU204462	EU204219	EU204538	KY828552
Trachymyrmex urichii	-	-	KC964657	KC964634	-
Trachymyrmex zeteki	EU204339	EU204580	EU204186	EU204505	-
Xerolitor explicatus	MG642983	MG642984	MG642985	MG642986	MG642987

Figure S1. Population-based cytogenetic banding analysis of $CAA_{(10)}$ tandem repeats. Scale bar = 5 um.

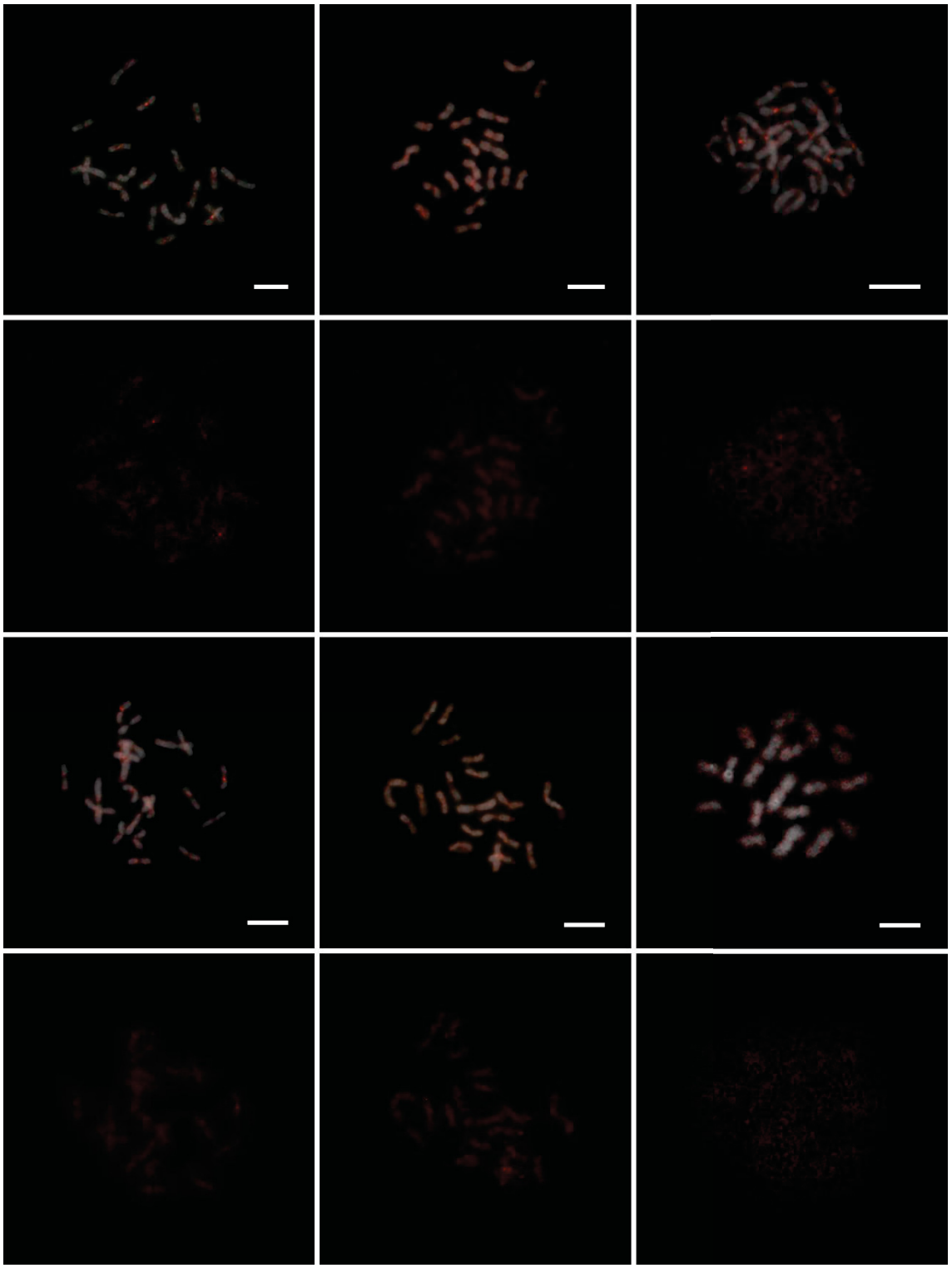


Figure S2. Population-based cytogenetic banding analysis of $GAG_{(10)}$ tandem repeats. Scale bar = 5 um.

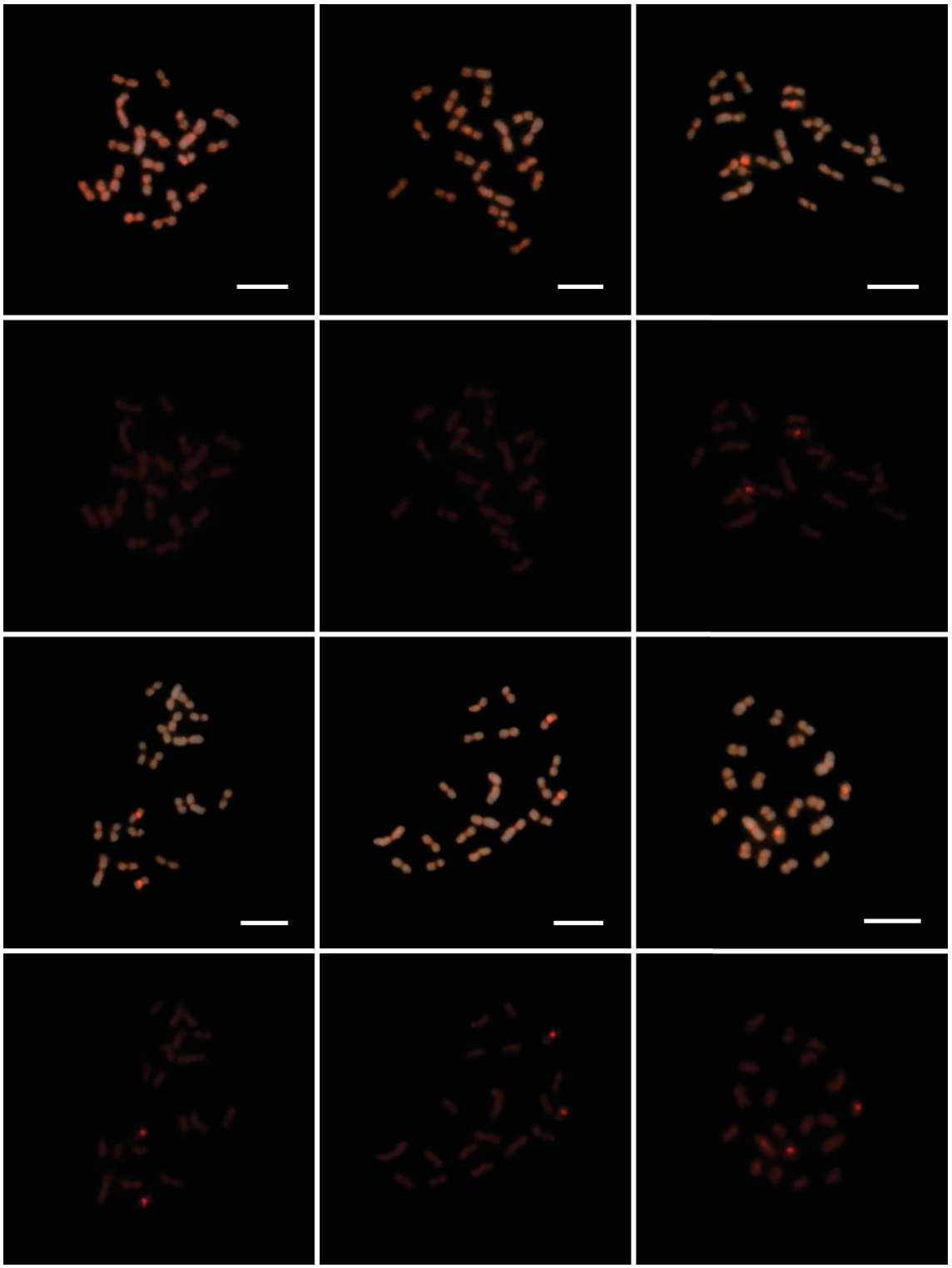
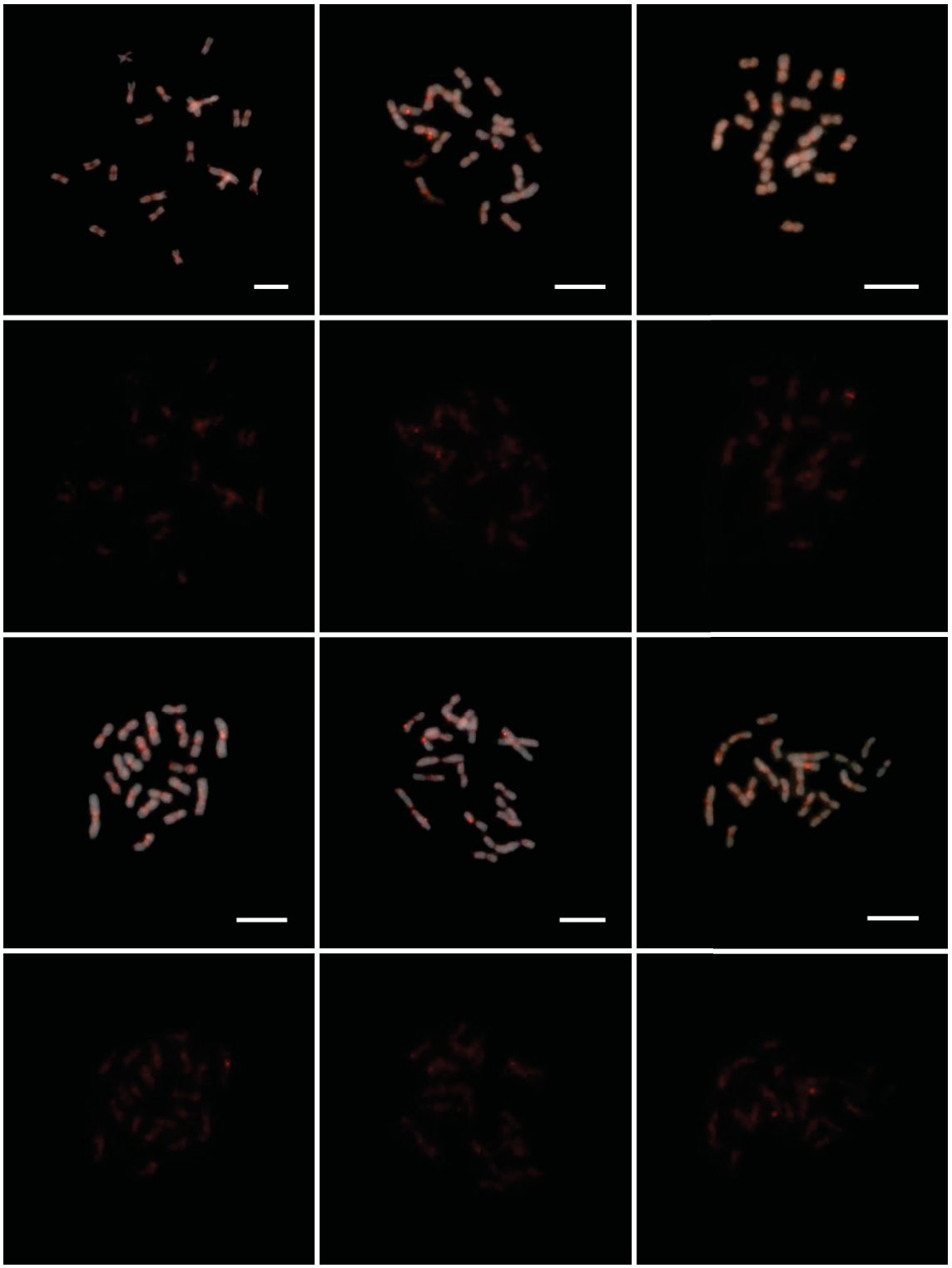


Figure S3. Population-based cytogenetic banding analysis of $CGG_{(10)}$ tandem repeats. Scale bar = 5 um.



CAPÍTULO 3:

KARYOTYPE AND PUTATIVE CHROMOSOMAL INVERSION SUGGESTED BY INTEGRATION OF CYTOGENETIC AND MOLECULAR DATA OF THE FUNGUS-FARMING ANT *Mycetomoellerius iheringi* EMERY, 1888

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Karyotype and putative chromosomal inversion suggested by integration of cytogenetic and molecular data of the fungus-farming ant *Mycetomoellerius iheringi* Emery, 1888

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Abstract

Comparative cytogenetic analyses are being increasingly used to collect information on species evolution, for example, diversification of closely related lineages and identification of morphologically indistinguishable species or lineages. Here, we have described the karyotype of the fungus-farming ant *Mycetomoellerius iheringi* Emery, 1888 and investigated its evolutionary relationships on the basis of molecular and cytogenetic data. The *M. iheringi* karyotype consists of 2n = 20 chromosomes (2K = 18M + 2SM). We also demonstrated that this species has the classical insect TTAGG telomere organization. Phylogenetic reconstruction showed that *M. iheringi* is phylogenetically closer to *M. cirratus* Mayhé-Nunes & Brandão, 2005 and *M. kempfi* Fowler, 1982. We compared *M. iheringi* with other congeneric species such as *M. holmgreni* Wheeler, 1925 and inferred that *M. iheringi* probably underwent a major pericentric inversion in one of its largest chromosomes, making it submetacentric. We discussed our results in the light of the phylogenetic relationships and chromosomal evolution.

Keywords

chromosomal evolution, FISH, fungus growing, karyomorphometry, TTAGG, *Trachymyrmex*

Introduction

Fungus-farming ants (Formicidae: Myrmicinae: Attini) are exclusive to the New World and occur mainly in the Neotropical region, with some species found in the Nearctic region (Weber 1966; Rabeling et al. 2007). The most recently diverged species include the well-known leafcutter ants (genera *Atta* Fabricius, 1804 and *Acromyrmex* Mayr, 1865) as well as the genera *Xerolitor* Sosa-Calvo et al., 2018, *Sericomyrmex* Mayr, 1865 and *Trachymyrmex* Forel, 1893. Previous phylogenetic analyses have shown that the genus *Trachymyrmex* is paraphyletic (e.g., Schultz and Brady 2008; Sosa-Calvo et al. 2018; Micolino et al. 2019a). However, this taxonomic complication was recently resolved by multilocus phylogenetic analyses with a comprehensive number of species (Solomon et al. 2019). Thus, a new systematic arrangement of three clades was proposed as follows: *Mycetomoellerius* Solomon et al. 2019 (former *Iheringi* group), *Paratrachymyrmex* Solomon et al., 2019 (former *Intermedius* group), and *Trachymyrmex* (based on the type species *Trachymyrmex septentrionalis* McCook, 1881). Nevertheless, *Trachymyrmex sensu stricto*, largely containing North American species, is still most prominently studied (e.g., Rabeling et al. 2007; Seal et al. 2015; Sánchez-Peña et al. 2017).

Cytogenetics encompasses the study of chromosomes that may have direct implications on species evolution, such as the identification of cryptic species and diversification of closely related lineages (White 1978; King 1993). In general, ants exhibit one of the largest chromosomal variability among organisms (reviewed by Lorite and Palomeque 2010), leading to the hypothesis that chromosomal rearrangements, i.e., Robertsonian fissions and fusions (known major rearrangements that can change the chromosomal number within lineages), actively contributed to the diversification of ants (Imai et al. 1988, 2001; Cardoso et al. 2018a). Despite the large number of species in the three genera formerly included into "*Trachymyrmex*" (about 60 species, see above), there is limited cytogenetic information on this ant group. To date, only seven species have been karyotyped, three of which have not been identified to the species level (see Table 1). On the basis of the available data, the described chromosomal numbers appear to be stable within the three genera, ranging from 2n = 12 to 2n = 22 and predominantly comprising metacentric chromosomes (reviewed by Cardoso et al. 2018a).

Table 1. Former "*Trachymyrmex*" species with their described karyotypes. 2n: diploid chromosome number; (n): haploid chromosome number; 2K: karyotype formula; Locality: sampling site; M: metacentric chromosomes; SM: submetacentric chromosomes.

Species	2n (n)	2K	Locality	References
*Mycetomoellerius fuscus**	18 (9)	16M + 2SM	Minas Gerais State, Brazil	Barros et al. (2013a)
Mycetomoellerius holmgreni	20 (10)	20M	Minas Gerais State, Brazil	Barros et al. (2018)
Mycetomoellerius iheringi	20 (10)	18M + 2SM	Santa Catarina State, Brazil	Present study
Mycetomoellerius relictus	20 (10)	20M	Minas Gerais State, Brazil	Barros et al. (2013b)
Trachymyrmex septentrionalis	20 (10)	20M	Barro Colorado Island, Panama	Murakami et al. (1998)
"*Trachymyrmex*" sp. 1	12 (6)	12M	Barro Colorado Island, Panama	Murakami et al. (1998)
"*Trachymyrmex*" sp. 2	18 (9)	18M	Barro Colorado Island, Panama	Murakami et al. (1998)
"*Trachymyrmex*" sp. 3	22 (11)	18M + 4SM	Minas Gerais State, Brazil	Barros et al. (2013b)

* current junior synonym of *M. urichii*.

Mycetomoellerius iheringi Emery, 1888, the type species of the genus, is a species endemic to South America, and it occurs mainly in the southern regions. The exclusive characteristic of *M. iheringi* is the finely striated discal area of the mandibles, which sets it apart from the congeneric species *Mycetomoellerius kempfi* Fowler, 1982 (Mayhé-Nunes and Brandão 2005). A feature of *M. iheringi* biology that facilitates field identification is the subterranean nest in the sand with a slim opening (Mayhé-Nunes and Brandão 2005). Some groups have been identified by morphological similarities within the former "*Trachymyrmex*", including the *Iheringi* group that also includes *Mycetomoellerius holmgreni* Wheeler, 1925 whose karyotype has been already described (Mayhé-Nunes and Brandão 2005; Barros et al. 2018). This fact allows cytogenetic comparisons with *M. iheringi*. However, the phylogenetic position of *M. iheringi* has not yet been described; only the relationship between its fungal cultivars has been reported (see Solomon et al. 2019).

Here, we have described the *M. iheringi* karyotype on the basis of karyomorphometric analysis and fluorescence *in situ* hybridization (FISH) with a telomeric probe. In addition, we identified the phylogenetic position of *M. iheringi* and examined its relationship with other species of the genus. We have discussed our results in the light of chromosomal evolution among fungus-farming ants.

Material and methods

Colony sampling

Colonies of *M. iheringi* were collected from the Restinga environment of the Brazilian Atlantic coast at Joaquina Beach, Florianópolis, Santa Catarina State, Brazil (27°37'44"S; 48°26'52"W). A total of five distantly spaced colonies were sampled. Such colonies were maintained *in vivo* at the Laboratório de Genética Evolutiva e de Populações, Universidade Federal de Ouro Preto, Brazil, according to the protocol established by Cardoso et al. (2011).

Chromosome preparation and FISH mapping

Metaphase chromosomes from the brain ganglia of pre-pupal larvae were obtained using the method of Imai et al. (1988). The ganglia were dissected under a stereomicroscope and incubated in hypotonic solution containing 1% sodium citrate and 0.005% colchicine for 60 min, and consecutively dissociated and fixed on stereoscopic microscope slides in acetic acid: ethanol: distilled water (3:3:4) and acetic acid: ethanol (1:1). Subsequently, the metaphase chromosomes were examined under a phase-contrast microscope and stained with 4% Giemsa stain dissolved in Sorensen's buffer, pH 6.8, to determine the chromosome number and morphology. We classified the chromosomes according to the nomenclature proposed by Levan et al. (1964), which is based on the ratio of the chromosomal arms (*r*), given by centromere posi-

tion. The chromosomes were classified into metacentric (r = 1.0–1.7), submetacentric (r = 1.7–3.0), subtelocentric (r = 3.0–7.0), and acrocentric (r > 7.0) categories, as modified by Crozier (1970). The metaphase chromosomes were measured using IMAGE-PRO PLUS software (Media Cybernetics, LP, USA), and the values were calibrated by the scale bar and transferred to EXCEL (Microsoft, Redmond, WA, USA). In addition, the degree of variation and karyotype measurement were validated using statistical tests, according to Cristiano et al. (2017).

FISH experiments were performed as previously described by Kubat et al. (2008), with detailed modifications for ants by Micolino et al. (2019a). For the hybridizations, we used the $TTAGG_{(6)}$ telomeric motif, which has fine conservation in most insects and the advantage of being able to detect chromosomal rearrangements such as telomere-related inversions and fusions. The $TTAGG_{(6)}$ probe was directly labeled with Cy3 at the 5' terminal during synthesis (Sigma, St. Louis, MO, USA). The summarized technique involves several saline washes, alcohol dehydration, and formamide denaturation, until hybridization with the probe. For visualization, the metaphase chromosomes were stained with 4',6-diamidino-2-phenylindole (DAPI Fluoroshield, Sigma-Aldrich) in an antifade solution. The metaphase chromosomes were analyzed under an OLYMPUS BX53 epifluorescence microscope with OLYMPUS CELLSENS IMAGING software (Olympus American, Inc., Center Valley, PA, USA), using WU (330–385 nm) and WG (510–550 nm) filters for DAPI and rhodamine, respectively. About 10–20 metaphases were analyzed in both cytogenetic analyses, and the images were edited with ADOBE PHOTOSHOP CC software.

DNA extraction, sequencing, and phylogenetic analysis

We extracted the DNA from *M. iheringi* ant workers, according to the standard CTAB/chloroform technique (Sambrook and Russell 2001). We sequenced the fragments of four nuclear genes, *elongation factor 1-alpha-F1* (EF1α-F1), *elongation factor 1-alpha-F2* (EF1α-F2), *wingless* (Wg), and *long-wavelength rhodopsin* (LWRh), and one mitochondrial gene, *cytochrome c oxidase I* (COI) (GenBank accession numbers: MT174160–MT174169). The primers used to generate the sequence data are listed in Table 2. Polymerase chain reaction was performed using a final volume of 25 μL, according to the manufacturer's instructions (Promega, Madison, WI, USA). The amplification conditions and sequencing were based on the methodology outlined in previous studies (see Schultz and Brady 2008, Cardoso et al. 2015a, b, Ward et al. 2015).

The gene fragments were aligned and concatenated using MEGA7 software (Kumar et al. 2016) and incorporated into the dataset of Solomon et al. (2019). The phylogeny was inferred using the maximum likelihood criterion in RAxML (Stamatakis 2014) by using the simultaneous best-tree search and rapid bootstrapping analysis (1000 replicates) with the GTR + G model of evolution. The generated tree and branch labels were visualized using FIGTREE software (Rambaut 2009).

Table 2. Primers used for sequencing four nuclear (*EF1a-F1*, *EF1a-F2*, *Wg* and *LW Rh*) and one mitochondrial (*COI*) gene fragments in the fungus-farming ant *Mycetomoellerius iheringi*.

	Primer	Sequence 5' to 3'	Source
EF1a-F1	1424F	GCGCCKGCGGCTCTCACCACCGAGG	Brady et al. (2006)
	1829R	GGAAGGCCTCGACGCACATMGG	Brady et al. (2006)
EF1a-F2	557F	GAACGTGAACGTGGTATYACSAT	Brady et al. (2006)
	1118R	TTACCTGAAGGGGAAGACGRAG	Brady et al. (2006)
LW Rh	LR143F	GACAAAGTKCCACCRGARATGCT	Ward and Downie (2005)
	LR639ER	YTTACCGRTTCCATCCRAACA	Ward and Downie (2005)
Wg	wg578F	TGCACNGTGAARACYTGCTGGATGCG	Ward and Downie (2005)
	wg1032R	ACYTCGCAGCACCARTGGAA	Abouheif and Wray (2002)
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)

Results

Cytogenetic data

The karyotype of *M. iheringi* has 2n = 20 chromosomes (Fig. 1). Our karyomorphometric analysis revealed that this karyotype consists of nine metacentric pairs and one submetacentric pair; the karyotype formula is 2K = 18M + 2SM, and the fundamental number is FN = 40. The total average length of all chromosomes (i.e., of the diploid karyotype) was estimated to be 82.51 ± 0.52 µm. The average chromosome length ranged from 5.77 ± 0.91 µm to 3.37 ± 0.4 µm (Table 3). The telomere distribution of the $TTAGG_{(6)}$ motif was displayed at both ends of all *M. iheringi* chromosomes (Fig. 2a). No signals for interstitial telomeric sites (ITS) were detected using this probe. Moreover, DAPI staining revealed that both arms of all chromosomes were completely labeled, i.e., mostly A-T rich, whereas the centromeric region showed no labeling for this fluorochrome (Fig. 2b).

Molecular data

The maximum likelihood phylogeny showed *M. iheringi* as the sister species of a lineage defined as *Mycetomoellerius* n.sp. nr *cirratus* (see Solomon et al. 2019) (bootstrap value, PB = 90). The clade composed of *M. cirratus* Mayhé-Nunes & Brandão, 2005 + *M. kempfi* (PB = 98) forms the sister group of *M. iheringi* + *M.* n.sp. nr *cirratus* (PB = 88). The species *M. holmgreni* previously diverged from the aforementioned clades (PB = 89), and *M. papulatus* Santschi, 1922 was estimated to be the most basal of the "*Iheringi* group" (PB = 93) (Fig. 3).

Discussion

Here, we have provided the karyotypic description of the fungus-farming ant *Mycetomoellerius iheringi*, which has 2n = 20 chromosomes; we presented its phylogenetic

Figure 1. Mitotic metaphase of *Mycetomoellerius iheringi* with 2n = 20 chromosomes and its karyotypic morphology. M: metacentric chromosomes; SM: submetacentric chromosomes. Scale bar: 5 μm.

Table 3. Karyomorphometric analysis of the chromosomes of *Mycetomoellerius iheringi*. TL: total length; L: long arm length; S: short arm length; RL: relative length; *r*: arm ratio (= L/S); Σ: total average length of all chromosomes or Karyotype lenght (KL).

Chromosome	TL	L	S	RL	*r*	Classification
1	5.77±0.91	3.03±0.48	2.74±0.43	6.97±0.34	1.1±0.05	Metacentric
2	5.46±0.75	2.86±0.46	2.6±0.32	6.61±0.24	1.1±0.08	Metacentric
3	5.09±0.66	3.02±0.41	2.08±0.27	6.17±0.29	1.46±0.09	Metacentric
4	4.71±0.53	2.67±0.29	2.04±0.28	5.72±0.34	1.32±0.12	Metacentric
5	4.38±0.49	2.38±0.29	1.99±0.29	5.31±0.2	1.21±0.18	Metacentric
6	4.2±0.46	2.3±0.23	1.91±0.27	5.1±0.15	1.22±0.14	Metacentric
7	4.07±0.46	2.24±0.2	1.83±0.33	4.94±0.16	1.26±0.21	Metacentric
8	4.01±0.44	2.3±0.26	1.72±0.26	4.87±0.16	1.32±0.19	Metacentric
9	3.89±0.43	2.19±0.3	1.7±0.18	4.72±0.11	1.31±0.14	Metacentric
10	3.83±0.45	2.16±0.3	1.67±0.17	4.65±0.06	1.3±0.11	Metacentric
11	3.78±0.43	2.15±0.28	1.63±0.2	4.59±0.1	1.32±0.15	Metacentric
12	3.73±0.41	2.07±0.3	1.66±0.15	4.53±0.15	1.25±0.15	Metacentric
13	3.7±0.39	2.03±0.26	1.67±0.19	4.5±0.14	1.22±0.14	Metacentric
14	3.66±0.4	2.08±0.24	1.58±0.2	4.44±0.13	1.33±0.14	Metacentric
15	3.58±0.35	2.01±0.28	1.57±0.13	4.35±0.13	1.29±0.17	Metacentric
16	3.54±0.38	2.01±0.26	1.54±0.17	4.3±0.12	1.32±0.16	Metacentric
17	3.51±0.4	2.04±0.19	1.47±0.25	4.26±0.13	1.41±0.16	Metacentric
18	3.37±0.4	1.94±0.29	1.43±0.12	4.09±0.11	1.36±0.13	Metacentric
19	4.29±1.1	2.74±0.68	1.56±0.42	5.15±0.72	1.77±0.06	Submetacentric
20	3.94±0.59	2.51±0.37	1.43±0.22	4.76±0.25	1.76±0.03	Submetacentric
Σ	**82.51±0.52**					

position in the clade of the "*Iheringi* group". Considering the cytogenetic data available from fungus-farming ants, we observed a numerical constancy among the karyotypes of the lineages that diverged most recently (i.e., leafcutter ants of the genera *Atta* and *Acromyrmex*), suggesting this karyotypic characteristic is shared by the relatively recent lineages. *Trachymyrmex septentrionalis*, a sister clade of leafcutter ants, has 2n = 20 metacentric chromosomes, equal to those of two *Mycetomoellerius* species, *M. holmgreni* and *M. relictus* Borgmeier, 1934 (see Table 1). All *Atta* species karyotyped to

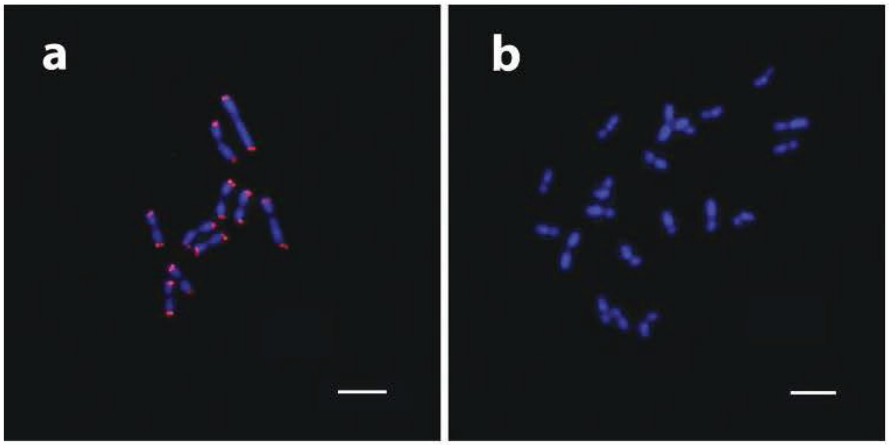

Figure 2. DAPI-stained *Mycetomoellerius iheringi* chromosomal metaphases **a** FISH mapping of the $TTAGG_{(6)}$ telomeric motif on haploid metaphase **b** chromosomes uniformly stained with DAPI fluorochrome, except for the centromeric region. Scale bar: 5 μm.

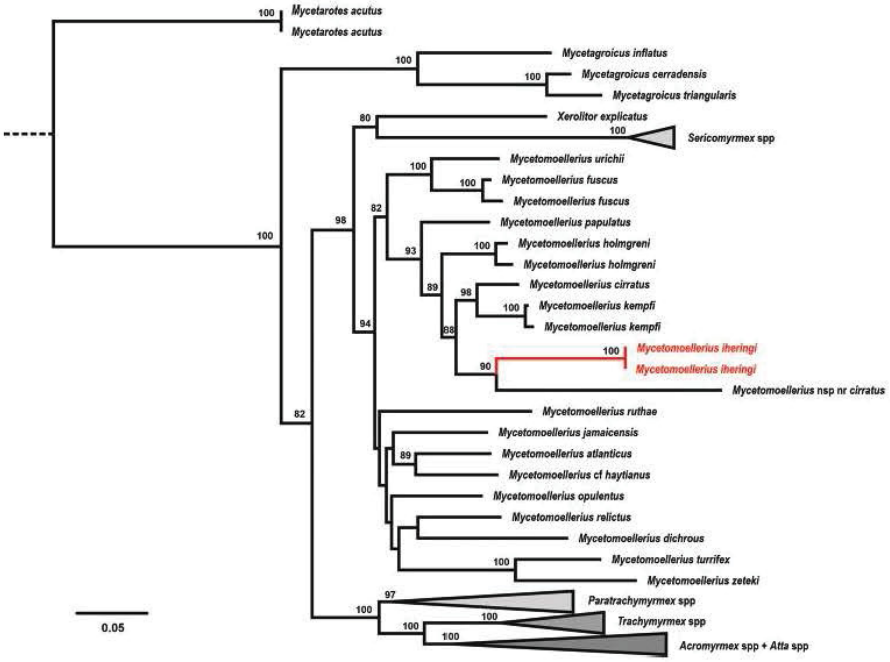

Figure 3. Maximum-likelihood phylogeny of "higher" fungus-farming ants generated in RAxML. *Mycetomoellerius iheringi* is indicated in red. Node numbers represent the bootstrapping values after 1000 replications; values < 80 are not shown. Scale bar indicates nucleotide substitutions per site.

date have 2n = 22 chromosomes, and most *Acromyrmex* species have 2n = 38 (reviewed by Cardoso et al. 2018a). In other Hymenoptera species, such as stingless bees of the tribe Meliponini Lepeletier, 1836, this scenario can also be seen in the genera with a conserved chromosome number (Travenzoli et al. 2019).

In the new taxonomic status, *Mycetomoellerius* is composed of about 30 described species (Solomon et al. 2019), but only four have known karyotypes and, interestingly, a prevalence of metacentric chromosomes (see Table 1). The species *M. iheringi* and *M. holmgreni* are closely related morphologically (Mayhé-Nunes and Brandão 2005), and, as we have shown, *M. holmgreni* diverged previously from *M. iheringi*. Moreover, both species co-occur in southern Brazilian sand-dune habitats (Cardoso and Schoereder 2014). Importantly, the karyotypes of these two species are similar: they have analogous karyotype measurements and DAPI-staining pattern as well the chromosomal number 2n = 20, differing by only one pair of submetacentric chromosomes (Barros et al. 2018; Cardoso et al. 2018b). A likely, and the most parsimonious, scenario for explaining such cytogenetic differences would involve at least one major chromosomal rearrangement. Therefore, we suggest a pericentric inversion occurred in one of the larger *M. iheringi* chromosomes, resulting in the current karyotype morphology. Such chromosomal rearrangement could have occurred in any lineage of the clades underlying *M. holmgreni*; however, such lineages should be karyotyped to verify this hypothesis. The base chromosome number, defined as the haploid number present in the initial lineage of a monophyletic clade, may be directly related to the chromosomal variability within that clade (Guerra 2008). Thus, the assumption of this major inversion is attributable to the fact that *M. holmgreni* has a karyotype formed by only metacentric chromosomes, which becomes a putative ancestral characteristic of the underlying lineages, such as *M. iheringi*.

The application of classical and molecular cytogenetic techniques, such as chromosomal banding and FISH mapping, has increasingly contributed to comparative evolutionary studies. Because of new ant cytogenetic data, valuable information is being collected and correlated to their evolution and exceptional chromosomal diversity. For instance, fusion and fission rearrangements have been proposed to play a crucial role in the diversification of the fungus-farming ants of the genus *Mycetophylax* Emery, 1913 (Cardoso et al. 2014; Micolino et al. 2019b). Indeed, chromosomal changes may be directly related to the speciation process for a range of taxa (Rieseberg 2001; Faria and Navarro 2010). In particular, inversions are abundant in natural populations and can have several evolutionary implications, such as adaptation and divergence of lineages (Ayala and Coluzzi 2005; Wellenreuther and Bernatchez 2018). Inversion polymorphisms may contribute to speciation by reducing recombination and consequently protecting genomic regions from introgression (Hoffmann and Rieseberg 2008). Moreover, a model has predicted that closely related lineages that co-occur in a region could readily differ by one or more inversions because such lineages would persist longer in the face of gene flow than in the absence of these inversions (Noor et al. 2001). Our data support such a model, mainly because the species *M. iheringi* and *M. holmgreni* live sympatrically and are phylogenetically close.

The rich karyotypic diversity of ants deserves special attention. Inversion polymorphisms, for example, have been reported in many ant species. For example, intrapopulational polymorphism has been detected in the *Iridomyrmex gracilis* Lowne, 1865 complex. Such populations with the same chromosome number but distinct karyotype structures have led authors to propose that a pericentric inversion occurred in a metacentric chromosome, making it acrocentric (n = 6M + 1SM + 1A to n = 5M + 1SM + 2A) (Crozier 1968). The chromosome number and morphology of *Pachycondyla* Smith, 1858 are variable; their karyotypes show a predominance of submetacentric and acrocentric chromosomes, which allows the interpretation that fission and pericentric inversions (where metacentric chromosomes turn acrocentric or vice versa) would be the most frequent chromosomal rearrangements in the evolution of this genus and even contribute to the speciation processes (Mariano et al. 2012). The intraspecific chromosomal variability in social organization (monogyny vs. polygyny) found in the fire ant *Solenopsis invicta* Buren, 1972 can also be explained by at least one large inversion, which would account for a lack of recombination over more than half of the two heteromorphic "social chromosomes" (Wang et al. 2013).

Another interesting finding was reported in *Mycetomoellerius fuscus* Emery, 1894 (current junior synonym of *M. urichii* Forel, 1893, see Micolino et al. 2019a for discussion), a species with a geographic distribution similar to *M. iheringi* and *M. holmgreni* and found largely in southern South America (Brandão and Mayhé-Nunes 2007). They are phylogenetically closer than previously expected (Micolino et al. 2019a; Solomon et al. 2019). *Mycetomoellerius fuscus* has a chromosomal morphology of eight metacentric pairs and a submetacentric pair (2n = 18) (Barros et al. 2013a). As the submetacentric pair is the biggest chromosome of the karyotype, there could have been a Robertsonian fusion rearrangement, followed by a pericentric inversion, making it submetacentric. The other few species of "*Trachymyrmex*" with the described karyotype (see Table 1) do not allow us to picture a full scenario for the karyoevolution of the genera. Further, unidentified specimens vary relatively widely from 2n = 12 to 2n = 22. The karyotype 2n = 12 presented by Murakami et al. (1998) is quite intriguing, as this unidentified specimen could be a key piece to understanding the chromosomal evolution of the clade to which it belongs. We emphasize that specimens submitted for cytogenetic analysis should be taxonomically identified. The non-identification of a specific sample triggers a series of problems, such as in the comparison with sister groups and eventual karyoevolutionary trajectories.

Our karyomorphometric approach was used primarily to reveal the chromosomal morphology of *M. iheringi*. Besides, future karyomorphometric comparisons among populations or even closely related lineages may serve as a basis for a possible delimitation of incipient lineages. For example, populations of *M. holmgreni* distributed on a North/South continuum of its distribution area diverged significantly in the length of their chromosomes, and the results were supported by flow cytometry analyses of the genome size (Cardoso et al. 2018b). Further, those populations were later identified to differ in the proportion of repetitive DNA by using FISH with microsatellite probes (Micolino et al. 2019a) Thus, the authors demonstrated the importance of using a

standardized karyomorphometric approach coupled with genome size estimation to identify hidden chromosomal variations (see Cardoso et al. 2018b).

Finally, we used a FISH probe of the highly conserved TTAGG telomeric sequence in most insects (reviewed by Kuznetsova et al. 2020) to test the assumption that the putative inversion rearrangement occurred in *M. iheringi* and involved the telomere. However, we did not observe any signal for the probe at the interstitial telomeric sites, which would denote inversion involving the telomere. Indeed, the TTAGG sequence also seems to be fairly conserved in ants (Lorite et al. 2002), including fungus-farming ants such as *Acromyrmex striatus* Roger, 1863 (Pereira et al. 2018), *Mycetophylax* spp. (Micolino et al. 2019b), and *M. holmgreni* (Micolino et al. 2019a). In conclusion, we have described another ant species with the TTAGG sequence conserved in its telomeres, and we suggest a significant chromosomal mechanism, a major pericentric inversion, most likely occurred in *M. iheringi* and could have been involved in its diversification process.

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CAPÍTULO 4:

CYTOGENETIC INSIGHTS INTO THE HIGH KARYOTYPE CONSERVATION OF ARGENTINE LEAFCUTTER ANT LINEAGES

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Cytogenetic insights into the high karyotype conservation of argentine leafcutter ant lineages

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Abstract

Leafcutter ants are considered the most important herbivores in terrestrial environments throughout the Neotropics. *Acromyrmex striatus* Roger, 1863 is estimated to be the sister clade of the remaining leafcutter ants, and part of this assumption is supported by morphological and cytogenetic characteristics shared with both *Atta* and *Acromyrmex*. Our objective here was to analyze cytogenetically Argentine populations of *A. striatus*, in addition to the description of the karyotype of its sister species, *A. silvestrii*, by means an integrative approach of classical and molecular cytogenetics. Our results show that the karyotypes of these two species are very similar, with identical number and structure (2n=22 and KF=20M+2SM), and location of repeated sequences on the chromossomes. We suggest that both species diverged relatively recently and are unmistakably sisters, due to the many shared characteristics, including the highly conserved karyotypes. Such karyotype conservation indicates that closely related ant species can also diverge without deep chromosomal changes during the process. Overall, our results provide valuable information about their possible kinship relations.

Key words: *Acromyrmex*, Chaco, chromosomes, evolution, leafcutter ants.

Introduction

Leafcutter ants comprise species from genera *Acromyrmex* and *Atta* have an obligatory symbiosis with specific basidiomycete fungi that ants grow the fungus for food in elaborate gardens. Worker ants provide fresh plant material into the fungal gardens that produce specialized hyphal tips (gongylidia) as the primary food source for the entire colony (HÖLLDOBLER & WILSON 2011). Leafcutter ants evolved about 20 million years ago to become the dominant herbivores of terrestrial environments in the Neotropical region (BRANSTETTER & al. 2017), which has also led them to be of high economic importance due to considerable losses for a variety of crops (HÖLLDOBLER & WILSON 2011, ZANETTI & al. 2014).

Despite the prominent karyotypic diversity within the Formicidae family, which ranges from 2n=2 to 2n=120 (reviewed in LORITE & PALOMEQUE 2010), each of the two genera of leafcutter ants has a mostly conserved chromosome number. Available cytogenetic data for *Atta* species display a regularity in both chromosome number and structure, all with 2n=22. On the other hand, *Acromyrmex* species display

predominantly 2n=38, but with very large variability in the structure of their chromosomes (reviewed in CARDOSO & al. 2018a). Interestingly, *Acromyrmex striatus* Roger, 1863 has the same karyotype number as the *Atta* species (2n=22) including most metacentric chromosomes, though differs in the GC-rich blocks pattern (CRISTIANO & al. 2013). This iconic species also shares morphological characters with both genera; three pairs of spines on promesonotum shared with *Acromyrmex* (only two pairs of spines in *Atta*), and smooth gastral tergum shared with *Atta* (whereas *Acromyrmex* has a tuberculate gaster) (MAYHÉ-NUNES 1991). Phylogenetic analyzes point out that *A. striatus* clade is the sister group of the remaining leafcutter ants (CRISTIANO & al. 2013, BRANSTETTER & al. 2017), well supported by karyotypic information setting the ancestral number of leafcutter ants as 2n=22 (PEREIRA & al. 2018).

Acromyrmex striatus is restricted to grassland habitats in subtropical and temperate zones southern South America, occurring on the southern Brazilian coast (known as Restinga ecosystem), on sandy soils across the Pampas and Chaco, and from the southernmost part of Paraguay to the temperate savannas of Low Monte in Argentina (KEMPF 1972; compiled in JANICKI & al. 2016). Recent paleodistribution analyzes have identified that the potential distribution of the species may have gone through oscillations arising from the last glacial periods, from which it would have expanded dramatically from the Pampas to the grasslands of Argentina (CRISTIANO & al. 2016). Thus, it is assumed that *A. striatus* populations from Chaco may be newer and slightly different from older Brazilian ecosystems.

The genus *Acromyrmex* itself is quite diverse comprising 34 valid species plus 29 subspecies (BOLTON 2020). This large number of subspecies is accentuated mainly due to the fact that it does not support the widely accepted biological meaning of "species" as well the failure of some molecular studies to identify traditional subspecies as phylogenetically distinct (MAYR 1982, PHILLIMORE & OWENS 2006). *Acromyrmex striatus* had a subspecies which was later raised to species, namely *A. silvestrii* Emery, 1905 which still includes a subspecies, *A. silvestrii bruchi* Forel, 1912 (BOLTON 2020). These lineages are found exclusively in subtropical and temperate grasslands, mainly in the Chaco biome in Argentina, and are even live sympatrically with *A. striatus* (CUEZZO 1998, JANICKI & al. 2016). Therefore, karyotype description of any of these lineages would shed light on the evolution of the group and may give valuable indications of kinship.

Karyotype changes, including chromosome number and structure, are speculated to promote species diversification events through chromosomal rearrangements that may later result in barriers to gene flow (RIESEBERG 2001, FARIA & NAVARRO 2010). In ants, such considerations can occur at various levels, including intraspecific dissimilarities involving the maintenance of different karyotypes in the same population, many of them between sister species or closely related species (e.g., IMAI & al. 1977, CROSLAND & al. 1988, HIRAI & al. 1996, CARDOSO & al. 2014, MICOLINO & al. 2019a). Populational intraspecific variation is increasingly being identified for a range of ants, associated with the identification of cryptic species by cytotaxonomy. For example, such findings were obtained by fluorescence in situ hybridization (FISH) based molecular cytogenetics using microsatellites and/or ribosomal DNA probes (MICOLINO & al. 2019a, 2019b), as well as standardized chromosome measurement analyzes (i.e., karyomorphometry) (CARDOSO & al. 2018b). Such comparative analyzes looking for karyotype variations among geographically isolated populations could help to understand the evolution of the distribution of a particular organism or population. Natural species obviously do not respect the boundaries established by mankind, but due to traditionally dubious taxonomy and slightly contrasting nesting sites, it is worth cytogenetically examining *A. striatus* populations from the Argentine Chaco, seeking a minor distinction from their putative older Brazilian relatives.

Here, we still consider that the specimens from Argentine belong to *A. striatus* taxa, but it is being revised by Cristiano & al. (manuscript in preparation). The aim of this study was to cytogenetically analyze *A. striatus* populations located in the Chaco biome in Argentina, as well to describe the hitherto unknown karyotype of *A. silvestrii*. To achieve this, we used a karyomorphometric approach and FISH chromosomal mapping with ribosomal, telomeric and microsatellite probes.

Material and methods

Colonies sampling

The colonies of *A. striatus* populations were collected along the Argentine Chaco at the following sampling points within the Córdoba Province: Córdoba (coordinates), Ischilín (coordinates) and Punilla (coordinates). Colonies of *A. silvestrii* were collected around the Chamical city belonging to La Rioja Province, Argentina (coordinates). All sampled

colonies are under the domain of the Dry Chaco ecoregion, set in areas of Tropical and Subtropical Grasslands, Savannas and Shrublands, according to DINERSTEIN & al. (2017).

Chromosome preparations

Metaphase chromosomes were obtained using brain ganglia of pre-pupae larvae dissected in colchicine hypotonic solution (0.005%) according to IMAI & al. (1988), with modifications described by CARDOSO & al. (2012). Then the metaphases were conventionally stained with 4% Giemsa® solution diluted in Sørensen's buffer (pH 6.8) to determine chromosome number and morphology. Karyotype structure was described by the chromosomal arm ratio proposed by LEVAN & al. (1964) and classified according to their centromeric position: metacentric (M), submetacentric (SM), subtelocentric (ST) and acrocentric (A). The ten best metaphases with chromosomal integrity and evident, non-overlapping centromeres were measured by the Image Pro Plus® software (Media Cybernetics, LP, USA). Chromosomes were evaluated according to the following characteristics: total length (TL), long arm length (L), short arm length (S), long arm to short arm ratio (r=L/S), and relative length of chromosomes (RL), according specified by CRISTIANO & al. (2017). Karyotypes were assembled by the Corel® PHOTO-PAINT software.

Fluorescence in situ hybridization (FISH)

FISH procedure for mapping repetitive DNA sequences through 18S rDNA, telomeric $TTAGG_{(6)}$ and microsatellite $GA_{(15)}$ probes was performed according to the partial description by KUBAT & al. (2008), with the appropriate modifications specified and detailed by MICOLINO & al. (2019b). The $TTAGG_{(6)}$ and $GA_{(15)}$ probes were a priori labeled with Cy3 at the 5' end during synthesis (Sigma, St. Louis, MO, USA), while the 18S rDNA probe was obtained by PCR amplification (see MICOLINO & al. 2019b). The summarized process involved several washes with saline solutions, followed by denaturation with formamide and dehydration by ethanol until hybridization to the probe in question. After overnight maintenance, the chromosome slides were again washed and dehydrated to remove excess probe, and then the final assembly process in DAPI Antifading solution (DAPI Fluoroshield, Sigma-Aldrich). The final step consisted of analyzing the chromosome slides under an Olympus BX53 epifluorescence microscope using the WU (330–385 nm) and WG (510–550 nm) filters for DAPI and

rhodamine, respectively. Chromosome images were obtained using a MX10 digital camera attached to the microscope and using CellSens software. Subsequently, the metaphase photos were edited by Adobe Photoshop CC® software.

Results

All *A. striatus* populations displayed metaphases with diploid chromosome number of 2n=22, consisting of ten metacentric chromosomes pairs and one submetacentric pair; their karyotype formula was 2K=20M+2SM and fundamental number of NF=44 (Figure 1). Likewise, the *A. silvestrii* karyotype displays exactly the same characteristics as *A. striatus* (Figure 1d). The karyomorphometric data of *A. striatus* chromosomes were presented in this work, as follows: the total chromosome length for population "A" ranged from 3.88±0.48 μm to 2.33±0.33 μm, while the total length of all chromosomes was 59.35 μm; population "B" presented total chromosome length ranging from 3.99±0.58 μm to 2.38±0.35 μm and total length of all chromosomes of 60.35 μm; and in population "C", the total length of chromosomes ranged from 4.05±0.63 μm to 2.34±0.42 μm, while the total length of all chromosomes was 59.35 μm (Table 1). In the species *A. silvestrii*, chromosome measurement ranged from 3.98±0.52 μm to 2.61±0.29 μm with total length of all chromosomes of 60.29 μm (Table 1).

FISH chromosomal mapping performed for the three Argentine populations of *A. striatus* and the *A. silvestrii* species displayed no significant difference in either number or brightness of the of 18S and TTAGG probes. The probe for the 18S rDNA cluster was labeled on only one chromosomal pair in all samples, which was located in the interstitial region of the long arms of the second pair of metacentric chromosomes (Figure 2a). The $TTAGG_{(6)}$ telomeric repeat distribution pattern was restricted to the ends in both arms on all chromosomes pairs. Also, no signal for interstitial telomeric sites (ITS) was observed (Figure 2b). The $GA_{(15)}$ microsatellite probe provided a rich band pattern in the subtelomeric regions of some specific chromosomes, extending more interstitially across both chromosomal arms, with some signals remarkably stronger than the others. Weaker markings can be observed on the smaller chromosomes, suggesting shortage of this repeat in these ones. In general, the $GA_{(15)}$ probe produced a scattered distribution along the chromosomes. This pattern was recurrent for all samples analyzed (Figure 2c).

Discussion

Our comparative cytogenetics showed that the diploid number of chromosomes in representatives of the three *A. striatus* populations as well as in the species *A. silvestrii* was invariably 2n=22. The karyomorphometric analysis revealed that the karyotype in all samples consists of ten metacentric pairs and one submetacentric pair. We describe for the first time the karyotype of *A. silvestrii*, a prospective sister lineage belonging to what we call here "Striatus group". Ongoing phylogenetic analyzes support this degree of closeness (CRISTIANO & al. in preparation). We also demonstrate that *A. striatus* and *A. silvestrii* are closely related from their well-conserved karyotypes, of which showed no differences in our cytogenetic surveys. We therefore suggest that both species have diverged relatively recently, due to many shared and conserved characteristics, including their karyotypes.

The results of karyotype length in *A. striatus* corroborate the previous findings of PEREIRA & al. (2018) and differ a little from those of CRISTIANO & al. (2013), which ranged from 5.78±0.15 μm to 1.77±0.05 μm and a total length of 78.67 μm. A possible explanation for this incongruence is that the karyomorphometry protocol in our group has undergone successive improvements and standardizations, making it increasingly close to the appropriate one and further corroborates genome size estimation by flow cytometry (see CARDOSO & al. 2018b). The karyotype of *A. silvestrii* showed a pronounced structural and numerical similarity with *A. striatus*, including in the length of the chromosomes. Population-based chromosomal studies may reveal often unreached intraspecific diversity either by morphological (i.e., cryptic species) or molecular (i.e., weak phylogenetic signal) methods (e.g., TALAVERA & al. 2013, LUKHTANOV & SHAPOVAL, 2017). For instance, a karyomorphometric analysis on *Mycetomoellerius holmgreni* fungus-farming ant populations revealed significant differences in karyotype and centromere length, suggesting that this ant species might be undergoing centromere drive (CARDOSO & al. 2018b). In fungi, chromosome length polymorphism (CLP) has been identified for a range of strains and it has also been shown that variable tandem repeats, such as rDNA genes, could be a source of CLPs (ZOLAN 1995). Such repetitive sequences are considered to be involved in translocation events through ectopic recombination, which could result in mutated chromosomes that benefit the organism, ultimately leading to speciation (BUSH & al. 1977). In addition, subtelomeric sequences are generally composed of a

mosaic of repeat units, so that recombination among subtelomeric repeats can lead to chromosomal extremity variability and result in CLPs (CORCORAN & al. 1988, ZOLAN 1995). Another CLP-related condition would be that a cross between any lineages with different length chromosomes could generate offspring with new chromosome sizes, provided that the new karyotypes were composed of viable gene combinations (ZOLAN & al. 1994). Given the possibilities, we include molecular cytogenetic analyzes corresponding to the ribosomal and telomeric sequences to attempt to correlate with the assumptions raised above.

Our results on the physical mapping of the 18S rDNA cluster and TTAGG telomeric motif were consistent with the previously described data for *A. striatus* in the Brazilian ecosystems of Restinga and Pampas (TEIXEIRA & al. 2017, PEREIRA & al. 2018). An interesting cytogenetic feature of *A. striatus* is the location of the 18S rDNA cluster, identified in the interstitial region of a pair of metacentric chromosomes, similar to those found for *Atta* species (TEIXEIRA & al. 2017). Likewise, the location of the 18S rDNA cluster in *A. silvestrii* was indistinguishable from *A. striatus*. The rDNA is one of the most conservative fractions of the eukaryotic genome and ribosomal RNA genes have changed minimally throughout evolutionary history (RASKINA & al. 2008). While there is this conservatism, rDNA is a strong source of genome instability, which their dynamics is an indicator of significant intra-genomic processes (RASKINA & al. 2004). It is therefore somewhat apparent that *A. striatus* would have the ancestral karyotype structure of the leafcutter ants' clade, in terms of the number and the location of the 18S rDNA on the chromosomes. Clearly this assumption has greater support from molecular data (e.g., CRISTIANO & al. 2013, PEREIRA & al. 2018), although it is noteworthy that an integrative approach (cytogenetic and molecular data) involving extensive sampling would remarkably support such a point.

The TTAGG telomeric repeat is considered to be the ancestral motif of insects (FRYDRYCHOVÁ & al. 2004). In general, the order Hymenoptera has the TTAGG repeat retained in its representatives, although it has been suggested that it was putatively lost in the ancestor of Apocrita with at least two subsequent independent recovers (MENEZES & al. 2017, GOKHMAN & KUZNETSOVA 2018). One of them has occurred in Formicidae and has been increasingly identified in ant telomeres (e.g., LORITE & al. 2002, PEREIRA & al. 2018, MICOLINO & al. 2019a, 2019b). One of the applications of telomeric probes has been in the recognition of fusion rearrangements, which can also identify population polymorphisms (RUIZ-HERRERA

& al. 2008). Preliminary analyzes with the $TTAGG_{(6)}$ probe in *A. striatus* found no interstitial telomeric signals, denoting that 2n=22 is the most likely ancestral karyotype of the leafcutter ants and is a plesiomorphic feature shared between *A. striatus* and *Atta* species (PEREIRA & al. 2018). We observed markings for the TTAGG motif exclusively at the ends of chromosomes in all samples of the "Striatus group" analyzed here, suggesting their putative conservation within these ant lineages.

FISH mapping results regarding the microsatellite $GA_{(15)}$ suggest that there is no distinguishable variation among samples, despite subtly stronger markings on some chromosome pairs. Such accumulation of these repeated sequences was observed in the subtelomeric region in both chromosomal arms. In fact, microsatellites repeats can be arranged in well-defined clusters on chromosomes. For example, 15 species of stingless bees of the genus *Melipona* displayed $GA_{(15)}$ repeats in predominantly telomeric blocks (TRAVENZOLI & al. 2019). Intraspecific cytogenetic polymorphisms have recently been found in the ant *Mycetomoellerius holmgreni*, showing distinct well-defined blocks of the microsatellite $GA_{(15)}$ in populations situated to the north and south of their occurrence area, denoting a potential geographic and/or reproductive isolation between them (MICOLINO & al. 2019b). Also, birds generally have preferential accumulation of microsatellite repeats restricted to the centromeric and telomeric regions. In particular, woodpeckers showed different distribution patterns of microsatellite sequences on the Z sex chromosome, including the dinucleotide $GA_{(15)}$ (DE OLIVEIRA & al. 2017). On the other hand, there are many findings showing such entirely scattered repeats in both heterochromatic and euchromatic regions on chromosomes (e.g., CIOFFI & al. 2011, CUNHA & al. 2015, PALACIOS-GIMENEZ & al. 2015). In sum, all these findings suggest the particularly dynamic nature of microsatellite sequences making them good cytogenetic markers mainly when they occur non-dispersively across chromosomes.

As each organism presents its own karyotype, the chromosome number and structure are interesting for taxonomic studies, so that closely related species tend to have karyotypes more similar than phylogenetically distant ones (SUMNER 2003). This was seen in *A. silvestrii*, whose karyotype number and structure is identical to that of *A. striatus*. Besides, such species resemble each other strongly morphologically, making it difficult to identify and differentiate between them. The very similar karyotypes of the two species confer an additional characteristic, in which closely related ant species can diverge without deep chromosomal changes during the process. As no structural

changes at the chromosomal level were identified, we suggest that perhaps changes related to genome function or regulation are more linked to divergence, but this would be considered for further research.

The recognized chromosomal properties of the "Striatus group" resemble karyotypic data in *Trachymyrmex* sensu stricto (all karyotyped species have 2n=20), mainly by the predominance of metacentric chromosomes and the absence of acrocentric ones (reviewed in CARDOSO & al. 2018a). Considering the phylogenetic position of these two clades, we can suggest a likely evolutionary trajectory focusing on chromosome changes. The karyotype differentiation of the "Striatus group" towards *Trachymyrmex* species is found by the difference of one extra chromosomal pair and the presence of a pair of submetacentric chromosomes. In this sense, there could have been a chromosomal fission in the ancestral karyotype followed by a pericentric inversion, changing the karyotype to its current state of 2n=22 comprising ten pairs of metacentric chromosomes and one submetacentric pair. A similar scenario about the role of chromosomal inversions in the lineages diversification process was also assumed for closely related species of the genus *Mycetomoellerius* (MICOLINO & al. in revision). Overall, we provide new karyotype data on *A. striatus* populations and on the species *A. silvestrii* – the two species known to belong to the "Striatus group". Such data may be useful in any future comparative cytogenetic analyzes. Our results also provide insights into their possible phylogenetic relationships hitherto unavailable.

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Table 1: Karyomorphometric analyzes of the chromosomes from three different populations of *A. striatus* (Pop. A – Córdoba, Pop. B – Ischilín and Pop. C – Punilla); and the *A. silvestrii* species. Chromosomes total length (TL) and karyotype length (KL) for each sample studied here.

Chromosome	**Pop. A TL(µM)**	**Pop. B TL(µM)**	**Pop. C TL(µM)**	***A. silvestrii* TL(µM)**	**Classification**
1	3.88±0.48	3.99±0.58	4.05±0.63	3.98±0.52	Metacentric
2	3.76±0.51	3.9±0.59	3.85±0.61	3.89±0.51	Metacentric
3	3.43±0.47	3.57±0.44	3.44±0.51	3.56±0.47	Metacentric
4	3.3±0.43	3.43±0.38	3.31±0.5	3.37±0.51	Metacentric
5	3.12±0.39	3.17±0.44	3.12±0.42	2.96±0.42	Metacentric
6	3.02±0.36	3.04±0.41	3.01±0.42	2.86±0.44	Metacentric
7	2.97±0.38	2.94±0.38	2.97±0.4	2.77±0.39	Metacentric
8	2.9±0.4	2.9±0.38	2.89±0.41	2.7±0.37	Metacentric
9	2.86±0.4	2.87±0.36	2.81±0.41	2.66±0.37	Metacentric
10	2.79±0.4	2.82±0.35	2.76±0.36	2.61±0.38	Metacentric
11	2.74±0.38	2.76±0.33	2.7±0.34	2.58±0.37	Metacentric
12	2.72±0.38	2.72±0.35	2.65±0.35	2.53±0.35	Metacentric
13	2.67±0.39	2.65±0.36	2.59±0.35	2.49±0.34	Metacentric
14	2.61±0.38	2.6±0.38	2.55±0.36	2.44±0.34	Metacentric
15	2.48±0.33	2.47±0.29	2.46±0.34	2.4±0.32	Metacentric
16	2.37±0.26	2.32±0.26	2.31±0.37	2.36±0.31	Metacentric
17	1.94±0.24	2.01±0.32	2.01±0.25	2.31±0.33	Metacentric
18	1.82±0.19	1.83±0.34	1.86±0.26	2.27±0.33	Metacentric
19	1.63±0.22	1.64±0.24	1.69±0.26	2.2±0.33	Metacentric
20	1.57±0.21	1.54±0.25	1.52±0.2	1.96±0.34	Metacentric
21	2.44±0.33	2.53±0.38	2.46±0.37	2.78±0.3	Submetacentric
22	2.33±0.33	2.38±0.35	2.34±0.42	2.61±0.29	Submetacentric
∑(KL)	**59.35**	**60.08**	**59.35**	**60.29**	

Figure 1: Conventional staining of mitotic metaphases and their respective karyotypes assembled by morphological similarity of three populations of *A. striatus* from the Argentine Chaco (a – Córdoba, b – Ischilín and c – Punilla); and *A. silvestrii* species. M: metacentric chromosomes. SM: submetacentric chromosomes. Scale bar: 5 μm.

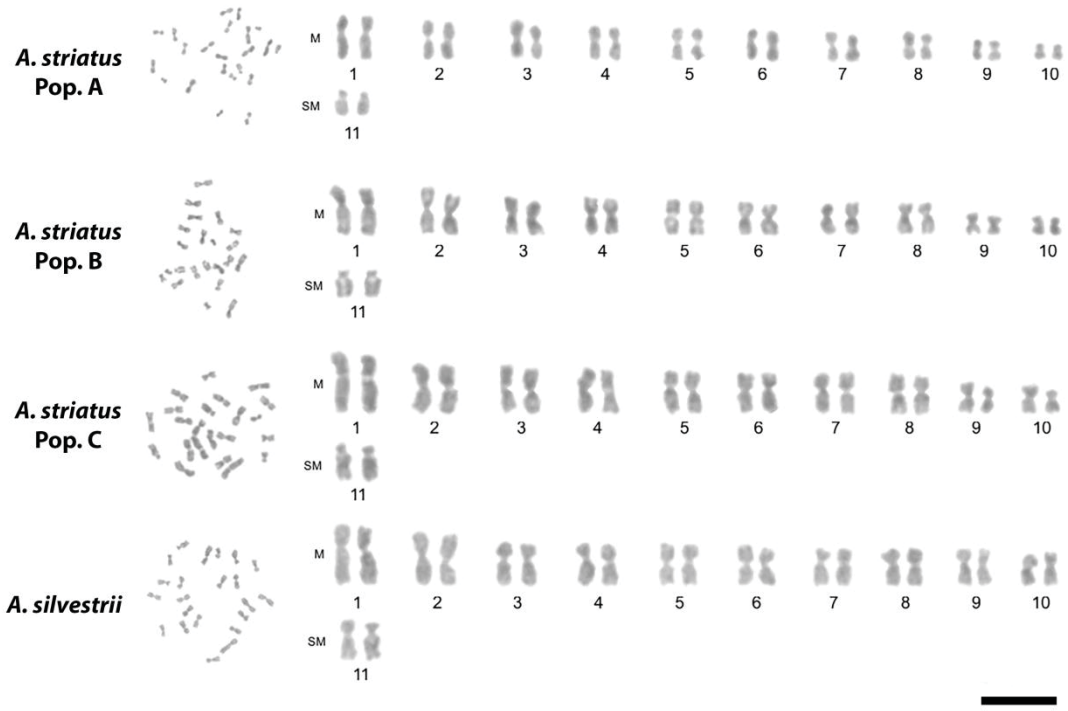
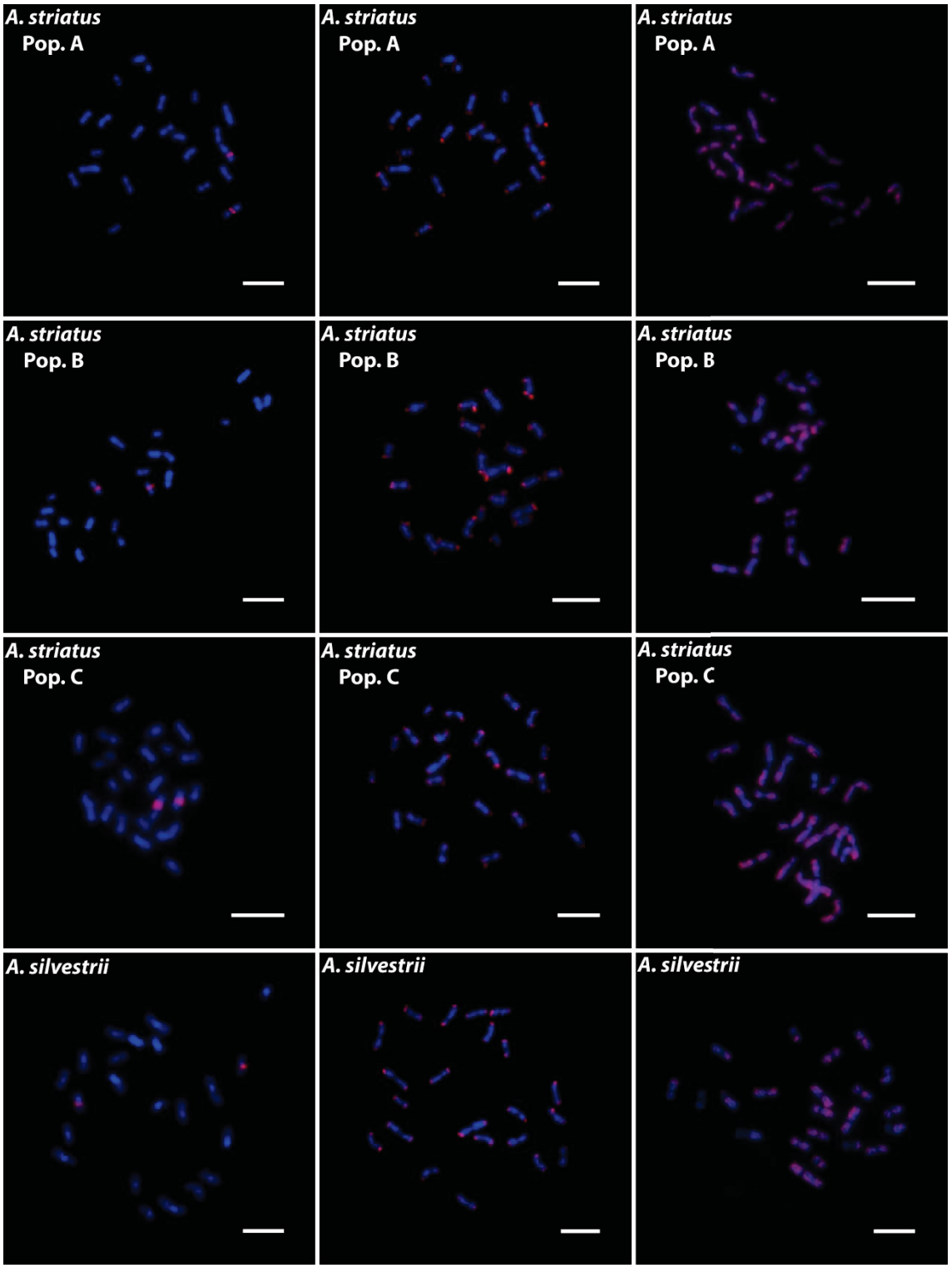


Figure 2: DAPI-stained chromosomal metaphases from three populations of A. striatus and species A. silvestrii. Left column: FISH mapping of the rDNA 18S cluster (in red). Center column: FISH mapping of the TTAGG(6) telomeric motif (in red). Right column: FISH mapping of microsatellite GA(15) (in red). Scale bar: 5 μm.



CAPÍTULO 5:

EVOLUTIONARY AND BIOGEOGRAPHIC INSIGHTS INTO "*Trachymyrmex*" FUNGUS-FARMING ANTS

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Evolutionary and biogeographic insights into "*Trachymyrmex*" fungus-farming ants

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Abstract

Recent multiloci phylogenetic analyses have resolved the formerly paraphyletic status of *Trachymyrmex* s.l., indicating two new genera: *Mycetomoellerius* and *Paratrachymyrmex*. However, many of their lineages and evolutionary history are unknown. Here, we used available morphological and molecular data to uncover their evolutionary relationships and possible biogeographical routes that reflect current geographic distributions. We estimated that *Mycetomoellerius* appeared in the Middle Miocene and that the Northern Grasslands (mostly the Cerrado biome) were its ancestral range. We then traced how these lineages reached their current geographical distributions, including those in the southern temperate grasslands and the Caribbean islands. We also speculate that a large transition range between the Cerrado and Amazon biomes could have been a center of diversification especially for the *Mycetomoellerius* lineages. *Paratrachymyrmex* would have appeared in the Amazonian Rainforest in the Early Miocene and its dispersion to Middle America could be linked to intercontinental land connections. *Trachymyrmex* s.s. would have arisen more recently in the Late Miocene as a result of an earlier dispersion to Middle America and spread through the southernmost Neartic region. We emphasized the resolution by using combined morphological and molecular data in phylogenetic analyses and using published data to connect their evolutionary relationships with the natural history of fungus-farming ants.

Introduction

Fungus-farming ants (Myrmicinae: Attini) established a symbiotic relationship with the Basiodiomycete fungi about 60 million years ago (Myr) (Schultz and Brady, 2008; Branstetter et al., 2017). Currently, about 260 extant species of fungus-farming ants are recognized across 19 genera (Bolton, 2020). They play a fundamental role in their respective environments, in addition to their unique role in fungiculture (Mueller et al., 2001). Fungus-farming ants are responsible for much of the nutrient cycling, as many species use the decomposing material to serve as food for the symbiotic fungi (Hölldobler and Wilson, 1990). The “higher” fungus-farming ants grow their fungus with the type of substrate (including plant debris, parts of flowers, seeds, and arthropod

feces) that is specialized for free-living leucocoprineous fungi. In contrast, leafcutter ants (genera *Atta* and *Acromyrmex*) culture their gardens with freshly cut leaf material (Hölldobler and Wilson, 1990).

The former genus *Trachymyrmex* s.l. was one of the most diverse groups within the fungus-farming ants and comprised species with a variety of unique morphological variations. The taxonomy of this group is challenging, although some researchers have performed a series of taxonomic reviews (Mayhé-Nunes and Brandão, 2002; 2005; 2007). To elucidate their phylogenetic relationships, Brandão and Mayhé-Nunes (2007) reconstructed a phylogeny using maximum-parsimony analyses with 50 characters from external worker morphology and 32 taxa. They suggested that *Trachymyrmex* s.l. was a monophyletic group, including species that shared unique microtuberculate mesosomal projections. However, several phylogenies based on molecular data including genomic scale have reconstructed *Trachymyrmex* s.l. as a paraphyletic grade, which gives rise to the ancestor of the sister genera *Xerolitor* and *Sericomyrmex*, as well as the ancestor of the leafcutter ants (e.g., Schultz and Brady, 2008; Branstetter et al., 2017; Sosa-Calvo et al., 2018).

To solve the taxonomic issue in *Trachymyrmex* s.l., multiloci phylogenetic analyses were recently performed with an extensive number of species and a new systematic arrangement, embracing two new genera: *Mycetomoellerius* and *Paratrachymyrmex,* was proposed (Solomon et al., 2019). *Trachymyrmex* s.s. was restricted to the sister clade of leafcutter ants and largely comprised of North American species (Solomon et al., 2019). The genus *Mycetomoellerius* is highly diverse, currently comprising 29 valid species, besides almost eight new species that have been suggested (Solomon et al., 2019). *Mycetomoellerius* can be divided into three major clades, which can be placed into the previously reported groups, namely the “Iheringi group” (six valid species), the “Jamaicensis group” (three valid species), and the “Opulentus group” (five valid species) (Solomon et al., 2019; see also Brandão and Mayhé-Nunes, 2007). The sampling of *Mycetomoellerius* in previous phylogenetic analyses was low, so the relationship with other species of the genus remains to be resolved.

The contribution of phylogenetic analyses using molecular and morphological data has been demonstrated in a range of species (see Wortley and Scotland, 2006). Such analyses have also supported the studies on exclusive morphological synapomorphies (e.g., Bertolani et al., 2014; Rocha et al., 2019). This integrative approach is useful in divergence dating, which uses morphological data to infer the

placement of fossils alongside the extant taxa in a "total-evidence" analysis (Ronquist et al., 2012a). Also, the existing biogeographic information can provide indications to possible adaptive characteristics that appeared in certain lineages over time (e.g., Clouse et al., 2015; Economo et al., 2015).

In this study, we inferred the phylogenetic and evolutionary relationships of three major clades of fungus-farming ants, namely *Mycetomoellerius*, *Paratrachymyrmex*, and *Trachymyrmex* s.s. For this, we took advantage of the previously published data based on both DNA sequences and morphological characters for a range of these species. We also reconstructed the divergence times for the major clades identified here and estimated the ancestral range of their lineages.

Materials and methods

Data acquisition

We downloaded the DNA sequences commonly used for ants (Schultz and Brady, 2008; Solomon et al., 2019; Micolino et al., 2020) from the GenBank: (i) four nuclear genes, *elongation factor 1-alpha-F1* (EF1α-F1), *elongation factor 1-alpha-F2* (EF1α-F2), *wingless* (Wg) and *long-wavelength rhodopsin* (LWRh); (ii) one mitochondrial gene, *cytochrome c oxidase I* (COI), and (iii) introns of EF1α-F1, Wg, and LWRh from 38 species of "higher" fungus-farming ants, including the genera *Mycetagroicus* (as outgroup), *Xerolitor*, *Sericomyrmex*, *Mycetomoellerius*, *Paratrachymyrmex*, and *Trachymyrmex* s.s. (Table S1). For morphological data, we imported the character matrix, from Brandão and Mayhé-Nunes (2007) for 50 external morphological worker characters compiled from 32 ant species belonging to the genera *Mycetomoellerius*, *Paratrachymyrmex*, and *Trachymyrmex* s.s. (Tables S2 and S3).

Phylogenetic signal test

To test the phylogenetic signal of the morphological data and thus be able to use them alongside with the molecular data in an integrative phylogenetic framework, we carry out two independent approaches commonly used in evolutionary ecology studies. For this, we generated a preliminary phylogenetic tree based only on morphological data using Bayesian inference by using the MrBayes 3.2.5 (Ronquist et al., 2012b). All

subsequent calculations were performed with R 3.6.3 (R Development Core Team, 2020). First, we estimated Abouheif's C_{mean} with the function abouheif.moran and the method oriAbouheif for the proximity matrix (package *adephylo*) according to Münkemüller et al. (2012). Abouheif's C_{mean} test (Abouheif, 1999) is an autocorrelation index and uses Moran's *I* statistic with a new matrix of phylogenetic proximities, which does not relate to branch length but focuses on topology and has a non-zero diagonal (Pavoine et al., 2008; Münkemüller et al., 2012). Second, we estimated Pagel's λ using the 'fitDiscrete' function (Yang, 2006) in the package *geiger* according to Münkemüller et al. (2012). Pagel's λ test (Pagel, 1999) assumes a Brownian motion model of trait evolution, which is based on likelihood optimization and is the transformation of the phylogeny that ensures the best fit of trait data to a Brownian motion model (Münkemüller et al., 2012). Based on these results we coupled morphological and molecular data matrixes to estimate our phylogenetic hypotheses.

Phylogenetic analyses

The downloaded DNA sequences were aligned and concatenated manually by using the MEGA7 (Kumar et al., 2016). Data were partitioned and modeled using the PartitionFinder 2.1.1 (Lanfear et al., 2016) under the Bayesian Information Criterion (BIC) with 18 input data blocks consisting of the first, second, and third codon positions of the coding regions of each of the five gene fragments plus the introns from EF1α-F1, LWRh, and WG, and with a user tree resulting from an unpartitioned maximum likelihood best-tree analysis conducted in RAxML 8.0.14 (Stamatakis, 2014). The data were partitioned into eight parts, as shown in Table 1. A partition was added to the gene matrix for morphological data. Before the analysis, we applied a topology constraint by which each species containing the morphological data was restricted to the clades of their respective genera. Nine partitions, each with its evolutionary model, were used in the Bayesian analysis by using the MrBayes 3.2.5 (Ronquist et al., 2012b). Two simultaneous independent Monte Carlo Markov chain (MCMC) runs were performed with nchains = 8, temp = 0.05 and at least 20 million generations were performed, with sampling every 1000 generations. We set a critical value for the topological convergence diagnostic (stopval = 0.005) in order to streamline the analysis. We also set a fraction of 20% of the initial samples to be discarded when the convergence

diagnostics reached the determined value (relburnin = yes, burninfrac = 0.20). The tree-generated and posterior probabilities were visualized in FigTree 1.4.3 (Rambaut, 2009).

Divergence dating

To estimate the divergence time of the major clades of *Trachymyrmex* s.l., we used only our molecular data matrix, which was incorporated into the matrix from Micolino et al. (2019). We used BEAST 2.5.2 (Bouckaert et al., 2018) under the Fossilized Birth-Death (FBD) process (Heath et al., 2014) using an uncorrelated, log normal relaxed clock model (Drummond et al., 2006). Fossil calibration and root age in the tree prior were set as specified by Micolino et al. (2019). We set the nucleotide substitution model as GTR + I + G for all genes. The MCMC chains were run in two independent analyses for 100 million generations each, with sampling every 1000 generations. Convergence, mixing and effective sample sizes (ESS > 200) were checked using the Tracer 1.7.1 (Rambaut et al., 2018). Sampled trees in each run were combined into a single file using LogCombiner 2.5.2, removing the first 10% of the trees in each run as burn-in. A maximum clade credibility tree was generated in the TreeAnnotator 2.5.2. Next, all fossils were removed from the tree using the FullToExtantTreeConverter tool (implemented in BEAUti 2.5.2). The generated tree and credible intervals were visualized in FigTree 1.4.3 (Rambaut, 2009).

Historical biogeography

We reconstructed ancestral geographic ranges using a Bayesian method with BBM (Bayesian Binary MCMC) analyses implemented in RASP 4.1 (Yu et al., 2015). These methods accommodate phylogenetic uncertainty by averaging the ancestral reconstructions over a sample of user-supplied trees (Ronquist and Huelsenbeck, 2003; Yu et al., 2015). We coded each of the fungus-farming ant species in the dataset as occurring in: the Amazon Rainforest (A); Middle America (B); Northern America, including mainly the southern USA (C); the Brazilian Atlantic Forest (D); Northern Grasslands, including the Caatinga and the Cerrado biomes (E); and Southern Grasslands, including the Chaco and the Pampas biomes (F). The species distributions were taken from www.antmaps.org (Janicki et al., 2016). We used the BBM approach because it allows the estimation of only one ancestral range for the nodes. We therefore

constrained the maximum number of estimated areas in the ancestral ranges to one. BBM analysis was performed using an estimated model, F81 + G, with a null root distribution, and the MCMC were performed for 10 million generations, with sampling every 1000 generations.

Results

Phylogenetic analyses

We found statistically significant levels of phylogenetic signal ($p < 0.05$) in 40 characters according to Abouheif's C_{mean} and 41 characters according to Pagel's λ (Table S2). Five characters did not exhibit a significant amount of phylogenetic signal as measured by both Abouheif's C_{mean} and Pagel's λ. Thus, these five characters were removed from subsequent analyses.

Our Bayesian analysis of the combined morphological and molecular data showed varying statistical support for the nodes along the branches of the tree. The generated phylogenetic tree did not entirely represent the three major clades previously estimated for *Mycetomoellerius* (i.e., "Iheringi", "Jamaicensis", and "Opulentus" groups), but *M. ruthae* shown as the most basal lineage of the last two was statistically well-supported (posterior probability, *pp* = 1.0) (Fig. 1). We identified *M. pruinosus* and *M. tucumanus* as part of the "Iheringi group" and the "Urichii group" as their sister group, which comprises *M. agudensis* and *M. isthmicus*. *Mycetomoellerius oetkeri* was included in the "Jamaicensis group", while *M. compactus* and *M. farinosus* were estimated to be in the "Opulentus group". Within the genus *Paratrachymyrmex*, *P. intermedius* and *P. mandibularis* were estimated to be sister lineages, while *P. levis* was estimated to be more closely related to *P.* cf *carib* and *P.* cf *irmgardae* lineages (Fig. 1). All genera represented in our dataset were recovered as monophyletic.

Divergence dating

The FBD-based divergence dating analysis recovered the origin of "higher" fungus-farming ants as 27.7 Myr (95% highest posterior density interval, HPD = 36.9–21.4 Myr) (Table 2; Fig. S1). The genus *Mycetomoellerius* was estimated to have emerged about 20.6 Myr (HPD = 27.1–15.8 Myr), giving rise to two major clades. The

major clade I, comprised two minor clades defined here as “Iheringi group” and “Urichii group” that would have split around 19 Myr (HPD = 25.2–14.4 Myr), while their likely origins were estimated as 15.6 Myr (HPD = 21.2–11.8 Myr) and 10.3 Myr (HPD = 15.8–5.3 Myr), respectively. The major clade II would have emerged as 19.6 Myr (HPD = 25.6–15 Myr). The genus *Paratrachymyrmex* was reconstructed as 20.5 Myr (HPD = 27.6–15.2 Myr), while the genus *Trachymyrmex* s.s. as 11.4 Myr (HPD = 15.8–8 Myr). The divergence event between these two clades was recovered as 22.5 Myr (HPD = 30.2–17.1 Myr) (Table 2; Fig. S1).

Historical biogeography

In our Bayesian biogeographic reconstruction, we considered ten major clades and treated them as the probability of the ancestral range per node. BBM analyses estimated that the ancestral range of the most recent common ancestor of “higher” fungus-farming ants would have originated in an area including the Northern Grasslands and the Amazon Rainforest (*pp* = 0.49 and *pp* = 0.34, respectively) (Fig. 1). The ancestor of *Mycetomoellerius* would have appeared in the Northern Grasslands (*pp* = 0.78) and required two dispersion and one vicariance events to support the current geographic distributions. The major clade I as well as both “Urichii group” and “Iheringi group” would have had the Northern Grasslands as ancestral range (*pp* = 0.78, *pp* = 0.42, and *pp* = 0.87, respectively) (Fig. 1). Five dispersion and two vicariance events were estimated during the diversification of clade I, but the first divergence event would not have involved dispersion, indicating diversification within the area or sympatric range inheritance. The ancestral range of the major clade II of *Mycetomoellerius* was estimated to be ambiguous (Amazon Rainforest: *pp* = 0.55; Northern Grasslands: *pp* = 0.42) (Fig. 1) and would have involved dispersion events in all lineages. Our biogeographic reconstruction inferred that the ancestor of *Paratrachymyrmex* had its origin in the Amazon Rainforest (*pp* = 0.99), without associated dispersion events. The ancestor of *Trachymyrmex* s.s. was estimated to be from Northern America with intermediate support (*pp* = 0.56) (Fig. 1), again with no dispersion events at least in the early diversification.

Furthermore, “speciation within area” was estimated to have occurred more often in the Amazon Rainforest and in the Northern Grasslands, with 13 and 11 events, respectively. The largest dispersion events between areas were estimated to have

occurred from: the Amazon Rainforest to Middle America (7 times), the Amazon Rainforest to Northern Grasslands (7 times), Northern Grasslands to the Amazon Rainforest (7 times), and Northern Grasslands to the Brazilian Atlantic Forest (8 times). In contrast, the areas that received the most migrants were the Brazilian Atlantic Forest (14 times), Middle America (13 times), Northern Grasslands (11 times) and the Amazon Rainforest (8 times) (Fig 2).

Discussion

Phylogeny from combined morphological and molecular data

Our Bayesian analysis from combined morphological and molecular data yielded phylogenetic estimates consistent with previous multiloci reconstructions (see Solomon et al., 2019). Parsimony analysis using exclusively morphological data (Brandão and Mayhé-Nunes, 2007) was slightly inconsistent with the findings of this study. For example, some synapomorphies, such as the presence of a large welt on each side of lateral posterior region of the first gastric tergite in *M. ruthae* and *M. farinosus* have not been recovered. In fact, the phylogenetic position of *M. ruthae* is difficult to trace, as Solomon et al. (2019) estimated it to be part of a clade having *M. jamaicensis*, but with a very weak phylogenetic signal. However, we have reconstructed a more basal position with high support in the "*M. ruthae* clade", indicating a greater confidence. Noteworthy, *M. ruthae* has an exclusive *Escovopsis* species, one of the potentially devastating fungal parasites for fungus-farming ants (Seifert et al., 1995). We therefore recommend that *M. ruthae* deserves extra attention as a likely key species within this major *Mycetomoellerius* clade.

The combination of morphological and molecular data matrices bears the complication that the latter may override the phylogenetic signal of morphology because DNA sequences usually have more informative sites (Wortley and Scotland, 2006). This contradicts the previous phylogenies obtained from morphological and molecular data separately (Brandão and Mayhé-Nunes, 2007; Solomon et al., 2019). However, comparisons of empirical phylogenetic studies suggest that combined morphological and molecular sequence datasets increase the resolution levels of the tree structure (i.e., phylogenies with and without polytomies) when compared to the analyses of the molecular data alone (Wortley and Scotland, 2006). For instance, well-

supported genetic clusters with shared morphological characteristics revealed reticulated evolution in the plant genus *Ranunculus* that could be identified only with the combination of morphological and molecular data (Hörandl and Emadzade, 2012). Our results contradict the previous findings on the "*M. ruthae* clade" (Solomon et al., 2019), but offer support in most nodes. In this case, there are enough indications to suggest that such a combined analysis may increase the resolution and the support of the branches in the tree, when most lineages have both morphological and molecular data available. This assumption has been verified in resolving the phylogeny of butterflies and skippers (Wahlberg et al., 2005).

Mycetomoellerius clade II (see Fig. 1) seemed to be more obscure, as we have highlighted with the example of *M. ruthae*, but the phylogenetic relationships of the other lineages were quite congruent with Solomon et al. (2019). We managed to recover the sister lineages *M. iheringi* and *M. tucumanus* with strong support in the "Iheringi group", with *M. pruinosus* showing a closer relation with this clade than with *M. holmgreni*, as previously suggested (Brandão and Mayhé-Nunes, 2007). In the "Urichii group", an unusual finding refers to the close relationship between *M. agudensis* and *M. isthmicus*, since these species that are hardly found have very contrasting geographic distributions. The *Paratrachymyrmex* clade showed a relatively weak phylogenetic signal for all nodes. This was probably due to the placement of *P. phaleratus* within this genus, which was previously estimated (based on morphological characters) to belong to the genus *Mycetomoellerius* within the "Urichii group" (see Brandão and Mayhé-Nunes, 2007). We found that *P. cornetzi* and *P. bugnioni* were the underlying lineages, unlike the estimates by Solomon et al. (2019) that reconstructed them as sister lineages; although the basal split that gave rise to this sister group was strongly supported, the posterior probabilities (Bayesian analysis) and the bootstrap values (maximum likelihood) that uncovered this sister group were weak or null, respectively. The *Trachymyrmex* s.s. clade seemed to be well-defined, since the recently described *T. pakawa* species was identified as a sister lineage of *T. smithi* (Sánchez-Peña et al., 2017).

Evolutionary implications for "higher" fungus-farming ants

The evolutionary history of ants has been unfolded for a variety of species and genera, and has proved to be great prominence, frequently showing remarkable results.

For instance, dating analyses of the "higher" fungus-farming ants of the *Sericomyrmex* genus indicated that their lineages radiated rapidly in several morphologically distinguishable species that were widely distributed with only a small degree of associated genetic divergence (Ješovnik et al., 2016; 2017). Increased sampling of taxa improves the topology inferences and the accuracy of branch length estimates as well as using values inferred from the full dataset refines mean age estimates and decreases their variance (Soares and Schrago, 2012; Bromham et al., 2018). Therefore, we took advantage of the published data for many species of the former *Trachymyrmex* s.l. to trace the evolutionary trajectories of its major clades across the New World with fairly reliable expected accuracy.

The most recent common ancestor of all the "higher" fungus-farming ants was discovered to have originated about 27.7 Myr in the Oligocene in a region including the Northern Grasslands and the Amazon Rainforest (likely an ecological transition range between the Cerrado and the eastern Amazon; see discussion below). Importantly, the Oligocene is often considered to be a pivotal ecological transition period, and major changes during this time included a global expansion of grasslands, and a regression of tropical moist broadleaf forests (Graham, 2011). The genus *Mycetomoellerius* would have originated about 20.6 Myr in the Early Miocene, giving rise to two major clades and from there to all diversifications. The ancestral range was estimated to be the Northern Grasslands, most likely in the Cerrado biome due to an extensive sampling of species in this area (Vasconcelos et al., 2008), and would have involved both dispersion and vicariance events (Fig. 2). The ecological niche of the species of *Mycetomoellerius* ranges from the temperate grasslands of southern South America to the Caribbean islands (compiled in Janicki et al., 2016). It will be possible to accurately estimate the supposed routes taken by their lineages only when the sampling is close to the total number of species. Therefore, we focused on tracing the paths of specific clades.

In *Mycetomoellerius* clade I, the basal split between "Urichii group" and "Iheringi group" occurred in the Early Miocene (ca. 19 Myr) and much of the lineage diversification took place in the Middle Miocene, a turbulent period characterized by a long-term cooling of the global climate, punctuated by an extreme climatic optima (also known as the Mid–Miocene Climatic Optimum) (Frigola et al., 2018). We estimate that the *Mycetomoellerius* clade II also originated during the Early Miocene (ca. 19.6 Myr) as well as most of the diversification of their lineages would have arisen during the Middle Miocene. The ancestral ranges of these two major clades were estimated as the

Northern Grasslands (clade I) and the Amazon Rainforest (clade II) and may be associated with several successive Atlantic marine transgressions that were recorded in South America during the Middle Miocene (Hernández et al., 2005). Accordingly, during this time an open seaway separated terrestrial environments of southern South America from those farther north, spreading all over eastern Argentina, western Uruguay, southern Paraguay, and south-eastern Bolivia (Pascual et al., 1996; Ortiz-Jaureguizar and Cladera, 2006). Owing to this incident, the only emerged lands were those with high altitude, such as the mountains and plateaus in central, eastern, and southeastern Brazil, covering much of the Cerrado, the Atlantic Forest and the eastern Amazon Rainforest. We suggest that the *Mycetomoellerius* lineages would have expanded earlier and/or by areas still lifted towards north and south of their ancestral range. Thus, the ancestor of the "Iheringi group" would have colonized the Southern Grasslands likely coming from the area raised in southeastern South America during the Middle Miocene marine transgressions. In contrast, some lineages of clade II would have dispersed into the Amazon Rainforest till it reached both Middle America ("Opulentus group") and the Caribbean islands ("Jamaicensis group") (Fig. 2). During the Late Miocene–Early Pliocene (ca. 11–5 Myr), the widespread surface flooded by the sea was emerged succeeded by plains (Hoorn et al., 2010), which were so conspicuous that this period came to be known as "the Age of the Southern Plains" (see Ortiz-Jaureguizar and Cladera, 2006). The gradual clearance of the sea could have facilitated the lineages diversification, since such marine incursions are seen as major sources of vicariance processes in South America (de Queiroz, 2005). We conclusively demonstrate that both dispersion and vicariance events would be intrinsically involved in the diversification processes in this group of ants.

The basal split of the *Paratrachymyrmex* and *Trachymyrmex* s.s. (plus leafcutter ants) would have occurred at the Oligocene–Miocene boundary around 22.5 Myr somewhere in the Amazon Rainforest. This was a period of relative quiescence, apparently uneventful, but important because if the trend in extinctions and redistributions had continued unabated, we would have been living under a very different set of global ecosystems (Graham, 2011). *Paratrachymyrmex* was estimated to have arisen in the Early Miocene (ca. 20.5 Myr) without associated dispersion and vicariance events. Indeed, most of their lineages occur predominantly in the Amazon Rainforest; however, we can consider any kind of sympatric speciation due to the highly dynamic habitats found there. *Trachymyrmex* s.s. would have originated at the

beginning of the Late Miocene (ca. 11.4 Myr) in dry habitats of the Nearctic, either somewhere in the southern USA or in the northernmost region of Middle America, as suggested previously by Branstetter et al. (2017). Our results further indicate that some fungus-farming ants, such as *Mycetomoellerius* and *Paratrachymyrmex*, expanded their range into Middle America and consequently to the Nearctic multiple times (see also Branstetter et al., 2017) (Fig. 2). We estimate here that there could have been at least seven major dispersion events of some lineages to Middle America. Dispersion events from these lineages could be linked to geological evidence of an initial collision of the Panama Block and South America in the Early Miocene, resulting in extensive terrestrial landscapes, although not necessarily fully connected (Bacon et al., 2015). Indeed, one of the possibilities for the spreading of some lineages from South America to Middle America refers to early intermittent land bridges facilitating colonization and constituting a potential mechanism for speciation and colonization before the full closure of the Isthmus of Panama. Such connections played an important role in the dispersion of many taxa, including ants (Winston et al., 2016). However, it is worth to mention that the closure of the Isthmus of Panama is still a hotly debated issue (e.g., Jaramillo et al., 2017). Furthermore, our FBD-dating estimates, including leafcutter ants, are largely congruent with the robust phylogenomic study by Branstetter et al. (2017).

Our biogeographic findings regarding the likely dispersion and vicariance events of the lineages reflected some insights about diversification of these ants. The species dispersion events between areas were notable between the Amazon Rainforest and in the Northern Grasslands, particularly in *Mycetomoellerius* and *Paratrachymyrmex*. These biomes share borders in large areas of ecological transition, moving from an open dry habitat to a more humid forest and vice versa (Marimon et al., 2006). Such a transition range could have been crucial in the dispersion and colonization events, and may also promote the speciation processes. This leads to another interesting issue concerning to the estimated “speciation within area” events. Speciation within area is almost always inferred when adjacent species in a clade co-occur in at least one area, but are not restricted to sympatric speciation (Brooks, 2005). Since most of the sister species partially overlaps each other’s range, it is more parsimonious to assume that speciation within area would have occurred in a shared areas, followed by post-speciation dispersion (Halas et al., 2005). This “shared area” might well have been the Amazon/Cerrado transition range (Fig. 2). In fact, the Amazon Rainforest and the

Northern Grasslands have recovered major speciation within area events. The dynamics of these environments (e.g., landscape formation and volcanism) integrated with past climatic changes (e.g., temperature oscillations and sea level fluctuations), could have played a key role during the diversification of the *Mycetomoellerius* and *Paratrachymyrmex* lineages. In addition, it would also have striking influence on the dispersion routes to both north and south areas from their ancestral range. Under taxon pulse and taxon cycle models, species and their adaptations arise in "centers of diversification" and that distributional ranges of taxa periodically fluctuate around a more stable, continuously occupied center (Wilson, 1961; Erwin, 1981). Based in our findings, we hypothesize that this Amazon/Cerrado transition range could have been a center of diversification for these ant lineages. We also estimated that the areas that received the most migrants were the Atlantic Forest and Middle America, coming mostly from the Northern Grasslands and the Amazon Rainforest, respectively, which further supports our hypotheses raised above.

Conclusion

Overall, we identified additional phylogenetic relationships for the former *Trachymyrmex* s.l. (current genera *Mycetomoellerius*, *Pararachymyrmex* and *Trachymyrmex* s.s.) using previously available morphological and molecular data, thereby emphasizing the importance of advantaging existing data to uncover unknown facts. With the use of dating and biogeography methods to the tree of fungus-farming ants, we suggested likely routes traced by the major clades since their estimated origin in South America for dispersion to both ends of their geographic distributions into temperate regions of Southern Grasslands in Uruguay and Argentina to Middle America, including the Caribbean islands, and the Neartic region of Northern America. We further suggest that the ecotone between the dry Cerrado and the humid Amazon could have been a center of diversification for "higher" fungus-farming ants, mainly for the *Mycetomoellerius* lineages.

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List of tables:

Table 1. The 18 input data blocks, eight partitions and the respective models identified by PartitionFinder 2 and used in the Bayesian analyzes of the concatenated dataset.

Partition	**Data blocks**	**Best model**
p1	Wg pos2, EF1αF1, EF1αF2 pos1	TRN+I
p2	EF1αF1 pos2, EF1αF2 pos2	F81+I
p3	LwRh pos3, Wg pos3, EF1αF1 pos3, EF1αF2 pos3	TRNEF+G
p4	LwRh pos1, LwRh pos2, Wg pos1	K80+I
p5	COI pos3	HKY+I+G
p6	COI pos1	GTR+G
p7	COI pos3	GTR+I+G
p8	WG intron, LwRh intron, EF1αF1 intron	TVMEF

Table 2. Estimates of the divergence time of the most recent common ancestor of the major "higher" fungus-farming ants' clades. Crown group means the latest possible origin. HPD: highest posterior density interval. Divergence times in millions of years ago.

Major clades	**Crown group**	**HPD**	**Geological epoch**
"Higher" fungus-farming ants	27.75	36.9–21.4	Late Oligocene
Mycetomoellerius + (*Sericomyrmex* + *Xerolitor*)	22.93	30.4–17.6	Early Miocene
Sericomyrmex + *Xerolitor*	19.04	26.4–12.3	Early Miocene
Mycetomoellerius	20.66	27.1–15.8	Early Miocene
Clade I	19.00	25.2–14.4	Early Miocene
"Iheringi group"	15.66	21.2–11.8	Middle Miocene
"Urichii group"	10.34	15.8–5.3	Late Miocene
Clade II	19.64	25.6–15.1	Early Miocene
Paratrachymyrmex + (*Trachymyrmex* + leafcutter ants)	22.55	30.2–17.1	Early Miocene
Paratrachymyrmex	20.53	27.6–15.2	Early Miocene
Trachymyrmex	11.39	15.8–8.0	Late Miocene
Trachymyrmex + leafcutter ants	18.50	25.3–13.9	Early Miocene
Leafcutter ants (*Acromyrmex striatus* group)	17.93	-	Early Miocene
Leafcutter ants (*Atta* + *Acromyrmex*)	15.26	21.3–10.9	Middle Miocene

List of figures:

Figure 1. Phylogeny reconstructed from Bayesian analyzes of combined morphological and molecular data with cladogram depicted on the left and phylogram depicted on the right, and ancestral range estimation based on the Bayesian Binary MCMC method. The squares at the nodes indicate the estimated ancestral range as shown on the map of the American continent (on the left). The colors on the branches of the tree and in the species names highlight the major clades identified here. The numbers below the nodes indicate the posterior probabilities; values < 0.5 are not shown.

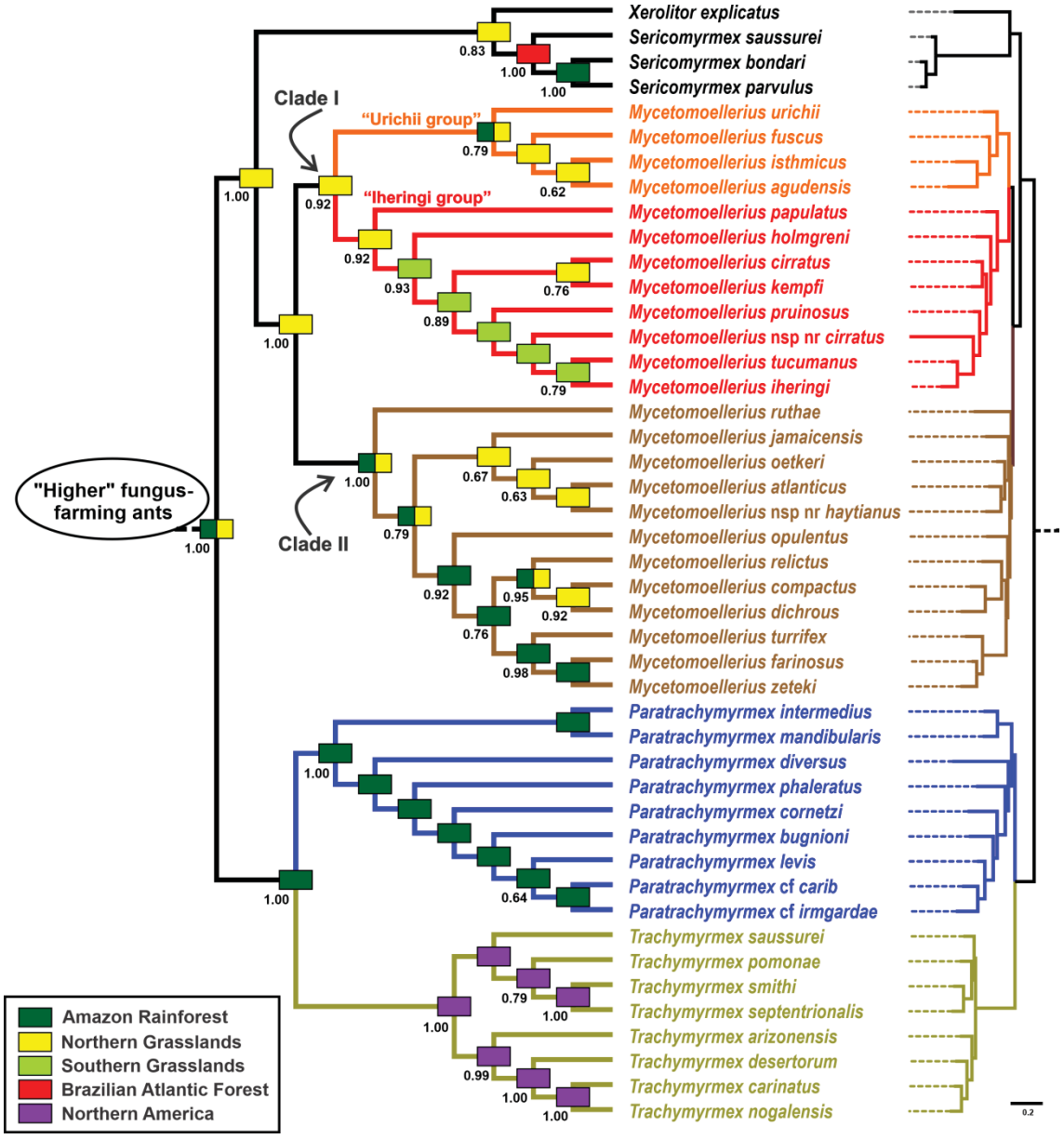
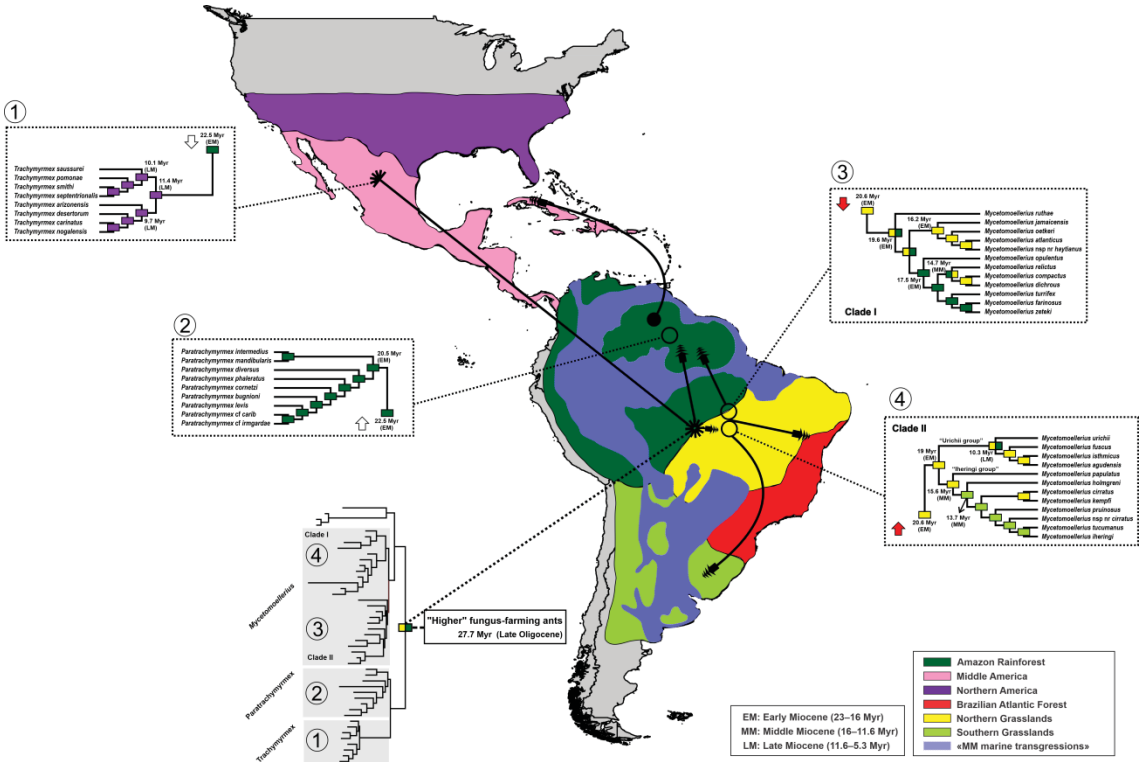


Figure 2. Historical biogeography and dispersion routes of major clades and lineages of *Mycetomoellerius*, *Paratrachymyrmex* and *Trachymyrmex* s.s. The map shows marine transgressions that crossed South America during the Middle Miocene. *Mycetomoellerius* would have dispersed further north into the Amazon Rainforest till it reached Middle America (most of clade II), and towards the south to the Southern Grasslands ("Iheringi group"). *Paratrachymyrmex* would have expanded its range into Middle America and Nearctic multiple times and *Trachymyrmex* would have originated in a Neartic region and diversified around.



Supplementary material

Table S1. Gene sequences of fungus-farming ants analyzed for this study and the respective GenBank accession numbers.

Taxon	**EF1αF1**	**EF1αF2**	**LwRh**	**Wg**	**COI**
Mycetagroicus cerradensis	MK600309	MK599983	MK600124	MK600215	–
Mycetagroicus inflatus	MK600310	MK599984	MK600125	MK600216	MK600058
Mycetagroicus triangularis	EU204371	EU204612	EU204537	MK600217	–
Mycetomoellerius atlanticus	MK600312	MK599986	MK600127	MK600220	MK600060
Mycetomoellerius cirratus	MK600313	MK599987	MK600128	MK600221	MK600061
Mycetomoellerius dichrous	MK600314	MK599988	MK600129	MK600222	MK600062
Mycetomoellerius fuscus	MK600318	MK599992	MK600133	MK600226	MK600066
Mycetomoellerius holmgreni	MK600321	MK599995	MK600136	MK600229	MK600069
Mycetomoellerius iheringi	**to submit**	**to submit**	**to submit**	**to submit**	**to submit**
Mycetomoellerius jamaicensis	MK600369	MK600044	MK600182	MK600291	MK600112
Mycetomoellerius kempfi	MK600327	MK600001	MK600142	MK600235	MK600074
Mycetomoellerius nsp nr *cirratus*	MK600336	MK600010	MK600151	MK600246	–
Mycetomoellerius nsp nr *haytianus*	MK600338	MK600012	MK600153	MK600248	MK600084
Mycetomoellerius opulentus	MK600339	MK600013	MK600154	MK600250	MK600085
Mycetomoellerius papulatus	EU204338	EU204579	EU204504	MK600251	MK600086
Mycetomoellerius relictus	MK600340	MK600014	MK600155	MK600252	MK600087
Mycetomoellerius turrifex	MK600379	MK600054	MK600192	MK600304	MK600120
Mycetomoellerius urichii	MK600381	MK600056	MK600194	MK600306	MK600121
Mycetomoellerius zeteki	MK600343	MK600017	MK600158	MK600254	–
Paratrachymyrmex bugnioni	EU204322	EU204564	EU204489	MK600258	–
Paratrachymyrmex cf *carib*	MK600345	MK600019	MK600160	MK600261	MK600091
Paratrachymyrmex cf *irmgardae*	MK600346	MK600020	MK600161	MK600262	MK600092
Paratrachymyrmex cornetzi	MK600350	MK600024	MK600165	MK600267	MK600096
Paratrachymyrmex diversus	MK600352	MK600026	MK600167	MK600270	MK600098
Paratrachymyrmex intermedius	EU204336	MK600027	EU204502	MK600271	–
Sericomyrmex bondari	MK600359	MK600034	MK600173	MK600279	–
Sericomyrmex parvulus	MK600360	MK600035	MK600174	MK600281	–
Sericomyrmex saussurei	MK600361	MK600036	–	MK600282	–

Trachymyrmex arizonensis	EU204388	EF013526	EF013655	MK600286	–
Trachymyrmex carinatus	MK600365	MK600040	MK600178	MK600287	MK600108
Trachymyrmex desertorum	MK600368	MK600043	MK600181	MK600290	MK600111
Trachymyrmex nogalensis	MK600371	MK600046	MK600184	MK600293	–
Trachymyrmex pomonae	MK600373	MK600048	MK600186	MK600295	MK600113
Trachymyrmex ruthae	MK600374	MK600049	MK600187	MK600296	MK600114
Trachymyrmex saussurei	MK600341	MK600015	MK600156	MK600297	MK600088
Trachymyrmex septentrionalis	MK600376	MK600051	MK600189	MK600300	MK600117
Trachymyrmex smithi	MK600378	MK600053	MK600191	MK600303	MK600119
Xerolitor explicator	MG642983	MG642984	MG642986	MG642985	–

Table S2. Worker morphological characters, character coding and the results of the phylogenetic signal analyses, including *p*-value, according to Abouheif's *C*mean and Pagel's λ inferences for the genera *Mycetomoellerius*, *Paratrachymyrmex* and *Trachymyrmex* s.s.

Character	**Abouheif *C*mean**	***p*-value**	**Pagel's λ**	***p*-value**
1. Pilosity of the gaster and femora: (0) only hairs, without fine pubescence; (1) dense, long dark hairs mixed with an extremely low, fine and abundant light pubescence.	0.715401786	0.001	0.999934	<0.001
2. Discal area of mandibles: (0) smooth; (1) finely striated; (2) coarsely striated.	0.445962702	0.001	0.999934	<0.001
3. Lateral borders of frontal lobes: (0) semicircular; (1) sub-triangular; (2) triangular.	0.320185467	0.007	0.94284	<0.001
4. Accessory lobes or teeth at the base of frontal lobes: (0) absent; (1) present.	0.249363426	0.020	0.351961	0.0675377
5. Anterior border of frontal lobes: (0) straight; (1) convex; (2) concave.	0.154386104	0.082	0.802617	0.0819158
6. Posterior border of frontal lobes: (0) straight; (1) convex; (2) concave.	0.095346188	0.193	0.430271	0.0342405
7. Lateral borders of frontal lobes: (0) smooth; (1) crenulated.	0.758033907	0.001	0.999934	<0.001
8. Frontal carina: (0) not reaching the preoccipital (posterior) margin of head; (1) reaching the preoccipital margin.	0.485961914	0.002	0.999934	<0.001
9. Frontal and preocular carina: (0) ending separated; (1) ending together.	0.303000710	0.012	0.459936	0.00511784
10. Preocular carina: (0) strongly curved; (1) vertical.	0.714843750	0.001	0.999934	<0.001
11. Preocular carina: (0) unique; (1)	-0.032132056	0.932	<0.001	1

double.				
12. Scrobe: (0) absent; (1) present.	0.591122159	0.001	0.999934	<0.001
13. Scrobe: (0) opened; (1) closed.	0.589612269	0.001	N/A	N/A
14. Apical tubercle of the antennal scrobes: (0) absent; (1) present.	0.588727679	0.001	0.999934	<0.001
15. Basal lobes of the antennal scapes: (0) absent; (1) present.	0.716517857	0.001	0.999934	<0.001
16. Basal lobes of antennal scapes: (0) not transversely broadened; (1) transversely broadened pointing mesad (2) transversely broadened pointing to both sides.	0.607782543	0.001	N/A	N/A
17. Anterior surface of antennal scapes: (0) smooth; (1) weakly microtuberculate; (2) notably microtuberculate.	0.607606422	0.001	0.924202	<0.001
18. Length of antennal scapes: (0) surpassing weakly the posterolateral corners of head; (1) surpassing notably the posterolateral corners; (2) not surpassing the posterolateral corners.	0.559389772	0.001	0.958559	<0.001
19. Preoccipital (posterior) margin of head: (0) distinctly notched; (1) almost straight.	0.246837798	0.022	0.805578	0.0475252
20. Preoccipital spines: (0) larger than the tubercles of the preoccipital lobes; (1) nearly the length of the tubercles of the preoccipital lobes; (2) absent or vestigial.	0.598065997	0.001	0.999934	<0.001
21. Supraocular projections: (0) absent or vestigial; (1) present.	0.669082126	0.001	0.999934	<0.001
22. Supraocular projections: (0) tooth or spine-like; (1) microtuberculate swelling.	0.516511563	0.002	N/A	N/A
23. Dorsal projections of mesosoma:	0.483870968	0.023	0.999934	<0.001

(0) smooth spine- or tooth-like; (1) microscopically multituberculate swelling, tooth- or spine-like.				
24. Median pronotal projections: (0) present; (1) absent or vestigial.	0.299479167	0.016	0.837623	0.027969
25. Number of median pronotal projections: (0) two; (1) one.	0.223453581	0.035	N/A	N/A
26. Lateral pronotal projections: (0) spine-like; (1) tooth-like; (2) microscopically multituberculate or multidentate swelling.	0.289428711	0.017	0.999934	<0.001
27. Lateral pronotal projections in frontal view: (0) directed upwards and outwards; (1) laterally directed.	0.377278646	0.003	0.935246	0.00208112
28. Inferior pronotal corner: (0) unarmed, obtusely angulate or rounded; (1) armed with a tooth or spine.	0.267645474	0.038	0.393652	0.381033
29. Size of projection on the inferior pronotal corner: (0) weakly projected; (1) notably projected.	0.107113487	0.161	N/A	N/A
30. Shape of projection on the inferior pronotal corner: (0) triangular; (1) spine-like; (2) rounded.	0.210769080	0.051	N/A	N/A
31. Anterior mesonotal projections: (0) nearly of the length of the pronotal lateral ones; (1) notably shorter than pronotal lateral ones; (2) notably longer than pronotal lateral ones.	0.252705011	0.028	0.999782	0.00357068
32. Apex of projection on the inferior pronotal corner: (0) blunt; (1) acute.	0.140976563	0.116	N/A	N/A
33. Anterior mesonotal projections: (0) spine-like; (1) microscopically	0.243017578	0.025	0.674592	0.0119226

multituberculate or multidentate swelling; (2) semicircular multidentate ridge from above.				
34. Shape of median pronotal projections: (0) spine-like; (1) ridge or multituberculate swelling.	0.007638889	0.380	<0.001	1
35. Posterior mesonotal projections: (0) present; (1) absent or vestigial.	0.253684843	0.021	0.656265	0.00758526
36. Shape of posterior mesonotal projections: (0) spine-like; (1) ridge or multituberculate tumulus.	0.217059796	0.035	N/A	N/A
37. Pilosity of mesopleura: (0) vestigial or absent; (1) present.	0.243915264	0.037	0.462962	0.151252
38. Projection on the inferior margin of mesopleura: (0) absent; (1) present.	-0.039870690	0.496	<0.001	1
39. Projection on the superior margin of mesopleura: (0) absent; (1) present.	0.365946691	0.004	0.999934	0.0008716
40. Shape of projection on the superior margin of mesopleura: (0) small tooth or triangular spine; (1) large lobe.	0.151806641	0.094	N/A	N/A
41. Projections at the meeting of basal and declivous faces of propodeum: (0) as long as the larger projections of promesonotum; (1) shorter than promesonotal projections; (2) longer than promesonotal projections.	0.166491890	0.064	<0.001	1
42. Projections at the meeting of basal and declivous faces of propodeum: (0) as long as the projections of basal face; (1) longer than the projections of basal face; (2) tooth-like, nearly of the length of	0.233559138	0.030	0.999934	0.00196617

pronotal lateral ones.				
43. Petiolar node: (0) unarmed; (1) with a pair of teeth; (2) with two pairs of teeth.	0.273643494	0.018	0.999934	0.00419879
44. Petiolar node from above: (0) longer than broad; (1) as long as broad.	0.300667318	0.008	0.675639	0.00394377
45. Postpetiole from above: (0) distinctly transverse; (1) as long as broad.	0.274528720	0.015	0.900605	0.00637438
46. Posterior border of postpetiole: (0) straight; (1) superficially excised; (2) notably excised.	0.301842731	0.013	0.999934	0.00335332
47. Hairs of the first gastric tergite: (0) strongly curved, hook-like; (1) in two shapes: straight or weakly curved medially and hook-like; (2) straight.	0.443897519	0.003	0.999934	<0.001
48. Tubercles on the basal third of the first gastric tergite: (0) clearly in four longitudinal rows; (1) more or less in four longitudinal rows; (2) randomly distributed; (3) inconspicuous, but with notable ridge on each side.	0.356867386	0.005	0.999925	0.00135226
49. Large welt on each side of lateral posterior region of the first gastric tergite: (0) absent; (1) present.	0.200390625	0.099	<0.001	1
50. First gastric sternite: (0) without hook-like hairs; (1) with hook-like hairs; (2) in two shapes: straight or weakly curved medially and hook-like.	0.290149007	0.011	0.999934	<0.001

Table S3. Data matrix of 31 taxa of the "higher" fungus-farming ants (genera *Mycetomoellerius*, *Paratrachymyrmex* and *Trachymyrmex*) and 50 morphological characters of workers. Adapted from Brandão and Mayhé-Nunes (2007).

Taxon	
M. agudensis	0 0 2 0 0 0 1 0 1 1 1 1 0 1 0 - 1 0 0 0 1 1 1 0 1 0 0 1 1 1 0 0 0 0 1 0 0 0 1 1 1 1 0 0 1 0 1 0 0 1
M. compactus	1 0 0 0 1 1 1 0 1 1 0 1 0 1 0 - 0 0 0 2 1 1 1 1 - 0 0 0 - - 1 - 1 1 1 - 1 0 0 - 1 1 0 0 0 2 2 2 0 1
M. dichrous	1 0 1 0 0 0 0 0 0 1 0 0 - 0 0 - 0 0 0 2 1 1 1 1 - 2 0 0 - - 2 - 0 0 0 0 1 0 0 - 1 2 0 0 0 2 2 3 0 0
M. farinosus	0 0 0 1 1 1 1 1 1 1 0 1 1 1 0 - 1 2 0 0 1 0 1 0 1 0 0 1 1 1 0 1 0 0 0 0 0 0 0 - 0 0 2 1 0 0 1 2 1 1
M. fuscus	0 0 0 1 1 1 1 1 1 1 0 1 1 1 0 - 1 0 0 0 1 0 1 0 1 0 0 1 1 0 0 1 0 0 0 0 0 0 1 0 2 0 2 0 1 0 1 0 0 1
M. holmgreni	0 1 1 0 0 2 0 0 0 1 0 1 0 0 1 1 2 1 0 0 1 1 1 0 0 0 1 1 1 1 0 1 0 1 1 - 0 0 1 0 2 1 1 1 1 0 1 1 0 1
M. iheringi	0 1 2 0 2 0 0 0 0 1 0 1 0 0 1 2 2 1 1 0 1 1 1 0 1 0 0 1 1 0 1 0 1 0 1 - 0 0 1 0 0 1 2 1 0 0 1 3 0 1
M. isthmicus	0 1 0 1 1 1 1 0 0 1 0 1 0 1 0 - 0 0 0 0 1 0 1 0 0 0 0 0 - - 0 - 0 0 0 0 1 0 0 - 1 1 2 0 1 0 1 0 0 1
M. jamaicensis	0 0 0 0 2 1 1 1 0 1 0 1 0 1 0 - 2 1 0 0 1 0 1 0 1 0 0 1 1 1 0 0 0 0 1 - 0 0 0 - 0 0 1 1 1 0 0 1 0 1
M. oetkeri	0 0 0 0 1 1 0 0 0 1 0 1 0 1 0 - 0 1 1 0 1 0 1 0 1 0 0 1 0 0 0 1 0 0 0 0 0 1 1 0 0 0 2 1 0 0 0 2 0 1
M. opulentus	1 2 0 0 0 1 1 0 1 1 0 1 0 1 0 - 0 0 0 2 1 0 1 0 1 1 1 1 1 0 2 1 0 0 0 1 1 0 0 - 1 0 0 0 0 0 2 2 0 0
M. papulatus	0 0 1 0 0 2 0 0 1 1 0 1 0 0 0 - 1 1 0 1 1 0 1 0 1 0 1 1 1 0 1 1 1 1 1 - 0 0 1 0 0 0 2 1 0 0 1 2 0 1
M. pruinosus	0 0 2 0 0 0 0 0 0 1 0 1 0 0 1 0 2 1 0 0 1 0 1 0 0 0 1 1 0 0 1 1 1 0 1 - 1 0 1 0 0 1 2 1 0 0 1 1 0 1
M. relictus	1 1 0 0 1 1 1 0 0 1 0 1 0 1 0 - 0 0 1 2 1 0 1 1 - 0 0 1 1 0 1 1 1 0 0 0 0 0 0 - 0 0 2 1 0 0 2 0 0 0
M. ruthae	0 0 0 1 1 1 1 0 1 1 0 1 0 1 0 - 1 2 0 0 1 0 1 0 0 0 0 1 1 0 1 1 2 0 1 - 0 0 0 - 0 0 2 0 0 1 2 2 1 2
M. tucumanus	0 0 1 0 2 2 0 0 0 1 0 1 0 0 1 2 1 1 0 0 1 1 1 0 0 0 0 1 1 1 1 0 1 0 1 - 1 0 1 0 0 1 2 1 0 0 1 3 0 1
M. turrifex	0 0 0 0 1 1 1 1 1 1 0 1 1 1 0 - 1 2 1 0 1 0 1 0 1 0 0 1 1 0 1 1 1 0 1 - 0 0 0 - 0 0 2 1 0 0 1 2 0 1

M. urichii	0 0 0 0 1 1 1 1 1 1 0 1 1 1 0 - 1 1 1 0 1 0 1 0 1 0 1 1 1 0 0 1 0 0 0 0 0 0 1 0 2 0 2 1 0 0 1 0 0 1
M. zeteki	0 0 0 1 1 1 1 1 0 1 0 1 0 1 0 - 1 2 0 0 1 0 1 0 1 0 0 1 1 1 0 1 0 0 1 - 0 0 0 - 0 0 2 0 0 0 0 1 0 1
P. bugnioni	0 2 0 0 2 0 0 0 0 1 0 1 0 0 0 - 0 0 0 1 0 - 1 0 0 1 1 1 1 1 0 0 1 0 1 - 0 0 0 - 0 0 1 1 1 0 0 2 0 1
P. cornetzi	0 0 1 0 0 0 0 0 0 1 0 0 - 0 0 - 0 0 1 1 0 - 1 0 0 1 1 1 1 1 0 1 1 0 1 - 0 1 0 - 1 1 1 1 1 1 1 0 0 1
P. diversus	0 1 1 0 0 0 0 0 0 0 0 0 - 0 0 - 0 1 1 0 1 1 1 0 0 0 1 1 1 2 1 0 1 0 1 - 0 0 1 0 1 0 1 1 0 1 0 2 0 1
P. irmgardae	0 2 1 0 0 0 0 0 0 1 0 1 0 0 0 - 0 0 0 2 0 - 1 1 - 1 1 1 1 1 1 0 1 1 1 - 0 0 0 - 1 0 1 1 1 0 0 2 0 1
P. levis	0 2 0 0 0 0 0 0 0 1 0 1 0 0 0 - 0 0 1 1 0 - 1 0 0 1 1 1 0 0 1 0 1 1 1 - 0 0 1 0 1 1 1 1 1 0 0 2 0 1
P. mandibularis	0 2 2 0 0 0 0 0 0 0 0 0 - 0 0 - 0 1 0 0 0 - 1 0 0 1 1 1 0 0 2 1 0 0 0 0 0 0 0 - 0 0 2 0 1 0 0 2 0 1
P. phaleratus	0 1 0 0 1 1 1 1 1 1 0 1 1 0 0 - 1 0 1 0 1 1 1 0 0 0 0 1 0 0 1 1 1 0 0 0 0 1 1 0 0 0 2 0 1 0 1 1 0 1
T. arizonenensis	0 2 1 0 2 2 0 0 0 0 0 0 - 0 0 - 0 1 1 1 0 - 1 1 0 0 0 1 1 1 0 0 0 0 1 - 0 0 0 - 1 1 2 1 0 0 1 2 0 1
T. nogalensis	0 2 2 0 2 0 0 0 0 0 0 0 - 0 0 - 0 1 1 1 0 - 1 1 - 0 0 1 1 1 0 1 0 0 0 1 0 0 0 - 1 1 2 0 0 1 1 2 0 1
T. saussurei	0 2 2 0 0 0 0 0 0 0 0 0 - 0 0 - 0 1 1 1 1 0 1 0 0 0 0 1 1 1 0 1 0 0 0 0 0 0 1 0 0 0 2 1 1 1 0 2 0 1
T. smithi	0 2 0 0 0 0 0 0 0 0 0 0 - 0 0 - 0 0 1 1 0 - 1 0 0 0 1 1 1 0 1 1 1 0 1 - 0 0 1 0 0 0 2 1 0 1 0 3 0 1
T.septentrionalis	0 2 1 0 0 0 0 0 0 0 0 0 - 0 0 - 0 1 1 0 1 0 1 0 0 0 0 1 0 0 0 1 0 0 1 - 0 0 1 0 0 0 2 1 0 1 0 2 0 1

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