GISELE AMARO TEIXEIRA

ORGANIZAÇÃO CROMOSSÔMICA E DINÂMICA EVOLUTIVA DE SEQUÊNCIAS REPETITIVAS EM FORMIGAS CULTIVADORAS DE FUNGOS (MYRMICINAE: ATTINI: ATTINA)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

Orientadora: Denilce Meneses Lopes

Coorientadoras: Luísa Antônia Campos Barros
Tânia Maria Fernandes Salomão

VIÇOSA – MINAS GERAIS
2022

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T

T266o
2022

Teixeira, Gisele Amaro, 1993-

Organização cromossômica e dinâmica evolutiva de sequências repetitivas em formigas cultivadoras de fungos (Myrmicinae: Attini: Attina) / Gisele Amaro Teixeira. – Viçosa, MG, 2022.

1 tese eletrônica (113 f.): il. (algumas color.).

Texto em português e inglês.

Orientador: Denilce Meneses Lopes.

Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Biologia Geral, 2022.

Inclui bibliografia.

DOI: https://doi.org/10.47328/ufvbbt.2022.295

Modo de acesso: World Wide Web.

1. Formigas - Genética. 2. Heterocromatina. 3. Microssatélites (Genética). 4. DNA. 5. Telômero. I. Lopes, Denilce Meneses, 1978-. II. Universidade Federal de Viçosa. Departamento de Biologia Geral. Programa de Pós-Graduação em Biologia Celular e Estrutural. III. Título.

CDD 22. ed. 595.796

Bibliotecário(a) responsável: Bruna Silva CRB6/2552

GISELE AMARO TEIXEIRA

ORGANIZAÇÃO CROMOSSÔMICA E DINÂMICA EVOLUTIVA DE SEQUÊNCIAS REPETITIVAS EM FORMIGAS CULTIVADORAS DE FUNGOS (MYRMICINAE: ATTINI: ATTINA)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

APROVADA: 16 de maio de 2022.

Assentimento:

Gisele Amaro Teixeira

Gisele Amaro Teixeira
Autora

Denilce Meneses Lopes
Orientadora

Dedico aos meus pais Imaculada e Edgard,
ao meu esposo Sebastião,
ao meu irmão Geovane

AGRADECIMENTOS

Agradeço primeiramente a Deus por me dar força sempre para superar todas as dificuldades e obstáculos e guiar meus caminhos.

Aos meus pais Edgard e Imaculada pelo amor incondicional, por me incentivarem a dedicar aos estudos desde pequena e por sempre apoiarem na busca por meus objetivos.

Ao meu Amor Sebastião por estar sempre ao meu lado me incentivando, dando apoio, carinho, e paciência nos momentos difíceis. Você me inspira sempre a ser uma pessoa melhor.

Ao meu irmão Geovane pela sua amizade e companheirismo.

À minha orientadora Profa. Denilce Menezes pela orientação, confiança no meu trabalho, ensinamentos, valiosa amizade nesses anos de doutorado e contribuição para meu crescimento pessoal e profissional.

À minha coorientadora Profa. Luísa Barros pela valiosa orientação desde a minha graduação, conselhos, ensinamentos, valiosa amizade, e pela contribuição para meu crescimento pessoal e profissional. Seu amor e dedicação pela pesquisa são fontes de inspiração para mim.

Ao Prof. Hilton Aguiar pela valiosa amizade, por todos os ensinamentos, conselhos, por sempre me ajudar desde a minha graduação e contribuição para meu crescimento pessoal e profissional.

As amigas Jaqueline, Marina, Thaís, Natália, Camila e Priscila pelas boas conversas, ótimos cafés e ótima convivência no laboratório.

Ao Prof. Lúcio Campos pelos ensinamentos, conselhos, boas conversas, e amizade.

A Profa. Mara Tavares pelos ensinamentos, amizade, boas conversas e oportunidade de parceria em artigos.

Ao Prof. Jacques Delabie pela identificação das espécies.

Aos professores Mara Tavares, Cléa Mariano, Hilton Aguiar, Vander Tosta por terem aceitado compor a minha banca de defesa de tese.

A todos os professores da graduação em Ciências Biológicas, do mestrado em Genética e Melhoramento e do doutorado em Biologia Celular e Estrutural que contribuíram para a minha formação profissional.

A Pós-graduação em Biologia Celular e Estrutural pela oportunidade de cursar o doutorado.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Ao Programa de Auxílio ao Pesquisador (PAPESQ) da Universidade Federal do Amapá pelo apoio financeiro.

A Universidade Federal de Viçosa pela infraestrutura fornecida, em especial aos laboratórios de Biologia Molecular de Insetos e Beagle pelo suporte técnico.

Enfim, a todos que direta ou indiretamente me ajudaram a conquistar mais essa etapa, os meus sinceros agradecimentos.

RESUMO

TEIXEIRA, Gisele Amaro, D.Sc., Universidade Federal de Viçosa, maio de 2022. **Organização cromossômica e dinâmica evolutiva de sequências repetitivas em formigas cultivadoras de fungos (Myrmicinae: Attini: Attina).** Orientadora: Denilce Meneses Lopes. Coorientadoras: Luísa Antônia Campos Barros e Tânia Maria Fernandes Salomão.

DNA repetitivo compõe uma porção substancial do genoma eucariótico e é importante para sua estabilidade, evolução, regulação e arquitetura. Formigas cultivadoras de fungos (subtribo Attina, clados Paleoattina e Neoattina) mostram altas taxas de rearranjos estruturais em seus genomas, com contrações e expansões gênicas, que foram necessárias para o estabelecimento de seu estilo de vida baseado em sociedades agrícolas. Processos que levaram a diversificação genômica em Attina também podem ter atuado sobre sequências repetitivas, destacando a importância do estudo do DNA repetitivo nesse grupo. Consistente com essa hipótese, dados citogenéticos em Attina mostram que sequências repetitivas heterocromáticas são ricas em GC, o que é um traço incomum em Formicidae. Assim, essa tese focou no estudo do DNA repetitivo em Attina a partir de uma abordagem citogenética molecular. Considerando que a maior parte dos estudos disponíveis envolvendo sequências repetitivas para Attina bem como para Formicidae, estão disponíveis para genes rDNA, no capítulo I dados sobre mapeamento de genes rDNA em formigas foram compilados e resultados inéditos de 13 espécies neotropicais são apresentados. Os dados mostraram que um único sítio de rDNA intracromossômico é a característica mais frequente e provavelmente ancestral em formigas, e que a localização cromossômica de clusters rDNA influencia na restrição/dispersão desses genes no cariótipo. No capítulo II, caracterizamos as espécies de Neoattina *Cyphomyrmex transversus*, *Sericomyrmex maravalhas* e *Mycetomoellerius relictus*, através de citogenética clássica, molecular e bandamentos. *Cyphomyrmex transversus* e *S. maravalhas* mostram cariótipos inéditos em seus respectivos gêneros. As três espécies apresentam heterocromatina rica em GC, o que reforça a hipótese de origem comum dessa heterocromatina em Attina. Dados obtidos associados a estudos prévios sugerem que um único sítio de rDNA intracromossômico é plesiomórfico em Attina e que inversões parecem ser importantes para mudar a posição desses genes no cariótipo. No capítulo III, foi produzido uma sonda C_0t-DNA (sequências altamente e moderadamente repetitivas) de *M. relictus* que foi hibridizada nos cariótipos de espécies de Paleoattina e Neoattina. Os resultados indicam pelo menos cinco eventos evolutivos que levaram à diferenciação da heterocromatina rica em GC em Attina desde seu provável surgimento no ancestral da subtribo. Adicionalmente, mapeamos as sequências repetitivas $(GA)_{15}$ e $(TTAGG)_6$ em espécies dos dois clados que mostraram resultados mais homogêneos localizadas na eucromatina e telômeros, respectivamente. No capítulo IV, o cariótipo da formiga cortadeira *Atta cephalotes* foi caracterizado por citogenética clássica, bandamentos cromossômicos e mapeamento físico de diferentes sequências repetitivas (teloméricas, microssatélites e genes rDNA 18S). *Atta cephalotes* mostrou um cariótipo conservado em relação as demais espécies de *Atta*, e os possíveis fatores que influenciam essa conservação cromossômica no gênero são discutidos. Os primeiros *insights* sobre a diversidade e organização cromossômica de vários DNAs repetitivos são apresentados em formigas cortadeiras, úteis para estudos futuros comparativos. Os marcadores citogenéticos utilizados nesta tese em diferentes espécies de Attina indicam padrões distintos de diversificação para o DNA repetitivo localizado na heterocromatina, enquanto o DNA repetitivo da eucromatina e telômeros parece ser mais conservado na subtribo.

Palavras-Chave: Formigas. Heterocromatina. Microssatélites. DNA. Telômero.

ABSTRACT

TEIXEIRA, Gisele Amaro, D.Sc., Universidade Federal de Viçosa, May, 2022. **Chromosomal organization and evolutionary dynamics of repetitive sequences in fungus-farming ants (Myrmicinae: Attini: Attina).** Advisor: Denilce Meneses Lopes. Co-advisors: Luísa Antônia Campos Barros and Tânia Maria Fernandes Salomão.

Repetitive DNA constitutes a substantial portion of the eukaryotic genome and are important for its stability, evolution, regulation, and architecture. Fungus farming ants (Attina subtribe, Paleoattina and Neoattina clades) show high rates of structural rearrangements in their genomes, with gene contractions and expansions, which were necessary for the establishment of their lifestyle based on agriculture. Processes that led to genomic diversification in Attina may also have acted on repetitive sequences, highlighting the importance of studying repetitive DNA in this group. Consistent with this hypothesis, cytogenetic data in Attina show that repetitive heterochromatic sequences are rich in GC, which is an uncommon trait in Formicidae. Thus, this thesis focused on the study of repetitive DNA in Attina from a molecular cytogenetic approach. Considering that most of studies involvig repetitive sequences available for Attina as well as Formicidae are available on rDNA genes, in chapter I data on mapping rDNA genes in ants were compiled and unpublished results from 13 Neotropical species are presented. The data showed that a single intrachromosomal rDNA site is the most frequent and probably ancestral feature in ants, and that the chromosomal location of rDNA clusters influences the restriction/dispersion of these genes in the karyotype. In chapter II, we characterized the Neoattina species *Cyphomyrmex transversus*, *Sericomyrmex maravalhas* and *Mycetomoellerius relictus*, through classical and molecular cytogenetics, and chromosomal banding. *Cyphomryemx transversus* and *S. maravalhas* show new karyotypes in their respective genera. The three species present GC-rich heterochromatin, which reinforces the hypothesis of a common origin of this heterochromatin in Attina. Data obtained in association with previous studies suggest that a single intrachromosomal rDNA site is plesiomorphic in Attina and that inversions seem to be important to change the position of these genes in the karyotype. In chapter III, a C_0t-DNA probe (highly and moderately repetitive sequences) from *M. relictus* was produced and hybridized in the karyotypes of species from Neoattina and Paleoattina clades. The results indicate at least five evolutionary events that led to the differentiation of GC-rich heterochromatin into Attina since its probable emergence in the subtribe's ancestor. Additionally, the repetitive sequences $(GA)_{15}$ and $(TTAGG)_6$ were mapped in species of the two clades that showed more homogeneous results located in euchromatin and telomeres, respectively. In chapter IV, the karyotype of the leaf-cutting ant *Atta cephalotes* was characterized by classical cytogenetics, chromosomal banding and physical mapping of different repetitive sequences (telomeric, microsatellites and 18S rDNA genes). *Atta cephalotes* showed a conserved karyotype in relation to other *Atta* species, and the possible factors that influence this chromosomal conservation in the genus are discussed. The first insights into the chromosomal diversity and organization of the various repetitive DNAs are presented in leaf-cutting ants, useful for future comparative studies. The cytogenetic markers used in this thesis in different Attina species indicate distinct patterns of diversification for the repetitive DNA located in heterochromatin while the repetitive DNA of euchromatin and telomeres seems to be more conserved in the subtribe.

Keywords: Ants. Heterochromatin. Microsatelites. DNA. Telomeres.

SUMÁRIO

1 - INTRODUÇÃO GERAL

Uma grande porção do genoma de eucariotos é composta por diferentes sequências altamente e moderadamente repetitivas organizadas em tandem ou dispersas localizadas na heterocromatina ou eucromatina, como famílias multigênicas, microssatélites, minissatélites, DNA satélites, DNA telomérico, e elementos transponíveis (Martins et al. 2011; López-Flores e Garrido-Ramos 2012). Além da função gênica observada nas famílias multigênicas (ex.: genes ribossomais e histonas), atualmente é consenso que o DNA repetitivo pode ter papéis importantes no genoma, incluindo na sua organização e estabilidade arquitetônica, na regulação gênica, segregação cromossômica e na origem de rearranjos cromossômicos (Shapiro e Sternberg 2005; Martins et al. 2011; Biscotti et al. 2015a; Koo et al. 2016). Além disso, sequências repetitivas podem estar relacionadas diretamente com a evolução de regiões cromossômicas específicas como centrômeros e telômeros (Biscotti et al. 2015b; Shatskikh et al. 2020; Thakur et al. 2021), e de cromossomos inteiros como cromossomos sexuais e cromossomos Bs (Martins et al. 2011; Chalopin et al. 2015; Hobza et al. 2015; Ruiz-Ruano et al. 2017). O DNA repetitivo também pode estar envolvido na evolução gênica uma vez que podem servir como matéria prima para novos genes ou variantes de genes que podem estar relacionados com adaptações ambientais (revisão em Paço et al. 2019; Sayah et al. 2004; Aminetzach et al. 2005; revisão em González e Petrov 2009; revisão em Gilbert et al. 2021). A notável importância funcional, estrutural e evolutiva do DNA repetitivo sobre o genoma instiga o estudo de sequências repetitivas nos mais variados organismos.

Desde a década de 1980, a citogenética molecular tem permitido o estudo mais resolutivo da estrutura cromossômica representando grandes avanços nos estudos citogenéticos. Uma das técnicas de citogenética molecular mais empregada é a hibridização *in situ* fluorescente (FISH) que provou ser uma ferramenta eficaz e precisa para mapear fisicamente sequências de DNA específicas dentro dos cromossomos, permitindo a construção de mapas citogenéticos das espécies (revisão em Levsky e Singer, 2003; Liehr, 2017; Martins et al. 2011). Estudos citogenéticos moleculares têm mapeado as diferentes sequências repetitivas nos cariótipos das espécies e têm sido notavelmente importantes para a compreensão da composição, origem, função, organização e padrões evolutivos do DNA repetitivo no genoma de eucariotos (Martins et al. 2011; Biscotti et al. 2015a).

Umas das sequências repetitivas amplamente mapeadas com a técnica de FISH são os genes ribossomais 45S, que se apresentam em várias cópias in tandem no genoma dos eucariotos. Cada cópia do gene rDNA 45S é constituída, nessa ordem para a maioria dos organismos, pelos genes ribossomais 18S, 5.8S e 28S, os espaçadores internos transcritos entre esses genes, os espaçadores externos transcritos flanqueando os genes 18S e 28S, espaçador intergênico não transcrito a montante, além da região do promotor que contém duas sequências importantes para a formação do complexo transcricional, o elemento de controle a jusante e o núcleo promotor (Sumner 2003, Symonová 2019). Uma característica notável desses genes é que suas sequências codificantes são altamente conservadas em eucariotos devido a sua importância funcional permitindo comparações entre táxons distantes. Entretanto, os espaçadores intergênicos variam em sequência e comprimento (Sumner 2003; Symonová 2019).

Os genes rDNA 45S fazem parte das regiões organizadoras de nucléolo (NORs) e são localizados em porções do DNA que após sua condensação, usualmente aparecem como constrições secundárias nos cromossomos metafásicos (Sumner 2003). Variações no número de sítios e localização de genes ribossomais são bem documentados em diferentes grupos de eucariotos, com espécies apresentando os clusters de rDNA em um ou mais pares cromossômicos localizados próximos a telômeros, centrômeros ou em posições intersticiais (Sochorová et al. 2018; Roa and Guerra 2012; Dutrillaux e Dutrillaux 2012; Menezes et al. 2021). A compilação de dados cromossômicos referentes a distribuição de genes ribossomais em diferentes organismos tem mostrado um padrão de associação entre a localização cromossômica e o número sítios de genes rDNA presentes no cariótipo (um único sítio de rDNA em regiões intracromossômicas e múltiplos sítios de rDNA em regiões terminais). Os estudos propõem que a localização cromossômica influencia a dispersão desses genes rDNA no cariótipo devido a ocorrência de rearranjos, como translocações, trocas desiguais e mecanismos de recombinação ectópica (entre cromossomos não homólogos), que podem levar à dispersão gênica ou aumento do tamanho das matrizes in tandem de rDNA no genoma, serem mais prováveis nas regiões terminais/subterminais dos cromossomos e serem incomums nas regiões intracromossômicas (Martins and Wasko 2004; Nguyen et al. 2010; Roa and Guerra 2012; Dutrillaux et al. 2016; Gerbault-Seureau et al. 2017; Hirai 2020).

Outras sequências repetitivas que têm sido estudadas do ponto de vista citogenético molecular são os microssatélites, pequenas matrizes de repetições em tandem, também denominadas SSR (*Simple Sequence Repeats*) ou STR (*Short Tandem Repeats*) presentes no genoma de todos os organismos já estudados (Sumner 2003, Ellegren 2004). Os microssatélites evoluem rapidamente, principalmente em termos de variabilidade no comprimento das sequências repetitivas e, portanto, são importantes marcadores genéticos (Li et al. 2002; Ellegren 2004). Diferentes mecanismos podem estar envolvidos na origem, propagação ou eliminação de microssatélites no genoma, como o deslizamento da DNA polimerase durante a replicação ou reparo do DNA, crossing-over desigual ou transposição, que influenciam seus padrões cromossômicos (Li et al. 2002; Ellegren 2004; Zhang 2004; Ruiz-Ruano et al. 2015). A distribuição cromossômica dessas sequências é altamente variável em eucariotos, podendo estar localizados na eucromatina ou heterocromatina de cromossomos autossômicos ou sexuais, microcromossomos ou cromossomos B, agrupados em regiões específicas ou espalhados por todo o comprimento do cromossomo. Isso indica que os microssatélites podem se acumular de maneira diferente e seguir caminhos evolutivos divergentes, o que é útil para entender a evolução do genoma das espécies (Palacios-Gimenez e Cabral-de-Mello 2015; Piscor e Maltempi 2016; Augstenová et al. 2018; Gunski et al. 2019; Marchioro et al. al. 2020).

A constituição de telômeros, responsáveis por evitar a fusão e degradação de terminais cromossômicos, nos diferentes eucariotos também tem sido investigada através da técnica de FISH mostrando que essas regiões cromossômicas são compostas de repetições específicas de DNA (revisão em Gomes et al. 2010; revisão em Watson e Riha 2010). O motif repetitivo hexonucleotídico $(TTAGGG)_n$ é conservado na maioria dos animais (Metazoa), no entanto variações nas sequências teloméricas são observadas em alguns grupos como em nemátodos que tem a sequência telomérica $(TTAGGC)_n$, enquanto a maioria dos artrópodes apresenta $(TTAGG)_n$ em seus telômeros (revisão em Gomes et al. 2010). Especificamente em insetos, $(TTAGG)_n$ é considerado o motif ancestral, embora tenha sido perdido independentemente em diferentes grupos que mostram variações na estrutura telomérica (Kuznetsova et al. 2019; Lukhtanov 2022). Por exemplo, algumas espécies de Coleoptera mostram a sequência telomérica $(TCAGG)_n$, enquanto em Diptera observa-se uma divergência maior na composição telomérica com espécies apresentando retrotransposons, repetições terminais longas complexas (>120 pb) ou repetições curtas (14, 16, and 22 bp). Além disso, inserções de outras sequências (retrotranposons não-LTR e repetições de ordem maior) dentro da matriz telomérica $(TTAGG)_n$ são observadas em algumas espécies incluídas em Hemiptera, Coleoptera, Lepidoptera e Apidae (revisão em Kuznetsova et al. 2019). Recentemente, utilizando análises genômicas de 180 espécies de 8 ordens diferentes de insetos, Lukhtanov (2022) forneceu dados inétidos para telômeros de insetos mostrando a presença de 19 novas variantes de motifs curtos de 1 a 17 bp. Além disso, repetições teloméricas longas e repetições intercaladas com retrotransposons específicos foram observados em novas espécies. Essa notável diversidade da composição e estrutura telomérica em insetos destaca a importância de estudos dessas regiões cromossômicas nesse grupo de organismos.

Sequências moderadamente e altamente repetitivas anônimas (fração C_0t-DNA) também têm sido utilizadas como sondas na FISH. Essas sequências são obtidas pela técnica de cinética de reassociação C_0t-DNA que se baseia na velocidade mais rápida com que sequências repetitivas se renaturam em relação às sequências de cópias únicas do genoma (Zwick et al. 1997; Martins et al. 2011). O procedimento consiste na fragmentação de DNA genômico total em altas temperaturas, desnaturação desse DNA genômico fragmentado (95ºC), reanelamento do DNA (65ºC por tempo específico), e finalmente o tratamento com a enzima S1 nuclease que irá degradar as sequências de cópia única que ainda permaneceram como fita simples (Zwick et al. 1997). Vários estudos conduzidos em diferentes organismos, por exemplo besouros, abelhas, cigarrinhas, peixes e lagartos, têm utilizado a fração C_0t-DNA (sequências moderadamente e altamente repetitivas do genoma) como marcador da heterocromatina, uma vez que essas regiões cromossômicas são geralmente enriquecidas com sequências repetitivas (Cabral-de-Mello et al. 2011; Terêncio et al. 2015; Anjos et al. 2016; Carvalho et al. 2016; Cunha et al. 2020). Esses estudos mostraram que a fração C_0t-DNA é uma ferramenta útil para entender os eventos de diferenciação e evolução da heterocromatina bem como os padrões de organização de sequências repetitivas nos genomas de espécies relacionadas (Carvalho et al. 2016; Cunha et al. 2020).

Em Formicidae, estudos citogenéticos moleculares empregando a técnica de FISH têm focado no mapeamento de genes ribossomais desde os primeiros estudos conduzidos em formigas australianas (Hirai et al. 1994; 1996), e vem aumentando consideravelmente nos últimos anos (ex.: Mariano et al. 2008; Santos et al. 2016; Aguiar et al. 2017; Barros et al. 2016, 2021, 2022; Micolino et al. 2019a, b, 2022; Teixeira et al. 2020). Outras sequências repetitivas também tem sido mapeadas com a FISH em diferentes espécies de formigas como a sequência telomérica canônica de insetos $(TTAGG)_n$ (Meyne et al. 1995; Pereira et al. 2018; Micolino et al. 2019a, b, 2020, 2022; Castro et al. 2020; Barros et al. 2021), e mais recentemente, microssatélites em três espécies de formigas cultivadoras de fungos (Barros et al. 2018; Micolino et al. 2019a, 2022).

Dentre os Formicidae, as formigas cultivadoras de fungos (subtribo Attina) são particularmente notáveis por cultivarem um fungo simbionte para sua alimentação (Weber 1966; Schultz e Brady 2008; revisão em Mehdiabadi e Schultz 2010), e em troca fornecem ao fungo nutrição, propagação para novos locais e proteção contra microorganismos parasitários (Weber 1966; Little et al. 2005). A subtribo Attina forma um grupo monofilético com origem há 50-65 milhões de anos nas florestas tropicais da América do Sul (Schultz e Brady 2008; Nygaard et al. 2016; Branstetter et al. 2017) e são exclusivas do Novo Mundo com distribuição predominantemente neotropical (Mayhé-Nunes e Jaffe 1998). Atualmente, Attina inclui aproximadamente 250 espécies descritas agrupadas em 20 gêneros (Bolton 2022). De acordo com dados moleculares, os gêneros são divididos em dois clados irmãos monofiléticos: Paleoattina (*Apterostigma*, *Mycocepurus* e *Myrmicocrypta*) e Neoattina (os 17 gêneros restantes) (Sosa-Calvo et al. 2018; Solomon et al. 2019; Cristiano et al. 2020).

Essas formigas podem ser agrupadas em cinco sistemas agrícolas: agricultura basal é o sistema ancestral observado em gêneros de Paleoattina e Neoattina basais, agricultura de fungos corais observado em *Apterostigma* grupo *pillosum* (Paleoattina), agricultura de levedura observado na maioria das espécies de *Cyphomyrmex* (Neoattina), agricultura superior generalizada observada nos gêneros *Sericomyrmex, Xerolitor, Mycetomoellerius, Trachymyrmex, Paratrachymyrmex* (Neoattina) e a forma mais derivada de agricultura, a de corte de folhas, que incluem os gêneros de formigas cortadeiras *Amoimyrmex*, *Acromyrmex* e *Atta* (Neoattina) (Mehdiabadi e Schultz 2010). De maneira geral, as espécies de formigas cultivadoras de fungos (com exceção das formigas cortadeiras) são pequenas e monomórficas, com cor escura e sem brilho e entram em tanatose quando perturbadas, as rainhas são inseminadas com único macho e seus ninhos são profundos, demandando grandes esforços de coleta. Contrastamente, formigas cortadeiras mostram além da forma mais derivada de agricultura utilizando somente material vegetal fresco para o cultivo do fungo simbionte, outras características de história-natural derivadas como castas trabalhadoras maiores e polimórficas, rainhas inseminadas por múltiplos machos e grandes colônias (Mehdiabadi e Schultz 2010; Sosa-Calvo et al. 2015).

Do ponto de vista citogenético, mais de 50 táxons de formigas cultivadoras de fungos incluídas em 12 gêneros já foram estudadas, e uma ampla diversidade cariotípica é observada na subtribo com o número cromossômico variando de 2n=8 em *Mycocepurus goeldii* (Forel, 1893) (Paleoattina) e *Mycocepurus* sp. até 2n=64 em *Mycetophylax lectus* (Forel, 1911) (como *Cyphomyrmex lectus*, Neoattina) (Murakami et al. 1998; Barros et al. 2010; revisão em Mariano et al. 2019). Variações no número cromossômico também são observadas dentro da maioria dos gêneros estudados (revisão em Mariano et al. 2019; Barros et al. 2021; 2022; Cardoso e Cristiano 2022). Isso contrasta com o gênero de formigas cortadeiras *Atta* em que se observa, até o momento, uma notável conservação cariotípica entre as espécies estudadas de grupos filogenéticos distintos considerando todos os caracteres citogenéticos analisados (número, morfologia cromossômica, localização de heterocromatina, de regiões ricas em bases GC e de genes ribossomais 18S) (Barros et al. 2014, 2015; Teixeira et al. 2017; revisão em Mariano et al. 2019).

Uma característica citogenética notável observada em formigas cultivadoras de fungos através de técnicas de bandamentos cromossômicos é a presença de heterocromatina rica em pares de bases GC em todos os cromossomos de várias espécies incluídas em Paleoattina e Neoattina. (Barros et. al. 2010, 2013, 2018, 2022; revisão em Mariano et al. 2019). Heterocromatina rica em GC é um traço incomum em formigas em geral. Barros et al. (2018) sugerem que essa heterocromatina rica em GC pode ter se originado no ancestral comum da subtribo, uma hipótese que necessita ser melhor investigada. Variações na composição da heterocromatina já foram observadas em algumas espécies como nas Neoattina *Atta* spp. e *Acromyrmex* spp. que possuem apenas as regiões de genes ribossomais ricas em GC (Barros et al. 2014, 2015, 2016, 2021; Teixeira et al. 2017), em *Amoimyrmex striatus* (Roger, 1863), *Acromyrmex niger* (Smith, 1858) e *Acromyrmex rugosus* (Smith, 1858) que mostram heterocromatina rica em GC em alguns pares cromossômicos (Cristiano et al. 2013; Barros et al. 2016), e mais recentemente algumas linhagens derivadas de *Mycetophylax* spp. apresentaram heterocromatina pericentromérica rica em bases AT na maioria dos cromossomos (Cardoso et al. 2022).

Esses dados de bandamentos cromossômicos peculiares para Formicidae estão em acordância com os dados genômicos de formigas cultivadoras de fungos que mostram taxas de rearranjos estruturais notáveis em comparação com as de outras formigas e insetos já estudados, com contrações e expansões gênicas indicando que um novo material genético substancial se tornou disponível e foi necessário para o estabelecimento desse estilo de vida baseado em sociedades agrícolas (Nygaard et al. 2016). Os processos que levaram à diferenciação gênica também podem ter afetado as diferentes sequências repetitivas, mostrando a relevância desse grupo peculiar na compreensão da porção repetitiva do genoma das formigas.

A maior parte dos estudos citogenéticos moleculares disponíveis para formigas cultivadoras de fungos bem como para formigas em geral, são focados em genes ribossomais, e até o momento ainda não foi realizado uma compilação dos dados disponíveis. Assim, no primeiro capítulo, foi revisado todos os dados citogenéticos moleculares publicados até o ano de 2020 relacionados ao mapeamento de genes ribossomais (45S, 18S ou 28S) em Formicidae e novos dados de 13 espécies neotropicais de quatro subfamílias foram apresentados pela primeira vez. Esses dados foram utilizados para investigar se o número e a localização dos clusters de genes rDNA seguem um padrão específico ou foram distribuídos aleatoriamente nos cariótipos e assim entender a organização genômica e a dinâmica evolutiva desses genes em formigas.

No segundo capítulo, cariótipos de três espécies de formigas cultivadoras de fungos, *Cyphomyrmex transversus* Emery, 1894, *Mycetomoellerius relictus* (Borgmeier, 1934), e *Sericomyrmex maravalhas* Ješovnik e Schultz, 2017, foram caracterizados através de número, morfologia cromossômica, localização de heterocromatina, de regiões ricas em GC e de genes ribossomais. O objetivo do capítulo foi investigar a presença de heterocromatina rica em GC nessas espécies e fornecer insights para auxiliar na compreensão dos padrões de evolução cromossômica em seus respectivos gêneros.

No terceiro capítulo, uma sonda C_0t-DNA foi produzida a partir do genoma de *Mycetomoellerius relictus* (Borgmeier, 1934), pertencente a Neoattina, que possui a heterocromatina rica em GC em todos os seus cromossomos. Essa sonda foi hibridizada nas sequências genômicas de espécies dos três gêneros de Paleoattina e de diferentes gêneros de Neoattina, basais e derivados, para testar se as sequências ricas em GC são compartilhadas entre os dois clados e entender a dinâmica evolutiva da heterocromatina no genoma de formigas cultivadoras de fungos. Além disso, foi mapeado fisicamente o microssatélite $(GA)_{15}$ e a sequência telomérica ancestral de insetos $(TTAGG)_6$ em diferentes espécies de Paleoattina e Neoattina para investigar seus padrões de distribuição nos cariótipos dessas formigas. Esses dados fornecem os primeiros insights sobre a organização, diversidade e padrões evolutivos da porção repetitiva do genoma de Attina de uma perspectiva ampla.

No quarto capítulo, o cariótipo da formiga cortadeira *Atta cephalotes* foi caracterizado através de citogenética clássica, bandamentos cromossômicos e mapeamento físico de diferentes sequências repetitivas (18S rDNA, motivos teloméricos e microssatélites mono-, di- e tri- nucleotídeos). O objetivo deste capítulo foi testar a conservação cariotípica desta espécie em comparação com outras espécies de *Atta* e iniciar a compreensão da organização genômica e diversidade de diferentes seqüências repetitivas em formigas cortadeiras.

Referências Bibliográficas

AGUIAR, H.J.A.C., BARROS, L.A.C., ALVES, D.R., MARIANO, C.S.F., DELABIE, J.H.C. POMPOLO, S.G. (2017). Cytogenetic studies on populations of *Camponotus rufipes* (Fabricius, 1775) and *Camponotus renggeri* Emery, 1894 (Formicidae: Formicinae). PLoS One, 12(5): e0177702.

AMINETZACH, Y.T., MACPHERSON, J.M., PETROV, D.A. (2005). Pesticide resistance via transposition-mediated adaptive gene truncation in *Drosophila*. Science, 309(5735): 764-767.

ANJOS, A., ROCHA, G.C., PALADINI, A., MARIGUELA, T.C., CABRAL-DE-MELLO, D.C. (2016). Karyotypes and repetitive DNA evolution in six species of the genus *Mahanarva* (Auchenorrhyncha: Cercopidae). Cytogenetic and Genome Research, 149(4): 321-327.

AUGSTENOVÁ, B., MAZZOLENI, S., KRATOCHVÍL, L., ROVATSOS, M. (2017). Evolutionary dynamics of the W chromosome in caenophidian snakes. Genes, 9(1): 5.

BARROS, L.A.C., AGUIAR, H.J.A.C., MARIANO, C.S.F., DELABIE, J.H.C., POMPOLO, S.G. (2010). Cytogenetic characterization of the lower-Attine *Mycocepurus goeldii* (Formicidae: Myrmicinae: Attini). Sociobiology, 56: 57–66.

BARROS, L.A.C., AGUIAR, H.J.A.C., MARIANO, C.S.F., DELABIE, J.H.C., POMPOLO, S.G. (2013). Cytogenetic characterization of the ant *Trachymyrmex fuscus* Emery, 1934 (Formicidae: Myrmicinae: Attini) with the description of a chromosomal polymorphism. In Annales de la Société entomologique de France 49(4): 367-373.

BARROS, L.A.C., TEIXEIRA, G.A., AGUIAR, H.J.A.C., MARIANO, C.S.F., DELABIE, J.H.C., POMPOLO, S.G. (2014). Banding patterns of three leafcutter ant species of the genus Atta (Formicidae: Myrmicinae) and Chromosomal Inferences. Florida Entomologist, 97(4): 1694–1701.

BARROS, L.A.C., AGUIAR, H.J.A.C., TEIXEIRA, G.A., MARIANO, C.S.F., TEIXEIRA, M.C., DELABIE, J.H.C. et al. (2015) Cytogenetic data on the threatened leafcutter ant *Atta robusta* Borgmeier, 1939 (Formicidae: Myrmicinae: Attini). Comptes Rendus Biologies, 338(10): 660–665.

BARROS, L.A.C., AGUIAR, H.J.A.C., MARIANO, C.S., ANDRADE-SOUZA, V., COSTA, M.A., DELABIE, J.H.C. ET AL. (2016). Cytogenetic data on six leafcutter ants of the genus *Acromyrmex* Mayr, 1865 (Hymenoptera, Formicidae, Myrmicinae): insights into chromosome evolution and taxonomic implications. Comparative Cytogenetics, 10(2): 229–243.

BARROS, L.A.C., TEIXEIRA, G.A., AGUIAR, H.J.A.C., LOPES, D.M. AND POMPOLO, S.G. (2018). Cytogenetic studies in *Trachymyrmex holmgreni* Wheeler, 1925 (Formicidae: Myrmicinae) by conventional and molecular methods. Sociobiology, 65(2): 185–190.

BARROS, L.A.C., AGUIAR, H.J.A.C., TEIXEIRA, G. A., SOUZA, D.J., DELABIE, J.H.C., MARIANO, C.S.F. (2021). Cytogenetic studies on the social parasite *Acromyrmex ameliae* (Formicidae: Myrmicinae: Attini) and its hosts reveal chromosome fusion in *Acromyrmex*. Zoologischer Anzeiger, 293: 273-281.

BARROS, L.A.C., RABELING, C., TEIXEIRA, G.A., MARIANO, C.S.F., DELABIE, J.H.C., AGUIAR, H.J.A.C. (2022). Decay of homologous chromosome pairs and discovery of males in the thelytokous fungus-growing ant *Mycocepurus smithii*. Scientific Reports, 12(1): 1-16.

BISCOTTI, M.A., OLMO, E., HESLOP-HARRISON, J.P. (2015a). Repetitive DNA in eukaryotic genomes. Chromosome Research, 23: 415–420.

BISCOTTI, M.A., CANAPA, A., FORCONI, M., OLMO, E., BARUCCA, M. (2015b). Transcription of tandemly repetitive DNA: functional roles. Chromosome Research, 23(3): 463-477.

BOLTON, B. (2022), An online catalog of the ants of the world. http://antcat.org. Accessed 05 april 2022.

BRANSTETTER, M.G., JEŠOVNIK, A., SOSA-CALVO, J., LLOYD, M. ., FAIRCLOTH, B.C., BRADY, S.G., SCHULTZ, T.R. (2017). Dry habitats were crucibles of domestication in the evolution of agriculture in ants. Proceedings of the Royal Society B: Biological Sciences, 284(1852): 20170095.

CABRAL-DE-MELLO, D.C., MOURA, R.D.C., SOUZA MELO, A., MARTINS, C. (2011). Evolutionary dynamics of heterochromatin in the genome of *Dichotomius* beetles based on chromosomal analysis. Genetica, 139(3): 315-325.

CARDOSO, D.C., CRISTIANO, M.P. (2021). Karyotype Diversity, Mode, and Tempo of the Chromosomal Evolution of Attina (Formicidae: Myrmicinae: Attini): Is There an Upper Limit to Chromosome Number? Insects, 12(12): 1084.

CARDOSO, D.C., MOURA, M.N., CRISTIANO, M.P. (2022). Dynamic development of AT-rich heterochromatin has followed diversification and genome expansion of psammophilous *Mycetophylax* (Formicidae: Attini: Attina). Insect Molecular Biology, https://doi.org/10.1111/imb.12759.

CARVALHO, N.D., CARMO, E., NEVES, R.O., SCHNEIDER, C.H., GROSS, M.C. (2016). Differential repetitive DNA composition in the centromeric region of chromosomes of Amazonian lizard species in the family Teiidae. Comparative Cytogenetics, 10(2): 203.

CASTRO, C.P.M., CARDOSO, D.C., MICOLINO, R., CRISTIANO, M.P. (2020). Comparative FISH-mapping of TTAGG telomeric sequences to the chromosomes of leafcutter ants (Formicidae, Myrmicinae): is the insect canonical sequence conserved? Comparative Cytogenetics, 14(3): 369–385.

CHALOPIN, D., VOLFF, J. N., GALIANA, D., ANDERSON, J. L., SCHARTL, M. (2015). Transposable elements and early evolution of sex chromosomes in fish. Chromosome research, 23(3): 545-560.

CRISTIANO, M.P., CARDOSO, D.C., FERNANDES-SALOMÃO, T.M. (2013). Cytogenetic andmolecular analyses reveal a divergence between *Acromyrmex striatus* (Roger, 1863) and other congeneric species: taxonomic implications. PLoS One, 8(3): e59784.

CRISTIANO, M.P., CARDOSO, D.C., SANDOVAL-GÓMEZ, V.E., SIMÕES-GOMES, F.C. (2020). *Amoimyrmex* Cristiano, Cardoso & Sandoval, gen. nov. (Hymenoptera: Formicidae): a new genus of leaf-cutting ants revealed by multilocus molecular phylogenetic and morphological analyses. Austral Entomology, 59(4): 643-676.

CUNHA, M.S., CAMPOS, L.A.O., LOPES, D.M. (2020). Insights into the heterochromatin evolution in the genus *Melipona* (Apidae: Meliponini). Insectes Sociaux, 67(3): 391-398.

DUTRILLAUX, A.M. AND DUTRILLAUX, B. (2012). Chromosome analysis of 82 species of Scarabaeoidea (Coleoptera), with special focus on NOR localization. Cytogenetic and Genome Research, 136(3): 208–219.

DUTRILLAUX, A.M., CARTON, B., CACHEUX, L. AND DUTRILLAUX, B. (2016). Interstitial NORs, fragile sites, and chromosome evolution: a not so simple relationship-the example of *Melolontha melolontha* and genus *Protaetia* (Coleoptera: Scarabaeidae). Cytogenetic and Genome Research, 149(4): 304–311.

ELLEGREN, H. (2004). Microsatellites: simple sequences with complex evolution. Nature reviews genetics, 5(6): 435-445.

GERBAULT-SEUREAU, M., CACHEUX, L. AND DUTRILLAUX, B. (2017). The relationship between the (In-) stability of NORs and their chromosomal location: the example of Cercopithecidae and a short review of other primates. Cytogenetic and Genome Research, 153(3): 138–146.

GILBERT, C., PECCOUD, J., CORDAUX, R. (2021). Transposable elements and the evolution of insects. Annual Review of Entomology, 66: 355-372.

GOMES, N.M., SHAY, J.W., WRIGHT, W.E. (2010). Telomere biology in Metazoa. FEBS letters, 584(17): 3741-3751.

GONZÁLEZ, J., PETROV, D.A. (2009). The adaptive role of transposable elements in the *Drosophila* genome. Gene, 448(2): 124-133.

GUNSKI, R.J., KRETSCHMER, R., DE SOUZA, M.S., DE OLIVEIRA FURO, I., BARCELLOS, S.A., COSTA, A.L., et al. (2019). Evolution of bird sex chromosomes narrated by repetitive sequences: Unusual W chromosome enlargement in *Gallinula melanops* (Aves: Gruiformes: Rallidae). Cytogenetic and Genome Research, 158(3): 152-159.

HIRAI, H. (2020) Chromosome dynamics regulating genomic dispersion and alteration of Nucleolus Organizer Regions (NORs). Cells, 9(4): 971–984.

HIRAI, H., YAMAMOTO, M.T., OGURA, K., SATTA, Y., YAMADA, M., TAYLOR, R.W. et al. (1994). Multiplication of 28S rDNA and NOR activity in chromosome evolution among ants of the *Myrmecia pilosula* species complex. Chromosoma, 103(3): 171–178.

HIRAI, H., YAMAMOTO, M.T., TAYLOR, R.W., IMAI, H.T. (1996). Genomic dispersion of 28S rDNA during karyotypic evolution in the ant genus *Myrmecia* (Formicidae). Chromosoma, 105: 190–196.

HOBZA, R., KUBAT, Z., CEGAN, R., JESIONEK, W., VYSKOT, B., KEJNOVSKY, E. (2015). Impact of repetitive DNA on sex chromosome evolution in plants. Chromosome Research, 23(3): 561-570.

KOO, D.H., ZHAO, H., JIANG, J. (2016). Chromatin-associated transcripts of tandemly repetitive DNA sequences revealed by RNA-FISH. Chromosome Research, 24(4): 467–480.

KUZNETSOVA, V., GROZEVA, S., GOKHMAN, V. (2020). Telomere structure in insects: A review. Journal of Zoological Systematics and Evolutionary Research, 58(1): 127-158.

LEVSKY, J.F. AND SINGER, R.H. (2003) Fluorescence *in situ* hybridization: past, present and future. Journal of Cell Science, 116: 2833–2838.

LI, Y.C., KOROL, A.B., FAHIMA, T., BEILES, A., NEVO, E. (2002). Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Molecular ecology, 11(12): 2453-2465.

LIEHR, T. (2017) "Classical cytogenetics" is not equal to "banding cytogenetics". Molecular Cytogenetics, 10: 1–3.

LÓPEZ-FLORES, I., GARRIDO-RAMOS, M.A. (2012). The repetitive DNA content of eukaryotic genomes. Reptitive DNA, 7:1–28, 2012.

LUKHTANOV, V.A. (2022). Diversity and evolution of telomere and subtelomere DNA sequences in insects. bioRxiv, 1-25.

MARCHIORO, P., CAMPOS, L.A., LOPES, D.M. (2020). First Record of a B Chromosome in *Polybia fastidiosuscula* Saussure (Vespidae) and Investigation of Chromatin Composition Through Microsatellite Mapping. Cytogenetic and genome research, 160(11-12): 711-718.

MARIANO, C.S.F., BARROS, L.A.C., VELASCO, Y.M., GUIMARÃES, I.N., POMPOLO, S.G., DELABIE, J.H.C. (2019). Citogenética de hormigas de la región neotropical. In: Fernández, F., Guerrero, R., and Delsinne, T., (Eds.) Hormigas de Colombia. Botoá: Universidad Nacional de Colombia, pp 131–157.

MARIANO, C.S.F., POMPOLO, S.G., BARROS, L.A.C., MARIANO-NETO, E., CAMPIOLO, S., DELABIE, J.H.C. (2008). A biogeographical study of the threatened ant *Dinoponera lucida* Emery (Hymenoptera: Formicidae: Ponerinae) using a cytogenetic approach. Insect Conservation and Diversity, 1(3): 161–168.

MARTINS, C., CABRAL-DE-MELLO, D.C., VALENTE G.T., MAZZUCHELLI J., OLIVEIRA S.G., PINHAL, D. (2011). Animal genomes under the focus of cytogenetics. New York, USA: Nova Science.

MARTINS, C., WASKO, A.P. (2004). Organization and evolution of 5S ribosomal DNA in the fish genome. In: Williams, C.R., (Ed.) Focus on genome research. New York: Nova Science Publishers Inc, pp. 335–363.

MAYHÉ-NUNES, A.J., JAFFÉ, K. (1998). On the biogeografy of Attini (Hymenoptera: Formicidae). Ecotropicos, 11(1): 45-54.

MEHDIABADI, N.J., SCHULTZ, T.R. (2010). Natural history and phylogeny of the fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). Myrmecological News, 13: 37-55.

MENEZES, R. S., CABRAL-DE-MELLO, D.C., MILANI, D., BARDELLA, V.B., & ALMEIDA, E.A. (2021). The relevance of chromosome fissions for major ribosomal DNA dispersion in hymenopteran insects. Journal of Evolutionary Biology, 34(9): 1466-1476.

MEYNE, J., HIRAI, H., IMAI, H.T. (1995) FISH analysis of the telomere sequences of bulldog ants (Mymecia: Formicidae). Chromosoma, 104: 14–18.

MICOLINO, R., CRISTIANO, M.P., TRAVENZOLI, N.M., LOPES, D.M. CARDOSO, D.C. (2019a) Chromosomal dynamics in space and time: evolutionary history of *Mycetophylax* ants across past climatic changes in the Brazilian Atlantic coast. Scientific Reports, 9(1): 1–13.

MICOLINO, R., CRISTIANO, M.P., CARDOSO, D.C. (2019b). Population-based cytogenetic banding analysis and phylogenetic relationships of the neotropical fungus-farming ant *Trachymyrmex holmgreni* Wheeler, 1925. Cytogenetic and Genome Research, 159(3): 151–162.

MICOLINO, R., CRISTIANO, M.P., CARDOSO, D.C. (2020). Karyotype and putative chromosomal inversion suggested by integration of cytogenetic and molecular data of the fungus-farming ant *Mycetomoellerius iheringi* Emery, 1888. Comparative Cytogenetics, 14(2): 197–210.

MICOLINO, R., BALDEZ, B.C.L., SÁNCHEZ-RESTREPO, A.F., CALCATERRA, L., CRISTIANO, M.P., CARDOSO, D.C. (2022). Karyotype structure and cytogenetic markers of *Amoimyrmex bruchi* and *Amoimyrmex silvestrii*: Contribution to understanding leaf-cutting ant relationships. Genome, 65(1): 43-51.

MURAKAMI, T., FUJIWARA, A., YOSHIDA, M.C. (1998). Cytogenetics of ten ant species of the tribe Attini (Hymenoptera, Formicidae) in Barro Colorado Island, Panama. Chromosome Science, 2: 135-139.

NGUYEN, P., SAHARA, K., YOSHIDO, A. AND MAREC, F. (2010). Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). Genetica, 138: 343–354.

NYGAARD, S., HU, H., LI, C., SCHIØTT, M., CHEN, Z., YANG, Z., et al. (2016). Reciprocal genomic evolution in the ant–fungus agricultural symbiosis. Nature Communications, 7(1): 1-9.

PAÇO, A., FREITAS, R., VIEIRA-DA-SILVA, A. (2019). Conversion of DNA sequences: From a transposable element to a tandem repeat or to a gene. Genes, 10(12): 1014.

PALACIOS-GIMENEZ, O.M., CABRAL-DE-MELLO, D.C. (2015). Repetitive DNA chromosomal organization in the cricket *Cycloptiloides americanus*: a case of the unusual X1X20 sex chromosome system in Orthoptera. Molecular Genetics and Genomics, 290(2): 623-631.

PEREIRA, T.T.P., REIS, A.C.C., CARDOSO, D.C., CRISTIANO, M.P. (2018). Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger, 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera). Comparative Cytogenetics, 12(1): 13–26.

PISCOR, D., PARISE-MALTEMPI, P.P. (2016). Microsatellite organization in the B chromosome and A chromosome complement in *Astyanax* (Characiformes, Characidae) species. Cytogenetic and Genome Research, 148(1): 44-51.

ROA, F., GUERRA, M. (2012). Distribution of 45S rDNA sites in chromosomes of plants: structural and evolutionary implications. BMC Evolutionary Biology, 12(1): 1–13.

RUIZ-RUANO, F.J., CABRERO, J., LÓPEZ-LEÓN, M.D., CAMACHO, J.P.M. (2017). Satellite DNA content illuminates the ancestry of a supernumerary (B) chromosome. Chromosoma,126(4): 487-500.

RUIZ-RUANO, F.J., CUADRADO, Á., MONTIEL, E.E., CAMACHO, J.P.M., LÓPEZ-LEÓN, M.D. (2015). Next generation sequencing and FISH reveal uneven and nonrandom microsatellite distribution in two grasshopper genomes. Chromosoma, 124(2): 221-234.

SANTOS, I.S., MARIANO, C.S.F., DELABIE, J.H.C., COSTA, M.A., CARVALHO, A.F. AND SILVA, J.G. (2016). "Much more than a neck": karyotype differentiation between *Dolichoderus attelaboides* (Fabricius, 1775) and *Dolichoderus decollatus* F. Smith, 1858 (Hymenoptera: Formicidae) and karyotypic diversity of five other Neotropical species of *Dolichoderus* Lund, 1831. Myrmecological News, 23: 61–69.

SAYAH, D.M., SOKOLSKAJA, E., BERTHOUX, L., LUBAN, J. (2004). Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature, 430: 569–573.

SCHULTZ, T.R., BRADY, S.G. (2008). Major evolutionary transitions in ant agriculture. Proceedings of the National Academy of Sciences, 105(14): 5435-5440.

SHAPIRO J.A., STERNBERG, R.V. (2005). Why repetitive DNA is essential to genome function. Biological reviews of the Cambridge Philosophical Society, 80(2): 227–250.

SHATSKIKH, A.S., KOTOV, A.A., ADASHEV, V.E., BAZYLEV, S.S., OLENINA, L.V. (2020). Functional significance of satellite DNAs: Insights from *Drosophila.* Frontiers in Cell and Developmental Biology, 8: 312.

SOCHOROVÁ, J., GARCIA, S., GÁLVEZ, F., SYMONOVÁ, R. AND KOVARˇÍK, A. (2018). Evolutionary trends in animal ribosomal DNA loci: introduction to a new online database. Chromosoma, 127: 141–150.

SOLOMON, S.E., RABELING, C., SOSA-CALVO, J., LOPES, C.T., RODRIGUES, A., VASCONCELOS, H.L. et al. (2019). The molecular phylogenetics of *Trachymyrmex* Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of ’higher attine’ ant agriculture. Systematic Entomology, 44(4): 939–956.

SOSA-CALVO, J., JESOVNIK, A., OKONSKI, E., SCHULTZ, T.R. (2015). Locating, collecting, and maintaining colonies of fungus-farming ants (Hymenoptera: Myrmicinae: Attini). Sociobiology, 62(2): 300-320.

SOSA-CALVO, J., SCHULTZ, T.R., JEŠOVNIK, A., DAHAN, R.A., RABELING, C. (2018). Evolution, systematics, and natural history of a new genus of cryptobiotic fungus-growing ants. Systematic Entomology, 43(3): 549-567.

SUMNER, A.T. (2003). Chromosomes: Organization and Function. North Berwick, United Kingdom: Blackwell Publishing.

SYMONOVÁ, R. (2019). Integrative rDNAomics—Importance of the oldest repetitive fraction of the eukaryote genome. Genes, 10(5): 345–360.

TEIXEIRA, G.A., BARROS, L.A.C., AGUIAR, H.J.A.C., POMPOLO, S.G. (2017). Comparative physicalmapping of 18S rDNA in the karyotypes of six leafcutter ant species of the genera *Atta* and *Acromyrmex* (Formicidae: Myrmicinae). Genetica, 145: 351–357.

TEIXEIRA, G.A., BARROS, L.A.C., LOPES, D.M., AGUIAR, H.J.A.C. (2020). Cytogenetic variability in four species of *Gnamptogenys* Roger, 1863 (Formicidae: Ectatomminae) showing chromosomal polymorphisms, species complex, and cryptic species. Protoplasma, 257: 549–560.

TERENCIO, M.L., SCHNEIDER, C.H., GROSS, M.C., CARMO, E.J., NOGAROTO, V., ALMEIDA, M.C., et al. (2015). Repetitive sequences: the hidden diversity of heterochromatin in prochilodontid fish. Comparative Cytogenetics, 9(4): 465-481.

THAKUR, J., PACKIARAJ, J., HENIKOFF, S. (2021). Sequence, chromatin and evolution of satellite DNA. International Journal of Molecular Sciences, 22(9): 4309.

WATSON, J.M., RIHA, K. (2010). Comparative biology of telomeres: where plants stand. FEBS letters, 584(17): 3752-3759.

WEBER, N.A. (1966). Fungus-Growing Ants. Science, 153: 587–604.

ZHANG, D.X. (2004). Lepidopteran microsatellite DNA: redundant but promising. Trends in Ecology & Evolution, 19(10): 507-509.

ZWICK, M.S., HANSON, R.E., ISLAM-FARIDI, M.N., STELLY, D.M., WING, R.A., PRICE, H.J., MCKNIGHT, T.D. (1997). A rapid procedure for the isolation of C_0t-1 DNA from plants. Genome, 40(1): 138-142.

Capítulo I

Evolutionary insights into the genomic organization of major ribosomal DNA in ant chromosomes

Artigo publicado na revista *Insect Molecular Biology*

Insect Molecular Biology

Insect Molecular Biology (2021) doi: 10.1111/imb.12699

Evolutionary insights into the genomic organization of major ribosomal DNA in ant chromosomes

G. A. Teixeira*†, H. J. A. C. de Aguiar‡, F. Petitclerc§, J. Orivel§, D. M. Lopes† and L. A. C. Barros‡

**Programa de Pós-graduação em Biologia Celular e Estrutural, Universidade Federal de Viçosa, Viçosa, Brazil; †Laboratório de Citogenética de Insetos, Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, Brazil; ‡Universidade Federal do Amapá, Campus Binacional, BR 156, n° 3051, Bairro Universidade, Oiapoque, 68980-000, Brazil; and §CNRS, UMR EcoFoG, AgroParisTech, CIRAD, INRA, Université de Guyane, Université des Antilles, Campus Agronomique, Kourou, France*

Abstract

The major rDNA genes are composed of tandem repeats and are part of the nucleolus organizing regions (NORs). They are highly conserved and therefore useful in understanding the evolutionary patterns of chromosomal locations. The evolutionary dynamics of the karyotype may affect the organization of rDNA genes within chromosomes. In this study, we physically mapped 18S rDNA genes in 13 Neotropical ant species from four subfamilies using fluorescence *in situ* hybridization. Furthermore, a survey of published rDNA cytogenetic data for 50 additional species was performed, which allowed us to detect the evolutionary patterns of these genes in ant chromosomes. Species from the Neotropical, Palearctic, and Australian regions, comprising a total of 63 species from 19 genera within six subfamilies, were analysed. Most of the species (48 out of 63) had rDNA genes restricted to a single chromosome pair in their intrachromosomal regions. The position of rDNA genes within the chromosomes appears to hinder their dispersal throughout the genome, as translocations and ectopic recombination are uncommon in intrachromosomal regions because they can generate meiotic abnormalities. Therefore, rDNA genes restricted to a single chromosome pair seem to be a plesiomorphic feature in ants, while multiple rDNA sites, observed in distinct subfamilies, may have independent origins in different genera.

Keywords: evolution, ribosomal DNA, gene dispersion, Formicidae, chromosome rearrangements, physical mapping.

Introduction

Eukaryotic genomes have repetitive tandem sequences such as in the major ribosomal RNA genes (45S = 18S + 5.8S + 28S), herein denominated rDNA, which contain highly conserved genic sequences and are therefore useful as molecular genetic markers, allowing comparisons across distant taxa. However, intergenic spacers vary in both sequence and length (Long and Dawid, 1980; Sumner, 2003; Symonová, 2019). The 45S ribosomal genes are part of the nucleolus organizing regions (NORs) and are located in portions of the DNA that, after their condensation, usually appear as secondary constrictions on metaphase chromosomes (Sumner, 2003).

The silver nitrate impregnation technique (Ag-NOR banding) shows only transcriptionally active NORs by staining nuclear acid proteins involved in transcription (Howell and Black, 1980; reviewed by Sumner, 2003). Species with multiple rDNA clusters do not usually exhibit silver-staining in all clusters (reviewed by Sumner, 2003; Vicari *et al.*, 2008). However, it must be assumed that if a species has only a single NOR (or single 45S rDNA gene site), then it will be transcriptionally active, as evidenced for different organisms (Dobigny *et al.*, 2002; Barros *et al.*, 2015; Falcione *et al.*, 2018; Cholak *et al.*, 2020; Malimpensa *et al.*, 2020).

Intercellular and interindividual variations are frequently detected by the Ag-NOR banding method (Zurita *et al.*, 1997; Cross *et al.*, 2003; Walker *et al.*, 2014; Schmid *et al.*, 2017). However, the use of this technique in ant species has not produced reliable results owing to low repeatability, difficulty in obtaining good-quality markings, and the appearance of unspecified marks on heterochromatic regions of many

Correspondence: Hilton Jeferson Alves Cardoso de Aguiar, Universidade Federal do Amapá, Campus Binacional – Oiapoque, n° 3051, Bairro Universidade, 68980-000, Oiapoque, Amapá, Brazil. hilton@unifap.br

chromosomes, which makes using this procedure unsatisfactory and inconclusive (Imai *et al.,* 1992; Hirai *et al.*, 1994; Lorite *et al.*, 1997; Barros *et al.*, 2009, 2015; Aguiar *et al.*, 2017).

Since the 1980s, molecular cytogenetic tools have been used to study karyotypes. For example, fluorescence *in situ* hybridization (FISH) has proven to be an effective and precise tool for physically mapping specific DNA sequences within chromosomes (reviewed by Levsky and Singer, 2003; Liehr, 2017). The rDNA genes can be located in single or multiple chromosome pairs (Sochorová *et al.*, 2018). In several organisms, studies of these genes have pointed to chromosomal differences within species complexes (Mantovani *et al.*, 2005; Barbosa *et al.*, 2017; Dutra *et al.,* 2020) and between species with similar karyotypes (Panzera *et al.*, 2012; Golub *et al.*, 2015; Gokhman *et al.*, 2016). As a consequence, inferences can be made based on chromosomal rearrangements that shape the chromosomal evolution of a species (Roy *et al.*, 2005; Nguyen *et al.*, 2010; Britton-Davidian *et al.*, 2012; Cabral-de-Mello *et al.*, 2011; Dutrillaux and Dutrillaux, 2012; Roa and Guerra, 2012; Menezes *et al.*, 2019; Degrandi *et al.*, 2020).

In ants, the physical mapping of rDNA genes using the FISH technique was first described in Australian ants by Hirai *et al.* (1994, 1996). Since then, the number of studies mapping these genes has increased (Mariano *et al.*, 2008; Santos *et al.*, 2016; Micolino *et al.*, 2019a; Teixeira *et al.*, 2020) and other repetitive sequences, such as telomeres (Meyne *et al.*, 1995; Pereira *et al.*, 2018; Micolino *et al.*, 2020; Castro *et al.*, 2020), satellite DNA (Lorite *et al.*, 2004; Huang *et al.*, 2016), 5S ribosomal genes (Aguiar *et al.*, 2017), and microsatellites (Barros *et al.*, 2018; Micolino *et al.*, 2019b), have been mapped in the chromosomes using the FISH technique. To date, molecular cytogenetic studies on rDNA genes in ants have improved understanding of chromosomal evolution and phylogeny and provided taxonomic resolutions for different ant groups (Hirai *et al.*, 1994, 1996; Santos *et al.*, 2010, 2016).

In this study, we physically mapped 18S rDNA clusters using the FISH technique and verified if they were GC-rich in 13 Neotropical ant species from four subfamilies (Ectatomminae, Formicinae, Myrmicinae, and Ponerinae). In addition, we reviewed previous molecular cytogenetic data related to rDNA gene clusters (45S, 18S, or 28S) in ants. Using these data, we investigated whether the number and location of the ribosomal gene clusters followed a specific pattern or were randomly distributed in order to understand the genomic organization and evolutionary dynamics of these genes in ants.

Results

Chromosome mapping of 18S rDNA clusters in 13 Neotropical ant species

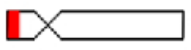
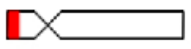
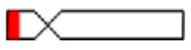
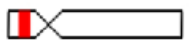
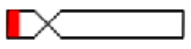
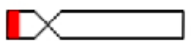
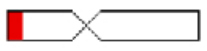
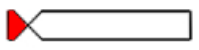
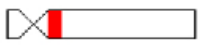
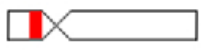
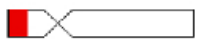
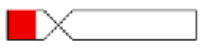
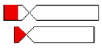
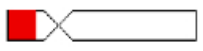
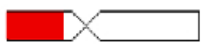
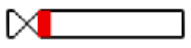
All of the studied ant species presented only a single 18S rDNA site that was colocalized with GC-rich regions (CMA_3^+) (Table 1), while AT-rich regions ($DAPI^+$) were not detected in any species. Most of the species presented GC-rich rDNA genes in the intrachromosomal regions (pericentromeric or interstitial), including *Pseudoponera gilberti* (Kempf, 1960) (Fig. 1A, S1A), *Anochetus targionii* Emery, 1894 (Fig. 1B, S1B), *Odontomachus haematodus* (Linnaeus, 1758) (Fig. 1C, D, S1C), *Odontomachus bauri* Emery, 1892 (Fig. 1E, S1D), *Pheidole germaini* Emery, 1896 (Fig. 2A, S2A), *Crematogaster longispina* Emery, 1890 (Fig. 2B, S2B), *Solenopsis geminata* (Fabricius, 1804) (Fig. 2C, D, S2C), *Myrmicocrypta* sp. (Fig. 2E, S2D), and *Acromyrmex echinatior* (Forel, 1899) (Fig. 2F). Additional GC-rich bands only occurred in *P. gilberti* in the pericentromeric region of the 3rd and 4th metacentric chromosome pairs (Fig. S1B). Differences in rDNA cluster sizes between homologous chromosomes were observed in heterozygous *O. bauri* individuals. One of the homologous chromosomes showed clusters approximately twice the size of those in the other chromosome (Fig. 1F, S1E). A male individual bearing a chromosome with minor GC-rich bands was analysed (Fig. S1F). Homozygous individuals with duplicated clusters were not observed.

The remaining species showed GC-rich 18S rDNA clusters across the entire chromosome arm, occupying either the long arm, as in *Gnamptogenys tortuolosa* (Smith, 1858) (Fig. 3A, S3A), or residing in the short arm, as in *Strumigenys diabola* Bolton, 2000 (Fig. 2G, S2E), *Camponotus atriceps* (Smith, 1858) (Fig. 3B, S3B), and *Gigantiops destructor* (Fabricius, 1804) (Fig. 3C, S3C, D). Heteromorphism of 18S rDNA clusters was detected in all of the analysed *C. atriceps* and *G. tortuolosa* individuals. In the latter species, the heteromorphism of the NOR resulted in differences in total size between homologous chromosomes, which changed their morphology such that one was submetacentric while the other was subtelocentric. In *G. destructor*, additional GC-rich bands were located in the interstitial region of the long arm of the largest subtelocentric chromosome pair (Fig. S3C, D).

Chromosome mapping review of rDNA clusters in ants

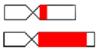
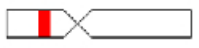
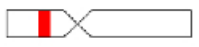
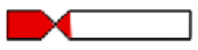
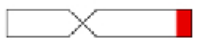
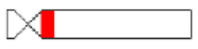
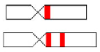
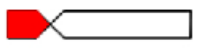
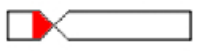
Cytogenetic data available in the literature related to the rDNA genes of 50 ant species from 12 genera and six subfamilies were reviewed (Table 1; Fig. 4). Most data were concentrated on Neotropic ants, with information on 33 species, while the Palearctic and Australian regions had data on only one and 16 species, respectively. A single rDNA site localized in the intrachromosomal region was observed in most species (Fig. 4A, B). However, *Camponotus renggeri* Emery, 1894, *Dinoponera gigantea* (Perty, 1833), and most of the studied *Myrmecia* species presented multiple rDNA sites over the entire short chromosome arm. The subfamily Myrmicinae possessed most of the rDNA data, and the Myrmeciinae subfamily showed

Table 1. Summary of the available molecular cytogenetic data concerning major ribosomal genes (45S, 28S or 18S) detected by FISH in ants [Colour table can be viewed at wileyonlinelibrary.com].

Ant species	2*n*/(*n*)	Localization of rDNA genes in karyotype	Ideogram	Co-localization CMA$_3$/rDNA	Reference CMA$_3$/rDNA
Neotropical ants					
Subfamily Myrmicinae					
Acromyrmex aspersus	38	Largest subtelocentric pair		Yes	Teixeira *et al.* (2017)
Acromyrmex coronatus	38	Largest subtelocentric pair		Yes	Barros *et al.* (2016)
Acromyrmex disciger	38	Largest subtelocentric pair		Yes	Barros *et al.* (2016
Acromyrmex echinatior	38	Largest subtelocentric pair		Yes	Barros *et al.* (2016)/ **Present study**
Acromyrmex niger	38	Largest subtelocentric pair		Yes[a]	Barros *et al.* (2016)
Acromyrmex striatus	22	2nd metacentric pair		Yes[a]	Cristiano *et al.* (2013)/ Teixeira *et al.* (2017)
Acromyrmex subterraneus molestans	38	Largest subtelocentric pair		Yes	Teixeira *et al.* (2017)
Atta bisphaerica	22	4th metacentric pair		Yes	Barros *et al.* (2014)/Teixeira *et al.* (2017)
Atta laevigata	22	4th metacentric pair		Yes	Barros *et al.* (2014)/Teixeira *et al.* (2017)
Atta robusta	22	4th metacentric pair		Yes	Barros *et al.* (2015)
Atta sexdens rubropilosa	22	4th metacentric pair		Yes	Barros *et al.* (2014)/Teixeira *et al.* (2017)
Crematogaster longispina	24	Largest metacentric pair		Yes	**Present study**
Mycetophylax conformis	30	11th metacentric pair		Yes	Cardoso *et al.* (2014)/ Micolino *et al.* (2019a)
Mycetophylax morschi	26	2nd submetacentric pair		–	Micolino *et al.* (2019a)
Mycetophylax morschi	28	7th metacentric pair		–	Micolino *et al.* (2019a)
Mycetophylax morschi	30	Acrocentric pair		–	Micolino *et al.* (2019a)
Mycetophylax simplex	36	Smallest metacentric pair		Yes	Cardoso *et al.* (2014)/ Micolino *et al.* (2019a)
Mycocepurus goeldii	8	2nd metacentric pair		Yes[a]	Barros *et al.* (2010)/Barros *et al.* (2012)
Myrmicocrypta sp.	30	9th metacentric pair		Yes	**Present study**
Pheidole germaini	22	Subtelocentric pair		Yes	**Present study**
Solenopsis geminata	32	Smallest submetacentric pair		Yes	**Present study**
Strumigenys diabola	40	4th submetacentric pair		Yes	**Present study**
Mycetomoellerius holmgreni	20	4th metacentric pair		Yes	Barros *et al.* (2018)
Subfamily Formicinae					
Camponotus atriceps[c]	40	2nd submetacentric pair		Yes	**Present study**
Camponotus cingulatus[c]	40	2nd submetacentric pair		Yes	Aguiar *et al.* (2017)
Camponotus renggeri	40	2nd submetacentric pair and medium-sized subtelocentric pair		Yes	Aguiar *et al.* (2017)
Camponotus rufipes[c]	40	2nd submetacentric pair		Yes	Aguiar *et al.* (2017)
Gigantiops destructor	(39)	8th metacentric pair		Yes[a]	**Present study**
Subfamily Ponerinae					
Anochetus altisquamis[c]	30	3rd submetacentric pair		–	Santos *et al.* (2010)
Anochetus horridus[c]	46	4th telocentric pair		–	Santos *et al.* (2010)
Anochetus targionii	30	7th metacentric pair		Yes	**Present study**
Dinoponera gigantea	82	Multiple pairs	-	–	Aguiar *et al.* (2011)
Dinoponera lucida	(59)	Largest pair		Yes	Mariano *et al.* (2008)
Odontomachus bauri[d]	44/(22)	2nd subtelocentric pair		Yes	**Present study**
Odontomachus haematodus	44	3rd subtelocentric pair		Yes	**Present study**

(*Continued*)

Table 1. Continued

Ant species	2*n*/(*n*)	Localization of rDNA genes in karyotype	Ideogram	Co-localization CMA$_3$/rDNA	Reference CMA$_3$/rDNA
Pseudoponera gilberti	22	Largest metacentric pair		Yes [a]	**Present study**
Subfamily Ectatomminae					
Gnamptogenys moelleri	34, 44	4th metacentric pair		Yes	Teixeira *et al.* (2020)
Gnamptongeys regularis [d]	26	Metacentric and submetacentric homologues		Yes	Teixeira *et al.* (2020)
Gnamptogenys striatula	32	5th metacentric pair		Yes	Teixeira *et al.* (2020)
Gnamptogenys striatula	34	4th metacentric pair		Yes	Teixeira *et al.* (2020)
Gnamptogenys triangularis	24	Largest metacentric pair		Yes	Teixeira *et al.* (2020)
Gnamptogenys tortuolosa [c]	44	Subtelocentric and submetacentric homologues		Yes	**Present study**
Subfamily Dolichoderinae					
Dolichoderus attelaboides	58	Largest submetacentric pair		Yes	Santos *et al.* (2016)
Dolichoderus bidens	18	Largest metacentric pair		Yes [a]	Santos *et al.* (2016)
Dolichoderus decollatus	38	2nd metacentric pair		Yes	Santos *et al.* (2016)
Dolichoderus diversus	22	Largest metacentric pair		Yes [a]	Santos *et al.* (2016)
Dolichoderus imitator	38	Largest metacentric pair		Yes	Santos *et al.* (2016)
Dolichoderus lutosus	10	2nd metacentric pair		Yes [a]	Santos *et al.* (2016)
Dolichoderus voraginosus	20	Largest metacentric pair		No [a]	Santos *et al.* (2016
Australian ants					
Subfamily Myrmeciinae					
Myrmecia banksi [c]	10	Smallest acrocentric pair		–	Hirai *et al.* (1994), (1996)
Myrmecia chasei	47	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia croslandi [d]	2, 3, 4 [b]	Acrocentric pair		–	Hirai *et al.* (1994), (1996)
Myrmecia forficata	52 [b], 54	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia fulvipes	48, 50	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia gulosa	38 [b]	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia haskinsorum	18	Multiple pairs	-	–	Hirai *et al.* (1994), (1996)
Myrmecia imaii [c]	8	Largest acrocentric pair		–	Hirai *et al.* (1994), (1996)
Myrmecia mandibularis	56 [b]	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia michaelseni	27	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia occidentalis	64 [b]	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia pavida	44	Multiple pairs	-	–	Hirai *et al.* 1996
Myrmecia pilosula	22 [b]	Acrocentric pair		–	Hirai *et al.* (1996)
Myrmecia pilosula	19, 23 [b], 27, 32	Multiple pairs	-	–	Hirai *et al.* (1994), (1996)
Myrmecia simillima	70 [b]	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia arnoldi	53, 55, 57, 59, 60[b], 64, 66	Multiple pairs	-	–	Hirai *et al.* (1996)
Myrmecia vindex	74, 76	Multiple pairs	-	–	Hirai *et al.* (1996)
Palearctic ant					
Subfamily Dolichoderinae					
Tapinoma nigerrimum	18	Largest submetacentric pair		Yes	Lorite *et al.* (1997)

[a] Additional markers CMA$_3^+$.
[b] Data showed by Hirai *et al.* (1996).
[c] Heteromorphism of rDNA clusters between the homologues.
[d] Polymorphism of rDNA clusters between the homologues
— unavailable data.

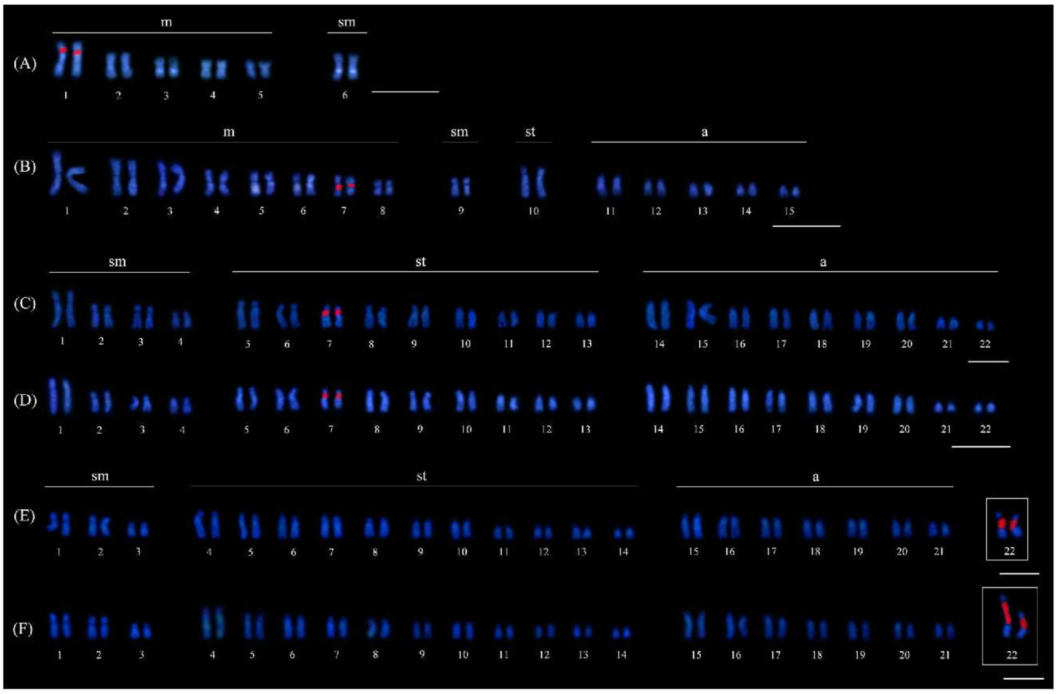


Figure 1. Fluorescence *in situ* hybridization with 18S rDNA probe (red blocks) of Ponerinae ants: (A) *Pseudoponera gilberti* ($2n = 12$), (B) *Anochetus targionii* ($2n = 30$), (C, D) *Odontomachus haematodus* ($2n = 44$, Brazil and French Guiana, respectively), (E, F) *Odontomachus bauri* ($2n = 44$). In the last species, the 18S rDNA site was located on the long arm of the polymorphic chromosomal pair. The boxes show a polymorphic subtelocentric pair in (E) in the homozygous state, and (F) in the heterozygous state. Bars = 5 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

a different pattern in relation to other subfamilies with multiple rDNA sites observed in the majority of species (Fig. 4C). *Dolichoderus voraginosus* Mackay, 1993 did not show any co-localization of 18S rDNA clusters and GC-rich bands. Fig. 5 summarizes the available data on the number and position of NORs in ant species and the phylogenetic relationships among these species and is based on the published molecular phylogenies.

Discussion

Patterns of rDNA clusters in the karyotypes of specific ant groups

Specific rDNA patterns can be observed in the karyotypes of some ant groups where several species have been studied. In species of the genus *Dolichoderus*, the chromosome numbers range from $2n$ = 10 to 58, and a single rDNA site in the interstitial region has been observed in the majority of the investigated species. However, there are two exceptions: *D. voraginosus* and *Dolichoderus attelaboides* (Fabricius, 1775), where the rDNA clusters are located in the terminal region of the long arm and in the short arm, respectively (Santos *et al*., 2016).

Despite the chromosomal variation observed in *Gnamptogenys* spp. ($2n$ = 24–44), they all have a single rDNA site in the intrachromosomal region (Teixeira *et al*., 2020). Even *Gnamptogenys moelleri* (Forel, 1912), a species with differing chromosome numbers between two populations ($2n$ = 34 and 44), has a single intrachromosomal rDNA site. *Gnamptogenys tortuolosa* is an exception, with rDNA clusters occurring over the entire long arm (this study). In the genus *Anochetus*, the chromosome number ranges from $2n$ = 30 to 46, and a single pericentromeric rDNA site has been observed (Santos *et al*., 2010).

All analysed fungus-farming ants (Attina) had a single rDNA site (Table 1). In the genus *Mycetophylax*, although chromosome number differs among species ($2n$ = 26–36), a single rDNA site is located in the pericentromeric or terminal region in all the species of the genus (Micolino *et al*., 2019a). The leaf-cutting ants are considered the most derived among the Attina species (Schultz and Brady, 2008). *Atta* spp. ($2n$ = 22) have a single rDNA site in the interstitial region and *Acromyrmex* spp. ($2n$ = 38)

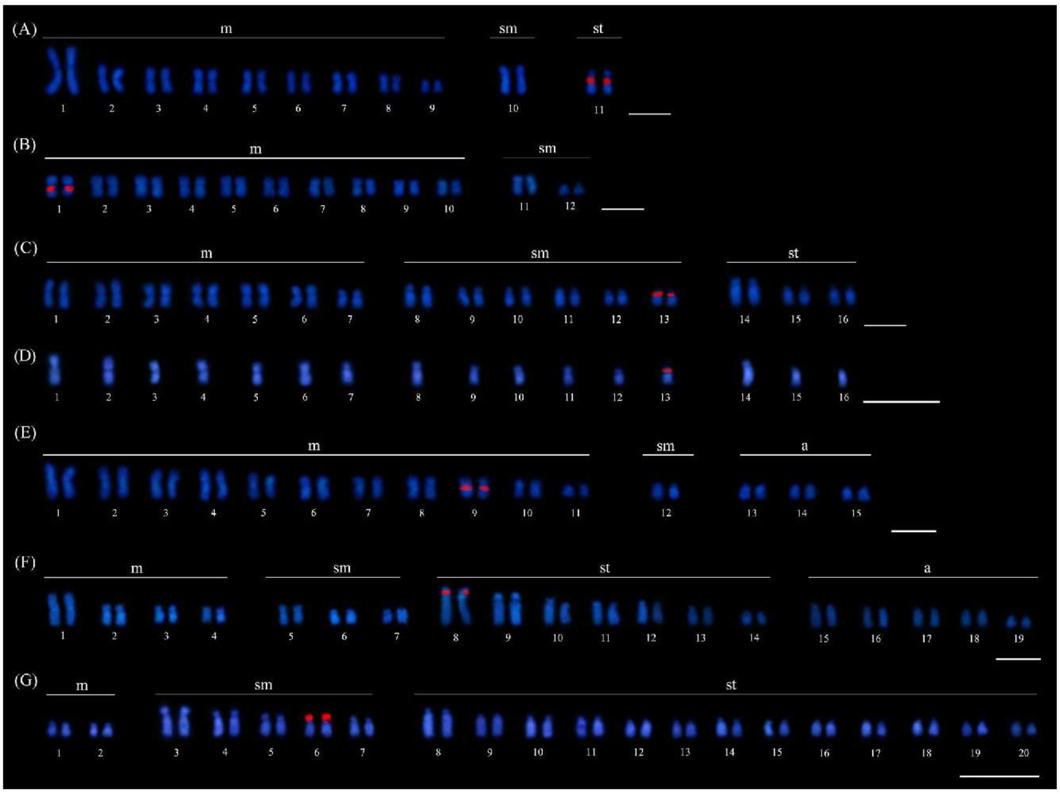


Figure 2. Fluorescence *in situ* hybridization with 18S rDNA probe (red blocks) of Myrmicinae ants: (A) *Pheidole germaini* ($2n = 22$), (B) *Crematogaster longispina* ($2n = 24$), (C) *Solenopsis geminata* ($2n = 32$) and (D) *Solenopsis geminata* ($n = 16$), (E) *Myrmicocrypta* sp. ($2n = 30$), (F) *Acromyrmex echinatior* ($2n = 38$), (G) *Strumigenys diabola* ($2n = 40$). Bars = 5 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

have a single rDNA site in the terminal region (Barros *et al.*, 2015, 2016; Teixeira *et al.*, 2017). *Acromyrmex striatus* (Roger, 1863), a sister group of the leaf-cutting ants, has $2n = 22$ and pericentromeric rDNA clusters that are not located on the same chromosome pair relative to that in *Atta* spp. (Cristiano *et al.*, 2013; Teixeira *et al.*, 2017). In *A. echinatior* ($2n = 38$), rDNA clusters are located in the interstitial region of the same pair as observed in other *Acromyrmex* spp. (this study).

The chromosome number of the Australian bulldog ants is highly variable, ranging from $2n = 2$ to 76 (Imai *et al.*, 1994). In addition, *Myrmecia* spp. present remarkable patterns of multiple 28S rDNA clusters that are highly dispersed throughout their genomes. The number of rDNA sites increases with the chromosome number of the species. This suggests several ribosomal gene amplification events have occurred in the different species of *Myrmecia* and that they have accumulated in karyotypes throughout the evolution of the genus. Only four out of 16 species from this monophyletic genus have the entire arm or intrachromosomal rDNA clusters restricted to a single pair of chromosomes (Hirai *et al.*, 1994, 1996; Hirai, 2020). This pattern is observed in species with small chromosome numbers, which suggests that a single NOR is plesiomorphic among *Myrmecia* (Hirai, 2020).

Carpenter ants (*Camponotus*) from the subgenus *Myrmothrix* have $2n = 40$ chromosomes, with its studied species having a single rDNA site in the terminal region. *Camponotus renggeri* is the only exception, having an additional rDNA cluster at the terminal region of a medium-sized subtelocentric pair (Aguiar *et al.*, 2017).

In the giant ants of the genus *Dinoponera*, two contrasting patterns have been observed: *D. gigantea* ($2n = 82$) has multiple rDNA sites located on its short chromosome arms (Aguiar *et al.*, 2011), whereas *Dinoponera lucida* Emery, 1901 has a higher chromosome number ($2n = 120$), but only a single rDNA site restricted to the

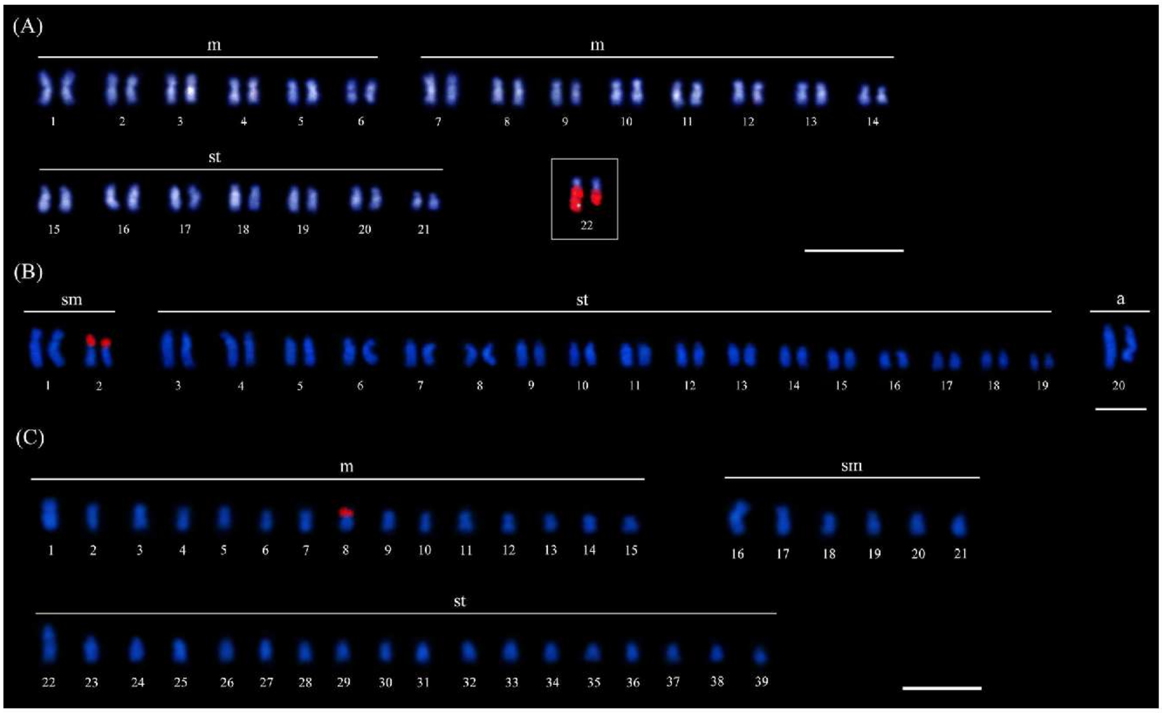


Figure 3. Fluorescence *in situ* hybridization with 18S rDNA probe (red blocks) of an Ectatomminae ant (A) *Gnamptogenys tortuolosa* ($2n = 44$), and Formicinae ants (B) *Camponotus atriceps* ($2n = 40$) and (C) *Gigantiops destructor* ($n = 39$). In *G. tortuolosa*, the box shows a remarkable size heteromorphism of 18S rDNA clusters between homologues. Bars: 5 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

intrachromosomal region of its largest chromosome pair (Mariano *et al.*, 2008).

Homeology patterns among chromosomal pairs bearing ribosomal genes can be detected in a few ant genera, such as *Gnamptogenys* (*striatula* group), *Camponotus* (*Myrmothrix*), *Atta*, and *Acromyrmex* (Table 1). However, it is speculative to infer such homeology patterns for the entire Formicidae family. Ants constitute an ultra-diverse monophyletic group with more than 13 800 described species (Bolton, 2020). They show a wide range of karyotype variation both in number ($2n = 2–120$) and chromosomal morphology (reviewed by Lorite and Palomeque, 2010; Mariano *et al.*, 2019).

Insights concerning the organizational patterns of ribosomal gene clusters in the ant genome

The mapping of the ribosomal gene clusters of 63 species distributed in 19 genera and six subfamilies, together with information on their phylogenetic relationships, demonstrated that a single pair of chromosomes bearing the GC-rich rDNA clusters is the most frequent trait among the studied species, regardless of the chromosome number (Table 1, Fig. 4A). Genomes carrying rDNA clusters in more than a single chromosome pair have been observed in non-related taxa, such as *D. gigantea* (Aguiar *et al.*, 2011), *C. renggeri* (Aguiar *et al.*, 2017), and *Myrmecia* spp. (Hirai *et al.*, 1994, 1996). We hypothesize that having a single rDNA site should be considered a plesiomorphic trait because multiple rDNA sites were observed in different non-related lineages that do not share exclusive common ancestry and appear *de novo* throughout the Formicidae family.

In eukaryotes, it is common to observe variations in the number of rDNA clusters and the location of these genes in the chromosomes within genera (Sánchez-Gea *et al.*, 2000; Gross *et al.*, 2010; Cabral-de-Mello *et al.*, 2011; Gokhman *et al.*, 2014; Mazzoleni *et al.*, 2018), among populations (Panzera *et al.*, 2014; Ferreti *et al.*, 2019; Menezes *et al.*, 2019), and sexes of the same species (Nakayama *et al.*, 2001; Šťáhlavský *et al.*, 2018). The possession of terminal rDNA clusters seems to be a common trait among mammals, fish, and molluscs, but less so in arthropods (Sochorová *et al.*, 2018). Within the ultra-diverse insect group, the location of rDNA clusters may follow distinct patterns in its two largest orders; terminal rDNA sites are more abundant in Coleoptera, whereas pericentromeric rDNA clusters are more frequent in Orthoptera (Sochorová *et al.*, 2018). In Formicidae, terminal rDNA sites are a less common feature and all species with multiple rDNA clusters show these genes in the entire short chromosome arms including terminal/subterminal

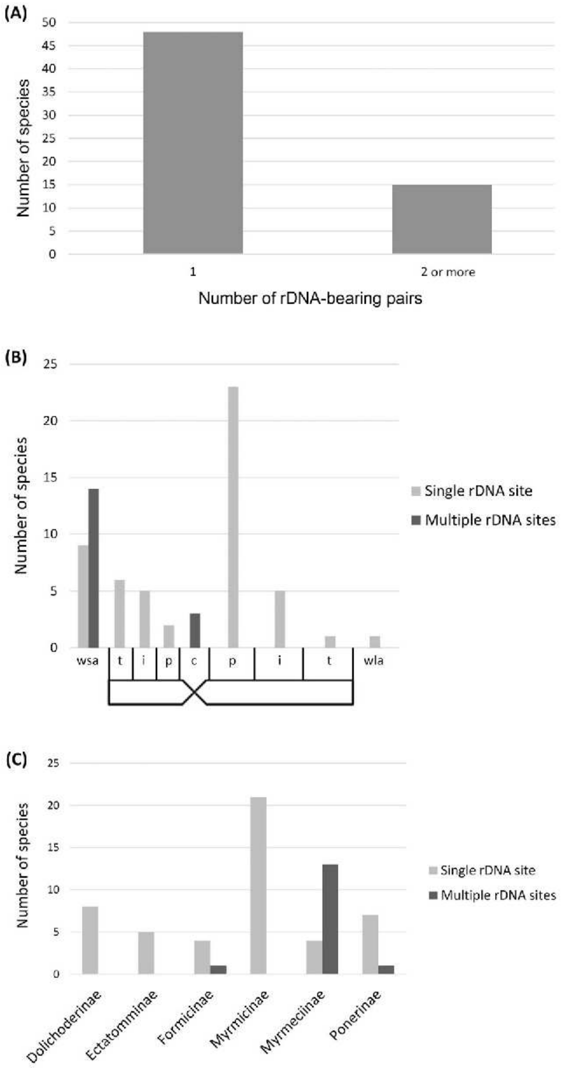


Figure 4. Summary of available molecular cytogenetic data (this study and in the literature) concerning rDNA genes in ant species: (A) number of species with single and multiple rDNA sites in a karyotype, (B) location of rDNA genes in chromosomes of ant species, considering the following chromosomal regions: c, centromeric region; p, pericentromeric region; i, interstitial region; t, terminal region; wsa, whole short arm; wla, whole long arm, and (C) number of species with single and multiple rDNA sites by subfamilies of Formicidae.

regions, such as *C. renggeri*, *D. gigantea*, and *Myrmecia* spp. (Aguiar *et al.*, 2011, 2017; Hirai *et al.*, 1994, 1996).

Different hypotheses have been proposed to explain the cytogenetic pattern (conservative or variable) of these rDNA clusters in the genome of several organisms. Such hypotheses are linked to the specific locations of these rDNA clusters on the chromosomes. Rearrangements, such as translocations, unequal exchange, and ectopic recombination mechanisms (i.e., between non-homologous chromosomes), which can lead to gene dispersion or increases in number in the genome, are more likely in the terminal/subterminal regions of chromosomes and are uncommon in the intrachromosomal regions (Martins and Wasko, 2004; Mantovani *et al.*, 2005; Nguyen *et al.*, 2010; Roa and Guerra, 2012; Hirai, 2020).

Based on rDNA mapping data in fish karyotypes, Martins and Wasko (2004) proposed that translocations are more likely to occur in telomeric regions because of their proximity within the interphase nucleus, which originates from the ordering of chromosomes according to Rabl's model. Effects due to the location of ribosomal genes in relation to their dispersion in the karyotype were also observed in Coleoptera (Dutrillaux *et al.*, 2016) and primates (Gerbault-Seureau *et al.*, 2017). These authors argued that translocations in the interstitial position could result in abnormal meiosis and, therefore, unbalanced gametes. In contrast, translocations in terminal positions may increase the number of rDNA genes in the genome. This would lead to fewer meiotic abnormalities and highlights the selection for interstitial rDNA site stability (Dutrillaux *et al.*, 2016; Gerbault-Seureau *et al.*, 2017).

Ectopic recombination is another mechanism suggested to explain the rDNA patterning in moths and butterflies (Nguyen *et al.*, 2010) and plants (Roa and Guerra, 2012). It is also included in the recent model proposed by Hirai (2020). In this model, two mechanisms are important: the "site effect" and the "molecular effect." The former allows terminal region associations due to the proximity of these regions in a meiotic bouquet. The "site effect" is a precondition for the "molecular effect," which refers to systems of affinity/non-affinity due to the similarity between rDNA sequences with other repetitive sequences. Thus, rDNA clusters in the terminal regions tend to associate with other repetitive sequences of non-homologous chromosomes more easily, facilitating the occurrence of ectopic recombination and dispersion of these genes in the genome (Hirai, 2020).

There are reports of species with multiple rDNA clusters associated with the centromeres of acrocentric chromosomes (Cazaux *et al.*, 2011). In the recent model proposed by Hirai (2020), the centromeric region of acrocentric chromosomes (chromosomes with a short and heterochromatic arm) that have rDNA genes associated with the centromere may behave as subterminal regions. Therefore, such an arrangement would also facilitate eventual associations of rDNA genes with other repetitive sequences and the occurrence of ectopic recombination, which leads to their dispersal in the genome (for details, see Hirai, 2020).

The ant rDNA chromosome evolution seems to be in accordance with the above-mentioned hypothesis about dispersal and NOR location because the single rDNA clusters of most studied species are interstitial or pericentromeric (Fig. 4B). In ant species, terminal rDNA clusters are

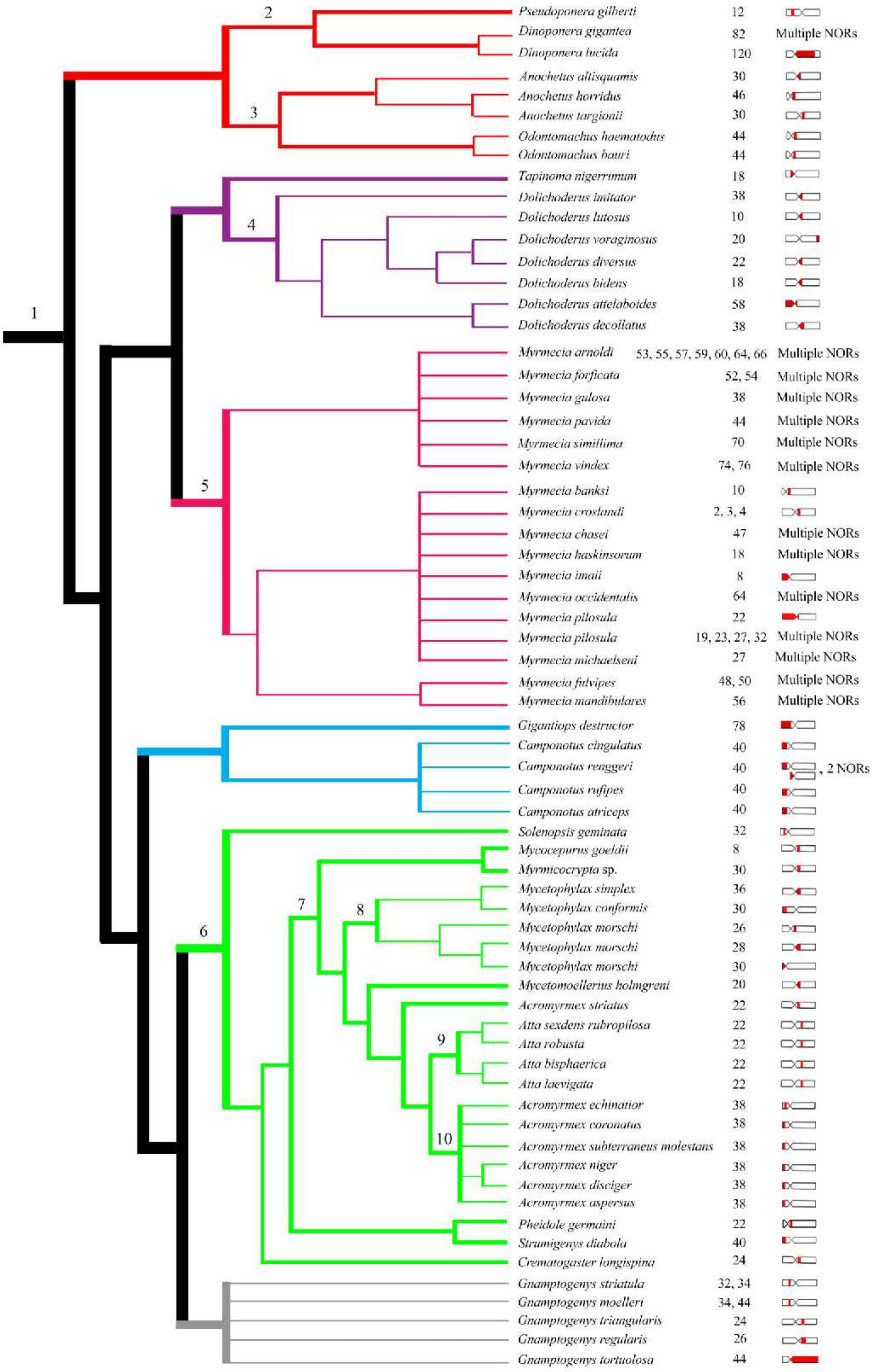


Figure 5. Summary of available data (this study and literature) concerning the number and position of rDNA genes in ant species, along with their degree of relatedness and diploid chromosome numbers. Ideograms of rDNA-bearing chromosomes show number and location (terminal, interstitial, pericentromeric or centromeric) of rDNA clusters (red) in the haploid complement. Phylogenetic relationships are based on 1—Moreau and Bell (2013), 2—Schmidt (2013), 3—Larabee *et al.* (2016), 4—Santos *et al.* (2016), 5—Hasegawa and Crozier (2006), 6—Ward *et al.* (2015), 7—Solomon *et al.* (2019), 8—Micolino *et al.* (2019a), 9—Bacci *et al.* (2009), 10—Queiroz EC (2015, unpublished data). Colours of phylogenetic branches indicate the following subfamilies: red, Ponerinae; purple, Dolichoderinae; pink, Myrmeciinae; blue, Formicinae; green, Myrmicinae; grey, Ectatomminae. [Colour figure can be viewed at wileyonlinelibrary.com].

Table 2. The relationship between Neotropical ant species studied using 18S rDNA FISH and their chromosome number

Ant species	Subfamily	Locality	2*n*
Acromyrmex echinatior (Forel, 1899)	Myrmicinae	Barro Colorado—Panamá	38
Anochetus targionii Emery 1894	Ponerinae	Campus Agronomique, Kourou—FG	30
Camponotus atriceps (Smith, 1858)	Formicinae	Viçosa—Minas Gerais—BR	40
Crematogaster longispina Emery, 1890	Myrmicinae	La Montagne des Singes—FG	24
Gigantiops destructor (Fabricius, 1804)	Formicinae	Sinnamary—FG	78
Gnamptogenys tortuolosa (Smith, 1858)	Ectatomminae	Sinnamary—FG	44
Myrmicocrypta sp.	Myrmicinae	Sinnamary—FG	30
Odontomachus bauri Emery, 1892	Ponerinae	Açailândia—Maranhão—BR	44
Odontomachus haematodus (Linnaeus, 1758)	Ponerinae	Campus Agronomique, Kourou—FG and Ubá—Minas Gerais—BR	44
Pheidole germaini Emery, 1896	Myrmicinae	Viçosa—Minas Gerais—BR	22
Pseudoponera gilberti (Kempf, 1960)	Ponerinae	Sinnamary—FG	12
Solenopsis geminata (Fabricius, 1804)	Myrmicinae	Sinnamary—FG	32
Strumigenys diabola Bolton 2000	Myrmicinae	Sinnamary—FG	40

FG, French Guiana; BR, Brazil.

prone to rearrangements that lead to their dispersal. *Camponotus renggeri* (Aguiar *et al.*, 2017), *D. gigantea* (Aguiar *et al.*, 2011), and *Myrmecia* spp. present multiple NORs in the entire short chromosome arms including terminal/subterminal regions, which facilitates the association of these genes with the heterochromatic sequences of other nonhomologous acrocentric chromosomes during meiosis and the subsequent occurrence of ectopic recombination (Hirai, 2020). This pattern may be applicable to different ant groups. In addition, inversions have been shown to change the position of rDNA genes in *A. echinatior* (this study), *Dolichoderus* spp. (Santos *et al.*, 2016), and *Myrmecia* spp. (Hirai *et al.*, 1996).

A single rDNA site located in the terminal region or entire chromosomal arm was observed in some ant species. The repetitive sequences in the subterminal/terminal chromosome regions probably do not form affinity systems with ribosomal genes (the so-called molecular effect; for details, see Hirai, 2020) in these species. Therefore, rDNA clusters are restricted to a single chromosomal pair. Future studies focusing on the characterization of repetitive sequences that make up the heterochromatin of these species will help clarify this hypothesis.

Size heteromorphisms are frequent in karyotypes where the rDNA clusters have terminal positions in the chromosomes, as reported in this study (*G. tortuolosa* and *C. atriceps*) as well as in other ants (Aguiar *et al.*, 2017) and insects in general (Cabral-de-Mello *et al.*, 2011; Maryańska-Nadachowska *et al.*, 2016; Andrade-Souza *et al.*, 2018). Subtle variations in the size of the rDNA clusters between homologous chromosomes can be observed as a result of late condensation during cell division (Sumner, 2003). However, large variations, such as those mentioned above, at the terminal region on the chromosome are usually related to duplications/deletions as a result of unequal exchange (Schubert and Lysack, 2011). It is believed that exchanges are less common in intrachromosomal regions (Hirai, 2020).

Size variations in the rDNA clusters can be observed when these genes are located in the interstitial/pericentromeric region of the chromosomes, as seen in *Gnamptogenys regularis* Mayr, 1870 (Teixeira *et al.*, 2020) and in *O. bauri* (this study). A different path seems to be involved in the evolution of these karyotypes compared to the rearrangements involved in terminal rDNA heteromorphisms. In these cases, the mechanism may be associated with the formation of extrachromosomal circular DNA (eccDNA), which is likely to form tandem repetitive sequences similar to the rDNA genes (Cohen and Segal, 2009). These eccDNAs may be lost, leading to deletions in the original rDNA sequences, or they may be replicated via a rolling circle mechanism and reintegrated into the original chromosome, producing duplications of these repetitive sequences (Cohen and Segal, 2009).

In ants as well as in other eukaryotes, rDNA clusters are located in GC-rich regions (Symonová, 2019) and, therefore, usually coincide with CMA_3^+ bands, possibly as a result of GC-biased gene conversion (gBGC) over the course of evolutionary time (Escobar *et al.*, 2011). This co-localization of GC-rich regions and rDNA was observed for all the ants studied to date (Table 1), with the exception of *D. voraginosus* (Santos *et al.*, 2016). However, GC-rich chromatin is not always an indication of ribosomal genes, as seen here in *G. destructor*, *P. gilberti*, and in some *Dolichoderus* spp. and fungus-farming ants (Table 1).

In insects, a compilation of previous data concerning rDNA genes has been conducted, for example, in moths and butterflies (Lepidoptera; Nguyen *et al.*, 2010), beetles (Coleoptera; Dutrillaux and Dutrillaux, 2012), and kissing bugs (Heteroptera; Panzera *et al.*, 2012). However, this is the first survey of Hymenoptera species. We have compiled available information and new data on 13 Neotropical ant species. In different organisms, including ants, the number and location of chromosomes bearing rDNA clusters within the genome follow general patterns that govern the modes of evolution for these genes (Martins and

Wasko, 2004; Nguyen *et al*., 2010; Dutrillaux *et al*., 2016; Gerbault-Seureau *et al*., 2017; Hirai, 2020; this study). We can conclude that having only a single pair of chromosomes bearing rDNA clusters is more common in the ant genome because of the pericentromeric/interstitial location of these genes on the chromosomes. Intrachromosomal regions are sites with low frequencies of rearrangements, such as non-Robertsonian translocations and ectopic recombination, and are therefore less prone to meiotic abnormalities. It should be assumed that the chromosomal location of rDNA clusters influences the dispersion of these genes within the karyotype.

Future studies will allow the mapping of rDNA genes in more ant taxa, including the other remaining subfamilies. Other repetitive sequences, such as 5S rDNA and histone genes, may also be mapped in ant species as a tool to investigate further patterns that reflect the relationship between chromosomal location and dispersion in the genome. Finally, a solid understanding of the evolutionary patterns of ribosomal gene dispersal in ant chromosomes may provide a comparative model for other insects.

Experimental procedures

Obtaining samples for analysis

Field surveys to collect ant colonies were performed in French Guiana, Brazil, and Panama (Table 2) from the following locations: *La Montagne des Singes*, Kourou (5.07225, −52.69407), Campus Agronomique, Kourou (5.17312, −52.65480), and Sinnamary (5.28482, −52.91403), all in French Guiana; Viçosa (−20.757041, −42.873516) and Ubá (−21.128880, −42.937646), both in Minas Gerais, Brazil, and Açailândia (−4.84200, −47.29667) in Maranhão, Brazil; and Barro Colorado Island (9.150000, −79.83333) in Panama. Sampling permit in Brazil was provided by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (SISBIO accession numbers 32 459 and 62 598). Adult specimens were identified by Dr. Jacques H. C. Delabie and deposited in the ant collection at the Laboratório de Mirmecologia do Centro de Pesquisas do Cacau (CPDC/Brazil), in Bahia, Brazil, under records #5802, #5803, and #5804.

In this study, previous molecular cytogenetic data related to rDNA genes (45S, 18S, or 28S) mapped by FISH from 50 ant species were used. For comparative analysis, the following traits were considered for each species: chromosome number, number of rDNA-bearing chromosomes, location of rDNA clusters in the karyotype, and co-localization of CMA$_3$ fluorochrome and rDNA clusters. Ag-NOR data were disregarded due to the highly variable patterns and unreliable results (for details, see Introduction).

Chromosome preparation

Mitotic metaphase chromosomes were obtained from the cerebral ganglia of larvae after meconium elimination, using colchicine hypotonic solution (0.005%) and fixatives according to the methods described by Imai *et al*. (1988).

Staining with fluorochromes chromomycin A$_3$ (CMA$_3$) and 4′6-diamidino-2-phenylindole (DAPI)

Metaphase chromosomes of all the species, except *A. echinatior*, were stained with the fluorochromes CMA$_3$ and DAPI for the detection of GC and AT-rich regions, respectively, based on the technique proposed by Schweizer (1980). The *A. echinatior* samples studied here correspond to the same colonies studied by Barros *et al*. (2016) and the CMA$_3$/DAPI staining in this species was performed by these authors.

Fluorescence in situ hybridization with rDNA 18S probe

The 18S rDNA probes were obtained by amplification via polymerase chain reaction (PCR) using *Melipona quinquefasciata* Lepeletier, 1836, rDNA primers 18SF1 (5′–GTC ATA GCT TTG TCT CAA AGA–3′) and 18SR1.1 (5′–CGC AAA TGA AAC TTT AAT CT–3′) Pereira JOP (2006, unpublished data) in the genomic DNA from the ant *Camponotus rufipes* (Fabricius, 1775). Gene amplification was performed following Pereira JOP (2006, unpublished data). The probes were labelled by an indirect method using digoxigenin-11-dUTP (Roche Applied Science, Mannheim, Germany), and the FISH signals were detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol.

The rDNA 18S genes were mapped by FISH, following the protocol of Pinkel *et al*. (1986). The slides were treated with RNase A (100 μg/ml) and kept in a moist chamber at 37°C for 1 h. After that, they were washed in 2 × SSC for 5 min, incubated in 5 μg/ml pepsin in 0.01 N HCl for 10 min, washed in 1 × PBS for 5 min, and dehydrated in 50%, 70% and 100% alcohol series for 2 min each. After this pretreatment, metaphase chromosomes were denatured in 70% formamide/2 × SSC at 75°C for 3 min, and 20 μl of hybridization mix including 200 ng of labelled probe, 2 × SSC, 50% formamide, and 10% dextran sulfate was denatured for 10 min at 85°C and added on preparations. The slides were kept in a moist chamber at 37°C overnight. Then, the slides were washed in 2 × SSC for 5 min; the detection solution including anti-digoxigenin-rhodamine was added on slides that were kept in a moist chamber at 37°C for 1 h. The slides were washed in 4 × SSC/Tween and dehydrated in an alcohol series. Finally, counterstaining with DAPI (DAPI Fluoroshield, Sigma Aldrich) was performed.

Chromosomal analysis

Chromosomes were arranged in order of decreasing size and based on the ratio of the chromosomes arm lengths (r = long arm/short arm), according to the classification proposed by Levan *et al*. (1964). The chromosomes were classified as m = metacentric (r = 1–1.7), sm = submetacentric (r = 1.7–3), st = subtelocentric (r = 3–7), and a = acrocentric ($r > 7$); they were organized using Adobe Photoshop® 21.1.1 and measured using Image Pro Plus®. Ideograms of the NOR-bearing chromosome/chromosomes (i.e., graphical representation of the chromosomes concerning the rDNA clusters) of the ant species were then designed with the Easy Idio software (Diniz and Xavier, 2006).

For the fluorochrome staining and FISH 18S rDNA technique, 30 metaphases from at least three individuals of each species were analysed. In the case of *O. bauri*, which presented a chromosomal polymorphism involving rDNA clusters, seven individuals were analysed (six females and one male). The metaphases were analysed and photographed using a fluorescence microscope, Olympus BX60, attached to an image system, QColor Olympus®, with the filters WB (450–480 nm), WU (330–385 nm), and WG (510–550 nm) for the fluorochromes CMA_3, DAPI, and rhodamine, respectively.

Phylogenetic relationships

The phylogenetic relationship among ant species was determined by associating with previously published molecular phylogenies. The resultant cladogram topology at the subfamily level was determined following Moreau and Bell (2013). The Poneroid clade topology was determined according to Schmidt (2013) and Larabee *et al.* (2016); the clade topology for the subfamily Dolichoderinae was determined according to Santos *et al.* (2016), and that for the subfamily Myrmicinae was determined according to Bacci *et al.* (2009), Ward *et al.* (2015), Queiroz EC (2015, unpublished data), Solomon *et al.* (2019), and Micolino *et al.* (2019a). The topology of the species groups within the subfamily Myrmeciinae was determined according to Hasegawa and Crozier (2006).

Acknowledgements

We would like to thank Dr. Cléa S. F. Mariano for kindly providing *Acromyrmex echinatior* samples, Dr. Jacques H. C. Delabie for species identification, and Dr. Luiz Fernando Gomes for his valuable assistance in the laboratory with *Odontomachus bauri* chromosome preparations. We also acknowledge Laboratório de Biologia Molecular de Insetos of the Universidade Federal de Viçosa (UFV) for technical support. GAT thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarships granted. Financial support for this study was provided by "Investissement d'Avenir" grants managed by the French Agence Nationale de la Recherche (DRIIHM ref. ANR-11-LABX-0010 and CEBA, ref. ANR-10-LABX-25-01), by the PO-FEDER 2014-2020, Région Guyane (BiNG, ref. GY0007194), and by the Programa de Auxílio ao Pesquisador – PAPESQ/UNIFAP/2019.

Data availability statement

All relevant data are within the paper.

References

Aguiar, H.J.A.C., Barros, L.A.C., Mariano, C.S.F., Delabie, J.H.C. and Pompolo, S.G. (2011) 45S rDNA localization for the giant Ant *Dinoponera gigantea* with evolutionary inferences for the *Dinoponera* genus (Formicidae: Ponerinae). *Sociobiology*, **57** (3), 607–620.

Aguiar, H.J.A.C., Barros, L.A.C., Alves, D.R., Mariano, C.S.F., Delabie, J.H.C. and Pompolo, S.G. (2017) Cytogenetic studies on populations of *Camponotus rufipes* (Fabricius, 1775) and *Camponotus renggeri* Emery, 1894 (Formicidae: Formicinae). *PLoS One*, **12**(5), e0177702.

Andrade-Souza, V., Duarte, O.M.P., Martins, C.C.C., Santos, I.S., Costa, M.G.C. and Costa, M.A. (2018) Comparative molecular cytogenetics of *Melipona* Illiger species (Hymenoptera: Apidae). *Sociobiology*, **65**(4), 696–705.

Bacci, M., Jr., Solomon, S.E., Mueller, U.G., Martins, V.G., Carvalho, A.O.R., Vieira, L.G.E. *et al.* (2009) Phylogeny of leafcutter ants in the genus *Atta* Fabricius (Formicidae: Attini) based on mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, **51**(3), 427–437.

Barbosa, P., Leal, E.V., Silva, M., Almeida, M.C., Moreira-Filho, O. and Artoni, R.F. (2017) Variability and evolutionary implications of repetitive DNA dynamics in genome of *Astyanax scabripinnis* (Teleostei, Characidae). *Comparative Cytogenetics*, **11** (1), 143–162.

Barros, L.A.C., Mariano, C.S.F., Pompolo, S.G. and Delabie, J.H. C. (2009) Hsc-FA and NOR bandings on chromosomes of the giant ant *Dinoponera lucida* Emery, 1901 (Hymenoptera: Formicidae). *Comparative Cytogenetics*, **3**, 97–102.

Barros, L.A.C., Aguiar, H.J.A.C., Mariano, C.S.F., Delabie, J.H.C. and Pompolo, S.G. (2010) Cytogenetic characterization of the lower-Attine *Mycocepurus goeldii* (Formicidae: Myrmicinae: Attini). *Sociobiology*, **56**, 57–66.

Barros, L.A.C., Aguiar, H.J.A.C., Andrade-Souza, V., Mariano, C. S.F., Delabie, J.H.C. and Pompolo, S.G. (2012) Occurrence of pre-nucleolar bodies and 45S rDNA location on the chromosomes of the ant *Mycocepurus goeldii* (Forel) (Formicidae, Myrmicinae, Attini). *Hereditas*, **149**, 50–54.

Barros, L.A.C., Teixeira, G.A., Aguiar, H.J.A.C., Mariano, C.S.F., Delabie, J.H.C. and Pompolo, S.G. (2014) Banding patterns of three leafcutter ant species of the genus *Atta* (Formicidae: Myrmicinae) and Chromosomal Inferences. *Florida Entomologist*, **97**(4), 1694–1701.

Barros, L.A.C., Aguiar, H.J.A.C., Teixeira, G.A., Mariano, C.S.F., Teixeira, M.C., Delabie, J.H.C. *et al.* (2015) Cytogenetic data on the threatened leafcutter ant *Atta robusta* Borgmeier, 1939 (Formicidae: Myrmicinae: Attini). *Comptes Rendus Biologies*, **338**(10), 660–665.

Barros, L.A.C., Aguiar, H.J.A.C., Mariano, C.S., Andrade-Souza, V., Costa, M.A., Delabie, J.H.C. *et al.* (2016) Cytogenetic data on six leafcutter ants of the genus *Acromyrmex* Mayr, 1865 (Hymenoptera, Formicidae, Myrmicinae): insights into chromosome evolution and taxonomic implications. *Comparative Cytogenetics*, **10**(2), 229–243.

Barros, L.A.C., Teixeira, G.A., Aguiar, H.J.A.C., Lopes, D.M. and Pompolo, S.G. (2018) Cytogenetic studies in *Trachymyrmex holmgreni* Wheeler, 1925 (Formicidae: Myrmicinae) by conventional and molecular methods. *Sociobiology*, **65**(2), 185–190.

Bolton, B. (2020) An online catalog of the ants of the world. https://antcat.org (accessed May 15, 2020).

Britton-Davidian, J., Cazaux, B. and Catalan, J. (2012) Chromosomal dynamics of nucleolar organizer regions (NORs) in the house mouse: micro-evolutionary insights. *Heredity*, **108**(1), 68–74.

Cabral-de-Mello, D.C., Oliveira, S.G., Moura, R.C. and Martins, C. (2011) Chromosomal organization of the 18S and 5S rRNAs and histone H3 genes in Scarabaeinae coleopterans: insights into the evolutionary dynamics of multigene families and heterochromatin. *BMC Genetics*, **12**, 88.

Cardoso, D.C., Pompolo, S.G., Cristiano, M.P. and Tavares, M.G. (2014) The role of fusion in ant chromosome evolution: insights from cytogenetic analysis using a molecular phylogenetic approach in the genus *Mycetophylax*. *PLoS One*, **9**(4), e95408.

Castro, C.P.M., Cardoso, D.C., Micolino, R. and Cristiano, M.P. (2020) Comparative FISH-mapping of TTAGG telomeric sequences to the chromosomes of leafcutter ants (Formicidae, Myrmicinae): is the insect canonical sequence conserved? *Comparative Cytogenetics*, **14**(3), 369–385.

Cazaux, B., Catalan, J., Veyrunes, F., Douzery, E.J. and Britton-Davidian, J. (2011) Are ribosomal DNA clusters rearrangement hotspots? A case study in the genus *Mus* (Rodentia, Muridae). *BMC Evolutionary Biology*, **11**(1), 124.

Cholak, L.R., Haddad, C.F. and Parise-Maltempi, P.P. (2020) Cytogenetic analysis of the genus *Thoropa* Cope, 1865 (Anura-Cycloramphidae) with evolutionary inferences based on repetitive sequences. *Genetics and Molecular Biology*, **43** (3), e20190364.

Cohen, S. and Segal, D. (2009) Extrachromosomal Circular DNA in Eukaryotes: possible involvement in the plasticity of tandem repeats. *Cytogenetic and Genome Research*, **124**, 327–338.

Cristiano, M.P., Cardoso, D.C. and Fernandes-Salomão, T.M. (2013) Cytogenetic and molecular analyses reveal a divergence between *Acromyrmex striatus* (Roger, 1863) and other congeneric species: taxonomic implications. *PLoS One*, **8**(3), e59784.

Cross, I., Vega, L. and Rebordinos, L. (2003) Nucleolar organizing regions in *Crassostrea angulata*: chromosomal location and polymorphism. *Genetica*, **119**(1), 65–74.

Degrandi, T.M., Gunski, R.J., Garnero, A.D.V., Oliveira, E.H.C.D., Kretschmer, R., Souza, M.S.D. *et al.* (2020) The distribution of 45S rDNA sites in bird chromosomes suggests multiple evolutionary histories. *Genetics and Molecular Biology*, **43**(2), e20180331.

Diniz, D. and Xavier, P.M. (2006) Easy Idio.

Dobigny, G., Ozouf-Costaz, C., Bonillo, C. and Volobouev, V. (2002) "Ag-NORs" are not always true NORs: new evidence in mammals. *Cytogenetic and Genome Research*, **98**(1), 75–77.

Dutra, R.T., Bitencourt, J., Netto, M.R.C.B., Paim, F.G., Sarmento-Soares, L.M. and Afonso, P.R.A.M. (2020) Chromosomal markers are useful to species identification in Rivulidae (Cyprinodontiformes, Aplocheiloidei), including the resolution of taxonomic uncertainties in a vulnerable species complex. *Zebrafish*, **17**(1), 48–55.

Dutrillaux, A.M. and Dutrillaux, B. (2012) Chromosome analysis of 82 species of Scarabaeoidea (Coleoptera), with special focus on NOR localization. *Cytogenetic and Genome Research*, **136**(3), 208–219.

Dutrillaux, A.M., Carton, B., Cacheux, L. and Dutrillaux, B. (2016) Interstitial NORs, fragile sites, and chromosome evolution: a not so simple relationship-the example of *Melolontha melolontha* and genus *Protaetia* (Coleoptera: Scarabaeidae). *Cytogenetic and Genome Research*, **149**(4), 304–311.

Escobar, J.S., Glémin, S. and Galtier, N. (2011) GC-biased gene conversion impacts ribosomal DNA evolution in vertebrates, angiosperms, and other eukaryotes. *Molecular Biology and Evolution*, **28**(9), 2561–2575.

Falcione, C., Hernando, A. and Bressa, M.J. (2018) Comparative cytogenetic analysis in *Erythrolamprus* snakes (Serpentes: Dipsadidae) from Argentina. *Anais da Academia Brasileira de Ciências*, **90**(2), 1417–1429.

Ferreti, A.B.S.M., Ruiz-Ruano, F.J., Milani, D., Loreto, V., Martí, D. A., Ramos, E. *et al.* (2019) How dynamic could be the 45S rDNA cistron? An intriguing variability in a grasshopper species revealed by integration of chromosomal and genomic data. *Chromosoma*, **128**, 165–175.

Gerbault-Seureau, M., Cacheux, L. and Dutrillaux, B. (2017) The relationship between the (In-) stability of NORs and their chromosomal location: the example of Cercopithecidae and a short review of other primates. *Cytogenetic and Genome Research*, **153**(3), 138–146.

Gokhman, V.E., Anokhin, B.A. and Kuznetsova, V.G. (2014) Distribution of 18S rDNA sites and absence of the canonical TTAGG insect telomeric repeat in parasitoid Hymenoptera. *Genetica*, **142**, 317–322.

Gokhman, V.E., Bolsheva, N.L., Govind, S. and Muravenko, O.V. (2016) A comparative cytogenetic study of *Drosophila* parasitoids (Hymenoptera, Figitidae) using DNA-binding fluorochromes and FISH with 45S rDNA probe. *Genetica*, **144**(3), 335–339.

Golub, N.V., Golub, V.B. and Kuznetsova, V.G. (2015) Variability of 18rDNA loci in four lace bug species (Hemiptera, Tingidae) with the same chromosome number. *Comparative Cytogenetics*, **9**(4), 513–522.

Gross, M.C., Schneider, C.H., Valente, G.T., Martins, C. and Feldberg, E. (2010) Variability of 18S rDNA locus among *Symphysodon* fishes: chromosomal rearrangements. *Journal of Fish Biologies*, **76**(5), 1117–1127.

Hasegawa, E. and Crozier, R.H. (2006) Phylogenetic relationships among species groups of the ant genus *Myrmecia*. *Molecular Phylogenetics and Evolution*, **38**, 575–582.

Hirai, H. (2020) Chromosome dynamics regulating genomic dispersion and alteration of Nucleolus Organizer Regions (NORs). *Cells*, **9**(4), 971–984.

Hirai, H., Yamamoto, M.T., Ogura, K., Satta, Y., Yamada, M., Taylor, R.W. *et al.* (1994) Multiplication of 28S rDNA and NOR activity in chromosome evolution among ants of the *Myrmecia pilosula* species complex. *Chromosoma*, **103**(3), 171–178.

Hirai, H., Yamamoto, M.T., Taylor, R.W. and Imai, H.T. (1996) Genomic dispersion of 28S rDNA during karyotypic evolution in the ant genus *Myrmecia* (Formicidae). *Chromosoma*, **105**, 190–196.

Howell, W.T. and Black, D.A. (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia*, **36**(8), 1014–1015.

Huang, Y.C., Lee, C.C., Kao, C.Y., Chang, N.C., Lin, C.C., Shoemaker, D. *et al.* (2016) Evolution of long centromeres in fire ants. *BMC Evolutionary Biology*, **16**, 189.

Imai, H., Taylor, R.W., Crosland, M.W. and Crozier, R.H. (1988) Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. *Japanese Journal of Genetics*, **63**, 159–185.

Imai, H.T., Hirai, H., Satta, Y., Shiroishi, T., Yamada, M. and Taylor, R.W. (1992) Phase specific Ag-staining of nucleolar organizer regions (NORs) and kinetochores in the Australian ant *Myrmecia croslandi*. *The Japanese Journal of Genetics*, **67**(6), 437–447.

Imai, H.T., Taylor, R.W. and Crozier, R.H. (1994) Experimental bases for the minimum interaction theory. I. Chromosome evolution in ants of the *Myrmecia pilosula* species complex (Hymenoptera: Formicidae: Myrmeciinae). *The Japanese Journal of Genetics*, **69**(2), 137–182.

Larabee, F.J., Fisher, B.K., Schmidt, C.A., Matos-Maraví, P., Janda, M. and Suarez, A.V. (2016) Molecular phylogenetics and diversification of trap-jaw ants in the genera *Anochetus* and *Odontomachus* (Hymenoptera: Formicidae). *Molecular Phylogenetics and Evolution*, **103**, 143–154.

Levan, A., Fredga, K., and Sandberg, A.A. (1964) Nomenclature for centromeric position on chromosomes. *Hereditas*, **52**(2), 201–220. http://dx.doi.org/10.1111/j.1601-5223.1964.tb01953.x.

Levsky, J.F. and Singer, R.H. (2003) Fluorescence *in situ* hybridization: past, present and future. *Journal of Cell Science*, **116**, 2833–2838.

Liehr, T. (2017) "Classical cytogenetics" is not equal to "banding cytogenetics". *Molecular Cytogenetics*, **10**, 1–3.

Long, E.O. and Dawid, I.B. (1980) Repeated genes in eukaryotes. *Annual Review of Biochemistry*, **49**(1), 727–764.

Lorite, P. and Palomeque, T. (2010) Karyotype evolution in ants (Hymenoptera: Formicidae), with a review of the known ant chromosome numbers. *Myrmecological News*, **13**(1), 89–102.

Lorite, P., Aránega, A.E., Luque, F. and Palomeque, T. (1997) Analysis of the nucleolar organizing regions in the ant *Tapinoma nigerrimum* (Hymenoptera, Formicidae). *Heredity*, **78**, 578–582.

Lorite, P., Carrillo, J.A., Tinaut, A. and Palomeque, T. (2004) Evolutionary dynamics of satellite DNA in species of the genus *Formica* (Hymenoptera, Formicidae). *Gene*, **332**, 159–168.

Malimpensa, G.D.C., Traldi, J.B., Martinez, J.D.F., Deon, G., Azambuja, M., Nogaroto, V. *et al.* (2020) Chromosomal diversification in two species of *Pimelodus* (Siluriformes: Pimelodidae): Comparative cytogenetic mapping of multigene families. *Zebrafish*, **17**(4), 278–286.

Mantovani, M., Abel, L.D. and Moreira-Filho, O. (2005) Conserved 5S and variable 45S rDNA chromosomal localization revealed by FISH in *Astyanax scabripinnis* (Pisces, Characidae). *Genetica*, **123**, 211–216.

Mariano, C.S.F., Pompolo, S.G., Barros, L.A.C., Mariano-Neto, E., Campiolo, S. and Delabie, J.C.H. (2008) A biogeographical study of the threatened ant *Dinoponera lucida* Emery (Hymenoptera: Formicidae: Ponerinae) using a cytogenetic approach. *Insect Conservation and Diversity*, **1**(3), 161–168.

Mariano, C.S.F., Barros, L.A.C., Velasco, Y.M., Guimarães, I.N., Pompolo, S.G. and Delabie, J.H.C. (2019) Citogenética de hormigas de la región neotropical. In: Fernández, F., Guerrero, R., and Delsinne, T., (Eds.) *Hormigas de Colombia*. Bogotá: Universidad Nacional de Colombia, pp. 131–157.

Martins, C. and Wasko, A.P. (2004) Organization and evolution of 5S ribosomal DNA in the fish genome. In: Williams, C.R., (Ed.) *Focus on genome research*. New York: Nova Science Publishers Inc, pp. 335–363.

Maryańska-Nadachowska, A., Anokhin, B.A., Gnezdilov, V.M. and Kuznetsova, V.G. (2016) Karyotype stability in the family Issidae (Hemiptera, Auchenorrhyncha) revealed by chromosome techniques and FISH with telomeric (TTAGG)n and 18S rDNA probes. *Comparative Cytogenetics*, **10**(3), 347–369.

Mazzoleni, S., Rovatsos, M., Schillaci, O. and Dumas, F. (2018) Evolutionary insight on localization of 18S, 28S rDNA genes on homologous chromosomes in Primates genomes. *Comparative Cytogenetics*, **12**(1), 27–40.

Menezes, R.S.T., Gazoni, T. and Costa, M.A. (2019) Cytogenetics of warrior wasps (Vespidae: *Synoeca*) reveals intense evolutionary dynamics of ribosomal DNA clusters and an unprecedented number of microchromosomes in Hymenoptera. *Biological Journal of the Linnean Society*, **126**(4), 925–935.

Meyne, J., Hirai, H. and Imai, H.T. (1995) FISH analysis of the telomere sequences of bulldog ants (*Mymecia*: Formicidae). *Chromosoma*, **104**, 14–18.

Micolino, R., Cristiano, M.P., Travenzoli, N.M., Lopes, D.M. and Cardoso, D.C. (2019a) Chromosomal dynamics in space and time: evolutionary history of *Mycetophylax* ants across past climatic changes in the Brazilian Atlantic coast. *Scientific Reports*, **9**(1), 1–13.

Micolino, R., Cristiano, M.P. and Cardoso, D.C. (2019b) Population-based cytogenetic banding analysis and phylogenetic relationships of the neotropical fungus-farming ant *Trachymyrmex holmgreni* Wheeler, 1925. *Cytogenetic and Genome Research*, **159**(3), 151–162.

Micolino, R., Cristiano, M.P. and Cardoso, D.C. (2020) Karyotype and putative chromosomal inversion suggested by integration of cytogenetic and molecular data of the fungus-farming ant *Mycetomoellerius iheringi* Emery, 1888. *Comparative Cytogenetics*, **14**(2), 197–210.

Moreau, C.S. and Bell, C.D. (2013) Testing the museum versus cradle tropical biological diversity hypothesis: phylogeny, diversification, and ancestral biogeographic range evolution of the ants. *Evolution*, **67-8**, 2240–2257.

Nguyen, P., Sahara, K., Yoshido, A. and Marec, F. (2010) Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). *Genetica*, **138**, 343–354.

Panzera, Y.1., Pita, S., Ferreiro, M.J., Ferrandis, I., Lages, C., Pérez, R. *et al.* (2012) High dynamics of rDNA cluster location in kissing bug holocentric chromosomes (Triatominae, Heteroptera). *Cytogenetics and Genome Research*, **38**(1), 56–67.

Panzera, F., Ferreiro, M.J., Pita, S., Calleros, L., Pérez, R., Basmadjián, Y. *et al.* (2014) Evolutionary and dispersal history of *Triatoma infestans*, main vector of Chagas disease, by chromosomal markers. *Infection, Genetics and Evolution*, **27**, 105–113.

Pereira, T.T.P., Reis, A.C.C., Cardoso, D.C. and Cristiano, M.P. (2018) Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger, 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera). *Comparative Cytogenetics*, **12**(1), 13–26.

Pinkel, D., Straume, T. and Gray, J.W. (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 2934–2938.

Roa, F. and Guerra, M. (2012) Distribution of 45S rDNA sites in chromosomes of plants: structural and evolutionary implications. *BMC Evolutionary Biology*, **12**(1), 1–13.

Roy, V., Monti-Dedieu, L., Chaminade, N., Siljak-Yakovlev, S., Aulard, S., Lemeunier, F. *et al.* (2005) Evolution of the chromosomal location of rDNA genes in two *Drosophila* species subgroups: *ananassae* and *melanogaster*. *Heredity*, **94**(4), 388–395.

Sánchez-Gea, J.F., Serrano, J. and Galián, J. (2000) Variability in rDNA loci in Iberian species of the genus *Zabrus* (Coleoptera: Carabidae) detected by fluorescence *in situ* hybridization. *Genome*, **43**, 22–28.

Santos, I.S., Mariano, C.S.F., Andrade, V., Costa, M.A., Delabie, J. H.C. and Silva, J.G. (2010) A Cytogenetic Approach to the Study of Neotropical *Odontomachus* and *Anochetus* Ants (Hymenoptera: Formicidae). *Annals of the Entomological Society of America*, **103**(3), 424–429.

Santos, I.S., Mariano, C.S.F., Delabie, J.H.C., Costa, M.A., Carvalho, A.F. and Silva, J.G. (2016) "Much more than a neck": karyotype differentiation between *Dolichoderus attelaboides* (Fabricius, 1775) and *Dolichoderus decollatus* F. Smith, 1858 (Hymenoptera: Formicidae) and karyotypic diversity of five other Neotropical species of *Dolichoderus* Lund, 1831. *Myrmecological News*, **23**, 61–69.

Schmid, M., Steinlein, C., Feichtinger, W. and Nanda, I. (2017) Chromosome banding in Amphibia. XXXV. Highly mobile nucleolus organizing regions in *Craugastor fitzingeri* (Anura, Craugastoridae). *Cytogenetic and Genome Research*, **152**(4), 180–193.

Schmidt, C. (2013) Molecular phylogenetics of ponerine ants (Hymenoptera: Formicidae: Ponerinae). *Zootaxa*, **3647**(2), 201–250.

Schubert, I. and Lysack, M.A. (2011) Interpretation of karyotype evolution should consider chromosome structural constraints. *Trends in Genetics*, **27**(6), 207–216.

Schultz, T.R. and Brady, S.G. (2008) Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, **105**(14), 5435–5440.

Schweizer, D. (1980) Simultaneous fluorescent staining of R bands and specific heterocromatic regions (DA/DAPI-bands) in human chromosomes. *Cytogenetics and Cell Genetics*, **27**, 190–193.

Sochorová, J., Garcia, S., Gálvez, F., Symonová, R. and Kovařík, A. (2018) Evolutionary trends in animal ribosomal DNA loci: introduction to a new online database. *Chromosoma*, **127**, 141–150.

Solomon, S.E., Rabeling, C., Sosa-Calvo, J., Lopes, C.T., Rodrigues, A., Vasconcelos, H.L. *et al.* (2019) The molecular phylogenetics of *Trachymyrmex* Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of 'higher attine' ant agriculture. *Systematic Entomology*, **44**(4), 939–956.

Šťáhlavský, F., Opatova, V., Just, P., Lotz, L.N. and Haddad, C.R. (2018) Molecular technique reveals high variability of 18S rDNA distribution in harvestmen (Opiliones, Phalangiidae) from South Africa. *Comparative Cytogenetics*, **12**(1), 41–59.

Sumner, A.T. (2003) In: *Chromosomes: Organization and Function*. North Berwick, United Kingdom: Blackwell Publishing.

Symonová, R. (2019) Integrative rDNAomics—Importance of the oldest repetitive fraction of the eukaryote genome. *Genes*, **10** (5), 345–360.

Teixeira, G.A., Barros, L.A.C., Aguiar, H.J.A.C. and Pompolo, S.G. (2017) Comparative physical mapping of 18S rDNA in the karyotypes of six leafcutter ant species of the genera *Atta* and *Acromyrmex* (Formicidae: Myrmicinae). *Genetica*, **145**, 351–357.

Teixeira, G.A., Barros, L.A.C., Lopes, D.M. and Aguiar, H.J.A.C. (2020) Cytogenetic variability in four species of *Gnamptogenys* Roger, 1863 (Formicidae: Ectatomminae) showing chromosomal polymorphisms, species complex, and cryptic species. *Protoplasma*, **257**, 549–560.

Vicari, M.R., Artoni, R.F., Moreira-Filho, O. and Bertollo, L.A.C. (2008) Diversification of a ZZ/ZW sex chromosome system in *Characidium* fish (Crenuchidae, Characiformes). *Genetica*, **134**(3), 311–317.

Walker, L.I., Soto, M.A. and Spotorno, Á.E. (2014) Similarities and differences among the chromosomes of the wild Guinea pig *Cavia tschudii* and the domestic Guinea pig *Cavia porcellus* (Rodentia, Caviidae). *Comparative Cytogenetics*, **8**(2), 153–167.

Ward, P.S., Brady, S.G., Fisher, B.L. and Schultz, T.R. (2015) The evolution of myrmicine ants: phylogeny and biogeography of a hyperdiverse ant clade (Hymenoptera: Formicidae). *Systematic Entomology*, **40**, 61–81.

Zurita, F., Sánchez, A., Burgos, M., Jiménez, R. and de la Guardia, R.D. (1997) Interchromosomal, intercellular and interindividual variability of NORs studied with silver staining and in *situ* hybridization. *Heredity*, **78**(3), 229–234.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Staining with chromomycin A_3 showing GC-rich regions (arrows) of Ponerinae ants: (A) *Pseudoponera gilberti* (2*n* = 12), (B) *Anochetus targionii* (2*n* = 30), (C) *Odontomachus haematodus* (2*n* = 44, French Guiana), (D, E) *Odontomachus bauri* showing the homozygous and heterozygous states, respectively (2*n* = 44), and (F) male of *Odontomachus bauri* with the smaller size regions rich in GC base pairs. Bars = 5 μm.

Figure S2 Staining with chromomycin A_3 showing GC-rich regions (arrows) of Myrmicinae ants: (A) *Pheidole germaini* (2*n* = 22), (B) *Crematogaster longispina* (2*n* = 24), (C) *Solenopsis geminata* (n = 16), (D) *Myrmicocrypta* sp. (2*n* = 30), (E) *Strumigenys diabola* (2*n* = 40). Bars = 5 μm.

Figure S3 Staining with chromomycin A_3 showing GC-rich regions (arrows) of an Ectatomminae ant (A) *Gnamptogenys tortuolosa* (2*n* = 44), and Formicinae ants (B) *Camponotus atriceps* (2*n* = 40) and (C, D) *Gigantiops destructor* (2*n* = 78, n = 39). Bars = 5 μm.

Supporting information – Evolutionary insights into the genomic organization of major ribosomal DNA in ant chromosomes.

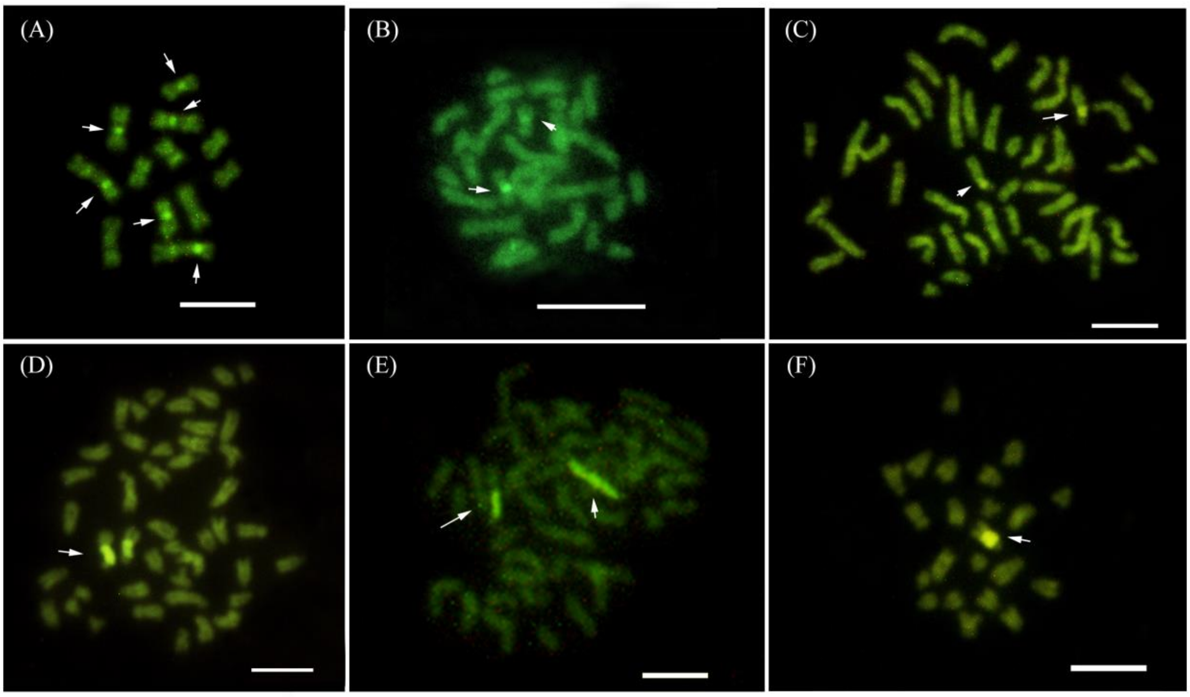


Figure S1. Staining with chromomycin A3 showing GC-rich regions (arrows) of Ponerinae ants: (A) *Pseudoponera gilberti* (2n = 12), (B) *Anochetus targionii* (2n = 30), (C) *Odontomachus haematodus* (2n = 44, French Guiana), (D, E) *Odontomachus bauri* showing the homozygous and heterozygous states, respectively (2n = 44), and (F) male of Odontomachus bauri with the smaller size regions rich in GC base pairs. Bars = 5 µm.

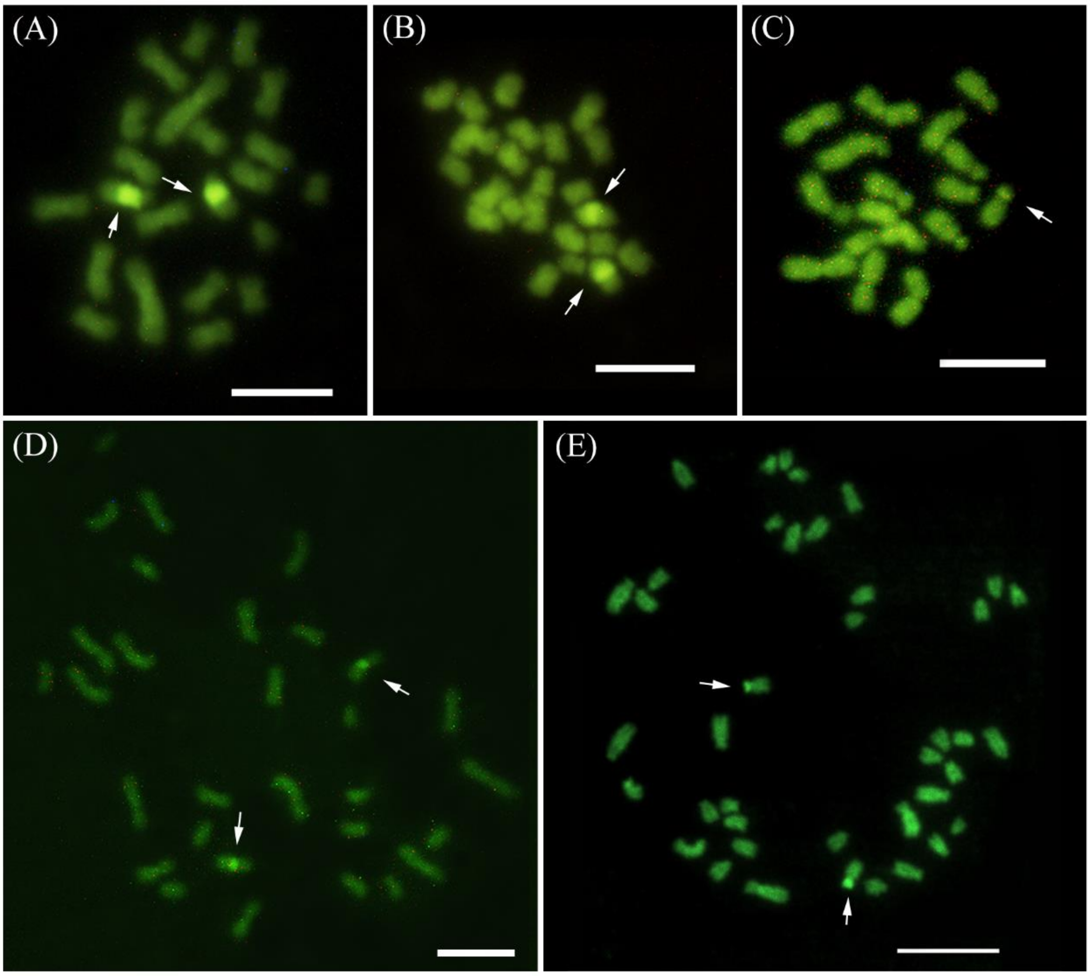


Figure S2. Staining with chromomycin A3 showing GC-rich regions (arrows) of Myrmicinae ants: (A) Pheidole germaini (2n = 22), (B) *Crematogaster longispina* (2n = 24), (C) *Solenopsis geminata* (n = 16), (D) *Myrmicocrypta* sp. (2n = 30), (E) *Strumigenys diabola* (2n = 40). Bars = 5 μm.

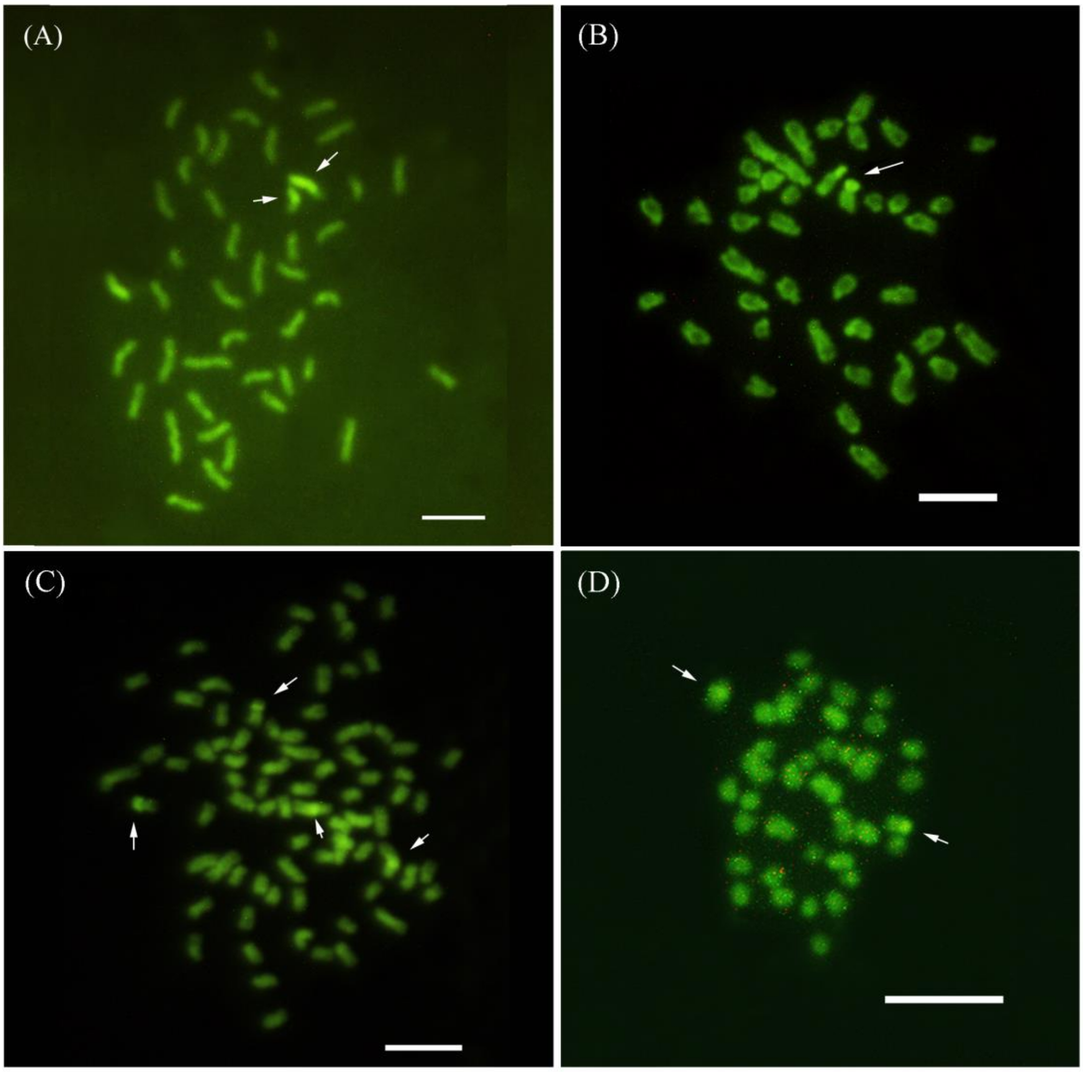


Figure S3. Staining with chromomycin A3 showing GC-rich regions (arrows) of an Ectatomminae ant (A) *Gnamptogenys tortuolosa* (2n = 44), and Formicinae ants (B) *Camponotus atriceps* (2n = 40) and (C, D) *Gigantiops destructor* (2n = 78, n = 39). Bars = 5 µm.

Capítulo II

Distribution of GC-rich heterochromatin and ribosomal genes in three fungus-farming ants (Myrmicinae, Attini, Attina): insights on chromosomal evolution

Artigo publicado na revista *Comparative Cytogenetics*

CompCytogen 15(4): 413–428 (2021)
doi: 10.3897/compcytogen.v15.i4.73769
https://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Distribution of GC-rich heterochromatin and ribosomal genes in three fungus-farming ants (Myrmicinae, Attini, Attina): insights on chromosomal evolution

Gisele Amaro Teixeira[1,2], Luísa Antônia Campos Barros[3],
Hilton Jeferson Alves Cardoso de Aguiar[3], Denilce Meneses Lopes[2]

1 *Programa de Pós-graduação em Biologia Celular e Estrutural, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil* **2** *Laboratório de Citogenética de Insetos, Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil* **3** *Universidade Federal do Amapá, Campus Binacional, n°3051, Bairro Universidade, 68980-000, Oiapoque, Amapá, Brazil*

Corresponding author: Luísa Antônia Campos Barros (luufv@yahoo.com.br)

Academic editor: Rodolpho Menezes | Received 31 August 2021 | Accepted 11 October 2021 | Published 25 November 2021

http://zoobank.org/DD774ADE-CFB0-4292-B229-5A27BE4E6EDA

Citation: Teixeira GA, Barros LAC, de Aguiar HJAC, Lopes DM (2021) Distribution of GC-rich heterochromatin and ribosomal genes in three fungus-farming ants (Myrmicinae, Attini, Attina): insights on chromosomal evolution. CompCytogen 15(4): 413–428. https://doi.org/10.3897/compcytogen.v15.i4.73769

Abstract

Cytogenetic studies on fungus-farming ants have shown remarkable karyotype diversity, suggesting different chromosomal rearrangements involved in karyotype evolution in some genera. A notable cytogenetic characteristic in this ant group is the presence of GC-rich heterochromatin in the karyotypes of some ancient and derivative species. It was hypothesized that this GC-rich heterochromatin may have a common origin in fungus-farming ants, and the increase in species studied is important for understanding this question. In addition, many genera within the subtribe Attina have few or no cytogenetically studied species; therefore, the processes that shaped their chromosomal evolution remain obscure. Thus, in this study, we karyotyped, through classical and molecular cytogenetic techniques, the fungus-farming ants *Cyphomyrmex transversus* Emery, 1894, *Sericomyrmex maravalhas* Ješovnik et Schultz, 2017, and *Mycetomoellerius relictus* (Borgmeier, 1934), to provide insights into the chromosomal evolution in these genera and to investigate the presence the GC-rich heterochromatin in these species. *Cyphomyrmex transversus* (2n = 18, 10m + 2sm + 6a) and *S. maravalhas* (2n = 48, 28m + 20sm) showed karyotypes distinct from other species from their genera. *Mycetomoellerius relictus* (2n = 20, 20m) presented the same karyotype as the colonies previously studied. Notably, *C. transversus* presented the lowest chromosomal number for the genus and a distinct karyotype from the other two previously observed for this species, showing the existence of a possible species complex and the need for its taxonomic revision. Chromosomal banding data revealed GC-rich heterochromatin in all three species, which increased the number of genera with this characteristic, supporting the hypothesis of a common origin of GC-rich heterochromatin in Attina. Although a single chromosomal pair carries rDNA genes in all studied species, the positions of these rDNA clusters varied. The rDNA genes were located in the intrachromosomal region in *C. transversus* and *M. relictus*, and in the terminal region of *S. maravalhas*. The combination of our molecular cytogenetic data and observations from previous studies corroborates that a single rDNA site located in the intrachromosomal region is a plesiomorphic condition in Attina. In addition, cytogenetic data obtained suggest centric fission events in *Sericomyrmex* Mayr, 1865, and the occurrence of inversions as the origin of the location of the ribosomal genes in *M. relictus* and *S. maravalhas*. This study provides new insights into the chromosomal evolution of fungus-farming ants.

Keywords

Biodiversity, chromatin, chromosomal rearrangements, Formicidae, karyotype evolution, molecular cytogenetics

Introduction

Fungus-farming ants, included in the subtribe Attina (sensu Ward et al. 2015), have an obligatory symbiotic relationship with fungi (Weber 1966). In this symbiosis, these ants cultivate the fungus for food and, in return, provide the fungus with nutrition, propagate it to new locations, and protect it against parasitic microorganisms (Weber 1966; Little et al. 2005). In this agricultural system, these ants use different types of substrates depending on the genus/species (reviewed by Mehdiabadi and Schultz 2010), and with this, they play important roles in natural ecosystems, such as dispersion and increasing the success of seed germination, soil structuring, and nutrient cycling (Leal and Oliveira 1998; Fernandez-Bou et al. 2019).

Several molecular phylogenetic studies have been conducted in Attina to address the relationships between genera and species (Schultz and Brady 2008; Ješovnik et al. 2017; Sosa-Calvo et al. 2017; Solomon et al. 2019). These phylogenies support the monophyly of the group, with an origin of approximately 50–60 million years ago (Schultz and Brady 2008; Nygaard et al. 2016; Sosa-Calvo et al. 2017). This group includes approximately 280 described taxa distributed in 20 genera (Bolton 2021), which are grouped into two monophyletic sister clades: Paleoattina (*Apterostigma* Mayr, 1865, *Mycocepurus* Forel, 1893, and *Myrmicocrypta* Smith, 1860) and Neoattina (the remaining 17 genera) (Sosa-Calvo et al. 2018; Solomon et al. 2019; Cristiano et al. 2020).

Some Attina genera have been extensively revised (Sosa-Calvo et al. 2017, 2018; Solomon et al. 2019; Cristiano et al. 2020), and in this scenario, cytogenetics is a tool that can help in taxonomic issues, since chromosomal rearrangements can lead to reduced gene flow between populations and reproductive isolation, playing an important role in speciation (Riesemberg 2001; reviewed by Faria and Navarro 2010). In addition to evolutionary, phylogenetic, and chromosomal patterns in different groups, cytogenetic studies on ants, using classical and molecular techniques, are important for the understanding of taxonomically challenging species (Mariano et al. 2012; Santos et al. 2016; Aguiar et al. 2017; Micolino et al. 2019a; Teixeira et al. 2021).

Cytogenetic data are available for 56 taxa of fungus-farming ants with representatives from 12 genera (reviewed by Mariano et al. 2019; Aguiar et al. 2020; Micolino et al. 2020; Barros et al. 2021) and the chromosome number observed for the group ranges from 2n = 8 in *Mycocepurus goeldii* (Forel, 1893) and *Mycocepurus* sp. to 2n = 64 in *Mycetophylax lectus* (Forel, 1911) (as *Cyphomyrmex lectus*) (reviewed by Mariano et al. 2019). A notable cytogenetic characteristic in this ant group is that some Paleoattina and Neoattina species have GC-rich heterochromatin in all chromosomes, with nucleotide composition yet to be determined but may have an origin in the common ancestor needing further investigation (Barros et al. 2018; reviewed by Mariano et al. 2019). Molecular cytogenetic studies using fluorescence *in situ* hybridization (FISH) for mapping ribosomal genes have already been performed in 17 taxa, including six genera showing a single chromosome pair carrying rDNA genes (reviewed in Teixeira et al. 2021).

According to available cytogenetic data, different chromosomal rearrangements have been proposed to explain karyotype evolution in some Attina genera. The occurrence of centric fissions, according to Minimum Interaction Theory (MIT) (Imai et al. 1994), was suggested to explain the remarkable karyotype variation in *Mycetarotes* Emery, 1913 (2n = 14 to 54), *Apterostigma* (2n = 20 to 46), *Cyphomyrmex* Mayr, 1862 (2n = 20 to 42), and in leaf-cutting ants, in which *Amoimyrmex striatus* (Roger, 1863) and *Atta* spp. present 2n = 22, and most *Acromyrmex* spp. show 2n = 38 (reviewed by Mariano et al. 2019; Barros et al. 2021). However, chromosomal fusion has been suggested as the origin of the derived karyotype from *Acromyrmex ameliae* De Souza et al. 2007 (2n = 36) (Barros et al. 2021). In *Mycetophylax* Emery, 1913, both chromosomal fusions and fissions are important for the karyotypic evolution of species (Micolino et al. 2019a). In addition, other mechanisms that do not change the chromosome number were proposed for some species as differential heterochromatin growth in *Acromyrmex* spp. (Barros et al. 2016), duplications of euchromatic regions by unequal crossing-over or non-homologous translocations in *Mycetomoellerius urichii* (Forel, 1893) (as *Trachymyrmex fuscus* Emery, 1934) (Barros et al. 2013a), paracentric inversion in *Acromyrmex echinatior* (Forel, 1899) (Barros et al. 2016; Teixeira et al. 2021) and pericentric inversion in *Mycetomoellerius iheringi* (Emery, 1888) (Micolino et al. 2020).

There are different possible mechanisms involved in the karyotype evolution of Attina genera, highlighting the need to increase the number of studied species for more robust inferences (Barros et al. 2013b, 2018). The remaining genera of fungus-farming ants have little or no cytogenetically studied species; therefore, the processes that shaped their chromosomal evolution remain obscure. Therefore, using classical and molecular cytogenetic techniques, we determined the karyotypes of three fungus-farming ants – *Cyphomyrmex transversus* Emery, 1894, *Mycetomoellerius relictus* (Borgmeier, 1934), and *Sericomyrmex maravalhas* Ješovnik et Schultz, 2017 – to investigate the presence of GC-rich heterochromatin in these species and understand the patterns of chromosomal evolution in their respective genera as well as in Attina in general.

Material and methods

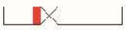
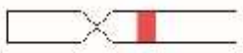
Colonies of *C. transversus*, *M. relictus*, and *S. maravalhas* were collected in Viçosa, in the Minas Gerais state, Brazil (-20.757041, -42.873516) (Table 1). Sampling permission was given by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (SISBIO accession number 32459). Adult vouchers were identified by Dr. Jacques H. C. Delabie and deposited in the myrmecological collection of the Centro de Pesquisas do Cacau at the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC), in Bahia, Brazil.

Mitotic metaphases were obtained from cerebral ganglia of larvae after meconium elimination accordingly to Imai et al. (1988). Chromosome number and morphology of metaphases were analyzed using conventional 4% Giemsa staining. Chromosomes were arranged in order of decreasing size, measured and classified according to the methodology proposed by Levan et al. (1964) that is based on the ratio of the chromosome arm lengths (r = long arm/short arm). The chromosomes were classified as m = metacentric (r = 1–1.7), sm = submetacentric (r = 1.7–3), st = subtelocentric (r = 3–7) and a = acrocentric (r > 7). Chromosomes were organized using Adobe Photoshop CS6 and measured using Image Pro Plus.

The heterochromatin distribution pattern was observed by C-banding technique according to Sumner (1972), with adaptations of Barros et al. (2013b). Metaphases were stained with the fluorochromes chromomycin A_3 (CMA_3) and 4'6-diamidino-2-phenylindole (DAPI), to the detection of GC and AT-rich regions, respectively based on the technique proposed by Schweizer (1980).

The ribosomal 18S gene clusters were detected by FISH, following the protocol of Pinkel et al. (1986) with the use of the 18S rDNA probes obtained via PCR amplification. The genomic DNA from the ant *Camponotus rufipes* (Fabricius, 1775) was used for amplification of 18S rDNA using the primers 18SF1 (5'-GTC ATA GCT TTG TCT CAA AGA-3') and 18SR1.1 (5'-CGC AAA TGA AAC TTT TTT AAT CT-3') (Pereira 2006). These primers amplify the initial portion of 18S rDNA (for details see Menezes et al. 2021). Gene amplification followed Pereira (2006). 18S rDNA probes were labeled by an indirect method using digoxigenin-11-dUTP (Roche Applied Science, Mannheim, Germany), and the FISH signals were detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol.

Table 1. Species of fungus-farming ants cytogenetically analyzed in the present study collected in Viçosa, Minas Gerais, Brazil. Species, total number of colonies and individuals; diploid chromosome numbers; diploid karyotype formulae, presence of GC-rich heterochromatin, and idiogram showing the location of 18S rDNA genes in the karyotype.

Species	Col. / Ind.	2n	Karyotype formulae	GC-rich Het	rDNA 18S location
Cyphomyrmex transversus	1 / 6	18	10m + 2sm + 6a	Yes	
Mycetomoellerius relictus	2 / 7	20	20m	Yes	
Sericomyrmex maravalhas	2 / 14	48	28m + 20sm	Yes	

Chromosomes from ten metaphases of each taxon were measured in order to determine the chromosomal morphology. For C-banding, fluorochrome staining, and FISH techniques, at least 30 metaphases of each taxon were analyzed. The metaphases were photographed using an epifluorescent microscope Olympus BX60 attached to an image system QColor Olympus with the filters WB (450–480 nm), WU (330–385 nm), and WG (510–550 nm) for the fluorochromes CMA_3, DAPI, and rhodamine, respectively.

Results

The chromosome numbers and karyotypic formulae observed in the three fungus-farming ant species were as follows: 2n = 18 (10m + 2sm + 6a) in *C. transversus* (Fig. 1a), 2n = 20 (20m) and n = 10 (10m) in *M. relictus* (Fig. 1b, c), and 2n = 48 (28m + 20sm) in *S. maravalhas* (Fig. 1d).

Heterochromatin was observed in the centromeric/pericentromeric regions of all chromosomes besides short arms of acrocentric chromosomes in *C. transversus* (Fig. 2a). *Mycetomoellerius relictus* presented heterochromatic bands in the centromeric regions of all chromosomes (Fig. 2b). In *S. maravalhas*, heterochromatin was observed in the centromeric and pericentromeric regions of metacentric chromosomes, and short arms of the 7th, 10th, and 13th metacentric and all submetacentric pairs (Fig. 2c). Most of the heterochromatic regions showed GC-rich patterns in all three species (Fig. 3).

The three species showed a single pair of chromosomes bearing rDNA clusters. The 18S ribosomal gene clusters were mapped in the pericentromeric region of the short arm of the 2nd metacentric pair in *C. transversus* (Fig. 4a), in the interstitial region of the long arm of the 5th metacentric pair in *M. relictus* (Fig. 4b, c), and in the terminal region of the short arm of the 7th metacentric pair in *S. maravalhas* (Fig. 4d).

Discussion

The association of cytogenetic and molecular data provided insights into the karyotype evolution of the three genera of fungus-farming ants in this study. In *Sericomyrmex*, the molecular phylogeny proposed by Ješovnik et al. (2017) showed that *S. maravalhas*, a new species recently described by Ješovnik and Schultz (2017), belongs to the *scrobifer* clade, which is basal to the other existing clade, the *amabilis*. Ješovnik and Schultz (2017) highlighted that the distribution data of *S. maravalhas* are clearly incomplete. This is the first report of this species in the Atlantic rainforest since its known occurrence, to this date, was restricted to Cerrado habitats.

Sericomyrmex maravalhas (*scrobifer* clade) has a basal position to *Sericomyrmex amabilis* Wheeler, 1925 (*amabilis* clade) (Ješovnik et al. 2017). The former species has 2n = 48, with more submetacentric chromosomes (this study), whereas the latter species has 2n = 50 with only metacentric chromosomes (Murakami et al. 1998). It is possible to

418 Gisele Amaro Teixeira et al. / CompCytogen 15(4): 413–428 (2021)

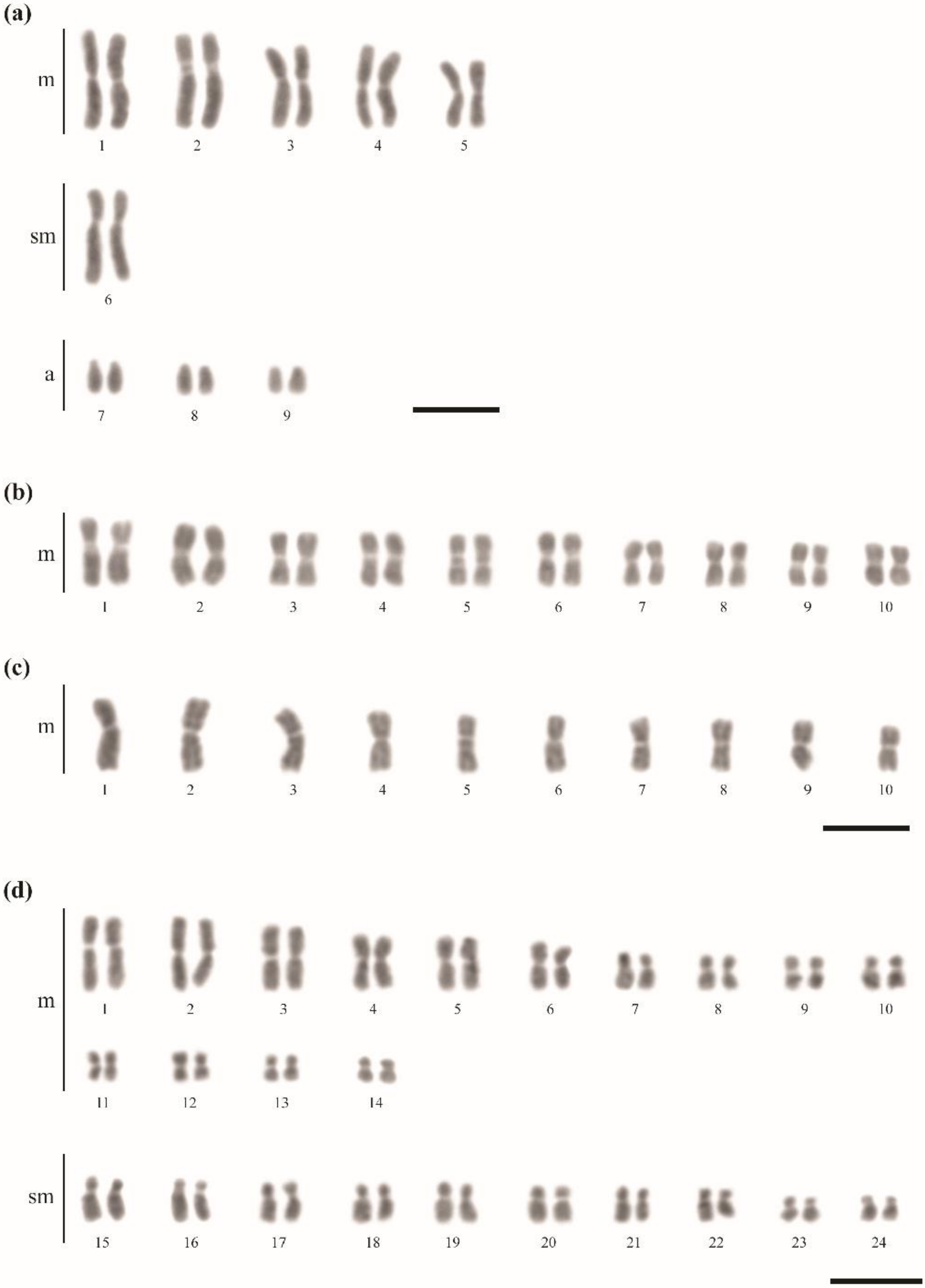


Figure 1. Karyotypes of fungus-farming ants **a** *Cyphomyrmex transversus* (2n = 18, 10m + 2sm + 6a) **b, c** *Mycetomoellerius relictus* (2n = 20, 20m and n = 10, 10m), and **d** *Sericomyrmex maravalhas* (2n = 48, 28m + 20sm). Scale bars: 5 µm.

Figure 2. Heterochromatic patterns after C-banding technique in the karyotypes of the studied fungus-farming ants **a** *Cyphomyrmex transversus* (2n = 18) **b** *Mycetomoellerius relictus* (2n = 20), and **c** *Sericomyrmex maravalhas* (2n = 48). Dark blocks indicate heterochromatin in the centromeric/pericentromeric regions and short arms of the chromosomes. Scale bars: 5 µm.

suggest an increase in chromosome number from 2n = 48 to 2n = 50. Additionally, the heterochromatic pattern on the short arms of the submetacentric/metacentric chromosomes of *S. maravalhas* is a strong indicator of centric fission events during the karyotype evolution in *Sericomyrmex*. The absence of subtelocentric/acrocentric chromosomes in the karyotype of *S. maravalhas*, which has also been observed in *S. amabilis*

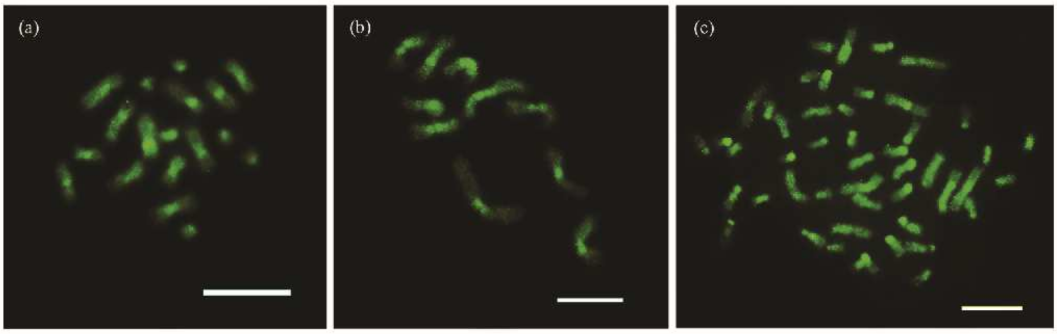


Figure 3. GC-rich chromatin patterns using Chromomycin A_3 fluorochrome on metaphases of the studied fungus-farming ants **a** *Cyphomyrmex transversus* (2n = 18) **b** *Mycetomoellerius relictus* (n = 10), and **c** *Sericomyrmex maravalhas* (2n = 48). The GC-rich bands in the centromeric/pericentromeric regions and short arms of the chromosomes are colocalized with heterochromatic blocks. Scale bars: 5 µm.

and *Sericomyrmex* sp. (Murakami et al. 1998; Barros et al. 2013b), can be associated with tandem growth of heterochromatin for telomeric stability after fission, which should have changed the chromosome's morphology from acrocentric to submetacentric/metacentric. These events of heterochromatin growth may have contributed to differences in chromosomal morphology observed in *S. maravalhas* in relation to *Sericomyrmex* sp. and *S. amabilis*. A similar mechanism has also been suggested to explain interspecific chromosomal variations in leaf-cutting ants *Acromyrmex* (Barros et al. 2016) and trap-jaw ants *Odontomachus* (Aguiar et al. 2020).

The molecular phylogeny of *Mycetomoellerius*, proposed by Solomon et al. (2019), showed two main clades. One clade includes *M. urichii* with 2n = 18 chromosomes (Barros et al. 2013a), *Mycetomoellerius holmgreni* (Wheeler, 1925), and *M. iheringi*, both of which have 2n = 20 chromosomes (Barros et al. 2018; Cardoso et al. 2018; Micolino et al. 2020; Table 2). *Mycetomoellerius relictus* belongs to the other clade and has 2n = 20 chromosomes (present study; Barros et al. 2013b). *Mycetomoellerius* sp. (as *Trachymyrmex* sp.) from the Atlantic rainforest has 2n = 22 (Barros et al. 2013b). Therefore, an ancestor of *Mycetomoellerius* with the chromosome number between 2n = 18–22 and with a predominance of metacentric chromosomes seems likely.

The cytogenetic data obtained in this study for *C. transversus* (2n = 18) showed the lowest chromosome number for this genus. This karyotype is different from the other two previously studied karyotypes in French Guiana (2n = 24) and Brazil (2n = 42) (Mariano et al. 2019; Aguiar et al. 2020; Table 2). The chromosomal morphology also differs among the three karyotypes of *C. transversus*, with a notable increase in the number of acrocentric pairs in the karyotype from São Paulo-Brazil, which has a higher chromosome number. These data suggest that *C. transversus* may be a species complex and, therefore, cytogenetic data highlight the need for taxonomic revision of this species. Based on cytogenetic studies available for *Cyphomyrmex*, Mariano et al. (2019) suggested that centric fissions play a major role in the karyotype evolution within this genus due to an increase in acrocentric chromosome pairs in species with high chromosome numbers. Further molecular phylogenetic studies associated with

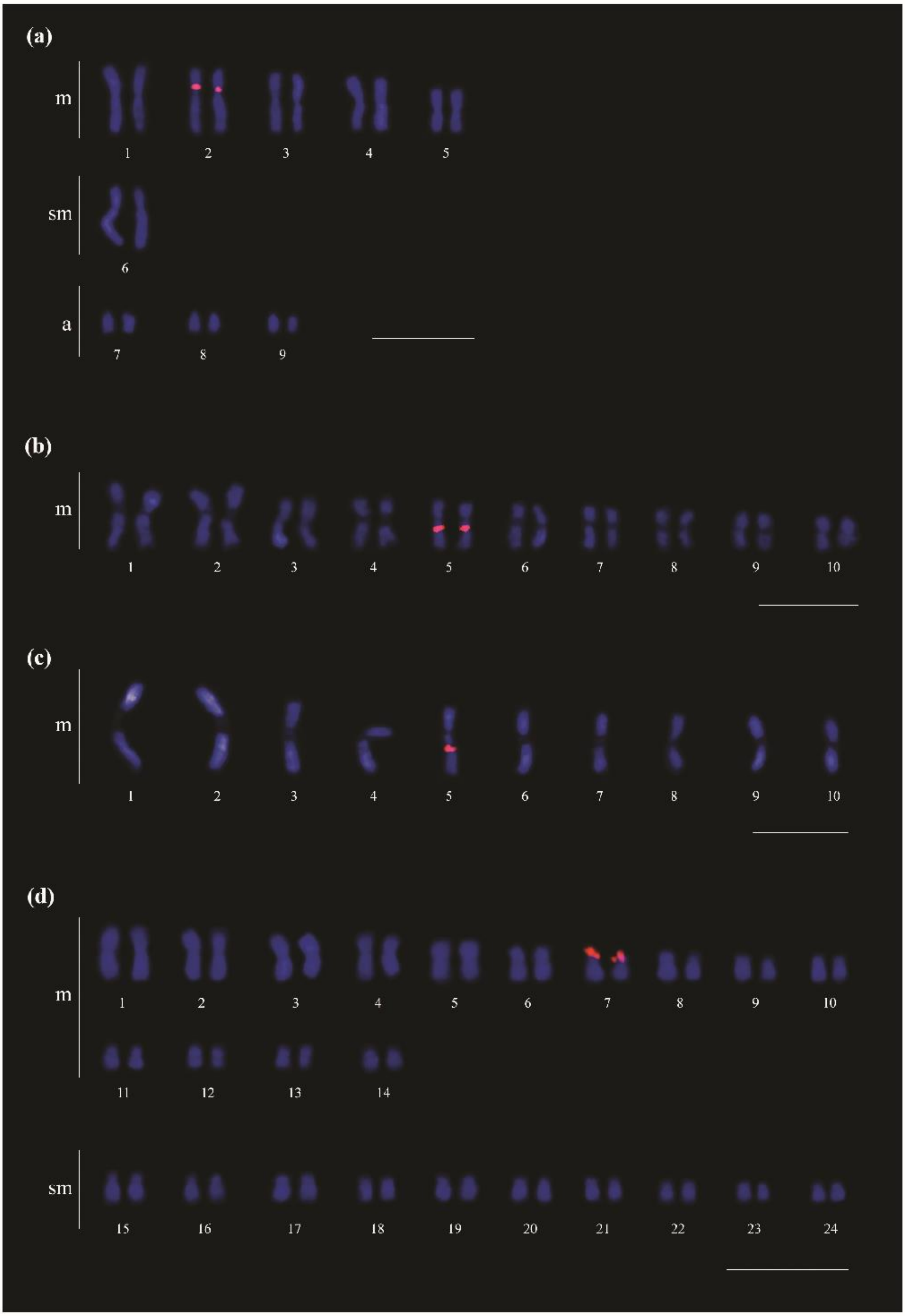


Figure 4. 18S rDNA clusters (red blocks) location on the karyotypes of the studied fungus-farming ants **a** *Cyphomyrmex transversus* (2n = 18) **b, c** *Mycetomoellerius relictus* (2n = 20, n = 10), and **d** *Sericomyrmex maravalhas* (2n = 48). Scale bars: 5 μm.

Table 2. Summary of available cytogenetic data in the literature and this study for the genera of fungus-farming ants *Cyphomyrmex*, *Sericomyrmex*, and *Mycetomoellerius*. Species, localities, chromosome numbers: diploid (2n)/haploid (n), diploid karyotype formulae, and references. The terminology used for karyotype formulae is in accordance to the published data.

Species	Localities	2n/(n)	Karyotype formulae	References
Cyphomyrmex				
C. costatus	Panama	20	20M	Murakami et al. (1998)
C. cornutus	French Guiana	22	10M + 12SM	Mariano et al. (2011)
C. rimosus	Panama	32	28M + 4A	Murakami et al. (1998)
C. transversus	French Guiana	24/(12)	14m + 6sm + 4a	Aguiar et al. (2020)
C. transversus	SP - Brazil	42	42A	Mariano et al. (2019)
C. transversus	MG - Brazil	18	10m + 2sm + 6a	**Present study**
Cyphomyrmex sp. §	MG - Brazil	32	14M + 18A	Mariano et al. (2019)
Sericomyrmex				
S. amabilis	Panama	50	50M	Murakami et al. (1998)
S. maravalhas	MG - Brazil	48	28m + 20sm	**Present study**
Sericomyrmex sp.	MG - Brazil	50/(25)	44m + 6sm	Barros et al. (2013b)
Mycetomoellerius				
*M. urichii**	MG - Brazil	18	16m + 2sm	Barros et al. (2013a)
M. holmgreni	MG - Brazil	20	20m	Barros et al. (2018) / Cardoso et al. (2018)
M. iheringi	SC - Brazil	20	18M + 2SM	Micolino et al. (2020)
M. relictus	MG - Brazil	20/(10)	20m	Barros et al. (2013b) / **Present study**
Mycetomoellerius sp.†	MG - Brazil	22	18m + 4sm	Barros et al. (2013b)

* As *Trachymyrmex fuscus* in Barros et al. (2013a); † According to new revision by Solomon et al. (2019); § *Cyphomyrmex* sp. group *rimosus*. MG: Minas Gerais State; SP: São Paulo State; SC: Santa Catarina State.

cytogenetic data will help in the discussion of the karyotype evolution of this genus and the taxonomy of *C. transversus*.

Regarding heterochromatin constitution, the three species of the present study showed GC-rich heterochromatin, as evidenced by the colocalization of the heterochromatic and CMA_3^+ bands. These data were first reported in *Sericomyrmex* and *Cyphomyrmex*. Other fungus-farming ants showed the same heterochromatic composition such as *M. goeldii* (Paleoattina) (Barros et al. 2010), *M. urichii* (Barros et al. 2013a), and *M. holmgreni* (Barros et. al. 2018), included in Neoattina. This pattern is not common in ants, with few examples in the *Dolichoderus* genus, which belongs to another subfamily (Santos et al. 2016). Barros et al. (2018) suggested that GC-rich heterochromatin observed in different species of Attina, with representatives in Paleoattina and Neoattina, may have a common origin within the subtribe. The heterochromatic pattern rich in GC observed in this study supports this hypothesis, increasing the number of genera with this characteristic. Further investigation of the chromatin composition of these species should corroborate this hypothesis.

The physical mapping of rDNA genes showed a single chromosome pair bearing these genes for the three species in this study. This pattern is similar to that observed for other fungus-farming ants, which is suggested to be a plesiomorphic characteristic in Formicidae (reviewed by Teixeira et al. 2021), and aculeate Hymenoptera as well (Menezes et al. 2021). Regarding the location of these rDNA genes on the chromosomes in Attina, most species presented these genes in the intrachromosomal region (pericentromeric or interstitial). This characteristic is observed in ancient species such

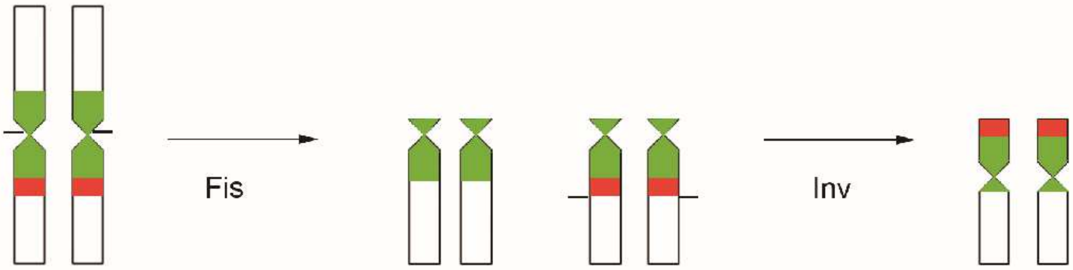


Figure 5. Diagram of origin of terminal rDNA clusters in metacentric chromosome from *Sericomyrmex maravalhas*, considering its ancestor with intrachromosomal rDNA clusters. Black bars: chromosomal breaks; Fis: centric fission; Inv: pericentric inversion; Green blocks: GC-rich regions; Red blocks: 18S ribosomal clusters.

as *M. goeldii*, *Myrmicocrypta* sp., *Mycetophylax* spp., and *C. tranversus*, in the transition species *M. holmgreni* and *M. relictus*, and leaf-cutting ants, most derived from the group, *Am. striatus* and *Atta* spp. (reviewed by Teixeira et al. 2021; this study). These data suggest that the intrachromosomal position of rDNA genes seems to be a plesiomorphic character in fungus-farming ants.

However, in *S. maravalhas*, the rDNA clusters were mapped in the terminal region of the heterochromatic short arm of the 7th metacentric pair (see Figs 2c, 4c). Considering an ancestor with a low chromosome number and intrachromosomal rDNA clusters, after centric fission events, the occurrence of pericentric inversion would change the pericentromeric rDNA genes to the terminal positions, as observed in *S. maravalhas* (Fig. 5). In some other fungus-farming ants, rDNA genes are also located in the terminal region, such as *Acromyrmex* spp. (Barros et al. 2016; Teixeira et al. 2017), *Mycetophylax conformis* (Mayr, 1884), and *M. morschi* (Emery, 1888) (2n = 30) (Micolino et al. 2019a), which are species with derived karyotypes within their respective phylogenetic branches. In the case of *M. conformis*, the terminal rDNA cluster located on the metacentric chromosome (Micolino et al. 2019a) seems to represent a derived pattern, explained by a single paracentric inversion, considering its ancestor with intrachromosomal rDNA clusters. The rDNA terminal location in *Acromyrmex* seems to be a derived condition among leaf-cutting ants (Barros et al. 2021).

In addition, a difference in the location of rDNA clusters was observed between *M. relictus* in this study and *M. holmgreni* (Barros et al. 2018; Micolino et al. 2019b). The former showed 18S rDNA clusters located in the interstitial region of the 5th metacentric pair while the latter presented these genes in the pericentromeric region of the 4th metacentric pair (Barros et al. 2018; Micolino et al. 2019b). This difference may reflect the phylogenetic position of these species, as they are included in distinct branches of *Mycetomoellerius*, in which *M. holmgreni* has a basal position to *M. relictus* (Solomon et al. 2019). However, the size variation between the 4th and 5th metacentric pairs was very subtle in *M. relictus* (see Fig. 4b, c). This suggests homeology of the chromosome pair carrying rDNA clusters between *M. relictus* and *M. holmgreni*. Therefore, the difference in the location of ribosomal genes between *M. relictus* and *M. homlgreni* may be the result of paracentric inversion. In addition, the occurrence of a paracentric inversion involving rDNA genes has already been observed in leaf-cutting ant *A. echinatior* (Teixeira et al. 2021). Thus, inversions seem to be important rearrangements that generate changes in the position of rDNA genes in the karyotype of fungus-farming ants.

Conclusions

In this study, the distribution of 18S ribosomal genes and GC-rich heterochromatin in *Sericomyrmex* and *Cyphomyrmex*, which were reported for the first time, suggest the origin of this heterochromatin in the common ancestor of Attina. The karyotype observed in *C. tranversus* shows the lowest chromosomal number for the genus, and chromosomal variability among populations of the species highlights the need for taxonomic revision of this species using an integrative approach. Although *Sericomyrmex* spp. are morphologically complex (Ješovnik and Schultz 2017), karyotype differences were observed in this study, highlighting cytogenetics as an important tool for integrative taxonomy. Cytogenetic data obtained for *S. maravalhas* suggested centric fission events during chromosomal evolution in *Sericomyrmex*. Inversions seem to be involved in the origin of location of 18S ribosomal genes in *M. relictus* and *S. maravalhas*. Therefore, this study provides new insights into chromosomal evolution in *Sericomyrmex*, *Cyphomyrmex*, and *Mycetomoellerius*. Our data suggest that chromosomal rearrangements have contributed to the species diversification in Attina. We also believe that the increase in the number of species studied using classical and molecular cytogenetic techniques will continue to contribute to discussions about the evolution of fungus-farming ants.

Acknowledgements

We are grateful to Dr. Jacques H. C. Delabie for species identification. GAT thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted. This study was supported by Programa de Auxílio ao Pesquisador PAPESQ/UNIFAP.

References

Aguiar HJAC, Barros LAC, Alves DR, Mariano CSF, Delabie JHC, Pompolo SG (2017) Cytogenetic studies on populations of *Camponotus rufipes* (Fabricius, 1775) and *Camponotus renggeri* Emery, 1894 (Formicidae: Formicinae). PLoS ONE 12(5): e0177702. https://doi.org/10.1371/journal.pone.0177702

Aguiar HJAC, Barros LAC, Silveira LI, Petitclerc F, Etienne S, Orivel J (2020) Cytogenetic data for sixteen ant species from North-eastern Amazonia with phylogenetic insights into three subfamilies. Comparative Cytogenetics 14(1): 43–60. https://doi.org/10.3897/CompCytogen.v14i1.46692

Barros LAC, Aguiar HJAC, Mariano CSF, Delabie JHC, Pompolo SG (2010) Cytogenetic characterization of the lower-Attine *Mycocepurus goeldii* (Formicidae: Myrmicinae: Attini). Sociobiology 56: 57–66.

Barros LAC, Aguiar HJAC, Mariano CSF, Delabie JHC, Pompolo SG (2013a) Cytogenetic characterization of the ant *Trachymyrmex fuscus* Emery, 1934 (Formicidae: Myrmicinae: Attini) with the description of a chromosomal polymorphism. Annales de la Société Entomologique de France 49: 367–373. https://doi.org/10.1080/00379271.2013.856201

Barros LAC, Mariano CSF, Pompolo SG (2013b) Cytogenetic studies of five taxa of the tribe Attini (Formicidae: Myrmicinae). Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics 66(1): 59–64. https://doi.org/10.1080/00087114.2013.780443

Barros LAC, Aguiar HJAC, Mariano CSF, Andrade-Souza V, Costa MA, Delabie JHC, Pompolo SG (2016) Cytogenetic data on six leafcutter ants of the genus *Acromyrmex* Mayr, 1865 (Hymenoptera, Formicidae, Myrmicinae): insights into chromosome evolution and taxonomic implications. Comparative Cytogenetics 10: 229–243. https://doi.org/10.3897/CompCytogen.v10i2.7612

Barros LAC, Teixeira GA, Aguiar HJAC, Lopes DM, Pompolo SG (2018) Cytogenetic studies in *Trachymyrmex holmgreni* Wheeler, 1925 (Formicidae: Myrmicinae) by conventional and molecular methods. Sociobiology 65(2): 185–190. https://doi.org/10.13102/sociobiology.v65i2.2214

Barros LAC, Aguiar HJAC, Teixeira GA, Souza DJ, Delabie JHC, Mariano CSF (2021) Cytogenetic studies on the social parasite *Acromyrmex ameliae* (Formicidae: Myrmicinae: Attini) and its hosts reveal chromosome fusion in *Acromyrmex*. Zoologischer Anzeiger 293: 273–281. https://doi.org/10.1016/j.jcz.2021.06.012

Bolton B (2021) An online catalog of the ants of the world. http://antcat.org [Accessed 15 January 2021]

Cardoso DC, Heinze J, Moura MN, Cristiano MP (2018) Chromosomal variation among populations of a fungus-farming ant: implications for karyotype evolution and potential restriction to gene flow. BMC Evolutionary Biology 18(1): 1–10. https://doi.org/10.1186/s12862-018-1247-5

Cristiano MP, Cardoso DC, Sandoval-Gómez VE, Simões-Gomes FC (2020) *Amoimyrmex* Cristiano, Cardoso & Sandoval, gen. nov. (Hymenoptera: Formicidae): a new genus of leaf-cutting ants revealed by multilocus molecular phylogenetic and morphological analyses. Austral Entomology 59(4): 643–676. https://doi.org/10.1111/aen.12493

Faria R, Navarro A (2010) Chromosomal speciation revisited: rearranging theory with pieces of evidence. Trends in Ecology & Evolution 25(11): 660–669. https://doi.org/10.1016/j.tree.2010.07.008

Fernandez-Bou AS, Dierick D, Swanson AC, Allen MF, Alvarado AGF, Artavia-León A, Carrasquillo-Quintana O, Lachman DA, Oberbauer S, Pinto-Tomás AA, Rodríguez-Reyes Y, Rundel P, Schwendenmann L, Zelikova TJ, Harmon TC (2019) The role of the ecosystem engineer, the leaf-cutter ant *Atta cephalotes*, on soil CO_2 dynamics in a wet tropical rainforest. Journal of Geophysical Research: Biogeosciences 124(2): 260–273. https://doi.org/10.1029/2018JG004723

Imai H, Taylor RW, Crosland MW, Crozier RH (1988) Modes of spontaneous chromossomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. Japanese Journal of Genetics 63: 159–185. https://doi.org/10.1266/jjg.63.159

Imai HT, Taylor RW, Crozier RH (1994) Experimental bases for the minimum interaction theory. Chromosome evolution in ants of the *Myrmecia pilosula* species complex (Hymenoptera: Formicidae: Myrmeciinae). Japanese Journal of Genetics 69: 137–182. https://doi.org/10.1266/jjg.69.137

Ješovnik A, Schultz TR (2017) Revision of the fungus-farming ant genus *Sericomyrmex* Mayr (Hymenoptera, Formicidae, Myrmicinae). Zootaxa 670: 1–109. https://doi.org/10.3897/zookeys.670.11839

Ješovnik A, Sosa-Calvo J, Lloyd MW, Branstetter MG, Fernández F, Schultz TR (2017) Phylogenomic species delimitation and host-symbiont coevolution in the fungus-farming ant genus *Sericomyrmex* Mayr (Hymenoptera: Formicidae): ultraconserved elements (UCEs) resolve a recente radiation. Systematic Entomology 42: 523–542. https://doi.org/10.1111/syen.12228

Leal IR, Oliveira PS (1998) Interactions between fungus-growing ants (Attini), fruits, and seeds in cerrado vegetation in southeast Brazil. Biotropica 30: 170–178. https://doi.org/10.1111/j.1744-7429.1998.tb00052.x

Levan A, Fredga K, Sandberg A (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Little AEF, Murakami T, Mueller UG, Currie CR (2005) Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biology Letters 2(1): 12–16. https://doi.org/10.1098/rsbl.2005.0371

Mariano CSF, Santos IS, Groc S, Leroy C, Malé PJ, Ruin-González MX, Cerdan P, Dejean A, Delabie JHC (2011) The karyotypes of *Gigantiops destructor* (Fabricius) and other ants from French Guiana (Formicidae). Annales de la Société Entomologique de France 47(1–2): 140–146. https://doi.org/10.1080/00379271.2011.10697705

Mariano CSF, Pompolo SG, Silva JG, Delabie JHC (2012) Contribution of cytogenetics to the debate on the paraphyly of *Pachycondyla* spp. (Hymenoptera, Formicidae, Ponerinae). Psyche: A Journal of Entomology 2012: 1–9. https://doi.org/10.1155/2012/973897

Mariano CSF, Barros LAC, Velasco YM, Guimarães IN, Pompolo SG, Delabie JHC (2019) Citogenética de hormigas de la región neotropical. In: Fernández F, Guerrero R, Delsinne T (Eds) Hormigas de Colombia. Universidad Nacional de Colombia, Bogotá, 131–157. [In Spanish]

Mehdiabadi NJ, Schultz TR (2010) Natural history and phylogeny of the fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). Myrmecological News 13: 37–55.

Menezes RS, Cabral-de-Mello DC, Milani D, Bardella VB, Almeida EA (2021) The relevance of chromosome fissions for major ribosomal DNA dispersion in hymenopteran insects. Journal of Evolutionary Biology 34(9): 1466–1476. https://doi.org/10.1111/jeb.13909

Micolino R, Cristiano MP, Travenzoli NM, Lopes D, Cardoso DC (2019a) Chromosomal dynamics in space and time: evolutionary history of *Mycetophylax* ants across past climatic changes in the Brazilian Atlantic coast. Scientific Reports 9(1): 1–13. https://doi.org/10.1038/s41598-019-55135-5

Micolino R, Cristiano MP, Cardoso DC (2019b) Population-based cytogenetic banding analysis and phylogenetic relationships of the neotropical fungus-farming ant *Trachymyrmex holmgreni* Wheeler, 1925. Cytogenetic and Genome Research 159(3): 151–161. https://doi.org/10.1159/000503913

Micolino R, Cristiano MP, Cardoso DC (2020) Karyotype and putative chromosomal inversion suggested by integration of cytogenetic and molecular data of the fungus-farming ant *Mycetomoellerius iheringi* Emery, 1888. Comparative Cytogenetics 14(2): 197–210. https://doi.org/10.3897/CompCytogen.v14i2.49846

Murakami T, Fujiwara A, Yoshida MC (1998) Cytogenetics of ten ant species of the tribe Attini (Hymenoptera, Formicidae) in Barro Colorado Island, Panama. Chromosome Science 2: 135–139.

Nygaard S, Hu H, Li C, Schiøtt M, Chen Z, Yang Z, Xie Q, Ma C, Deng Y, Dikow RB, Rabeling C, Nash DR, Wcislo WT, Brady SG, Schultz TR, Zhang G, Boomsma JJ (2016) Reciprocal genomic evolution in the ant–fungus agricultural symbiosis. Nature Communications 7(1): 1–9. https://doi.org/10.1038/ncomms12233

Pereira JOP (2006) Diversidade genética da abelha sem ferrão *Melipona quinquefasciata* baseada no sequenciamento das regiões ITS1 parcial e 18S do DNA ribossômico nuclear. Thesis, Universidade Federal do Ceará, Fortaleza, Brazil, 141 pp. [In Portuguese]

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences of the United States of America 83: 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Riesemberg LH (2001) Chromosomal rearrangements and speciation. Trends in Ecology & Evolution 7(1): 351–358. https://doi.org/10.1016/S0169-5347(01)02187-5

Santos IS, Mariano CSF, Delabie JHC, Costa MA, Carvalho AF, Silva JG (2016) "Much more than a neck": karyotype differentiation between *Dolichoderus attelaboides* (Fabricius, 1775) and *Dolichoderus decollatus* F. Smith, 1858 (Hymenoptera: Formicidae) and karyotypic diversity of five other Neotropical species of *Dolichoderus* Lund, 1831. Myrmecological News 23: 61–69.

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. Proceedings of the National Academy of Sciences of the United States of America 105: 5435–5440. https://doi.org/10.1073/pnas.0711024105

Schweizer D (1980) Simultaneous fluorescent staining of R bands and specific heterocromatic regions (DA/DAPI-bands) in human chromosomes. Cytogenetics and Cell Genetics 27: 190–193. https://doi.org/10.1159/000131482

Solomon SE, Rabeling C, Sosa-Calvo J, Lopes CT, Rodrigues A, Vasconcelos HL, Bacci Jr M, Mueller UG, Schultz TR (2019) The molecular phylogenetics of *Trachymyrmex* Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of 'higher-attine' ant agriculture. Systematic Entomology 44(4): 939–956. https://doi.org/10.1111/syen.12370

Sosa-Calvo J, Ješovnik A, Vasconcelos HL, Bacci Jr M, Schultz TR (2017) Rediscovery of the enigmatic fungus-farming ant "*Mycetosoritis*" *asper* Mayr (Hymenoptera: Formicidae):

implications for taxonomy, phylogeny, and the evolution of agriculture in ants. PLoS ONE 12: e0176498. https://doi.org/10.1371/journal.pone.0176498

Sosa-Calvo J, Schultz TR, Ješovnik A, Dahan RA, Rabeling C (2018) Evolution, systematics, and natural history of a new genus of cryptobiotic fungus-growing ants. Systematic Entomology 43: 549–567. https://doi.org/10.1111/syen.12289

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 83: 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

Teixeira GA, Barros LAC, Aguiar HJAC, Pompolo SG (2017) Comparative physical mapping of 18S rDNA in the karyotypes of six leafcutter ant species of the genera *Atta* and *Acromyrmex* (Formicidae: Myrmicinae). Genetica 145: 351–357. https://doi.org/10.1007/s10709-017-9970-1

Teixeira GA, Aguiar HJAC, Peticlerc F, Jerome O, Lopes DM, Barros LAC (2021) Evolutionary insights into the genomic organization of major ribosomal DNA in ant chromosomes. Insect Molecular Biology 30(3): 340–354. https://doi.org/10.1111/imb.12699

Ward PS, Brady SG, Fisher BL, Schultz TR (2015) The evolution of Myrmicinae ants: phylogeny and biogeography of a hyperdiverse ant clade (Hymenoptera: Formicidae). Systematic Entomology 40: 61–81. https://doi.org/10.1111/syen.12090

Weber NA (1966) Fungus-Growing Ants. Science 153: 587–604. https://doi.org/10.1126/science.153.3736.587

ORCID

Gisele Amaro Teixeira https://orcid.org/0000-0002-7106-5798
Luísa Antônia Campos Barros https://orcid.org/0000-0002-1501-4734
Hilton Jeferson Alves Cardoso de Aguiar https://orcid.org/0000-0001-7738-1460
Denilce Meneses Lopes https://orcid.org/0000-0001-7209-4411

Capítulo III

Multiple heterochromatin diversification events in the genome of fungus-farming ants: insights from repetitive sequences

Artigo publicado na revista *Chromosoma*

Chromosoma
https://doi.org/10.1007/s00412-022-00770-7

ORIGINAL ARTICLE

Multiple heterochromatin diversification events in the genome of fungus-farming ants: insights from repetitive sequences

Gisele Amaro Teixeira[1,2] · Luísa Antônia Campos Barros[3] · Hilton Jeferson Alves Cardoso de Aguiar[3,4] · Denilce Meneses Lopes[2]

Received: 23 November 2021 / Revised: 18 January 2022 / Accepted: 21 February 2022

Abstract
A substantial portion of the eukaryotic genome includes repetitive DNA, which is important for its stability, regulation, and architecture. Fungus-farming ant genomes show remarkable structural rearrangement rates that were necessary for the establishment of their agriculture-based lifestyle, highlighting the relevance of this peculiar group in understanding the repetitive portion of ant genome. Chromosomal banding studies are in accordance with genomic data because they show that repetitive heterochromatic sequences of basal and derivative Attina species are GC-rich, an uncommon trait in Formicidae. To understand the evolutionary dynamics of heterochromatin in Attina, we compared GC-rich heterochromatin patterns between the Paleoattina and Neoattina clades of this subtribe. To this end, we hybridized the Mrel-C_0t probe (highly and moderately repetitive DNA) obtained from *Mycetomoellerius relictus*, Neoattina with GC-rich heterochromatin, in karyotypes of Paleoattina and Neoattina species. Additionally, we mapped the repetitive sequences $(GA)_{15}$ and $(TTAGG)_6$ in species of the two clades to investigate their organization and evolutionary patterns in the genome of Attina. The Mrel-C_0t probe marked the heterochromatin in *M. relictus*, in other *Mycetomoellerius* spp., and in species of *Mycetarotes*, *Cyphomyrmex*, and *Sericomyrmex* (Neoattina). In *Mycetomoellerius urichii*, only pericentromeric heterochromatin was marked with Mrel-C_0t. No marking was observed in Paleoattina species or in *Atta* and *Acromyrmex* (Neoattina). These results indicated that different evolutionary events led to heterochromatin differentiation in Attina. The most likely hypothesis is that GC-rich heterochromatin arose in the common ancestor of the two clades and accumulated various changes throughout evolution. The sequences $(GA)_{15}$ and $(TTAGG)_6$ located in euchromatin and telomeres, respectively, showed more homogeneous results among the species.

Keywords Attina · C_0t-DNA · Microsatellites · Chromosome evolution · Telomeres · Biodiversity

Introduction

A substantial portion of the eukaryotic genome includes moderately and highly repetitive sequences, such as microsatellites, minisatellites, satellite DNA, some multigene families, and transposable elements (Charlesworth et al. 1994; Martins et al. 2011; López-Flores and Garrido-Ramos 2012). Comparative molecular cytogenetic studies using *in situ* hybridization are extremely important for understanding the composition, origin, function, organization, and evolutionary patterns of repetitive DNA in the eukaryotic genome (Martins et al. 2011; Biscotti et al. 2015). Studies in several ant groups involving satellite DNA (e.g., Lorite et al. 2002, 2004, 2017) and transposable elements (e.g., Krieger and Ross 2003; Palomeque et al. 2006; Lorite et al. 2012; Santlorente et al. 2020), via comparative evolutionary molecular analysis, have remarkably contributed to the understanding

---

✉ Luísa Antônia Campos Barros
luufv@yahoo.com.br

[1] Programa de Pós-Graduação em Biologia Celular e Estrutural, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570-900, Brazil

[2] Laboratório de Citogenética de Insetos, Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, Minas Gerais 36570-900, Brazil

[3] Universidade Federal do Amapá, Campus Binacional – Oiapoque, n°3051, Bairro Universidade, Oiapoque, Amapá 68980-000, Brazil

[4] Programa de Pós-graduação em Biodiversidade Tropical, Universidade Federal do Amapá, 68.903-419 Macapá, Brazil

of the repetitive portion of the Formicidae genome. However, studies using molecular cytogenetic approaches are scarce in ants.

Fungus-farming ants (subtribe Attina) are a monophyletic group with origin at approximately 50–60 million years ago, and they cultivate a symbiotic fungus for nourishment (Schultz and Brady 2008; Nygaard et al. 2016). These ants have remarkable ecological importance because they help in seed dispersion and improve the success rate of seed germination, soil structuring, and nutrient cycling (Leal and Oliveira 1998; Leal et al. 2014). In addition, some species are economically important because they are pests in agricultural areas and cultivated pastures (Della Lucia and Souza 2011). The subtribe includes approximately 250 described species and 20 genera (Bolton 2021), which are divided into two monophyletic sister clades: Paleoattina (*Apterostigma* Mayr, 1865, *Mycocepurus* Forel, 1893 and *Myrmicocrypta* Smith, 1860) and Neoattina (the remaining 17 genera) (Sosa-Calvo et al. 2018; Solomon et al. 2019; Cristiano et al. 2020). Furthermore, these ants can be grouped according to agricultural systems in which leaf-cutter ants have the most derived form of agriculture within Attina using only fresh vegetation for symbiotic fungus cultivation (reviewed by Mehdiabadi and Schultz 2010). Fungus-farming ants (except leaf-cutter ants) are inconspicuous ants with small size; move slowly, with dark color and dull; and enter into thanatosis when disturbed; besides their nests are deep, being a big challenge the collection of the colonies which demands great field efforts for many species (Sosa-Calvo et al. 2015).

Most available molecular cytogenetic data on fungus-farming ants are focused on ribosomal genes (reviewed by Teixeira et al. 2021a, b). Recently, other repetitive sequences of the genome have been mapped in a few species, for example, telomeric DNA in *Mycetophylax* spp. (Micolino et al. 2019a), *Mycetomoellerius* spp. (Micolino et al. 2019b; 2020), *Amoimyrmex striatus* (Roger, 1863) (Pereira et al. 2018), *Acromyrmex* spp., *Atta sexdens* (Linnaeus, 1758) (Castro et al. 2020), and *Acromyrmex ameliae* De Souza et al. (2007) (Barros et al. 2021) and microsatellites in *Mycetomoellerius holmgreni* (Wheeler, 1925) (Barros et al. 2018; Micolino et al. 2019b). Microsatellites have a highly variable distribution and can be located in the euchromatin or heterochromatin of autosomal or sex chromosomes, micro-chromosomes, or B chromosomes, clustered in specific regions or scattered throughout the entire chromosome length. This indicates that microsatellites can accumulate differently and follow divergent evolutionary paths, which is useful for understanding genome evolution (Milani and Cabral-de-Mello 2014; Augstenová et al. 2018; Travenzoli et al. 2019; Gunski et al. 2019; Marchioro et al. 2020).

Repetitive sequences, such as satellite DNA or mobile elements, usually compose the constitutive heterochromatin in eukaryotes (Martins et al. 2011; reviewed by López-Flores and Garrido-Ramos 2012). Chromosomal banding data indicate that the sequences that constitute the heterochromatin of some species of fungus-farming ants are rich in GC-base pairs (Table 1), which is an uncommon feature in ant species studied so far (reviewed by Teixeira et al. 2021a, b). In Attina, GC-rich heterochromatin in all chromosomes was observed in ***Mycocepurus goeldii*** (Forel, 1893) and *Apterostigma auriculatum* Wheeler, 1925, belonging to Paleoattina, and in species of ***Mycetarotes***, ***Cyphomyrmex***, ***Sericomyrmex***, and ***Mycetomoellerius***, belonging to Neoattina (Barros et al. 2010, 2013a, 2018; Barros 2014; Teixeira et al. 2021a, b). Barros et al. (2018) suggested that this GC-rich heterochromatin may have originated in the common ancestor of the subtribe and, thus, may be an informative trait for the construction of a phylogenetic hypothesis. However, some species, such as ***Myrmicocrypta*** sp., *Atta* spp., and *Acromyrmex* spp. (reviewed by Teixeira et al. 2021a, b), have only GC-rich ribosomal gene regions, while other species such as *Am. striatus*, *Acromyrmex niger* (Smith, 1858), and *Acromyrmex rugosus* (Smith, 1858) (Cristiano et al. 2013; Barros et al. 2016) have GC-rich heterochromatin in a few chromosomal pairs. These data show a process of differentiation in the composition of heterochromatin in these species. Thus, it would be important to carry out further cytogenetic studies in fungus-farming ants to gain a comprehensive understanding of heterochromatin composition among Attina species (Barros et al. 2018; reviewed by Mariano et al. 2019).

Several studies have used the C_0t-1 DNA fraction (moderately and highly repetitive sequences of the genome) as heterochromatic region marker in different organisms, such as beetles, bees, spittlebugs, fishes, and lizards (Cabral-de-Mello et al. 2011; Terencio et al. 2015; Anjos et al. 2016; Carvalho et al. 2016; Cunha et al. 2020). These studies showed that the C_0t-1 DNA fraction is a useful tool for understanding the events of heterochromatin differentiation and the organization patterns of repetitive sequences in the genomes of related species (Carvalho et al. 2016; Cunha et al. 2020).

Table 1 Summary of cytogenetic data on patterns of heterochromatin and correspondence of heterochromatin (HC)/GC-rich region distribution patterns in karyotypes of fungus-farming ant species available in the literature

Species	Heterochromatin pattern	HC/GC co-localized	References
Paleoattina clade			
Apterostigma madidiense Weber, 1938	M^{c}, SM^{sa}, ST^{sa}, A^{sa}	-	Barros (2014)
Apterostigma auriculatum Wheeler, 1925	$M^{c\text{-}pc}$, $SM^{c\text{-}pc}$	Yes	Barros (2014)
Mycocepurus goeldii (Forel, 1893)	$M^{c,\ pc}$	Yes	Barros et al. (2010)
Myrmicocrypta sp.	-	No*	Teixeira et al. (2021a)
Neoattina clade			
Acromyrmex ameliae De Souza et al. 2007	M^{c}, ST^{sa}, A^{sa}	No*	Barros et al. (2021)
Acromyrmex aspersus (Smith, 1858)	M^{c}, SM^{sa}, ST^{sa}, A^{sa}	No*	Barros (2014)
Acromyrmex balzani (Emery, 1890)	M^{c}, SM^{sa}, ST^{sa}, A^{sa}	No*	Barros et al. (2016)
Acromyrmex echinatior (Forel, 1899)	M^{c}, SM^{sa}, ST^{sa}, A^{sa}	No*	Barros et al. (2016)
Acromyrmex niger (Smith, 1858)	M^{c}, SM^{sa}, ST^{sa}, A^{sa}	No†	Barros et al. (2016)
Acromyrmex rugosus (Smith, 1858)	M^{c}, SM^{sa}, ST^{sa}, A^{sa}	No†	Barros et al. (2016)
Acromyrmex subterraneus brunneus (Forel, 1912)	M^{c}, ST^{sa}, A^{sa}	No*	Barros et al. (2021)
Acromyrmex subterraneus molestans Santschi, 1925	M^{c}, SM^{sa}, ST^{sa}, A^{sa}	No*	Teixeira et al. (2017)
Acromyrmex subterraneus subterraneus (Forel, 1893)	M^{c}, ST^{sa}, A^{sa}	No*	Barros et al. (2021)
Amoimyrmex striatus (Roger, 1863)	$M^{c,\ pc}$, $SM^{c,\ t}$	No†	Cristiano et al. (2013)
Atta bisphaerica Forel, 1908	M^{c}, SM^{c}, ST^{c}	No*	Barros et al. (2014)
Atta laevigata (Smith, 1858)	M^{c}, SM^{c}, ST^{c}	No*	Barros et al. (2014)
Atta robusta Borgmeier, 1939	M^{c}, SM^{c}, ST^{c}	No*	Barros et al. (2015)
Atta sexdens rubropilosa Forel, 1908	M^{c}, SM^{c}, ST^{c}	No*	Barros et al. (2014)
Neoattina clade			
Cyphomyrmex transversus Emery, 1894	M^{c}, SM^{c}, A^{sa}	Yes	Teixeira et al. (2021b)
Mycetarotes carinatus Mayhé-Nunes, 1995	$M^{c,\ pc}$, SM^{pc}	Yes	Barros (2014)
Mycetomoellerius urichii (Forel, 1893)§	$M^{c,\ pc}$, $SM^{c,\ pc}$	Yes	Barros et al. (2013a)
Mycetomoellerius holmgreni (Wheeler, 1925)	M^{c}	Yes	Barros et al. (2018)
Mycetomoellerius relictus (Borgmeier, 1934)	M^{c}	Yes	Barros et al. (2013a), Teixeira et al. (2021b)
Mycetomoellerius sp.	M^{c}, SM^{c}	-	Barros (2014)
Sericomyrmex maravalhas Ješovnik and Schultz, 2017	$M^{c,\ pc}$, M^{sa}, SM^{sa}	Yes	Teixeira et al. (2021 b)
Sericomyrmex sp.	$M^{c,\ pc}$, M^{sa}, SM^{sa}	Yes	Barros (2014)

Chromosomal types: **M**-metacentric, **SM**-submetacentric, **ST**-subtelocentric, **A**-acrocentric;

Chromosomal regions: **c**-centromeric, **pc**-pericentromeric, **sa**-short arm, **t**-terminal

*: GC-rich heterochromatin restricted to ribosomal genes location

†: GC-rich heterochromatin located in few chromosome pairs

-: data not available.

§: is the current synonym of *Trachymyrmex fuscus*

The genome of fungus-farming ants show notable structural rearrangement rates compared to those of other ants and insects already studied, with gene contractions and expansions indicating that substantial new genetic material became available and was necessary for the establishment of this lifestyle based on agricultural societies (Nygaard et al. 2016). The processes that led to genetic differentiation may also have affected repetitive sequences, showing the relevance of this peculiar group in understanding the repetitive portion of the ant genome. Thus, we produced a C_0t-DNA probe from the genome of *Mycetomoellerius relictus* (Borgmeier, 1934), belonging to Neoattina, which has the GC-rich heterochromatin on all its chromosomes. We hybridized this probe in the genome sequences of species of three genera

Table 2 Fungus-farming ant taxa from different locations, with its diploid/ (haploid) chromosome numbers, used in this study for physical mapping of microsatellite $(GA)_{15}$, telomeric motif $(TTAGG)_6$ or Mrel-C_0t probe (moderately and highly repetitive sequences from *Mycetomoellerius relictus* DNA)

Species	Locality	Coordinates	2n/(n)	Physical mapping		
				$(GA)_{15}$	**$(TTAGG)_6$**	**Mrel-C_0t**
Paleoattina clade						
Apterostigma madidiense Weber, 1938	Viçosa, MG, Brazil	– 20.757041, – 42.873516	(23)	P—DE	P—T	P—NM
Apterostigma steigeri Santschi, 1911	Viçosa, MG, Brazil	– 20.757041, – 42.873516	22	NP	NP	P—NM
Mycocepurus goeldii (Forel, 1893)	Viçosa, MG, Brazil	– 20.757041, – 42.873516	8	P – DE^{c}	P—T	P—NM
Myrmicocrypta sp.	Sinnamary, French Guiana	5.28482, – 52.91403	30	NP	NP	P—NM
Neoatttina clade						
Acromyrmex ameliae De Souza et al. 2007	Paraopeba, MG, Brazil	– 19.761728, – 44.4157997	36	P—DE	NP*	NP
Acromyrmex aspersus (Smith, 1858)	Viçosa, MG, Brazil	– 20.757041, – 42.873516	38	P—DE	NP	NP
Acromyrmex balzani (Emery, 1890)	Viçosa, MG, Brazil	– 20.757041, – 42.873516	38	P—DE	P—T	NP
Acromyrmex echinatior (Forel, 1899)	Barro Colorado Island, Panama	9.1562356, – 79.8802166	38	P—DE	P—T	P—NM
Acromyrmex niger (Smith, 1858)	Viçosa, MG, Brazil	– 20.757041, – 42.873516	38	P—DE	P—T	P—NM
Acromyrmex rugosus (Smith, 1858)	Paraopeba, MG, Brazil	– 19.761728, – 44.4157997	38	P—DE	NP	P—NM
Acromyrmex subterraneus brunneus (Forel, 1912)	Paraopeba, MG, Brazil	– 19.761728, – 44.4157997	38	P—DE	P—T	NP
Acromyrmex subterraneus molestans Santschi, 1925	Viçosa, MG, Brazil	– 20.757041, – 42.873516	38	P—DE	P—T	NP
Acromyrmex subterraneus subterraneus (Forel, 1893)	Paraopeba, MG, Brazil	– 19.761728, – 44.4157997	38	P—DE	NP	NP
Atta bisphaerica Forel, 1908	Lavras, MG, Brazil	– 21.27134, – 45.1467128	22	P—DE	NP	P—NM
Atta robusta Borgmeier, 1939	Aracruz, ES, Brazil	– 19.188083, – 40.2735622	22	P—DE	P—T	P—NM
Atta sexdens rubropilosa Forel, 1908	Ponte Nova, MG, Brazil	– 20.4206913, – 43.052746	22	P—DE	P—T	NP
Neoatttina						
Cyphomyrmex transversus Emery, 1894	Viçosa, MG, Brazil	– 20.757041, – 42.873516	18	P—DE	P—T	P—CH^{gc}
Mycetarotes carinatus Mayhé-Nunes, 1995	Viçosa, MG, Brazil	– 20.757041, – 42.873516	14	P—DE	NP	P—CH^{gc}
Mycetomoellerius urichii (Forel, 1893)†	Paraopeba, MG, Brazil	– 20.757041, – 42.873516	18	P—DE	NP	P—CH^{gc-pc}
Mycetomoellerius holmgreni (Wheeler, 1925)	Itutinga, MG, Brazil	– 21.283333, -44.650000	20	NP*	NP*	P—CH^{gc}
Mycetomoellerius relictus (Borgmeier, 1934)	Viçosa, MG, Brazil	– 20.757041, – 42.873516	20	P—DE	P—T	P—CH^{gc}

Table 2 (continued)

Species	Locality	Coordinates	2n/(n)	Physical mapping		
				$(GA)_{15}$	**$(TTAGG)_6$**	**Mrel-C_0t**
Mycetomoellerius sp.	Viçosa, MG, Brazil	− 20.757041, − 42.873516	22	P—DE	NP	P—CH^{gc}
Sericomyrmex maravalhas Ješovnik and Schultz, 2017	Viçosa, MG, Brazil	− 20.757041, − 42.873516	48	P—DE	NP	P—CH^{gc}
Sericomyrmex sp.	Viçosa, MG, Brazil	− 20.757041, − 42.873516	50	P – DE^{C}	P—T	P—CH^{gc}

*previously published data

† is the current synonym of *Trachymyrmex fuscus*

Brazilian states: MG – Minas Gerais; ES – Espírito Santo;

P: physical mapping performed in this study

NP: physical mapping not performed in this study

Chromosomal localization: DE, dispersed in euchromatin; DE^{C}, dispersed in euchromatin with additional clustering in some regions; T, telomeres; NM, no marking; CH^{gc}, co-localized with GC-rich heterochromatin; $CH^{gc, pc}$, co-localized with pericentromeric GC-rich heterochromatin

of Paleoattina and different Neoattina genera, basal and derivatives, to test whether GC-rich sequences are shared between the two clades and understand the evolutionary dynamics of heterochromatin in the genome of fungus-farming ants. Furthermore, we physically mapped the microsatellite $(GA)_{15}$ and the insect canonical telomeric sequence $(TTAGG)_6$ in different species of Paleoattina and Neoattina to investigate their distribution patterns in the karyotypes of these ants. Our data provide the first insights into the organization, diversity, and evolutionary patterns of the repetitive portion of the Attina genome from a broad perspective.

Materials and methods

Ant species sampling and chromosomal preparation

Colonies of 24 taxa of fungus-farming ants, with representatives of Paleoattina and Neoattina, were collected in different localities in Brazil, French Guiana, and Panama (Table 2). Species sampling authorizations were given by ICMBio/SISBIO in Brazil (accession number: 31861, 32,459, and 62,598) and by ABSCH-CNA-FR-240495–2 in French Guiana (accession number: TREL1820249A/61). Ant vouchers (workers) were deposited in the reference collection at the Laboratório de Mirmecologia, Centro de Pesquisas do Cacau (CPDC/Brazil), in Bahia, Brazil.

The mitotic metaphases from the fungus-farming ant species were obtained from cerebral ganglia of the larvae after meconium elimination, according to the protocol described by Imai et al. (1988).

Chromosomal mapping through fluorescence *in situ* hybridization (FISH) of different repetitive sequences

C_0t-DNA samples enriched with moderately and highly repetitive sequences of *M. relictus* (Neoattina) were obtained using the renaturation kinetics technique (Zwick et al. 1997). *Mycetomoellerius relictus* was chosen for two important reasons: (1) it has a GC-rich heterochromatin on all its chromosomes (Barros et al. 2013b; Teixeira et al. 2021b), and (2) it is a species with available chromosomal banding data that is phylogenetically closer to the leaf-cutting ant species, a more derivative group from the Neoattina clade (Solomon et al. 2019), which shows GC-rich heterochromatin restricted to the ribosomal gene region or in a few chromosomal pairs (reviewed by Teixeira et al. 2021a).

The C_0t-DNA probe from *M. relictus*, herein called Mrel-C_0t, was obtained according to the protocol described by Zwick et al. (1997), with modifications in the DNA precipitation step by Cunha et al. (2020). The reannealing time used was 8 min. Total DNA from *M. relictus* was extracted with a Qiagen DNeasy Blood and Tissue (cod. 69,506) extraction kit, according to the manufacturer's manual. The Mrel-C_0t probe was labeled by an indirect method with digoxigenin-11-dUTP using the kit DIG-Nick Translation Mix (Roche Applied Science, Mannheim, Germany), and the FISH signals were detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol.

In addition, other repetitive sequences were mapped to the karyotypes of fungus-farming ants. Microsatellite $(GA)_{15}$ and telomeric motif $(TTAGG)_6$ were used as

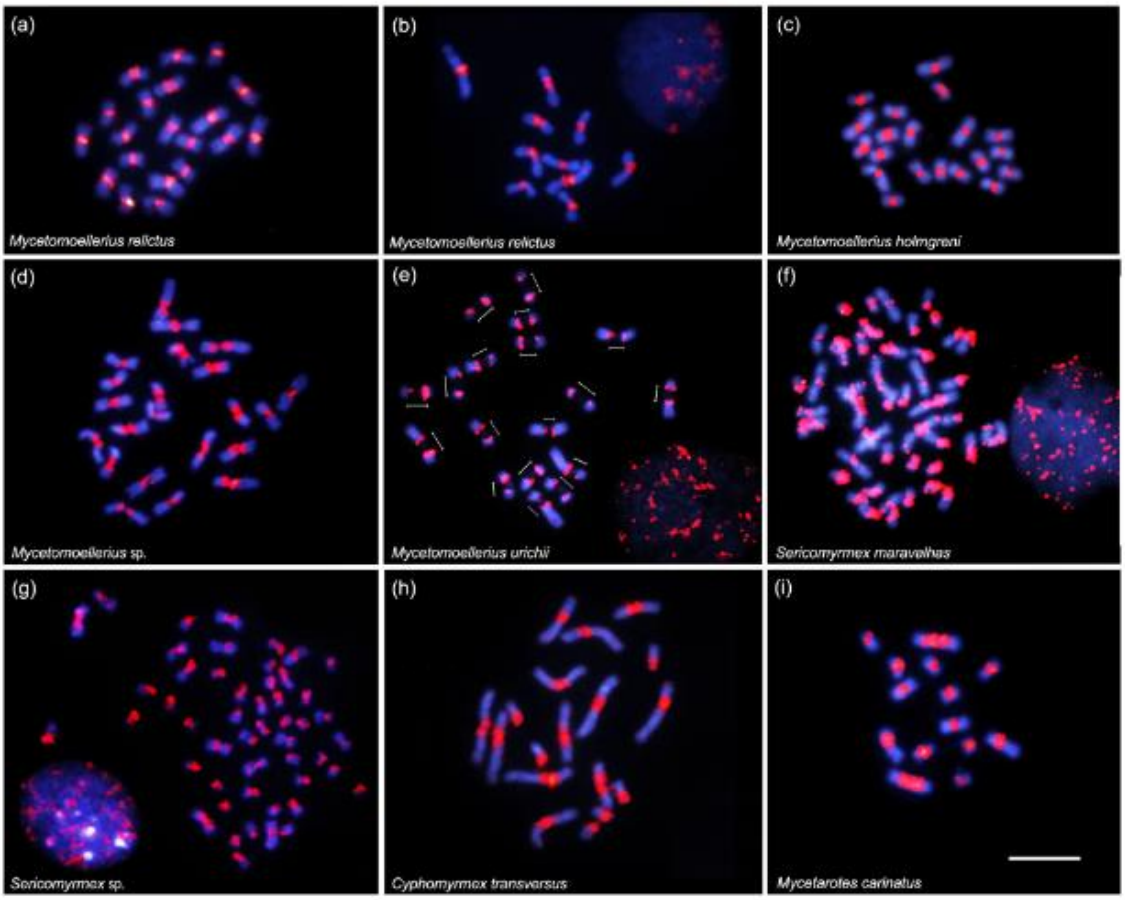


Fig. 1 Fluorescence *in situ* hybridization (FISH) using the C_0t-DNA probe from *Mycetomoellerius relictus* (red regions) and counterstained with DAPI (blue regions) in different species of fungus-farming ants included in the Neoattina clade: (**a**) and (**b**) female and male of *M. relictus* (2n=20, n=10), respectively, (**c**) *Mycetomoellerius holmgreni* (2n=20), (**d**) *Mycetomoellerius* sp. (2n=22), (**e**) *Mycetomoellerius urichii* (2n=18), (**f**) *Sericomyrmex maravalhas* (2n=48), (**g**) *Sericomyrmex* sp. (2n=50), (**h**) *Cyphomyrmex transversus* (2n=18), and (**i**) *Mycetarotes carinatus* (2n=14). Dimension lines in (e) indicate centromeric and pericentromeric regions. Bar: 5 µm

probes directly labeled with Cyanine-3 (Cy3) at the 5' terminus (Sigma, St. Louis, MO, USA).

The mapping of the repetitive sequences was performed by Fluorescence *in situ* Hybridization (FISH) following the protocol of Pinkel et al. (1986) with modifications: metaphasic chromosomes were denatured in 70% formamide/2X SSC at 75 °C for 5 min; the probes were hybridized with chromosomes in 20 µL of hybridization mix (200 ng of labeled probe, 2 × SSC, 50% formamide, and 10% dextrane sulfate); the hybridization mix was heated for 10 min at 85°C; and the slides were kept in a moist chamber at 37°C overnight. After, the slides with Mrel-C_0t probe were incubated in a detection solution containing anti-digoxygenin-rhodamine for 1h in a humid chamber at 37°C. In the case of the probes $(GA)_{15}$ and $(TTAGG)_6$, this incubation step with the antibody on the second day was omitted. Then, the slides were washed in 4 × SSC/Tween and dehydrated in an alcohol series 50%, 70%, and 100%. Lastly, the chromosomes were counterstained with DAPI (DAPI Fluoroshield, Sigma Aldrich).

Chromosomal analysis

At least 30 metaphases per species were analyzed to determine the karyotypic patterns obtained with the different repetitive probes. The metaphases were

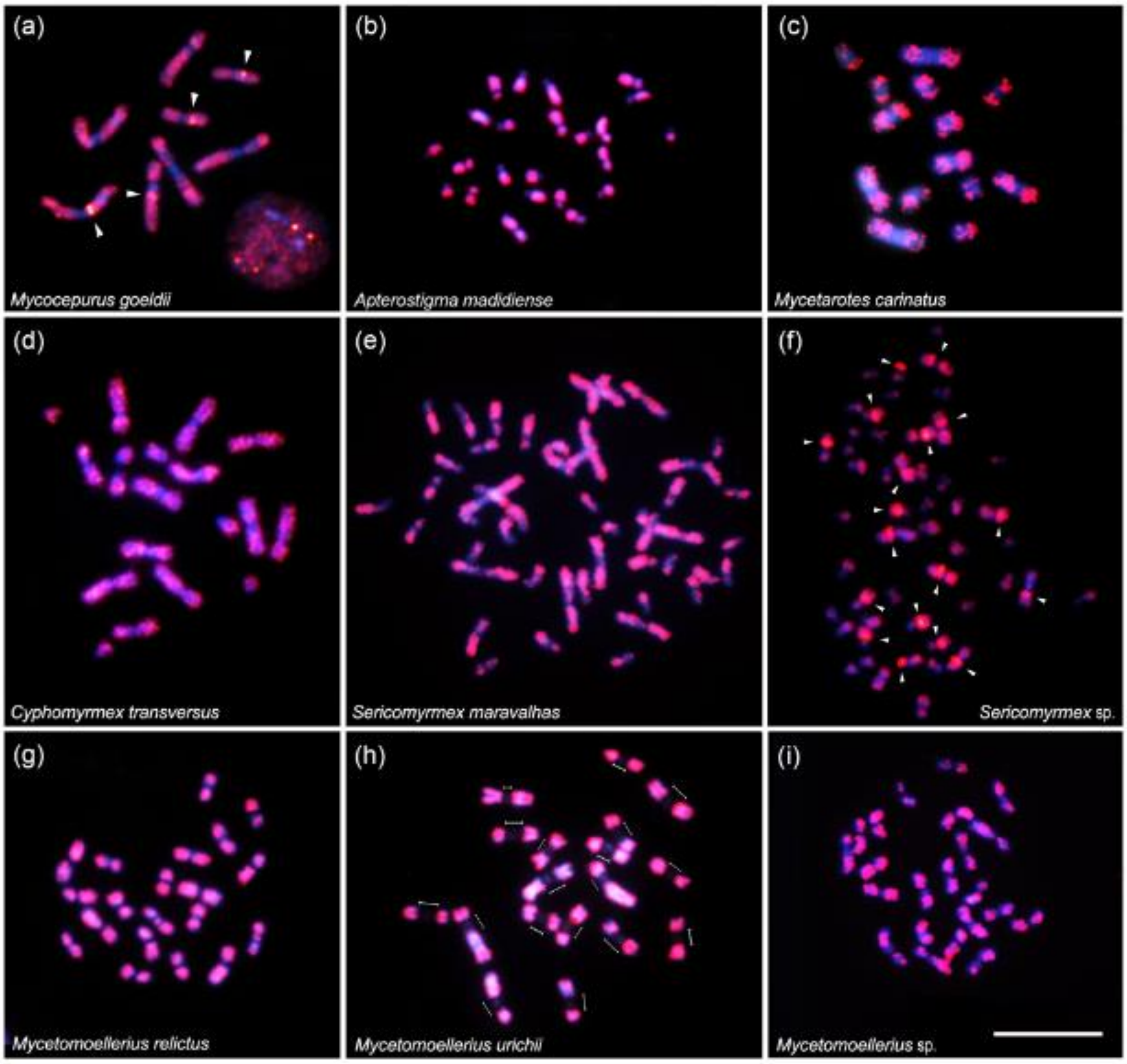


Fig. 2 Fluorescence *in situ* hybridization (FISH) using the $(GA)_{15}$ microsatellite probe (red regions) and counterstained with DAPI (blue regions) in different species of fungus-farming ants included in Paleoattina (**a**) *Mycocepurus goeldii* (2n=8) and (**b**) *Apterostigma madidiense* (n=23), and in Neoattina (**c**) *Mycetarotes carinatus* (2n=14), (**d**) *Cyphomyrmex transversus* (2n=18), (**e**) *Sericomyrmex maravalhas* (2n=48), (**f**) *Sericomyrmex* sp. (2n=50), (**g**) *Mycetomoellerius relictus* (2n=20), (**h**) *Mycetomoellerius urichii* (2n=18) and (**i**) *Mycetomoellerius* sp. (2n=22). Arrowheads in (**a**) and (**f**) indicate additional clustering to the scattered distribution of microsatellite $(GA)_{15}$. Dimension lines in (**h**) indicate centromeric and pericentromeric regions. Bar: 5 µm

photographed using an epifluorescence microscope Olympus BX53F attached to an Olympus MX10 camera, and CellSens image capture software. The filters WU (330–385 nm) and WG (510–550 nm) were used to analyze the fluorochromes DAPI and rhodamine and Cy3, respectively. Images were analyzed and processed using Adobe Photoshop® CS6 software.

Phylogenetic relationships

The phylogenetic relationship among fungus-farming ants was plotted in a figure constructed in accordance with molecular phylogenetic hypotheses, which are exclusive to specific groups within Attina, from previously published studies. The phylogenetic topology at

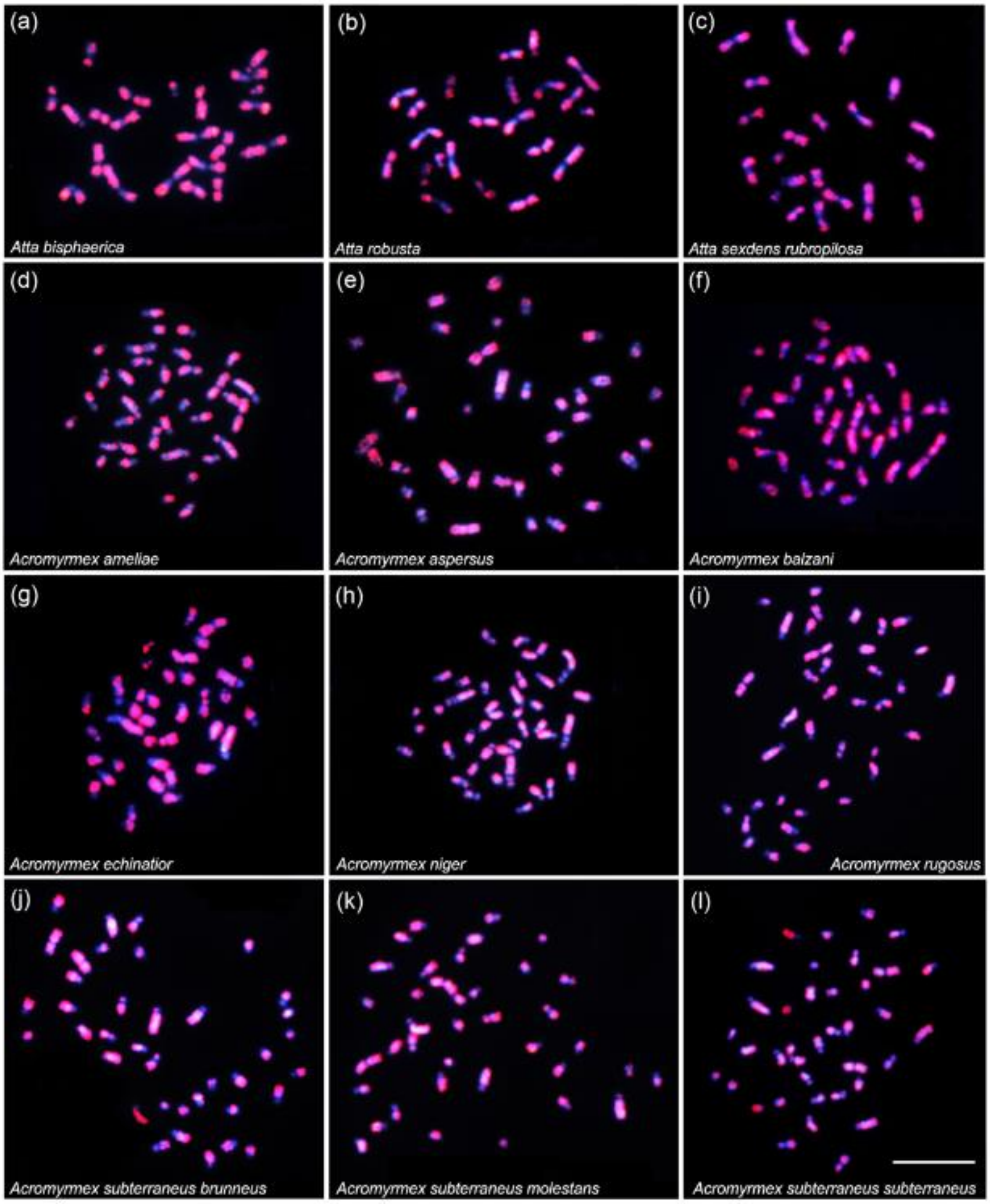


Fig. 3 Fluorescence *in situ* hybridization (FISH) using the $(GA)_{15}$ microsatellite probe (red regions) and counterstained with DAPI (blue regions) in different species of fungus-farming ants included in Neoattina: (**a**) *Atta bisphaerica* (2n=22), (**b**) *Atta robusta* (2n=22), (**c**) *Atta sexdens rubropilosa* (2n=22), (**d**) *Acromyrmex ameliae* (2n=36) (**e**) *Acromyrmex aspersus* (2n=38), (**f**) *Acromyrmex balzani* (2n=38), (**g**) *Acromyrmex echinatior* (2n=38), (**h**) *Acromyrmex niger* (2n=38), (**i**) *Acromyrmex rugosus* (2n=38), (**j**) *Acromyrmex subterraneus brunneus* (2n=38), (**k**) *Acromyrmex subterraneus molestans* (2n=38) and (l) *Acromyrmex subterraneus subterraneus* (2n=38). Bar: 5 µm

the genus level and among *Mycetomoellerius* species was determined according to Solomon et al. (2019), while the relationships within *Atta* and *Acromyrmex* followed Bacci et al. (2009) and Cristiano et al. (2020), respectively. Since there are no conflicting topologies among these phylogenetic hypotheses, a broad picture was constructed to help understand karyotype evolution among Attina species.

Results

The Mrel-C_0t probe marked the heterochromatic regions of all the chromosomes of *M. relictus*, *M. holmgreni*, *Mycetomoellerius* sp., and of the other Neoattina species *Sericomyrmex maravalhas* Ješovnik and Schultz, 2017, *Sericomyrmex* sp., *Cyphomyrmex transversus* Emery, 1894, and *Mycetarotes carinatus* Mayhé-Nunes, 1995 (Fig. 1). In *Mycetomoellerius urichii* (Forel, 1893) (current synonym of *Trachymyrmex fuscus*), only the pericentromeric heterochromatin of both chromosomal arms showed markings with the Mrel-C_0t probe (Fig. 1e). These results were compared with the C-banding data of these species obtained in previous studies (Table 1, Fig. S1), and it was confirmed that the Mrel-C_0t probe hybridized the heterochromatic regions. Markings with the Mrel-C_0t probe were neither observed in any species of Paleoattina, namely *Mycc. goeldii*, *Apterostigma steigeri* Santschi, 1911, *Apterostigma madidiense* Weber, 1938, and *Myrmicocrypta* sp. (Fig. S2) nor in the Neoattina species *Atta bisphaerica* Forel, 1908, *Acromyrmex balzani* (Emery, 1890), *Acromyrmex echinatior* (Forel, 1899), *Ac. niger*, and *Ac. rugosus* (Fig. S3).

Two distribution patterns of the microsatellite $(GA)_{15}$ were observed in the studied ants: (1) most species showed dispersed markings only in the euchromatic regions of the chromosomes (Figs. 2 and 3), and (2) additional clustering to the dispersed distribution was observed in *Mycc. goeldii*, in pair 4 and in one of the homologous of pairs 1 and 3 (Figs. 2 and S4a), and in *Sericomyrmex* sp., in nine metacentric chromosome pairs (Figs. 2f and S4b). These results were compared with the C-banding data of these species obtained in previous studies (Table 1) confirming that this microsatellite was not present in heterochromatic regions.

The $(TTAGG)_6$ probe marked the telomeres of both chromosomal arms of all species studied, and no interstitial markings were observed (Fig. 4).

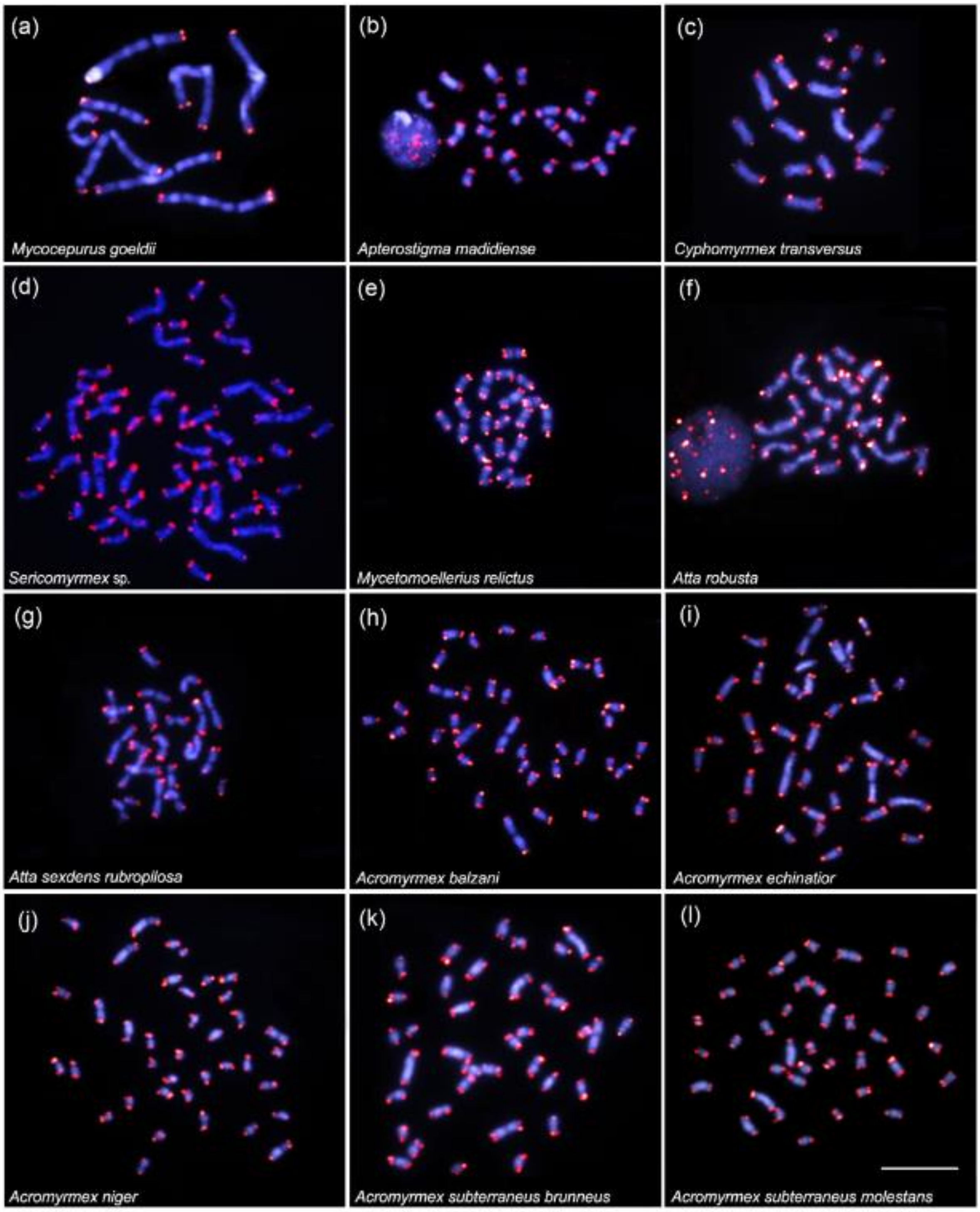
Discussion

Heterochromatin evolutionary dynamics in the genome of fungus-farming ants

The results of this study, obtained with the Mrel-C_0t probe associated with previously published chromosomal banding data (Table 1), revealed different evolutionary events that led to heterochromatin diversification among and within the clades of fungus-farming ants. It was possible to recognize at least five events of heterochromatin differentiation: (1) between the clades Paleoattina and Neoattina, (2) in the Paleoattina genus *Myrmicocrypta*, (3) in the Neoattina species *Mycetomoellerius urichii*, (4) in *Atta* and *Acromyrmex* genera derived from Neoattina, and (5) within *Acromyrmex* (Fig. 5).

Chromosomal banding data show that different species of the Paleoattina and Neoattina clades have GC-rich heterochromatin in all chromosomes (Table 1), a characteristic uncommon in ants (reviewed by Teixeira et al. 2021a). Markings with the Mrel-C_0t probe were observed in Neoattina species that had GC-rich heterochromatin, including *Mycetomoellerius*, *Sericomyrmex*, and in two other basal genera *Cyphomyrmex* and *Mycetarotes*, indicating that GC-rich repetitive sequences are shared among Neoattina genera. Interestingly, none of the Paleoattina analyzed species showed markings with the Mrel-C_0t probe. These data indicate differentiation of the GC-rich heterochromatin between Paleoattina and Neoattina, and therefore, this region consists of different repetitive sequences in these two clades (event 1, Fig. 5). The most likely hypothesis for the GC-rich heterochromatin origin is that it originated in the common ancestor of Attina and accumulated various changes over time, leading to its diversification into the two clades. This hypothesis is supported by the fact that the genera *Myrmicocrypta* (Paleoattina), *Atta*, and *Acromyrmex* (Neoattina) do not have GC-rich heterochromatin, are derived within their clades, and do not share exclusive common ancestry (Fig. 5). Alternatively, the difference in the constitution of the repetitive sequences could be explained by the independent arising of this GC-rich heterochromatin in the two Attina clades, which would be a less likely hypothesis because this type of heterochromatin is rare in ants (reviewed by Teixeira et al. 2021a).

Furthermore, within Paleoattina, available chromosomal banding data suggest a differentiation in heterochromatin composition among genera (event 2, Fig. 5),


(a)
Mycocepurus goeldii
(b)
Apterostigma madidiense
(c)
Cyphomyrmex transversus
(d)
Sericomyrmex sp.
(e)
Mycetomoellerius relictus
(f)
Atta robusta
(g)
Atta sexdens rubropilosa
(h)
Acromyrmex balzani
(i)
Acromyrmex echinatior
(j)
Acromyrmex niger
(k)
Acromyrmex subterraneus brunneus
(l)
Acromyrmex subterraneus molestans

◂**Fig. 4** Fluorescence *in situ* hybridization (FISH) using $(TTAGG)_6$ telomeric probe (red regions) and counterstained with DAPI (blue regions) in different species of fungus-farming ants included in Paleoattina (**a**) *Mycocepurus goeldii* (2n = 8) and (**b**) *Apterostigma madidiense* (n = 23), and in Neoattina (**c**) *Cyphomyrmex transversus* (2n = 18), (**d**) *Sericomyrmex* sp. (2n = 50), (**e**) *Mycetomoellerius relictus* (2n = 20), (**f**) *Atta robusta* (2n = 22), (**g**) *Atta sexdens rubropilosa* (2n = 22), (**h**) *Acromyrmex balzani* (2n = 38), (**i**) *Acromyrmex echinatior* (2n = 38), (**j**) *Acromyrmex niger* (2n = 38), (**k**) *Acromyrmex subterraneus brunneus* (2n = 38) and (**l**) *Acromyrmex subterraneus molestans* (2n = 38). Bar: 5 µm

because *Mycc. goeldii* and *Ap. auriculatum* showed GC-rich heterochromatin in all chromosomes (Barros et al. 2010; Barros 2014), whereas *Myrmicocrypta* sp. showed GC-rich ribosomal gene sites restricted only to a single chromosome pair (Teixeira et al. 2021a). Our data strengthen the importance of heterochromatin comprehension for the evolution of Attina, and further cytogenetic studies on Paleoattina species could provide more information on the evolutionary patterns of heterochromatin in this clade.

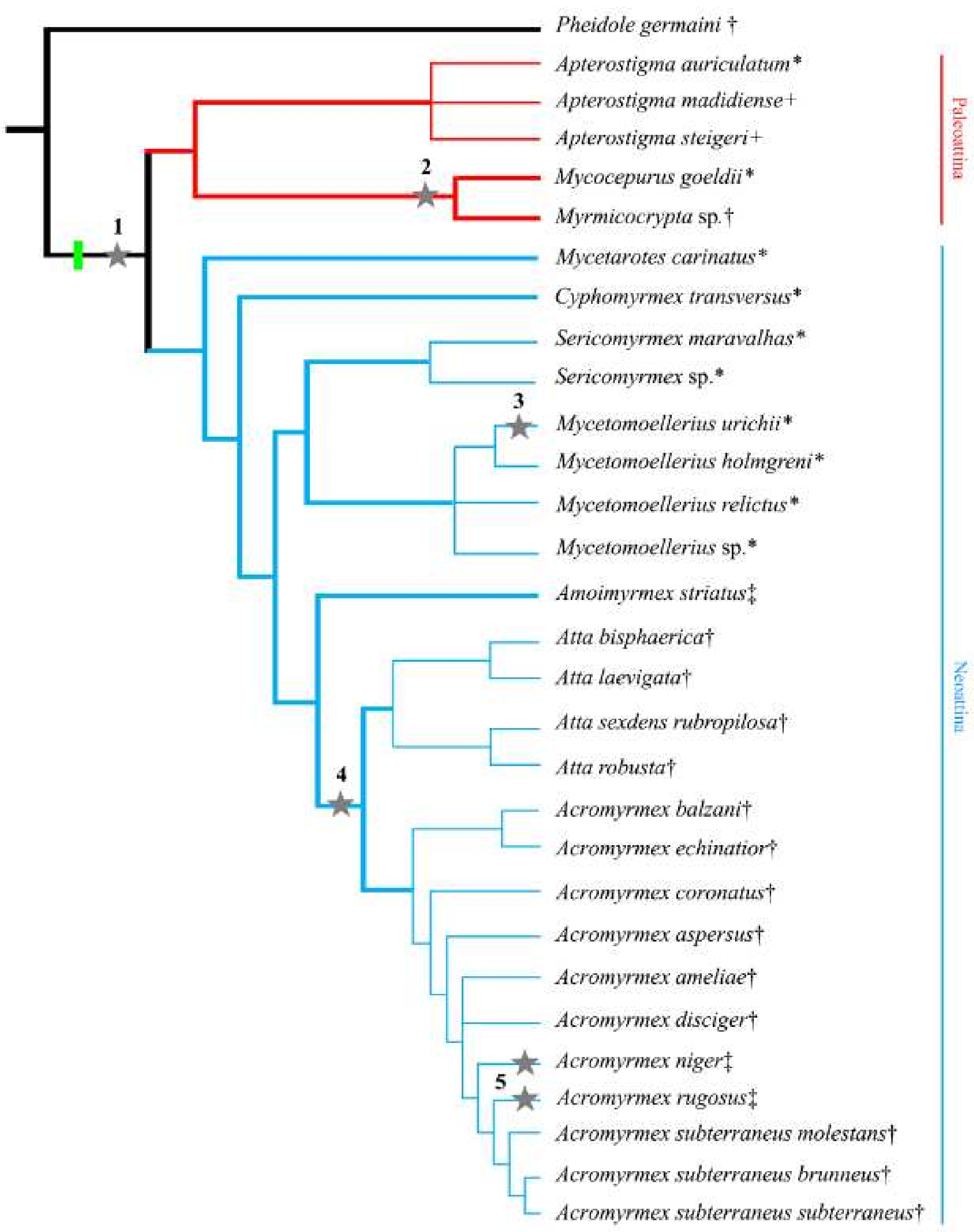
The third heterochromatin diversification event can be observed in the Neoattina species *M. urichii*, which showed a different pattern from the Mrel-C_0t probe, marking only the pericentromeric heterochromatin of the chromosomes. This indicates that this pericentromeric heterochromatin shares sequences with the repetitive DNA pool from *M. relictus*, whereas the centromeric heterochromatin has a distinct composition (event 3, Fig. 5). Previously, Barros et al. (2013a) highlighted this divergent pattern in *M. urichii* and showed that GC-rich heterochromatin is located in the centromeric and pericentromeric regions of the chromosomes, whereas in the other *Mycetomoellerius* spp., the heterochromatin is restricted to the centromeric region (Table 1). A possible hypothesis to explain the distinct composition of heterochromatin observed in *M. urichii* would be a change inside of the centromeric repetitive array of this species and its subsequent amplification by DNA polymerase slip, rolling circle replication with re-insertion of sequence or unequal crossing-over, resulting in centromeric array expansion. This would increase the extent of heterochromatin on chromosomes and shift the repetitive sequences shared with repetitive pools from *M. relictus* to the pericentromeric region (Fig. 6).

Evidences for centromere expansion has been suggested in some ants (Huang et al. 2016; Cardoso et al. 2018), although experiments with specific markings for centromeric proteins have not yet been performed in these studies. For example, centromere expansion has been observed in some *Solenopsis* fire ant species, with a significant increase in the copy number of CenSol, the most common satellite of the genome that constitutes the centromeric DNA in these species (Huang et al. 2016). Centromere expansion has also been suggested in Neoattina ant *M. holmgreni* due to variations in the length of DAPI-negative centromeric regions among different populations (Cardoso et al. 2018).

Centromeric expansion has been explained in ants by the centromere drive (Huang et al. 2016; Cardoso et al. 2018). This model, which is based on the asymmetry of female meiosis, predicts that longer centromeres would have a selective advantage in their transmission to the egg by providing a greater chromosomal extension for the binding of spindle proteins. This advantage would have negative effects on male meiosis because the four meiotic products become gametes, which may increase the chances of non-disjunction of chromosomes, leading to the production of unbalanced gametes. Mitigation of deleterious effects and restoration of meiotic balance can occur by positive selection of mutations in centromeric proteins that lead to more extensive binding in smaller centromeres allowing fixation of centromere expansion, or in heterochromatin-binding proteins that associate with expanded repetitive DNA in longer centromeres suppressing centromere expansion (Henikoff et al. 2001; Malik 2009). However, because ant males are haploid, this advantage of a longer centromere would not be a problem and would be a potentially common process in these insects and other hymenopterans (Huang et al. 2016; Cardoso et al. 2018). Thus, the rapid evolution of centromeric DNA, both in sequence and size, is a prediction of the centromere drive model (Henikoff et al. 2001; Malik 2009) and has been observed in different organisms, including ants (Melters et al. 2013; Zhang et al. 2014; Huang et al. 2016; Cardoso et al. 2018).

The data obtained in this study with the Mrel-C_0t probe in *M. urichii*, besides the length of DAPI-negative centromeric/pericentromeric regions observed in this species was greater than that in the other three *Mycetomoellerius* spp. (Fig. S5), suggest the hypothesis of occurrence of variations in the centromere sequence with its subsequent expansion in *M. urichii*, representing a possible case of long centromeres in ants. Alternatively, the suppression of long centromeres could have occurred in *M. urichii* through the binding of non-centromeric proteins in its pericentromeric heterochromatin, which has a nucleotide composition shared with the centromeric heterochromatin of other *Mycetomollerius* species. Future studies involving the characterization of sequences that constitute the centromeric/pericentromeric heterochromatin in this species, as well as experiments with markings for centromeric proteins, will be needed to clarify these hypotheses.

Some other heterochromatin diversification events were also observed within the Neoattina clade. The


Pheidole germaini †
Apterostigma auriculatum*
Apterostigma madidiense+
Apterostigma steigeri+
Mycocepurus goeldii*
Myrmicocrypta sp.†
Mycetarotes carinatus*
Cyphomyrmex transversus*
Sericomyrmex maravalhas*
Sericomyrmex sp.*
Mycetomoellerius urichii*
Mycetomoellerius holmgreni*
Mycetomoellerius relictus*
Mycetomoellerius sp.*
Amoimyrmex striatus‡
Atta bisphaerica†
Atta laevigata†
Atta sexdens rubropilosa†
Atta robusta†
Acromyrmex balzani†
Acromyrmex echinatior†
Acromyrmex coronatus†
Acromyrmex aspersus†
Acromyrmex ameliae†
Acromyrmex disciger†
Acromyrmex niger‡
Acromyrmex rugosus‡
Acromyrmex subterraneus molestans†
Acromyrmex subterraneus brunneus†
Acromyrmex subterraneus subterraneus†
1
2
3
4
5
Paleoattina
Neoattina

◂**Fig. 5** Phylogenetic relationships of fungus-farming ant species showing different evolutionary events that led to heterochromatin diversification among the Attina clades since its appearance in the common ancestor of the subtribe (vertical green bar). The events of heterochromatin differentiation, indicated by gray stars, occurs in: (1) between clades Paleoattina and Neoattina, (2) in Paleoattina genus *Myrmicocrypta*, (3) in Neoattina species *Mycetomoellerius urichii*, (4) in *Atta* and *Acromyrmex*, genera more derived from Neoattina, and (5) within *Acromyrmex* (*Ac. rugosus* and *Ac. niger*). Phylogenetic relationships based on Solomon et al. (2019), Bacci et al. (2009) and Cristiano et al. (2020). Thicker and thinner lines represent phylogenetic relationships among genera and species, respectively. Symbols represent: * GC-rich heterochromatin, † GC-rich regions restricted to ribosomal genes, ‡ GC-rich heterochromatin in few chromosome pairs, and + no available banding data

absence of markings with the Mrel-C_0t probe in the genera most derived from Neoattina *Atta*, and *Acromyrmex* confirms the differentiation of heterochromatin composition in comparison to that in the other basal Neoattina genera (event 4, Fig. 5). These results were expected because in these genera, only the ribosomal gene region is rich in GC, which is restricted to a chromosome pair (Barros et al. 2014, 2015, 2016; Teixeira et al. 2017). However, in some *Acromyrmex* spp., *Ac. rugosus*, and *Ac. niger*, a few GC-rich heterochromatic regions other than ribosomal genes were observed (Barros et al. 2016), which were not tagged with a Mrel-C_0t probe. These results indicate that the GC-rich heterochromatin from *Ac. niger* and *Ac. rugosus* has an origin independent of the heterochromatin observed in the other Neoattina species, representing a derived condition (event 5, Fig. 5). Additionally, these data evidence the lineage structuration in *Acromyrmex* and future molecular cytogenetic studies associated with a broad molecular phylogeny would be useful to investigate the origin of this GC-rich heterochromatin within *Acromyrmex*.

Atta and *Acromyrmex* present the most derived form of agriculture within Attina using only fresh vegetation for symbiotic fungus cultivation, in addition to other derivative characteristics such as polymorphic worker castes with complex division of labor, multiple-inseminated founding queens, and large colonies (reviewed by Mehdiabadi and Schultz 2010). The notable distinction in heterochromatin composition observed in *Atta* and *Acromyrmex* compared to the other Neoattina genera is in accordance with the natural-history data. However, little evidence of substantial genic or transcriptomic changes was observed in the ancestral lineages of leaf-cutting ants compared to other Neoattina species (Nygaard et al. 2016). Considering that repetitive sequences may have important roles in the genome, including those in its architectonic organization, gene regulation, replication, DNA repair, chromosomal rearrangements, and chromosome segregation (Shapiro and Sternberg 2005; Martins et al. 2011; Koo et al. 2016; Allshire and Madhani 2018), further studies involving repetitive DNA in fungus-farming ant species may shed some light on processes that led to the establishment of this derivative agriculture form in Attina.

Chromosomal mapping of microsatellite $(GA)_{15}$ and canonical telomeric insect sequence $(TTAGG)_6$ in karyotypes of fungus-farming ants

The other repetitive sequence probes used in this study showed more homogeneous results in the chromosomes of fungus-farming ants. The microsatellite $(GA)_{15}$ mapping, associated with the C-banding data available to date (Table 1), revealed that this sequence is located in the euchromatic regions of 21 analyzed taxa, which is common in other insects (Milani and Cabral-de-Mello 2014; Ruiz-Ruano et al. 2015; Palacios-Gimenez and Cabral-de-Mello 2015; Travenzoli et al. 2019). Although the $(GA)_{15}$ was present only in euchromatin, two distinct distribution patterns of this microsatellite were observed. The dispersed pattern was the most frequent among the species of different genera of Paleoattina and Neoattina. However, additional clustering of the scattered pattern was observed in *Mycc. goelldi*, *Sericomyrmex* sp. (present study), and in some populations of *M. holmgreni* (Micolino et al. 2019b). The combination of these cytogenetic data with the molecular phylogenies of Attina genera (Ješovnik et al. 2017; Solomon et al. 2019) suggests that $(GA)_{15}$ clustering in specific chromosomal regions seems to be a derived character.

Microsatellites show rapid evolution, mainly in terms of variability in the length of the repetitive sequences, which are important genetic markers (Li et al. 2002; Ellegren 2004). Different mechanisms, such as slippage of DNA polymerase during DNA replication or repair and unequal crossing-over, may be involved in the expansion or elimination of microsatellites in the genome (Li et al. 2002; Ellegren 2004). Furthermore, associations between microsatellites and transposable elements in different organisms have been observed, which indicates that mobile elements may also be involved in the genesis and genomic propagation of microsatellites (Ellegren 2004; Zhang 2004; Ruiz-Ruano et al. 2015). Thus, mechanisms such as DNA polymerase slip, DNA recombination, or transposition may be involved in $(GA)_{15}$ clustering in some regions of the karyotype of these fungus-farming ant species.

Some studies have suggested a possible role of microsatellites in the regulation of gene activity, particularly when located in promoter or intronic regions. These sequences can affect transcription, translation, and gene expression levels (reviewed by Li et al. 2002). In

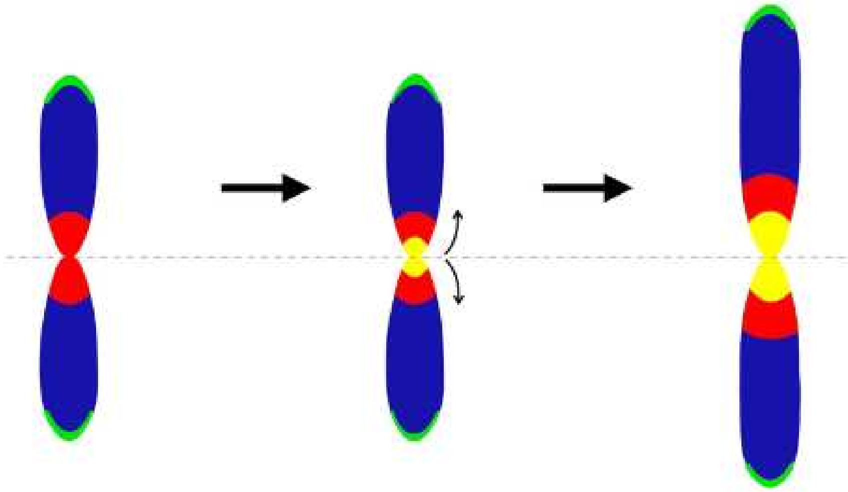


Fig. 6 Illustration of centromere expansion hypothesis in *Mycetomoellerius urichii*. The amplification of the new centromeric sequences (Yellow) pushes the ancestral centromeric sequences (Red) to pericentromeric regions. Telomeric sequences $(TTAGG)_6$ are shown in green

addition, microsatellites co-localized with gene regions, such as 5S and histones, for example, have been observed in molecular cytogenetic studies (Ruiz-Ruano et al. 2015, Piscor and Parise-Maltempi 2016). This shows that the clustering of microsatellites in specific chromosomal regions may suggest some functional role of these sequences in the Attina genome. Cytogenetic mapping of other repetitive sequences, such as transposable elements and multigene families (histones and 5S rDNA), in fungus-farming ants may elucidate this hypothesis.

Regarding the telomeric composition of the chromosomes of fungus-farming ants, the telomeric repetitive motif $(TTAGG)_6$ was detected in all telomeres of the species in this study. The telomeric sequence $(TTAGG)_n$ is considered to be the ancestral motif of insect telomeres with a possible origin from the sequence $(TTAGGG)_n$, the vertebrate canonical telomeric repeat (Frydrychová et al. 2004; Vítková et al. 2005; reviewed by Kuznetsova et al. 2019). However, the $(TTAGG)_n$ sequence was lost independently in different groups of insects and reappeared in others (review in Kuznetsova et al. 2019). In ants, the $(TTAGG)_n$ sequence has been conserved, with available data focusing mainly on Old World species (reviewed by Kuznetsova et al. 2019). Recent studies have been performed on fungus-farming ant species, including Neoattina, *Mycetophylax* spp. (Micolino et al. 2019a), *Mycetomoellerius* spp. (Micolino et al. 2019b, 2020), *Am. striatus* (Pereira et al. 2018), *Acromyrmex* spp., *A. sexdens* (Castro et al. 2020), and *Ac. ameliae* (Barros et al. 2021). Our data extend the (TTAGG) repetitive motif sampling in the telomeres of fungus-farming ants, as well as to the New World region, adding genera of Paleoattina (*Mycocepurus* and *Apterostigma*) and other genera of Neoattina (*Sericomyrmex* and *Cyphomyrmex*), showing that this canonical telomeric insect sequence is conserved in Attina.

No interstitial marking $(TTAGG)_n$ was observed in the species in this study or in previously available data for the subtribe (Pereira et al. 2018; Micolino et al. 2019a, b, 2020; Castro et al. 2020; Barros et al. 2021). Telomeric sequences located interstitially on chromosomes may be indicative of chromosomal fusion in an ancestral karyotype (Lin and Yan 2008; Olsson et al. 2018). According to the available cytogenetic data, chromosomal fissions seem to play a more important role in the karyotype evolution of most Attina genera (reviewed by Mariano et al. 2019). However, some reports suggest chromosomal fusion to explain chromosomal evolution, as in the derived karyotype of *Ac. ameliae* (Barros et al. 2021) and some phylogenetic branches in *Mycetophylax* (Micolino et al. 2019a). Such fusions (Robertsonian translocations) involve loss of genetic material, including telomeres, which would explain the absence of interstitial telomeric markings in these species (Micolino et al. 2019a; Barros et al. 2021).

Final remarks

This is the first large-scale study to analyze repetitive sequences in the karyotypes of different ant species with chromosomal markers for euchromatin and heterochromatin. Specifically, our study provides, with the unprecedented use of C_0t-DNA technique in ants, insights into the heterochromatin evolution in fungus-farming ants, indicating the occurrence of distinct differentiation events in its composition among Attina species. The presence of distinct patterns of $(GA)_{15}$ distribution among the studied species suggests that this microsatellite may be a promising chromosomal marker. Finally, our data show that the insect canonical telomeric sequence $(TTAGG)_6$ is conserved in this subtribe. Future studies focusing on the characterization of the repetitive sequences that constitute the heterochromatin in Paleoattina and Neoattina species will be important to investigate other possible diversification events and to extend the mapping of other microsatellites, enabling us to verify whether they follow a general trend of dispersed distribution, similar to $(GA)_{15}$, or other evolutionary patterns, and thus, continuing to contribute to the knowledge of the Attina genome.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00412-022-00770-7.

Acknowledgements We would like to thank Dr. Cléa S. F. Mariano for kindly providing *Acromyrmex echinatior* samples, Dr. Jacques H. C. Delabie for species identification, and Jérôme Orivel for the use of laboratory to process the *Myrmicocrypta* sp. samples. We also acknowledge Laboratório de Sistemética Molecular (Beagle) of the Universidade Federal de Viçosa (UFV) and Marina Souza da Cunha for technical support. GAT thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarships granted. Financial support for this study was provided by the Programa de Auxílio ao Pesquisador – PAPESQ/UNIFAP.

Author contributions All the authors were involved in the project design, data collection, and data interpretation; GAT performed the experiments; All the authors participated in writing the paper, read, and approved the final manuscript.

Funding Financial support for this study was provided by the Programa de Auxílio ao Pesquisador – PAPESQ/UNIFAP and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Data Availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval Ethical approval was not required for this study in accordance with local/national guidelines.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

References

Allshire RC, Madhani HD (2018) Ten principles of heterochromatin formation and function. Nat Rev Mol Cell Biol 19(4):229–244. https://doi.org/10.1038/nrm.2017.119

Anjos A, Rocha GC, Paladini A, Mariguela TC, Cabral-de-Mello DC (2016) Karyotypes and repetitive DNA evolution in six species of the genus *Mahanarva* (Auchenorrhyncha: Cercopidae). Cytogenet Genome Res 149(4):321–327. https://doi.org/10.1159/000450730

Augstenová B, Mazzoleni S, Kratochvíl L, Rovatsos M (2018) Evolutionary dynamics of the W chromosome in caenophidian snakes. Genes 9(1):5. https://doi.org/10.3390/genes9010005

Bacci M Jr, Solomon SE, Mueller UG, Martins VG, Carvalho AO, Vieira LG, Silva-Pinhati ACO (2009) Phylogeny of leafcutter ants in the genus *Atta* Fabricius (Formicidae: Attini) based on mitochondrial and nuclear DNA sequences. Mol Phylogenet Evol 51(3):427–437. https://doi.org/10.1016/j.ympev.2008.11.005

Barros LAC (2014) Evolução cromossômica da tribo Attini (Formicidae: Myrmiciane). Universidade Federal de Viçosa, Brazil, Thesis

Barros LAC, Aguiar HJAC, Mariano CSF, Delabie JHC, Pompolo SG (2010) Cytogenetic characterization of the lower-Attine *Mycocepurus goeldii* (Formicidae: Myrmicinae: Attini). Sociobiology 56:57–66

Barros LAC, Aguiar HJAC, Mariano CSF, Delabie JHC, Pompolo SG (2013a) Cytogenetic characterization of the ant *Trachymyrmex fuscus* Emery, 1934 (Formicidae: Myrmicinae: Attini) with the description of a chromosomal polymorphism. Ann Soc Entomol Fr 50:1–7. https://doi.org/10.1080/00379271.2013.856201

Barros LAC, Mariano CSF, Pompolo SG (2013b) Cytogenetic studies of five taxa of the tribe Attini (Formicidae: Myrmicinae). Caryologia 66(1):59–64. https://doi.org/10.1080/00087114.2013.780443

Barros LAC, Teixeira GA, Aguiar HJAC, Mariano CSF, Delabie JHC, Pompolo SG (2014) Banding patterns of three leafcutter ant species of the genus *Atta* (Formicidae: Myrmicinae) and chromosomal inferences. Fla Entomol 97:1694–1701. https://doi.org/10.1653/024.097.0444

Barros LAC, Aguiar HJAC, Teixeira GA, Mariano CSF, Teixeira MC, Delabie JHC, Pompolo SG (2015) Cytogenetic data on the threatened leafcutter ant *Atta robusta* Borgmeier, 1939 (Formicidae: Myrmicinae: Attini). C R Biol 338(10):660–665. https://doi.org/10.1016/j.crvi.2015.07.006

Barros LAC, Aguiar HJAC, Mariano CSF, Andrade-Souza V, Costa MA, Delabie JHC, Pompolo SG (2016) Cytogenetic data on six leafcutter ants of the genus *Acromyrmex* Mayr, 1865 (Hymenoptera, Formicidae, Myrmicinae): insights into chromosome evolution and taxonomic implications. Comp Cytogenet 10:229–243. https://doi.org/10.3897/CompCytogen.v10i2.7612

Barros LAC, Teixeira GA, Aguiar HJAC, Lopes DM, Pompolo SG (2018) Cytogenetic studies in *Trachymyrmex holmgreni* Wheeler, 1925 (Formicidae: Myrmicinae) by conventional and molecular methods. Sociobiology 65(2):185–190. https://doi.org/10.13102/sociobiology.v65i2.2214.

Barros LAC, Aguiar HJAC, Teixeira GA, Souza DJ, Delabie JHC, Mariano CSF (2021) Cytogenetic studies on the social parasite *Acromyrmex ameliae* (Formicidae: Myrmicinae: Attini) and its hosts reveal chromosome fusion in *Acromyrmex*. Zool Anz 293:273–281. https://doi.org/10.1016/j.jcz.2021.06.012

Biscotti MA, Olmo E, Heslop-Harrison JP (2015) Repetitive DNA in eukaryotic genomes. Chromosome Res 23:415–420. https://doi.org/10.1007/s10577-015-9499-z

Bolton B (2021) An online catalog of the ants of the world. http://antcat.org. Accessed 05 February 2021.

Cabral-de-Mello DC, Moura RDC, Souza AM, Martins C (2011) Evolutionary dynamics of heterochromatin in the genome of *Dichotomius* beetles based on chromosomal analysis. Genetica 139(3):315–325. https://doi.org/10.1007/s10709-011-9551-7

Cardoso DC, Heinze J, Moura MN, Cristiano MP (2018) Chromosomal variation among populations of a fungus-farming ant: implications for karyotype evolution and potential restriction to gene flow. BMC Evol Biol 18(1):146. https://doi.org/10.1186/s12862-018-1247-5

Carvalho ND, Carmo E, Neves RO, Schneider CH, Gross MC (2016) Differential repetitive DNA composition in the centromeric region of chromosomes of Amazonian lizard species in the family Teiidae. Comp Cytogenet 10(2):203–217. https://doi.org/10.3897/CompCytogen.v10i2.7081

Castro CPM, Cardoso DC, Micolino R, Cristiano MP (2020) Comparative FISH-mapping of TTAGG telomeric sequences to the chromosomes of leafcutter ants (Formicidae, Myrmicinae): is the insect canonical sequence conserved? Comp Cytogenet 14(3):369–385. https://doi.org/10.3897/CompCytogen.v14i3.52726

Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371(6494):215–220. https://doi.org/10.1038/371215a0

Cristiano MP, Cardoso DC, Fernandes-Salomão TM (2013) Cytogenetic and molecular analyses reveal a divergence between *Acromyrmex striatus* (Roger, 1863) and other congeneric species: taxonomic implications. PLoS ONE 8(3):e59784. https://doi.org/10.1371/journal.pone.0059784

Cristiano MP, Cardoso DC, Sandoval-Gómez VE, Simões-Gomes FC (2020). *Amoimyrmex* Cristiano, Cardoso & Sandoval, gen. nov. (Hymenoptera: Formicidae): a new genus of leaf-cutting ants revealed by multilocus molecular phylogenetic and morphological analyses. Austral Entomol 59(4):643–676. https://doi.org/10.1111/aen.12493.

Cunha MS, Campos LAO, Lopes DM (2020) Insights into the heterochromatin evolution in the genus *Melipona* (Apidae: Meliponini). Insectes Soc 67(3):391–398. https://doi.org/10.1007/s00040-020-00773-6

Della Lucia TMC, Souza DJ (2011) Importância e História de Vida das Formigas-Cortadeiras. In: Della Lucia TMC (ed) Formigas-Cortadeiras: da biotecnologia ao manejo, 1st edn. Editora UFV, Vicosa, Brazil, pp 13–26.

Ellegren H (2004) Microsatellites: simple sequences with complex evolution. Nat Rev Genet 5(6):435–445. https://doi.org/10.1038/nrg1348

Frydrychová R, Grossmann P, Trubac P, Vítková M, Marec FE (2004) Phylogenetic distribution of TTAGG telomeric repeats in insects. Genome 47(1):163–178. https://doi.org/10.1139/g03-100

Gunski RJ, Kretschmer R, Souza MS, Oliveira FI, Barcellos SA, Costa AL et al (2019) Evolution of bird sex chromosomes narrated by repetitive sequences: unusual W chromosome enlargement in *Gallinula melanops* (Aves: Gruiformes: Rallidae). Cytogenet Genome Res 158(3):152–159. https://doi.org/10.1159/000501381

Henikoff S, Ahmad K, Malik HS (2001) The centromere paradox: stable inheritance with rapidly evolving DNA. Science 293(5532):1098–1102. https://doi.org/10.1126/science.1062939

Huang YC, Lee CC, Kao CY, Chang NC, Lin CC, Shoemaker D, Wang J (2016) Evolution of long centromeres in fire ants. BMC Evol Biol 16(1):1–14. https://doi.org/10.1186/s12862-016-0760-7

Imai H, Taylor RW, Crosland MW, Crozier RH (1988) Modes of spontaneous chromossomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. Jpn J Genet 63:159–185. https://doi.org/10.1266/jjg.63.159

Ješovnik A, Sosa-Calvo J, Lloyd MW, Branstetter MG, Fernández F, Schultz TR (2017) Phylogenomic species delimitation and host-symbiont coevolution in the fungus-farming ant genus *Sericomyrmex* Mayr (Hymenoptera: Formicidae): ultraconserved elements (UCEs) resolve a recent radiation. Syst Entomol 42:523–542. https://doi.org/10.1111/syen.12228

Koo DH, Zhao H, Jiang J (2016) Chromatin-associated transcripts of tandemly repetitive DNA sequences revealed by RNA-FISH. Chromosome Res 24(4):467–480. https://doi.org/10.1007/s10577-016-9537-5

Krieger MJB, Ross KG (2003) Molecular evolutionary analyses of mariners and other transposable elements in fire ants (Hymenoptera: Formicidae). Insect Mol Biol 12(2):155–165. https://doi.org/10.1046/j.1365-2583.2003.00397.x

Kuznetsova V, Grozeva S, Gokhman V (2019) Telomere structure in insects: A review. J Zool Syst Evol Res 58(1):127–158. https://doi.org/10.1111/jzs.12332

Leal IR, Oliveira PS (1998) Interactions between fungus-growing ants (Attini), fruits, and seeds in cerrado vegetation in southeast Brazil. Biotropica 30:170–178. https://doi.org/10.1111/j.1744-7429.1998.tb00052.x

Leal IR, Wirth R, Tabarelli M (2014) The multiple impacts of leaf cutting ants and their novel ecological role in human modified neotropical forests. Biotropica 46:516–528. https://doi.org/10.1111/btp.12126

Li YC, Korol AB, Fahima T, Beiles A, Nevo E (2002) Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Mol Ecol 11(12):2453–2465. https://doi.org/10.1046/j.1365-294X.2002.01643.x

Lin KW, Yan J (2008) Ending in the middle: current knowledge of interstitial telomeric sequences. Mutat Res 658:95e110. https://doi.org/10.1016/j.mrrev.2007.08.006

López-Flores I, Garrido-Ramos MA (2012) The repetitive DNA content of eukaryotic genomes. Reptitive DNA 7:1–28. https://doi.org/10.1159/000337118

Lorite P, Carrillo JA, Tinaut A, Palomeque T (2002) Comparative study of satellite DNA in ants of the *Messor* genus. Gene 297(1–2):113–122. https://doi.org/10.1016/s0378-1119(02)00875-2.

Lorite P, Carrillo JA, Tinaut A, Palomeque T (2004) Evolutionary dynamics of satellite DNA in species of the genus Formica (Hymenoptera, Formicidae). Gene 332:159–168. https://doi.org/10.1016/j.gene.2004.02.049

Lorite P, Maside X, Sanllorente O, Torres MI, Periquet G, Palomeque T (2012) The ant genomes have been invaded by several types of mariner transposable elements. Naturwissenschaften 99(12):1007–1020. https://doi.org/10.1007/s00114-012-0982-5

Lorite P, Muñoz-López M, Carrillo JA, Sanllorente O, Vela J, Mora P et al (2017) Concerted evolution, a slow process for ant satellite DNA: study of the satellite DNA in the *Aphaenogaster* genus (Hymenoptera, Formicidae). Org Divers Evol 17(3):595–606. https://doi.org/10.1007/s13127-017-0333-7

Malik HS (2009) The centromere-drive hypothesis: a simple basis for centromere complexity. In: Ugarkovic D (ed) Centromere: Structure and Evolution, 1st edn. Springer, Berlin, Heidelberg, pp 33–52

Marchioro P, Campos LAO, Lopes DM (2020) First Record of a B Chromosome in *Polybia fastidiosuscula* Saussure (Vespidae) and Investigation of Chromatin Composition Through Microsatellite Mapping. Cytogenet Genome Res 160:711–718. https://doi.org/10.1159/000513641

Mariano CSF, Barros LAC, Velasco YM, Guimarães IN, Pompolo SG, Delabie JHC (2019) Citogenética de hormigas de la región neotropical. In: Fernández F, Guerrero R, Delsinne T (eds), 1st edn. Hormigas de Colombia. Universidad Nacional de Colombia, Bogotá, pp 131–157.

Martins C, Cabral-de-Mello DC, Valente GT, Mazzuchelli J, Oliveira SG, Pinhal D (2011). Animal genomes under the focus of cytogenetics. Nova Science, New York, USA.

Mehdiabadi NJ, Schultz TR (2010) Natural history and phylogeny of the fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). Myrmecol News 13:37–55

Melters DP, Bradnam KR, Young HA, Telis N, May MR, Ruby JG et al (2013) Comparative analysis of tandem repeats from hundreds of species reveals unique insights into centromere evolution. Genome Biol 14(1):1–20. https://doi.org/10.1186/gb-2013-14-1-r10

Micolino R, Cristiano MP, Travenzoli NM, Lopes DM, Cardoso DC (2019a) Chromosomal dynamics in space and time: evolutionary history of *Mycetophylax* ants across past climatic changes in the Brazilian Atlantic coast. Sci Rep 9(1):1–13. https://doi.org/10.1038/s41598-019-55135-5

Micolino R, Cristiano MP, Cardoso DC (2019b) Population-Based Cytogenetic Banding Analysis and Phylogenetic Relationships of the Neotropical Fungus-Farming Ant *Trachymyrmex holmgreni* Wheeler, 1925. Cytogenet Genome Res 159(3):151–161. https://doi.org/10.1159/000503913

Micolino R, Cristiano MP, Cardoso DC (2020) Putative chromosomal inversion clue in the fungus-farming ant *Mycetomoellerius iheringi* Emery, 1888. Comp Cytogenet 14(2):197–210. https://doi.org/10.3897/CompCytogen.v14i2.49846

Milani D, Cabral-de-Mello DC (2014) Microsatellite organization in the grasshopper *Abracris flavolineata* (Orthoptera: Acrididae) revealed by FISH mapping: remarkable spreading in the A and B chromosomes. PLoS ONE 9(5):e97956. https://doi.org/10.1371/journal.pone.0097956

Nygaard S, Hu H, Li C, Schiøtt M, Chen Z, Yang Z et al (2016) Reciprocal genomic evolution in the ant–fungus agricultural symbiosis. Nat Commun 7(1):1–9. https://doi.org/10.1038/ncomms12233

Olsson M, Wapstra E, Friesen CR (2018) Evolutionary ecology of telomeres: a review. Ann NY Acad Sci 1422(1): 5e28. https://doi.org/10.1111/nyas.13443.

Palacios-Gimenez OM, Cabral-de-Mello DC (2015) Repetitive DNA chromosomal organization in the cricket *Cycloptiloides americanus*: a case of the unusual X 1 X 2 0 sex chromosome system in Orthoptera. Mol Genet Genom 290(2):623–631. https://doi.org/10.1007/s00438-014-0947-9

Palomeque T, Carrillo JA, Muñoz-López M, Lorite P (2006) Detection of a mariner-like element and a miniature inverted-repeat transposable element (MITE) associated with the heterochromatin from ants of the genus *Messor* and their possible involvement for satellite DNA evolution. Gene 371(2):194–205. https://doi.org/10.1016/j.gene.2005.11.032

Pereira TTP, Reis ACCC, Cardoso DC, Cristiano MP (2018) Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger, 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera). Comp Cytogenet 12(1):13–26. https://doi.org/10.3897/CompCytogen.v12i1.21799

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. PNAS USA 83:2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Piscor D, Parise-Maltempi PP (2016) Microsatellite organization in the B chromosome and A chromosome complement in *Astyanax* (Characiformes, Characidae) species. Cytogenet Genome Res 148(1):44–51. https://doi.org/10.1159/000444728

Ruiz-Ruano FJ, Cuadrado Á, Montiel EE, Camacho JPM, López-León MD (2015) Next generation sequencing and FISH reveal uneven and nonrandom microsatellite distribution in two grasshopper genomes. Chromosoma 124(2):221–234. https://doi.org/10.1007/s00412-014-0492-7

Sanllorente O, Vela J, Mora P, Ruiz-Mena A, Torres MI, Lorite P, Palomeque T (2020) Complex evolutionary history of Mboumar, a mariner element widely represented in ant genomes. Sci Rep 10(1):1–10. https://doi.org/10.1038/s41598-020-59422-4

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. PNAS USA 105:5435–5440. https://doi.org/10.1073/pnas.0711024105

Shapiro JA, Sternberg RV (2005) Why repetitive DNA is essential to genome function. Biol Rev Camb Philos Soc 80(2):227–250. https://doi.org/10.1017/s1464793104006657

Solomon SE, Rabeling C, Sosa-Calvo J, Lopes CT, Rodrigues A, Vasconcelos HL, Bacci M Jr, Mueller UG, Schultz TR (2019) The molecular phylogenetics of *Trachymyrmex* Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of 'higher-attine' ant agriculture. Syst Entomol 44(4):939–956. https://doi.org/10.1111/syen.12370

Sosa-Calvo J, Jesovnik A, Okonski E, Schultz TR (2015) Locating, collecting, and maintaining colonies of fungus-farming ants (Hymenoptera: Myrmicinae: Attini). Sociobiology 62(2):300-320. https://doi.org/10.13102/sociobiology.v62i2.300-320

Sosa-Calvo J, Schultz TR, Ješovnik A, Dahan RA, Rabeling C (2018) Evolution, systematics, and natural history of a new genus of cryptobiotic fungus-growing ants. Syst Entomol 43:549–567. https://doi.org/10.1111/syen.12289

Teixeira GA, Barros LAC, Aguiar HJAC, Pompolo SG (2017) Comparative physical mapping of 18S rDNA in the karyotypes of six leafcutter ant species of the genera *Atta* and *Acromyrmex* (Formicidae: Myrmicinae). Genetica 145:351–357. https://doi.org/10.1007/s10709-017-9970-1

Teixeira GA, Aguiar HJAC, Petitclerc F, Orivel J, Lopes DM, Barros LAC (2021a) Evolutionary insights into the genomic organization of major ribosomal DNA in ant chromosomes. Insect Mol Biol 30(3):340–354. https://doi.org/10.1111/imb.12699

Teixeira GA, Barros LAC, Aguiar HJAC, Lopes DM (2021b) Distribution of GC-rich heterochromatin and ribosomal genes in three fungus-farming ants (Myrmicinae, Attini, Attina): insights on chromosomal evolution. Comp Cytogen 15(4):413–428. https://doi.org/10.3897/10.3897/compcytogen.v15.i4.73769

Terencio ML, Schneider CH, Gross MC, Carmo EJ, Nogaroto V, Almeida MC et al (2015) Repetitive sequences: the hidden diversity of heterochromatin in prochilodontid fish. Comp Cytogen 9(4):465–481. https://doi.org/10.3897/CompCytogen.v9i4.5299

Travenzoli NM, Lima BA, Cardoso DC, Dergam JA, Fernandes-Salomão TM, Lopes DM (2019) Cytogenetic analysis and chromosomal mapping of repetitive DNA in *Melipona* species (Hymenoptera, Meliponini). Cytogenet Genome Res 158(4):213–224. https://doi.org/10.1159/000501754

Vítková M, Král J, Traut W, Zrzavý J, Marec F (2005) The evolutionary origin of insect telomeric repeats (TTAGG)n. Chromosome Res 13(2):145–156. https://doi.org/10.1007/s10577-005-7721-0

Zhang DX (2004) Lepidopteran microsatellite DNA: redundant but promising. Trends Ecol Evol 19(10):507–509. https://doi.org/10.1016/j.tree.2004.07.020

Zhang H, Koblížková A, Wang K, Gong Z, Oliveira L, Torres GA et al (2014) Boom-bust turnovers of megabase-sized centromeric DNA in *Solanum* species: rapid evolution of DNA sequences associated with centromeres. Plant Cell 26(4):1436–1447. https://doi.org/10.1105/tpc.114.123877

Zwick MS, Hanson RE, Islam-Faridi MN, Stelly DM, Wing RA, Price HJ, McKnight TD (1997) A rapid procedure for the isolation of C 0 t-1 DNA from plants. Genome 40(1):138–142. https://doi.org/10.1139/g97-020

Supplementary information - Multiple heterochromatin diversification events in the genome of fungus-farming ants: insights from repetitive sequences

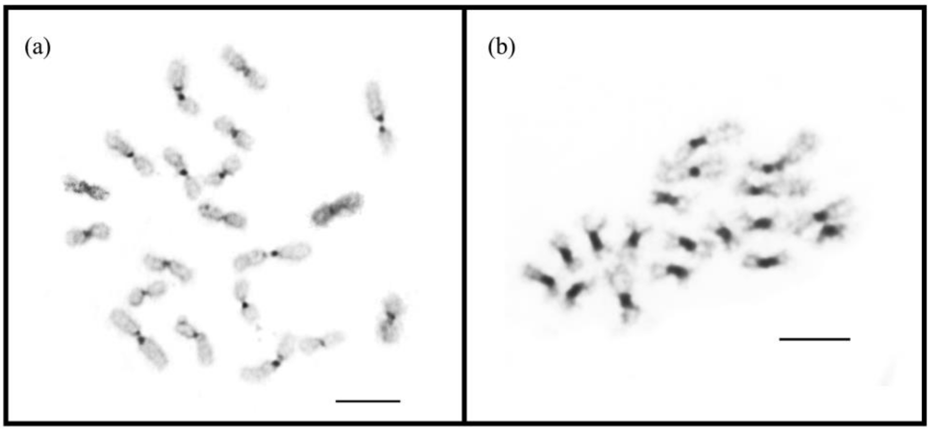


Fig. S1 Female metaphases submitted to C-banding technique showing heterochromatin location (dark regions) in different species of fungus-farming ants included in the Neoattina clade: (a) centromeric regions of *Mycetomoellerius relictus* (2n = 20), and (b) centromeric and pericentromeric regions of *Mycetomoellerius urichii* (2n = 18). Bars: 5 µm.

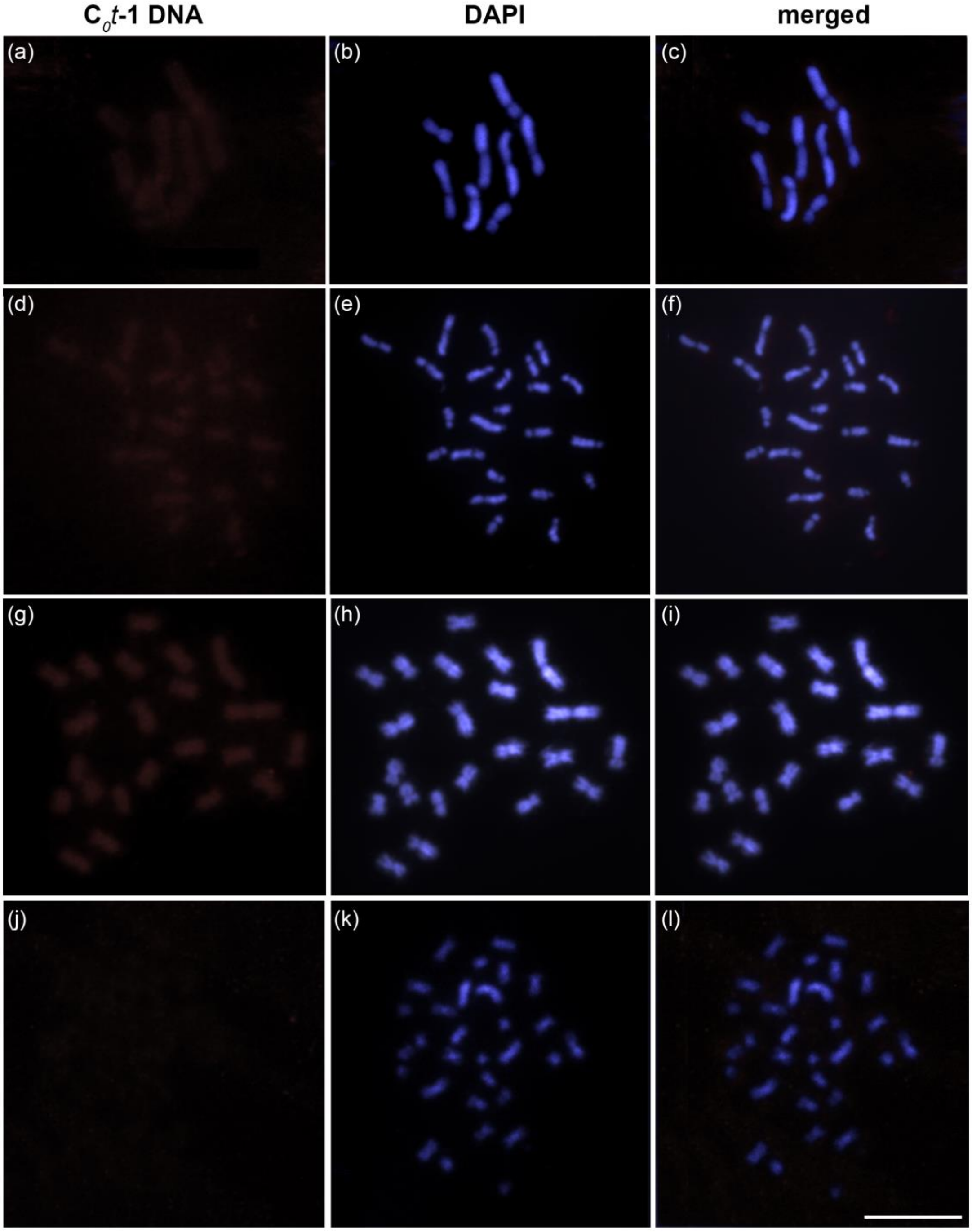


Fig. S2 Fluorescence *in situ* hybridization (FISH) using C_0t-DNA probe from *Mycetomoellerius relictus* and counterstained with DAPI (blue regions) in different species of fungus-farming ants included in the Paleoattina clade: (a), (b), (c) *Mycocepurus goeldii* (2n = 8), (d), (e), (f) *Apterostigma madidiense* (n = 23), (g), (h), (i) *Apterostigma steigeri* (2n = 22) and (j), (k), (l) *Myrmicocrypta* sp. (2n = 30). Bar: 5 µm

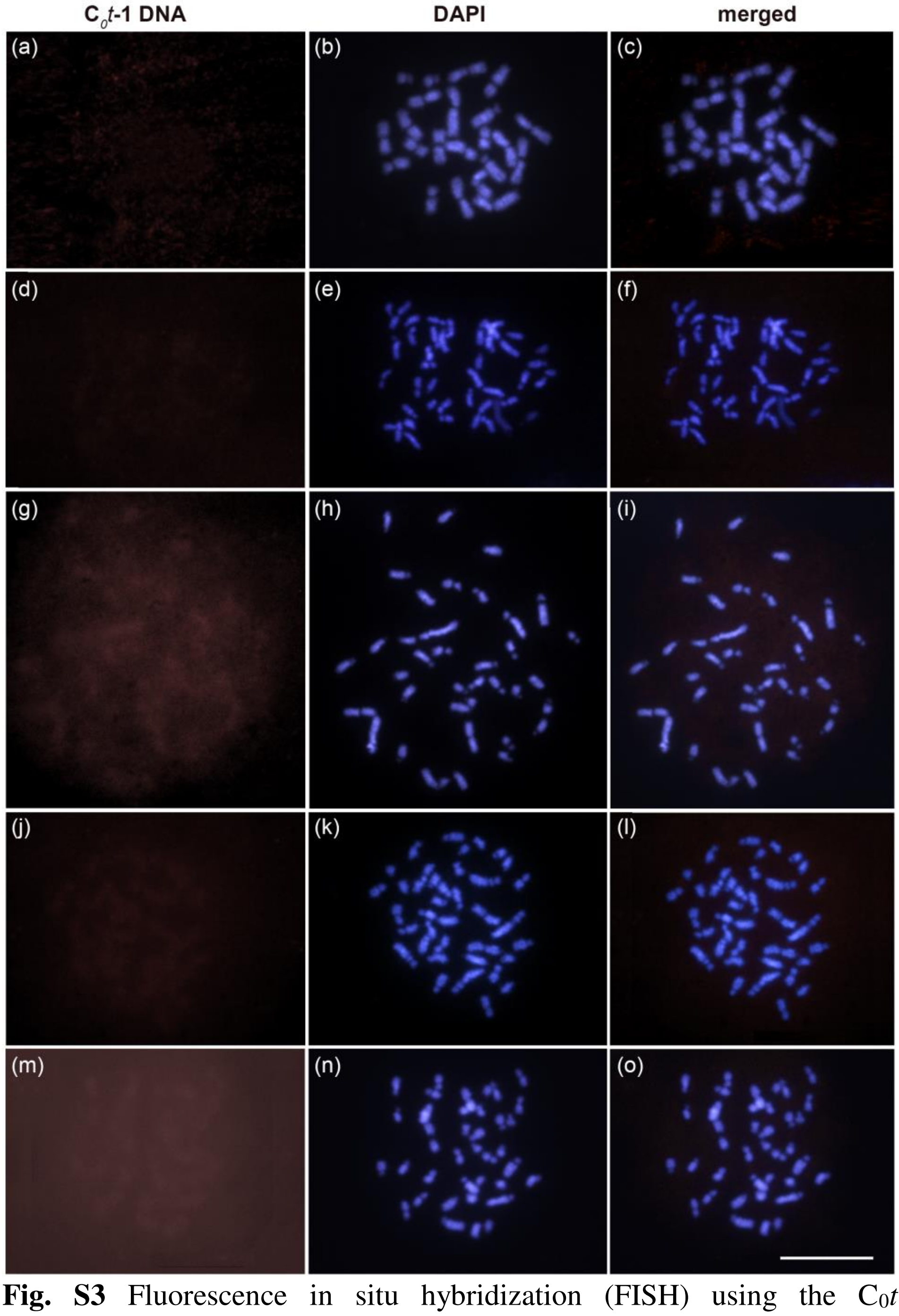


Fig. S3 Fluorescence in situ hybridization (FISH) using the C$_0t$-DNA probe from Mycetomoellerius relictus and counterstained with DAPI (blue regions) in different species of fungus-farming ants included in the Neoattina clade: (a), (b), (c) *Atta bisphaerica* (2n = 22), (d), (e), (f) *Acromyrmex balzani* (2n = 38), (g), (h), (i) *Acromyrmex echinatior* (2n = 38), (j), (k), (l) *Acromyrmex niger* (2n = 38) and (m), (n), (o) *Acromyrmex rugosus* (2n = 38). Bar: 5 µm

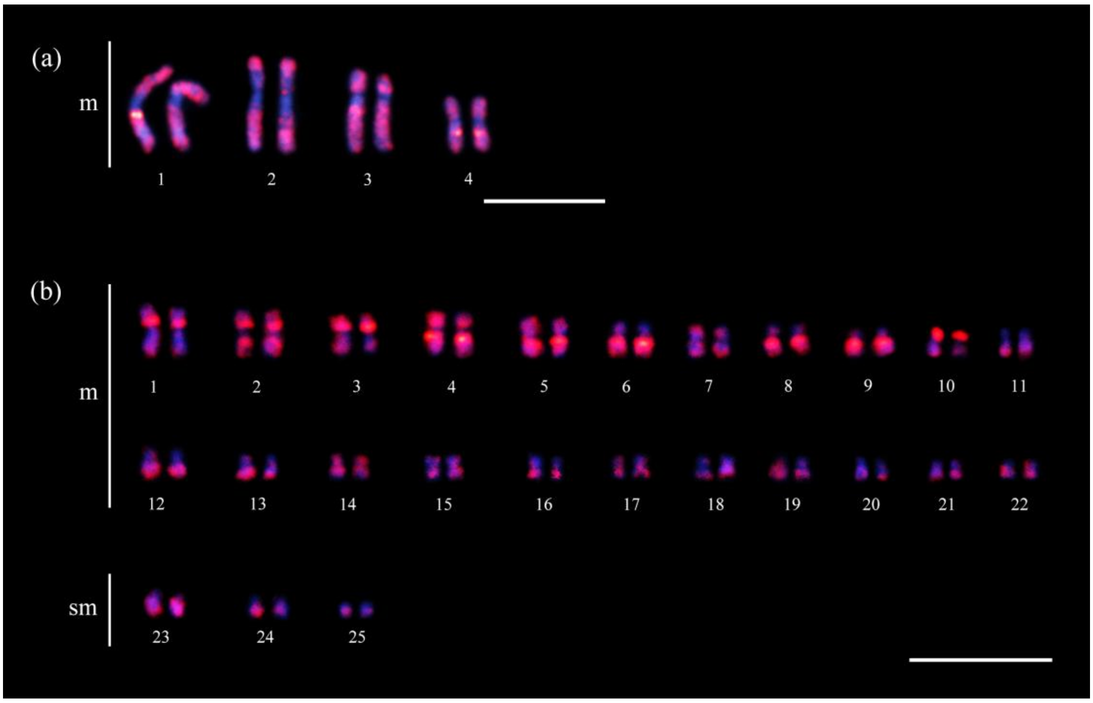


Fig. S4 Female karyotypes of (a) *Mycocepurus goeldii* (2n = 8) and (b) *Sericomyrmex* sp. (2n = 50) submitted to fluorescence *in situ* hybridization (FISH) with the (GA)15 microsatellite probe (red regions) and counterstained with DAPI (blue regions). Bars: 5 µm

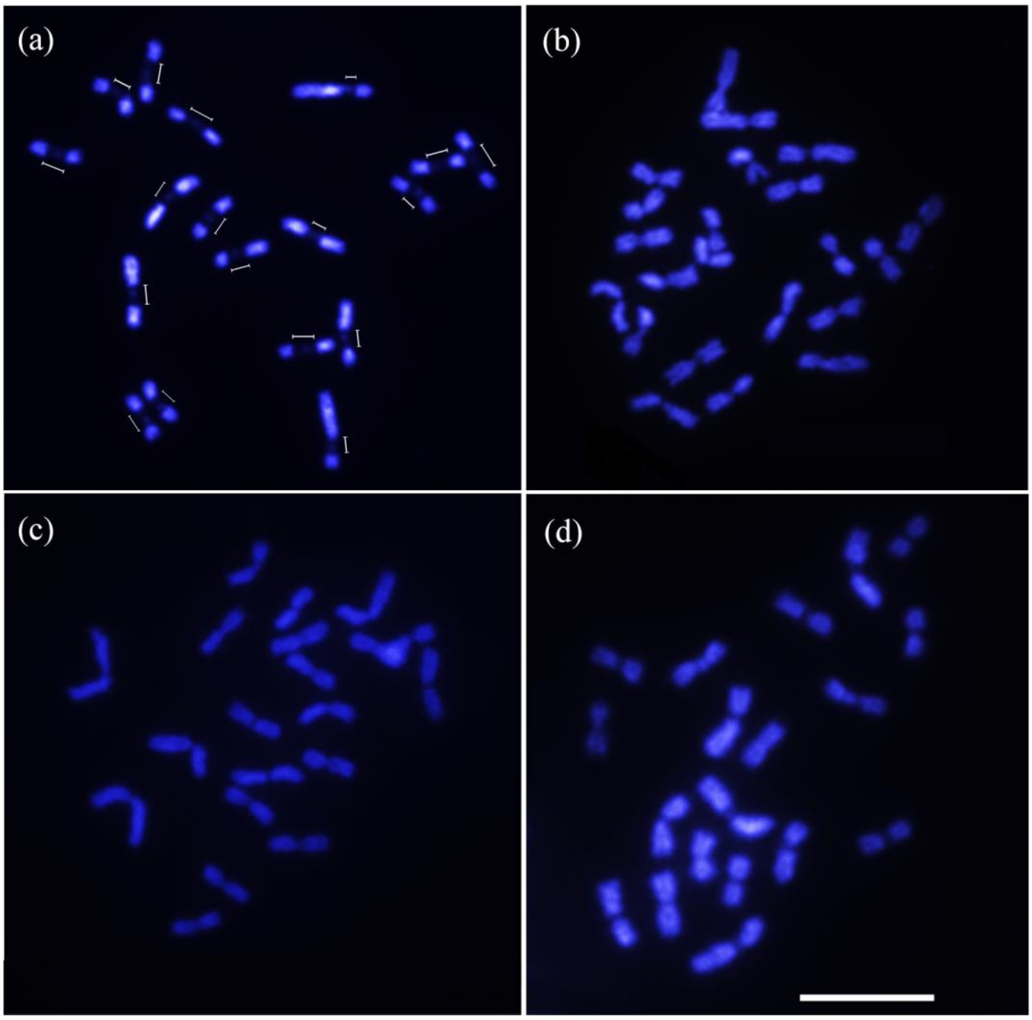


Fig. S5 Metaphases of *Mycetomoellerius* spp. submitted to DAPI fluorochrome: (a) *Mycetomoellerius urichii* (2n = 18), (b) *Mycetomoellerius* sp. (2n = 22), (c) *Mycetomoellerius holmgreni* (2n = 20), and (d) *Mycetomoellerius relictus* (2n = 20). Dimension lines in (a) indicate centromeric and pericentromeric regions. Bar: 5 µm

Capítulo IV

Karyotype conservation and genomic organization of repetitive sequences in the leaf-cutting ant *Atta cephalotes* (Linnaeus, 1758) (Formicidae: Myrmicinae)

Artigo submetido na revista *Genome* em dezembro/2021

Karyotype conservation and genomic organization of repetitive sequences in the leaf-cutting ant *Atta cephalotes* (Linnaeus, 1758) (Formicidae: Myrmicinae)

Gisele Amaro Teixeira[a,b], Luísa Antônia Campos Barros[c]§*, Linda Inês Silveira[d], Jérôme Orivel[e], Denilce Meneses Lopes[a,b], Hilton Jeferson Alves Cardoso de Aguiar[c,f]

[a] Programa de Pós-graduação em Biologia Celular e Estrutural, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570-900, Brazil.

[b] Laboratório de Citogenética de Insetos, Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil.

[c] Universidade Federal do Amapá, Campus Binacional – Oiapoque, n°3051, Bairro Universidade, Oiapoque, Amapá, 68980-000, Brazil.

[d] Programa de Pós-graduação em Biologia Animal, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570-900, Brazil.

[e] CNRS, UMR EcoFoG, AgroParisTech, CIRAD, INRAE, Université de Guyane, Université des Antilles, Campus Agronomique, BP 316, 97379, Kourou Cedex, France.

[f] Programa de pós graduação em Biodiversidade Tropical, Universidade Federal do Amapá, Campus Marco Zero do Equador, Macapá, Amapá, 68.903-419, Brazil.

§ GA Teixeira and LAC Barros contributed equally to this work.

* Corresponding author
Luísa Antônia Campos Barros; E-mail: luufv@yahoo.com.br

Highlights

Atta cephalotes showed a conserved chromosome number of 2n=22, like other *Atta* species.

Heterochromatic GC-rich regions and 18S rDNA sites appeared to be conserved in different *Atta* species.

Five microsatellites showed scattered but nonrandom distribution exclusively among euchromatic regions.

Our data corroborate karyotype conservation in *Atta* using classical and molecular cytogenetics.

Heterochromatin location may play an important role in karyotype conservation in *Atta* species.

Abstract

Leaf-cutting ants are among the New World's most conspicuous and studied ant species due to their notable ecological and economic role. Cytogenetic studies carried out in *Atta*, including species of three of the four phylogenetic groups, show remarkable karyotype conservation among the species. Molecular cytogenetic studies have enabled the understanding of the diversity, origin, organization, and evolutionary patterns of repetitive DNA in the eukaryotic genome. We performed classical cytogenetics and physical mapping of different repetitive sequences (18S rDNA, telomeric motifs, and microsatellites) in the leaf-cutting ant *Atta cephalotes* (Linnaeus, 1758), the type species of the genus, which belongs to the *Atta sensu stricto* group. Our goal was to test the karyotype conservation in *Atta* and to start to understand the genomic organization and diversity regarding repetitive sequences of leaf-cutting ant karyotypes. *Atta cephalotes* showed 2n=22 chromosomes with the karyotypic formula 18m+2sm+2st. The heterochromatin followed a centromeric pattern, and the GC-rich regions and 18S rDNA clusters were co-located interstitially in the 4th metacentric pair. All these cytogenetic characteristics were observed in other *Atta* species that had previously been studied. Physical mapping with repetitive probes allows for a better understanding of the chromatin composition of the chromosomes. We demonstrated that $(TTAGG)_n$ is the telomeric motif in *A. cephalotes* as observed in other ants, while the $(TTAGGG)_n$ and $(TCAGG)_n$ telomeric motifs are not present in their chromosomes, for which mapping was performed for the first time in the subtribe Attina. The $A_{(30)}$, $(GA)_{15}$, $(CA)_{15}$, $(GAG)_{10}$, and $(CAA)_{10}$ microsatellites showed a scattered distribution exclusively on euchromatic areas of the chromosomes, covering nearly the entirety of the chromosome lengths except for the heterochromatic centromeric regions. In the present study, we determined the karyotype of the leaf-cutting ant *A. cephalotes*, confirming the karyotype conservation in *Atta* using classical and molecular cytogenetic techniques. Evolutionary implications regarding the conservation of the chromosome number in leaf-cutting ants are also discussed. Additionally, several microsatellites and different telomeric sequences were mapped on the chromosomes of *A. cephalotes*, thus contributing to the first insights into genomic organization and diversity of repetitive sequences in leaf-cutting ants, which may be useful in further comparative studies.

Keywords: Attina, cytogenetic markers, fluorochromes, euchromatin, chromosomal evolution, FISH

Introduction

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

Currently, leaf-cutting ants include 53 valid species distributed in three genera: *Atta* Fabricius, 1804; *Acromyrmex* Mayr, 1865; and *Amoimyrmex* Cristiano et al. 2020 (reviewed by Bolton, 2021). Due to their notable characteristics mentioned above, these ants have been the focus of several research lines, including cytogenetics (Table 1; reviewed by Barros et al., 2021; Micolino et al., 2022). Particularly for *Atta*, cytogenetic data are available for five species (two subspecies) (Table 1), with representatives in three of the four monophyletic groups defined by molecular phylogenies previously proposed (Bacci et al., 2009; Barrera et al., 2021). *Atta sexdens* (Linnaeus, 1758) and *Atta robusta* Borgmeier, 1939 are included in the *Neoatta* group, *Atta bisphaerica* Forel, 1908 in the *Epiatta* group, and *Atta colombica* Guérin-Méneville, 1844 in the *Atta sensu stricto* group. No cytogenetic data is available for the ancestral *Archeatta* group, which is restricted to the Caribbean and North/Central America (Barrera et al., 2021).

Thus far, all of the *Atta* species submitted to chromosomal measurements have a diploid chromosome number of 2n=22 and the karyotype formula 18m+2sm+2st (Table 1); therefore, they are considered as having a conserved karyotype (Barros et al., 2014, 2015; Aguiar et al., 2020). In addition, distinctive GC-rich chromatin was detected in the 4th metacentric chromosome pair, which co-localized in the secondary constriction of *A. bisphaerica*, *A. laevigata*, *A. sexdens rubropilosa*, and *A. robusta* (Barros et al., 2014, 2015). This distinct GC-rich chromatin corresponds to the 18S ribosomal sites (Barros et al., 2015; Teixeira et al., 2017). *Atta cephalotes* also belongs to the *Atta sensu stricto* group (Bacci et al., 2009; Barrera et al., 2021) and is the type species of the genus; it is widely distributed in the Neotropical region (Janicki et al., 2016) and has high environmental importance since it is a deep forest species and, therefore, not a major agricultural pest (Jaffe and Vilela, 1989;

Urbas et al., 2007; Forti et al., 2020). Despite having its genomic data available at DDBJ/EMBL/GenBank (Suen et al., 2011), no information regarding the karyotype configuration of *A. cephalotes* has been published thus far. Within the *Atta sensu stricto* group, *A. colombica* is the only species that has been karyotyped (Murakami et al., 1998); however, information concerning the physical mapping of its chromosomes is lacking. Despite the importance of *A. colombica* karyological data, it is challenging to compare the karyotype to that of other *Atta* species since it was published over 20 years ago and image quality is highly limited.

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

In ants, reports of SSRs are scarce and, so far, are only available for the fungus-farming ants *Mycetomoellerius holmgreni* (Wheeler, 1925) (Barros et al., 2018; Micolino et al., 2019), *Amoimyrmex bruchi* (Forel, 1912), and *Amoimyrmex silvestrii* (Emery, 1905) (Micolino et al., 2022). Scattered distribution of the $(GA)_{15}$ microsatellite was observed exclusively in the euchromatin of the chromosomes (Barros et al., 2018), although specific blocks with differences among some populations were also reported for these species (Micolino et al., 2019, 2022). In contrast, the $(GAG)_{10}$, $(CAA)_{10}$, and $(CGG)_{10}$ microsatellites did not show specific bands in *M. holmgreni* (Micolino et al., 2019). Additionally, other repetitive sequences, such as telomeric sequences, have been mapped on ant chromosomes, including leaf-cutting ants; they show that the TTAGG motif constitutes their telomeres (Meyne et al., 1995; Lorite et al., 2002; Pereira et al., 2018; Castro et al., 2020; Teixeira et al., in prep.).

In the present study, we performed classical cytogenetics and physical mapping of repetitive sequences (18S rDNA, telomeric motifs, and microsatellites mono-, di-, and tri-nucleotides) on the chromosomes of the leaf-cutting *A. cephalotes*. Our goal was to test the karyotype conservation of this species compared to other *Atta* species and start to understand the genomic organization and diversity of repetitive sequences in leaf-cutting ants.

Table 1. Summary of cytogenetic data available, including this study, for leaf-cutting ants of the genus *Atta*, organized in monophyletic groups according to Bacci et al. (2009) and Barrera et al. (2021). Diploid chromosome number (2n), diploid karyotypic formula, heterochromatin distribution, 18S rDNA chromosome location and references

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

&karyotypic formula using chromosome measurements according to the classification provided by Levan et al (1964).

Chromosome types: M/m - metacentric, sm - submetacentric, st - subtelocentric, A – acrocentric

Materials and Methods

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

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

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

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

Table 2. Summary of the chromosomal hybridization signals of microsatellites and telomeric sequences detected in chromosomes of *Atta cephalotes* using FISH technique. SP: scattered pattern of the FISH signal; T: telomeric region; – no hybridization signal

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

The mapping of the repetitive sequences was performed by Fluorescence *in situ* Hybridization (FISH) following the protocol of Pinkel et al. (1986) with modifications: metaphase chromosomes were denatured in 70% formamide/2X SSC at 75°C for 3 min; the probes were hybridized with chromosomes in 20 µL of hybridization mix (200 ng of labeled probe, 2× SSC, 50% formamide, and 10% dextrane sulfate); The hybridization mix was heated for 10 min at 85° C, and the slides were kept in a moist chamber at 37°C overnight. After, the slides with rDNA 18S probe were incubated in a detection solution containing anti-digoxygenin-rhodamine for 1 hour in a humid chamber at 37°C. In the case of microsatellite and telomeric probes, this incubation step with the antibody on the second day was omitted. Then, the slides were washed in 4×SSC/Tween and dehydrated in an alcohol series 50%, 70%, 100%. Lastly, the chromosomes were counterstained with DAPI (DAPI Fluorshield, Sigma Aldrich).

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

The phylogenetic relationship among *Atta* spp. was determined according to previously published molecular phylogeny from Bacci et al. (2009) and Barrera et al. (2021).

Results

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

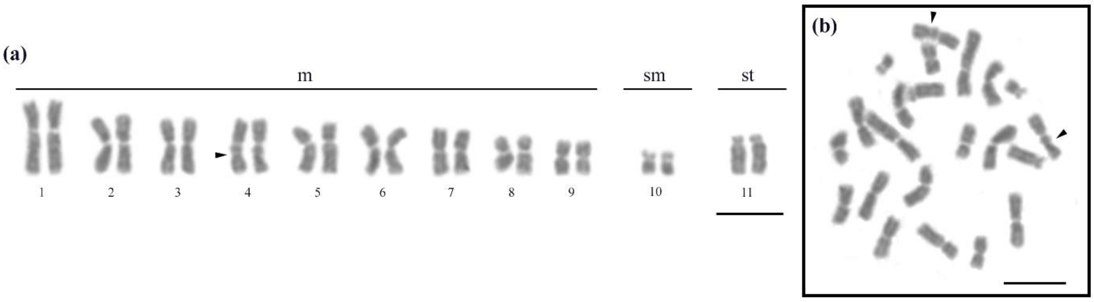


Figure 1. Diploid karyotype and metaphase of *Atta cephalotes* female (2n=22; 2n=18m+2sm+2st). Arrowhead indicates the presence of a secondary constriction in the fourth metacentric pair. Bars=5µm.

Table 3. Karymorphometrical analyses of *Atta cephalotes* female. The values (µm) represent an average obtained from 10 metaphases. p: short arm length; q: long arm length; TL: total length (p + q); RL: relative length (RL = TL*100/ΣTL); r: arm ratio (q / p). Chromosomal classification according to Levan et al. (1964)

Chromosomes	**p**	**q**	**TL**	**RL**	**r**	**Classification**
1	2.21 ± 0.29	3.19 ± 0.44	5.39 ± 0.70	6,73%	1.45 ± 0.11	Metacentric
1	2.17 ± 0.24	3.22 ± 0.42	5.40 ± 0.65	6,75%	1.48 ± 0.09	Metacentric
2	2.08 ± 0.36	2.47 ± 0.33	4.55 ± 0.66	5,68%	1.20 ± 0.13	Metacentric
2	2.06 ± 0.29	2.43 ± 0.32	4.49 ± 0.59	5,61%	1.19 ± 0.08	Metacentric
3	1.86 ± 0.23	2.40 ± 0.27	4.26 ± 0.49	5,32%	1.30 ± 0.10	Metacentric
3	1.90 ± 0.27	2.38 ± 0.25	4.27 ± 0.49	5,33%	1.27 ± 0.13	Metacentric
4	1.76 ± 0.26	2.32 ± 0.29	4.08 ± 0.54	5,10%	1.32 ± 0.06	Metacentric
4	1.68 ± 0.21	2.35 ± 0.31	4.04 ± 0.49	5,05%	1.40 ± 0.14	Metacentric
5	1.57 ± 0.13	2.30 ± 0.35	3.87 ± 0.45	4,83%	1.47 ± 0.16	Metacentric
5	1.53 ± 0.16	2.29 ± 0.33	3.82 ± 0.47	4,77%	1.50 ± 0.13	Metacentric
6	1.65 ± 0.19	2.21 ± 0.31	3.85 ± 0.48	4,85%	1.32 ± 0.10	Metacentric
6	1.59 ± 0.18	2.09 ± 0.25	3.75 ± 0.41	4,68%	1.26 ± 0.10	Metacentric
7	1.55 ± 0.19	1.91 ± 0.27	3.46 ± 0.43	4,32%	1.23 ± 0.12	Metacentric
7	1.66 ± 0.19	1.85 ± 0.28	3.41 ± 0.44	4,26%	1.19 ± 0.11	Metacentric
8	1.55 ± 0.15	1.73 ± 0.18	2.95 ± 0.31	3,68%	1.43 ± 0.13	Metacentric
8	1.56 ± 0.14	1.75 ± 0.17	2.89 ± 0.28	3,61%	1.50 ± 0.13	Metacentric
9	1.22 ± 0.13	1.63 ± 0.19	2.72 ± 0.32	3,40%	1.22 ± 0.06	Metacentric
9	1.16 ± 0.14	1.49 ± 0.23	2.66 ± 0.36	3,32%	1.28 ± 0.08	Metacentric
10	0.58 ± 0.08	1.18 ± 0.14	1.75 ± 0.20	2,19%	2.07 ± 0.29	Submetacentric
10	0.57 ± 0.10	1.19 ± 0.14	1.76 ± 0.20	2,20%	2.13 ± 0.39	Submetacentric
11	0.64 ± 0.11	2.72 ± 0.33	3.36 ± 0.40	4,20%	4.30 ± 0.63	Subtelocentric
11	0.70 ± 0.16	2.64 ± 0.30	3.34 ± 0.44	4,17%	3.87 ± 0.52	Subtelocentric
			ΣTL = 80,01			

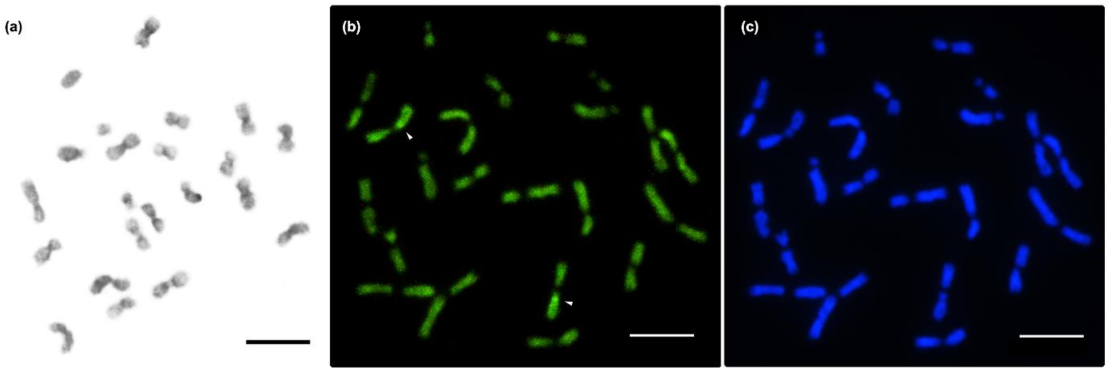


Figure 2. Diploid metaphases of *Atta cephalotes* female (2n=22) submitted to: (a) C-banding showing small centromeric heterochromatic blocks (dark regions), (b) and (c) staining with fluorochromes Chromomycin A_3 (CMA_3) and 4’6- diamidino-2-phenylindole (DAPI), respectively. Arrowheads in (b) indicate GC-rich regions. Bars=5µm.

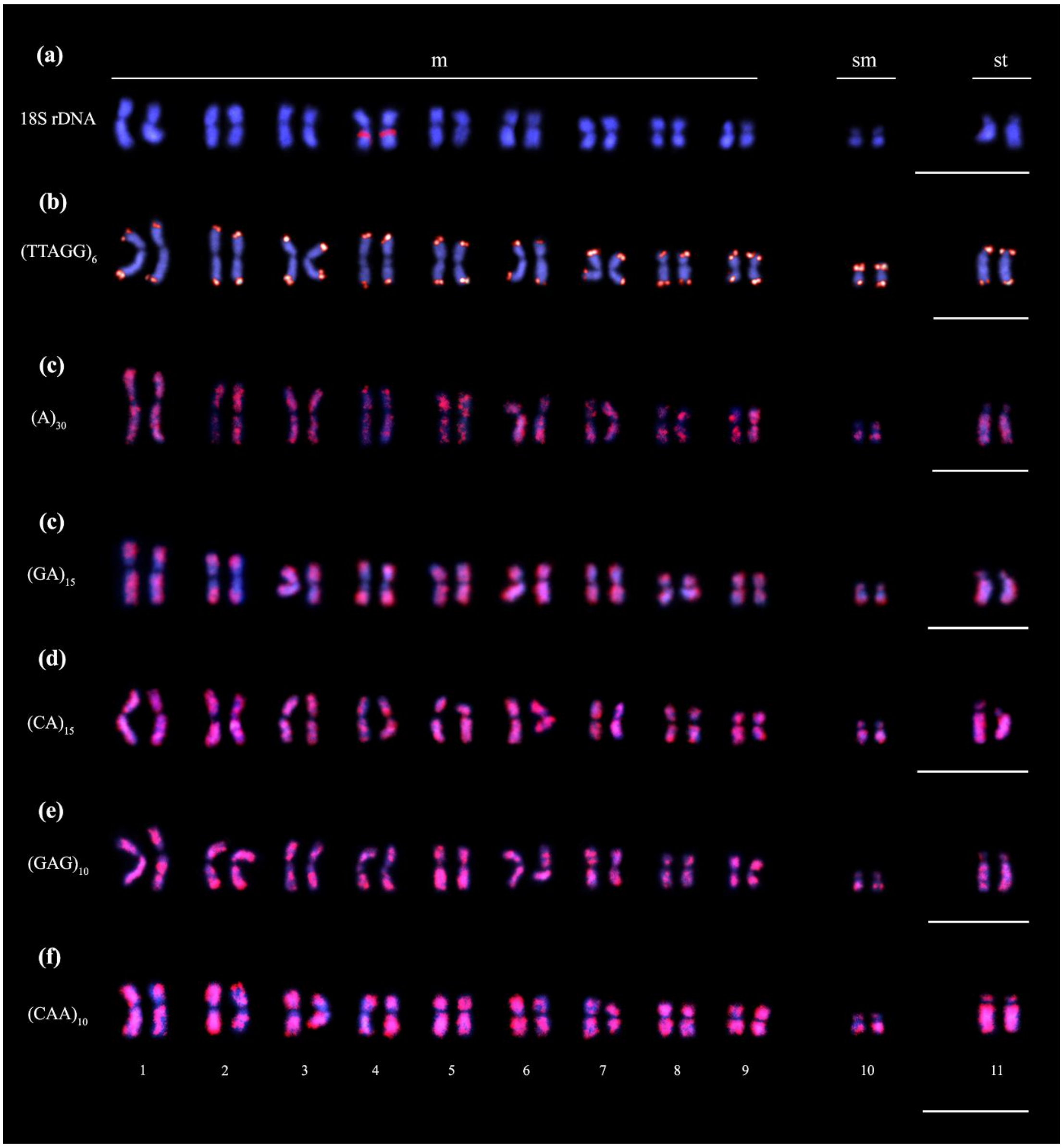


Figure 3. Diploid karyotypes of *Atta cephalotes* female (2n=22) submitted to Fluorescence *in situ* hybridization (FISH) with different repetitive probes (red regions): (a) 18S rDNA genes, (b) $(TTAGG)_6$, (c) $(A)_{30}$, (d) $(GA)_{15}$, (e) $(CA)_{15}$, (f) $(GAG)_{10}$, (g) $(CAA)_{10}$. Bars=5µm.

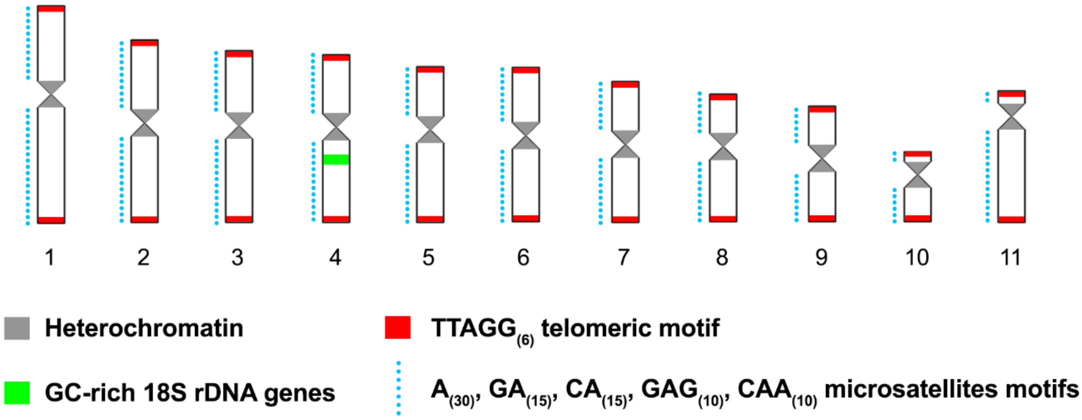


Figure 4. Ideogram showing the *Atta cephalotes* haploid complement with all the chromosomal markers analyzed in the present study.

Discussion

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

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

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

Considering that insects are highly diverse with regard to their telomere structures (Kuznetsova et al., 2019), the presence of such different repetitive sequences associated or not with the telomeres should be investigated in fungus-farming ant species. Traces of the (TTAGGG)n motif, the canonical telomeric sequence in vertebrates, have been previously observed in some chromosomes of bulldog ants through FISH (Meyne et al., 1995) and even in the fire ant *Solenopsis invicta* Buren, 1972 (Myrmicinae) through sequencing analysis (Wurm et al., 2011). The (TCAGG)n motif has been detected in telomeres of some clades of the order Coleoptera (reviewed by Kuznetsova et al., 2019) and has recently been characterized as a microsatellite located in the centromeric heterochromatin of the social wasp *P. fastidiosuscula* (Marchioro et al., 2021). Our physical mapping data showed that such telomeric sequence variations are absent in any portion of the *A. cephalotes* chromosomes. However, we cannot discard the possibility that these motifs may be present at low levels that might be detectable only through genome sequencing analyses. The presence of different mechanisms of telomeric maintenance may also lead to variations in the ancestral (TTAGG) motif along with insect evolution (Kuznetsova et al., 2019).

By contrast, the telomeric canonic motif TTAGG(n) of insects showed strong signals at the end of all the chromosomes of *A. cephalotes*, as in other ant species (Meyne et al., 1995; Lorite et al., 2002), including leaf-cutting ants (Pereira et al., 2018; Castro et al., 2020, Barros et al. 2021, Micolino et al. 2022). Interstitial telomeric sequences can be considered relicts of the fusions of ancestral chromosomes (Lin and Yan, 2008; Olsson et al., 2018). However, no interstitial telomere signal was detected on the chromosomes of *A. cephalotes* as expected since it has the same chromosome number proposed for the ancestor of leaf-cutting ants ($2n = 22$) (Pereira et al., 2018).

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

In the present study, the heterochromatin of *A. cephalotes* had no trace of several microsatellites, such as $(GA)_{15}$, $(CA)_{15}$, $(TAT)_{10}$, $(CGG)_{10}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(TCAGG)_{6}$. However, these microsatellites are present in the heterochromatic regions of other Hymenoptera species, like the bees *Frieseomelitta* species and *M. seminigra merrilae*, and the wasp *P. fastidiosuscula* (Santos et al., 2018; Barbosa et al., 2021; Marchioro et al., 2020). Either these microsatellites were located in euchromatin, or there were no signals produced on the *A. cephalotes* chromosomes. These results suggest that the heterochromatin in *A. cephalotes* comprises other types of repetitive sequences, such as satellite DNA, or that it has low levels or other microsatellites undetectable by FISH and therefore warrant further investigations.

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

Conserved chromosome numbers have been observed in two other genera of leaf-cutting ants. *Acromyrmex* species show predominantly 2n=38 chromosomes (reviewed by Barros et al., 2021), with the single exception of *Acromyrmex ameliae* De Souza et al. 2007. This social parasite displays 2n=36 chromosomes, probably due to the Robertsonian chromosome translocation, representing a derivation from the genus. Chromosomal data on *Amoimyrmex* showed that *Am. bruchi*, *Am. silvestrii*, and *Amoimyrmex striatus* (Roger, 1863) have the same chromosome number, 2n=22 (Cristiano et al. 2013, Micolino et al. 2022). Distinct ant groups like *Odontomachus* (*haematodus* group), with 2n=44, and *Camponotus* (*Myrmothrix*) (reviewed by Mariano et al., 2019), with 2n=40, also show a conserved chromosome number. However, in all these genera, other cytogenetic characteristics vary between species. For example, variations in chromosome morphology were observed between *Acromyrmex* species (reviewed Barros et al., 2021) and *Odontomachus* species (Aguiar et al., 2020). *Camponotus* species (*Myrmothrix*) have different 18S rDNA site numbers (Aguiar et al., 2017), and *Amoimyrmex* species show distinct microsatellite $(GA)_{15}$ patterns (Micolino et al., 2022). Furthermore, according to our perceptions based on both the karyotype figure and the chromosome measurements of *Am. bruchi* and *Am. silvestrii,* as presented in Micolino et al. (2022), other differences also seem to exist. The total chromosome sizes of the 9th and 10th metacentric pairs seem to be smaller than the 8th chromosome pair in *Am. silvestrii*. However, the 8th, 9th, and 10th metacentric pairs seem to have the same size in *Am. bruchi*. Measurement data provided by Micolino et al. (2022) confirm these differences. Our observations highlight additional karyotype variations between these *Amoimyrmex* species.

Our results corroborate the karyotype conservation in three of the four *Atta* groups, including chromosome number and morphology, heterochromatin pattern, location of GC-rich region, 18S rDNA clusters, and the $GA_{(15)}$ microsatellite, contributing with data on another *Atta* phylogenetic group (*Atta sensu stricto*) (Fig. 5). Karyotype conservation as observed in *Atta*, which includes different chromosome information and distinct species and groups (Fig. 5), have not yet been described in other genera.

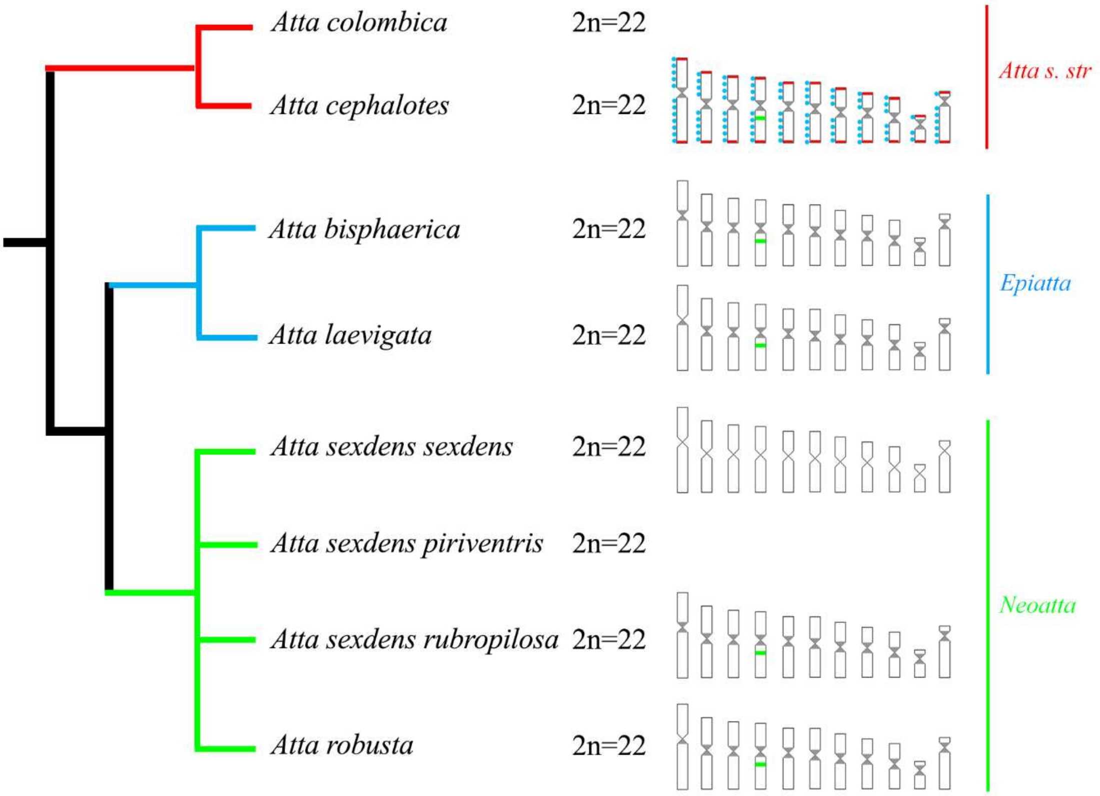


Figure 5. Summary of available cytogenetic data (this study and literature) from *Atta* species included in three of the four monophyletic groups, namely *Neoattina*, *Epiatta* and *Atta sensu stricto*, defined in the molecular phylogeny proposed by Bacci et al. (2009) and Barrera et al. (2021). Ideograms show chromosome morphology, heterochromatin (gray), and 18S rDNA clusters (green), telomeres (red) and microsatellites $(A)_{30}$, $(GA)_{15}$, $(CA)_{15}$, $(GAG)_{10}$ e $(CAA)_{10}$ (blue dotted line) in the haploid complement. Phylogenetic relationships among the species are based on Bacci et al. (2009) and Barrera et al. (2021).

It has been suggested that heterochromatin plays an essential role in ant karyotype evolution by dispersing in the karyotype and increasing the blocks in the chromosomes (Imai, 1991). According to the recent model proposed by Hirai (2020), the physical associations of distal heterochromatic blocks in the meiotic prophase favor its dispersion in karyotype. However, intrachromosomal heterochromatic blocks do not form associations. *Acromyrmex* species show a higher heterochromatin distribution by the C-banding technique that is located in some intrachromosomal regions but predominantly in small, short chromosome arms (Barros et al., 2016), denominated by Imai (1991) as pseudo-acrocentric. The location of these heterochromatic blocks in *Acromyrmex* seems to follow Hirai's model, leading to heterochromatin dispersion and variations in the chromosome morphology among the species. Heterochromatin variations display an important role in karyotype evolution in *Acromyrmex* (Barros et al., 2016). In contrast, *Atta* species show smaller heterochromatin blocks restricted to intrachromosomal regions (Barros et al., 2014, 2015), which may influence the karyotype conservation among species.

Cytogenetic data indicate a wide increase and diversification in the chromosome number and morphology in ant genera of the ancient subfamilies, such as Ponerinae and the Australian Myrmeciinae. In contrast, smaller variations are observed within genera of the derived subfamilies Dolichoderinae, Formicinae, and Myrmicinae (Mariano et al., 2019). According to these authors, geographic barriers or recent diversification time did not drastically influence the karyotypes, especially with regard to the chromosome number found in these ants. Thus, another explanation for the conserved chromosome number would be the recent radiation and restricted geographic distribution of the leaf-cutting ants (Barrera et al., 2021).

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

Acknowledgements

We are grateful to Dr. Jacques H. C. Delabie for species identification, Laboratório de Sistemática Molecular Beagle of the Universidade Federal de Viçosa for the technical support provided and Frédéric Petitclerc for valuable assistance. GAT thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted. Financial support for this study was provided by “Investissement d’Avenir” grants managed by the French Agence Nationale de la Recherche (DRIIHM ref. ANR-11-LABX-0010 and CEBA, ref. ANR-10-LABX-25-01), by the PO-FEDER 2014-2020, Région Guyane (BiNG, ref. GY0007194), and by the Programa de Auxílio ao Pesquisador – PAPESQ/UNIFAP.

References

Aguiar, H.J.A.C., Barros, L.A.C., Alves, D.R., Mariano, C.S.F., Delabie, J.H.C., Pompolo, S.G., 2017. Cytogenetic studies on populations of *Camponotus rufipes* (Fabricius, 1775) and *Camponotus renggeri* Emery, 1894 (Formicidae: Formicinae). PLoS ONE 12(5), e0177702. https://doi.org/10.1371/journal.pone.0177702.

Aguiar, H.J.A.C, Barros, L.A.C., Silveira, L.I., Petitclerc, F., Etienne, S., Orivel, J., 2020. Cytogenetic data for sixteen ant species from North-eastern Amazonia with phylogenetic insights into three subfamilies. Comp. Cytogenet. 14(1), 43–60. https://doi.org/10.3897/CompCytogen.v14i1.46692.

Allshire, R.C., Madhani, H.D., 2018. Ten principles of heterochromatin formation and function. Nat. Rev. Mol. Cell Biol. 19(4), 229-244. https://doi.org/10.1038/nrm.2017.119.

Bacci, M. Jr., Solomon, S.E., Mueller, U.G., Martins, V.G., Carvalho, A.O.R., Vieira, L.G.E., Silva-Pinhati, A.C.O., 2009. Phylogeny of leafcutter ants in the genus *Atta* Fabricius (Formicidae: Attini) based on mitochondrial and nuclear DNA sequences. Mol. Phylogenet. Evol. 51(3), 427–437. https://doi.org/10.1016/j.ympev.2008.11.005.

Barbosa, I.C.O., Schneider, C.H., Goll, L.G., Feldberg, E., Carvalho-Zilse, G.A., 2021. Chromosomal mapping of repetitive DNA in *Melipona seminigra merrillae* Cockerell, 1919 (Hymenoptera, Apidae, Meliponini). Comp. Cytogenet. 15, 77-87. https://doi.org/10.3897/CompCytogen.v15i1.56430.

Barrera, C.A., Sosa-Calvo, J., Schultz, T.R., Rabeling, C., Bacci Jr, M., 2021. Phylogenomic reconstruction reveals new insights into the evolution and biogeography of *Atta* leaf-cutting ants (Hymenoptera: Formicidae). Syst. Entomol. https://doi.org/10.1111/syen.12513.

Barros, L.A.C., Mariano, C.S.F., Pompolo S.G., 2013. Cytogenetic studies of five taxa of the tribe Attini (Formicidae: Myrmicinae). Caryologia 66(1), 59-64. https://doi.org/10.1080/00087114.2013.780443.

Barros, L.A.C., Teixeira, G.A., Aguiar, H.J.A.C., Mariano, C.S.F, Delabie, J.H.C., Pompolo, S.G., 2014. Banding patterns of three leafcutter ant species of the genus *Atta* (Formicidae: Myrmicinae) and chromosomal inferences. Fla. Entomol. 97(4), 1694–1701. https://doi.org/10.1653/024.097.0444.

Barros, L.A.C., Aguiar, H.J.A.C., Teixeira, G.A., Mariano, C.S.F., Teixeira, M.C., Delabie, J.H.C., Pompolo, S.G., 2015. Cytogenetic data on the threatened leafcutter ant *Atta robusta* Borgmeier, 1939 (Formicidae: Myrmicinae: Attini). C. R. Biol. 338(10), 660–665. https://doi.org/10.1016/j.crvi.2015.07.006.

Barros, L.A.C., Aguiar, H.J.A.C., Mariano, C.S.F., Andrade-Souza, V., Costa, M.A., Delabie, J.H.C., Pompolo, S.G., 2016. Cytogenetic data on six leafcutter ants of the genus *Acromyrmex* Mayr, 1865 (Hymenoptera, Formicidae, Myrmicinae): insights into chromosome evolution and taxonomic implications. Comp. Cytogenet. 10, 229-243. https://doi.org/10.3897/CompCytogen.v10i2.7612.

Barros, L.A.C., Teixeira, G.A., Aguiar, H.J.A.C., Lopes, D.M., Pompolo, S.G., 2018. Cytogenetic studies in *Trachymyrmex holmgreni* Wheeler, 1925 (Formicidae: Myrmicinae) by conventional and molecular methods. Sociobiology 65(2), 185–190. https://doi.org/10.13102/sociobiology.v65i2.2214.

Barros, L.A.C., Aguiar, H.J.A.C., Teixeira, G.A., Souza, D.J., Delabie, J.H.C., Mariano, C.D.S.F., 2021. Cytogenetic studies on the social parasite *Acromyrmex ameliae* (Formicidae: Myrmicinae: Attini) and its hosts reveal chromosome fusion in *Acromyrmex*. Zool. Anz. 293, 273-281. https://doi.org/10.1016/j.jcz.2021.06.012.

Bolton, B. 2021. An online catalog of the ants of the world. http://antcat.org. (accessed 15 March 2021).

Brandão, C.R.F., Mayhé-Nunes, A.J., Sanhudo, C.E.D., 2011. Taxonomia e filogenia das cortadeiras, in: Della Lucia, T.M.C. (Ed.), Formigas-cortadeiras da biotecnologia ao manejo. Editora da Universidade Federal de Viçosa, Viçosa, pp. 27–47.

Castro, C.P.M., Cardoso D.C, Micolino, R., Cristiano, M.P., 2020. Comparative FISH-mapping of TTAGG telomeric sequences to the chromosomes of leafcutter ants (Formicidae, Myrmicinae): is the insect canonical sequence conserved? Comp. Cytogenet. 14(3), 369-385. https://doi.org/10.3897/CompCytogen.v14i3.52726.

Correia, J.P.S.O., Mariano, C.S.F., Delabie, J.H.C., Lacau, S., Costa, M.A., 2016. Cytogenetic analysis of *Pseudoponera stigma* and *Pseudoponera gilberti* (Hymenoptera: Formicidae: Ponerinae): a taxonomic approach. Fla. Entomol. 99(4), 718-721. https://doi.org/10.1653/024.099.0422.

Cristiano, M.P., Cardoso, D.C., Fernandes-Salomão, T.M., 2013. Cytogenetic and molecular analyses reveal a divergence between *Acromyrmex striatus* (Roger, 1863) and other congeneric species: taxonomic implications. PLoS One 8(3), e59784. https://doi.org/10.1371/journal.pone.0059784.

Cuadrado, A., Cardoso, M., Jouve, N., 2008. Physical organization of simple sequence repeats (SSRs) in Triticeae: structural, functional and evolutionary implications. Cytogenet. Genome Res. 120, 210–219. https://doi.org/10.1159/000121069.

Della Lucia, T.M.C., Gandra, L.C., Guedes, R.N., 2014. Managing leaf-cutting ants: peculiarities, trends and challenges. Pest Manag. Sci. 70, 14–23. https://doi.org/10.1002/ps.3660.

Elizeu, A.M., Travenzoli, N.M., Ferreira, R.P., Lopes, D.M., Tavares, M.G., 2021. Comparative study on the physical mapping of ribosomal genes and repetitive sequences in *Friesella schrottkyi* (Friese 1900) (Hymenoptera: Apidae, Meliponini). Zool. Anz. 292, 225-230. https://doi.org/10.1016/j.jcz.2021.04.006.

Ellegren, H., 2004. Microsatellites: simple sequences with complex evolution. Nat. Rev. Genet. 5(6), 435-445. https://doi.org/10.1038/nrg1348.

Fadini, M.A.M., Pompolo, S.G., 1996. Cytogenetics of some ant species of the tribe Attini (Hymenoptera, Formicidae) from the region of Viçosa, MG. Bra. J. Genet. 19, 53-55.

Forti, L.C., Rando, J.S., Silva Camargo, R., Moreira, A.A., Castellani, M.A., Leite, S.A., Souza, K.K.A., Caldato, N., 2020. Occurrence of Leaf-Cutting and Grass-Cutting Ants of the Genus *Atta* (Hymenoptera: Formicidae) in Geographic Regions of Brazil. Sociobiology 67(4), 514-525. https://doi.org/10.13102/sociobiology.v67i4.5741.

Gokhman, V.E., 2009. Karyotypes of parasitic wasps. First ed. Springer.

Hirai, H., 2020. Chromosome dynamics regulating genomic dispersion and alteration of nucleolus organizer regions (NORs). Cells 9(4), 971. https://doi.org/10.3390/cells9040971.

Hölldobler, B., Wilson, E.O., 1990. The ants. First ed. Harvard University Press, Cambridge.

Imai, H., Taylor, R.W., Crosland, M.W., Crozier, R.H., 1988. Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. Jpn. J. Genet. 63, 159-185. https://doi.org/10.1266/jjg.63.159.

Imai, H.T., 1991. Mutability of constitutive heterochromatin (C-bands) during eukaryotic chromosomal evolution and their cytological meaning. Jpn. J. Genet 66(5), 635-661. https://doi.org/10.1266/jjg.66.635.

Jaffe, K., Vilela, E., 1989. On Nest Densities of the Leaf-Cutting Ant *Atta cephalotes* in Tropical Primary Forest. Biotropica 21(3), 234–236. https://doi.org/10.2307/2388649.

Janicki, J., Narula, N., Ziegler, M., Guénard, B., Economo, E.P., 2016. Visualizing and interacting with large-volume biodiversity data using client–server web-mapping applications: The design and implementation of antmaps.org. Ecol. Inform. 32, 185-193. https://doi.org/10.1016/j.ecoinf.2016.02.006.

Kuznetsova, V., Grozeva, S., Gokhman, V., 2020. Telomere structure in insects: A review. J. Zool. Syst. Evol. Res. 58(1), 127-158. https://doi.org/10.1111/jzs.12332.

Levan, A., Fredga, K., Sandberg, A.A., 1964. Nomenclature for centromeric position on chromosomes. Hereditas 52(2), 201–220. http://dx.doi.org/10.1111/j.1601-5223.1964.tb01953.x.

Lin, K.W., Yan, J., 2008. Ending in the middle: Current knowledge of interstitial telomeric sequences. Mutat. Res. 658, 95–110. https://doi.org/10.1016/j.mrrev.2007.08.006.

Lopes, D.M., Travenzoli, N.M., Fernandes, A., Campos, L.A., 2020. Different Levels of Chromatin Condensation in *Partamona chapadicola* and *Partamona nhambiquara* (Hymenoptera, Apidae). Cytogenet. Genome Res. 160(4), 206-213. https://doi.org/10.1159/000507835.

López-Flores I, Garrido-Ramos MA (2012) The repetitive DNA content of eukaryotic genomes. Repetitive DNA 7, 1-28. https://doi.org/10.1159/000337118.

Lorite, P., Carrillo, J. A., & Palomeque, T., 2002. Conservation of (TTAGG) n telomeric sequences among ants (Hymenoptera, Formicidae). J. Hered. 93(4), 282-285. https://doi.org/10.1093/jhered/93.4.282.

Marchioro, P., Campos, L.A.O., Lopes, D.M., 2020. First record of B chromosome in *Polybia fastidiosuscula* Saussure, (Vespidae) and investigation of chromatin composition through microsatellite mapping. Cytogen. Genome Res. 160(11-12), 711-718. https://doi.org/10.1159/000513641.

Mariano, C.S.F., Barros, L.A.C., Velasco, Y.M., Guimarães, I.N., Pompolo, S.G., Delabie, J.H.C., 2019. Citogenética de hormigas de la región neotropical, in: Fernández, F., Guerrero, R., Delsinne, T. (Eds.), Hormigas de Colombia. Universidad Nacional de Colombia, Bogotá, pp. 131–157.

Martins, C., Cabral-de-Mello, D.C., Valente, G.T., Mazzuchelli, J., Oliveira, S.G., Pinhal, D., 2011. Animal genomes under the focus of cytogenetics. First ed. Nova Science, New York.

Meyne, J., Hirai, H., Imai, H.T., 1995. FISH analysis of the telomere sequences of bulldog ants (Myrmecia: Formicidae). Chromosoma 104(1), 14–18. https://doi.org/10.1007/BF003 52221.

Micolino, R., Cristiano, M.P., Cardoso, D.C., 2019. Population-based cytogenetic banding analysis and phylogenetic relationships of the neotropical fungus-farming ant *Trachymyrmex holmgreni* Wheeler, 1925. Cytogen. Genome Res. 159(3), 151-162. https://doi.org/10.1159/000503913.

Micolino, R., Baldez, B.C.D.L., Sánchez Restrepo, A.F.F., Calcaterra, L., Cristiano, M.P., Cardoso, D.C., 2022. Karyotype structure and cytogenetic markers of *Amoimyrmex bruchi* and *Amoimyrmex silvestrii*: the contribution to understanding leafcutting ant relationships. Genome 65(1), 43-51. https://doi.org/10.1139/gen-2021-0044.

Milani, D., Cabral-de-Mello, D.C., 2014. Microsatellite organization in the grasshopper *Abracris flavolineata* (Orthoptera: Acrididae) revealed by FISH mapping: remarkable spreading in the A and B chromosomes. PLoS One 9(5), e97956. https://doi.org/10.1371/journal.pone.0097956.

Murakami, T., Fujiwara, A., Yoshida, M.C., 1998. Cytogenetics of ten ant species of the tribe Attini (Hymenoptera, Formicidae) in Barro Colorado Island, Panama. Chromosome Sci. 2, 135-139.

Olsson, M., Wapstra, E., Friesen, C.R., 2018. Evolutionary ecology of telomeres: a review. Ann. N. Y. Acad. Sci. 1422(1), 5–28. https://doi.org/10.1111/nyas.13443.

Palacios-Gimenez, O. M., Cabral-de-Mello, D. C. (2015). Repetitive DNA chromosomal organization in the cricket *Cycloptiloides americanus*: a case of the unusual X 1 X 2 0 sex chromosome system in Orthoptera. Mol. Genet. Genom. 290(2), 623-631. https://doi.org/10.1007/s00438-014-0947-9.

Palacios-Gimenez, O.M., Carvalho, C.R., Ferrari Soares, F.A., Cabral-de-Mello, D.C., (2015). Contrasting the chromosomal organization of repetitive DNAs in two Gryllidae crickets with highly divergent karyotypes. PLoS One 10(12), e0143540. https://doi.org/10.1371/journal.pone.0143540.

Pereira, J.O.P., 2006. Diversidade genética da abelha sem ferrão *Melipona quinquefasciata* baseada no sequenciamento das regiões ITS1 parcial e 18S do DNA ribossômico nuclear. Thesis. Universidade Federal do Ceará, Brazil.

Pereira, T.T.P., Reis, A.C.C.C., Cardoso, D.C., Cristiano, M.P., 2018. Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger, 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera). Comp. Cytogenet. 12(1), 13–26. https://doi.org/10.3897/CompCytogen.v12i1.21799.

Piccoli, M.C.A., Bardella, V.B., Cabral-de-Mello, D.C., 2018. Repetitive DNAs in *Melipona scutellaris* (Hymenoptera: Apidae: Meliponidae): chromosomal distribution and test of multiple heterochromatin amplification in the genus. Apidologie 49, 497–504. http://dx.doi.org/10.1007/s13592-018-0577-z.

Pidoux, A.L., Allshire, R.C., 2005. The role of heterochromatin in centromere function. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 360(1455), 569–579. https://doi.org/10.1098/rstb.2004.1611.

Pinkel, D., Straume, T., Gray, J.W., 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Natl. Acad. Sci. U.S.A. 83, 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Ruiz-Ruano, F.J., Cuadrado, Á., Montiel, E.E., Camacho, J.P.M., López-León, M.D., 2015. Next generation sequencing and FISH reveal uneven and nonrandom microsatellite distribution in two grasshopper genomes. Chromosoma 124, 221–234. https://doi.org/10.1007/s00412-014-0492-7.

Santos, J.M., Diniz, D., Rodrigues, T.A.S., Cioffi, M.B., Waldschmidt, A.M., 2018. Heterochromatin distribution and chromosomal mapping of microsatellite repeats in the genome of *Frieseomelitta* stingless bees (Hymenoptera: Apidae: Meliponini). Fla. Entomol. 101(1), 33-39. https://doi.org/10.1653/024.101.0107.

Santos-Colares, M.C., Viégas, J., Martino Roth, M.G, Loeck, A.E., 1997. Preparation of mitotic chromosomes of leaf-cutting ants from the genera *Atta* and *Acromyrmex*. Brazilian J. Genet. 20, 25-27.

Schweizer, D., 1980. Simultaneous fluorescent staining of R bands and specific heterochromatic regions (DA/DAPI-bands) in human chromosomes. Cytogenet. Cell Genet. 27, 190–193. https://doi.org/10.1159/000131482.

Suen, G., Teiling, C., Li, L., Holt, C., Abouheif, E., Bornberg-Bauer, E., et al., 2011. The genome sequence of the leaf-cutter ant *Atta cephalotes* reveals insights into its obligate symbiotic lifestyle. PLoS Genet. 7(2), e1002007. https://doi.org/10.1371/journal.pgen.1002007.

Sumner, A.T., 1972. A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. 83, 304-306. https://doi.org/10.1016/0014-4827(72)90558-7.

Swanson, A.C., Schwendenmann, L., Allen, M.F., Aronson, E.L., Artavia-León, A., Dierick, D., et al., 2019. Welcome to the *Atta* world: A framework for understanding the effects of leaf-cutter ants on ecosystem functions. Funct. Ecol. 33(8), 1386-1399. https://doi.org/10.1111/1365-2435.13319.

Teixeira, G.A., Barros, L.A.C., Aguiar, H.J.A.C., Pompolo, S.G., 2017. Comparative physical mapping of 18S rDNA in the karyotypes of six leafcutter ant species of the genera *Atta* and *Acromyrmex* (Formicidae: Myrmicinae). Genetica 145, 351–357. https://doi.org/10.1007/s10709-017-9970-1.

Teixeira, G.A., Aguiar, H.J.A.C., Peticlerc, F., Jerome, O., Lopes, D.M., Barros, L.A.C., 2021. Evolutionary insights into the genomic organization of major ribosomal DNA in ant chromosomes. Insect Mol. Biol. 30(3), 340-354. https://doi.org/10.1111/imb.12699.

Travenzoli, N.M., Barbosa, I.C.O., Carvalho-Zilse, G.A., Salomão, T.M.F., Lopes, D.M., 2019a. Karyotypic description and repetitive DNA chromosome mapping of *Melipona interrupta* Latreille, 1811 (Hymenoptera: Meliponini). Caryologia 72(2), 91-95. https://doi.org/10.13128/cayologia-239.

Travenzoli, N.M., Lima, B.A., Cardoso, D.C., Dergam, J.A., Salomão, T.M.F., Lopes, D.M., 2019b. Cytogenetic analysis and chromosomal mapping of repetitive DNA in *Melipona* species (Hymenoptera, Meliponini). Cytogenet. Genome Res. 158(4), 213-224. https://doi.org/10.1159/000501754.

Urbas, P., Araújo Jr, M. V., Leal, I. R., Wirth, R., 2007. Cutting more from cut forests: edge effects on foraging and herbivory of leaf-cutting ants in Brazil. Biotropica 39(4), 489-495. https://doi.org/10.1111/j.1744-7429.2007.00285.x.

Velasco, Y.A.M., Delabie, J.H.C., Costa, M.A., Lacau, S., Mariano, C.D.S.F., 2014. Studies on the karyotype of the ant *Pachycondyla harpax* (Formicidae: Ponerinae: Ponerini) in southern Bahia, Brazil. Fla Entomol. 97(3), 1049-1055. https://doi.org/10.1653/024.097.0307.

Wurm, Y., Wang, J., Riba-Grognuz, O., Corona, M., Nygaard, S., Hunt, B.G., et al., 2011. The genome of the fire ant *Solenopsis invicta*. PNAS USA 108 (14), 5679-5684. https://doi.org/10.1073/pnas.1009690108.

Zhang, D.X., 2004. Lepidopteran microsatellite DNA: redundant but promising. Trends Ecol. Evol. 19(10), 507-509. https://doi.org/10.1016/j.tree.2004.07.020.

6 - CONCLUSÕES GERAIS

Os diferentes capítulos nos permitiram concluir que:

- Os dados de mapeamento físico de genes rDNA de 63 espécies de formigas, incluindo as cultivadoras de fungos, distribuídas em 19 gêneros e 6 subfamílias associados com as informações sobre suas relações filogenéticas apoiam a hipótese de um único sítio de genes rDNA por cariótipo haploide ser a característica plesiomórfica em Formicidae.

- Múltiplos sítios de rDNA foram observados em linhagens de formigas não relacionadas que não compartilham um ancestral comum exclusivo, o que sugere amplificações independentes de rDNA nesses grupos;

- Os dados de mapeamento físico de genes rDNA 18S em formigas revelou uma relação entre localização cromossômica e a dispersão desses genes no cariótipo. A presença de um único par de cromossomos contendo os genes rDNA é o caráter mais comum em formigas devido à localização intracromossômica desses genes nos cromossomos, pois são locais menos propensos a rearranjos, como recombinação ectópica ou translocações não-recíprocas, que poderiam promover a dispersão de genes rDNA no genoma. No entanto, associações entre regiões terminais de cromossomos não homólogos poderia favorecer a ocorrência de tais rearranjos levando a dispersão de genes rDNA pelos cromossomos, como observado nas espécies de formigas com rDNA terminais em vários cromossomos;

- Inversões parecem ser rearranjos importantes que geram mudanças na posição dos genes de rDNA no cariótipo de formigas cultivadoras de fungos como observado neste estudo para *Acromyrmex echinatior*, *Mycetomoellerius relictus* e *Sericomyrmex maravalhas*;

- *Sericomyrmex maravalhas* mostrou um cariótipo inédito em relação as outras espécies do gênero estudadas e a associação dos dados citogenéticos com a filogenia molecular sugerem um aumento do número cromossômico de 2n=48 para 2n=50 através de fissão cromossômica. Os dados de bandamentos cromossômicos obtidos para a espécie que mostram heterocromatina nos braços curtos dos cromossomos também sugerem a ocorrência de fissões ao longo da evolução do gênero.

- O cariótipo obtido para *Cyphomyrmex transversus* mostra o menor número cromossômico para o gênero e difere dos observados para a mesma espécie em duas localidades diferentes, o que sugere que *C. transversus* seja um complexo de espécies e destaca a necessidade de sua revisão taxonômica;

- Nossos dados mostraram a presença de heterocromatina rica em GC em espécies de Neoattina que ainda não tinham sido analisadas pertencentes a gêneros diferentes (*Sericomyrmex*, *Cyphomyrmex* e *Mycetomoellerius*), reforçando a hipótese de origem comum dessa heterocromatina com composição peculiar em Attina.

- O uso da técnica de C_0t-DNA em formigas associado a dados prévios de bandamentos cromossômicos para a subtribo forneceu *insights* sobre a evolução da heterocromatina em formigas cultivadoras de fungos, indicando a ocorrência de pelo menos cinco eventos distintos de diferenciação da composição da heterocromatina em Attina: (1) entre os clados Paleoattina e Neoattina, (2) no gênero Paleoattina *Myrmicocrypta*, (3) na espécie Neoattina *Mycetomoellerius urichii*, que pode ser um possível caso de *drive* centromérico, (4) nos gêneros derivados de Neoattina *Atta* e *Acromyrmex* e (5) dentro de *Acromyrmex* (*A. rugosus* e *A. niger*);

- A sequência telomérica canônica de insetos $(TTAGG)_6$ foi observada nos telômeros de diferentes espécies de Paleoattina e Neoattina indicando que esse motif é conservado em Attina;

- O microssatélite $(GA)_{15}$ foi localizado exclusivamente na eucromatina de todas as espécies de diferentes gêneros de Paleoattina e Neoattina, sendo o padrão de distribuição disperso o mais frequente enquanto as bandas adicionais observadas em algumas espécies parecem ser uma condição derivada. Mecanismos como crossing-over desigual, deslizamento da DNA polimerase durante a replicação ou reparo do DNA ou transposição podem estar envolvidos na origem dessas bandas adicionais de $(GA)_{15}$;

- Diferentes microssatélites mono, di e tri-nucleotídicos apresentaram distribuição dispersa exclusivamente em toda a eucromatina dos cromossomos de *Atta cephalotes.* Nenhuma marcação foi obtida para as regiões heterocromáticas com os microssatélites testados, o que sugere que a heterocromatina dessa espécie pode ser constituída de outros tipos de sequências repetitivas como DNA satélite ou elementos transponíveis.

- *Atta cephalotes* mostrou um cariótipo conservado em relação as demais espécies de *Atta* já estudadas considerando todos os caracteres citogenéticos analisados. Essa conservação cariotípica pode estar relacionada com a localização centromérica da heterocromatina observada nas espécies de *Atta*, que não favorece associações físicas entre as sequências repetitivas heterocromáticas de cromossomos não homólogos durante a meiose, e assim impede a dispersão de tais sequências e variações cromossômicas entre as espécies. Outros fatores que podem estar relacionados com a conservação cariotípica entre as espécies de *Atta* seriam a radiação recente e a distribuição geográfica restrita.