DANON CLEMES CARDOSO

HISTÓRIA EVOLUTIVA DAS ESPÉCIES DO GÊNERO *Mycetophylax* EMERY, 1913 (HYMENOPTERA: FORMICIDAE): FORMIGAS ENDÊMICAS DE RESTINGA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

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Aprovada: 25 de julho de 2013

Prof. Antonio José Mayhé Nunes

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Prof. Jorge A. Dergam dos Santos

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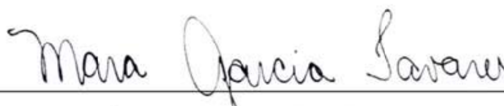

Prof^a^. Mara Garcia Tavares
(Orientadora)

À minha família, novamente dedico.
E a todas as pessoas presentes em minha vida que de alguma forma contribuíram pela realização deste objetivo.

“There are no eternal facts, as there are no absolute truths.”

“There are no facts, only interpretations.”

Friedrich Nietzsche

— Nossa condição não é tão angustiante assim...

— Como não?

— Atravessamos o espaço numa bola que não controlamos, num universo que não entendemos, por uma razão que não sabemos para um fim que não veremos.

— Sim, mas fora isso...

As Cobras – Luis Fernando Verissimo

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Resumo

CARDOSO, Danon Clemes, D.Sc., Universidade Federal de Viçosa, Julho de 2013. **História evolutiva das espécies do gênero *Mycetophylax* Emery, 1913 (Hymenoptera: Formicidae): formigas endêmicas de restinga**. Orientadora: Mara Garcia Tavares Coorientadores: José Henrique Schoereder e Tânia Maria Fernandes-Salomão.

As formigas do gênero *Mycetophylax* pertencem à tribo Attini e são restritas a ecossistemas de dunas da costa do oceano Atlântico. Estas formigas são um grupo taxonômico interessante para o estudo dos efeitos das mudanças climáticas e geológicas do passado sobre a evolução dos habitats abertos costeiros da Mata Atlântica, conhecidos como restinga. Assim, o principal objetivo deste trabalho foi conduzir um estudo sobre a evolução do gênero *Mycetophylax* por meio de diferentes abordagens, a fim de fornecer informações sobre a diversificação e evolução destes organismos em ambientes abertos de dunas. Para atingir nossos objetivos conduzimos estudos de filogenia molecular, citogenética, e genética de populações, em um contexto evolutivo. As espécies foram caracterizadas citogeneticamente e seus cromossomos foram submetidos às técnicas de bandamento C e coloração por fluorocromos. A integração desses dados a uma hipótese filogenética permitiu a inferida de uma hipótese de evolução cromossômica para o gênero *Mycetophylax*. A análise filogeográfica de *M. simplex* permitiu testar o impacto das mudanças paleoambientais sobre a história das populações da costa brasileira sul e sudeste do Atlântico. Além disso, avaliamos como os eventos de regressão e transgressão marinha influenciou a estrutura demográfica desta espécie ao longo de sua área de distribuição. A hipótese filogenética inferida a partir dos genes nucleares *wingless* e *long wave rhodopsin* sugerem que o gênero *Mycetophylax,* em sua atual designação, é monofilético. As espécies *M. simplex* e *M. conformis* agruparam em um ramo que é grupo irmão de *M. morschi* e todas as espécies formam um grupo monofilético com alto suporte estatístico. *M. morschi* apresentou dois cariótipos, com colônias 2n=26 e 2n=30. *M. conformis* e *M. simplex* apresentaram o número diploide de cromossomos igual a 30 e 36, respectivamente. Observou-se que os resultados dos padrões de bandamento cromossômico concordam com a hipótese de filogenia molecular inferida para o gênero. O cariótipo ancestral estimado por meio da abordagem filogenética bayesiana foi n=17. Este resultado sugere que eventos de fusão cromossômica estiveram envolvidos na redução dos

cariótipos de *M. morschi* e *M. conformis* e fissão cromossômica em *M. simplex*. A genealogia do gene mitocondrial citocromo oxidase I (COI) de *M. simplex* sugeriu uma baixa estrutura filogeográfica. Os haplótipos estiveram distribuídos em toda a sua área de distribuição ao longo da costa do Atlântico. Não foram identificadas barreiras evidentes ao fluxo gênico entre as populações de *M. simplex* e elas parecem ter experimentado um longo período de estabilidade demográfica até expansões populacionais recentes. Estas expansões foram coincidentes com os níveis mais baixos do oceano durante o Quaternário. Esses resultados oferecem evidências adicionais convincentes de que os habitats abertos da costa do Atlântico Sul foram ambientes dinâmicos que influenciaram a diversificação e distribuição das espécies.

Abstract

CARDOSO, Danon Clemes, D.Sc., Universidade Federal de Viçosa, July of the 2013. **Evolutionary history of species of the genus *Mycetophylax* Emery, 1913 (Hymenoptera: Formicidae): ants endemic of restinga**. Advisor: Mara Garcia Tavares. Co-advisors: José Henrique Schoereder and Tânia Maria Feranandes-Salomão.

The ants of the genus *Mycetophylax* belongs to the tribe Attini and are endemic to sand dune coastal environments of Atlantic Ocean. These ants are an ideal group to examine the impact of geological and climatic changes over the past on the evolution of open habitats associated with Atlantic Forest, known as restinga. The main aim of this thesis is to evaluate the evolution of the genus *Mycetophylax* by means of different approaches in order to provide insights about the diversification of these ants on the harsh environment of coastal sand dunes. In order to achieve our objectives, the three species of the genus were studied using molecular phylogenetics, cytogenetics and populations genetics, under an evolutionary context. The three species were cytogenetically characterized and the chromosomes were submitted to banding techniques (C-banding and Fluorochrome staining). Using an integrative approach we inferred a chromosome evolution hypothesis for the genus *Mycetophylax*. A phylogeographic stud was carried out with the species *M. simplex* to test the relative impact of palaeoenvironmental changes on the population history of south and southeast Brazilian Atlantic Coast. Besides, we tested how the marine regression and introgression had facilitated the gene flow from south to northern distribution of this ant. *M. simplex* and *M. conformis* appear as a sister group of *M. morschi* and the genus are monophyletic, as suggested by published morphological analysis. *M. morschi* showed a polymorphic number of chromosomes, with colonies showing 2n=26 and colonies 2=30 chromosomes. *M. conformis* presented a diploid chromosome number of 30 chromosomes, while *M. simplex* showed 36 chromosomes. The banding patterns were agreement with the molecular phylogenetic hypothesis inferred based on nuclear protein-coding genes. The ancestral haploid chromosome number inferred by Bayesian phylogenetic approach of *Mycetophylax* genus was 17. These results also claimed that fusions were responsible for the evolutionary reduction in chromosome numbers of *M. conformis* and *M. morschi* karyotypes and a fission rearrangement towards *M. simplex* karyotype.

The mitochondrial DNA (mtDNA) gene genealogy of *M. simplex* suggested a shallow phylogeographic structure with haplotypes distributed throughout their distribution range along the Atlantic Coast. There was no evident genetic break between populations and they seem to have experienced a long period of demographic stability until population expansions that were coincident with the lowest level of the sea during the quaternary. Our findings offer compelling evidences that the coastal open habitats of South Atlantic were an evolutionary dynamic environment underpinning species diversity and distribution.

Introdução Geral

A biologia evolutiva e a biogeografia buscam entender a diversificação e distribuição espaço-temporal da biodiversidade. Ambas foram tradicionalmente baseadas nas variações morfológicas observáveis entre os organismos. Esta abordagem muito provavelmente nos levou por muito tempo a subestimar os níveis reais de diversidade. O advento da biologia molecular trouxe a oportunidade de explorar a diversidade biológica por outra perspectiva, ao nível de moléculas. Hoje, podemos mensurar a diversidade mesmo dentro de espécies, podemos quantificar a diversidade, rastrear o movimento dos organismos inferindo eventos de migração e emigração, bem como reconstruir padrões históricos de dispersão e demografia (Freeland et al., 2011). A diversidade genética pode ser avaliada em um contexto evolutivo por meio do estudo das relações filogenéticas das linhagens genealógicas e sua relação com a distribuição geográfica. Este é o principal objetivo da filogeografia, que une elementos da genética de populações e filogenia molecular sob um contexto histórico e espacial (Avise, 2000), possibilitando assim a formulação de hipóteses biogeográficas.

Uma das questões centrais da filogeografia na região Neotropical é qual a importância das oscilações climáticas e eventos geológicos do passado em moldar sua atual biodiversidade (Hewitt, 2004). Atualmente estão compreendidos no Neotrópico os sete *hotspots* mais diversos do mundo. Embora haja um crescente número de estudos, nosso conhecimento acerca desta imensa diversidade é escasso. Alguns mecanismos de diversificação das espécies têm sido propostos e ressaltam a importância das oscilações climáticas no Quaternário que levaram a divergência das espécies por alopatria e parapatria (Carnaval et al., 2009). Estes eventos de diversificação estão principalmente apoiados na hipótese dos refúgios Pleistocênicos. Os refúgios eram fragmentos florestais que se mantinham em regiões onde a umidade era preservada e neles as espécies persistiam durante os períodos de climas secos e frios. Estas condições ocorriam nos períodos glaciais, apontados como uma consequência de variações na órbita terrestre ao redor do Sol, conhecida como teoria de Croll-Milankovitch (revisado em Hewitt, 2000). No entanto, recentemente a hipótese de refúgios pleistocênicos tem sido questionada por não explicar todos os padrões de diversidade encontrados no Neotrópico (Fitzpatrick et al., 2009; Thomé et al., 2010; Tonini et al., 2013).

A despeito da utilidade dos estudos de filogeografia para o entendimento e conservação da biodiversidade, e das formigas como componente ecológico importante da fauna dos diversos ecossistemas, poucos estudos filogeográficos têm sido conduzidos na América do Sul e no Brasil com estes organismos (Ahrens et al., 2005; Solomon et al., 2008; Resende et al., 2010).

Na região neotropical destaca-se o grupo de formigas da tribo Attini, que ao longo de sua evolução, estabeleceu a agricultura por meio do cultivo de fungos Basidiomicetos há mais de 50 milhões de anos atrás (Schultz & Brady, 2008). É notável a evolução de tal comportamento, visto que este é conhecido apenas para três grupos de animais, incluindo o homem (Mueller et al., 2005). As formigas cultivadoras de fungo, como são conhecidas, dependem obrigatoriamente da manutenção do fungo simbionte para alimentação da colônia (Weber, 1972). A cultura do fungo pode depender da utilização de vários recursos e baseado no comportamento de forrageamento das formigas, a maioria dos Attini pode ser incluída em duas guildas tróficas básicas. As formigas cortadeiras herbívoras dos gêneros *Atta* e *Acromyrmex*, que cultivam seu fungo simbionte utilizando grandes quantidades de material vegetal fresco cortado, e as não cortadeiras, que mantêm seus jardins de fungos utilizando detritos vegetais, fezes e animais em decomposição (Weber, 1972).

Baseado em caracteres morfológicos únicos e suportados por dados moleculares (Schultz & Brady, 2008), as espécies de Attini podem ainda ser separadas em dois clados monofiléticos denominados Paleoattini e Neoattini, inicialmente identificadas por Kusnezov, (1963). O primeiro incluem as espécies basalmente divergentes *Mycocepurus*, *Myrmicocrypta* e *Apterostigma*, enquanto, o último inclui todas as outras linhagens *Kalathomyrmex*, *Mycetarotes*, *Mycetosoritis*, *Mycetophylax*, *Cyphomyrmex*, *Mycetagroicus*, *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex* e *Atta* (Schultz & Brady, 2008).

Apesar de importância ecológica e científica para o estudo da evolução da cultura do fungo, o gênero *Mycetophylax* é um dos menos estudados (Klingenberg & Brandão, 2009). Segundo Kempf (1972), *Mycetophylax* consistiria de 15 espécies encontradas somente em zonas áridas. No entanto, recentemente Klingenberg & Brandão (2009) realizaram a revisão taxonômico do grupo, e concluíram com base em caracteres morfológicos, que o gênero é composto por apenas três espécies: *M. conformis*, *M. simplex* e *M. morschi.* Ainda segundo estes autores, o gênero *Mycetophylax* ocorre apenas em ecossistema de restinga, ao longo da costa do Atlântico Sul.

A espécie *M. conformis* ocorre desde o caribe até o estado de São Paulo. Enquanto a espécie *M. simplex*, por sua vez, ocorre nos estados do Rio Grande do Sul e Santa Catarina (Klingenberg et al., 2007; Cardoso et al. 2010). Por outro lado, Albuquerque et al. (2005), constataram que Weber (1982) indicava o estado de São Paulo como o limite de ocorrência do gênero *Mycetophylax*. Apesar disso, Kempf (1972) em seu catálogo de formigas da região Neotropical já havia registrado *M. simplex* no estado do Rio Grande do Sul. Isto indica que os dados sobre sua distribuição são controversos e requerem maiores estudos para completa compreensão. As espécies *M. simplex* e *M. conformis* parecem não sobreporem suas áreas de ocorrência, entretanto, ocorrem simpatricamente com *M. morschi*. A espécie *M. morschi*, previamente incluída no gênero *Cyphomyrmex,* é encontrada nas restingas desde o estado do Rio de Janeiro até o Rio Grande do Sul (Klingenberg & Brandão, 2009).

Por serem organismos adaptados a ambientes abertos e restritos a região de dunas em restinga, as espécies do gênero *Mycetophylax* são uma excelente oportunidade para a avaliação de hipóteses filogeográficas alternativas de diversificação e distribuição das espécies na região Neotropical. De fato, os estudos filogeográficos têm sido conduzidos com espécies associadas e restritas a ambientes úmidos de floresta. A análise limitada a estas espécies não permite uma completa apreciação dos processos condicionantes da biodiversidade de um bioma tão heterogêneo quanto a Mata Atlântica.

Adicionalmente, estudos citogenéticos e sobre o tamanho de genoma tem sido ferramentas úteis no estudo da diversidade de espécies e contribuído significativamente, para o entendimento da história evolutiva de vários grupos de organismos (Soldán & Putz, 2000; Pellegrino et al., 2005; Schubert, 2007). O tamanho do genoma dentro de uma espécie tende a ser constante, assim como certas características dos cromossomos, como número, tamanho, forma e composição de DNA. Assim, como todas essas características estão sujeitas a mudanças evolutivas e relacionadas com a especiação (King, 1993), tem sido empregadas no estudo das relações filogenéticas das espécies.

Diversos estudos citogenéticos foram realizados em Formicidae e, de modo geral, estes sugerem que as mudanças nos cariótipos acompanharam a diversificação das espécies e gêneros em formigas (revisado por Lorite & Palomeque, 2010). Modificações cariotípicas causados por fissões cromossômicas parecem ter tido um papel maior do que fusões, inversões e translocações na evolução do cariótipo de Formicidae (Lorite & Palomeque, 2010).

Embora poucos estudos tenham avaliado o tamanho de genoma em formigas (Tsutsui et al., 2008; Ardila-Garcia et al., 2010), estes mostraram que assim como os cromossomos, este caractere evoluiu continuamente neste grupo de organismos. No entanto, mudanças no tamanho do genoma parecem não estar correlacionados com o aumento do número cromossômico (Lorite & Palomeque, 2010).

O principal objetivo da presente tese de doutorado foi estudar a história evolutiva e relações filogenéticas do grupo de formigas endêmicas de restinga do gênero *Mycetophylax.* Os resultados e a abordagem multidisciplinar utilizada no presente trabalho devem contribuir para um melhor entendimento da interação dos diversos fatores que moldaram a evolução e diversificação de Attini. Eles também contribuirão para o entendimento do estabelecimento destes organismos e da fungicultura em ecossistemas abertos não florestais.

Para alcançar esse objetivo, a tese está estruturada em três capítulos que, embora estejam interconectados, foram escritos como unidades de leitura independente.

No primeiro capítulo construiu-se uma hipótese filogenética, com caracteres moleculares, para *Mycetophylax.* Esta análise incluiu as três espécies do gênero, coletadas em diferentes locais da sua distribuição geográfica. Analisando dois genes nucleares, testou-se o *status* monofilético de *Mycetophylax,* determinado pela avaliação das características morfológicas, e reafirmou-se a inclusão da espécie previamente incluída no gênero *Cyphomyrmex.*

No segundo capítulo discutiu-se a organização do genoma das três espécies do ponto de vista citogenético. Estabeleceu-se o cariótipo das espécies e utilizaram-se diferentes técnicas de coloração e bandamento com o objetivo de avaliar as relações filogenéticas do gênero *Mycetophylax.* Utilizou-se uma abordagem filogenética para a formulação de uma hipótese de evolução cromossômica e dos possíveis eventos históricos envolvidos nas mudanças cariotípicas observadas, utilizando-se o relógio molecular.

Por último, no capítulo três, acessou-se a estrutura genética populacional de *Mycetophylax simplex*, considerando-se toda sua área de distribuição ao longo da costa do Atlântico. O intuito dessa análise era verificar o efeito dos fatores históricos em moldar a distribuição atual desta espécie. Por meio de sequências de DNA do gene mitocondrial citocromo oxidase I (COI) e do gene nuclear "wingless" avaliou-se se as populações de *M.*

simplex permaneceram demograficamente estáveis durante o tempo evolutivo e se estas foram afetadas pelas transgressões e regressões oceânicas.

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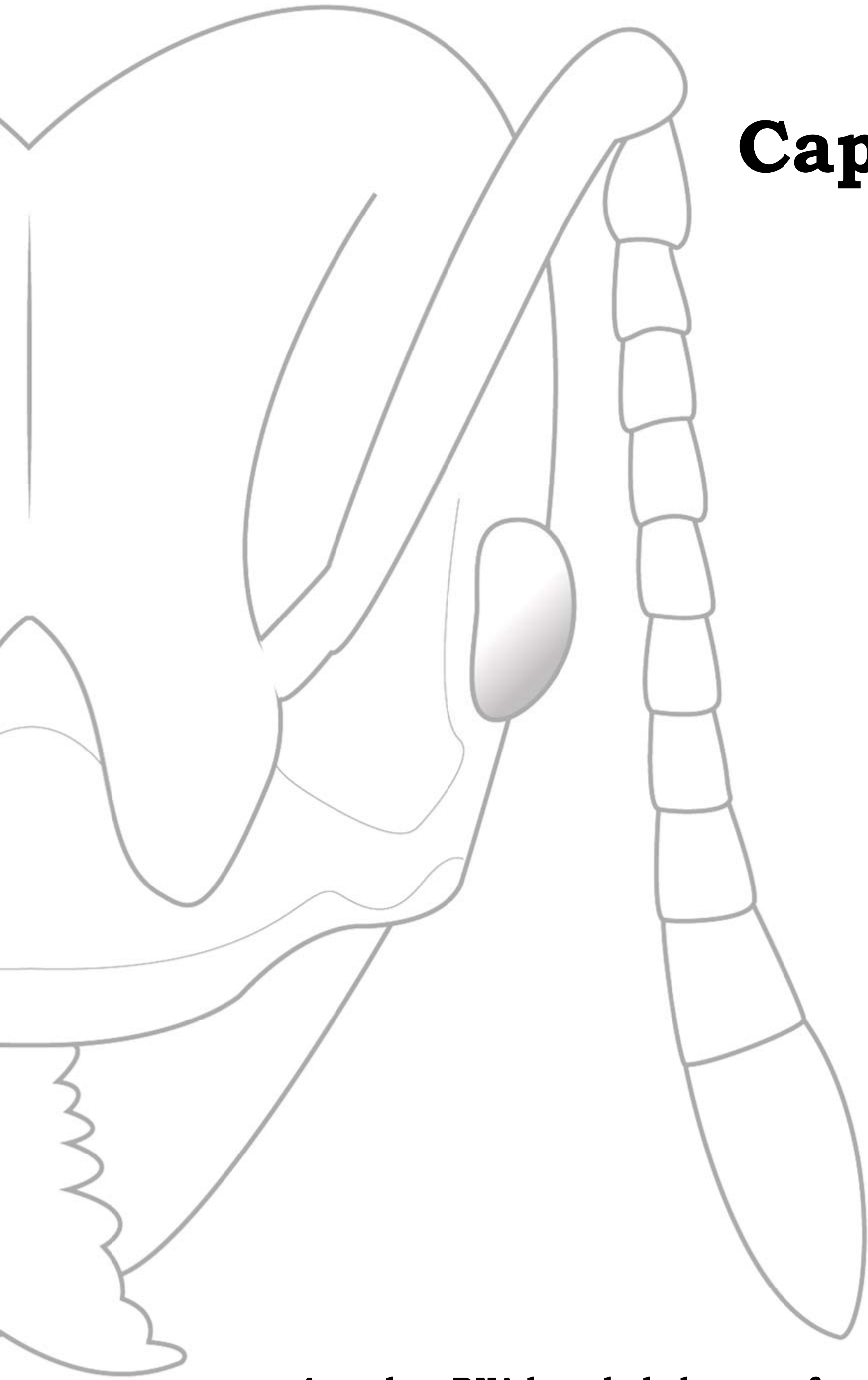

Capítulo I

A nuclear DNA based phylogeny of endemic sand dune ants of the genus *Mycetophylax* Emery, 1913: how morphology is reflected in molecular data

A nuclear DNA based phylogeny of endemic sand dune ants of the genus *Mycetophylax* Emery, 1913: how morphology is reflected in molecular data

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Abstract

Molecular methods have substantially advanced our knowledge about ant systematics in the past few years. Here, we infer the molecular phylogeny of sand dune ants of the genus *Mycetophylax*, Emery 1913 (Formicidae: Myrmicinae: Attini) using 730 base pairs of DNA sequences of the two nuclear genes longwave rhodopsin and wingless. Our analyses indicate that *Mycetophylax* is monophyletic, as suggested by its morphological characters. *M. morschi*, previously considered a species of *Cyphomyrmex* due to a scrobe-like impressed area on the head, forms a well-supported cluster with the two other species of *Mycetophylax*, *M. conformis* and *M. simplex*. Our analysis yields the first comprehensive phylogeny of *Mycetophylax* based on molecular data and includes specimens from localities within a wide distributional range as well as all species belonging to the genus following the recent taxonomic revision.

Key-Words: Formicidae, Attini, molecular phylogeny, evolution, fungus-growing ants

Introduction

Ants are a large and ecologically successful group of insects ubiquitously occurring in diverse ecosystems and habitats throughout the world. Over 12.500 species are currently known (Agosti and Johnson, 2013), all belonging to the monophyletic family Formicidae. Ant taxonomy and systematic has advanced in recent years, providing us good clues about phylogenetic relationships. Moreover, taxonomic reviews have provided a more comprehensive picture of the number of species within genera due to the description of new species or new synonyms (Mayhé-Nunes and Brandão, 2007; Rabeling et al., 2007; Klingenberg and Brandão, 2009; Sosa-Calvo and Schultz, 2010).

Mycetophylax Emery 1913 is a genus of the tribe Attini (Formicidae: Myrmicinae), which, like another genera of this tribe, grows Basidiomycota fungi and utilizes them as their main food source. Until recently, more than 15 species and subspecies (plus four synonyms) had been described as members of the *Mycetophylax* genus (Bolton et al., 2006). However, Klingenberg and Brandão (2009) synonymized most of these or transferred them to other

genera, so that the only remaining species form a relatively homogenous group, characterized by a distinctly smooth mesosoma without spines or only rounded protuberances and a subtriangular head without psammophore. Considering these criteria, only three species remained in the genus, *M. morschi* (Emery, 1888), *M. conformis* (Mayr, 1884) (type species) and *M. simplex* (Emery, 1888) (Klingenberg and Brandão, 2009). Based on comparative morphological traits, the authors suggested that the *Mycetophylax* genus is monophyletic.

As previous molecular phylogenetic reconstructions of the Attini did not include *M. simplex* or specimens of the three species from different localities, we here use a comprehensive phylogeny of *Mycetophylax* based on molecular data to test the proposed monophyly of the genus.

Material and Methods

Taxon Sampling and DNA Extraction

Samples of 17 colonies of the three species of *Mycetophylax* were collected along the South Atlantic coast, based on their previously published distribution area (Klingenberg and Brandão, 2009; Cardoso and Cristiano, 2010; Cardoso et al., 2012). Additionally, we sampled specimens of the *Apterostigma* sp. *pilosum* complex, *Apterostigma steigeri* and *Sericomyrmex parvulus* in Viçosa, MG, Brazil, and *Mycocepurus goeldii* in Araranguá, SC, Brazil. Samples of *Trachymyrmex fuscus* from Rio Claro, SP, Brazil, were kindly provided by Prof. Dr. Odair C. Bueno. Additional sequences of other Attini genera and out-group were obtained from GenBank. Sample size, locations, and accession numbers are listed in Table 1.

Genomic DNA extraction from one worker per colony was performed according to the standard CTAB/chloroform techniques (Sambrook and Russell, 2001) or following a modified phenol-chloroform protocol (Fernandes-Salomão et al., 2005). Nuclear sequences were obtained for the wingless (WG) and longwave rhodopsin (LW) genes, using previously published primers (Ward and Downie, 2005; Brady et al., 2006). These loci have been successfully sequenced in previous phylogenetic studies on ants and particularly the wingless locus was shown to be informative at the species- and genus-levels (Schultz and Brady, 2008; Mehdiabadi et al., 2012).

DNA Amplification, Sequencing and Phylogenetic Analysis

Polymerase chain reaction (PCR) was performed in a final volume of 25 μL (2U of GoTaq® Flexi DNA Polymerase (Promega), dNTPs (0.25 mM each), $MgCl_2$ (2.5 mM), reaction buffer (1x), a pair of primers (0.48 μM each) and 1 mL of DNA). The thermocycler conditions during the amplification reaction were 2 min denaturation at 94ºC, followed by 35 cycles of 94ºC for 1 min, 60ºC (for LW) or 55ºC (for WG) for 1 min, and 72ºC for 1 min, with a final extension at 72ºC for 7 min. Purified PCR products were sequenced directly using the same primers for amplification by Macrogen Inc., South Korea (www.macrogen.com).

The chromatograms were evaluated and edited using the program Consed (Gordon et al., 1998). The 17 sequences of LW and WG were separately aligned with sequences of five other Attini sequences obtained in this study and eleven sequences downloaded from GenBank (Table 1 for accession numbers). Next, sequences were concatenated and analyzed by translation into amino acids using the program MEGA 5.0 (Tamura et al., 2011). The intron of the LW gene was excluded from the alignment.

In order to select the substitution model of DNA evolution that fitted best to each gene under Akaike's Information Criterion (AIC) we used Modeltest 3.6 (Posada and Crandall, 1998). Taking into account these parameters, a maximum likelihood (ML) tree was constructed with PAUP 4.0 (Swofford, 2003), with boot-strapping of 1000 replicates. Bayesian analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) for phylogenetic inference after using MrModeltest 2.2 (Nylander 2004) to estimate the nucleotide substitution model under AIC. The Bayesian analyses consisted of two independent runs of ten million generations each, sampled every 1000 generations and an appropriate burn-in was determined using Tracer v1.4 (Rambaut and Drummond, 2007). A total of 25% of the tree was burned out to produce a single consensus topology that was visualized using the FigTree v1.4 program (Rambaut, 2009). The topologies inferred by both methods were tested to check the congruence using the Shimodaira-Hasegawa test implemented in PAUP 4.0.

Results

Partial sequences were obtained for the LW (384 bp without intron) and WG (346 bp) genes, resulting in an alignment of 730 base pairs comprising 19 sequences from specimens of *Mycetophylax* (17 obtained in this study plus two downloaded from the GenBank), 15 sequences of other Attini ants and one out-group. The alignment included 208 variable sites and 150 parsimony informative sites. The substitution models selected for LW (HKY + G) and WG (GTR + G) were used in the Maximum Likelihood and Bayesian analysis. The topologies from both phylogenetic methods were statistically equivalent (S-H test, $p = 0.236$).

Figure 1 shows the Bayesian consensus phylogeny based on the concatenated sequences. Our phylogenetic analysis unambiguously supports the monophyly of the genus *Mycetophylax* with higher statistical support as a sister group of the genus *Cyphomyrmex*. *M. morschi* is clearly nested within the genus *Mycetophylax*, as currently suggested based on morphological traits (Klingenberg and Brandão, 2009). This result was also supported when both nuclear markers were analyzed separately (data not shown) and by both methods of phylogenetic reconstruction.

Discussion

The purpose of this study was to assess the monophyly of the genus *Mycetophylax* as presently recognized by morphological traits. Our results clearly supported the findings based on morphological traits described by Klingenberg and Brandão (2009). All species and specimens of the genus *Mycetophylax* from different localities throughout their distributional range fell into a well-supported monophyletic clade. This result is in agreement with previous molecular phylogenetic hypotheses based on a study with *M. morschi* (denominated *Cyphomyrmex*) and *M. conformis*, though only one specimen from each species was included in the study (Schultz and Brady, 2008). Our molecular phylogenetic reconstruction included six additional specimens of *M. morschi* and five of *M. conformis* from different localities, and six specimens of *M. simplex*, which was not included in the previous molecular phylogenetic analysis (see Wetterer et al., 1998; Schultz and Brady, 2008). *M. morschi*, of which some taxonomists still believe that it should be considered as member

of the genus *Cyphomyrmex*, was unambiguously placed in the genus *Mycetophylax*.

We also observed that the inclusion of *M. simplex* in the analysis changed the relationship between *M. conformis* and *M. morschi*. In the Bayesian consensus phylogeny reconstructed with these three species, *M. morschi* appeared as sister group of a well-supported cluster comprising *M. simplex* and *M. conformis* (posterior probability (PP) = 0.99). Early during the evolutionary time, the genus *Mycetophylax* seems to have diverged into two different lineages. One of these lineages evolved into *M. morschi*, while the other diversified into *M. conformis* and *M. simplex*. This relationship is also supported by the morphological similarity between *M. conformis* and *M. simplex* that can be easily distinguished from *M. morschi* that bears a scrobe-like depression on head (see Klingenberg and Brandão, 2009).

The genus *Cyphomyrmex* is likely the sister group of the genus *Mycetophylax*. Although this cluster does not show statistical support in Maximum Likelihood analysis, it is well-supported by Bayesian analysis and is in agreement with a previous report (Schultz and Brady, 2008). Moreover, the species *Kalathomyrmex emeryi,* which previously was included in the genus *Mycetophylax,* clearly branches off early in the tree, even with the inclusion of *M. simplex* in the analysis. Considering the morphological distinctiveness of *K. emeryi* due to psammophore setae on the clypeus (Klingenberg and Brandão, 2009) and its basal position on the tree, we agree that *K. emeryi* should be considered a distinct monotypic genus, as defined in the taxonomic revision conducted by Klingenberg and Brandão (2009). The other species removed from the genus *Mycetophylax* was not included in the analysis, however *Paramycetophylax bruchi* also presents some morphological distinctions from *Mycetophylax* (see Klingenberg and Brandão, 2009). Its phylogenetic position requires further analysis.

Mycetophylax seems to be a small genus with marked geographic distribution. It is restricted to the sand dune environments of the Atlantic coast. *M. conformis* and *M. simplex* are parapatric throughout most of their range (Cardoso et al., 2012). This pattern suggests that speciation between them could have been facilitated by vicariant events. The coast of Brazil is known to have been profoundly remodeled during the Quaternary (Dillenburg and Hesp, 2009). The sand dune fields along the coast were modified due to marine introgressions and regressions, which could have created islands that isolated populations and might have promoted speciation. However, *M. morschi*

is sympatric with the other two species. The high phylogenetic resolution (phylogenetic signal) among the *Mycetophylax* species may reflect that there has been sufficient time for the accumulation of shared derived nucleotide substitutions among lineages and that supports the observed monophyly.

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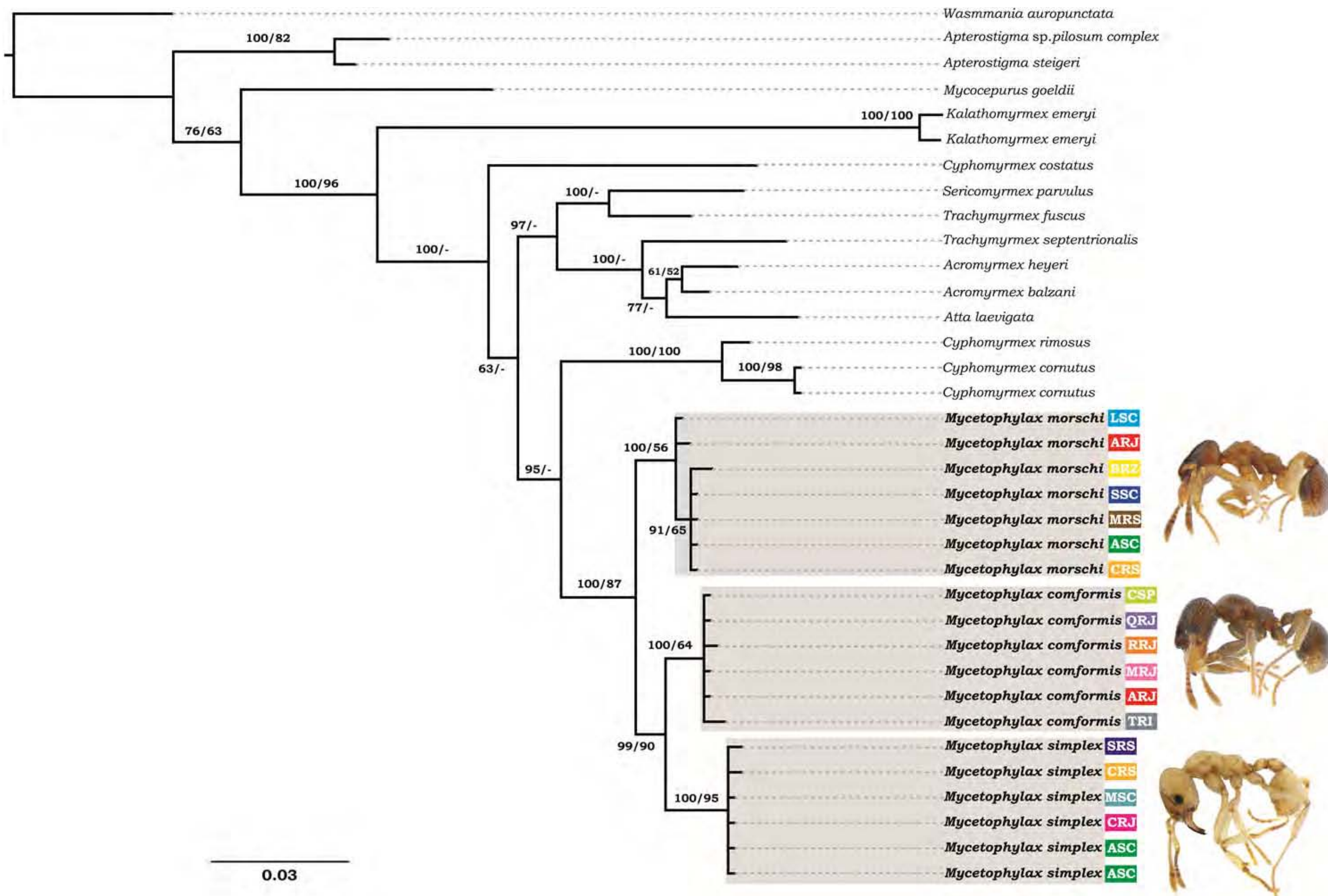
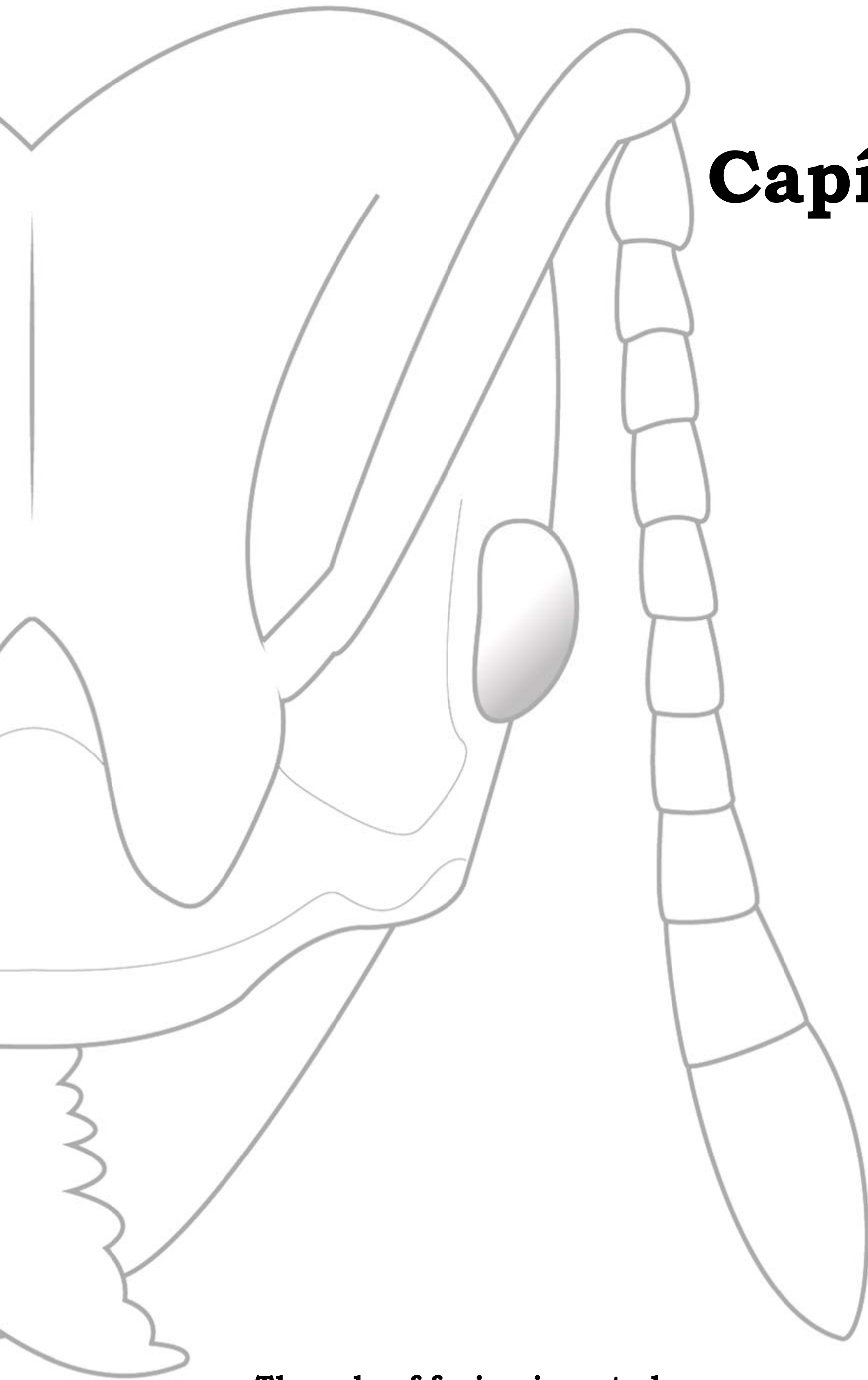


Figure 1 – Bayesian phylogenetic consensus tree of combined wingless and long-wave rhodopsin gene sequences. The first numbers at branches are Bayesian posterior probabilities followed by bootstrap values ("-"<50) calculated from the maximum likelihood analysis of 1000 sequence replicates. Three branches are proportional to mutational events sampled in sequences alignment (scale bar). Codes at tips indicate sampled localities as given in table 1.

Table 1 - Nuclear DNA phylogeny of the genus *Mycetophylax*. Species, codes, collecting localities and accession numbers of the sequences included in the phylogenetic analysis.

Species	Code	Locality of sampled specimens	GenBank Accession Number	
			LW Rhodopsin	Wingless
Mycetophylax morschi	CRS	Chuí/RS – Brazil	KC964626	KC964647
Mycetophylax morschi	MRS	Mostardas/RS – Brazil	KC964625	KC964645
Mycetophylax morschi	ASC	Araranguá/SC – Brazil	KC964621	KC964646
Mycetophylax morschi	LSC	Laguna/SC – Brazil	KC964622	KC964648
Mycetophylax morschi	SSC	São Franc. do Sul/SC – Brazil	KC964624	KC964644
Mycetophylax morschi	ARJ	Angra dos Reis/RJ – Brazil	KC964623	KC964643
Mycetophylax conformis	ARJ	Angra dos Reis/RJ – Brazil	KC964616	KC964638
Mycetophylax conformis	MRJ	Manbucaba/RJ – Brazil	KC964617	KC964639
Mycetophylax conformis	CRJ	Maricá/RJ – Brazil	KC964618	KC964640
Mycetophylax conformis	QRJ	Quissamã/RJ – Brazil	KC964619	KC964641
Mycetophylax conformis	CSP	Caraguatatuba/SP – Brazil	KC964620	KC964642
Mycetophylax simplex	CRS	Cassino/RS – Brazil	KC964631	KC964653
Mycetophylax simplex	SRS	São José do Norte/RS – Brazil	KC964632	KC964654
Mycetophylax simplex	BSC	Arroio do Silva/SC – Brazil	KC964629	KC964652
Mycetophylax simplex	ASC	Araranguá/SC - Brazil	KC964627	KC964649
Mycetophylax simplex	ASC	Araranguá/SC - Brazil	KC964628	KC964650
Mycetophylax simplex	CRJ	Cabo Frio/RJ - Brazil	KC964630	KC964651
Apterostigma sp. pilosum complex	-	Viçosa/MG - Brazil	KC964637	KC964658
Apterostigma steigeri	-	Viçosa/MG - Brazil	KC964636	KC964659

Mycocepurus goeldii	-	Araranguá/SC - Brazil	KC964635	KC964655
Serycomyrmex parvulus	-	Viçosa/MG - Brazil	KC964633	KC964656
Trachymyrmex fuscus	-	Rio Claro/SP-Brazil	KC964634	KC964657
Acromyrmex octospinosus	-	-	EU204465/ EU204222[b]	EU204145
Acromyrmex versicolor	-	-	EF013534/ EF013534	EF013662
Acromyrmex heyeri	-	-	EU204529/ EU204286	EU204210
Acromyrmex landolti	-	-	EU204530/ EU204287	EU204211
Acromyrmex lundi	-	-	EU204497/ EU204254	EU204178
Acromyrmex balzani	-	-	EU204490/ EU204247	EU204170
Atta laevigata	-	-	EU204481/ EU204238	EU204161
Cyphomyrmex rimosus	-	-	EU204466/ EU204223	EU204146
Cyphomyrmex cornutus	-	-	EU204521/ EU204278	EU204202
Cyphomyrmex cornutus			EU204532/ EU204289	EU204213
Cyphomyrmex costatus	-	-	EU204488/ EU204245	EU204168
Trachymyrmex septentrionalis		-	EU204503/ EU204260	EU204184
Mycetophylax conformis	TRI	Trinidade e Tobago	EU204486/ EU204243	EU204166
Mycetophylax morschi	BRZ	Brazil	EU204531/ EU204288	EU204212
Kalathomyrmex emeryi	-	-	EU204478/ EU204235	EU204158
Kalathomyrmex cf. emeryi	-	-	EU204524/ EU204281	EU204205
Wasmannia auropunctata	-	-	EU204483/ EU204240	EU204163



Capítulo II

The role of fusion in ant chromosome evolution: insights from cytogenetic analysis using a molecular phylogenetic approach in the genus *Mycetophylax*

The role of fusion in ant chromosome evolution: insights from cytogenetic analysis using a molecular phylogenetic approach in the genus *Mycetophylax*

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Abstract

The genus *Mycetophylax* Emery, 1913 is a small monogynous basal Attini ant (Formicidae: Myrmicinae), endemic to sand dunes along the Brazilian coastlines (Restinga). A recent taxonomic revision validates three species, *Mycetophylax morschi* (Emery, 1888), *M. conformis* (Mayr, 1884) and *M. simplex* (Emery, 1888). In this paper, we cytogenetically characterized all species that belongs to the genus and analyzed the karyotypic evolution of *Mycetophylax* in the context of a molecular phylogeny and ancestral character state reconstruction. *M. morschi* showed a polymorphic number of chromosomes, with colonies showing 2n=26 and 2n=30 chromosomes. *M. conformis* presented a diploid chromosome number of 30 chromosomes, while *M. simplex* showed 36 chromosomes. The C-banding pattern revealed lower heterochromatin content in both cytotypes of *M. morschi*, which were mainly centromeric. Furthermore, *M. conformis and M. simplex* showed positive blocks in pericentromeric and centromeric regions and some completely heterochromatic short arms. Sequential staining with DA/DAPI/CMA_3 revealed that heterochromatin in *M. conformis* and *M. simplex* is AT^+ rich, and one pair showed a positive CMA_3 band. Our analysis suggest that the ancestral haploid chromosome number of *Mycetophylax* genus was 17 (Likelihood framework) or 18 (Bayesian framework). The analysis also confirmed that fusions were responsible for the evolutionary reduction in chromosome numbers of *M. conformis* and *M. morschi* karyotypes whereas fission determines the *M. simplex* karyotype. These results obtained show the importance of fusions in chromosome changes in Formicidae and how a phylogenetic background can be used to reconstruct hypotheses about chromosomes evolution.

Keywords: chromosome evolution, karyotype, molecular phylogeny, comparative methods, fungus-growing ants

Introduction

The Attini tribe belongs to the Myrmicinae subfamily and comprises ants that are known to engage in a symbiosis with a *Basidiomycota* fungus, which is their main food source. They are restricted to the New World and are primarily distributed in the Neotropics, where they achieve their greatest diversity. Currently, the tribe comprises more than 230 described species grouped into 14 genera (Schultz & Brady 2008, Klingenberg & Brandão 2009). Although some systematic studies on Attini tribe have been conducted, information is scarce regarding the majority of the groups and the taxonomy of many species still requires revision. Indeed, recent revisionary studies have permitted the identification of sibling species (Schultz et al., 2002), description of new species (Sosa-Calvo & Schultz, 2010), and even the description of a new genus (Klingenberg & Brandão, 2009).

The genus *Mycetophylax* is a small monogynous basal Attini that just recently has gained more attention (Kligenberg & Brandão, 2009, Cardoso et al., 2011, 2012). About 15 species, subspecies and varieties were coded to the genus *Mycetophylax* (Kempf, 1972). Following the taxonomic revision based on morphological systematics, the majority of those species were synonymized and some others were included into *Mycetophylax*, originally belonging to the Attini genus *Cyphomyrmex* Mayr, 1862. Currently the genus *Mycetophylax* is composed of three valid species, *M. conformis* (Mayr, 1884), *M. morschi* (Emery, 1888) and *M. simplex* (Emery, 1888). However, some issues concerning the occurrence of sibling species within *Mycetophylax* and the status of *M. morschi* within the genus are still undefined.

Chromosomes can display different sizes, shapes and compositions of DNA and there is ample evidence that chromosome changes can contribute to speciation (King, 1993). All these characteristics make them a reliable criterion in evolutionary and taxonomic studies. Karyotype descriptions and chromosomal comparative analyses are an important independent tool for taxonomy and understanding chromosome evolution, particularly when relying on phylogenetic tree (Guerra 2012). The ready availability of DNA sequences and advances in molecular phylogenetic analysis have allowed researchers to infer the relationships of ants that can be used in an integrative cytogenetic approach. Sequences of protein-coding nuclear genes have been shown to be useful for resolving phylogenetic relationships within genera and between related species in fungus growing ants (Schultz & Brady 2008; Mehdiabadi et al., 2012). Analysis of wingless and long-wave rhodopsin gene

sequences led to the recent molecular phylogenetic hypothesis of the *Mycetophylax* genus (Cardoso et al., 2013 - Chapter 1), which was in agreement with the morphological features. Recently, the nuclear content of the three species were estimated by flow cytometry, data that provided noteworthy information at a higher level than the species level (Cardoso, 2012), but information concerning the *Mycetophylax* karyotype is no longer available.

Thus, our aim was to provide the first characterization of *Mycetophylax* species karyotype, including chromosome number, morphology, heterochromatin location and chromatin AT/GC richness. We discuss the evolutionary dynamics of the karyotypes within the genus considering the recently published phylogeny. Additionally, lineage-specific rearrangements leading to different chromosome numbers in *Mycetophylax* were tested using ancestral character state reconstruction. For this purpose we used two recently developed different approaches by Mayrose et al. (2010) based on Maximum Likelihood and Bayesian methods in order to put forward insights about chromosome evolution in ants.

Materials and Methods

Biological material and chromosome preparation

Colonies of the three species were collected from sand dunes throughout their occurrence area along the Brazilian Atlantic Coast, from Rio Grande do Sul State to Rio de Janeiro State between December 2009 and March 2011. The colonies of *M. simplex* (19 colonies) were collected on beaches in the States of Rio Grande do Sul, Santa Catarina, Paraná and Rio de Janeiro. *M. conformis* (21 colonies) were collected on beaches in the States of Rio de Janeiro and São Paulo, while *M. morschi* (32 colonies) were collected in all the states mentioned. Following collection, the colonies were transported to the laboratory and reared following the protocol described by Cardoso et al. (2011) until brooding occurred. When available, at least ten individuals from each colony were used in cytogenetic analyses. All the ants collected were preserved in ethanol and confirmation of species identification was performed by Rodrigo Feitosa, at the Museum of Zoology of the University of São Paulo (*Museu de Zoologia da Universidade de São Paulo*, MUZSP), where vouchers were also deposited.

Metaphase spreads were prepared from the cerebral ganglia of post-defecant larvae, according to protocol proposed by Imai et al. (1988). The cerebral ganglion was dissected in colchicine-hypotonic solution (0.005%) under a stereoscopic microscope, transposed to a new drop of same solution and incubated under light protection for one hour until slide preparation (see Imai et al., 1988 for detailed procedure). The slide with metaphases were examined under a phase contrast microscope and stained with 4% Giemsa solution in Sorensen's buffer, pH 6.8, to determine chromosome number and morphology. We classified the chromosomes following a modified nomenclature based on the proposed by Levan et al. (1964), which is based on four types of centromeric position: acrocentric (A), subtelocentric (ST), submetacentric (SM) and metacentric (M).

C-banding and Fluorochrome staining

In order to determine the distribution pattern of heterochromatin, the BSG (barium hydroxide/saline/Giemsa) banding technique was performed, essentially following the method described by Sumner (1972), with modifications in the duration of treatment with $Ba(OH)_2$, as proposed by Pompolo and Takahashi (1990). Sequential fluorochrome staining with chromomycin A3 / distamycin A / 4'-6'-diamindino-2-phenylindole (CMA3/DA/DAPI) was conducted according to Schweizer (1980) in order to characterize CG and AT richness region on chromosomes. The slides were analyzed under an epifluorescence microscope (Olympus BX 60) equipped with a digital camera system (Q color 3 Olympus®). The fluorescent signals were analyzed with different filters: WB filter (450 to 480 nm) for the fluorochrome CMA3 and WU filter (330 to 385 nm) for the fluorochrome DAPI.

Chromosome evolution analysis

In order to infer and support the patterns and processes underpinning chromosomal evolution in *Mycetophylax* an integrative cytogenetic and molecular phylogeny study was conducted. To determine the direction of chromosomal changes (i.e. fusion versus fission) that occurred in the genus *Mycetophylax*, Attini species with known karyotypes were used as an out-group. Thus, two different methods were performed with the purpose of outlining a chromosome evolution hypothesis for this genus. The software

ChromEvol 1.3 (Mayrose et al., 2010) was used to infer the chromosome evolution model and haploid ancestral states (chromosome numbers) by Maximum Likelihood and Bayesian methods, relying on previously published phylogenetic hypotheses (Appendix 1).

The molecular phylogenetic tree for *Mycetophylax*, on which the haploid ancestral states were inferred in this work, was based on the wingless and long-wave rhodopsin matrix of Cardoso et al. (2013). We reconstruct the Bayesian tree from that study using selected taxa that were cytogenetically characterized or that have their karyotype known, using the same settings parameters and substitution model to run MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). ChromEvol 1.3 was carried out relied on this reconstructed tree, and evaluates eight mechanisms of chromosome evolution hypotheses, taking into account: dysploidy, the increase or decrease in chromosomes; duplications, whole-genome duplication; and demi-duplication, a mechanism that facilitates the transition from an *n* chromosome to 1.5 *n*. The last feature was not evaluated in our analysis, since it is only widespread and common in plants. All the parameters were adjusted to the data following the recommendation of Mayrose et al. (2010). The models and their null hypotheses were analyzed with 10,000 simulations and the one that best fit the data set was selected under the Akaike information criterion (AIC).

Subsequently, to define a more complete chromosome evolution hypothesis, the node ages of *Mycetophylax* were estimated. This analysis was performed to determine when the possible splits between lineages occurred, to assess the information of chromosome changes in a geological and evolutionary context. Thus, molecular dating of *Mycetophylax* lineages were estimated using previously reported nuclear clock calibrations for Attini ants (Schultz & Brandy, 2008). In a matrix for the genes *wingless* and *long-wave rhodopsin* downloaded from the NCBI GenBank (see Appendix 1), we included sequences of cytogenetically characterized *Mycetophylax* species. The nuclear genes matrix was analyzed under a Bayesian framework and uncorrelated lognormal-relaxed clock model in BEAST v. 1.6.1. (Drummond & Rambaut, 2007) as described by Rabeling et al. (2011).

Results

Karyotype analysis and chromosome banding

The three species of the genus *Mycetophylax* investigated present different chromosome numbers and karyotype morphologies (Fig. 1). *M. morschi* showed populations with two distinct chromosome sets. Populations from Rio Grande do Sul and Rio de Janeiro State presented diploid chromosome numbers equal to 2n=30 or 2n=26 (n=13), whereas all the populations from Santa Catarina, Paraná and São Paulo State showed 2n=26. No hybrids were found. *M. morschi* presented an almost bimodal karyotype that consisted of seven large and six small chromosome pairs for populations with 2n=26 chromosomes, and six large and nine small pairs in populations with 2n=30 chromosomes (Fig. 1a and b). Both *M. morschi* karyotypes displayed nine metacentric pairs and one acrocentric pair; however *M. morschi* 2n=26 showed only three submetacentric pairs, whereas specimens with 2n=30 showed five. In karyotypes with 2n=26, one strongly and two weakly stained regions were observed in the terminal portion of the long arm of chromosome 3 and chromosomes 2 and 5, respectively. These regions may correspond to secondary constrictions. Similarly, a secondary constriction on chromosome 3 was observed in the karyotype 2n=30.

In all of the metaphases analyzed, *M. conformis* presented 2n= 30 (n=15) and a chromosome complement composed of eleven metacentric and four submetacentric pairs, morphologically similar and relatively small, except for the first metacentric pair, which was larger (Fig. 1c). A secondary constriction was observed on the second pair of metacentric chromosomes.

M. simplex showed the largest chromosome number of the genus, 2n=36 (n=18). It was composed by ten metacentric and eight submetacentric pairs (Fig. 1 d). The first metacentric and submetacentric chromosomes (pairs 1 and 11) were large and the remainders were from medium to small in size.

The species showed different patterns of heterochromatin distribution (Fig. 2). In *M. morschi* karyotypes, the heterochromatin is quite evident and can be distinguished in a few chromosomes in the centromeric region (Fig. 2a and b). *M. conformis* and *M simplex* showed conspicuous heterochromatin blocks in the centromeric and pericentromeric regions of most chromosomes, as well as on the short arms of two in *M. conformis* and eight chromosomes pairs (pairs 4, 6, 7, 9, 10, 16, 17 and 18) in *M. simplex* (Fig. 2c and d).

The sequential fluorochrome staining revealed positive GC rich blocks (CMA_3^+) in only one pair, on the telomeric region, in *M. conformis* and on the pericentromeric region in *M. simplex* (Fig. 2e, f). DAPI showed general banding pattern coincident with the C-bands, indicating that the heterochromatin is AT rich (Fig. 2g and h). *M. morschi* did not show any GC or AT rich regions in either karyotype, since the chromosomes were stained uniformly. The results obtained are summarized in Table 1.

Chromosome evolution

The results obtained in the analysis of chromosome evolution suggested that the best supported model of the process underpinning chromosome change was the hypothesis with constant gain, loss and duplication (log likelihood = -56.9052, AIC = 119.81). The rate parameters estimated in the best model were 16.52 for loss (δ), 7.01 for gain (λ) and 0.40 for duplication (ρ). The total inferred chromosome loss events were 181.41, gain 72.91 and duplication 1.70. These results revealed the occurrence of polyploidization events and suggested that whole karyotype duplication could have occurred during the chromosome evolution of these species. The main events inferred were loss (fusion) and gain (fission), which showed P.P.>0.5. In the Bayesian analysis, the haploid chromosome number at the most recent common ancestor (MRCA) of *Mycetophylax* with highest posterior probability (PP) was n=17 and in the ML analysis the most likely haploid number was n=18 (Figure 3).

To describe an evolutionary scenario for chromosome evolution inferred by ChromEvol to *Mycetophylax*, we focused on the haploid chromosome numbers estimated in the Bayesian method, since this method provides posterior probabilities (PP) as a statistical parameter. From the MRCA of the *Mycetophylax* species, the chromosome number decreased, becoming n=15 (PP= 43) and subsequently, n=13 in the branch leading to *M. morschi*. Likewise, in the branch leading to *M. conformis*, the chromosome number decreased to n=15 (PP=63). In the case of *M. simplex*, the haploid chromosome number increased to n=18 (PP=74).

The Bayesian time-calibrated tree allowed us to infer that the *Mycetophylax* species diverged from *Cyphomyrmex* during the Miocene, around ~13 Ma (95%CI= 8.49-18.91, Fig. 5). This divergence was probably followed by chromosome changes. Considering the three species of *Mycetophylax*, *M.*

morschi split early, around 9.1 Ma (95%CI= 5.75-14.34), whereas *M. conformis* and *M. simplex* split around ~6.62 Ma (95%CI= 3.22-10.32). The initial emergence of *M. morschi* karyotypes was estimated to have occurred during the Pleistocene, around ~2.29 Ma (95%CI= 0.31-4.72).

Discussion

We detected wide chromosomal variability among the species of the genus *Mycetophylax*. The three species analyzed showed different karyotypes and, in fact, we verified two different diploid chromosome numbers for *M. morschi.* However, the karyotypes did not show significant geographic structuring, since they were found in both the northern and southern occurrence areas of this species. In two sampled localities, Cabo Frio beach in the State of Rio de Janeiro and Chuí beach in the State of Rio Grande do Sul, we detected the karyotypes n=13 and n=15, though not living sympatrically on the same beach. Moreover, despite the large number of colonies analyzed, we did not find hybrid karyotypes. Thus, we suggest that these two karyotypes denote two different lineages throughout the distribution of *M. morschi.* These results are in agreement with the general rule that changes in the karyotype occur throughout species diversification (Lorite & Palomeque, 2010). As show in their review, usually when species from one genus are cytogenetically analyzed they show polymorphic karyotypes regarding both number and morphology.

The cytogenetic data available for Attini, however, are so scarce that it is premature to assume that the chromosome numbers verified for the genus *Mycetophylax* are in agreement with the common chromosome counts reported for this tribe. Its sister group, *Cyphomyrmex*, presents only three species with known karyotypes and they show different haploid chromosome numbers: *C. costatus* and *C. cornutus* have a low chromosome number of n=10 and n=11, respectively, whereas *C. rimosus* presents n=16 chromosomes. Other genera of Attini also display similar variance in the chromosome number, with the exception of the leafcutter ants, which are reported to have a more homogenous chromosome number, with the majority of the species that have been analyzed presenting n=19 in *Acromyrmex* and all species in *Atta* presenting a haploid number of 11 chromosomes (Mariano et al., 2011)

Both karyotypes of *M. morschi* showed nine pairs of metacentric chromosomes and one acrocentric pair, whereas the other two species presented only metacentric and submetacentric chromosomes. According to

molecular phylogenetic analysis (Cardoso et al., 2013), the genus *Mycetophylax* is divided into two major linages. One is composed only by the species *M. morschi* and the other comprises *M. conformis* and *M. simplex*. Since the last two species are more closely related to each other, the majority of metacentric and submetacentric chromosomes may be a characteristic shared by *M. conformis* and *M. simplex.* Furthermore, the pair of acrocentric chromosomes common to the karyotypes of *M. morschi* may be a symplesiomorphic chromosomal character retained from the ancestor that was lost in the lineage that diversified into *M. simplex* and *M. conformis*. On the other hand, this chromosome rearrangement could be one of the karyotypical characters that differentiate *M. morschi* from the others.

The different karyotypes verified in *M. morschi* can be explained by chromosome rearrangements that change both chromosome morphology and diploid number, probably through centric fission. For instance, a biarmed chromosome breaks apart at the centromere and produces two telomeric chromosomes, followed by pericentromeric inversions or chromatin growth, resulting in the two new submetacentric chromosomes found in karyotypes n=15. This mechanism would have occurred at least twice to produce the two additional submetacentric pairs in the *M. morschi* lineage n=15. Indeed, based on the cytogenetic study of *Myrmecia* spp. (*pilosula* group), Imai et al. (1994) proposed "*the minimum-interaction theory*", wherein the karyotype changes tend towards increasing the number of chromosomes in order to minimize the threats of deleterious rearrangements due to the interaction of chromosomes within the nucleus. In general, this model predicts an increase in chromosome number due to centric fission, followed by chromatin addition (mainly heterochromatin) or pericentrometric inversions (for details see Imai et al., 1994; Imai et al., 2001). According to this hypothesis the ancestral karyotype of *M. morschi* would be n=13, reaching n=15 by mean of fission rearrangements.

However, based on our analysis of chromosome evolution, the recovered ancestral haploid chromosome number between karyotypes of *M. morschi* was n=15, suggesting that the karyotype n=13 probably arose due to tandem fusion from the karyotype with n=15 chromosomes. The karyotypes do not show any absence of the medium size chromosomes, which would be expected in the case of centric fission from 13 to 15 haploid chromosomes, or acrocentric chromosomes in the karyotypes n=15, which could have occurred in the case of centric fusion from n=15 chromosomes to n=13. The minimum

interaction theory predicts that fission is likely to be the main rearrangement shaping chromosome evolution in ants, but does not reject the importance of fusions. This mechanism would lead the karyotype to chromosome number reduction due to a random occurrence, followed by retention and positive selection if it provided short-term advantages (see Imai et al., 1986; Imai et al., 1994). Likewise, the contemporary haploid chromosome number of *M. conformis* seems to be produced by fusion, decreasing from n=17 to n=15. The estimated ancestral haploid chromosome number between *M. confomis* and *M. simplex* was n=17, which is also the ancestral state estimated for the genus *Mycetophylax*. Thus, the karyotype number verified for *M. simplex* may have evolved due to centric fission instead of fusion, since it shows a haploid number of 18 chromosomes.

Ants with high chromosome numbers (n > 12) tend to show Robertsonian polymorphisms as the main rearrangements during the evolution of their karyotypes (Imai et al., 1986). Thus, the chromosome numbers can decrease through centric fusion or increase through centric fission. Currently, the majority of works that evaluate karyotype evolution within genera advocate in favor of centric fission and pericentric inversions as the main chromosomal rearrangements determining karyotypes, e.g. regarding Ponerinae ants, the suggestion is that chromosome changes occurred in the evolution of the genera *Odontomachus* and *Anochetus* (Santos et al., 2010). The authors explained that centric fission is the principal evolutionary force acting on the karyotypes of *Odontomachus*, resulting in a larger, more stable karyotype mainly composed of subtelocentric chromosomes, compared with *Anochetus*, which is characterized by extreme karyotype diversification ranging from n=12 to n=23 and mainly composed of metacentric chromosomes. However, tandem fusion has been proposed to drive karyotype differentiation in a few cases (reviewed by Lorite & Palomeque, 2010). In *Myrmecia pilosula*, this chromosome rearrangement was used to explain the origin of a long metacentric chromosome through the fusion of a subtelocentric and an acrocentric chromosome (Imai & Taylor, 1989). Chromosome fusion was also suggested to be involved in the genus *Acromyrmex*, due the decrease in the chromosome number, from n=19 to n=18, in *Acromyrmex ameliae* (Barros, 2010). Notwithstanding, tandem fusion has also been reported to be involved in chromosome rearrangements for great number of other animals, including grasshoppers (Warchałowska-Śliwa et al., 2013; Hemp et al., 2013), wasps (Gokhman, 2009) and bats (Rodrigues et al., 2003).

The C-banding technique and fluorochrome staining confirmed the cytotaxonomic groups distinguished by chromosome morphology analysis. Both karyotypes of *M. morschi* showed minimal amounts of heterochromatin restricted to the centromeric region and uniform staining for fluorochrome CMA_3 and DAPI. In contrast, *M. conformis* and *M. simplex* comprise a distinct group with intermediate to large amounts of heterochromatin and evident AT- and CG-rich regions. The banding patterns shared by *M. conformis* and *M. simplex* suggest that their chromosomes underwent rearrangements following the split from a common ancestor related to *M. morschi*. Moreover, the amount of heterochromatin found in *M. simplex* is in agreement with the higher DNA content of this species (Cardoso et al., 2012). The DAPI banding was coincident with the C-banding blocks in both species, suggesting that the heterochromatin is AT-base-pair-rich. The main constitute of heterochromatin is repeated sequences, such as satellite DNA. It has been suggested that these sequences are the most rapidly evolving component of the genome and thus contribute to the rearrangements (Lorite & Palomeque, 2010). Hence, the specific heterochromatic pattern observed in the species studied here may be the result of a long-term evolutionary process with tandem duplication, transposable elements, as well as epigenetic factors, such as methylation of DNA, thought to play an important role in compacting and organizing the genome into distinct chromatic domains (Ma et al., 2005; Grewal & Jia, 2007).

Based on fluorochrome staining, we were also able to identify other synapomorphic chromosomal characters shared by *M. conformis* and *M. simplex*. One pair of chromosomes of these two species showed positive CG-rich regions, absent in *M. morschi*. These GC-positive blocks were located in the telomeric region in *M. conformis* and in the pericentromeric region in *M. simplex*. This difference can be explained by paracentric inversion. It has been thought that for ants and some other insects the chromosome pair bearing this GC-rich positive block is associated with the nucleolus organizer region (NOR) (Cardoso et al., 2012). This suggests that the rDNA sequences present in these chromosomes are interspaced by sequences rich in CG base pairs. This pattern has also been observed in other animals, such as fishes (Vidotto et al., 2004).

The interspecific karyotype variability found among the species of the genus *Mycetophylax* could be associated with the biological environment where these species are restricted. As mentioned above, these species are confined to sand dune habitats along the Atlantic coast. This area is known to

have been strongly influence during the Quaternary, having been remodeled due to periods of transgression and regression of the sea level (Dillenburg & Hesp, 2009). Our results suggested that the genus *Mycetophylax* diverged from *Cyphomyrmex* during the middle Miocene (~13 Ma) and diversified into the current species between the end of the Miocene and the beginning of the Pliocene. These periods are marked by deep modifications in the landscape that could facilitate the isolation of populations, culminating in the accumulation of chromosome mutations that could favor speciation and the evolution of new taxa. We suggest that an ancestor of these species was distributed along the Atlantic coast and later, due to transgressive movements of the sea, the geographic distribution was split by rising sea levels, producing barriers and sandy islands where the speciation process took place. The distinct karyotypes of *M. morschi* arose during the Pleistocene, a period extensively reported to have influenced the diversification of species in the Brazilian Atlantic Forest (Carnaval & Moritz, 2008), which includes its coastline. Highly intra and inter-specific karyotype variability is also reported for the genus *Ctenomys* (Freitas, 2006), a subterranean rodent that has habitat requirements and a distribution pattern similar to *Mycetophylax*. Thus, the sand dune environments on the Atlantic Coast of Brazil and their geological history could have acted as a trigger for chromosomal rearrangements and the subsequent speciation in these areas.

This is the first comprehensive cytogenetic description and evolutionary analysis of an Attini genus based on molecular data and provides a baseline for future comparative and integrative studies. Based on our chromosome evolution approach and cytogenetic banding techniques, we hypothesized that fusions instead of fission could be involved in the chromosome evolution of the *Mycetophylax* genus. These chromosome rearrangements likely took place by involving complete genetic isolation of the two major lineages within *Mycetophylax* that therefore established their own evolutionary strategies. One of these lineages diversified into the *M. morschi* group complex and the other diversified into *M. simplex* and *M. conformis*. Overall, the results presented in this study confirm that tandem fusion could very well participate in chromosome ant evolution. It is important that studies involving cytogenetic data within genus and between related genera are continued and that these studies take into account molecular phylogenic methods in the evaluation of the cytogenetic data.

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Conflict of interest

The authors declare no conflict of interest

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Figure and Tables:

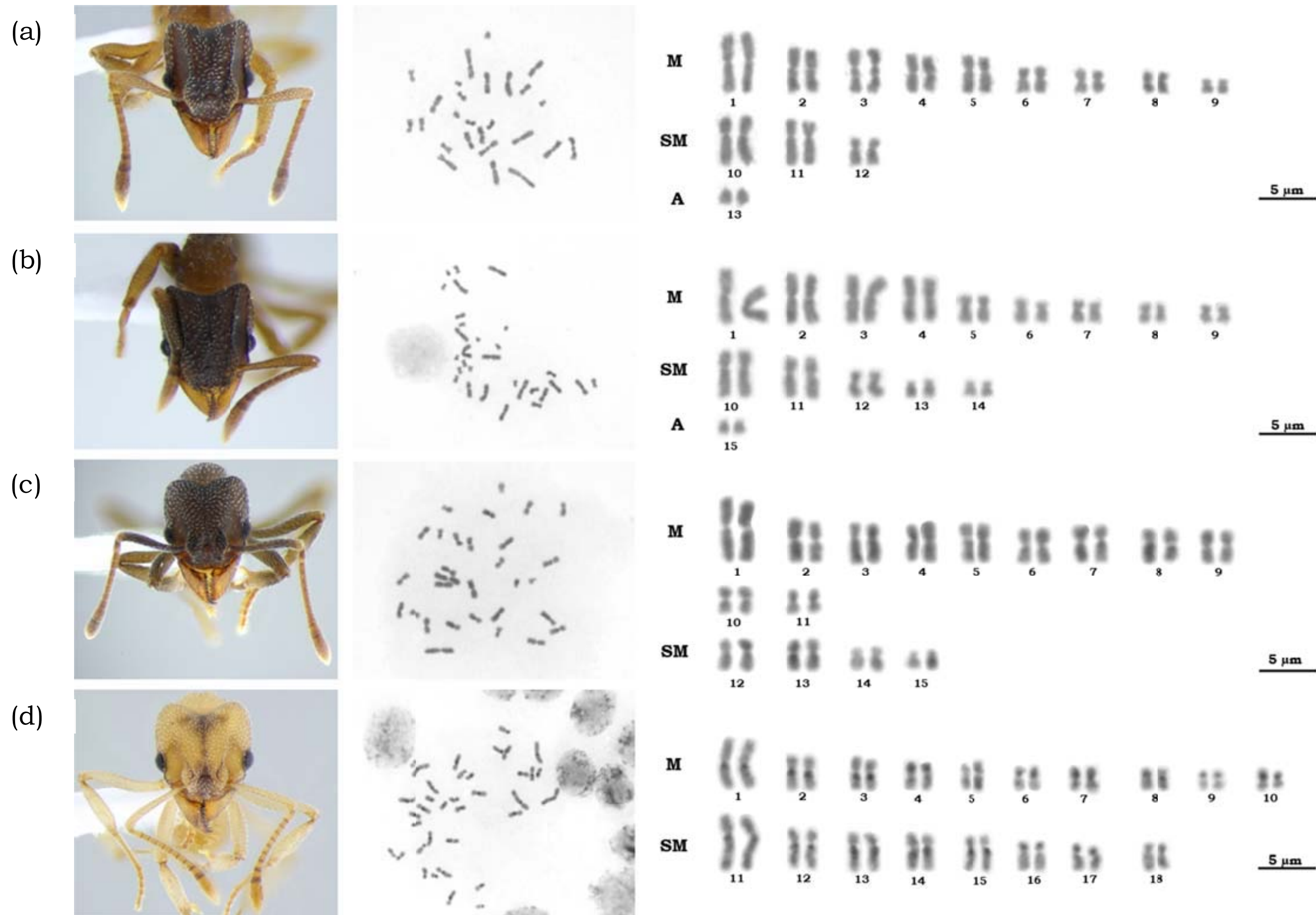

Figure 1 – Auto-montage images, metaphases and diploid karyotypes of *Mycetophylax morschi* 2n=26 (a) and 2n=30 (b), *Mycetophylax conformis* (c) and *Mycetophylax simplex* (d). M= metacentric, SM= submetacentric and A= acrocentric.

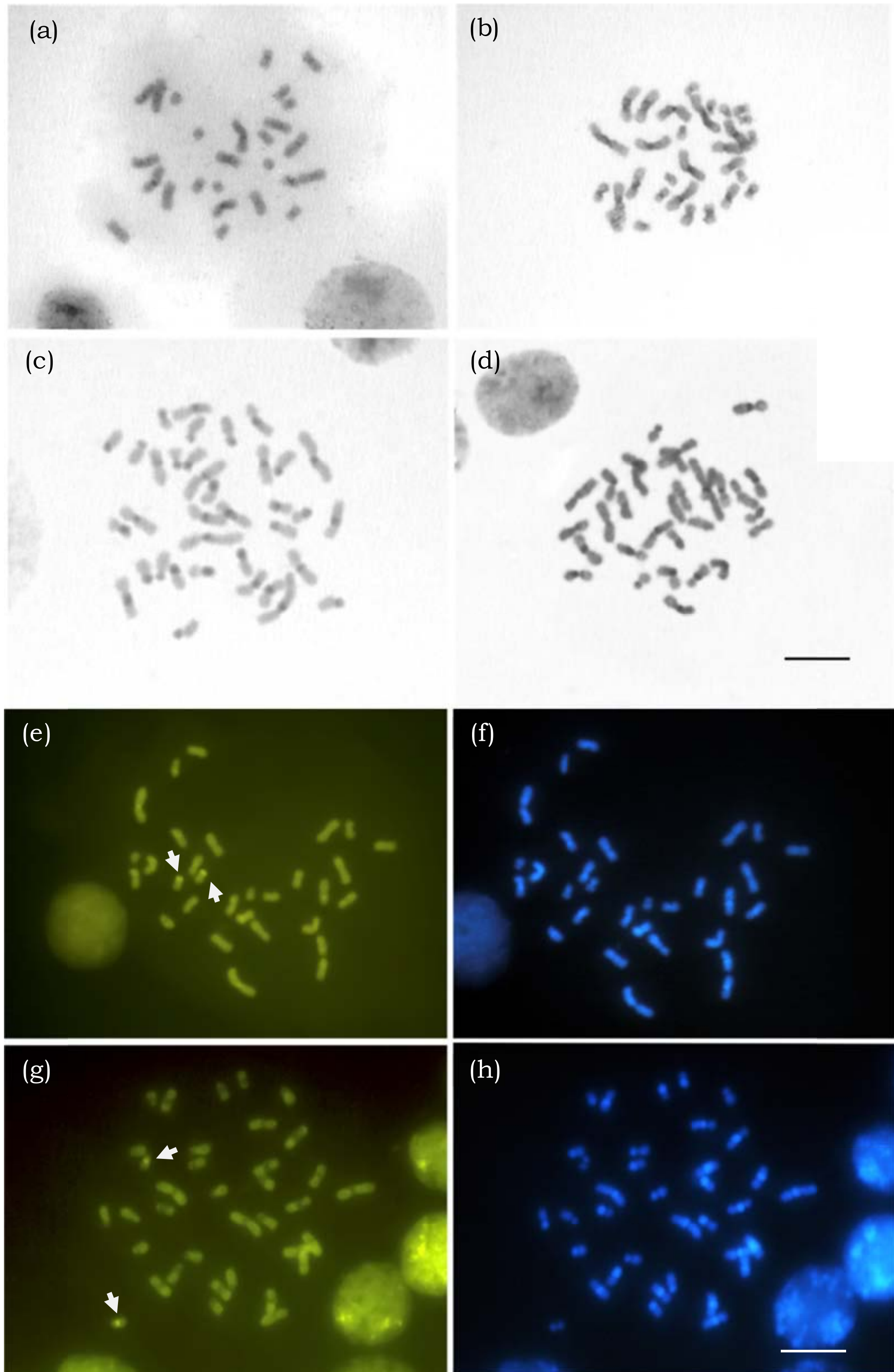

Figure 2 - Metaphase of *M. morschi* 2n=26 (a), 2n=30 (b); *M. simplex* (c) *M. conformis* (d) submitted to C-banding technique denoting the heterochromatic positive bands (dark grey). Metaphase of *M. conformis* (e) and (f) and *M. simplex* (g) and (h) stained with fluorochromes CMA_3 and DAPI, respectively. The arrows indicate the positive staining for CMA_3. DAPI positivity was in agreement with the C-banding pattern. Bar = 5μm.

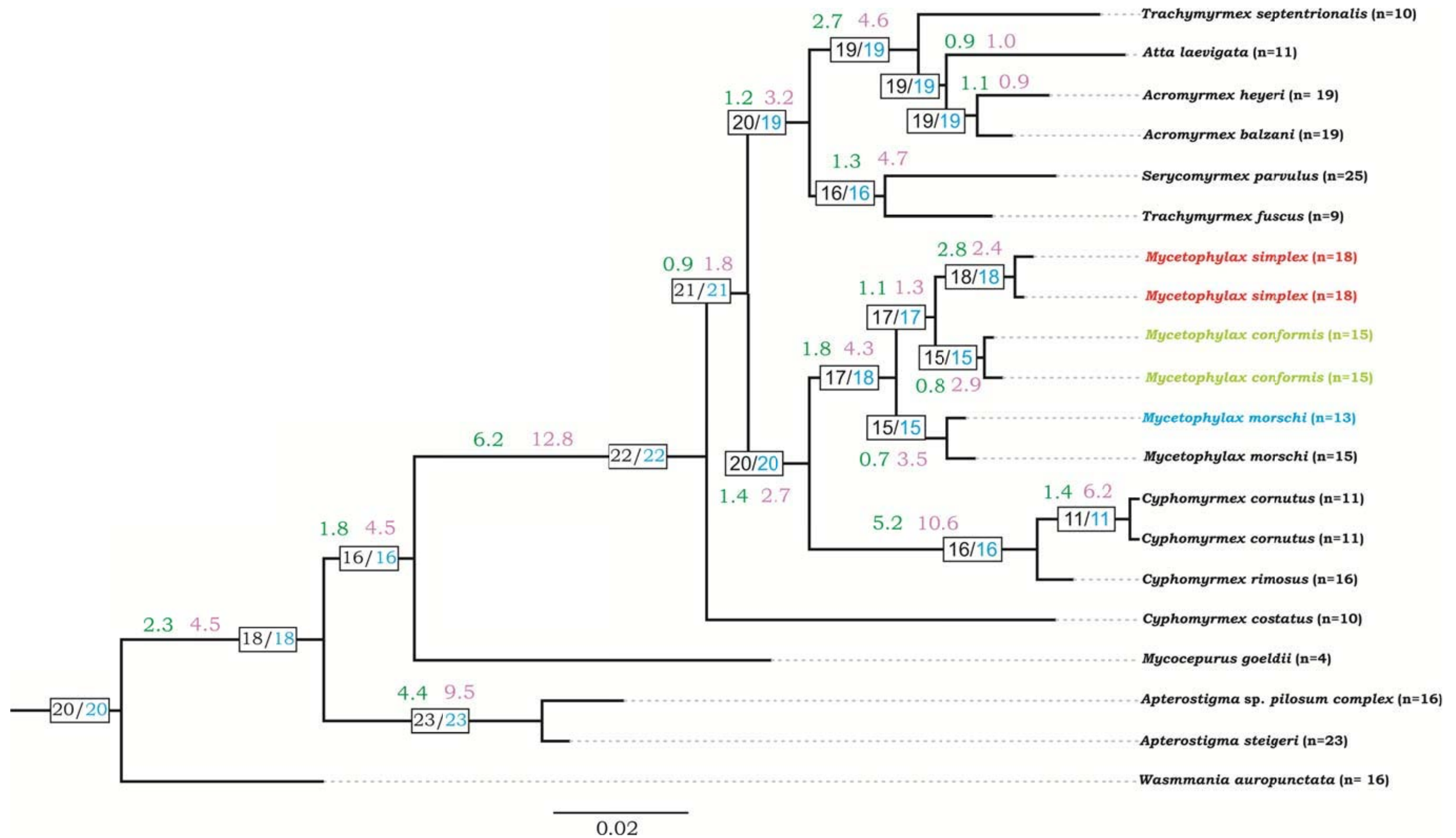

Figure 3 - Chromosome number evolution and inferred ancestral chromosome state in the genus *Mycetophylax* inferred under Bayesian and Maximum likelihood optimization, including other Attini ants and an outgroup. Boxes at the nodes present the inferred ancestral haploid chromosome number for each node by Bayesian and ML analysis, respectively. Numbers at the tips are the known haploid chromosome numbers of species. Green numbers at the branches represent the inferred frequency of gain events (fission) and purple loss events (fusion) that had a probability > 0.5.

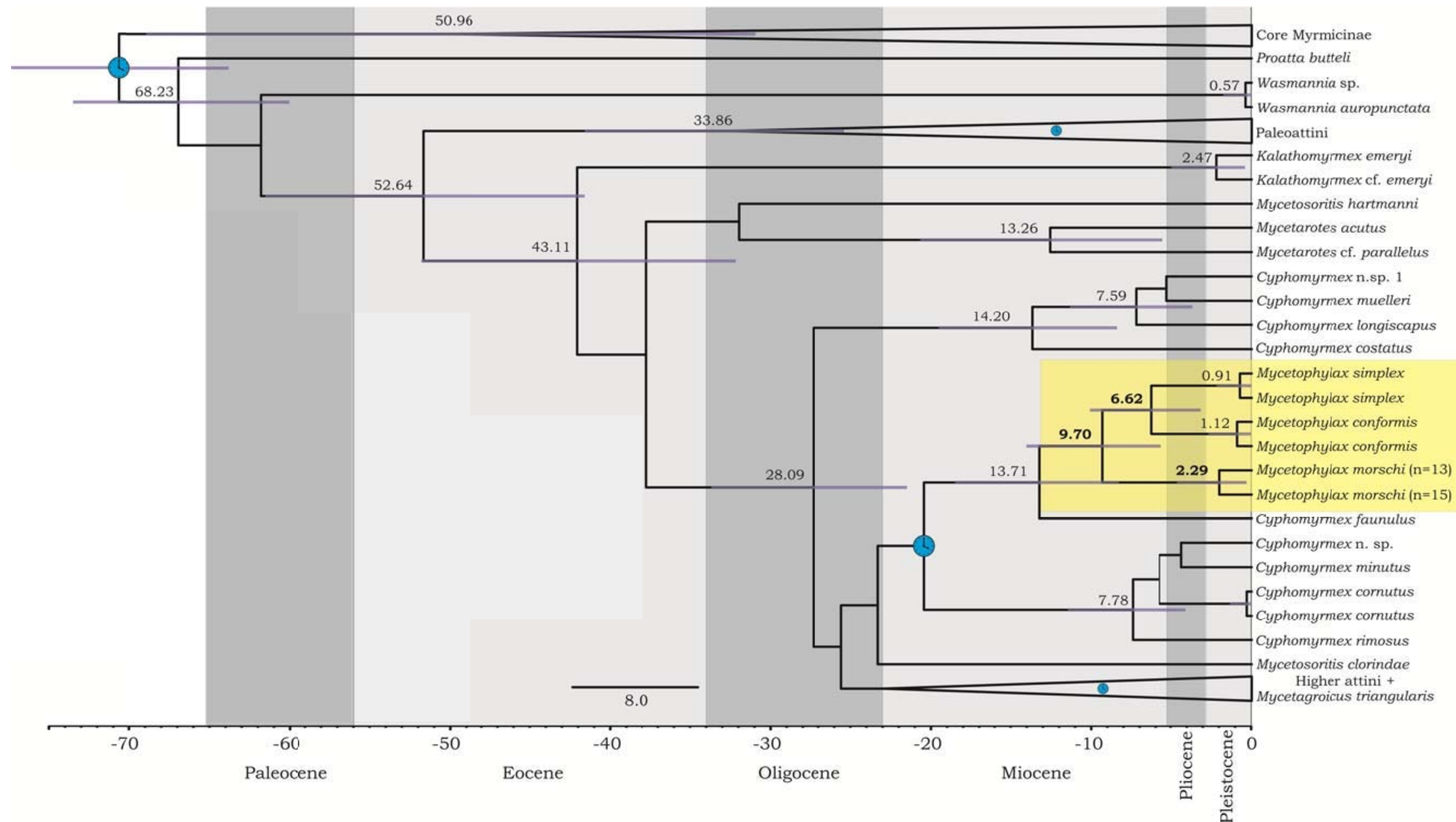

Figure 4 - Bayesian time-calibrated maximum clade-credibility tree using a relaxed clock. Calibration points are indicated with blue clocks where two are suppressed within the Paleoattini and Higher Attini clade (for calibration point details see Schultz and Brady, 2008). The numbers on the upper branches are the inferred age of the nodes (yellow shaded area denotes the genus *Mycetophylax*), while the 95% credibility intervals are indicated as purple bars on the nodes.

Table 1 - Cytogenetic data of the *Mycetophylax* species. Summary of chromosome number, chromosome morphology and banding patterns of observed karyotypes of all the specimens analyzed here.

	Chromosome number (2n)	**Chromosome morphology**			**C-banding positive blocks**			**Fluorochrome staining**	
		M	SM	A	C	PC	SA	AT+ bands	GC+ bands
M. simplex	36	10	8	no	yes	yes	yes	yes	yes
M. conformis	30	11	4	no	yes	yes	yes	yes	yes
M. morschi	30	9	5	1	yes	no	no	no	no
M. morschi	26	9	3	1	yes	no	no	no	no

M: metacentric; SM: submetacentric; A: acrocentric.

C: centromeric; PC: pericentromeric; SA: Short arm.

Supplement Material

Appendix 1 – GenBank accession numbers of specimens used for phylogenetic inference in molecular clock analysis and chromosome evolution (in bold) reconstruction.

Taxa	**opsin exon 1**	**opsin exon 2**	**wingless**
Acromyrmex octospinosus	EU204465	EU204222	EU204145
Acromyrmex versicolor	EF013534	EF013534	EF013662
Acromyrmex heyeri	**EU204529**	**EU204286**	**EU204210**
Acromyrmex landolti	EU204530	EU204287	EU204211
Acromyrmex lundi	EU204497	EU204254	EU204178
Acromyrmex lundi	EU204526	EU204283	EU204207
Acromyrmex lundi	EU204527	EU204284	EU204208
Acromyrmex balzani	**EU204490**	**EU204247**	**EU204170**
Apterostigma auriculatum	EF013549	EF013549	EF013677
Apterostigma pilosum complex sp. 4	EU204514	EU204271	EU204195
Apterostigma auriculatum	EU204484	EU204241	EU204164
Apterostigma cf. *goniodes*	EU204513	EU204270	EU204194
Apterostigma collare	EU204540	EU204297	EU204221
Apterostigma dentigerum	EU204515	EU204272	EU204196
Apterostigma dorotheae	EU204500	EU204257	EU204181
Apterostigma manni	EU204485	EU204242	EU204165
Apterostigma new sp.	EU204533	EU204290	EU204214
Apterostigma pilosum complex sp.1	EU204501	EU204258	EU204182
Atta cephalotes	EU204516	EU204273	EU204197
Atta laevigata	**EU204481**	**EU204238**	**EU204161**
Atta mexicana	EU204491	EU204248	EU204171
Atta texana	EU204525	EU204282	EU204206
Cyphomyrmex rimosus	**EU204466**	**EU204223**	**EU204146**
Cyphomyrmex cornutus	**EU204521**	**EU204278**	**EU204202**
Cyphomyrmex cornutus	**EU204532**	**EU204289**	**EU204213**
Cyphomyrmex costatus	**EU204488**	**EU204245**	**EU204168**
Cyphomyrmex faunulus	EU204487	EU204244	EU204167
Cyphomyrmex longiscapus	EU204496	EU204253	EU204177
Cyphomyrmex minutus	EU204508	EU204265	EU204189
Cyphomyrmex muelleri	EU204535	EU204292	EU204216
Cyphomyrmex new sp.	EU204520	EU204277	EU204201
Cyphomyrmex new sp.	EU204534	EU204291	EU204215
Mycetarotes acutus	EU204517	EU204274	EU204198
Mycetarotes cf.	EU204474	EU204231	EU204154
Mycetoagroicus triangularis	EU204537	EU204294	EU204218
Kalathomyrmex cf.emeryi	EU204524	EU204281	EU204205
Kalathomyrmex emeryi	EU204478	EU204235	EU204158
Mycetosoritis clorindae	EU204536	EU204293	EU204217
Mycetosoritis hartmanni	EU204479	EU204236	EU204159

Mycocepurus tardus	EU204507	EU204264	EU204188
Mycocepurus smithi	EU204477	EU204234	EU204157
Mycocepurus smithi	EU204523	EU204280	EU204204
Mycocepurus curvispinosus	EU204509	EU204266	EU204190
Myrmica sp.	EU204472	EU204229	EU204152
Myrmica striolagaster	EF013598	EF013598	EF013726
Myrmicocrypta infuscata	EF013600	EF013600	EF013728
Myrmicocrypta buenzlii	EU204510	EU204267	EU204191
Myrmicocrypta ednaella	EU204539	EU204296	EU204220
Myrmicocrypta sp.	EU204506	EU204263	EU204187
Myrmicocrypta urichi	EU204471	EU204228	EU204151
Myrmicocrypta new sp.	EU204522	EU204279	EU204203
Pogonomyrmex sp.	EU204492	EU204249	EU204172
Proatta butteli	EU204495	EU204252	EU204176
Pseudoatta new sp.	EU204493	EU204250	EU204174
Sericomyrmex cf.parvulus	EU204467	EU204224	EU204147
Trachymyrmex arizonensis	EF013655	EF013655	EF013783
Trachymyrmex bugnioni	EU204470	EU204227	EU204150
Trachymyrmex cf.intermedius	EU204502	EU204259	EU204183
Trachymyrmex cf. zeteki	EU204505	EU204262	EU204186
Trachymyrmex cornetzi	EU204468	EU204225	EU204148
Trachymyrmex diversus	EU204469	EU204226	EU204149
Trachymyrmex new sp.	EU204499	EU204256	EU204180
Trachymyrmex smithi	EU204538	EU204295	EU204219
Trachymyrmex irmgardae	EU204489	EU204246	EU204169
Trachymyrmex opulentus	EU204498	EU204255	EU204179
Trachymyrmex papulatus	EU204504	EU204261	EU204185
Trachymyrmex septentrionalis	**EU204503**	**EU204260**	**EU204184**
Wasmannia auropunctata	**EU204483**	**EU204240**	**EU204163**
Wasmannia sp.	EU204528	EU204285	EU204209
***Apterostigma pilosum complex* sp.**	**KC964637**		**KC964658**
Apterostigma steigeri	**KC964636**		**KC964659**
Mycocepurus goeldii	**KC964635**		**KC964655**
Serycomyrmex parvulus	**KC964633**		**KC964656**
Trachymyrmex fuscus	**KC964634**		**KC964657**
***Mycetophylax simplex* SRS**	**KC964632**		**KC964654**
***Mycetophylax simplex* CRJ**	**KC964630**		**KC964651**
***Mycetophylax morschi* ASC**	**KC964621**		**KC964646**
***Mycetophylax morschi* ARJ**	**KC964623**		**KC964643**
***Mycetophylax conformis* CRJ**	**KC964618**		**KC964640**
***Mycetophylax conformis* ARJ**	**KC964616**		**KC964638**

Capítulo III

Phylogeography of the endemic sand dune ant species *Mycetophylax simplex* (Formicidae, Attini): evidences for demographic and range expansion during the Quaternary

Phylogeography of the endemic sand dune ant species *Mycetophylax simplex* (Formicidae, Attini): evidences for demographic and range expansion during the Quaternary

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Abstract

Aim: Several species inhabiting forests experienced demographic and range contractions over glacial periods in the past. However, species living outside these wet habits have been shown to present a variable number of responses to Quaternary events. Here we investigated the genetic diversity distribution of the endemic sand dune ant *Mycetophylax simplex* across its whole range along the Brazilian coast.

Location: Southern and south-eastern sandy dune environments ("restinga") along the Atlantic Forest (AF) biome. AF represents an excellent opportunity to draw a phylogeographical hypothesis due to its wide range of vegetation types that includes open habitats covered predominantly by herbaceous and shrubby plants, strongly influenced by sea.

Method: We used partial sequences of the mitochondrial gene cytochrome oxidase I from 108 specimens to assess the phylogeography and demographic history of this species. To achieve this we performed different methods of phylogenetic and standard population genetic analysis.

Results: The observed genetic diversity distribution and historical demographic profile suggested that *M. simplex* does not agree to the expected scenario for others AF species, but rather underwent demographic and range expansion during glacial periods. This was confirmed by shallow phylogeographic structure along the populations analyzed with isolation by distance effect. Our coalescent approach indicated two starting pulses in the population expansion that was coincident with the low sea-level during the Quaternary. Such demographic events were likely shaped by the enlargement of shorelines during marine transgression/regression and habitat release/evolution.

Main conclusion: Our overall results are in agreement with log-term persistence showed by coastal sand dune and grassland species. Beyond that, we add an important framework about how both, glacial and interglacial, events could positively affect species distribution and diversification along south Atlantic Coast.

Key-words: Brazil, South America, Phytogeography, sand dunes, ants, marine transgression

Introduction

Climatic fluctuations during the late Quaternary associated with the last glacial maximum (LGM) have had a strong impact on the current distribution of many animal and plant species worldwide. Climate change not only affected the landscape of continental areas but also the sea level, which in turn shaped coastal landscapes by forming land bridges, islands, sand coastal plains, as well as connecting and separating areas (Hewitt, 2000; Dillenburg & Hesp, 2009). All these climate-linked processes may have influenced the evolutionary history of the species, especially those inhabiting coastal areas (Crottini et al., 2012, Barker et al., 2012).

Pollen data, fossil records, and palaeoclimatic data indicate that numerous taxa in the Northern hemisphere were restricted during the period of glaciation to southern or eastern refugia (Stewart & Lister, 2001), and phylogeographic studies show how expansion from these sites has molded their distribution today (Hewitt, 2004; Hewitt, 2011; Vialatte et al., 2008, Widmer et al., 2012). In contrast, less is known about these phenomena in the Southern hemisphere, where the glacial refugia hypothesis has only recently been formally evaluated (Carnaval & Moritz, 2008; Carnaval et al., 2009). It is presently the most common used mechanism to explain the current distribution of species diversity in the Brazilian Atlantic Forest (AF), the second largest Neotropical forest after the Amazon rainforest.

AF covers more than 3.300 km along the eastern coast of Brazil. It has received worldwide attention because of its high biodiversity and great level of endemism and was determined as one of the 25 world biodiversity hotspots with high priority in conservation (Myers, 2000, Carnaval et al., 2009). The AF presents a wide range of vegetation types with conspicuous changes across landscapes, which include open habitats covered predominantly by herbaceous and shrubby plants, which develop on marine deposits ("Restinga"). Despite of an increasing number of phylogeographic studies, the knowledge about the evolutionary history of AF is still limited and controversial (Grazziton et al., 2006; Carnaval & Moritz, 2008, Fitzpatrick, et al., 2009, Thomé et al., 2010; Resende et al, 2010, Pinheiro et al., 2011, Ribeiro et al., 2011, Tonini et al., 2013). The refugia hypothesis has gained

support by increasing number of phylogeographical studies that attempt to explain the high diversity in AF (Grazziton et al., 2006, Thomé et al., 2010, Resende et al., 2010). Basically, it states that during glaciation, when the climate was drier in the Southern hemisphere, forests contracted and persisted only in moister areas. These became refugia for humidity-dependent species. By vicariant processes, these refugia promoted speciation and therefore, today they harbor a higher genetic diversity and endemism than areas that did not serve as refugia (Hewitt 2000).

The retreat of forests and the changed global climate conditions may have allowed the expansion of drought-tolerant biomes (Behling & Negrelle 2001; Behling 2002). These authors suggested that open and dry vegetation in southern and eastern regions of South America, including those that occur along Atlantic coast, expanded during the drier periods throughout climatic oscillations. Compared to species restricted to refugia, species associated with open habitats may have experienced recurrent shifts in their distribution, with populations mixing or separating from each other with the cyclical shrinking or expanding of forests. Alternatively, their distribution may have remained largely unchanged during these historical events. While several species associated with wet forest environments have been used to infer the evolutionary processes occurring during the Quaternary in the Atlantic Forest, only a few studies have addressed organisms – exclusively plants – associated with dry environments (see Pinheiro et al., 2011).

Mycetophylax simplex is a small fungus-growing ant (Formicidae: Myrmicinae) endemic to sand dunes environment along the Brazilian coast, occurring from southern São Paulo State to Rio Grande do Sul (Cardoso et al., 2012). Although this ant exhibits a wide distribution, it is restricted to specialized habitats (sand dunes), making it a good model organism to test different phylogeographical scenarios relative to open / dry coastal environments. Thus, the aim of this study is to evaluate the genetic relationship among populations of *M. simplex* across-its whole distribution and to infer how the Quaternary oscillations have affected genetic diversity and structure of its populations. Based on DNA sequences of the mitochondrial gene cytochrome oxidase (COI) and the nuclear gene wingless, we aimed to test if (i) *M. simplex* underwent a persistent range or population expansion during the climatic oscillations of the Quaternary and if and how (ii) its demographic history was affected by the cyclic changes in the sea level.

Material and Methods

Sampling and laboratory procedures

A total of 108 colonies of *M. simplex,* spanning its whole distribution area, were collected over the period between January and September 2011 (Figure 1). The geographical references and sample size of all sampled sites are given in the Table 1. The specimens were preserved in 100% ethanol until DNA extraction. We extracted the whole genomic DNA from one individual per colony, totalizing 108 sequenced individuals. To ensure that estimates of population differentiation and diversity were reliable, adjacent sampled sites (beaches) were merged into a unique population based on dispersal capability of Attini, and then comprising sample sizes with at least eight colonies.

Genomic DNA was extracted using proteinase K digestion followed by a standard CTAB protocol (Sambrook & Russell, 2001). Fragments of the mitochondrial gene cytochrome oxidase subunit I (COI) and the nuclear gene wingless, both known to be useful in intra-specific studies (Medihabadi et al., 2012) were amplified by PCR using four previously published primers LCO1490 (Folmer et al., 1994) and Ben (Kronauer et al., 2004) for COI and Wg578F (Ward et al., 2005) and Wg1032R (Abouheif et al., 2002) for wingless. The fragments of COI ranged from 800 to 950 bp long and comprised an overlapping region that resulted in assembled sequences typically with 1,100 bp. Fragments of wingless had 386 bp to 411 bp, resulting in an assembled sequence with 373 bp.

Amplification of the genes was conducted in 25 µL final volume reactions containing: $MgCl_2$ (2.5mM), buffer reaction (1x; Promega), dNTPs (1 mM each), a pair of primers (0.48 µM each), *Taq* polymerase (2 U of GoTaq® Flexi DNA Polymerase) and 1 µL of template DNA. PCR cycling conditions was as follows: initial denaturation for 2 min at 94°C, then 35 cycles of 94°C for 1 min, an annealing temperature of 50°C or 55°C for 1 min for COI and wingless, respectively, 72°C for 2 min, and then a final extension at 72°C for 7 min. Finally, PCR products were purified and sequenced by Macrogen Inc. (www.macrogen.com) directly using the same primers of amplification in an ABI PRISM 3700 sequencing system. Singletons were checked by sequencing a second individual from the same colony when available.

Analysis

The chromatograms of each gene were evaluated and edited separately using the program Consed (Gordon et al., 1998). Afterwards, they were analyzed by translation into amino acids using the program MEGA 5.0 (Tamura et al., 2011). All sequences were deposited in GenBank. The wingless gene was assumed to be single-copy because only one band was observed on gels after the amplification by PCR. Since wingless did not show intraspecific variation throughout the sampled populations, subsequent analysis was conducted only with the mitochondrial gene COI. Further evaluation of additional nuclear genes (long-wave rhodopsin and abdominal A) showed similar results (data not shown).

Sequence variation was analyzed by mean of Mega 5.0 software and the diversity parameters, including nucleotide diversity (π) and haplotype diversity (h) were computed using the software DNASP 5 (Tamura et al., 2011) for each population and for the complete data set.

In order to assess the correlation between genetic diversity and geographical distances among sampling populations, i.e. testing for isolation-by-distance (IBD), we carried out a Mantel test. For this we used the program ALLELES IN SPACE – AIS (Miller, 2005) to test correlation between log-transformed genetic and geographic distance of individuals. The genetic differentiation among *M. simplex* populations was measured by means of F-statistics (Excoffier et al., 1992). Pairwise comparisons of F_{ST} between populations were first calculated using the program ARLEQUIN 3.5 (Excoffier & Lischir, 2010). By this we could identify barriers to genetic flow among populations of *M. simplex*.

We also carried out a spatial analysis of molecular variance using the program SAMOVA 1.0 (Dupanloup et al., 2002) to search for partitions of sampling sites that were genetically homogenous but maximally differentiated from each other. Based on a pre-specified *K* this method uses simulated annealing procedures to seek for a better clustering option that can be defined between groups of populations by the among group genetic variation coefficient (FCT). Analyses were conducted five times to check consistency and based on 1000 simulated annealing steps with the number of *K* groups increasing from 2 to 9. Thus, we could identify the clustering of samples that yielded the largest, and most significant, F_{CT} for a given *K*.

The phylogenetic relationships among *M. simplex* haplotypes were investigated using two methods: the Median Joining network algorithm implemented in the NETWORK 4.6 (http://www.fluxus-engineering.com) and the statistical parsimony procedure for phylogenetic network estimation, with 95% criterion for a parsimonious connection applied in TCS 1.21 (Clement et al., 2000). Based on this, we could determine if gene genealogy matches with geographically separated populations. This analysis also provided a more accurate estimate of intraspecific gene genealogies even in cases of shallow genetic divergence among haplotypes (Posada & Crandall, 2001). We also used Bayesian inference performed in MRBAYES 2.3 (Ronquist & Huelsenbeck, 2003) to infer gene genealogies among unique haplotypes of *M. simplex* in order to evaluate nodal support and assess the monophyletic status of this species. We selected the model of nucleotide evolution for each codon that best fit to our data set using the Akaike's information criterion (AIC) implemented in MRMODELTEST 2.3 (Nylander, 2004). The Bayesian analyses consisted of two independent runs of ten million generations each, sampled every 1000 generations. The convergence among runs was verified by the average standard deviation of split frequencies that was <0.01. Then, mixing and appropriated burn-in was determined using Tracer v1.4 (Rambaut & Drummond, 2007). A total of 25% of the tree was burned out to produce a consensus topology. Finally, the Bayesian topology was visualized using the FIGTREE v1.3.1 program (Rambaut, 2009).

Trends in the demographic history of *M. simplex* populations were investigated using three different approaches. First, under the assumption of neutrality deviations in Tajima's and Fu's *FS* statistics we tested for past population expansions. A negative value in both statistic tests would reflect either purifying selection in a population at mutation-drift equilibrium, or deviations from mutation-drift equilibrium, resulting from population expansion. Second, we observed the distribution of pairwise nucleotide differences among haplotypes and tested the deviation from the expected mismatch distribution under sudden and spatial model by means of a generalized least-squares method and Harpending's *h* statistic. Both analyses were carried out in Arlequin 3.5. Third, we used a Bayesian skyline plots (BSP) approach (Drummond et al., 2005), implemented in BEAST 1.6.1 (Drummond & Rambaunt, 2007), with the aim to recover changes in the effective population size (Ne) over time. For this, we first estimated a mutation rate for the genus *Mycetophylax* from the COI sequences and fossil calibration of the

molecular phylogeny of the tribe Attini (Mehdiabadi et al., 2012). Mutation rate estimation was performed under an uncorrelated lognormal-relaxed clock model using the model of sequence evolution GTR+I+Γ with three partitions (codons 1, 2, and 3) with a random starting tree underneath a Yule speciation process as tree prior. Based on *Cyphomyrmex rimosus* group fossils, to the root node was given a lognormal distribution with mean of 1.6, standard deviation of 1.0 and offset equal 15, as described in Mehdiabadi et al., (2012). The results from three independent runs of 50 million generations sampled every 5,000 and with a burning of 15 million were combined in TRACER 1.4.1 and checked for adequate mixing of the MCMC chains by mean of values effective sample size (ESS). Finally, we used the mutation rate calculated above on our intraspecific COI data set in order to construct the timing and magnitude of changes in the effective population size using the Bayesian Skyline (BS) method (Drummond et al., 2005). This model was used to estimate the effective population size through time since the most recent common ancestor of *M. simplex.* Therefore, BEAST software had its molecular clock set to Uncorrelated Log-normal under a Relaxed Clock and analyses were performed using the SRD06 with distinct rates of sequence evolution for each codon partition. Results from three independent runs with 50 000 000 generations each, from which the initial 10% were burned out were combined and checked in Tracer 1.4.1 as described below.

Results

Sequence variation, phylogenetic relationships and structure

A total of 845 base pairs of the gene COI were sequenced and comprised 32 unique haplotypes widely sampled across the distribution range of *M. simplex*. From these resolved nucleotide sites we observed 32 polymorphic sites, being 19 singleton variables, 13 parsimony informative and a bias against guanidine (T: 36.2%. C: 20.3%, A: 31.9% G: 11.6%). All nucleotide substitutions were located at the third position of the codon and transitions (*Ti*) were proportional to transversion (*Tv*), thereby the ratio of *Ti:Tv* was 0.73, suggesting no saturation in relation to substitution. Overall haplotype and nucleotide diversity was 0.865 ± 0,022 and 0.00346 ± 0,00019 (mean ± SD), respectively (Table 2). In Bayesian and ML phylogenetic analysis (data not shown), posterior probabilities and bootstrapping values indicated that *M.*

simplex formed a well-supported monophyletic clustering and that all specimens evaluated throughout the distribution could be considered as belonging to the same species.

The Median Joining haplotype network was in agreement with the statistical parsimony network. The gene genealogy among the haplotypes considering a 95% threshold for the probability of a parsimonious connection was achieved, so all the haplotypes were joined in the same single network (Figure 2). The network did not indicate divergent clades within genealogies of *M. simplex* and substantial genetic diversity could be seen due to many unique haplotypes. Two major haplotype represented 50% of the individuals: H3 represented 27.7% of the individual and seems to be widespread across the species range, whereas the haplotype H11 was almost restricted to the northern range of the species distribution and was separated from the other by missing intermediate haplotypes (Figure 2). Large portions of the found haplotypes were singleton (Table 1), but no more than two nucleotide substitutions separated immediate haplotypes (Figure 2).

There was no apparent association between genetic structure and geography concerning the haplotypes, since they were widespread within the bounders of *M. simplex* distribution (Figure 2 and 3). The most frequent haplotype (H3) could be found in populations covering fully the southern and northern studied areas (Figure 3).

However, considering the pairwise F_{ST} comparisons between populations, we could identify major genetic drifts along the analyzed populations of *M. simplex*. Population from the southern distribution and from the north (starting from Itapirubá/Garopaba/Pinheira – SC6) showed no significant genetic differentiation and lower F_{ST} values among them (Table 3), suggesting that gene flow should be occurring among them. However, the gene flow between these populations is restricting leading to some genetic structure among *M. simplex* haplotypes. Thus, we could identify that the haplotypes of *M. simplex* are shallowly structured into three major population groups: Southern populations including RS1 and RS2 (green shading Table 3), Core-East populations including RS3, SC4 and SC5 (yellow shading Table 3) and finally, the Northern population, were including the remainder ones (red shading Table 3).

In line with these results, the Mantel test showed that genetic and geographic distance are slightly correlated (r = 0.075, p = 0.0007), suggesting that there is a weak degree of isolation by distance. Furthermore, the spatial

analysis of molecular variance implemented in SAMOVA was not able to identify possible breaks among populations. All parameters showed small range of variation (Figure 4) and any F_{CT} calculated for a given K group suggested better explaining about the genetic structure of *M. simplex*. The F_{CT} values slightly differed from each other (0.22-0.26) and did not show any trend of increasing or decreasing with *K*. Besides, the major proportion of variance was within-population variation, ranging from 76% to 83% (Figure 4b).

Demographic history

We observed a shallow phylogeographic structure in *M. simplex* populations; therefore the historical demography analysis was conducted for the complete set of haplotypes. The neutrality tests allowed us to identify some statistical significant signatures for expanding population for both tests. The extremely negative and significant values of Tajima's *D* (-1.47062, $P = 0.0422$) and Fu's *FS* (-21.59803 $P = 0.0001$) suggested that haplotype frequencies differ from that expected from a neutral population. Yet, the distribution of pairwise nucleotide differences between haplotypes had a bimodal shape in the mismatch distribution (Figure 5a), which is expected for populations that have shown constant population demography. However, both models of population expansion could not be rejected. Harpending's raggedness *h* statistic (R_2) and the sum of square differences (SSD) supported a close fit to the observed distribution under a pure demographic expansion model (SDD = 0.00754, p = 0.62760; R_2 = 0.01864, p = 0.86400) and under a sudden spatial expansion model (SSD = 0.00905, p = 0.40100; R_2 = 0.01864, p = 0.84750).

The tMRCA for all *M. simplex* haplotypes was recovered to be 0.197 Mya (with 95% highest posterior density, HPD, of: 0.699-0.355). The analysis of Bayesian Skyline Plot provided an additional strong support for the evidence of past population expansion (Figure 5c). The results suggested that *M. simplex* underwent a long-term period of demographic population stability since the tMRCA until ~70,000 years ago. We could observe that the expansion had a second increasing cline around 25,000 years ago, corroborating the observed mismatch distribution.

Discussion

The impact of Quaternary climatic fluctuation and geological events underpinning the biodiversity of Brazilian Atlantic Forest has been object of discussion of innumerous studies based on the evaluation of the genetic structure of wet associated environment species (WAES). Here, we aimed to contribute to this discussion by exploring the population genetic structure under a phylogeographic perspective of *M. simplex*, a not WAES ant species.

A shallow phylogeographic pattern was observed throughout the distribution of *M. simplex* along the Brazilian Atlantic Coast. In fact, the genealogical relationships among haplotypes were not associated with geographically separated sampled sites. Further, the spatial analysis of population structure implemented by SAMOVA could not recognize a congruent grouping that better explains the observed genetic structure of *M. simplex*. In sum, our results suggest that *M. simplex* reveal low levels of genetic drift and that genetic bottleneck due to extinction are restrict, since these two processes would decrease genetic diversity and increase genetic differentiation among populations.

Clearly recognizable geographical barriers could not be identified across the extensive sandy coastal plain where *M. simplex* occurs, from southern São Paulo to Chuí in Rio Grande do Sul State. For instance, long water bodies as rivers and the *estuarine* complex of the Paranaguá Bay apparently does not to impair the gene flow among northern areas, since population from Florianópolis (SC) to "Ilha Comprida" (SP) were genetically similar.

Contrasting phylogeographic patterns have been reported for species associated to wet environments in AF, where rivers systems had played an important role as physical barriers to gene flow (Pellegrino et al., 2005, Resende et al., 2010). Patterns of genetic diversity geographically structured have also been postulated as a consequence to glacial refugia in AF when forests were fragmented during glacial periods (Carnaval et al., 2009; Thomé et al., 2010). Under this scenario it would be expected that a long-term isolation of species in multiple fragments would shed genetic signatures (i.e. higher genetic variability than recolonized areas), resulting in reciprocally monophyletic linages in current populations.

In the analysis through *M. simplex* lineages we did not observed such signatures or monophyletic lineages among haplotypes. Indeed, the widespread North-South division pattern generally reported for other AF

species (e.g. Grazziotin et al., 2006; Resende et al., 2010) was not observed in the genealogical relationships of *M. simplex* haplotypes. This suggests that long-term allopatric population divergence has not taken place in this sand dune restrict species. Our results also support the idea that barriers should have different effects on distinct organisms.

By contrast, an alternative hypothesis for the phylogeographic pattern observed for *M .simplex* is an enduring persistence of its populations along the seashore of the Atlantic coast and unrestricted gene-flow crossways South-Northern regions. In fact, Tropical forests (including wet AF) were reduced during the Quaternary ice ages, imposing the contraction of species distribution and vicariant process (Hewitt, 2000, 2004). Yet, the drier and cooler environment during the ice ages in the Neotropics promoted the expansion of open and scrub environments in southern Brazil which included the sand dunes areas along the Atlantic coast (Behling & Negrelle, 2001, Moraes et al., 2009). Since the dry sand dune environments are the current and restricted habitats where *M. simplex* lives, the absence of phylogeographic structure among the haplotypes of this species is in agreement with the geomorphologic history of this area, suggesting that *M. simplex* may have not experienced shifts in their distribution. This result is consistent with phylogeographic patterns reported for a coastal orchid species with similar range distribution (Pinheiro et al., 2011). Together, these results suggest that an alternative scenario such as the range persistence through the past climatic oscillations may have contributed to the distribution of coastal lowland species in AF. Furthermore, similar patterns have been reported to other sand dune or coastal species worldwide (Mora et al., 2006, Piñeiro et al., 2007, King et al., 2009, Frey et al., 2012).

The pairwise F_{ST} analysis allowed us to identify some breakdowns in gene flow amongst populations from the three major geographical regions (Table 03), likely as a result of IBD. Though weakly, there is some degree of isolation by distance among the populations of *M. simplex*. Besides, the haplotype H3 that are widely dispersed geographically are localized in interior of the network. This supports the known idea that high-frequent haplotypes connected by multiple connections are most likely to be pleisomorphic. Therefore, long-term persistence and historic conservation of ancestral polymorphism within haplotypes shared between distant localities, rather than current gene flow, is a more likely scenario to explain such distribution

pattern. Additionally, this could explain the uniformity of the nucleotide diversity values found across the entire north-southern species distribution.

Pleistocene sea levels fluctuated considerably through the glacial and interglacial periods during the Quaternary. During Pleistocene, the Coastal Plain of the southern region of South America was larger than now (Ab'sáber, 1990, 2001). Palaeo-geomorphological studies showed that sea-level was about 120 meters lower than the present (Figure 1 and 5b), what represents an elongation of the coast-line limits to ~100 kilometers (Corrêa, 1996, Behling & Negrelle, 2001). This enlargement exposed the lowland on the coast and may have represented an increase in niche availability to *M. simplex,* leading to the expansion of its populations. We found genetic signatures that supported this hypothesis. The negative and highly significant Fu's *FS* and Tajimas's *D* showed departures from the neutrality. Hence, the Mismatch distribution detected imprints of both sudden and spatial demographic expansion in the past population of *M. simplex*. The historical demographic reconstruction based on BS plot showed that *M. simplex* underwent a stable demographic period shifting to a persistent growth of effective population size in recent periods. The population growth started around 70,000 years ago and displays a slight increase at around 25,000 years ago. These results indicated that the process underpinning the current diversity of *M. simplex* happened during the middle-Pleistocene and intensified under different climatic and geological events imposed by the Last Glacial Maximum (LGM), at Late-Pleistocene.

It is noteworthy that both "*pulses*" in the historical demography of *M. simplex* populations are in agreement with the two lower historic sea levels during the Quaternary, and the overall *M. simplex* historic demographic growing started and persisted during the major period of sea level fall (see Figure 5b, c). A reasonable explanation for the observed phenomenon is that historic demographic expansion occurred due the habitat release by both sea regression and AF shrinking during cool dry climate seasons that extended the open and scrub vegetation in south Brazil (Behling & Negrelle, 2001). These consequently favored the suitable habitats for *M. simplex*. It is reasonable to assume that the cooler climatic conditions during the Pleistocene (lower around 3-7 °C), were not a constraint for the distribution of *M. simplex,* since this species seems to be intolerant to warm temperatures (Cardoso, pers. obs.).

Nowadays, this species presents an almost exclusively subtropical distribution, to the south of the Tropic of Capricorn (23° S), except by one

residual population at Cabo Frio beaches, in Rio de Janeiro state. The present distribution of *M. simplex* strengthens the important role of ocean transgression in shaping *M. simplex* distribution over the past. The northernmost limit of the current distribution is coincident with the major enlargement of the coastal lowland in Brazil, from southern Chuí in Rio Grande do Sul State to Cabo Frio in Rio de Janeiro State (Figure 1) (Corrêa, 1996, see also Hewitt, 2000). South Brazil had pronounced marine regression from its continental shelf that was strongly distinct from the northern region (Corrêa, 1996), resulting in exposed lowland. These land bridges may have enabled migration of many organisms across the latitudinal gradient by forming sand corridors or sand islands due to coastal deposits.

As mentioned before, resulting water bodies from regression and transgression of the sea or even the ocean should not represent an insurmountable obstacle for flying insects and may not impair the dispersion of ant species (Seal et al., 2011). However, it would be unreasonable to assume that sandy beaches corridors and sandy islands emerged promptly during Quaternary. Corrêa (1996) using sedimentary deposition analysis suggested that this region underwent at least three phases of sea stabilization up to the current level, lasting until 6,500 years ago. Thus, we could suggest that *M. simplex* had enough time to widespread across their distribution range until Cabo Frio beaches in Rio de Janeiro State, living in an almost panmictic population.

It is important to note that there is a gap in the current distribution of *M. simplex* on the stretch of the coast between southern São Paulo and Cabo Frio. Its exclusion from the south-east Brazilian coastline could be explained by three not exclusive hypotheses: (i) Holocene marine transgression drowned the suitable *M. simplex* habitats; (ii) open habitats were removed by the expansion of the forests towards the seashore, which is currently covered by tropical rain AF; (iii) Competition with congeneric species.

Overall, Rio de Janeiro is characterized by a rocky coastline with little development of transitional sedimentary coastal plains, due the proximity to the mountainous relief of Serra do Mar (Dias & Kjerfve, 2009). As an effect of Post Glacial Marine Transgression, the sea submerged the majority of sandy beach ridges; removing the *M. simplex* from this portion of Brazilian coastline. These ants could have survived in the region of Cabo Frio, protected from drowning by barriers that act as feasible refugia during the marine transgressive events. Cabo Frio and neighborhood is pointed out as the only

sector of this portion of the east coastline to present Coastal aeolian dunes (Dias & Kjerfve, 2009). These barriers are sand deposits from Pleistocene and also have been reported to act as a refugia spot for sand dune vegetation in south coast of Brazil (Pinheiro et al., 2011).

Conversely, the expansion of AF towards the Atlantic coast may have facilitated *M. simplex* exclusion by removing open and sandy habits turning these areas no longer suitable. Post glacial AF expansion together with the rise of the sea have imposed the shrinkage of sand dune habitats, mainly between the south latitude 23° and 24° (Souza et al., 2008). Starting on north coast of São Paulo State, *M. simplex* could compete with other congeneric species (*M. conformis)* for food and nesting sites since both species nest near to seashore and use debris from sparse vegetation to grow up its fungus garden (see Klingenberg et al., 2007 for nesting details). *M. conformis* seems to have higher ecological amplitude than *M. simplex*, being the former observed nesting also in shaded areas and workers performing tasks also during the day (Cardoso, pers. orbs.), whereas the later always nests on sunny open areas and is active only during dusk and overnight (Diehl-Fleig & Diehl, 2007). Thus the factors mentioned above may have reduced the local density of *M. simplex,* that culminated by been excluded due to competition pressure.

Phylogeographic studies regarding species associated with open and dry environments have attracted much less attention compared to the number of studies using organisms associated with forests as study model. Identifying explicit phylogeographical patterns and the factors underpinning the genetic structure are reasonably difficult, particularly for those species inhabiting historic and dynamic regions, as coastal sand dunes. Our findings indicate that *M. simplex* presents a complex evolutionary history consistent with shifts in the sea-level and changes in the distribution of dry vegetation on south-eastern Brazil. The diversification and expansion started in the mid-Pleistocene, during which major climatic changes occurred worldwide. Our results are in agreement with other studies of sand dunes species that indicate expansion during glacial periods, but is contrasting with others, suggesting that a single and wide model of Quaternary effects on the diversification and distribution of species is unrealistic.

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Figure and Tables:

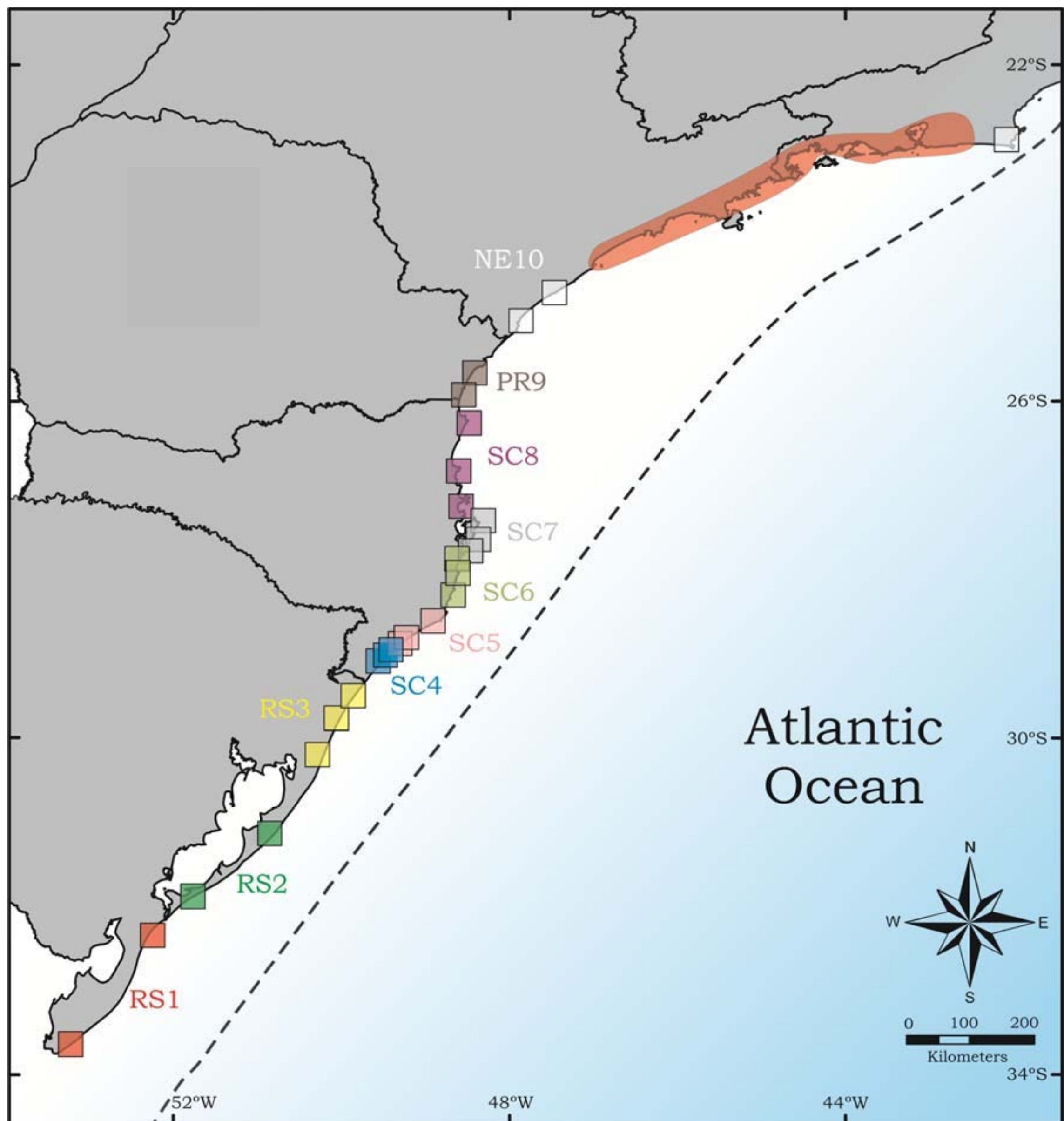


Figure 1 – Map showing the 27 sampled localities of herein depict *M. simplex* distribution from southern and southeast Atlantic coast of Brazil. Each color square represents one population (for details see table 1). The read highlighted area on the southeast coast is the gap in the distribution of *M. simplex* and the dashed line represents the limits of the sea–level during the last glacial maximum (approximately 21 Mya).

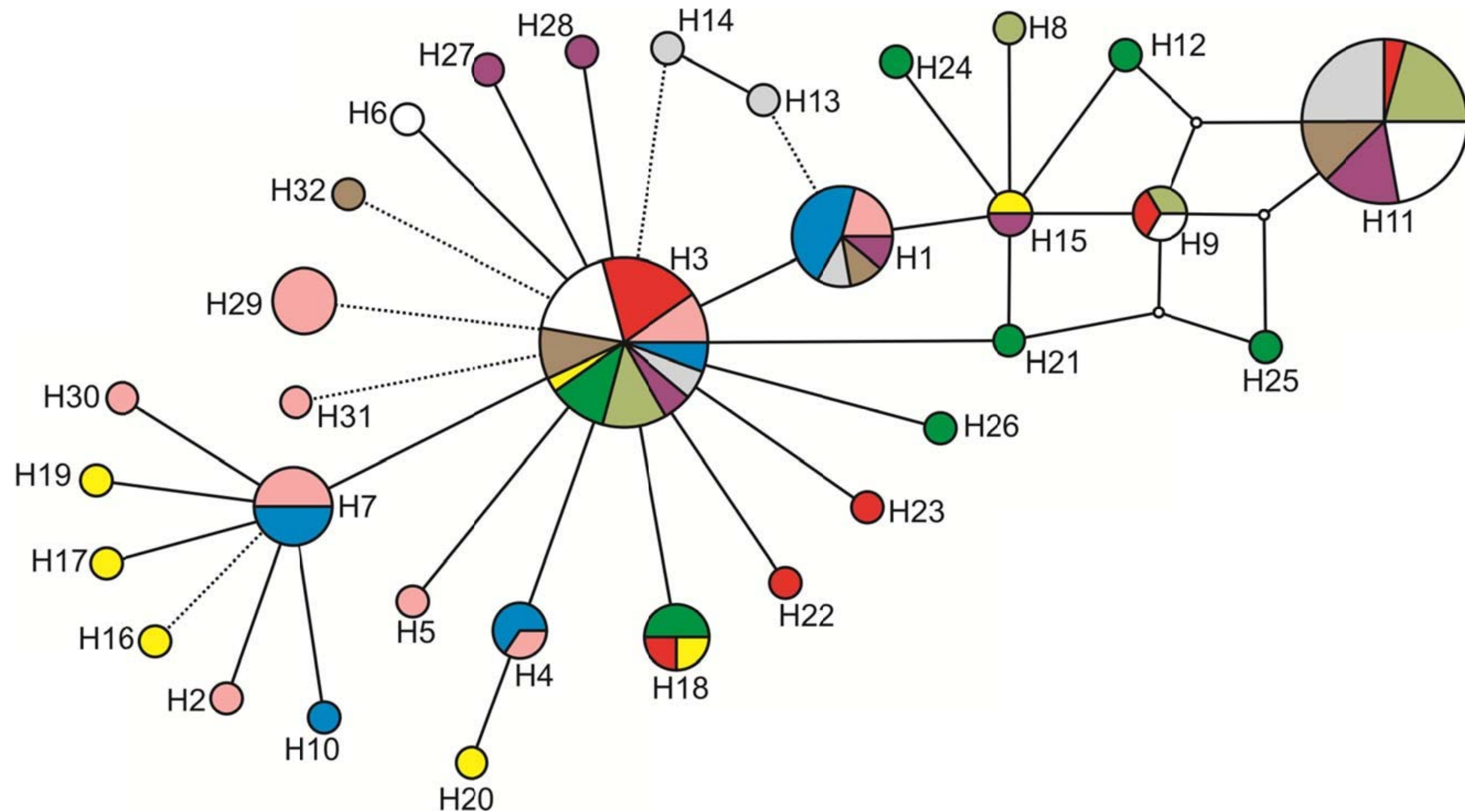


Figure 2 – Statistical parsimony haplotype network showing the phylogenetic relationship among 32 unique haplotypes observed among ten populations of *M. simplex*. The circles are the haplotypes and their size represents their frequencies in the total sample, small and white circles are missing estimated haplotypes. Each color corresponds to the populations given in table 1 and figure 1.

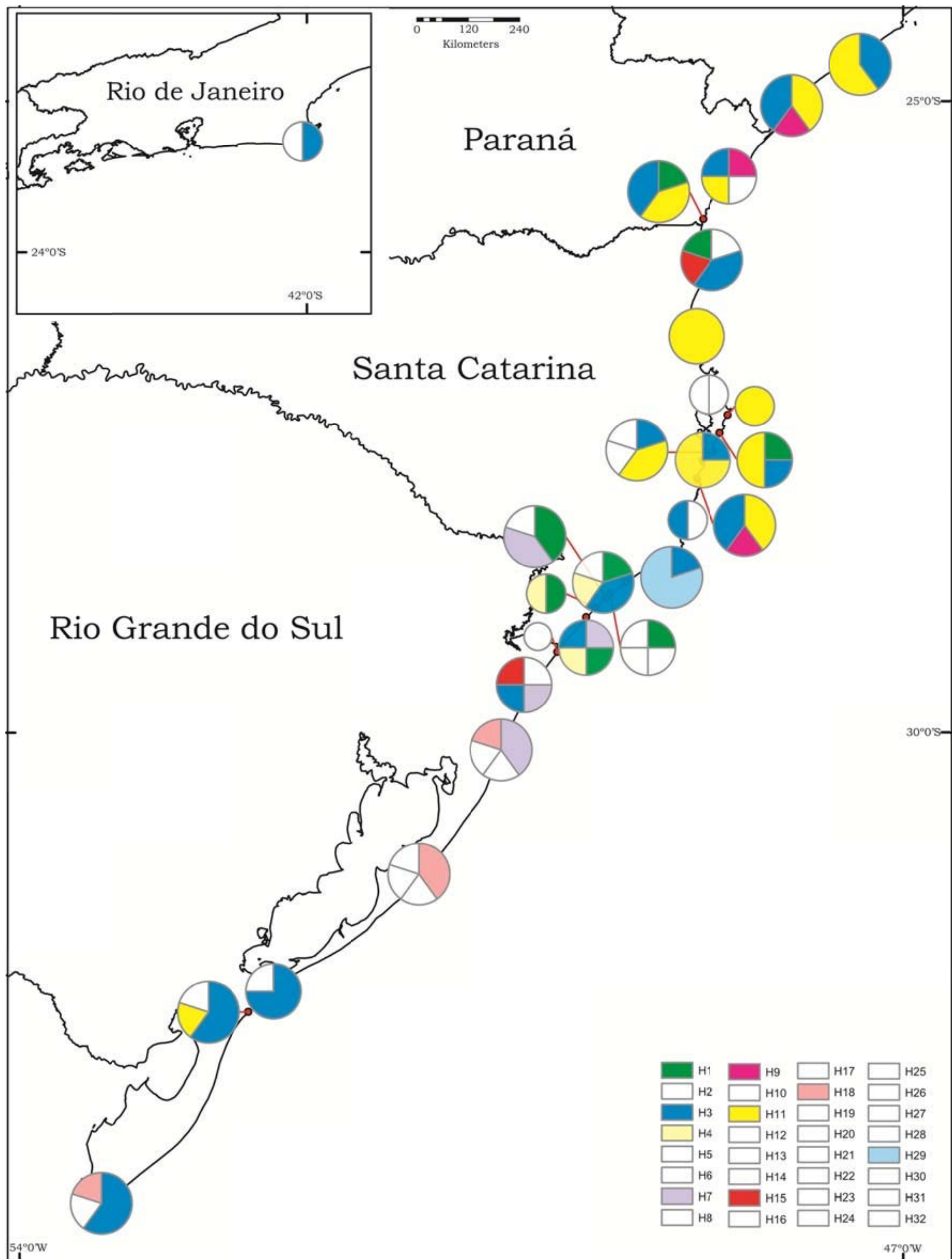


Figure 3 - Geographical distribution of all 32 cytochrome oxidase I (COI) unique haplotypes observed across the distribution of *M. simplex* along Atlantic Brazilian coast. Singletons were suppressed and are shown in white. Colors display frequent haplotypes distributed along throughout *M. simplex* distribution along Atlantic coast.

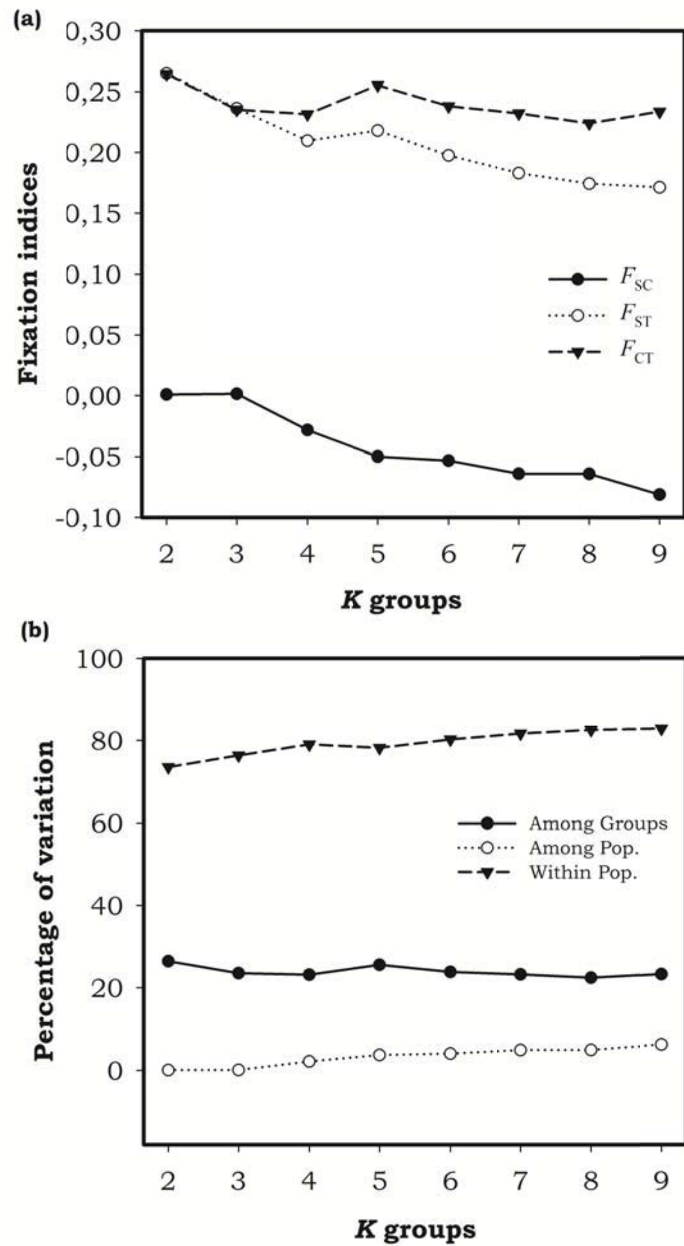


Figure 4 – Spatial analysis of molecular variance - SAMOVA of populations of *M. simplex*. (a) Fixation indices calculated and (b) percentage of genetic variation explained by each hierarchical level for the best grouping option for each pre-specified *K* groups.

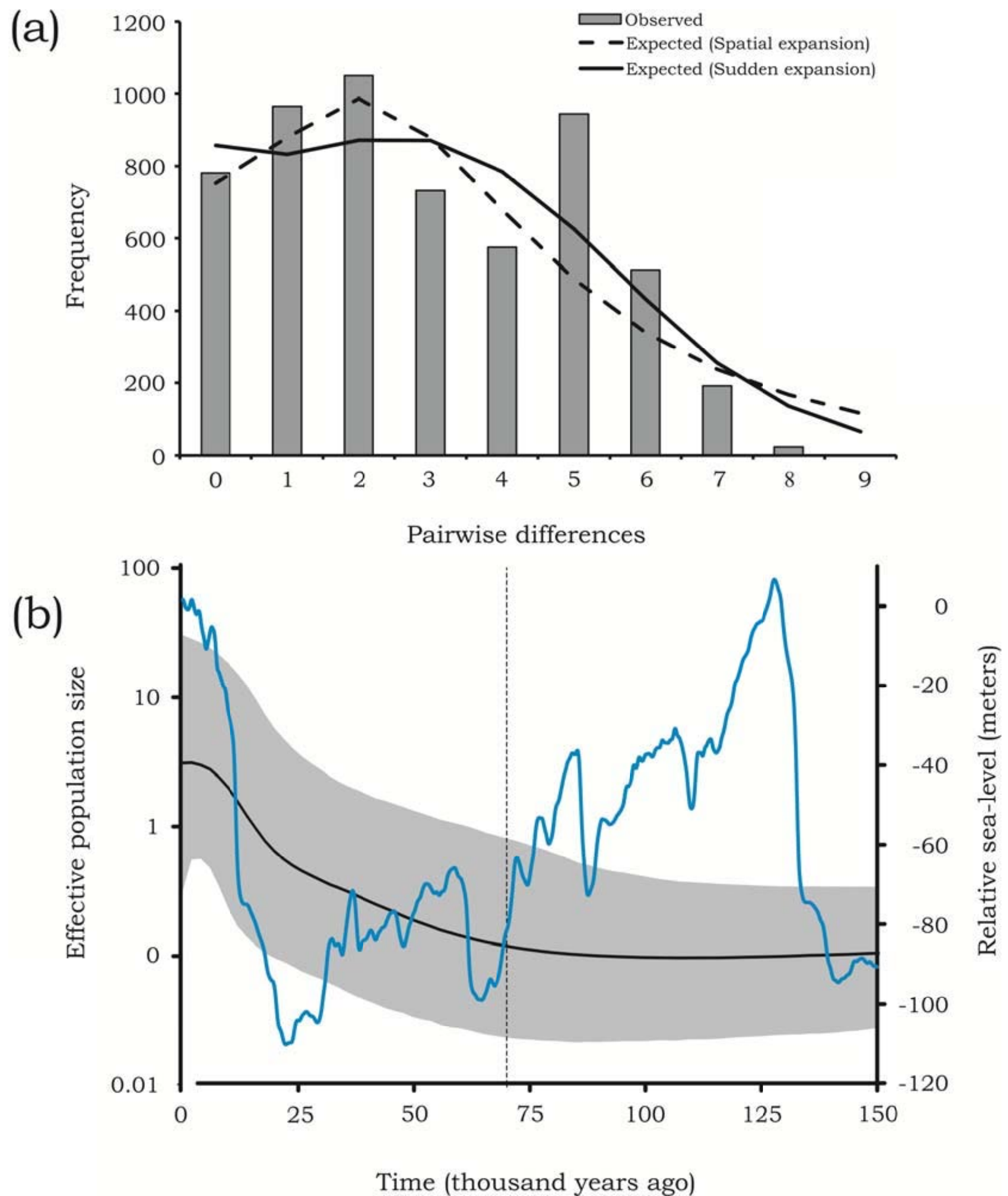


Figure 5 – Demographic history of *M. simplex* and relative sea level. (a) Pairwise mismatch distribution of the mtDNA sequences for total dataset. *M. simplex* presented a bimodal shaped distribution, but still it did not reject the spatial expansion model (SDD=0.0226 p= 0.3803). (b) Bayesian skyline plot showing the historical demography and complete reconstruction of female effective population size fluctuations throughout the time of *M. simplex*. Black line represents median estimate and the grey area the upper and lower 95% confident intervals. Dashed line indicates the beginning of demographic expansion. Blue line shows the sea-level during the last 150,000 years during the Quaternary (redrawn from Grant et al., 2012).

Table 1 – Population details, geographical location of the population encompassing 27 sampled localities (S=latitude, W=longitude) throughout the range distribution of *M. simplex* known and its haplotypes distribution.

Population	Locality	Coordinate		Haplotype (n)
		S	W	
RS1	Chuí	33° 43’	53° 21’	H5(3), H22(1), H26(1)
	Cassino	32° 13’	52° 11’	H5(3), H14(1), H27(1)
RS2	São José do Norte	32° 03’	51° 59’	H5(3), H29(1)
	Mostradas	31° 07’	50° 50’	H22(2), H25(1), H28(1), H30(1)
RS3	Cidreira	30° 07’	50° 11’	H2(2), H21(1), H22(1), H23(1)
	Curumim	29° 37’	49° 56’	H2(1), H5(1), H19(1), H20(1)
	Torres	29° 21’	49° 44’	H24(1)
SC4	Bal. Arroio do Silva	29° 00’	49° 26’	H1(1), H4(1)
	Bal. Gaivota	29° 11’	49° 35’	H1(1), H4(1), H3(1), H7(1)
	Araranguá	28° 57’	49° 22’	H1(2), H7(2), H10(1)
SC5	Ilhas	28° 54’	49° 19’	H1(1), H2(1), H30(1), H31(1)
	Bal. Rincão	28° 48’	49° 12’	H1(1), H3(2), H4(1), H5(1)
	Laguna	28° 36’	48° 50’	H3 (1), H29(4)
SC6	Itapirubá	28° 19’	48° 42’	H3(1), H8(1)
	Garopaba	27° 59’	48° 37’	H3(2), H9(1), H11(2)
	Pinheira	27° 50’	48° 35’	H3(1), H11(3)
SC7	Florianópolis – Moçambique	27° 29’	48° 23’	H11(2)
	Florianópolis – Joaquina	27° 37’	48° 27’	H1(1), H3(1), H11(2)
	Florianópolis – Pântano do Sul	27° 46’	48° 31’	H3(1), H11(2), H13(1), H14(1)
SC8	Gov. Celso Ramos	27° 19’	48° 32’	H27(1), H28(1)
	Navegantes	26° 51’	48° 38’	H11(4)
	São Francisco do Sul	26° 15’	48° 31’	H1(1), H3(2), H12(1), H15(1)
PR9	Guaratuba	25° 56’	48° 34’	H1(1), H3(2), H11(2)
	Pontal do Paraná	25° 40’	48° 27’	H3(1), H9(1), H11(1), H32(1)
NE10	Ilha Comprida - Cananéia	25° 02’	47° 53’	H3(2), H9(1), H11(2)
	Ilha Comprida - Iguapé	24° 42’	47° 28’	H3(2), H11(3)
	Cabo Frio	22° 54’	42° 02’	H3(1), H6(1)

Table 2 – Genetic diversity and neutrality tests for each population and with all populations of *M. simplex* together.

Populations	Nucleotide diversity (π) (± S.D.)	Haplotype diversity (*h*) (± S.D.)	Tajima's *D*	Fu's FS
RS1	0,00189 (0,00090)	0,667 (0,163)	-1,87333 (*P* = 0,0083)	-1,11562(*P* = 0,1609)
RS2	0,00224 (0,00054)	0,889 (0,091)	-0,6299 (*P* = 0,2859)	-2,32907(*P* = 0,0261)
RS3	0,00276 (0,00055)	0,933 (0,077)	-1,50661(*P* = 0,0632)	-4,46904(*P* = 0,0025)
SC4	0,00181 (0,00024)	0,818 (0,083)	0,43329 (*P* = 0,6969)	-1,02733(*P* = 0,1714)
SC5	0,00268 (0,00036)	0,890 (0,060)	-1,09063 (*P* = 0,1463)	-2,8844(*P* = 0,0302)
SC6	0,00336 (0,00039)	0,709 (0,099)	1,52257 (*P* = 0,9504)	1,62676(*P* = 0,8143)
SC7	0,00387 (0,00064)	0,709 (0,137)	1,49895 (*P* = 0,9408)	0,7727(*P* = 0,6626)
SC8	0,00346 (0,00042)	0,873 (0,089)	1,00501 (*P* = 0,8566)	-1,68615(*P* = 0,1229)
SC9	0,00368 (0,00059)	0,833 (0,098)	0,92757 (*P* = 0,8263)	0,12678(*P* = 0,4978)
NE10	0,00339 (0,00038)	0,697 (0,090)	1,68302 (*P* = 0,9613)	1,85074(*P* = 0,8387)
All populations	0,00346 (0,00019)	0,865 (0,022)	-1,47062 (*P* = 0,0422)	-21,59803(*P* = 0,0001)

Table 03 - F_{ST} values for pairwise comparison between population of *M. simplex* (lower left) and *p* values (upper right). Population names are given in the Table 1.

	RS1	**RS2**	**RS3**	**SC4**	**SC5**	**SC6**	**SC7**	**SC8**	**PR9**	**LE10**
RS1	-	0,54618	0,01228	0,04633	0,18008	0,01792	0,01594	0,04841	0,08653	0,0492
RS2	0,01856	-	0,01683	0,00515	0,0302	0,02624	0,01465	0,04742	0,09554	0,0592
RS3	**0,11411**	**0,13078**	-	0,27888	0,02287	0,00386	0,00337	0,00485	0,01297	0,00941
SC4	**0,09502**	**0,1619**	0,01451	-	0,08455	0,00356	0,00297	0,00752	0,01733	0,01129
SC5	0,02839	**0,093**	**0,10675**	0,07119	-	0,00069	0,0004	0,00168	0,00713	0,00614
SC6	**0,27381**	**0,24143**	**0,34481**	**0,34975**	**0,30795**	-	0,78299	0,92516	0,6338	0,83912
SC7	**0,29673**	**0,27747**	**0,36158**	**0,35894**	**0,32158**	0,05903	-	0,59489	0,43421	0,47352
SC8	**0,19818**	**0,17864**	**0,28682**	**0,26863**	**0,24282**	0,06991	0,03956	-	0,93931	0,77794
PR9	0,13621	0,12338	**0,23448**	**0,21937**	**0,1883**	0,06591	0,0298	0,08068	-	0,94852
LE10	**0,18098**	**0,16623**	**0,27856**	**0,27675**	**0,23074**	0,07182	0,03535	0,06859	0,0855	-

Bold values are significant at $P < 0.05$

5. Considerações Finais

Nos capítulos do presente estudo foram descritas as relações filogenéticas, a organização do genoma do ponto de vista citogenético e a evolução cromossômica das espécies endêmicas de restinga do gênero *Mycetophylax.* Além disso, foi descrito o padrão filogeográfico da espécie *M. simpl*ex associada aos ambientes de dunas da costa do Atlântico. Por meio do emprego destas diferentes abordagens busca-se contribuir para o entendimento da evolução destes organismos e da história dos ambientes costeiros de restinga. Podemos assim considerar:

1. O gênero *Mycetophylax* na sua atual designação apresenta-se como um grupo monofilético;

2. A espécie *M. morschi*, anteriormente incluída em *Cyphomyrmex*, indubitavelmente agrupa com *M. simplex* e *M. conformis*;

3. A hipótese filogenética com base em caracteres moleculares concorda com as relações estabelecidas com base em dados morfológicos publicados na literatura;

4. O número cromossômico verificado para colônias de *M. morschi* variou de 2n=26 a 2n=30 cromossomos, enquanto o número cromossômico diploide detectado para *M. conformis* e *M. simplex* foi de 30 e 36 cromossomos, respectivamente;

5. A variação cariotípica verificada para *M. morschi* foi atribuída a eventos de fusão cromossômica;

6. Os padrões de bandamento indicam que as espécies *M. simplex* e *M. conformis* são proximamente relacionadas e diferem dos cariótipos de *M. morschi* quanto à quantidade de heterocromatina e composição das bases nucleotídicas AT/CG;

7. A diversidade genética de *M. simplex* não foi correlacionada com a distribuição geográfica ao longo de sua área de distribuição;

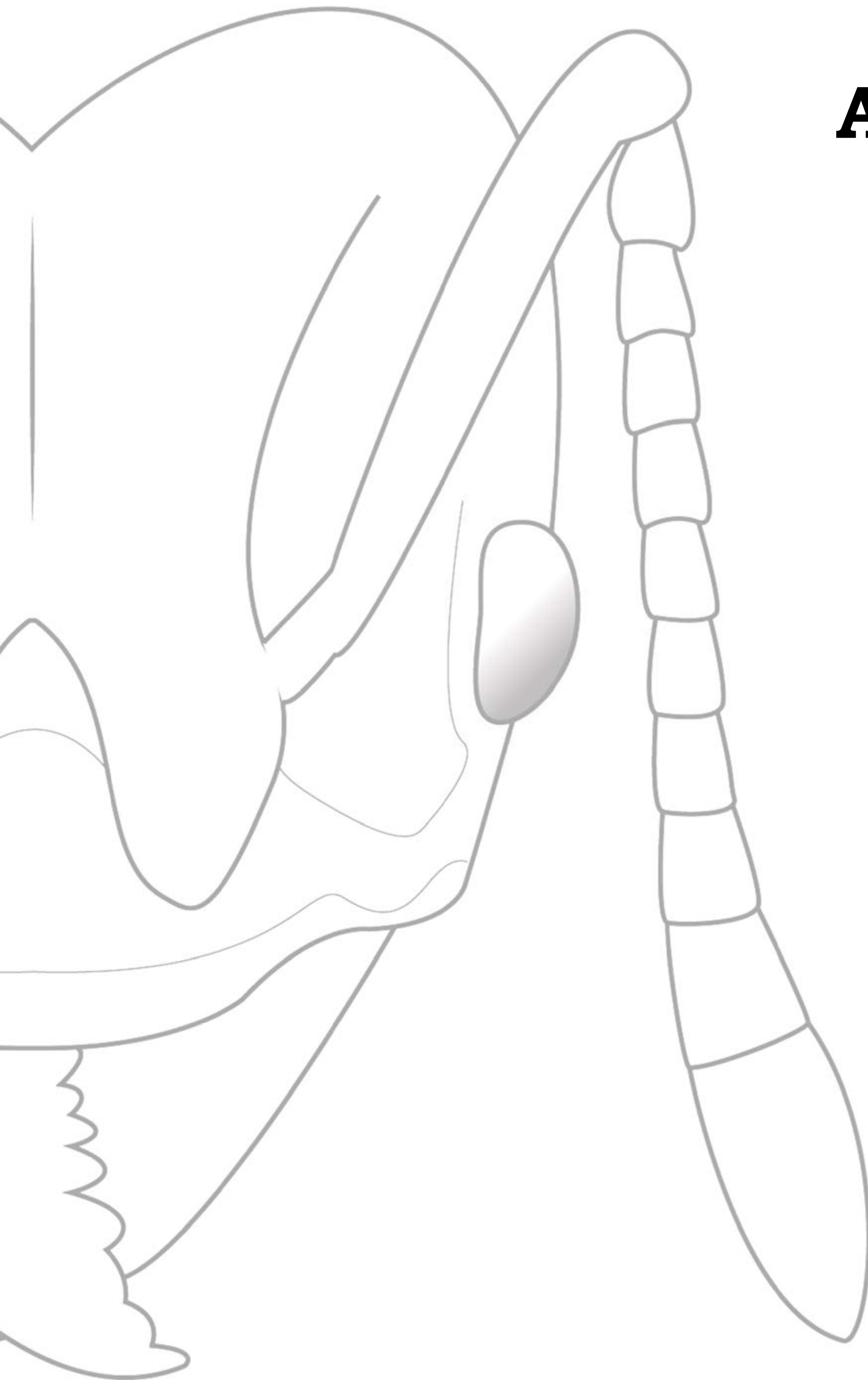
8. Haplótipos frequentes foram observados distribuídos ao longo de toda a costa sul e sudeste do Atlântico, onde *M. simplex* ocorre;

9. A análise de demografia histórica evidenciou que as populações de *M. simplex* passaram por um período de estabilidade populacional com recentes eventos de expansão demográfica;

10. Os eventos de expansão populacional foram coincidentes com os períodos mais baixos do nível dos oceânicos durante o Quaternário;

11. Os resultados obtidos na presente tese sugerem que a diversificação de *Mycetophylax* parece ter acompanhado as mudanças e evolução dos ambientes de restinga, que foi altamente dinâmico durante o Quaternário.

Anexos



Publicações Relacionadas

Embora não incluídos como parte integrante do conteúdo principal da tese, os artigos aqui relacionados estão a ela associados e foram publicados durante o desenvolvimento do projeto de doutorado:

Artigo I – Methodological remarks on rearing basal Attini ants in the laboratory for biological and evolutionary studies: overview of the genus *Mycetophylax*;

Artigo II – Co-occurrence of putatively allopatric species of the genus *Mycetophylax*: first record of *Mycetophylax simplex* (EMERY, 1888) (Hymenoptera: Formicidae) from Rio de Janeiro State, Brazil;

Artigo III – Estimation of nuclear genome size of the genus *Mycetophylax* Emery, 1913: evidence of no whole-genome duplication in Neoattini.

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Insectes Sociaux

RESEARCH ARTICLE

Methodological remarks on rearing basal Attini ants in the laboratory for biological and evolutionary studies: overview of the genus *Mycetophylax*

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Abstract Some studies require fresh biological material for their development. Ant colonies have been reared under laboratory conditions for scientific purposes, and several methodologies for leafcutter ants have been reported in the literature. However, these methods are not well adapted for rearing basal Attini. In this study, we proposed a methodology for rearing basal Attini species in the laboratory based on the evaluation of colonies of the genus *Mycetophylax*. The complete system consists of two round translucent polypropylene containers inserted one inside the other, where one serves as a chamber proper and the other as a foraging area. Both containers are sealed with their lids, protecting the environment against desiccation. From a total of 29 colonies collected in the field, 22 colonies survived for at least 30 weeks, and *Mycetophylax morschi* was the most adapted for rearing under laboratory conditions. The main problem with rearing basal Attini in the laboratory is the loss of moisture. Thus, the method applied here may be adopted for rearing other basal Attini, as well as other ant species very sensitive to moisture variation.

Keywords Rearing · Laboratory protocols · *Mycetophylax* · Attini · Formicidae

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Introduction

The genus *Mycetophylax* is a small monogynous basal Attini (Formicidae: Myrmicinae), endemic to sand dunes on Brazilian coastlines (Restinga). It has several characteristics considered basal, such as presenting a monomorphic worker caste, not cutting fresh leaves but instead collecting dry plant matter, feces and insect carcasses to supply its symbiotic fungi, and the fact that it forms small colonies with around a dozen to a hundred workers (Hölldobler and Wilson, 1990; Fernández-Marín et al., 2005; Lopes, 2007).

This genus has undergone a taxonomic revision by Klingenberg and Brandão (2009) and consists of three nominal species: *Mycetophylax morschi* (Emery, 1888), *M. conformis* (Mayr, 1884) and *M. simplex* (Emery, 1888). The three species have a marked geographic distribution. Two species, *M. conformis* and *M. simplex*, are parapatric, and their geographic distributions along the Atlantic coastline do not overlap. Otherwise, *M. morschi* is sympatric with the other two species, but it does not have common nesting sites, occurring farther from the ocean than *M. simplex* and *M. conformis* (Klingenberg et al., 2007). However, Cardoso et al. (2010) found *M. simplex* and *M. morschi* living in the same phytophysiognomies. The three species showed similar features including nest architecture, number of workers and fungus chambers (Klingenberg et al., 2007, pers. obs.).

In the course of developing a project on phylogeography and the karyotypic evolution of the *Mycetophylax* genus, we collected colonies of the species *M. simplex*, *M. conformis* and *M. morschi.* Since cytogenetic and molecular analyses require fresh biological material, we needed to keep the colonies alive. Several methodologies for rearing leafcutter ants have been reported in the literature (see Della-Lucia, 1993 for a review), but these methods were unsuccessful for rearing basal Attini (Klingenberg, 2006; Sanhudo et al.,

2008). In spite of this, Himler et al. (2009) have been successful in rearing Attini ant colonies in the laboratory for years; however, details of the methodology employed have not been reported. Thus, in this study we proposed a new methodology for rearing basal Attini species in the laboratory based on the evaluation of the colonies of the genus *Mycetophylax*.

Materials and methods

Sampling of colonies

We collected colonies of three species from sand dunes in the Santa Catarina (27°S, 49°W) and Rio de Janeiro states (23°S, 43°W) between December 2009 and January 2010. The colonies of *M. simplex* (13 colonies) were collected on beaches in Santa Catarina state, *M. conformis* (7 colonies) on beaches in Rio de Janeiro state, and *M. morschi* (9 colonies) were collected in both states. The nests were located by nest mounds, which can be easily recognized by their specific characteristics. The entrances are located on the top of a small sand turret and surrounded by a sand crater, except in the case of *M. morschi*, which constructs larger craters without a sand turret (Weber, 1982; Diehl-Fleig and Diehl, 2007). A hole of about 1 m in depth was excavated exactly 10 cm from the nest mound. Afterward, the sand walls of the hole were carefully removed until the fungus chamber had been exposed.

A total of 29 colonies were transported to the Universidade Federal de Viçosa and reared under laboratory conditions. Initially, the colonies were maintained alive using the protocols described by Weber (1972) for leafcutter ants, which employ two plastic containers connected by a tube. This protocol was used with some adaptations regarding size to take into account the proportions of basal Attini ants. The colonies were maintained in a climate-controlled chamber of the biological oxygen demand (BOD) type at a temperature of 25°C. The colonies rapidly declined, and due to this lack of success, we developed the protocol described below.

Outline of rearing system

The complete system consisted of two round translucent polypropylene containers inserted one inside the other, one of which served as a chamber proper and the other as a foraging area. The internal container ("chamber") had a volume of 175 mL (5.4 cm height and 8.5 cm diameter), and the fungus garden and all individuals were placed inside. A small hole was made in the base of the internal container, and this was then placed inside the external container, which had a volume of 500 mL (7.7 cm height and 11.9 cm diameter). Both containers were sealed with their lids, protecting the internal environment against desiccation. The lid of the internal container featured small holes with wet cotton wool above to keep the moisture level inside the system high (around 80% of the relative humidity) (Fig. 1). A 1 g piece of cotton wool moistened with 10 mL of water was used to cover the lid of the internal container.

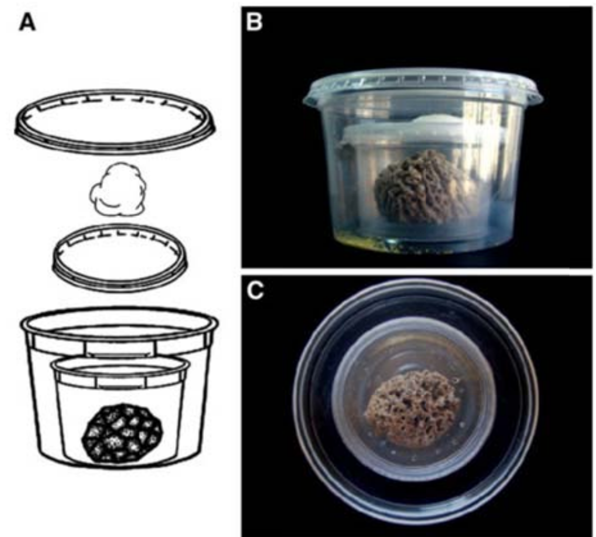

Fig. 1 Schematic drawing of the system used for rearing colonies of three *Mycetophylax* species in the laboratory (**a**). Pictures of the whole system: lateral view (**b**) and top view (**c**). Fungus chamber of *M. conformis*

The substrate offered for the growth of the fungus garden was placed in the forage arena (external container). We offered as substrate dried orange rind, flaked oats, triturated popcorn, dry grass and semi-dry grass cut into very small pieces. The fungus substrate was supplied three times a week, alternating between different types. All colonies were maintained at room temperature monitored by a thermo-hygrometer (Incoterm, mod. 9860.17.1.00). The colonies were cleaned once a week to remove the waste deposited by workers in the forage arena. At the same time, the moistened cotton wool was changed to avoid contamination with microorganisms or because workers had deposited waste on it.

Results and discussion

Out of the 29 colonies collected in the field, 22 colonies survived for at least 30 weeks and by using the method presented in this study. Eight of these colonies were of *M. morschi*, nine colonies were of *M. simplex* and five

colonies were of *M. conformis*. The colonies of *M. simplex* (four colonies) reared by the method of Weber (1972) survived for 4–5 weeks, but two colonies of *M. morschi* and one colony of *M. conformis* died quickly in a maximum of 1 week. At the end of 1 week, the fungus garden had lost its moisture completely, and all individuals had died. The death of the colonies was due to rapid desiccation as there is no feature within them to retain moisture, and the colonies were placed directly in the BOD incubator inside a single container connected to a forage arena by a tube (Weber, 1972).

This method is very similar to that adopted for rearing leafcutter ants, but in the latter species the moisture is conserved by the fungus garden itself, since their substrate is composed of fresh leaves (Hölldobler and Wilson, 1990; Lopes 2005). In contrast, *Mycetophylax* use a dry substrate to grow their fungus garden; therefore, the retention of moisture is critical. The average temperature of the fungus chamber in the field is 17 ± 2°C, and the relative moisture in the hole excavated (1 m depth) for colony collection was around 80%. Thus, we conclude that the colonies will survive in a tightly closed container, which should be able to conserve the moisture of the micro-environment. Consequently, we reduced the entire system to a single container, which included the fungus chamber, forage arena and a source of moisture. Moreover, as the foraging area and the fungus chamber are encased in a single container instead of two or more containers connected by tubes, a greater number of nests can be stored in a smaller space in the laboratory.

So far at least three colonies have survived for 37 weeks (one colony of *M. morschi* and two colonies of *M. simplex*) and the remainder for more than 30 weeks, which is enough time for the development of our studies. *M. morschi* was the most adapted species for rearing in the laboratory, and the fungus grew to occupy the entire volume of the internal container in 37 weeks. We offered as fungus garden substrate various plant sources (see "Materials and methods"), all of which were accepted by workers of both species. These substrates were offered one at a time tri-weekly. After 1 week, the colonies were cleaned due to the accumulation of excess substrate offered, as well as the accumulation of waste deposited by workers in the foraging area as a result of the cleaning of the fungus garden. During the experimental period, the colonies were wiped 30 times (once at the end of each week), and three colonies were cleaned 37 times (one colony of *M. morschi* and two colonies of *M. simplex*). Over a period of 1 week, the cotton wool was found to be damp, and the fungus garden had a wet and healthy appearance, similar to its appearance in the field. However, to avoid possible contamination of the cotton wool and to ensure that the moisture level was maintained, a new piece of wet cotton wool was introduced to the system during cleaning. In a few cases, waste was found deposited by the workers on the cotton wool.

Production of alate forms occurred in all species throughout rearing, but it was more conspicuous in *M. morschi* and *M. conformis*. Males arose earlier than gynes. Data regarding the biology of these species are scarce; hence, we do not know how many alate forms are produced and in which season it takes place. According to Klingenberg et al. (2007), there are differences between the three species concerning the number of gynes and males, but the number of alate forms was not correlated to the size of the colony population. During our excavations, we always found gynes and males, but in the summer the number of gynes was higher than in winter.

Based on the evidence presented in this study for the *Mycetophylax* genus and due to the fact that most basal Attini do not cut fresh material for the growth of their symbiotic fungi (Lopes, 2007), we believe that the main problem with rearing basal Attini in the laboratory is the loss of moisture. As the substrate offered for the growth of the fungus garden was accepted in this study by workers and was also accepted by other species (Sanhudo et al., 2008), we suggest that the method applied in this work may be adopted for the rearing of other basal Attini, as well as other ant species that are very much sensitive to moisture variation.

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Myrmecological News	16	57-59	Vienna, January 2012

Co-occurrence of putatively allopatric species of the genus *Mycetophylax*: first record of *Mycetophylax simplex* (EMERY, 1888) (Hymenoptera: Formicidae) from Rio de Janeiro State, Brazil

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Abstract

This work reports the first recorded presence of *Mycetophylax simplex* (EMERY, 1888) in Rio de Janeiro state in Brazil. It also describes the discovery of co-occurrence of the two species of the genus that were previously considered allopatric species. One colony was collected at Praia das Dunas beach in Cabo Frio, Rio de Janeiro state. The co-occurrence of the three species of the genus *Mycetophylax* indicates that the species are sympatric. The known distribution of the genus *Mycetophylax* is summarized and discussed.

Key words: Ants, Attini, Formicidae, species distribution, restinga.

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Introduction

All ants in the tribe Attini engage in a mutual symbiosis with their fungal cultivars, which serve as their main food source. This relationship has been the focus of several studies that seek to understand the origin and transitions of the fungus growing habit (MEHDIABADI & SCHULTZ 2010). The 230 described species the tribe Attini comprises were divided into five agricultural systems due to correlations among ants, fungal cultivar and fungal pathogen phylogenies (see SCHULTZ & BRADY 2008 and MEHDIABADI & SCHULTZ 2010). Lower agriculture is the most abundant agricultural system and more informative for the elucidation of the events related to the first steps in the evolution of the tribe (MUELLER 2002). However, the majority of natural history studies are concerned with higher agriculturists (MEHDIABADI & SCHULTZ 2010, MUELLER & al. 2010).

The genus *Mycetophylax* EMERY, 1913 (Formicidae: Myrmicinae: Attini) practices a lower agriculture system and consists of three nominal species – *Mycetophylax morschi* (EMERY, 1888), *M. conformis* (MAYR, 1884) and *M. simplex* (EMERY, 1888) – after a taxonomic revision by KLINGENBERG & BRANDÃO (2009). The three species appear to be restricted to sand dunes on Brazilian Atlantic ocean coastlines (restinga), and according to KLINGENBERG & al. (2007) the genus *Mycetophylax* has a marked geographic distribution, where two species, *M. conformis* and *M. simplex* are allopatric and their geographic distributions along the Atlantic coastline do not overlap, whereas *M. morschi* is sympatric with the other two species. However, like other lower agriculturist species, the distribution of *Mycetophylax* is poorly known. Here we report the first record of *Mycetophylax simplex* in Rio de Janeiro state and its co-occurrence with its congeneric putatively allopatric species *M. conformis*.

Methods

Samples analyzed in the present study were collected from sand dunes at Praia das Dunas beach (22° 54' S, 42° 02' W) in Cabo Frio, Rio de Janeiro state in September of 2010. The entrances of the colonies are located on the top of a small sand turret and surrounded by a sand crater, except in the case of *Mycetophylax morschi*, which constructs larger craters without a sand turret (WEBER 1982, DIEHL-FLEIG & DIEHL 2007). A hole of about one meter in depth was excavated exactly 10 cm from the nest mound. Afterwards, the sand walls of the hole were carefully removed until the fungus chamber had been exposed. In total, 12 colonies were excavated.

Workers and alates were used for identification using keys published by KLINGENBERG & BRANDÃO (2009) and by comparison with material collected by CARDOSO & CRISTIANO (2010), held in the reference collection of the Laboratório de Ecologia de Comunidades of the Universidade Federal de Viçosa, where all voucher specimens were deposited. In addition, the identification was confirmed by specialists (Rodrigo Feitosa, Museu de Zoologia da Universidade de São Paulo, and Vivian Sandoval, Universidade Federal de Viçosa) not involved in the present study.

Results and Discussion

In the course of developing a phylogeographical study of the *Mycetophylax* genus, we collected samples from colonies of the three species that belong to the genus throughout

the area of their known occurrence. Taking into account the species distribution described in the literature (KEMPF 1964, KEMPF 1972, DIEHL-FLEIG & DIEHL 2007, KLINGENBERG & al. 2007, KLINGENBERG & BRANDÃO 2009, CARDOSO & CRISTIANO, 2010, CARDOSO & al. 2010), and given that the nest entrances of *M. simplex* and *M. conformis* are similar (WEBER 1972, DIEHL-FLEIG & DIEHL 2007), we assumed that we were digging up a colony of *M. conformis*. However, the only chamber we found was of *M. simplex*. As a result, we obtained the first record of the occurrence of *M. simplex* in Rio de Janeiro state. In total 12 colonies were excavated in Cabo Frio, Rio de Janeiro, and five of these were *M. conformis*, six were *M. morschi* and one was *M. simplex*.

The genus *Mycetophylax* is the most poorly known and studied of the genera (KLINGENBERG & BRANDÃO 2009) in the Attini tribe. There are no records of *Mycetophylax* species in forest environments, and KLINGENBERG & al. (2007) reported, in a study of nest architecture, that *Mycetophylax* species are endemic to sand dune environments, known as restinga in Brazil. We agree with the endemic status for *Mycetophylax* species for South Atlantic coastline environments in Brazil, since the record for Paraguay cited by FOWLER (1980) is dubious and according to WILD (2007) probably refers to the common inland species *Kalathomyrmex emeryi* (FOREL, 1907). However, the limits of species occurrence throughout the area of their known distribution are controversial. WEBER (1982) reports that the *Mycetophylax* genus is distributed across the whole of South America, with its southern limit in the state of São Paulo, Brazil. Nevertheless, KEMPF published a list of Neotropical ant fauna in 1972, as well as records of *M. simplex* in São Lourenço, Rio Grande do Sul state, which is considerably further south than São Paulo state. Until relatively recently, however, *M. simplex* had not been recorded in Santa Catarina state, but KLINGENBERG & al. (2007) collected colonies of this species on Florianópolis Island and CARDOSO & CRISTIANO (2010) recorded it on the southern coast of Santa Catarina state (Fig. 1).

Regarding their surveys and data on the geographic distribution of *Mycetophylax* species obtained from collected material, KLINGENBERG & al. (2007) suggested that the species *M. simplex* and *M. conformis* are allopatric along Atlantic coastlines. These authors emphasize that *M. simplex* occurs in sand dunes at the beaches of Santa Catarina Island (Florianópolis) and Rio Grande do Sul states, and that *M. conformis* occurs in sand dunes at the beaches of Rio de Janeiro and São Paulo states. However, in this study we collected one colony of *M. simplex* in sand dunes of the Praia das Dunas beach at Cabo Frio, Rio de Janeiro state, Brazil, for the first time. The third species, *M. morschi*, occurs sympatrically with both other species of the genus, in all the states cited. Furthermore, KLINGENBERG & al. (2007) report that *M. morschi* do not have common nesting sites, occurring farther from the ocean than *M. simplex* and *M. conformis* (see KLINGENBERG & al. 2007). However, CARDOSO & al. (2010) found *M. simplex* and *M. morschi* living in the same phytophysiognomies.

In our study we recorded the co-occurrence of the three species of the genus in the same locality (Praia das Dunas beach), indicating that *Mycetophylax simplex* and *M. conformis* are not allopatric. Further, we refute the hypothesis proposed by KLINGENBERG & al. (2007) that the distribution of the *Mycetophylax* species results in a partitioning

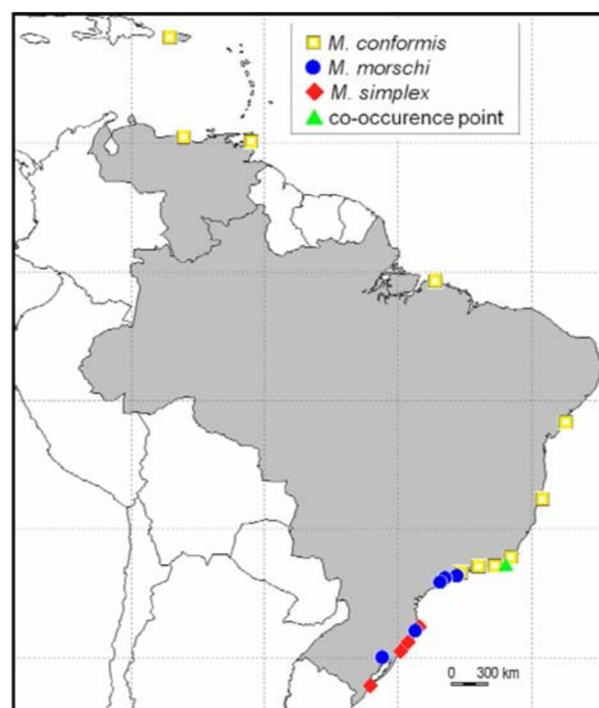

Fig. 1: New distribution map of the *Mycetophylax* species modified from KLINGENBERG & BRANDÃO (2009) with a highlighted co-occurrence of the three species in Cabo Frio (Rio de Janeiro) (green filled triangle): *M. simplex* (red filled diamonds), *M. conformis* (yellow open squares) and *M. morschi* (blue filled circles).

of the resource nesting space to reduce interspecific competition, since three species were found in the same nesting sites. Interspecific competition probably occurs among *Mycetophylax* species and evidence of this can be observed in the mean abundance shifts of these species in a composition study (CARDOSO & al. 2010). These authors report that *M. simplex* and *M. morschi* occur in the same restinga phytophysiognomies and that the mean abundance of *M. morschi* is higher when the mean abundance of *M. simplex* is lower or when this species is absent.

Finally, we suggest two hypotheses for the distribution of *Mycetophylax simplex*: (I) *M. simplex* has a distribution similar to that of the species *M. morschi* proposed by KEMPF (1964), which extends from southern Rio Grande do Sul state to Cabo Frio, Rio de Janeiro state; or (II) *M. simplex* had a similar distribution to *M. morschi* in the past and its current wide distribution is restricted to Santa Catarina and Rio Grande do Sul state, while it presents a relict population in Cabo Frio, Rio de Janeiro state. Other studies on the geographic distribution and phylogeography of the *Mycetophylax* species are presently being carried out by the authors and may elucidate the natural history of the *Mycetophylax* species.

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Paulo confirmed the identification of the ant species. We are grateful to Vivian Sandoval for valuable suggestions on the manuscript and two anonymous reviewers for constructive criticism. Thanks also to Dr. Gareth Cuttle for the English revision of this article.

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Genetics/Génétique

Estimation of nuclear genome size of the genus *Mycetophylax* Emery, 1913: Evidence of no whole-genome duplication in Neoattini

Estimation de la taille du génome nucléaire du genre Mycetophylax *Emery, 1913 : absence de preuve d'une duplication du génome chez une Neoattini*

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ABSTRACT

Genome size estimates and their evolution can be useful for studying the phylogenetic relationships and taxonomy of a particular group. In the present study, the genome sizes of the three species that comprise the *Mycetophylax* genus were estimated by flow cytometry (FCM). There was little variation in genome size among them. The mean haploid genome size value of male and female individuals of *Mycetophylax morschi* was 312.96 Mbp (0.32 pg) and that of *Mycetophylax conformis* and *Mycetophylax simplex* females were 312.96 Mbp (0.32 pg) and 381.42 Mbp (0.39 pg), respectively. At first glance, this variation could be related with the heterochromatin content. Our results, together with other previous reports, have contributed to our knowledge about Attini genome size and will be useful to improve the understanding of the evolution of this tribe. It will help select potential model species in Attini for future genomic and sequencing projects.

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Mots clés :
Taille du génome
Cytométrie en flux
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Fourmis
Formicidae

RÉSUMÉ

L'estimation de la taille des génomes et leur évolution peuvent être utiles pour comprendre les relations phylogénétiques et taxonomiques d'un groupe particulier. Dans cette étude, les tailles des génomes de trois espèces de fourmis du genre *Mycetophylax* ont été estimées par cytométrie en flux (CMF). Ces trois espèces présentent peu de variation de taille du génome. La valeur moyenne de la taille du génome haploïde mâle et femelle de *Mycetophylax morschi* était de 312,96 Mbp (0,32 pg) et celle des femelles de *Mycetophylax conformis* et *Mycetophylax simplex* de 312,96 Mbp (0,32 pg) et de 381,42 Mbp (0,39 pg), respectivement. Cette différence, à première vue, peut être liée à la teneur en hétérochromatine. Ces résultats, ainsi que d'autres études, contribuent à améliorer nos connaissances sur la taille du génome des *Attini* et peuvent être utiles pour la compréhension de l'évolution de cette tribu. Ils aideront à sélectionner de potentielles espèces modèles chez les *Attini* pour de futurs projets de séquençage et génomique.

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1. Introduction

The tribe Attini belongs to the Myrmicinae subfamily and comprises more than 230 described species. All ants in this tribe engage in a symbiosis with their fungal cultivars, which serve as their main food source. Recently, it was proposed that the Attini diverged from other ants approximately 50 million years ago (Schultz and Brady, [1]). These authors suggested that all the Attini genera can be separated into two monophyletic clades, the Paleoattini and Neoattini, [1]. These represent remarkable differences in morphological and biological features. The Paleoattini clade includes the basally divergent lineages, *Mycocepurus*, *Myrmicocrypta* and *Apterostigma;* while the Neoattini clade comprises all other genera of Attini including the more divergent ones, *Kalathomyrmex*, *Mycetarotes*, *Mycetosoritis*, *Mycetophylax*, *Cyphomyrmex*, *Mycetagroicus*, *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex* and *Atta*.

The genus *Mycetophylax* Emery, 1913 (Formicidae: Myrmicinae: Attini) is considered to occupy an intermediate position in the Neoattini clade. This position within the Attini phylogeny is critical for understanding the major evolutionary transitions of this group. The genus consists of three nominal species – *Mycetophylax morschi* (Emery, 1888), *Mycetophylax conformis* (Mayr, 1884) and *Mycetophylax simplex* (Emery, 1888) – after a taxonomic revision by Klingenberg and Brandão [2], and is distributed on sand dune environments from Caribbean beaches to southern Brazil (Klingenberg et al., [3]; Cardoso et al., [4,5]). Two species, *M. conformis* and *M. simplex* are allopatric in their major range, whereas *M. morschi* is sympatric with the other two species. However, these three species are found living together on the beaches of Cabo Frio, Rio de Janeiro State, Brazil [5].

Genome sizes have become an important evolutionary feature in biological studies and have gained great attention as genome size tends to be characteristic of a taxon and overall constant within a species (Swift, [6]). Additionally, it may be combined in phylogenetic studies. Moreover, some studies have shown positive correlation between genome size of organisms and eusociality (Koshikawa et al., [7]), parasitism (Johnston et al., [8]) or development (Gregory, [9]), showing that genome size may be an evolutionary constraint. However, these correlations between genome size and a particular character are not always clear and broadly applied. For example, Ardila-Garcia and Gregory [10] verified a positive correlation between genome size and body size in dragonflies but a negative one in damselflies. Moreover, sociality appears not to be related with small genomes in all Hymenoptera groups (Ardila-Garcia et al., [11]).

Ants form a well-defined and ecologically successful group within insects and are almost universally distributed. Of more than 12,000 ant species described so far, only 66 have their genome size estimated and from these, just seven belong to the Attini (Tsutsui et al., [12]; Ardila-Garcia et al., [11]). These studies have shown that ants display one of the lower genome sizes among the insects, with a mean genome size of 0.37 picogram (pg). This small value has been proposed to be a result of the high metabolic rates and complete metamorphosis (holometabolism) of ants [9], next to eusociality [11].

The purpose of this study was to determine the genome size (C-values, which represents the haploid DNA content in eukaryotic cells) of the species of the *Mycetophylax* genus for a better understanding of the genomic organization and genetic diversity of the genome size evolution of Attini. We are also interested in the comprehension of what evolutionary forces are involved in constraint and expansion of genome size in Attini.

2. Materials and methods

2.1. Experimental material

The specimens of *Mycetophylax* used in this investigation were obtained from several locations along the Brazilian sandy coastal plain between February and September 2010. At least five colonies of *M. morschi* and *M. simplex* were collected on sand dunes in the states of Santa Catarina and Rio de Janeiro, respectively. Ten colonies of *M. conformis* were collected on sand dunes of Rio de Janeiro State. Colonies were localized based on their specific characteristics, [13], and a hole of about 1 m in depth was excavated 10 cm from the nest mound. Afterward, the sand walls of the hole were carefully removed until the fungus chamber had been exposed. All colonies were kept alive under laboratory condition following protocols by Cardoso et al. [13], in order to obtain pupae, which were analyzed by flow cytometry.

2.2. Genome size by flow cytometry

The flow cytometry (FCM) analyses were carried out at the Laboratory of Cytogenetics and Cytometry, Department of General Biology, Federal University of Viçosa (UFV). The nuclear DNA content of three female pupae of all species and three males of *M. morschi* was measured using the C DNA content value of a female of *Scaptotrigona xantotricha* as internal standard, which had been successfully used to estimate DNA content of Hymenoptera (Tavares et al., [14]).

To obtain the nuclei suspension cells, pupae ganglia of the standard and samples were carefully dissected in physiologic solution (0.155 mM NaCl). The material was crushed 10 times with a pestle in a tissue grinder (Kontes Glass Company®) with 100 μL OTTO-I lysis buffer (Otto, [15]) containing 0.1 M citric acid (Merck®), 0.5% Tween 20 (Merck®) and 50 μg/mL RNAse (Sigma-Aldrich®), pH = 2.3. The suspension was adjusted to 1.0 mL with the same buffer, filtered through 30 μm nylon mesh (Partec®) and centrifuged at 100 g in microcentrifuge tubes for 5 min. The pellet was then incubated for 10 min in 100 μL OTTO-I lysis buffer and stained with 1.5 mL OTTO-I:OTTO-II (1:2) solution for 30 min (Loureiro et al. [16,17]), supplemented with 75 μM propidium iodide (PI Sigma®–excitation/emission wavelengths: 480–575/550–740 nm) and 50 μg/mL RNAse (Sigma-Aldrich®), pH = 7.8. The nuclear suspension was filtered through 20 μm diameter mesh nylon filter (Partec®) and maintained in the dark for 20 min.

For genome size estimates, the suspension was analyzed with a Partec PAS® flow cytometer (Partec®) equipped with a laser source (488 nm). PI fluorescence emitted from nuclei was collected through a RG 610 nm band-pass filter and converted to 1024 channels. The equipment was calibrated for linearity and aligned with microbeads and standard solutions according to the manufacturer's recommendations. FlowMax® software (Partec®) was used for data analyses. The standard nuclei peak was set to channel 200 and more than 10,000 nuclei were analyzed. Three independent replications were conducted and histograms with a coefficient of variation (CV) above 5% were rejected. The nuclear genome size average (pg) of each sample was measured according to the formula adapted from Doležel and Bartos [18] and subsequently converted to megabase pairs (1 pg = 978 Mbp) (Doležel et al., [19]).

In order to compare the genomes size of the *Mycetophylax* species with that of the Attini ants, we obtained data from Animal Genome Size Database (Gregory, [20]) and current literature. We used the Pearson correlation to examine the relationship between genome size and chromosome number of the species studied here and of those species of which chromosome number and DNA content had previously been reported. These analyses were performed using R program (R Development Core Team, [21]).

3. Results

The PI staining yielded reproducible, stable nuclear fluorescence without apparent interference, non-specific binding or other cellular material. The histograms of the nuclear DNA amount showed peaks corresponding to G0/G1 nuclei (2C DNA amount) and a minor peak representing nuclei in the G2 (4C DNA amount). However, the G0/G1 peaks were appropriately discriminated and showed CVs ranging from 3.02% to 4.51%, which are considered suitable for FCM measurements (Fig. 1). The mean genome size estimated values of male and female of *M. morschi* was 312.96 Mbp (1C = 0.32 pg). Indeed, these values were the same for populations with $2n = 26$ and 30 chromosomes. The mean genome size estimated of *M. conformis* and *M. simplex* females was 312.96 Mbp (1C = 0.32 pg) and 381.42 Mbp (1C = 0.39 pg), respectively (Table 1). Therefore, the mean haploid genome size of the genus *Mycetophylax* was 335.78Mbp (0.343 pg). The Pearson correlation test between genome sizes and chromosome number were highly significant in Attini ($r = 0.954$; $P = 0.0002289$; $n = 8$) (Fig. 2).

4. Discussion

The 1C DNA amount estimated to the *Mycetophylax* genus ranges from 0.32 pg to 0.39 pg. These values agree with some previously published values for ants (Tsutsui et al., [12]; Ardila-Garcia et al., [11]), that showed small genomes compared to other insects, [12], and are identical to those verified for other species of the subfamily Myrmicine [12], to which *Mycetophylax* belongs.

The low variation in genome size values among the species studied here are in agreement with the general pattern observed in other insect and non-insect families (Gregory and Herbert, [22]; Gregory and Shorthouse, [23]). These studies have demonstrated that interspecific variation in the DNA amount is lower than variation between genera and subfamilies. Tsutsui et al. [12] showed that the major dissimilarity is found between ant subfamilies, followed by genera. These authors suggested that the dissimilarity pattern observed may be a consequence of the relatively small sample size analyzed within some subfamilies and genera. However, there is no difference in this pattern when all 66 ants mean genome sizes estimated are considered together (Li and Heinz, [24]; Johnston et al., [8]; Aron et al., [25]; Sïrvo et al., [26]; Tsutsui et al., [12]; Ardila-Garcia et al., [11]).

Although *M. conformis* and *M. morschi* have identical genome sizes, the haploid genome size of *M. simplex* is larger than their congeneric relatives. An explanation to this can be the difference in the heterochromatin content (Cardoso et al., in preparation). Both *M. conformis* and *M. morschi* show a low heterochromatin content according to C-banding technique, whereas *M. simplex* shows large and visible heterochromatic blocks. The increase and/or decrease in the copy number of repetitive DNA sequences has been postulated as the main evolutionary force leading to differences in DNA amount among different organisms (Bennetzen et al., [27]; Boulesteix et al., [28]; Tavares et al., [14]). Additionally, Lorite and Palomeque [29] suggested that the most plausible explanation to differences between genome sizes among ant species is the discrepancy in the heterochromatin content, once it seems to be recurrent and not too harmful.

Even though it has been suggested that the chromosome number may be not correlated with genome size in ants (Lorite and Palomeque, [27]), we found a strong correlation between genome size and chromosome number in Attini. *M. simplex* has a higher chromosomal number ($2n = 36$) and a higher C-value than *M. conformis* and *M. moschi* ($2n = 30$ and $2n = 26/30$, respectively) (Cardoso et al. in preparation) while *Sericomyrmex amabilis* has the largest genome size and karyotype known. Taking the Minimum Interaction Theory (Imai et al., [30]) into account, the increase of the chromosome number by centric fission could consequently lead to the increase of DNA amount. This is because the break at the centromere of one metacentric chromosome into two telocentric chromosomes may contribute to the increase of genome size by heterochromatin growth given by the instability of the telocentric chromosome (Imai et al., [30,31]). This reinforces the suggestion that the increase of the genome size in these insects appears to be due to the increase in heterochromatin content being concomitant with changes in chromosome number. Although the same C-value was found for populations with different chromosome numbers of *M. morschi*, this should be due to similarities of heterochromatin content in these karyotypes. Both populations showed heterochromatin-positive blocks restricted to centromeric regions, suggesting that these two karyotypes may be formed by means of chromosomal rearrangements of the fusion and fission type, without heterochromatin and euchromatin growth.

Thus, within Attini there appears to be a strong positive correlation between the C-value and chromosome number.

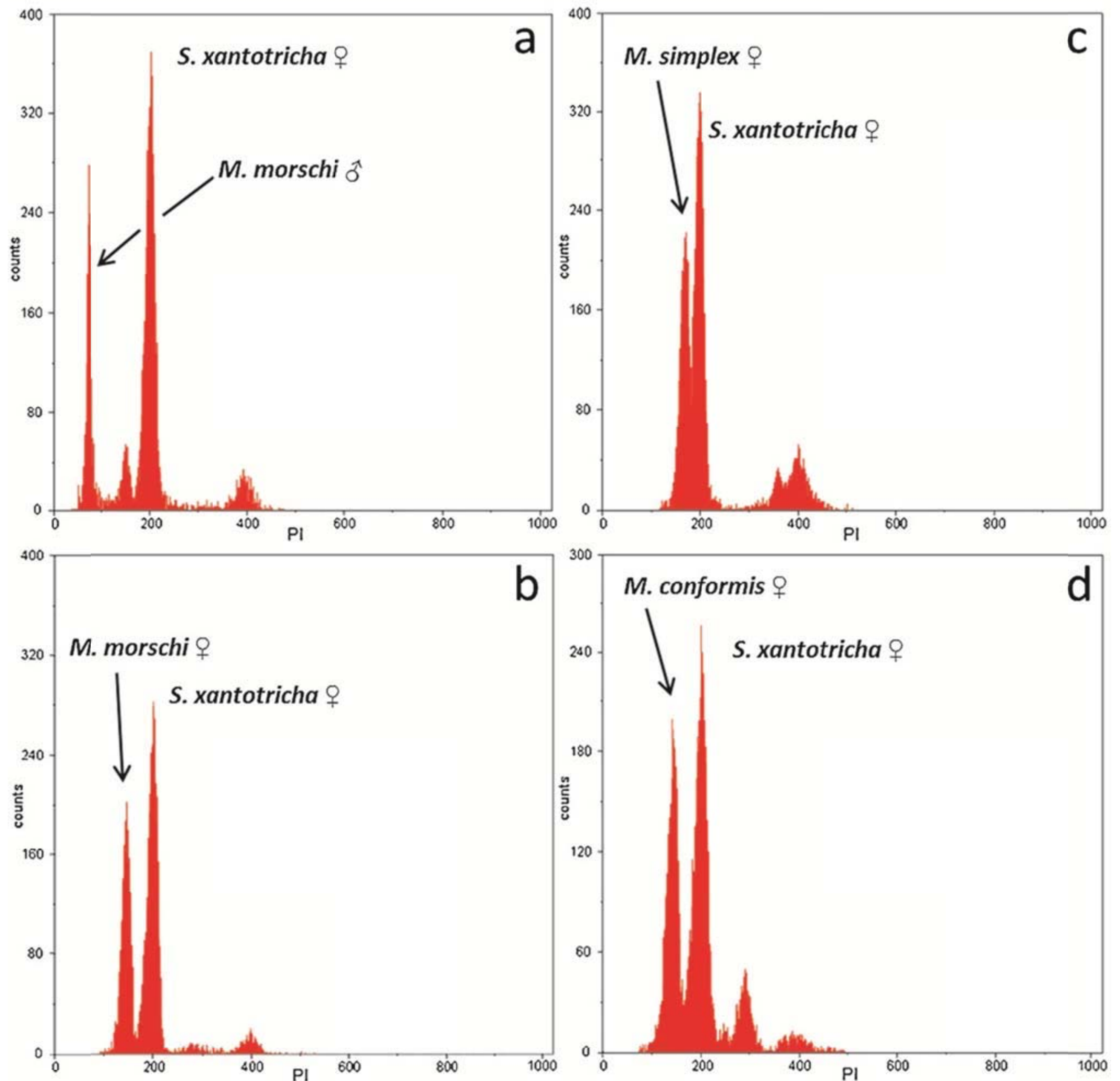

Fig. 1. Genome size DNA-histograms of *Mycetophylax* species through analysis of nuclear suspension of pupae cerebral ganglion tissue stained with PI. (a) Male *M. morschi* (1C = 0.32 pg, channel 73) and female *Scaptotrigona xantotricha* (internal standard 2C = 0.88 pg, channel 200). (b) Female *M. morschi* (2C = 0.64 pg, channel 145) and female *S. xantotricha* (internal standard 2C = 0.88 pg, channel 200). (c) Female *M. simplex* (2C = 0.78 pg, channel 177) and female *S. xantotricha* (2C = 0.88 pg, channel 200). (d) Female *M. conformis* (2C = 0.64 pg, channel 145) and female *S. xantotricha* (2C = 0.88 pg, channel 200).

However, this result must be judged with caution since it was established using a small sample set. Furthermore, there are frequent cases of great variation in DNA content despite karyotypic constancy (Ardila-Garcia and Gregory, [10]). For ants, centric fission followed by chromatin (heterochromatin and euchromatin) growth cannot provide a general explanation for the variation in genome size within the entire family. For example, in the subfamily Ponerinae, *Dinoponera australis* and *Ponera pennsylvanica* have a very similar genome size (555 and 592 Mbp, respectively), but very different chromosome numbers ($2n = 114$ and $2n = 12$, respectively) (Tsutsui et al., [12]). This suggests that many changes in the chromosome number in this group do not appear to have occurred concomitantly with changes in genome size, and should be a result of simple fissions or fusions without any changes in the DNA content. However, there are very few species of which chromosome number and genome size are known so far. Therefore the existence of a positive correlation between genome size and chromosome number in related and less divergent species cannot be rejected, and appears to be the case of the Attini tribe. Moreover, positive correlations between chromosomal

Table 1
Genome size estimates and chromosome number for the three *Mycetophylax* studied in this work and for the others Attini studied so far.

Species	Mean genome size (1C) (pg-Mbp)	Chromosome number	Genome sizes source work
Mycetophylax simplex	0.39 – 381.42	36[a]	This work
Mycetophylax morschi	0.32 – 312.96	26 and 30	This work
Mycetophylax conformis	0.32 – 312.96	30	This work
Mean	**0.343 – 335.78**		
Acromyrmex echinatior	0.342 – 335.0	36[b]	Sirviö et al. [26]
Atta cephalotes	0.306 – 300.1	n.a.	Tsutsui et al. [12]
Atta colombica	0.30 – 298.8	22[c]	Tsutsui et al. [12]
Atta texana	0.27 – 264.06	n.a.	Ardila-Garcia et al. [11]
Apterostigma dentigerum	0.65 – 636.4	n.a.	Tsutsui et al. [12]
Sericomyrmex amabilis	0.45 – 440.7	50[c]	Tsutsui et al. [12]
Trachymyrmex septentrionalis	0.25 – 244.5	20[c]	Ardila-Garcia et al. [11]
Mean	**0.367 – 359.94**		
Total mean	**0.36 – 352.69**		

n.a.: not available.
[a] Cardoso et al., in preparation.
[b] Sïrvio et al. [26].
[c] Murakami et al. [33].

number and genome size have been reported to other insects like beetles and damselflies (Gregory et al., [32]; Ardila-Garcia and Gregory, [10]).

Tsutsui et al. [12] observed that *Ectatomma tuberculatum* and *Apterostigma dentigerum* show a haploid genome size twice higher than that of their related species and suggested that whole-genome duplication may be involved in the genome size evolution of these two ant lineages. These authors also suggested that in order to clarify this hypothesis it would be necessary to evaluate other related species. Thus, considering that *Mycetophylax* is one of the three genera that split earlier than the remaining Neoattini genera (Schultz and Brady, [1]) and that our results give evidence that all *Mycetophylax* species showed a genome size around 330 Mbp, we found no evidence of whole-genome duplication in these lower agriculturist Neoattinis. Moreover, species more distant from the common ancestor of the Neoattini, as *Serycomyrmex* [1], show an intermediate genome size (440 Mbp) that does not correspond to twice the genome size of the lower Attini. Additionally, *Atta* and *Acromyrmex* (leaf-cutter agriculturists) show genome sizes around 300 Mbp (Table 1), suggesting that whole-genome duplication appears to be unlikely in Neoattini but if whole-duplication did take place in Paleoattini it most likely occurred after the separation of these groups.

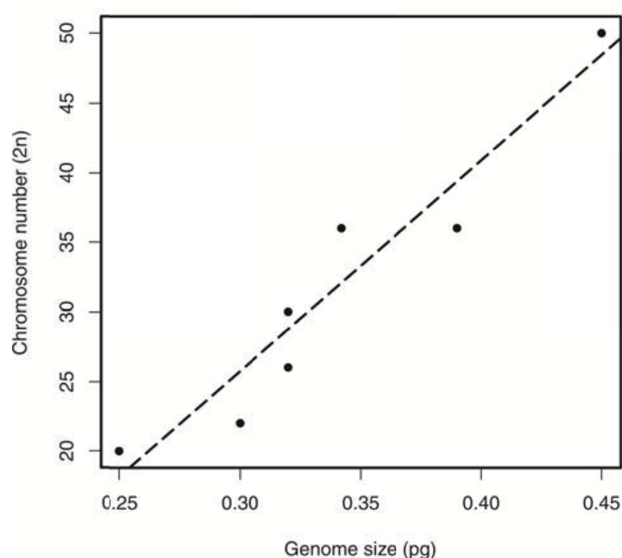

Fig. 2. Relationship between genome size and diploid chromosome number of Attini ants ($r = 0.9544073$, $df = 6$, $p = 0.0002289$). The species which chromosome number was available were included in the analysis and are showed in Table 1.

The estimated genome size of the *Mycetophylax* species presented here and those of all other Attini, which represent five from ten genera of the Neoattini clade, show a mean of around 321.15 Mbp. This suggests that a genome size around 300 Mbp may be the conserved genome size of the Neoattini cluster. On the other hand, considering the Paleoattini clade, the only genome size information available is that of *A. dentigerum* (636.4 Mbp). Thus, it is not possible to conclude the genome size of Paleoattini, as genome sizes of *Mycocepurus* and *Myrmicocrypta* have not yet been evaluated.

Our results show a nearly complete absence of variation in genome sizes of *Mycetophylax* species. Both the *M. morschi* and *M. conformis* population showed the same C-value, while *M. simplex* differs only in 0.07 pg. These C-values are in agreement with the results obtained for other Attini species. Moreover, the genome size estimated for the *Mycetophylax* genus and the other lineages of Neoattini suggest that the whole-genome duplication phenomena may have occurred only in the Paleoattini clade. The results obtained in this study improve our knowledge about the Attini genome size and will contribute to a better understanding of the natural history of this tribe.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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