

UNIVERSIDADE FEDERAL DO PARANÁ

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ESTUDOS MOLECULARES EM *Acromyrmex* Mayr, 1865

Tese apresentada como requisito parcial à obtenção do título de Doutora em Ciências Biológicas, pelo Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Entomologia, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Marcio Roberto Pie

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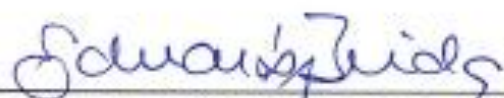
ELISIANE CASTRO DE QUEIROZ

"FILOGENIA MOLECULAR DAS ESPÉCIES BRASILEIRAS DE *Acromyrmex*,
Mayr, 1865)"

Tese aprovada como requisito parcial para obtenção do grau de "Doutor em
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DEDICO

À Deus

Aos meus amados pais, a quem devo toda minha formação

À minha família

Ao meu amado marido

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Resumo

Acromyrmex é um gênero taxonomicamente desafiador porque possui uma diversidade de táxons subespecíficos e apresenta grande importância ecológica e econômica. Descrições originais foram tradicionalmente baseadas em caracteres morfológicos muito variáveis, levando ao reconhecimento de dois subgêneros, 32 espécies e pelo menos 30 subespécies. Nossos objetivos foram inferir as relações internas em *Acromyrmex* com base em um estudo morfológico e uma filogenia molecular utilizando um marcador mitocondrial e marcadores nucleares, incluindo as espécies que ocorrem no Brasil, com destaque para as subespécies de *Acromyrmex subterraneus*. Delimitar as espécies (*Acromyrmex balzani* e *A. subterraneus*) utilizando métodos de delimitação recentemente desenvolvidos também foi nosso objetivo. Obtivemos sequências para 15 espécies de *Acromyrmex*, incluindo as três subespécies: *A. subterraneus subterraneus*, *A. subterraneus brunneus* e *A. subterraneus molestans* e inferimos uma filogenia molecular utilizando máxima verossimilhança e métodos de inferência Bayesiana. Além disso, foram examinados 123 espécimes de *A. subterraneus* para acessar o grau de variação na cor e morfologia entre as subespécies analisadas. Para a delimitação de espécies foram sequenciadas 66 amostras de *A. balzani* e *A. subterraneus* para o gene mitocondrial citocromo oxidase I (COI), e para três marcadores (EPIC-“exon-primed intron-crossing”). Nós estimamos árvores gênicas por inferência Bayesiana e usamos as mesmas para estimar hipóteses de relacionamento adicionais além daquelas da taxonomia atual, pelo método do modelo Yule coalescente (GMYC). Nós, então, estimamos árvores de espécies multiloci para cada hipótese de relação entre espécie, e comparamos os resultados de cada uma das hipóteses utilizando o método de Critério de informação Akaike através da cadeia de Markov Monte Carlo (AICM). Além disso, utilizamos o método implementado em Análise Bayesiana de dados de sequências genômicas sob o modelo coalescente (BP&P) para obter uma validação da melhor hipótese de espécie. A filogenia obtida sugere que os subgêneros são parafiléticos assim propomos serem abandonados, com *Moellerius* sendo proposto como sinônimo de *Acromyrmex*; a respeito das subespécies de *A. subterraneus*, não há consistência na variação morfológica entre *A. subterraneus* e *A. brunneus*, e estes dois apresentam uma divergência genética baixa, quando comparados a outras espécies de *Acromyrmex*. Por outro lado, *A. molestans* não só apresenta variação morfológica consistente, mas também um nível de divergência genética comparável àquele encontrado entre pares de espécies de *Acromyrmex* válidos. Diante desses resultados, sugerimos a elevação de *A. subterraneus molestans* para o status de espécie e a sinonímia de *A. subterraneus brunneus*, sob *A. subterraneus*. Finalmente, o gênero *Pseudoatta* é sinonimizado sob *Acromyrmex*. Para delimitação nossos resultados apontam para o reconhecimento de três espécies, *A. balzani*, *A. subterraneus subterraneus*, e *A. subterraneus molestans*. A história demográfica de *A. balzani*, que corta apenas gramíneas, é na maior parte constante, com exceção da tendência de um pequeno declínio entre 1-0,4 Ma para o presente, ao passo que a história demográfica de *A. subterraneus subterraneus*, que corta apenas dicotiledôneas e possui ninhos em habitats sombreados e úmidos, aponta para um aumento da população de 1,3 Ma para o presente. Embora não seja possível identificar um evento glacial ou interglacial como causa dessas variações populacionais, essas variações são consistentes com um aumento das áreas de florestas abertas durante o último 1 Ma.

Palavras-chave: Formicidae, Attini, taxonomia, DNA, filogeografia

Abstract

Acromyrmex is a taxonomically challenging genus because it has a variety of subspecific taxa of prominent ecological and economic importance. Original descriptions were traditionally based on very variable morphological characters, leading to the recognition of two subgenera, 32 species and at least 30 subspecies. Our objectives were to infer the internal relations in *Acromyrmex* based on a morphological study and a molecular phylogeny using mitochondrial and nuclear markers and including the species that occur in Brazil, with emphasis on the subspecies of *Acromyrmex subterraneus*. Delimit the species (*Acromyrmex balzani* and *A. subterraneus*) using methods of species delimitation recently developed was also our goal. We obtained sequences for 15 species of *Acromyrmex*, including the three subspecies: *A. subterraneus subterraneus*, *A. subterraneus brunneus*, and *A. subterraneus molestans*, and inferred a molecular phylogeny using maximum likelihood and Bayesian inference methods. Additionally, we examined 123 specimens of *A. subterraneus* to assess the degree of variation in color and morphology between the analyzed subspecies. For the delimitation of species were sequenced 66 samples of these species for the mitochondrial gene cytochrome oxidase I (COI), and for three exon-primed intron-crossing (EPIC) markers, and used these sequences for analyses of species delimitation and evolutionary demography. We estimated gene trees by Bayesian inference and used them to estimate additional species relationship hypotheses besides the ones of the current taxonomy by the method of the Generalized Mixed Yule Coalescent model (GMYC). We then estimated multilocus species trees for each species relationships hypotheses, and compared the results of each hypotheses by using the method of posterior simulation-based analogue of Akaike Information Criterion through Markov chain Monte Carlo (AICM). In addition we used the method implemented in Bayesian analysis of genomic sequence data under the multispecies coalescent model (BP&P) to validate the best supported species relationship hypothesis. The obtained phylogeny suggests that the subgenera are paraphyletic and could be abandoned, with *Moellerius* being proposed as a synonym of *Acromyrmex*; regarding the subspecies of *A. subterraneus*, there is no consistency in morphological variation between *A. subterraneus* and *A. brunneus*, and these two present a shallow genetic divergence when compared to other full species of *Acromyrmex*. On the other hand, *A. molestans* not only presents consistent morphological variation, but also a level of genetic divergence comparable to that found between pairs of valid species of *Acromyrmex*. Given these results, we elevate *A. subterraneus molestans* to the specific status and synonymize *A. subterraneus brunneus*, under *A. subterraneus*. Finally, the genus *Pseudoatta* is synonymized under *Acromyrmex*. For delimitation our results point to the recognition of three species in our sample, which are currently recognized as *A. balzani*, *A. subterraneus subterraneus*, and *A. subterraneus molestans*. The demographic history of *A. balzani*, a grass specialist, is mostly stable, except for tendency of a small decline from 1-0.4 Ma to the present, whereas the demographic history of *A. subterraneus subterraneus*, which dicotyledonous specialist cutter and nests in shaded and humid habitats, points for an increase in the population numbers from 1.3 Ma to the present. Although it is not possible to pinpoint one glacial or interglacial event as cause of these population variations, these variations are consistent with an increase in forests areas over more open habitats in the last 1 Ma.

Key words: Formicidae, ants, Attini, taxonomy, DNA

LISTA DE FIGURAS

Capítulo I - Molecular phylogeny and classification of *Acromyrmex* MAYR, 1865, with comments on related genera

- Figure 1:** Phylogeny of a subset of *Acromyrmex* species, as inferred by the analysis of one mitochondrial and three nuclear genes, and the partition scheme indicated in Table 3..... 21
- Figure 2:** Pairwise genetic distances between *Acromyrmex subterraneus* subspecies. The blue line indicates the mean and red lines indicate the corresponding confidence interval 22
- Figure 3:** Morphological variation in *Acromyrmex subterraneus* subspecies.... 23

Capítulo II - Species delimitation and phylogeography of the Neotropical leaf-cutter ants *Acromyrmex subterraneus* and *A. balzani*.

- Figure 1:** Localities sampled for *Acromyrmex balzani* (squares) and *Acromyrmex subterraneus* (stars) 45
- Figure 2:** Gene trees with the delimitation of GMYC for COI gene 52
- Figure 3:** Gene trees with the delimitation of GMYC for EPIC 346 marker..... 53
- Figure 4:** Gene trees with the delimitation of GMYC for EPIC 965 marker..... 54
- Figure 5:** A. Illustrative RaxML topology of all samples. B. Species hypothesis of the current taxonomy. C. Subspecies hypothesis of the current taxonomy. D. Species hypothesis obtained by the GMYC analysis of EPIC 346 marker. White squares indicates lack of samples. E. Species hypothesis obtained by the GMYC analysis of EPIC 965 marker. F. Species hypothesis obtained by the GMYC analysis of EPIC 1503 marker. G. Best species tree hypothesis according to AICM 56
- Figure 6:** Dated Bayesian inference tree for the studied *Acromyrmex* species.. 58
- Figure 7:** Bayesian skyride plots for the COI gene for A. *Acromyrmex balzani* and B. *Acromyrmex subterraneus subterraneus* 60

LISTA DE TABELAS

Capítulo I - Molecular phylogeny and classification of *Acromyrmex* MAYR, 1865, with comments on related genera

Table 1. Primers used for DNA amplification and sequencing.....17

Table 2. Partition scheme and DNA substitution models selected by PARTITIONFINDER using the Bayesian Information Criterion. ¹ = first position; ² = second position; ³ = third position 19

Table S1. Taxa included in the present study and their respective GenBank accession numbers.....36

Table S2. Morphological analysis of *Acromyrmex subterraneus* subspecies deposited in the collection of the Zoology Museum of São Paulo considering the key criteria Mayhé-Nunes 1991..... 38

Table S3. Morphological analysis of *Acromyrmex subterraneus* subspecies with samples obtained from donations and collect, deposited in the collection DZUP considering the key criteria Mayhé-Nunes 1991..... 40

Capítulo II - Species delimitation and phylogeography of the Neotropical leaf-cutter ants *Acromyrmex subterraneus* and *A. balzani*.

Table 1. Sample number, locality of collection, and GenBank accession numbers 46

Table 2. Primers used for PCR amplification and bidirectional sequencing.....47

Table 3. Comparison of the different species relationship hypotheses estimated in *BEAST using the posterior simulation-based analogue of Akaike Information Criterion through Markov chain Monte Carlo (AICM) 57

Table 4. Summary statistics of the tests of neutrality *Acromyrmex subterraneus subterraneus* and *Acromyrmex balzani* 59

Sumário

Introdução Geral	9
CAPÍTULO I - Molecular phylogeny and classification of <i>Acromyrmex</i> MAYR, 1865, with comments on related genera.....	13
1. Abstract	14
2. Introduction	14
3. Material and Methods.....	16
4. Results	19
5. Discussion	24
5.1. Taxonomic synopsis of <i>Acromyrmex</i> in Brazil	26
6. Acknowledgements	29
8. References	29
CAPÍTULO II -Species delimitation and phylogeography of the Neotropical leaf-cutter ants <i>Acromyrmex subterraneus</i> and <i>A. balzani</i>.....	41
1. Abstract	42
2. Introduction	43
3. Material and Methods.....	44
3.1. Population sampling and DNA sequencing	44
3.2. Species delimitation	48
3.3. Species divergence and demography	50
4. Results	51
4.1. Species delimitation	51
4.2. Species divergence and demography	57
5. Discussion	61
5.1. Phylogeographic patterns	62
6. Acknowledgements	64
7. References	64
Considerações Finais.....	72

Introdução Geral

A compreensão da história evolutiva dos seres vivos contribui para o conhecimento da diversidade biológica. A sistemática molecular busca compreender tais relações evolutivas entre os organismos utilizando como base sequências de DNA e outros marcadores moleculares (Graur & Li 2000). Por outro lado, a filogeografia busca compreender como fatores e processos históricos influenciaram na distribuição geográfica de uma linhagem genealógica (Avice 2000). A análise e a interpretação da distribuição espacial das linhagens requerem o processamento conjunto de informação de uma série de disciplinas, incluindo sistemática filogenética, genética de populações, etologia, demografia, paleontologia, geologia e modelos paleogeográficos e paleoclimáticos (Martins & Domingues 2010).

Porém, para compor e contar a história da biodiversidade da maneira mais fidedigna é necessário definir os limites dos táxons dos clados estudados. Tradicionalmente, dados morfológicos têm sido usados para definir limites entre espécies desde o surgimento da nomenclatura binomial no século XVIII, mas tal abordagem pode subestimar ou superestimar o número de táxons envolvidos, falhando em detectar espécies crípticas (ou convergentes na morfologia) e dando muito peso a pequenas variações morfológicas que não necessariamente refletem a evolução dos organismos. Tal subjetividade dos critérios para a interpretação da morfologia dos organismos deu origem a diversas definições do que é uma espécie, sendo que atualmente existe mais de 20 conceitos de espécie divididos em sete linhas de argumentação (biológica, ecológica, evolucionária, coesão fenotípica, filogenética, fenética e genotípica) (Queiroz 2007). Todas estas linhas estão em conformidade com um conceito geral unificado, segundo o qual as espécies são linhagens de metapopulação que evoluem separadamente, considerando essas linhas como critérios para avaliar a separação da linhagem (Queiroz 2007).

A proposta de unificação do conceito de espécie ainda não está totalmente esclarecida, assim esta ambiguidade na definição do que representa uma espécie representa um problema nos dias atuais de crise da biodiversidade, sendo estimada a existência de 8,7 milhões de espécies de eucariotos na Terra, dos quais aproximadamente 86% dos organismos terrestres e 91% dos marinhos ainda aguardam uma descrição formal como espécie (Mora et al. 2011). Nesse sentido, a obtenção e análise de dados genômicos ou moleculares trouxe o desenvolvimento de métodos para

delimitação de espécies com base na teoria coalescente, o que permite a aplicação de métodos estatísticos para avaliar tal problema. Mesmo assim, a delimitação de espécies é um campo integrativo, que depende cada vez mais de tipos de dados de diversas áreas da biologia (Fujita et al. 2012).

Dentro da diversidade biológica, as formigas (Formicidae) ocupam uma posição de destaque. Em algumas florestas tropicais a biomassa do grupo excede a de vertebrados (Ward 2006). São reconhecidas atualmente 13.000 espécies de formigas, porém tal número pode representar apenas metade das espécies de Formicidae (Bolton 2014). É estimado que as formigas tenham surgido ainda no Cretáceo (cerca de 100 milhões de anos atrás, Ma) (Ward et al. 2014), e desde então desenvolveram uma notável gama de comportamentos sociais, hábitos de forrageamento e associações com outros organismos (Hölldobler & Wilson 1990), o que tem gerado grande interesse científico e público.

As formigas podem ser divididas em dois clados, poneróide e formicóide, que possui a maioria das espécies conhecidas (Brown, 1954). Existem 16 subfamílias atuais de Formicidae, porém quatro destas representam c. 90% das espécies (Bolton 2014), com a subfamília Myrmicinae, a maior das quatro, incluindo cerca de metade das espécies descritas (Ward et al. 2014). Myrmicinae tem uma idade estimada em c. 98.6 Ma, para Atini c. 67 Ma (Ward et al. 2014). As formigas cultivadoras de fungo (*Atta* genus group) com idade de 55.6 Ma (Ward et al. 2014) são singulares, pois dependem obrigatoriamente do cultivo de jardins de fungos para alimentação (Schultz & Brady 2008). As espécies mais primitivas utilizam para tal cultivo detritos orgânicos, porém o ápice da agricultura nas formigas cultivadoras de fungo é encontrado nos gêneros *Acromyrmex* e *Atta*, os quais cortam e processam vegetação fresca como substrato para o fungo (Weber 1972; Hölldobler & Wilson 1990; Mehdiabadi & Schultz 2010). Estes dois gêneros, mais *Trachymyrmex* e *Sericomyrmex* apresentam as maiores colônias, maior tamanho dos indivíduos e maior complexidade social (Mehdiabadi & Schultz 2009) ocorrem nas Américas, com a maioria das espécies na região Neotropical (Hölldobler & Wilson 1990).

Os gêneros *Atta* e *Acromyrmex* são popularmente conhecidos como formigas cortadeiras, e utilizam muitas espécies de plantas para cultivar o fungo simbiote do qual se alimentam, assim causam prejuízos econômicos, principalmente em florestas plantadas ainda nos primeiros anos da cultura (Nickele et al. 2012, Cantarelli et al. 2008). Destes dois gêneros, *Acromyrmex* é o mais diverso, incluindo 32 espécies e 30

subespécies válidas (Bolton 2014). Tal diversidade de táxons subespecíficos sugere a possível existência de espécies crípticas não reconhecidas anteriormente, com *Acromyrmex* representando um sistema interessante para investigar a existência de espécies crípticas através de métodos filogenéticos e filogeográficos. Os objetivos deste estudo foram inferir as relações internas em *Acromyrmex* em dados moleculares e morfológicos. No primeiro Capítulo foi inferida uma filogenia molecular utilizando um marcador mitocondrial e marcadores nucleares, incluindo 15 espécies que ocorrem no Brasil, sendo realizada também uma avaliação das características morfológicas das subespécies de *Acromyrmex subterraneus*. No segundo Capítulo foram utilizados métodos de delimitação de espécies recentemente desenvolvidos, e ainda não testados para os Formicidae, para inferir sobre os limites específicos em *A. balzani* e *A. subterraneus* com base em marcadores mitocondriais e nucleares, e estimar a história demográfica das espécies envolvidas.

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Capítulo I

Molecular phylogeny and classification of *Acromyrmex* MAYR, 1865, with comments on related genera

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Abstract

Acromyrmex is a taxonomically challenging genus comprising 32 species of prominent ecological and economic importance. Original descriptions were traditionally based on very variable morphological characters, leading to the recognition of two subgenera and at least 30 subspecies. Our objectives were to infer the internal relations in *Acromyrmex* based on a morphological study and a molecular phylogeny using mitochondrial and nuclear markers and including the species that occur in Brazil, with emphasis on the subspecies of *Acromyrmex subterraneus* (FOREL, 1893). We obtained sequences for 15 species of *Acromyrmex*, including the three subspecies: *A. subterraneus*, *A. subterraneus subterraneus*, *A. subterraneus brunneus*, and *A. subterraneus molestans*, and inferred a molecular phylogeny using maximum likelihood and Bayesian inference methods. Additionally, we examined 123 specimens of *A. subterraneus* to assess the degree of variation in color and morphology between the analyzed subspecies. The obtained phylogeny suggests that (I) the above mentioned subgenera are paraphyletic and could be abandoned, with *Moellerius* being proposed as a synonym of *Acromyrmex*; (II) regarding the subspecies of *A. subterraneus*, there is no consistency in morphological variation between *A. subterraneus subterraneus* and *A. subterraneus brunneus*, and these two present a shallow genetic divergence when compared to other full species of *Acromyrmex*. On the other hand, *A. molestans* not only presents consistent morphological variation, but also a level of genetic divergence comparable to that found between pairs of valid species of *Acromyrmex*. Given these results, we propose to elevate *A. subterraneus molestans* (SANTSCHI, 1925) to the specific status and synonymize *A. subterraneus brunneus* (FOREL, 1911), under *A. subterraneus*. Finally, the genus *Pseudoatta* is synonymized under *Acromyrmex*.

Key words: Formicidae, ants, Attini, taxonomy, DNA

Introduction

There has been considerable progress in ant taxonomy over the past few decades following the integration of morphological and molecular datasets and the description of a variety of key taxa (e.g. RABELING & al. 2008; SOSA-CALVO & al. 2013; CSÖSZ & al. 2014; FERNÁNDEZ & al. 2014; SCHMIDT & SHATTUCK 2014). For instance, WARD & al.

(2014) investigated the evolutionary history of the hyperdiverse subfamily Myrmicinae and suggested that the diversification of this clade started about 100 million years ago (mya), initially being concentrated in the tropics and then spreading considerably, resulting in over 6,000 extant species found in almost all terrestrial ecosystems.

Within this subfamily, some of the most unique species are found in the *Atta* genera group, which includes ants that cultivate their own food resources (WEBER 1972; HÖLLDOBLER & WILSON 1990; CHAPELA & al. 1994; MUELLER & al. 1998, 2001). The habit of growing fungi as food is a hallmark of this group, which arose at about 50 mya (SCHULTZ & BRADY 2008). Within the *Atta* group, leaf-cutter ants of the genera *Atta* (FABRICIUS, 1804) and *Acromyrmex* cut fresh plant materials as substrate to cultivated fungus, whereas the remaining genera of the group use dead plant material, invertebrate carcasses, and/or insect frass as fungus substrate (WEBER 1972; HÖLLDOBLER & WILSON 1990; MEHDIABADI & SCHULTZ 2010). Higher attines also display the most populous colonies, the largest body sizes, the most pronounced polymorphism in the worker caste, and the greatest social complexity (HÖLLDOBLER & WILSON 1990; HUGHES & al. 2003; DELLA LUCIA & SOUZA 2011). Many higher attines are among the most important agricultural pests in the New World (HÖLLDOBLER & WILSON 1990; DELLA LUCIA 2011; MONTROYA-LERMA 2012; NICKELE & al. 2012).

The taxonomic history of *Acromyrmex* (Hymenoptera: Formicidae: Myrmicinae) went through several transitions, with its species at times being considered as part of *Formica* (LINNEAUS, 1758), *Atta* (FABRICIUS, 1804), *Myrmica* (LATREILLE, 1818), and *Oecodoma* (LATREILLE, 1818). *Acromyrmex* was formally erected by MAYR (1865) as a subgenus of *Atta*, having as type-specimen *Acromyrmex hystrix* (LATREILLE, 1802). FOREL (1893) described *Moellerius* as another subgenus of *Atta* that included *Acromyrmex landolti* (FOREL, 1884) and *Acromyrmex balzani* (EMERY, 1890). Later, EMERY (1913) elevated *Acromyrmex* to the status of genus, with two subgenera: *Acromyrmex* (*Acromyrmex*) and *Acromyrmex* (*Moellerius*). Currently the genus includes 32 species and 30 subspecies (BOLTON 2014). The genus was taxonomically revised by EMERY (1905), SANTSCHI (1925), GONÇALVES (1961), FOWLER (1988) and MAYHÉ-NUNES (1991). At the beginning of his work on the genus, Gonçalves (1961) found 22 species and 42 subspecies of *Acromyrmex* occurring in Brazil. After a detailed morphological study based on large series from several localities, Gonçalves eliminated the species whose diagnostic characters could not be reliably scored, including variation in coloration that were not invariant within the same nest, leading to the recognition of

19 species and eight subspecies occurring in Brazil. MAYHÉ-NUNES (1991) also reviewed the *Acromyrmex* species in Brazil and questioned the validity of the subspecies, suggesting the need for further studies. For instance, *Acromyrmex subterraneus* (FOREL, 1893), has three subspecies in Brazil: *A. subterraneus subterraneus*, *A. subterraneus brunneus* (FOREL, 1911), and *A. subterraneus molestans* (SANTSCHI, 1925) and in Argentina *Acromyrmex subterraneus ogloblini* (SANTSCHI, 1933) and in Peru *Acromyrmex subterraneus peruvianus* (BORGMEIER, 1940). *Acromyrmex subterraneus* is characterized by the large protruding eyes, inferior pronotal spine directed forward, anterior mesonotal spines and lateral pronotal spines long and subequal in length, and the gaster tubercles arranged in four longitudinal rows. The subspecies are distinguished by the light brown color of most workers of *A. subterraneus subterraneus*, dark brown to black coloration of *A. subterraneus brunneus*, with the inferior pronotal spine straight and directed forward in both subspecies. *A. subterraneus molestans* workers are light or dark brown, usually with the front of the head darker and inferior pronotal spine curved and pointed upwards (SANTSCHI 1925).

Given the difficulties in species delimitation and the delineation of species groups, molecular tools can provide valuable information to propose more robust taxonomic decisions. Therefore, the objectives of the present study were to infer a molecular phylogeny of a representative sample of the *Acromyrmex* species that occur in Brazil using mitochondrial and nuclear markers, with special emphasis on the subspecies of *Acromyrmex subterraneus*.

Material and methods

We obtained tissue samples from 15 species and three subspecies of *Acromyrmex* (Table S1), with vouchers being deposited in the Coleção Entomológica Padre Jesus Santiago Moure (DZUP - Table S1). We obtained sequences from three additional species of the genus and a set of outgroup species from GenBank (Table S1). The complete dataset included 53% of the species of *Acromyrmex* and 62.5% of the Brazilian species of the genus. We also examined morphological variation in 73 specimens of *Acromyrmex subterraneus* from an extensive and geographically widespread sample of specimens deposited in the Museu de Zoologia da Universidade

de São Paulo (MZSP) (Table S2), as well as 50 specimens that were either collected by the authors or received as donations, all of which deposited in the DZUP (Table S3).

Total DNA was extracted using DNeasy® kits (Qiagen Inc.) according to the manufacturer's instructions, and then examined for concentration and purity using a Nanodrop® spectrophotometer (concentrations between 7.0 and 68.7 ng/μl). DNA extracts were used to amplify four loci: the cytochrome c oxidase subunit I (COI), and the nuclear genes ribosomal RNA subunits 18S and 28S, and wingless (WG). Primers used for amplification are shown in Table 1. PCR amplifications were performed in a 25-μl volume, using two (μl) of DNA, 0.4 of Taq Polymerase, 1 X of PCR buffer, 2.0 mM of MgCl₂, 0.5 mM of dNTP's, and 0.6 μM of each primer. Thermocycling conditions were 3 min at 95 °C, 35 cycles of 94 °C during 45 s, 45° for 1 min, and 70 °C for 1 min, followed of a final extension at 72 °C for 5 min. PCR products were subject to electrophoresis on 1.5% agarose gels to check for product size and possible contaminants. Positive results were purified using isopropanol and sequenced on an ABI 3500 sequencer using BigDye® Terminator v. 3.1 (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions.

Table 1. Primers used for DNA amplification and sequencing.

Locus	Forward primer 5'-3'	Reverse primer 5'-3'	Reference
COI	LCO –	HCO -	FOLMER & al. 1994
	GGTCAACAAATCATAAAG ATATTGG	TAAACTTCAGGGTGACCAAAAAATCA	
18S	18S-5 –	18S-847 –	WIEGMANN & al. 2000
	TGGTTGATCCTGCCAGTAG	CACTCTAATTTKTTCAAAG	
28S	28S-4678 –	28S-5015 –	WARD &DOWNIE 2005
	GAAAGGCGTTGGTTGCTT	ACGGCTGTTACACGAA	
WG	Wg578 -	Wg1032 -	ABOUHEIF &WRAY 2002
	TGCACNGTGAARACYTGCTGGATGCG	ACYTCGCAGCACCARTGGAA	

All sequences were aligned separately for each gene using MAFFT (KATO & al. 2002). We did not include some of the COI *Acromyrmex* sequences available at GenBank that did not overlap with the fragment used in our study. Poorly-aligned regions of the resulting 18S and 28S gene alignments were removed using GBLOCKS (CASTRESANA 2000, TALAVERA &CASTRESANA 2007) using default settings for 18S,

and allowing position with gaps in less than 50% of the sequences to be selected in the final block for 28S. To avoid under- or overparameterization of the analyses, we used PARTITIONFINDER (LANFEAR & al. 2012) to choose the optimal partitioning scheme and DNA substitution models for each partition (Table 2). The Bayesian Information Criterion (BIC, SCHWARZ 1978) was used to select the partition scheme and DNA substitution models (restricted to those implemented in MRBAYES) given its higher performance in previous simulation in comparison with other criteria (LUO & al. 2010). Phylogenetic inference was carried out using two methods - Maximum Likelihood (ML) and Bayesian Inference (BI) - in the CIPRES Science Gateway v. 3.3 (MILLER & al. 2010). The ML analysis was performed using RAXML 8.0 (STAMATAKIS 2014) with the GTR+ Γ model for each of the partitions selected using PARTITIONFINDER, with support being estimated by 1,000 bootstrap replicates, whereas the BI analysis was performed in MrBayes 3.2 (HUELSENBECK & RONQUIST 2001, RONQUIST & HUELSENBECK 2003, RONQUIST & al. 2012) using the same partition scheme and the models selected by PARTITION FINDER. We conducted four replicates of the analysis, each with 10,000 generations and temperature of 0.08, sampling at each 1,000 generations. The results from the replicates were examined for convergence using TRACER 1.6 (RAMBAUT & al. 2013) and AWTY (WILGENBUSCH & al. 2004, NYLANDER & al. 2008). As all replicates were convergent, we combined the trees of the different replicates (except for the burn-in of 25%), and used them to produce a 50% majority rule consensus tree using the sumtrees script (SUKUMARAN & HOLDER 2010). Additionally, to assist with species delimitations, we calculated the uncorrected genetic distances between *Acromyrmex* species for each gene using MEGA 6.0 (TAMURA & al. 2013). The obtained values were used to estimate a mean genetic distance between the species and a 95% confidence interval that should include valid species.

Table 2. Partition scheme and DNA substitution models selected by PARTITIONFINDER using the Bayesian Information Criterion. ^a = first position; ^b = second position; ^c = third position.

Partitions	DNA substitution Model
COI ^a	GTR+Γ
COI ^b	HKY+Γ+I
COI ^c	HKY+Γ
18S	K80+Γ
28S	K80+Γ
WG ^a	GTR+Γ
WG ^b +WG ^c	K80+Γ

Results

The concatenated data matrix included 49 terminals (of which 27 were *Acromyrmex*) and 1,975 positions (COI: 671 base pairs, 18S: 633 b.p., 28S: 254 b.p., WG: 397 b.p.). The obtained tree is show in Fig. 1. There were two distinct groups within *Acromyrmex*, one including *A. balzani* (EMERY, 1890), *A. landolti*, and *A. fraticornis* (FOREL, 1909), and the other including the remaining species, except for *A. striatus* (ROGER, 1863), thus invalidating the subgenera name proposed by Emery: *Acromyrmex* (*Acromyrmex*) was reconstructed as polyphyletic, whereas *Acromyrmex* (*Moellerius*) appears paraphyletic. The species of the subgenus *Moellerius*: *A. balzani*, *A. heyeri* (FOREL, 1899), *A. landolti*, *A. striatus*, and *A. versicolor* (PERGANDE, 1893) are scattered throughout the phylogeny, with a clade including *A. balzani* and *A. landolti* plus *A. fraticornis*, *A. heyeri* forming a clade with *A. lundii* (GUÉRIN-MÉNEVILLE, 1838), *A. versicolor* being part of a polytomy, and *A. striatus* being basal with respect to all other *Acromyrmex*. Pairwise distances within and between *Acromyrmex* species are shown in Fig. 2, with the COI gene presenting the most distinctive values. All genes, except for the 18S, suggest that *A. subterraneus molestans* shows a high level of sequence divergence from *A. subterraneus subterraneus* and *A. subterraneus brunneus*.

The analysis of morphological characters revealed that coloration is not a reliable criterion to discriminate subspecies of *A. subterraneus*, given that it can vary

geographically among specimens of a subspecies, or even for different nests at the same locality in the case of *A. subterraneus subterraneus*. For *A. subterraneus subterraneus* 82.4% (n = 74) of the individuals were light brown and 17.6% dark brown. For *A. subterraneus molestans* all 13 specimens were light brown with the front of the head darkened. For *A. subterraneus brunneus* 40% of the specimens (n = 35) were light brown and 60% black. All examined specimens of *A. subterraneus molestans* presented an inferior pronotal spine curved and pointed upwards, while the individuals of *A. subterraneus subterraneus* and *A. subterraneus brunneus* presented the inferior pronotal spine straight and pointed forward (Fig. 3).

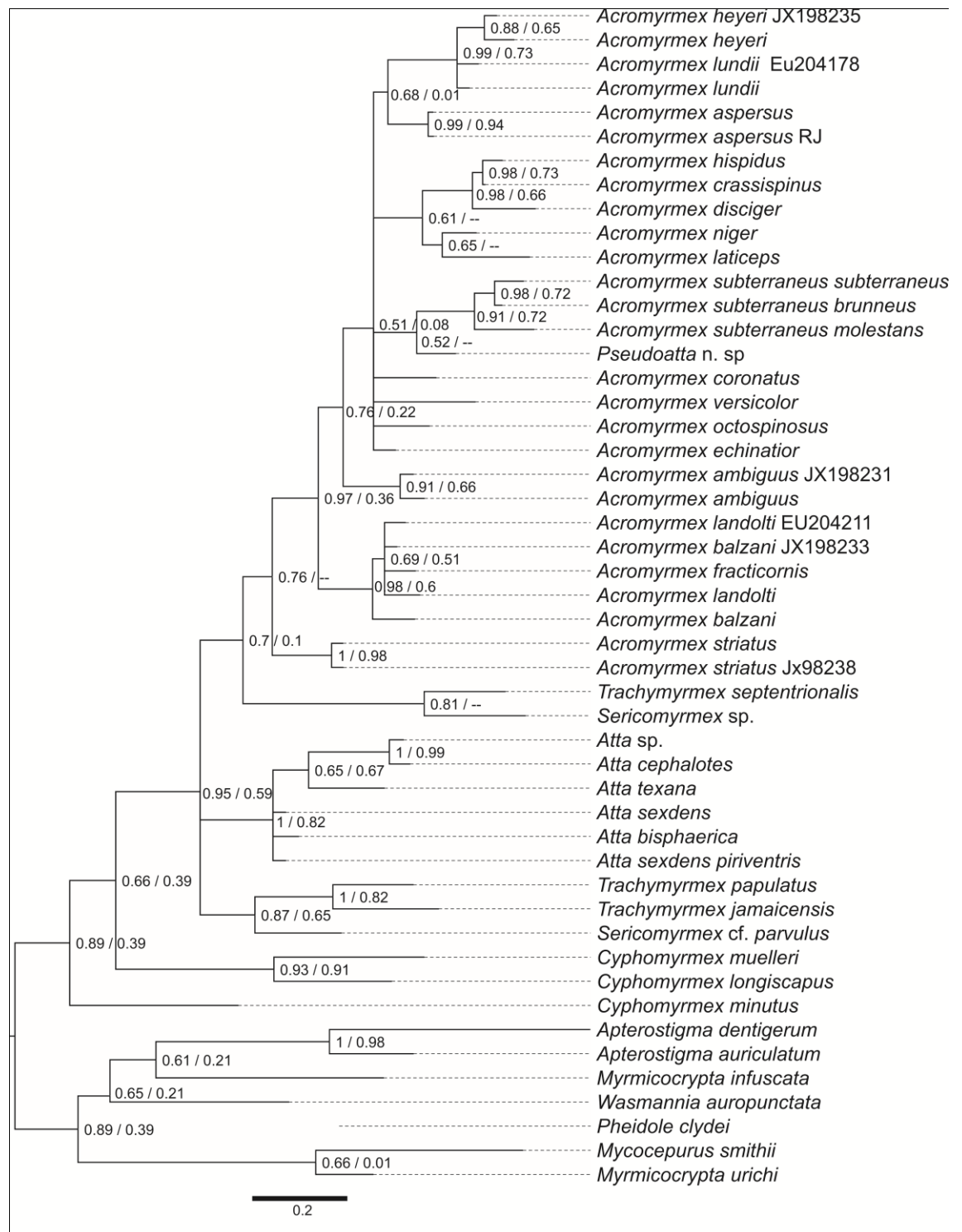


Figure 1: Phylogeny of a subset of *Acromyrmex* species, as inferred by the analysis of one mitochondrial and three nuclear genes, and the partition scheme indicated in Table 2. The topology was obtained by Bayesian Inference using MrBayes, being very similar to that found by maximum likelihood (ML) using RAxML. Node values are Bayesian posterior probabilities/bootstrap support (from 1,000 replicates), respectively. Nodes with -- were not present in the ML tree.

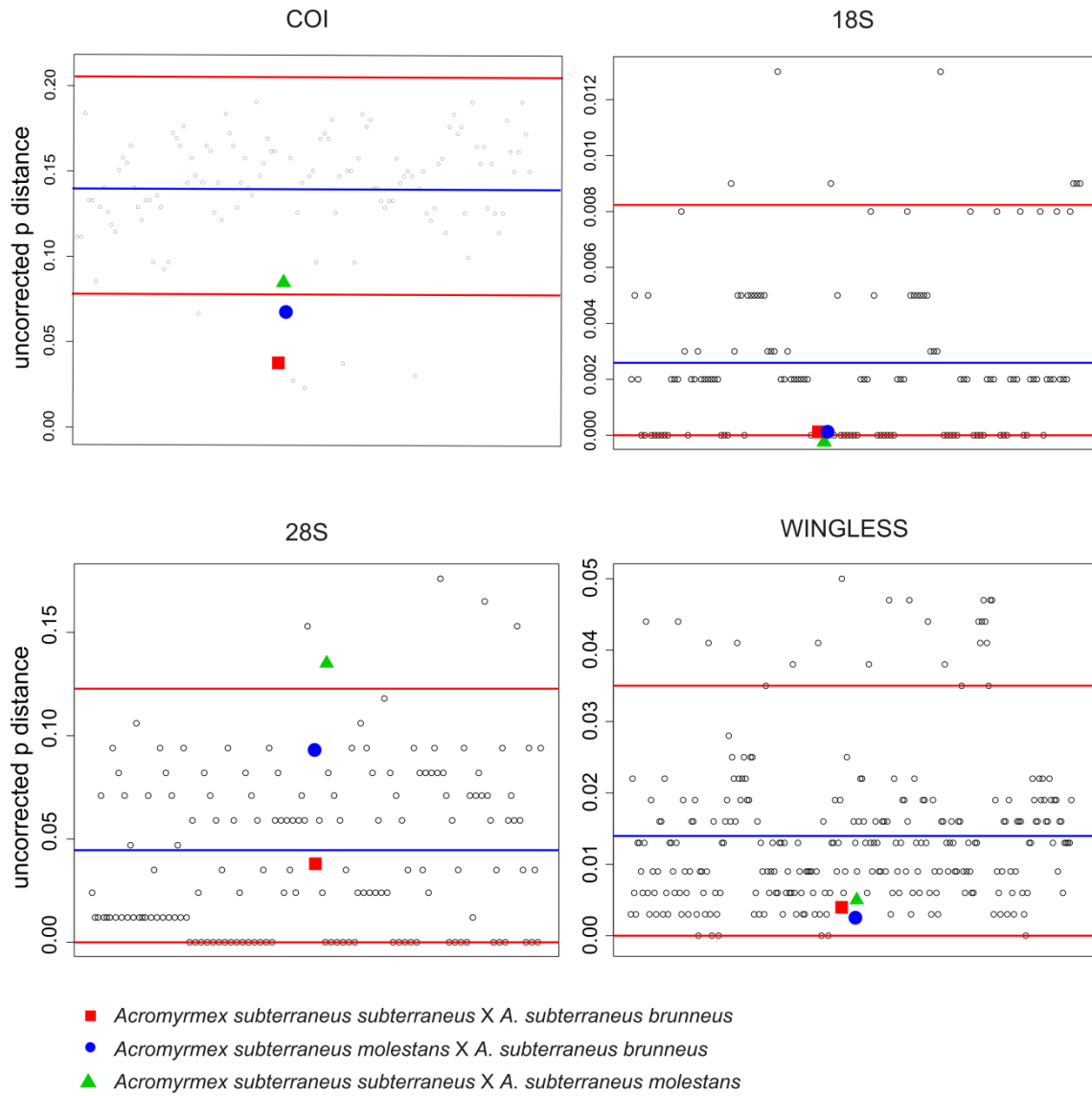


Figure 2: Pairwise genetic distances between *Acromyrmex subterraneus* subspecies. The blue line indicates the mean and red lines indicate the corresponding confidence interval.

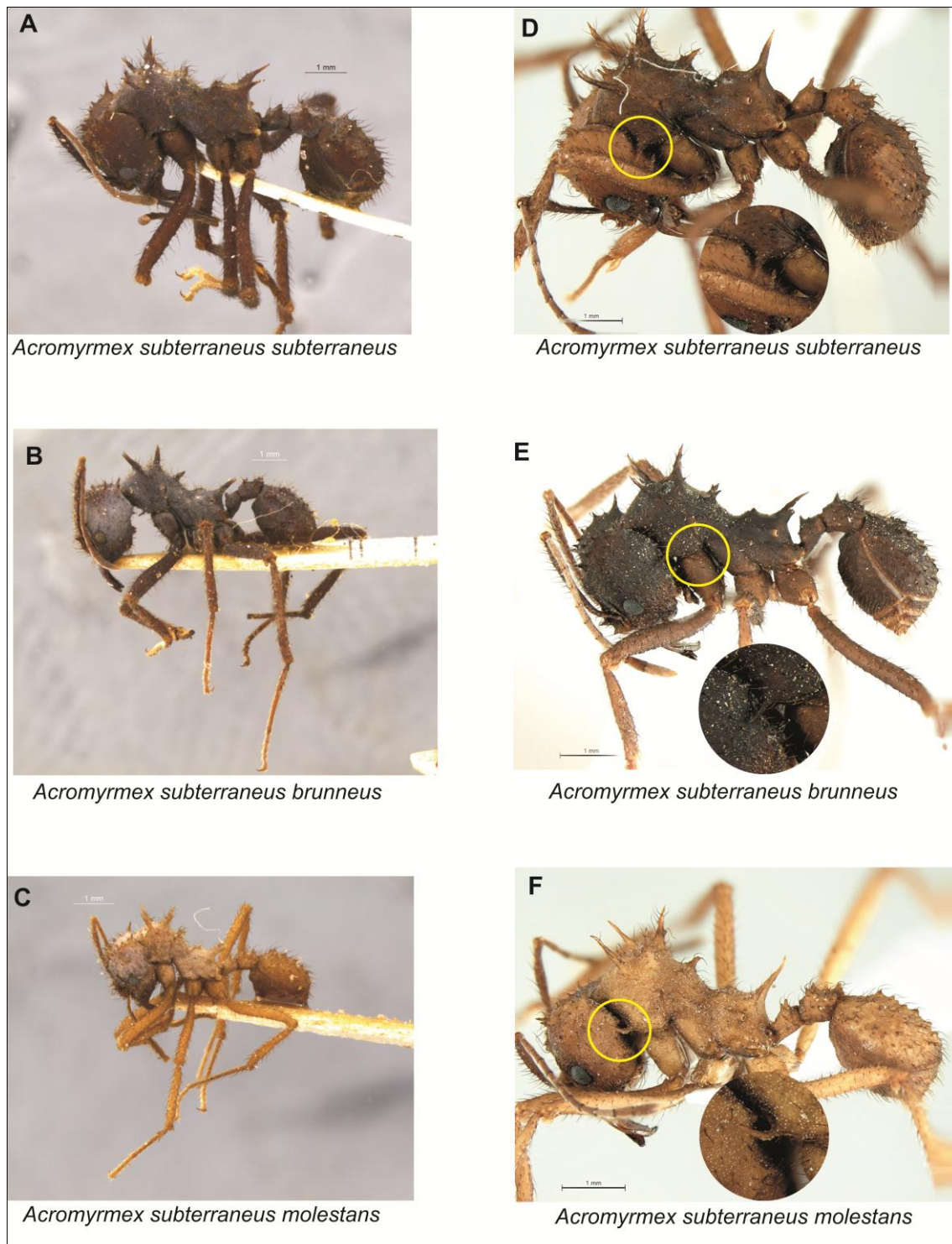


Figure 3: Morphological variation in *Acromyrmex subterraneus* subspecies. Figures A-C corresponds to images obtained from the Museu de Zoologia da Universidade de São Paulo and D-F are images from specimens obtained in the present study (Taxonline-UFPR). Circles correspond to the magnification of the inferior pronotal spine: D, C the inferior pronotal spine straight and directed forward and E: inferior pronotal spine curved and pointed upwards.

Discussion

Despite the low support of some basal nodes, our results agree with previous studies that found that *Pseudoatta* sp. is nested within *Acromyrmex* (SUMNER al. 2004, MEHDIABADI & SCHULTZ 2010, DELABIE & al. 2011, CRISTIANO & al. 2013). In addition, our topology is congruent with previous studies that found that *Acromyrmex*, *Atta*, and *Trachymyrmex* are closely related (WETTERER & al. 1998, SUMNER & al. 2004, SCHULTZ & BRADY 2008, MEHDIABADI & SCHULTZ 2010, SOSA-CALVO & al. 2013). However, we recovered *Acromyrmex striatus* as basal to the other species in this genus, differing from the results of CRISTIANO & al. (2013), which recovered *A. striatus* as basal to the clade that includes *Acromyrmex* and *Atta*. Moreover, even with a small sample size for *Trachymyrmex* and *Sericomyrmex*, we recovered these genera as polyphyletic, with *T. papulatus* (SANTSCHI, 1922) forming a clade with *Sericomyrmex parvulus* (FOREL, 1912), and *T. septentrionalis* (MCCOOK, 1881) forming a clade with *Sericomyrmex* sp. SCHULTZ & BRADY (2008). This result suggests the need for further studies to clarify the taxonomic status of the genera *Trachymyrmex* and *Sericomyrmex*. Within *Acromyrmex*, the subgenus *Moellerius* was proposed based on the absence of the supra-ocular spine found in *Acromyrmex* (*Acromyrmex*), in combination with shorter mandibles (EMERY 1913). Despite these morphological differences, *Acromyrmex* (*Moellerius*) was recovered as paraphyletic. SUMNER & al. (2004); SCHULTZ & BRADY 2008, CRISTIANO & al. (2013) recovered *Moellerius* as polyphyletic.

In relation to the subspecies of *Acromyrmex subterraneus*, the uncorrected pairwise genetic distances for the COI gene between *A. subterraneus subterraneus* and *A. subterraneus brunneus* was below the estimated 95% confidence interval obtained using the data for other currently recognized species of *Acromyrmex*. The described diagnosis between *A. subterraneus subterraneus* and *A. subterraneus brunneus* is based on coloration (darker in the latter) (GONÇALVES 1961, MAYHÉ-NUNES 1991), with no differences in the morphology of the inferior pronotal spine, or in the condition of the male genitalia (ANDRADE 2002). The existing differences in coloration between the two taxa are inconsistent, with light brown and dark brown colonies of *A. subterraneus brunneus* (ANDRADE 2002), which is also found in the examined specimens of this subspecies and those of *A. subterraneus subterraneus*. This variation could be associated with nest age, as suggested by field observations, with smaller workers and

lighter shades of brown in younger nests, with an increase in size and darker shades in older nests (ECQ per. obs.).

Some authors suggest that nests of *A. subterraneus subterraneus* and *A. subterraneus brunneus* differ in their external appearance, with the nests of *A. subterraneus subterraneus* being shallow, and covered by loose soil, whereas mounds of *A. subterraneus brunneus* nests includes also straws as cover (e.g. GONÇALVES 1961; DELABIE 1989). However, ANDRADE (2002) found nests of the latter subspecies covered only by loose soil, which leads to nest cover to be an inconsistent character to separate the two taxa. *Acromyrmex subterraneus* as a whole presents a widespread distribution, and the variation in nest construction between the two subspecies mentioned above could be due to climatic or soil conditions, as both are able to adapt to disturbed habitats.

With respect to the subspecies of *Acromyrmex subterraneus*, *A. subterraneus molestans* presented an uncorrected genetic distance from *A. subterraneus subterraneus* for the COI gene that is within the 95% confidence interval found between taxa currently recognized as different species of *Acromyrmex*, and even higher than the distance found between some pairs of species. Although the divergence between *A. subterraneus molestans* and *A. subterraneus brunneus* remained outside of the confidence interval, the latter forms a clade with *A. subterraneus subterraneus*, with the former being basal to this clade.

There are also morphological characteristics that allow the diagnosis between *A. subterraneus subterraneus* and *A. subterraneus molestans*. In the former, the inferior pronotal spine is straight and pointed forward, whereas in the latter it is curved and pointed upwards (GONÇALVES 1961, MAYHÉ-NUNES 1991). Moreover, the male genitalia in *A. subterraneus subterraneus* presents a gonocoxite large at the proximal region, and the volsella with the internal portion and two small protuberances in its curvature covered by bristles, with its extension (digit) strongly marked and without bristles. In *A. subterraneus molestans* the gonocoxite is larger and rounded at its proximal region, and its volsella narrower, with a marked curvature with a small protuberance without bristles, with a longer and narrower digit without bristles (ANDRADE 2002).

Given these considerations, we propose a series of taxonomic changes in *Acromyrmex*. First, we suggest that the use of subgenera for *Acromyrmex* should be abandoned as these are not monophyletic. In this sense, *Moellerius* is synonymized (**n.**

syn.) with *Acromyrmex*, eliminating the subgeneric names in this genus. The genus *Pseudoatta* is synonymized (**n. syn.**) under *Acromyrmex*, generating the **new combination** *Acromyrmex argentinus* (BRUCH, 1925) for the single species recognized for *Pseudoatta* so far.

Regarding the subspecies of *Acromyrmex subterraneus* occurring in Brazil, *A. subterraneus molestans* is elevated to species, *Acromyrmex molestans* Santschi, 1925 (**n. stat.**). Additionally, we synonymize *A. subterraneus brunneus* (**n. syn.**) under *A. subterraneus* (FOREL, 1893) generating a single specific taxon in Brazil, given that there are two more subspecies: *A. subterraneus oglobini* (SANTSCHI, 1933) from Argentina and *A. subterraneus peruvianus* (BORGMEIER, 1940) from Peru. Although the phylogeny is fragile as support for these taxonomic changes, the morphological study is sufficient for these to be made.

This paper represents the first attempt to clarify the somewhat chaotic taxonomic arrangement of *Acromyrmex* by the combined use of modern molecular methods and a morphological study. Our taxonomic proposals join current advances in an attempt to stabilize ant nomenclature and classification by suppressing subgeneric and subspecific names. We hope that this paper will encourage further examination and revision of this ecological and economic important ant genus.

Taxonomic synopsis of *Acromyrmex* in Brazil

Acromyrmex (MAYR, 1865)

= *Moellerius* (FOREL, 1893 **n. syn.**)

= *Pseudoatta* (GALLARDO, 1916 **n. syn.**)

Acromyrmex ambiguous (EMERY, 1888)

= *Acromyrmex ambiguus* var. *erectus* SANTSCHI, 1925)

Acromyrmex ameliae (DE SOUZA, SOARES & DELLA LUCIA, 2007)

Acromyrmex aspersus (SMITH, F., 1858)

= *Atta* (*Acromyrmex*) *mesonotalis* (EMERY, 1905)

= *Atta* (*Acromyrmex*) *mesonotalis* var. *inquirens* (FOREL, 1914)

= *Acromyrmex aspersus* var. *affinis* (SANTSCHI, 1925)

= *Acromyrmex aspersus* st. *mesonotalis* var. *clarus* (SANTSCHI, 1925)

Acromyrmex balzani (EMERY, 1890)

=*Sericomyrmex gallardoi* SANTSCHI, 1920)

=*Acromyrmex* (Moellerius) *landolti* var. *nivalis* (SANTSCHI, 1922)

=*Acromyrmex* (Moellerius) *landolti* var. *parens* (SANTSCHI, 1925)

Acromyrmex coronatus (FABRICIUS, 1804)

=*Atta* (*Acromyrmex*) *moeller* (FOREL, 1893)

=*Atta* (*Acromyrmex*) *moelleri* st. *meinerti* (FOREL, 1893)

=*Atta* (*Acromyrmex*) *moelleri* r. *modesta* (FOREL, 1901)

=*Acromyrmex coronatus* st. *andicola* var. *flavescens* (SANTSCHI, 1925)

=*Acromyrmex coronatus* st. *andicola* var. *medianus* (SANTSCHI, 1925)

=*Acromyrmex coronatus* st. *moelleri* var. *obscurior* (SANTSCHI, 1925)

=*Acromyrmex coronatus* st. *ochraceolus* (SANTSCHI, 1925)

=*Acromyrmex coronatus* st. *ochraceolus* var. *ornatus* (SANTSCHI, 1925)

Acromyrmex crassispinus (FOREL, 1909)

=*Acromyrmex nigrosetosa* var. *diabolica* (SANTSCHI, 1922)

=*Acromyrmex aspersus* var. *insularis* (SANTSCHI, 1925)

=*Acromyrmex diabolicus* var. *mediocris* (SANTSCHI, 1925)

=*Acromyrmex hispidus* st. *atratus* (SANTSCHI, 1925)

=*Acromyrmex hispidus* st. *formosus* var. *rufescens* (SANTSCHI, 1925)

=*Acromyrmex crassispinus* st. *rusticus* (SANTSCHI, 1925)

Acromyrmex diasi (GONÇALVES, 1983)

Acromyrmex disciger (MAYR, 1887)

Acromyrmex fracticorni (FOREL, 1909)

=*Acromyrmex* (Moellerius) *fracticornis* var. *jorgenseni* (FOREL, 1913)

Acromyrmex heyeri (FOREL, 1899)

=*Acromyrmex* (Moellerius) *heyeri* var. *gaudens* (SANTSCHI, 1925)

=*Acromyrmex* (Moellerius) *heyeri* var. *lillensis* (SANTSCHI, 1925)

Acromyrmex hispidus fallax (SANTSCHI, 1925)

Acromyrmex hystrix ajax (FOREL, 1909)

Acromyrmex landolti (FOREL, 1885)

=*Atta* (Moellerius) *landolti* r. *cloosae* FOREL, 1912)

=*Acromyrmex* (Moellerius) *balzani* var. *senex* (SANTSCHI, 1925)

- =*Acromyrmex (Moellerius) balzani* subsp. *planorum* (WEBER, 1937)
- Acromyrmex laticeps* (EMERY, 1905)
 - =*Acromyrmex laticeps* var. *hortulanus* (SANTSCHI, 1925)
- Acromyrmex lobicornis* (EMERY, 1888)
 - =*Acromyrmex bucki* (WASMANN, 1931)
 - =*Acromyrmex lobicornis* var. *rufidens* (SANTSCHI, 1933)
- Acromyrmex lobicornis ferrugineus* (EMERY, 1905)
- Acromyrmex lundii* (GUÉRIN-MÉNEVILLE, 1838)
 - =*Atta (Acromyrmex) pubescens* var. *bonariensis* (EMERY, 1905)
 - =*Atta (Acromyrmex) laticeps* var. *dubia* (FOREL, 1908)
 - =*Atta (Acromyrmex) lundii* var. *risii* (FOREL, 1908)
- Acromyrmex lundii carli* (GONÇALVES, 1961)
- Acromyrmex molestans* (SANTSCHI, 1925 **n. stat.**)
 - =*Acromyrmex (Acromyrmex) subterraneus* var. *eidmanni* (SANTSCHI, 1937)
- Acromyrmex niger* (SMITH, F., 1858)
 - =*Atta (Acromyrmex) nigra* r. *muticinoda* (FOREL, 1901)
 - =*Atta (Acromyrmex) muticinoda* var. *homalops* (EMERY, 1905)
 - =*Acromyrmex subterranea* var. *depressoculis* (FOREL, 1913)
- Acromyrmex nigrosetosus* (FOREL, 1908)
 - =*Atta (Acromyrmex) aspersa* subsp. *dimidiata* (FOREL, 1911)
 - =*Acromyrmex laticeps* st. *garbei* (SANTSCHI, 1925)
 - =*Acromyrmex nigrosetosa* st. *pulchella* (SANTSCHI, 1925)
- Acromyrmex nobilis* (SANTSCHI, 1939)
- Acromyrmex octospinosu* (REICH, 1793)
 - =*Atta (Acromyrmex) guentheri* (FOREL, 1893)
- Acromyrmex rugosus* (SMITH, F., 1858)
 - =*Oecodoma pallida* (SMITH, F. 1858)
 - =*Acromyrmex rugosus* st. *bigener* (SANTSCHI, 1925)
 - =*Acromyrmex rugosus* var. *vestitus* (SANTSCHI, 1925)
- Acromyrmex rugosus rochai* (FOREL, 1904)
 - =*Acromyrmex rugosus* var. *navarroi* (BORGMEIER, 1937)
- Acromyrmex striatus* (ROGER, 1863)

=*Acromyrmex (Moellerius) striatus* var. *laeviventris* (SANTSCHI, 1925)

Acromyrmex subterraneus (FOREL, 1893)

=*Acromyrmex subterraneus peruvianus* (BORGMEIER, 1940)

=*Acromyrmex subterraneus ogloblini* (SANTSCHI, 1933)

=*Acromyrmex subterraneus brunneus* (FOREL, 1912 **n. syn.**)

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Table S1. Taxa included in the present study and their respective Gen Bank accession numbers. Sequences obtained in the present study are indicated by an asterisk. Vouchers specimens were deposited at the Coleção Entomológica Padre Jesus Santiago Moure (DZUP). Letters in location names correspond to Brazilian states - PR: Paraná, RJ: Rio de Janeiro, SC: Santa Catarina, PA: Pará, MG: Minas Gerais, MS: Mato Grosso do Sul, SP: São Paulo.

Taxa	location	COI	18S	28S	WG
<i>Acromyrmex ambiguus</i>	Brazil: Matinhos/PR	*	*	*	*JX198231
<i>Acromyrmex aspersus</i>	Brazil: Rio das Ostras/RJ	*	*	*	*
<i>Acromyrmex balzani</i>	Brazil: Botucatu/SP	*	*	*	*JX198233
<i>Acromyrmex coronatus</i>	Brazil: Bandeirantes/PR	*	*	*	*
<i>Acromyrmex crassispinus</i>	Brazil: Rio Negrinho/SC	*	*	*	*
<i>Acromyrmex disciger</i>	Brazil: Itapoá/SC	*	*	*	*
<i>Acromyrmex fracticornis</i>	Brazil: Corumbá-MS	*	*	*	*
<i>Acromyrmex heyeri</i>	Brazil: Santa Maria/RS	*	*	*	*JX198235
<i>Acromyrmex hispidus</i>	Brazil: Monte Carlo/SC	*	*	*	*
<i>Acromyrmex landolti</i>	Brazil: Sooretama/ES	*	*	*	*EU204211
<i>Acromyrmex laticeps</i>	Brazil: São João do Araguaia-PA	*	*	*	*
<i>Acromyrmex lundii</i>	Brazil: Dom Pedrito/RS	*	*	*	*EU204178
<i>Acromyrmex niger</i>	Brazil: Santa Maria/RS	*	*	*	*
<i>Acromyrmex striatus</i>	Brazil: Santa Maria/RS	*	*	*	*JX198238
<i>Acromyrmex subterraneus</i>	Brazil: Sengés-PR	*	*	*	*
<i>subterraneus</i>					
<i>Acromyrmex subterraneus</i>	Brazil: Piracicaba-SP	*	*	*	*
<i>bruneus</i>					
<i>Acromyrmex subterraneus</i>	Brazil: Viçosa-MG	*	*	*	*
<i>molestans</i>					
<i>Acromyrmex echinator</i>					KC478095

Taxa	location	COI	18S	28S	WG
<i>Acromyrmex octospinosus</i>					EU204145
<i>Acromyrmex versicolor</i>			EF012826	EF012954	EF013662
<i>Acromyrmex</i> sp. nov.					EU204174
<i>Atta sexdens</i>					KC478101
<i>Atta bisphaerica</i>					KC478098
<i>Atta cephalotes</i>					EU204197
<i>Atta sexdens piriventris</i>					KC478100
<i>Atta sexdens rubropilosa</i>					KC478101
<i>Atta texana</i>					EU204206
<i>Apterostigma auriculatum</i>		AY398289	EF012841	EF012969	EF013677
<i>Apterostigma dentigerum</i>		AY398297			EU204196
<i>Cyphomyrmex longicarpus</i>		JQ617474			
<i>Cyphomyrmex minutus</i>		JX625155			
<i>Cyphomyrmex muelleri</i>		JQ617523			
<i>Mycocepurus smithii</i>		JN055269			
<i>Myrmicocrypta infusata</i>		AY398285			
<i>Myrmicocrypta urichi</i>					EU204151
<i>Pheidole clydei</i>		EF518329	EF012907	EF013035	EF013743
<i>Sericomyrmex</i> sp. MAS001		KC418743			
<i>Sericomyrmex</i> cf. <i>parvulus</i>					EU204147
<i>Trachymyrmex septentrionalis</i>					EU204184
<i>Trachymyrmex jamaicensis</i>			DQ35344		DQ353036
<i>Trachymyrmex papulatus</i>					EU204185
<i>Wasmannia auropunctata</i>			EF012950	EF013078	EU204163

*will be deposited in GenBank in the occasion of acceptance of the article.

Table S2. Morphological analysis of *Acromyrmex subterraneus* subspecies deposited in the collection of the Museu de Zoologia de São Paulo (DZUP), considering the key criteria Mayhé-Nunes 1991

Colection	Local	date	Subspecies	IPS-S	IPS-C	lbc	abc	dbc	bc	lbchd
Borgmeier	Alto da Serra-SP		<i>subterraneus</i>	X		X				
Borgmeier	Alto da Serra-SP		<i>subterraneus</i>	X		X				
Borgmeier	Est. Rio Grande-SP São Paulo-SP		<i>subterraneus</i>	X		X				
Borgmeier	Ipiranga	1917	<i>subterraneus</i>		X	X				
Borgmeier	Ilha São Sebastião São Paulo-SP	1915	<i>subterraneus</i>		X					
Borgmeier	Cachoeirinha	1939	<i>subterraneus</i>	X				X		
Borgmeier	Cruz Alta-RS Nova Wuertemberg		<i>subterraneus</i>	X		X				
Borgmeier	Cruz Alta-RS Nova Wuertemberg		<i>subterraneus</i>	X		X				
Borgmeier	Teresópolis-RJ		<i>subterraneus</i>	X		X				
Borgmeier	Campo Belo-RJ	21/X/1926	<i>subterraneus</i>		X	X				
Borgmeier	Campo Belo-RJ	21/X/1926	<i>subterraneus</i>	X		X				
Borgmeier	Rio Negro-PR	28/II/1924	<i>subterraneus</i>	X		X				
Borgmeier	Rio Negro-PR	22/X/1925	<i>subterraneus</i>	X		X				
Borgmeier	Rio Negro-PR	22/X/1925	<i>subterraneus</i>	X		X				
	Três Lagoas-MS	08-14/I/1970	<i>subterraneus</i>	X		X				
	Três Lagoas-MS	08-14/I/1971	<i>subterraneus</i>	X		X				
	Serra Caraça-MG	X/1961	<i>subterraneus</i>	X		X				
	Monsenhor Paulo-MG	5/XII/1956	<i>subterraneus</i>	X		X				
Borgmeier	Palmira-MG	8/IX/1925	<i>subterraneus</i>	X		X				
Borgmeier	Santa Teresa-ES	8/VII/1928	<i>subterraneus</i>	X		X				
Borgmeier	Santa Teresa-ES	8/VII/1928	<i>subterraneus</i>	X		X				
	Morro Pará-BA	I/1926	<i>subterraneus</i>	X		X				
	Encruzilhada-BA	XI/1974	<i>subterraneus</i>	X		X				
	50KM S Lábrea-AM	XI/1969	<i>brunneus</i>	X					X	
	Itabuna-BA	14/VII/1974	<i>brunneus</i>	X					X	
	Parque Sooretama – ES	30/VIII/1961	<i>brunneus</i>		X		X			
	Parque Sooretama – ES	30/VIII/1961	<i>brunneus</i>		X				X	
	Parque Sooretama – ES	30/VIII/1961	<i>brunneus</i>		X				X	
	Caruaru-PE	V/1972	<i>brunneus</i>		X	X				
Kempf	Rolândia-PR	10/XII/1956	<i>brunneus</i>		X				X	
Kempf	Rolândia-PR	10/XII/1956	<i>brunneus</i>		X		X			
Borgmeier	Rio de Janeiro-RJ	11/IX/1924	<i>brunneus</i>	X			X			
	Ilha Grande-RJ		<i>brunneus</i>		X	X				
	Itatiaia-RJ	VII/1954	<i>brunneus</i>	X		X				
	Itatiaia-RJ	VII/1954	<i>brunneus</i>	X		X				
	Petrópolis-RJ	19/XII/1969	<i>brunneus</i>	X		X				
Borgmeier	Gaspar-SC	1928	<i>brunneus</i>	X			X			
Borgmeier	B. Constant-MG	3/V/1915	<i>molestans</i>		X					X
Santschi	Itambacuri-MG	17/I/1958	<i>molestans</i>		X					X
	E. Luja-MG		<i>molestans</i>		X				X	
Borgmeier	Itambacuri-MG	17/I/1958	<i>molestans</i>		X					X
Borgmeier	E. Luja-MG	1922	<i>molestans</i>		X	X				X
Borgmeier	Mendes-RJ	1933	<i>molestans</i>		X	X				
Borgmeier	Itaipava-RJ	VII-1937	<i>molestans</i>	X		X				
	Serra do Araça-AM	IV-1975	<i>subterraneus</i>	X				X		
	Serra do Araça-AM	IV-1975	<i>subterraneus</i>	X				X		
Borgmeier	São Paulo-SP		<i>subterraneus</i>		X		X			
H. Fowler	Paraguay (Villet)	17/X/1975	<i>subterraneus</i>	X			X			
Kempf	Blumenau-SC	19/I/1972	<i>brunneus</i>		X		X			
	Campo Alegre-SC	20/V/1976	<i>brunneus</i>	X		X				
	Campo Alegre-SC	20/V/1976	<i>brunneus</i>	X				X		

Colection	Local	date	Subspecies	IPS-S	IPS-C	lbc	abc	dbc	bc	lbchd
Borgmeier	Blumenau-SC	19/I/1972	<i>brunneus</i>	X			X			
	Blumenau-SC	19/I/1972	<i>brunneus</i>	X				X		
		7-						X		
	Caraguatatuba-SP	14/VII/1962	<i>brunneus</i>	X						
	Praia Massaguaçu	28-						X		
	Caraguatatuba-SP	30/V/1962	<i>brunneus</i>		X					
	Campinas-SP		<i>brunneus</i>		X			X		
	Ilha dos Gatos	III/1970	<i>brunneus</i>	X				X		
	Anhembi-SP	14/II/1969	<i>brunneus</i>		X	X				
	Anhembi-SP	14/II/1969	<i>brunneus</i>	X			X			
	Niquelândia-GO						X			
	48°18'W 14°01'S	24/IX/1995	<i>brunneus</i>		X					
	Niquelândia-GO							X		
	48°18'W 14°01'S	24/IX/1995	<i>brunneus</i>		X					
	Luiz Antonio-SP						X			
	Reserva Jataí	23/V/1997	<i>brunneus</i>		X					
	Reserva Jataí	23/V/1997	<i>brunneus</i>		X					
	Reserva Jataí	23/V/1997	<i>brunneus</i>		X		X			
	Santa Rita de Cássia-BA	18/VII/1991	<i>brunneus</i>	X						
	Santa Rita de Cássia-BA	18/VII/1991	<i>brunneus</i>	X		X				
	Santa Rita de Cássia-BA	18/VII/1991	<i>brunneus</i>		X	X				
	Santa Rita de Cássia-BA	18/VII/1991	<i>brunneus</i>	X		X				
Kempf	Lassance-MG	13/IX/1985	<i>molestans</i>		X		X			
	Serra do Araça-AM	IV/1975	<i>subterraneus</i>	X				X		
	Serra do Araça-AM	IV/1975	<i>subterraneus</i>	X				X		
	Paraguai Pastoreo	26/IX/1975	<i>brunneus</i>		X	X				
	Yungas de Palmas -Bolivia	25/II/1949	<i>brunneus</i>	X				X		

IPS-S = inferior pronotal spine straight and directed forward, IPS-C = inferior pronotal spine curved and pointed upwards, lbc = light brown color, abc= average brown color, dbc = dark brown color, bc= black color, lbchd = light brown color with front of the head darker

Table S3. Morphological analysis of *Acromyrmex subterraneus* subspecies with samples obtained from donations and collect, deposited in the collection DZUP considering the key criteria Mayhé-Nunes 1991

Local	Date	Subspecies	IPS-S	IPS-C	lbc	abc	dbc	bc	lbchd
Bom Jardim-RJ	27/XI/2012	<i>subterraneus</i>	X		X				
Bom Jardim-RJ	27/XI/2012	<i>subterraneus</i>	X		X				
Bom Jardim-RJ	27/XI/2012	<i>subterraneus</i>	X		X				
Curitiba-PR	08/I/2013	<i>subterraneus</i>	X			X			
Curitiba-PR	08/I/2013	<i>subterraneus</i>	X			X			
Curitiba-PR	08/I/2013	<i>subterraneus</i>	X			X			
Curitiba-PR	13/V/2012	<i>subterraneus</i>	X		X				
Curitiba-PR	13/V/2012	<i>subterraneus</i>	X		X				
Curitiba-PR	13/V/2012	<i>subterraneus</i>	X		X				
Ilha Solteira-SP	24/I/2013	<i>subterraneus</i>	X		X				
Ilha Solteira-SP	24/I/2013	<i>subterraneus</i>	X		X				
Corumbá-MS	08/I/2013	<i>subterraneus</i>	X		X				
Corumbá-MS	08/I/2013	<i>subterraneus</i>	X		X				
Porto Seguro-BA		<i>subterraneus</i>	X					X	
Gandú-BA		<i>subterraneus</i>	X					X	
Viçosa-MG	19/II/2013	<i>molestans</i>		X	X				X
Viçosa-MG	19/II/2013	<i>molestans</i>		X	X				X
Viçosa-MG	20/II/2013	<i>molestans</i>		X	X				X
Viçosa-MG	20/II/2013	<i>molestans</i>		X	X				X
Campo do Tenente-PR	10/VI/2013	<i>subterraneus</i>	X		X				
Arapoti-PR	14/V/2013	<i>subterraneus</i>	X		X				
Campo do Tenente-PR	10/VI/2013	<i>subterraneus</i>	X		X				
Adrianópolis-PR	19/II/2013	<i>subterraneus</i>	X		X				
Cerro Azul-PR	20/II/2013	<i>subterraneus</i>	X		X				
Cerro Azul-PR	20/II/2013	<i>subterraneus</i>	X		X				
Campo do Tenente-PR	28/I/2013	<i>subterraneus</i>	X		X				
Cerro Azul-PR	20/II/2013	<i>subterraneus</i>	X		X				
Cerro Azul-PR	20/II/2013	<i>subterraneus</i>	X		X				
Adrianópolis-PR	19/II/2013	<i>subterraneus</i>	X		X				
Piracicaba-SP	21/III/2013	<i>brunneus</i>	X				X		
Três Barras-SC Rigesa	10/IV/2013	<i>subterraneus</i>	X		X				
Campo do Tenente-PR	10/VI/2013	<i>subterraneus</i>	X		X				
Campo do Tenente-PR	10/VI/2013	<i>subterraneus</i>	X		X				
Rio Negro-PR	06/VI/2013	<i>subterraneus</i>	X		X				
Sengés-PR	06/VI/2013	<i>subterraneus</i>	X		X				
Sengés-PR	07/VI/2013	<i>subterraneus</i>	X		X				
Sengés-PR	10/VI/2013	<i>subterraneus</i>	X		X				
Arapoti-PR	07/VI/2013	<i>subterraneus</i>	X		X				
Arapoti-PR	08/VI/2013	<i>subterraneus</i>	X		X				
Arapoti-PR	10/VI/2013	<i>subterraneus</i>	X		X				
Arapoti-PR	13/VI/2013	<i>subterraneus</i>	X		X				
Arapoti-PR	13/VI/2013	<i>subterraneus</i>	X		X				
Arapoti-PR	14/VI/2013	<i>subterraneus</i>	X		X				
Rio Negrinho-SC	10/X/2011	<i>subterraneus</i>	X		X				
Mafra-SC	27/VI/2013	<i>subterraneus</i>	X		X				
Mafra-SC	27/VI/2013	<i>subterraneus</i>	X		X				
Rio Claro-SP		<i>subterraneus</i>	X			X			
Morretes-PR	14/XII/2013	<i>subterraneus</i>	X					X	
Morretes-PR	14/XII/2013	<i>subterraneus</i>	X					X	
Paranaguá-PR		<i>brunneus</i>	X				X		

IPS-S = inferior pronotal spine straight and directed forward, IPS-C = inferior pronotal spine curved and pointed upwards, lbc = light brown color, abc= average brown color, dbc = dark brown color, bc= black color, lbchd = light brown color with front of the head darker

Capítulo II

Delimitação de espécies e filogeografia das formigas cortadeiras Neotropicais *Acromyrmex subterraneus* e *A. balzani*

Species delimitation and phylogeography of the Neotropical leaf-cutter ants *Acromyrmex subterraneus* and *A. balzani*.

Abstract

Mastering the knowledge of our biodiversity is a major current challenge, and while morphological data can underestimate the actual number of species in a given group, genetic data can be used to identify potentially cryptic species. Here we apply recently developed methods of species delimitation to two species of leaf-cutter ants (*Acromyrmex balzani* and *A. subterraneus*) that are considered agricultural pests. We sequenced 66 specimens of these species for the mitochondrial gene cytochrome oxidase I (COI), and for three exon-primed intron-crossing (EPIC) markers, and used these sequences for analyses of species delimitation and evolutionary demography. We estimated gene trees by Bayesian inference and used them to infer species limits using the method of the Generalized Mixed Yule Coalescent model (GMYC). We then estimated multilocus species trees for each species relationships hypotheses, and compared the results of each hypothesis using a method of posterior simulation-based analogue of Akaike Information Criterion through Markov chain Monte Carlo (AICM). In addition, we used the method implemented in Bayesian analysis of genomic sequence data under the multispecies coalescent model (BP&P) to validate the best-supported species relationship hypothesis. Our results points to the recognition of three species in our sample, which are currently recognized as *A. balzani*, *A. subterraneus subterraneus*, and *A. subterraneus molestans*. The demographic history of *A. balzani*, a grass specialist, is mostly stable, except for tendency of a small decline from 1-0.4 Ma to the present, whereas the demographic history of *A. subterraneus subterraneus*, a dicotyledonous specialist that nests in shaded and humid habitats, indicates a slight increase in population size from 1.3 Ma to the present. Although is not possible to pinpoint one glacial or interglacial event as cause of these population variations, these variations are consistent with an increase in forests areas over more open habitats over the past 1 My.

Introduction

Mastering the knowledge of our biodiversity is a major challenge for the 21st century. Species are traditionally identified and described using morphological characters, but there is concern about the possibility that this practice might underestimate the actual the number of species [1]. Genetic data have allowed the identification of several cryptic species and their phylogenetic affinities, as well as to obtain information about genetic diversity, gene flow, the existence of hybrids, and population demography. Moreover, recently several studies involved the development and application of statistical approaches to species delimitation based on genetic data [i.e. 2-5], with methods operating in the interface between populations and species, and incorporating a coalescent model [1, 6-8].

It has been estimated that there are between 2-8.5 million undescribed insect species [9]. Between the insects, the ants (Formicidae) are one of the groups that represent most of the biomass in several habitats, but for which there are a large number of previously unknown taxa still being described [10]. The fossil record dates the first appearance of ants in the Cretaceous (110-90 Million years ago [Ma]) [9], what makes the ants represent one of the most extensive ecological and evolutionary success of all eusocial insects [11, 12]. In the long evolutionary history of the ants, the group was surely affected by some of the geological and climatological changes that are described for the last 100 Ma [see 13]. While these events could have affected the historical distribution patterns of ant species, much more recently in the geological scale several taxa started to occur in habitats created by humans, with several being considered agricultural pests [11, 14-16].

Between the ants that have the higher impact in plant cultivation activities are the leaf-cutter ants of the genera *Atta* and *Acromyrmex* (Myrmicinae), which live in symbiosis with a fungus, using fresh vegetable matter to cultivate the fungus that are used for feeding the colony [11,17-20]. Of these two genera, *Acromyrmex* has the largest number of taxa, including 32 species and 30 subspecies that occur from southern United States until the south of South America, inhabiting tropical forests, savannas, and disturbed areas [21]. Traditionally the genus taxonomy was based only in morphological characters [e.g.22], which could have lead to an underestimate of the number of the species of *Acromyrmex*.

Among all species of *Acromyrmex*, highlight two with high economical importance, namely *A. subterraneus* Forel and *A. balzani* Emery. The former species cuts dicotyledonous and build large and populous nests, occurring in Argentina, Bolivia, Paraguay, with records in 50% of the Brazilian states, being absent only of northwestern Brazil [22-24]. In Brazil is subdivided in three subspecies: *A. subterraneus subterraneus*, *A. subterraneus brunneus* and *A. subterraneus molestans* Santschi. The diagnostic characters between these subspecies are considered ambiguous because are based on color and on a character said to be inconstant (inferior pronotal spine shape) [25], with a need to access the validity of these taxa [26]. On the other hand, *A. balzani* cuts exclusively grasses, being common fields and grasslands, including pastures, and coastal and/or urban areas, and significantly impairing the use of pastures [26, 22]. This species is known to occur in Paraguay and in the Brazilian states of Santa Catarina, São Paulo, Rio de Janeiro, Minas Gerais, Mato Grosso do Sul, Goiás, and Piauí [22-24], but its validity as a species is considered uncertain. *A. balzani* was described by Emery in 1890, collected in Asuncion, Paraguay (type locality). Gonçalves, 1961[22] downgraded the status of species of *A. balzani* to subspecies of *A. landolti* (Forel, 1885), although it has found characters that separate *A. balzani* of *A. landolti*. Fowler, 1988 [75] raised *A. balzani* to species status by considering the following diagnostic characters: small eyes, not prominent; spine pronotal median tuberciforme prominent, being more conspicuous than in *A. landolti*. Mayhé-Nunes, 1991[27] agrees with Gonçalves (1961) [22]. Given such considerations, the objectives of the present study were to analyze the specific delimitation of *A. subterraneus* and *A. balzani* using data from multiple loci and methods based on the coalescent theory [e.g. 1, 28], and to infer the demographic history of the delimited species.

Materials and Methods

Population sampling and DNA sequencing

Specimens were obtained through collections and donations between 2012 and 2013. Our analyses included 39 individuals of *Acromyrmex subterraneus* and 22 *Acromyrmex balzani*, representing, respectively, 16 and 8 localities in Brazil (Fig. 1). We collected sequence data (Table 1) for four loci (used primers are indicated in Table

2): one mitochondrial (COI) and three exon-primed intron-crossing (EPIC) markers (346, 965, 1503), that bind to conserved exonic regions flanking an intron, which tends to accumulate mutations at a higher rate than transcribed regions [29]. All sequences are deposited at GenBank (Table1).

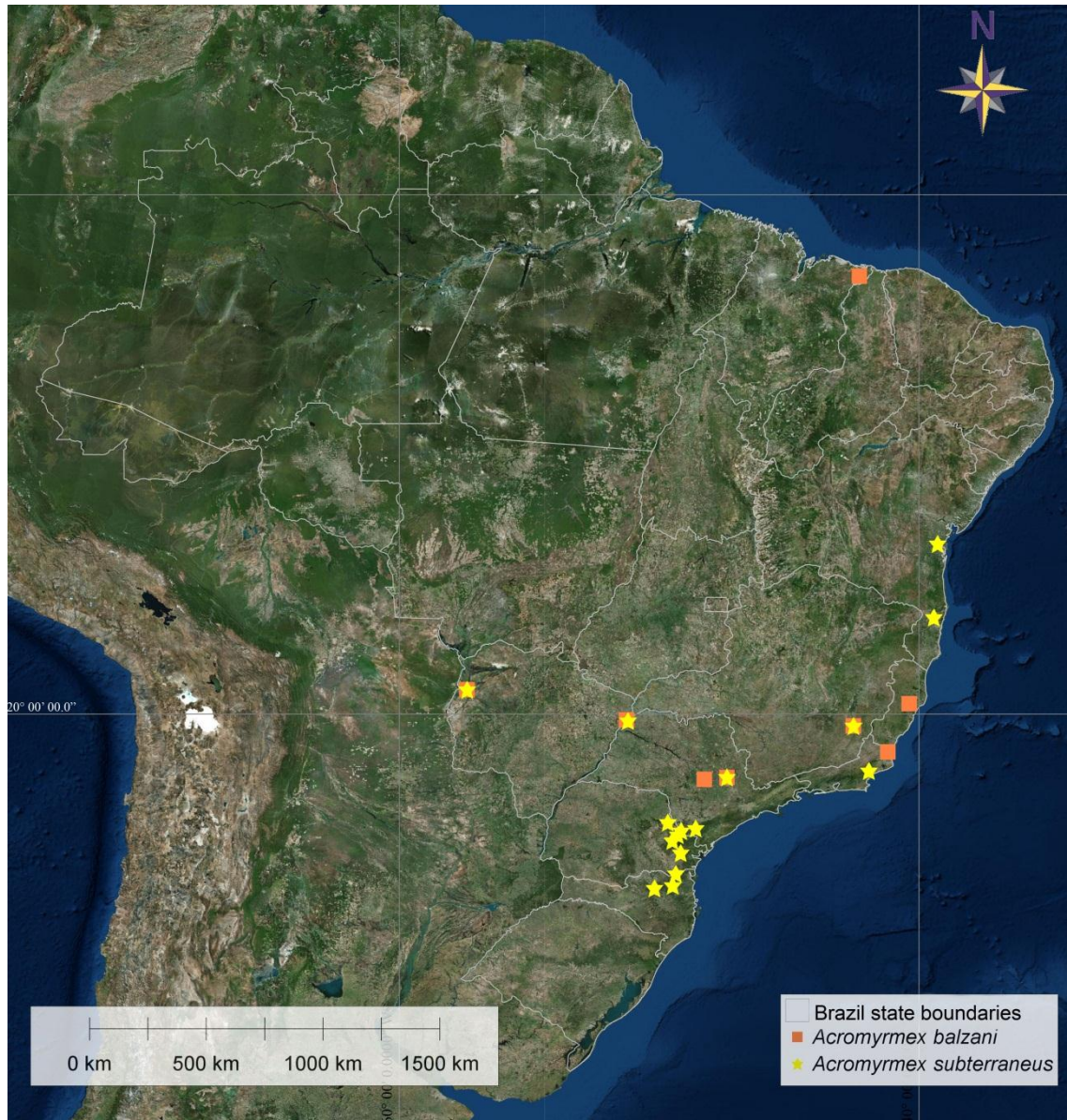


Fig. 1. Localities sampled for *Acromyrmex balzani* (squares) and *Acromyrmex subterraneus* (stars).

Table 1. Sample size, locality of collection, and GenBank accession numbers.

Nº	Specie	Locality	Coordinates	COI	EPIC 346	EPIC 965	EPIC 1503
1	<i>A.subterraneus subterraneus</i>	Bom Jardim-RJ	-41.9333 -22.1833	*	*	*	*
2	<i>A.subterraneus subterraneus</i>	Bom Jardim-RJ	-41.9333 -22.1833	*	*	*	*
3	<i>A.subterraneus subterraneus</i>	Bom Jardim-RJ	-41.9333 -22.1833	*	*	*	*
6	<i>A.subterraneus subterraneus</i>	Curitiba-PR	-49.15719 -24.49412	*	*	*	*
7	<i>A.subterraneus subterraneus</i>	Curitiba-PR	-49.15719 -24.49412	*	*	*	*
8	<i>A.subterraneus subterraneus</i>	Curitiba-PR	-49.15719 -24.49412	*	*	*	*
9	<i>A. subterraneus</i>	Ilha Solteira-SP	-51.20414 -20.25168	*	*	*	*
10	<i>A. subterraneus</i>	Ilha Solteira-SP	-51.20414 -20.25168	*	*	*	*
11	<i>A. subterraneus</i>	Corumbá-MS	-57.39285 -19.0589	*	*	*	*
13	<i>A. subterraneus</i>	Porto Seguro-BA	-39.4312 -16.27099	*	*	*	*
14	<i>A. subterraneus</i>	Gandú-BA	-39.28545 -13.43964	*	*	*	*
15	<i>A. subterraneus molestans</i>	Viçosa-MG	-42.5212 -20.45383	*	*	*	*
16	<i>Asubterraneus molestans</i>	Viçosa-MG	-42.5212 -20.45383	*	*	*	*
18	<i>A. subterraneus molestans</i>	Viçosa-MG	-42.5212 -20.45383	*	*	*	*
19	<i>A. subterraneus molestans</i>	Viçosa-MG	-42.5212 -20.45383	*	*	*	*
21	<i>A. subterraneus</i>	Arapoti-PR	-49.6667 -24.1667	*	*	*	*
22	<i>A. subterraneus</i>	Campo do Tenente-PR	-49.48014 -26.5949	*	*	*	*
23	<i>A. subterraneus</i>	Adrianópolis-PR	-48.59357 -24.39435	*	*	*	*
24	<i>A. subterraneus</i>	Cerro Azul-PR	-49.27969 -24.6441	*	*	*	*
25	<i>A. subterraneus</i>	Cerro Azul-PR	-49.27969 -24.6441	*	*	*	*
26	<i>A. subterraneus</i>	Campo do Tenente-PR	-49.48014 -26.5949	*	*	*	*
27	<i>A. subterraneus</i>	Cerro Azul-PR	-49.27969 -24.6441	*	*	*	*
28	<i>A. subterraneus</i>	Cerro Azul-PR	-49.27969 -24.6441	*	*	*	*
29	<i>A. subterraneus</i>	Adrianópolis-PR	-48.59357 -24.39435	*	*	*	*
49	<i>A. subterraneus brunneus</i>	Piracicaba-SP	-47.38856 -22.43499	*	*	*	*
50	<i>A. subterraneus</i>	Três Barras-SC	-50.18390 -26.6993	*	*	*	*
51	<i>A. subterraneus</i>	Campo do Tenente-PR	-49.48014 -26.5949	*	*	*	*
52	<i>A. subterraneus</i>	Campo do Tenente-PR	-49.48014 -26.5949	*	*	*	*
53	<i>A. subterraneus</i>	Rio Negro-PR	-49.16972 -25.34320	*	*	*	*
54	<i>A. subterraneus</i>	Sengés-PR	-49.49225 -24.8684	*	*	*	*
55	<i>A. subterraneus</i>	Sengés-PR	-49.49225 -24.8684	*	*	*	*
56	<i>A. subterraneus</i>	Sengés-PR	-49.49225 -24.8684	*	*	*	*
57	<i>A. subterraneus</i>	Arapoti-PR	-49.6667 -24.1667	*	*	*	*
58	<i>A. subterraneus</i>	Arapoti-PR	-49.6667 -24.1667	*	*	*	*

59	<i>A. subterraneus</i>	Arapoti-PR	-49.6667 -24.1667	*	*	*	*
60	<i>A. subterraneus</i>	Arapoti-PR	-49.6667 -24.1667	*	*	*	*
61	<i>A. subterraneus</i>	Arapoti-PR	-49.6667 -24.1667	*	*	*	*
62	<i>A. subterraneus</i>	Arapoti-PR	-49.6667 -24.1667	*	*	*	*
63	<i>A. balzani</i>	Sooretama-ES	-40.3740 -19.5956	*	*	*	*
64	<i>A. balzani</i>	Sooretama-ES	-40.3740 -19.5956	*	*	*	*
65	<i>A. balzani</i>	Sooretama-ES	-40.3740 -19.5956	*	*	*	*
66	<i>A. subterraneus</i>	Rio Negrinho-SC	-49.3578 -26.1463	*	*	*	*
30	<i>A. balzani</i>	Campo dos Goytacazes-RJ	-41.19464 -21.45268	*	*	*	*
31	<i>A. balzani</i>	Campo dos Goytacazes-RJ	-41.19464 -21.45268	*	*	*	*
32	<i>A. balzani</i>	Botucatu-SP	-48.25908 -22.51022	*	*	*	*
33	<i>A. balzani</i>	Botucatu-SP	-48.25908 -22.51022	*	*	*	*
34	<i>A. balzani</i>	Botucatu-SP	-48.25908 -22.51022	*	*	*	*
35	<i>A. balzani</i>	Botucatu-SP	-48.25908 -22.51022	*	*	*	*
36	<i>A. balzani</i>	Selvíria-MS	-51.25438 -20.21821	*	*	*	*
37	<i>A. balzani</i>	Selvíria-MS	-51.25438 -20.21821	*	*	*	*
38	<i>A. balzani</i>	Selvíria-MS	-51.25438 -20.21821	*	*	*	*
39	<i>A. balzani</i>	Selvíria-MS	-51.25438 -20.21821	*	*	*	*
40	<i>A. balzani</i>	Corumba-MS	-57.39285 -19.0589	*	*	*	*
41	<i>A. balzani</i>	Parnaíba-PI	-42.28923 -3.13702	*	*	*	*
42	<i>A. balzani</i>	Parnaíba-PI	-42.28923 -3.13702	*	*	*	*
43	<i>A. balzani</i>	Viçosa-MG	-42.52502 -20.45447	*	*	*	*
44	<i>A. balzani</i>	Viçosa-MG	-42.52502 -20.45447	*	*	*	*
45	<i>A. balzani</i>	Viçosa-MG	-42.52502 -20.45447	*	*	*	*
46	<i>A. balzani</i>	Viçosa-MG	-42.52502 -20.45447	*	*	*	*
47	<i>A. balzani</i>	Viçosa-MG	-42.52502 -20.45447	*	*	*	*
48	<i>A. balzani</i>	Piracicaba-SP	-47.38856 -22.43499	*	*	*	*

*will be deposited in GenBank in the occasion of acceptance of the article.

Table 2. Primers used for PCR amplification and bidirectional sequencing.

Primer	Length	TA*	Sequence (5'to 3')	References
LCO	25	45 °C	GGTCAA CAA ATC ATA AAG ATA TTGG	Folmer et al. 1994
HCO	26	45 °C	TAAACT TCA GGG TGA CCA AAA AATCA	Folmer et al. 1994
ant.346F	23	60 °C	GTGGTCCACCATCCGTKGGATCT	Ströher et al. 2013
ant.346R	26	60 °C	GGATTGTTTTGTGTAATCTGCGTTCG	Ströher et al. 2013
ant.965F	24	60 °C	AGTTCAAGGTTACCGGTGCCTAA	Ströher et al. 2013
ant.965R	25	60 °C	GAGAAGGYGAAYTTAAAGACTGATG	Ströher et al. 2013
ant.1503F	21	62 °C	GRTTYGCCTTCCAGGAGATCA	Ströher et al. 2013
ant.1503R	23	62 °C	AAGTAGTCCAGGCAGAACCACAC	Ströher et al. 2013

* annealing temperatures (TA)

Total DNA was extracted using DNeasy® kits (Qiagen Inc.) according to the manufacturer's instructions, and then examined for concentration and purity using a Nanodrop® spectrophotometer. PCR amplifications were performed in a volume of 25 µl, using two µl of DNA extracts (concentrations between 8.8 and 93.6 ng/µl), 0.4 of Taq Polymerase, 1 X of PCR buffer, 2.0 mM of MgCl₂, 0.5 mM of dNTP's, and 0.6 µM of each primer. Thermocycling conditions were 5 min at 95 °C, 35 cycles of 92 °C during 1min, 45° during 45s, and 70 °C for 2 min, followed of a final extension at 72 °C for 5 min. PCR products were subject to electrophoresis on agarose gel at 1.5% to check for product size and possible contaminants. Positive results were purified using isopropanol and sequenced on an ABI 3500 sequencer using BigDye® Terminator v. 3.1 (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions.

Species delimitation

In order to generate additional potential species delimitation hypotheses besides the ones offered by the current taxonomic status of the sampled species (*A. subterraneus* and *A. balzani*) and subspecies (*A. subterraneus subterraneus* and *A. subterraneus molestans* plus *A. balzani*), we performed a Bayesian Inference analysis for each loci using all available sequences in BEAST 2 [30] with a random local clock with rate set to 1, the HKY substitution model [31,32], and a coalescent prior of constant population size for 10,000,000 generations. The results were checked for convergence in TRACER 1.6 [33]. The consensus tree obtained for each loci was used to perform a Generalized Mixed Yule Coalescent (GMYC) approach to species delimitation [28] with a single threshold, with the results of this method being used as additional species relationships hypothesis to be tested using a multi-loci coalescent analysis. The GMYC method was proposed by T.G. Barraclough, and presented for the first time in [6, 34], being formally described by [28]. This single locus method tries to locate independent evolution (mutations that not spread rapidly in other species) at the tree branches [35-37]. The method uses a maximum likelihood model of branching between and within species on a gene tree, combining the Yule diversification model and an intraspecific genealogy based on a neutral coalescent model [28].

To test the species delimitation hypothesis proposed by GMYC, we performed a multilocus *BEAST analysis [38] for each species tree hypothesis in BEAST 2 [39]. This

methodology uses an integrated search for species and gene trees, allowing the comparison of hypothesis using a Bayesian approach that considers phylogenetic and genealogical uncertainty [e.g. 40]. We used only the individuals with sequences available for at least three loci, the HKY substitution model for each partition [41], a strict molecular clock, with COI rate set to 1 and other gene rates being estimated, with each analysis run for 100,000,000 generations, with sampling every 1,000. The results were checked for convergence in TRACER 1.6 [33]. The maximum credibility tree and 95% HPD of each analyzes was produced in TREEANOTATOR 2 [30]. The results of the *BEAST analyses were then compared to select the best species relationships model using the posterior simulation-based analogue of Akaike Information Criterion [42] through Markov chain Monte Carlo (AICM) proposed by [43], which were computed in TRACER 1.6.

Additionally, we performed analysis using Bayesian analysis of genomic sequence data under the multispecies coalescent model (BP&P 3) [1, 44] to assess the support for each species delimitation hypothesis. For these analyses were included 25 individuals of *A. subterraneus* and 18 of *A. balzani*, representing 11 and eight 8 localities, respectively (Tab.1). This method implements a reversible jump Markov Chain Monte Carlo algorithm to split and fuse different tree branches, generating posterior probabilities to the modified trees that correspond to a hypothesis [1]. Each locus was assigned a heredity scalar (mitochondrial = 0.25, nuclear autosome = 1.0), and a gamma prior $G(2, 2000; \text{mean} = 0.001)$ was used on the population size parameters (θ_s), with the age of the root of the species tree (τ_0) being assigned a gamma prior $G(30, 2000)$, while the locus rates were estimated using a random-rates model of Burgess & Yang [45], using a Dirichlet distribution with $\alpha = 2$. Each analysis consisted of a burn-in of 50,000 generations, plus 500,000 generations with sampling each 2. All schemes were run twice to confirm consistency, using both algorithm 0 and $\varepsilon = 2$ (equations 2 and 3 of [1]), and algorithm 1 with $\alpha = 2$ and $m = 1$ (equations 6 and 7 of [1]). We performed the analyses using different values of θ , to test if shallow ($G = 2, 2000$) or deep divergence ($G = 1, 10$) could influence the results.

Species divergence and demography

To obtain the age of the divergence between the species found in the best fit model we generated a dated tree for the COI gene using one taxa of each species plus *Atta laevigata* Smith as outgroup, with the tree being estimated in BEAST using a lognormal relaxed clock with the root of the tree being assigned a normal prior between 8-12 Million years ago (Ma) (mean = 10 Ma) for the divergence between *Acromyrmex* and *Atta* [46]. We analyzed if the defined species presented any pattern of population structure using a haplotypic network estimated by the median-joining (MJ) method [47] in the software NETWORK 4.6 [48], and the Bayesian clustering method implemented by software Structure [49, 50], but none of these analyses indicated the existence of any structure in the examined species (not shown).

For the species with large sample size (e.g. $n > 5$), the demographic histories of each gene were analyzed using Tajima's D [51] and Fu's F_s [52], where significant values for the statistics were found (respectively, $P < 0.05$, and $P < 0.02$), negative values can indicate population expansion, and positive values indicates population decline [51,52]. We also performed the R_2 test [53], where values near the lower end of the test distribution can indicate population expansion, and values near the higher end of the test distribution indicate population decline [54]. All these test were performed in the software DNASP v5 [55] using coalescent simulations with 1000 replicates.

Additionally we performed analyzes of population change over time for the COI gene using a Bayesian skyride plot [56] with a Gaussian Markov random field (GMRF) smoothing prior. This method differs from the related Bayesian skyline plot [57] by not requiring the specification of the expected number of population size changes in the history of the sample [58]. This method was implemented in BEAST 1.8 [59] due to the fact that it is not yet implemented in BEAST 2. We used a lognormal relaxed clock, and a lognormal prior for the for the root of each species corresponding to the 95% HPD and median values estimated in the dated tree estimated using the COI gene, with each analyzes being run for 10,000,000 generations, with sampling each 1,000, and a burn-in of 10%. The results were checked for convergence in TRACER 1.6 [33].

Results

We obtained sequences of 41 individuals of *Acromyrmex subterraneus*, with COI sequences for 26 individuals (355 base pairs), EPIC 346 for 36 (443 b.p.), EPIC 965 for 36 (333 b.p.), and EPIC 1503 for 38 (727 b.p.). For *A. balzani* we sequenced 24 individuals, with COI sequences for 18 individuals (427 b.p.), EPIC 346 for 20 (418 b.p.), EPIC 965 for 20 (286 b.p.), and EPIC 1503 for 20 (742 b.p.).

Species delimitation

The obtained gene trees from the initial BEAST analyses are shown in Fig. 2-4. These trees were used for the GMYC analyses to evaluate additional hypothesis of species relationships to be tested with the multilocus analyzes. For the COI gene tree, the GMYC method delimited eight clades in *A. subterraneus* and seven in *A. balzani*, with a $L_{obs} = 58.16685$, significantly superior to the likelihood of the null model ($L_0 = 52.75092$, $P < 0.005$), with a confidence interval of 7–16 clusters. The result for the EPIC 346 gene tree suggests the existence of two clades in *A. subterraneus* and four in *A. balzani*, also with likelihood significantly superior to the null model ($L_{obs} = 392.1296$, $L_0 = 385.6385$; $P < 0.001$), with a confidence interval of 3-9 clusters. The EPIC 965 gene tree produced a delimitation of three clades in *A. subterraneus* and one in *A. balzani*, with a confidence interval of 1-12 clusters, not being significantly better than the null model ($L_{obs} = 449.2506$, $L_0 = 448.3908$; $P = 0.4232$ n.s.). The results of the GMYC analysis for the EPIC 1503 gene tree delimited one cluster for *A. subterraneus* and one for *A. balzani*, with a confidence interval between 1-15 clusters, not being statistically significant ($L_{obs} = 501.4727$, $L_0 = 501.1345$, $P = 0.7131$ n.s.).

Fig 2. Gene trees with the delimitation of GMYC for gene COI and lineages through time plot. The red line in the plot represents the coalescence point. The branching rate to the left indicates species formation, and the values to the right indicate intraspecies diversification.

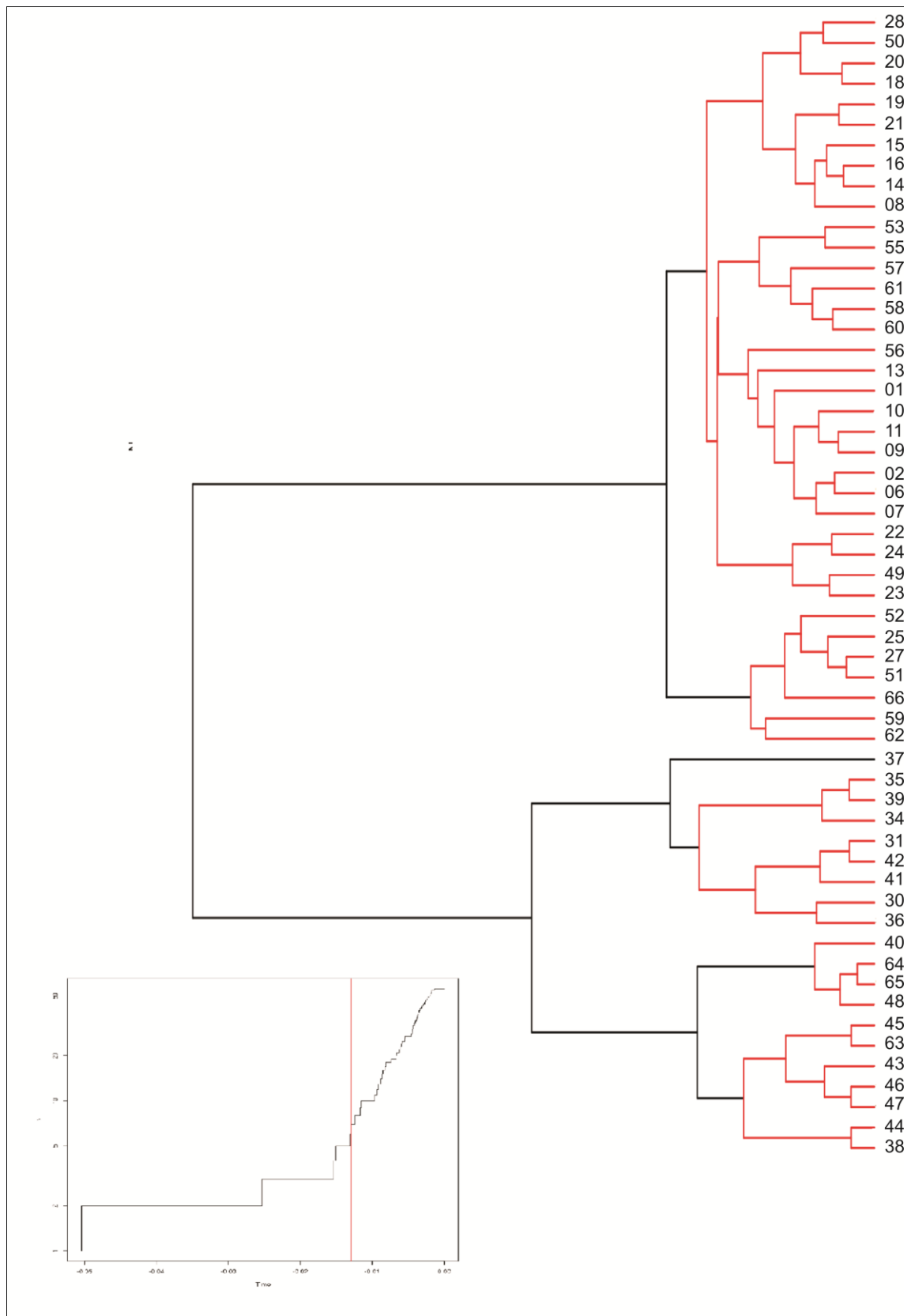


Fig 3. Gene trees with the delimitation of GMYC for gene EPIC 346 and lineages through time plot. The red line in the plot represents the coalescence point. The branching rate to the left indicates species formation, and the values to the right indicate intraspecies diversification.

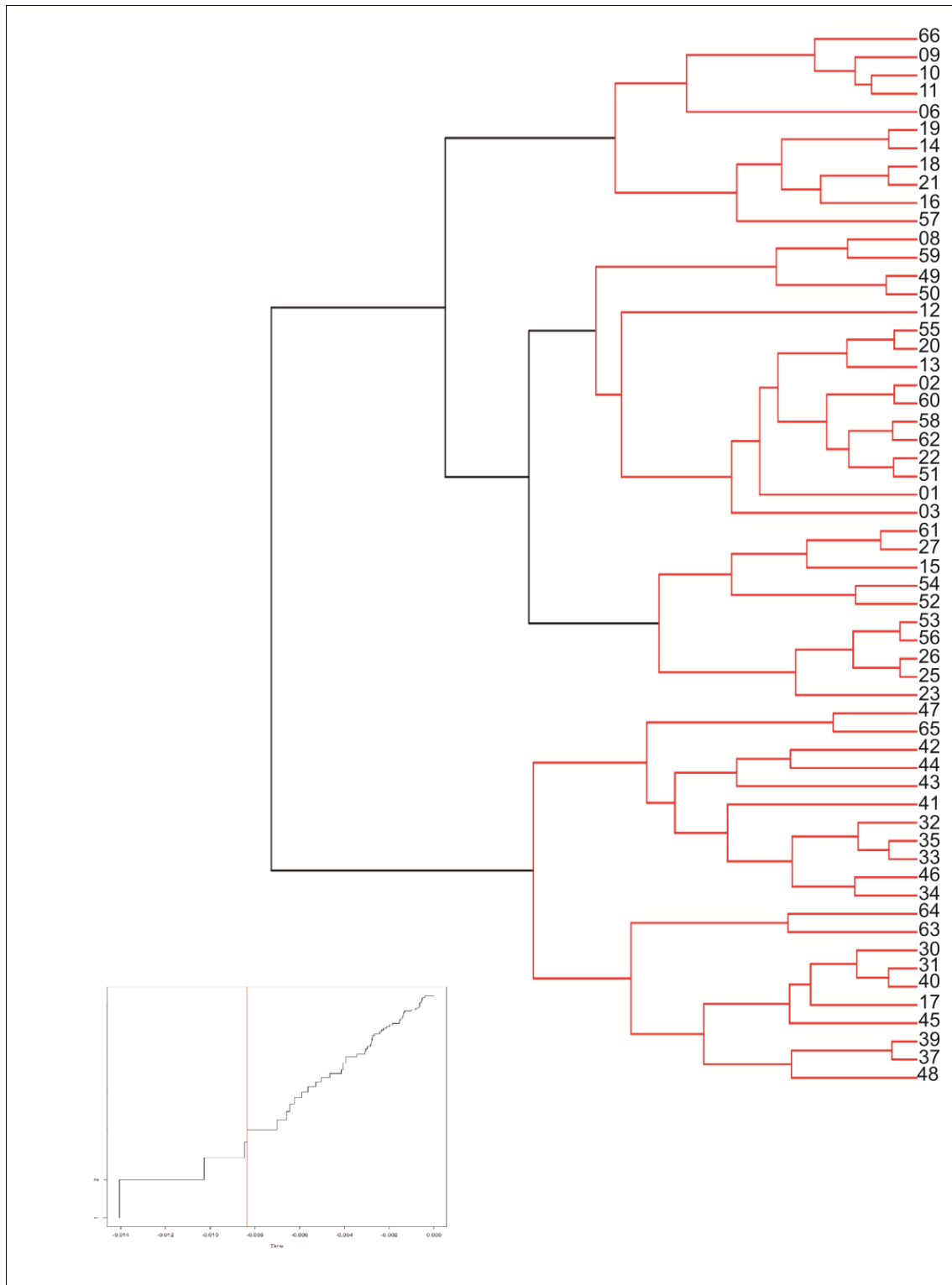


Fig 4. Gene trees with the delimitation of GMYC for gene EPIC 965 and lineages through time plot. The red line in the plot represents the coalescence point. The branching rate to the left indicates species formation, and the values to the right indicate intraspecies diversification.

We used the results of the delimitation (even the non-significant), as well as the two proposals based on traditional taxonomy, as guides to set *BEAST analyses using the proposed species relationship of each delimitation scheme (Fig. 5). The results of the *BEAST analyses were then compared using the AICM (Table 3). The best-fit model according to this criterion was the *subterraneus* subspecies scheme (*A. subterraneus subterraneus*, *A. subterraneus molestans*, and *A. balzani*). The BP&P analyzes also recovered a high posterior probability for this model under shallow and deep divergence (Fig. 5).

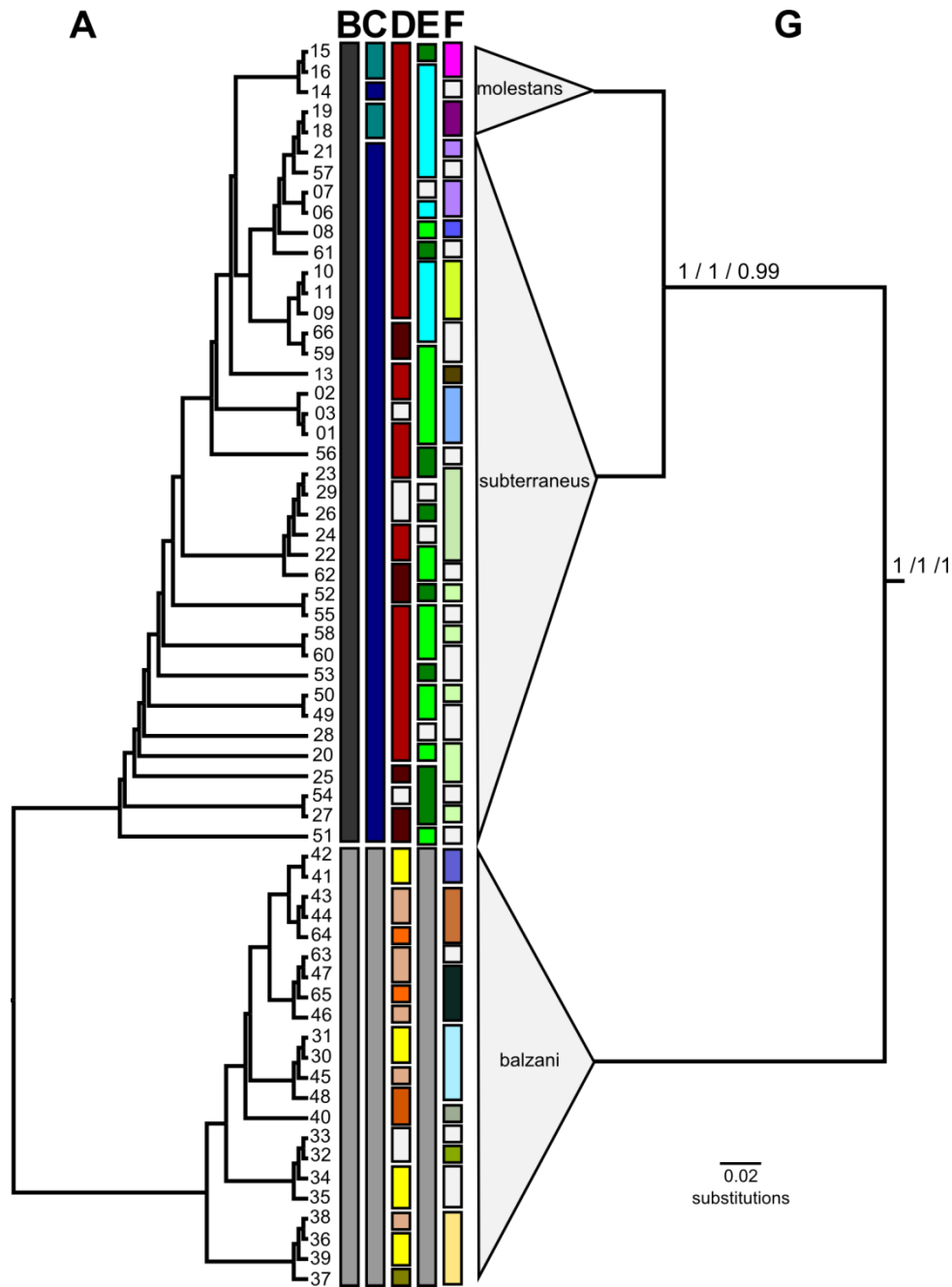


Fig. 5. A. Representative RaxML topology of all samples. B. Species hypothesis of the current taxonomy. C. Subspecies hypothesis of the current taxonomy. D. Species hypothesis obtained by the GMYC analysis of EPIC 346 marker. White squares indicates lack of samples. E. Species hypothesis obtained by the GMYC analysis of EPIC 965 marker. F. Species hypothesis obtained by the GMYC analysis of COI marker. G. Best species tree hypothesis according to AICM. Values at the nodes are, from left to right, *BEAST posterior probability, BP&P posterior probability for θ with $G = 2$, 2000, and BP&P posterior probability for θ with $G = 1$, 10.

Table 3. Comparison of the different species relationship hypotheses estimated in *BEAST using the posterior simulation-based analogue of Akaike Information Criterion through Markov chain Monte Carlo (AICM). S.E. estimated from 1,000 bootstrap replicates. The reported differences between the AICM estimates for the hypotheses indicates a better fit model of the row model in relation to the column's models.

	AICM	S.E.*	B	C	D	E	F
B	9560.03	+/- 0.764	-	-97.384	-78.689	-69.338	-41.629
C	9462.646	+/- 0.914	97.384	-	18.695	28.045	55.755
D	9481.341	+/- 0.261	78.689	-18.695	-	9.351	37.06
E	9490.692	+/- 0.458	69.338	-28.045	-9.351	-	27.71
F	9518.402	+/- 0.561	41.629	-55.755	-37.06	-27.71	-

*S. E. Standard error. B. Taxonomy species. C. Taxonomy subspecies D. GMYC EPIC 346. E. GMYC EPIC 965. F. GMYC COI.

Species divergence and demography

The obtained dated tree for the COI gene suggests that *A. balzani* diverged from *A. subterraneus* near 7.46 Ma (95% HPD 3.95-10.51 Ma), while *A. subterraneus subterraneus* and *A. subterraneus molestans* diverged more recently, at about 1.31 Ma (95% HPD 0.32-2.95 Ma) (Fig. 6). We performed additional analyses only for the clades of *A. balzani* and *A. subterraneus subterraneus* due to the low sample size for *A. subterraneus molestans*. In none of the two analyzed species there was signs of population structure, with clades in *A. subterraneus subterraneus* including samples from the states of Bahia, Minas Gerais, and Paraná, whereas in *A. balzani* there are clades including samples from states of Piauí, Minas Gerais, and Mato Grosso do Sul.

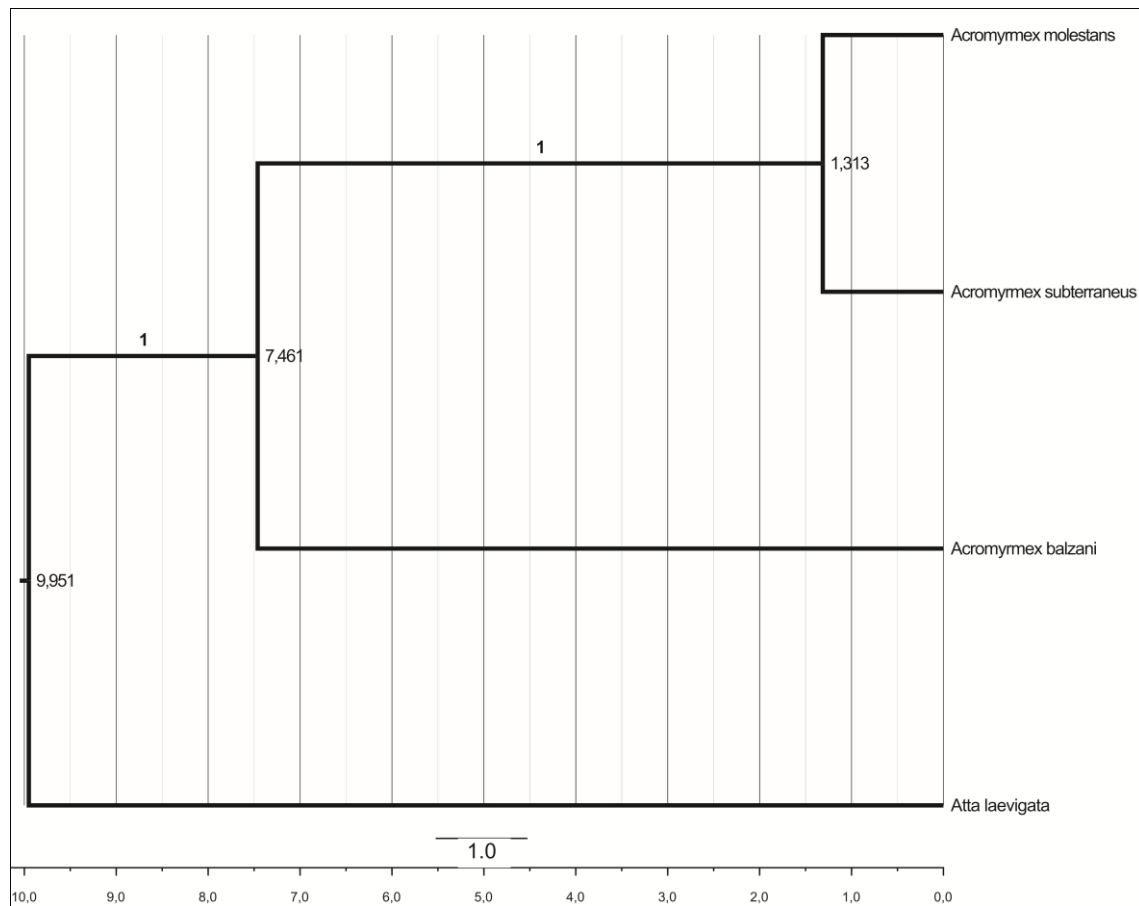


Fig. 6. Dated Bayesian inference tree for the studied *Acromyrmex* species. The tree was dated by setting a normal prior with mean 10 Ma (range from 8-12 Ma) for the divergence between *Acromyrmex* and *Atta* (see Methods, species divergence).

The results for the Tajima's D , Fu's F_s , and R_2 tests for the different loci of the two species are shown in Table 4. For *A. subterraneus subterraneus* according to D and F_s only the EPIC's 346 and 965 departed from neutrality, with the negative values indicating a possible population expansion. The R_2 tests for this species were significant for all genes, with all values being at the lower end of the test distribution, indicating population expansion. For *A. balzani* the D and F_s tests were mostly non-significant, with the exception of the F_s for the EPIC 965, which also indicates a possible population expansion. The R_2 tests for this species were significant for all genes, with its values being near the higher of the test distribution, indicating population decline. The Bayesian skyline plots for the COI gene of both species are shown in Fig. 7.

Table 4. Summary statistics of the tests of neutrality *Acromyrmex subterraneus subterraneus* and *Acromyrmex balzani*.

species/gene	n	H	Hd	π	Tajima's <i>D</i>	Fu's <i>F_s</i>	<i>R₂</i>
<i>Acromyrmex subterraneus subterraneus</i>							
COI	26	13	0.849	0.09172	-0.13472 P>0.57800	-0.07869 (P > 0.988)	0.11986* (P <0.001)
EPIC 346	36	36	1.000	0.01693	-0.11444 P>0.24500	-0.24303* (P <0.001)	0.11438* (P <0.001)
EPIC 965	36	20	0.900	0.01029	-0.01400 P>0.09500	-0.03875* (P <0.001)	0.11691* (P <0.001)
EPIC 1503	38	22	0.954	0.00797	-0.07245 P>0.17200	-0.06621 (P > 0.005)	0.11004* (P <0.001)
<i>Acromyrmex balzani</i>							
COI	18	12	0.954	0.05547	-0.10368 P>0.63700	0.08234 (P > 0.809)	0.13343* (P <0.001)
EPIC 346	20	5	0.747	0.00243	-0.03691 P>0.72400	-0.21209 (P > 0.169)	0.15568* (P <0.001)
EPIC 965	20	13	0.932	0.01019	-0.05382 P>0.05300	-0.03036* (P <0.001)	0.13783* (P <0.001)
EPIC 1503	20	7	0.795	0.00286	-0.08083 P>0.22100	0.10014 (P > 0.286)	0.14140* (P <0.001)

n, number of sequences; H, number of haplotypes; Hd, haplotype diversity; π , nucleotide diversity; results for the population expansion test *D*, Tajima's *D*; *F'*, Fu and Li's *F*; *D'*, Fu and Li's *D*; *F_s*, Fu's *F_s*.
*R₂** = statistically significant values.

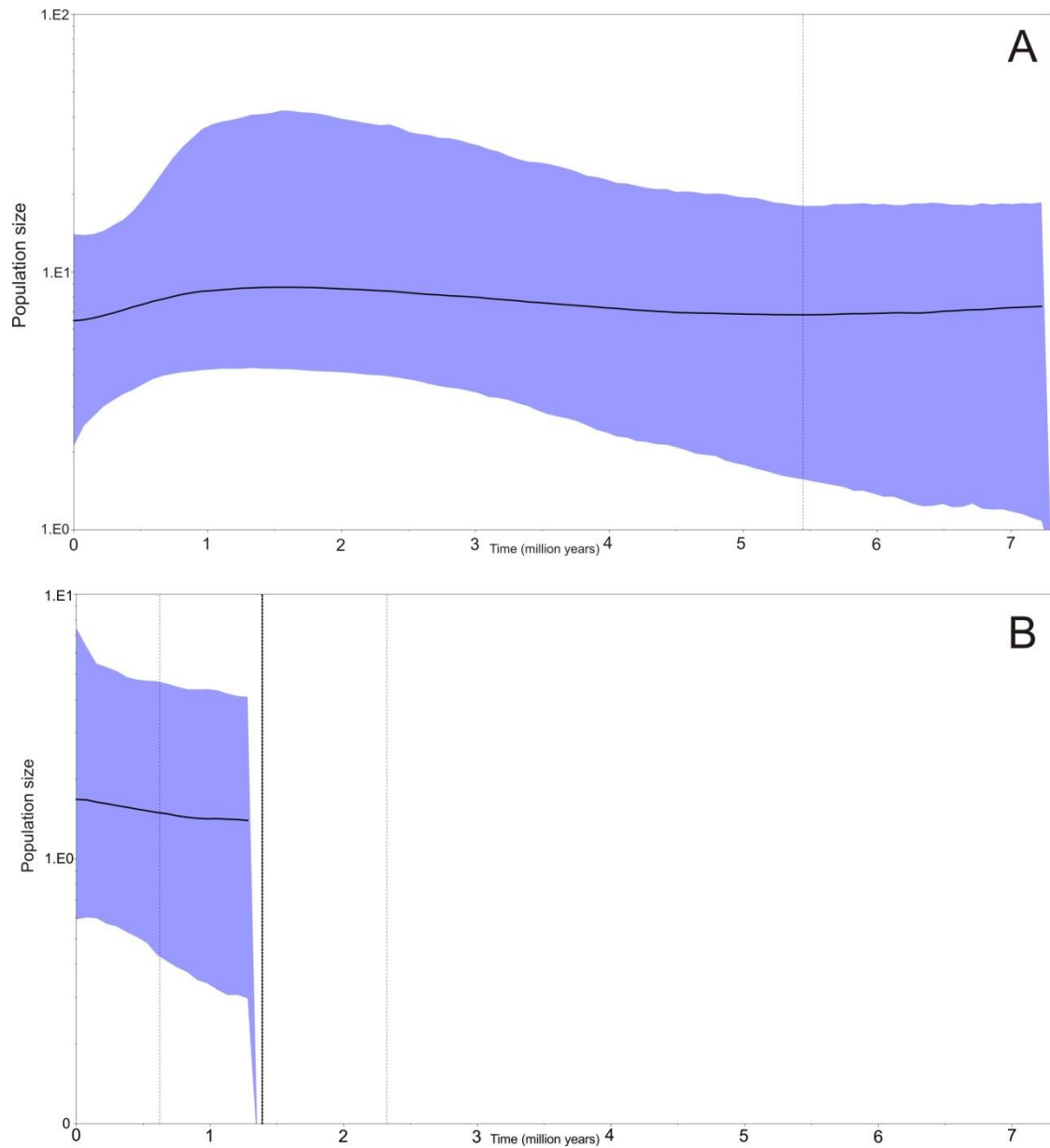


Fig. 7. Bayesian skyride plots for the COI gene for A. *Acromyrmex balzani* and B. *Acromyrmex subterraneus subterraneus*. Plots are based on median population values, with the dotted vertical lines indicating, from left to right, the lower 95% HPD for population age (only this one is visible in A), the median of the initial population age (bold dots), and the higher 95% for population age (visible only in B).

Discussion

Different methods of species delimitation have different assumptions and precision levels. Here we employed a maximum likelihood method (GMYC) as an *a priori* method to establish additional hypothesis besides the morphological ones to be tested using a multiloci dataset and a Bayesian approach that can compare non-nested models (AICM). We obtained similar results using an alternative Bayesian method (BP&P) already used by several authors [60-63]. Our results suggests the existence of cryptic species under the name *A. subterraneus*, with at least the taxon *molestans* being worthy of species level, whereas for *A. balzani* there were no indicatives of the existence of cryptic taxa.

The GMYC method was used by several authors for species delimitation [64-70]. In our analyses the results of the delimitation for the EPIC's 965 and 1503 were not statistically significant, possible due to the markers evolving too fast, and/or due to the coalescence between the clades had occurred before the species formation. The delimitations obtained for *A. subterraneus* by this method for the EPIC 346 and COI were not concordant with morphological classification, with the populations widespread in several clades without geographical structure. For *A. balzani* the delimitations obtained for the EPIC 346 and COI also were not concordant, with the first obtaining four clades that are coincident to geographically close populations that could have experienced a recent genetic flux, and the second obtaining seven clades, splitting more distant samples as Parnaíba (Piauí state), Corumbá (Mato Grosso state), and São Paulo, from the more southern ones.

The multilocus comparison of the multiple relationship hypotheses using species trees and AICM selected as the best fit model the one that recognized three taxa: *A. subterraneus subterraneus*, *A. subterraneus molestans*, and *A. balzani*. This result also presented a high posterior probability in the BP&P analysis. Most of the taxa under *A. subterraneus* were described based mainly in color [e.g. 71-73], but there are morphological differences between *A. subterraneus subterraneus* and *A. subterraneus molestans* (e.g. the inferior pronotal spine is straight and pointed forward, whereas in the latter it is curved and pointed upwards [27]). The two taxa also present some ecological differences, as the nests of the first are underground or covered by earth and located in humid and shady habitats, and of the second are constructed in open fields and are mainly covered by straws or earth motes [74]. Although we not included all subspecies

of *A. subterraneus* in our analyses, we consider that the molecular, morphological, and ecological evidences points to the recognition of *A. subterraneus molestans* as a full species.

Regarding *A. balzani*, the taxon was considered to be a subspecies of *Acromyrmex landolti* [22, 27], but Fowler [75] proposed the recognition of *A. balzani* as a valid species based on its worker morphology. Although we not included *A. landolti* in the present analyzes, previous studies indicates a recent divergence between this species and *A. balzani* [46], as in the case of *A. subterraneus subterraneus* and *A. subterraneus molestans*. We believe that if we had included *A. landolti* on our sampling it would not change the age of divergence between *A. subterraneus* and *A. balzani*, although surely it would change part of the demographic pattern found for *A. balzani*.

Phylogeographic patterns

As stated above, our sampling lacked some species of *Acromyrmex* that could have influenced the divergence age and population aspects estimated for *A. balzani*. Our results suggests a mostly stable population size for *A. balzani* between 7-3 Ma, followed by a small increase between 3-1 Ma, and a small decrease after 1 Ma. Using an age similar to the estimated by Schultz & Brady [46] for the root of *A. balzani*, with a mean age set to 2 Ma, does not change the overall pattern of the skyride for the species, except for compressing it to fit the interval set in the analysis (not show), with a small increase between 1-0.4 Ma, and a decrease after that. This species cuts only grasses [22,26], and is expected that *Acromyrmex* evolution is somehow correlated to the evolution of grasses, possibly due to common origin in Pleistocene [75].

There are records of fossil grasses in South America since the Eocene (a. 50 Ma) [77, 78], but the initial development of Neotropical savannas and *cerrados* with C₃ grasses is thought to have occurred at the end of the Oligocene (a. 28-25 Ma) [77]. Both genera of leaf-cutter ants, *Acromyrmex* and *Atta*, have an Upper Miocene age (8-12 Ma) [46], which predates the initial development of the savannas and *cerrados* in South America, but is coincident with the appearance of C₄ grasses at about 10 Ma, which later become dominant over C₃ grasses at about 7.6-5 Ma, with the first C₄ dominated diets in mammals appearing around 6.5-3.9 Ma [77, 79, 80]. The Upper Miocene was also the initial period of what was called the “Southern plains age” (SPA) (11-3 Ma), where open habitats dominated by grasses covered most of the southern part of the

continent (78, 81, 82). Thus, the initial development of the lineage that led to *A. balzani* coincides with the initial dominance of C₄ grasses, with a stable population during the whole SPA.

Regardless of the age used for the root of *A. balzani*, both values set the modifications of population sizes between the end of Upper Pliocene and the Pleistocene (3-0.4 Ma), which suggests that only more recent events could have influenced the species demography. In southern South America, the end of the SPA seems to be related to a decrease in the temperature and an increase in the aridity [88], coinciding with the beginning of the northern hemisphere glaciation (3.3-2.75 Ma) and the establishment of the bipolar glaciation between 2.8-1.9 Ma [84-86]. The currently available paleoclimate records for after the establishment of the bipolar glaciation points to a series of interglacial (six periods including the current one) and glacial periods (five periods) [87,88]. This makes difficult to pinpoint an specific event to account for such small population variations as seem in *A. balzani*, due to the fact that if there are population increases during glacial cycles (grassland expansion phase) and population reductions during interglacial cycles (forest expansion), one event can mask the effects of the other if their intensity is similar.

The divergence between *A. subterraneus subterraneus* and *A. subterraneus molestans* was of Pleistocene age (median 1.31 Ma), with a small but continuous increase in the population of *A. subterraneus subterraneus* after the split until the present. These taxa differ ecologically, as the first nests in humid and shaded habitats, while the second nests on open fields [74]. As *A. subterraneus subterraneus* differs in habitat from *A. balzani*, is expected that the two species present different demographic histories. The continuous increase in the population of *A. subterraneus subterraneus* contrasts with the small decrease in *A. balzani* over the same time, what suggests that over this period there was an increase in the habitat availability for the former species over areas where the latter had occurred previously. This is compatible with a scenario where forest expansion in Pleistocene favored the dispersion of associated Attini [89]. These results differs from the ones obtained by Solomon [90] for some species of the genus *Atta* as this author obtained older ages for the species studied (a. 13-5 Ma), which suggests that the responses of the species of the genera *Atta* and *Acromyrmex* to future climatic changes will be different. Although is not possible to pinpoint one glacial or interglacial event as cause of the population variation found in the studied *Acromyrmex*

species population variations, these variations are consistent with an increase in forests areas over more open habitats in the last 1 Ma.

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Considerações Finais

- Sugerimos que o subgênero *Moellerius* deixe de ser usado, sendo sinônimo de *Acromyrmex*;
- Sugerimos a elevação de *A. subterraneus molestans* a espécie e *A. subterraneus brunneus* sinonimizado sob *A. subterraneus subterraneus*.
- *Pseudoatta* sinonimizada sob *Acromyrmex*.
- As populações de *A. balzani* divergiram de *A. subterraneus* no Mioceno, próximo a 7.46 Ma (95% HPD 3.95-10.51 Ma), com uma constante expansão populacional entre 7-3 Ma, que coincide com a expansão de gramíneas C4, seguida de um pequeno aumento entre 3-1 Ma, e uma pequena queda após 1 Ma.
- *A. subterraneus subterraneus* e *A. subterraneus molestans* divergiram no Pleistoceno, entre 1,31 Ma (95% HPD 0,32-2,95 Ma), com um aumento pequeno, mas contínuo na população de *A. subterraneus subterraneus* após o desdobramento até o presente.

As ferramentas moleculares e os métodos de delimitação utilizados contribuíram para elucidar algumas questões taxonômicas em *Acromyrmex* já levantadas por outros autores, mas que aguardavam confirmação. Em nossas análises o GMYC indicou hipóteses adicionais para as relações específicas, porém estas hipóteses não foram validadas pelos outros métodos utilizados, e quando considerado a morfologia, essas delimitações propostas não foram biologicamente plausíveis. Os métodos de delimitação aqui utilizados, pela primeira vez empregados em formigas, revelaram uma perspectiva diferente da tradicional de espécies, porém as ferramentas computacionais utilizadas são complexas, em especial para o método BP&P, que varias vezes ficou preso em locais de verossimilhança sub-ótima, não analisando todas as topologias possíveis para alguns esquemas de delimitação de espécies utilizados (EPIC 965 e 1503). Assim, se considera que análises de delimitação de espécies devem ser validadas com mais de um método, mas considerando uma taxonomia integrada.