MARIANA NEVES MOURA

DIVERSIDADE E EVOLUÇÃO DO TAMANHO DE GENOMAS: UMA PERSPECTIVA EM FORMICIDAE E BROMELIACEAE

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

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APROVADA: 23 de março de 2018.

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(Coorientador)

Maykon Passos Cristiano
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"Nothing in biology makes sense except in the light of Evolution"

Theodosius Dobzhansky

A meus pais, Antônio Carlos e Marisa,
com todo meu amor e admiração, dedico.

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RESUMO

MOURA, Mariana Neves, D.Sc., Universidade Federal de Viçosa, março de 2018**. Diversidade e evolução do tamanho de genomas: uma perspectiva em Formicidae e Bromeliaceae.** Orientador: Maykon Passos Cristiano. Coorientador: Danon Clemes Cardoso.

O tamanho do genoma, também denominado conteúdo de DNA, quantidade de DNA ou DNA C-valor, foi descrito como um traço que "encontra-se exclusivamente na interseção do fenótipo e genótipo" e o tamanho do genoma em eucariotos varia em cinco ordens de magnitude, com uma distribuição enviesada em torno de valores pequenos, cerca de 2 picogramas (pg). Na família Formicidae o método para estimar o tamanho do genoma ainda é pouco difundido, o que torna difícil comparar estudos e compreender a evolução desse traço. Neste estudo, reunimos e investigamos a informação sobre o tamanho do genoma em formigas, compilando o conteúdo de DNA estimado. Analisamos também o método aplicado, o tipo de tecido utilizado e o padrão interno. Todos os valores foram colocados em uma árvore filogenética e os valores médios das subfamílias foram então comparados estatisticamente (Capítulo 1). Padronizamos um protocolo de citometria de fluxo (FCM) em formigas; analisamos, entre os padrões internos atualmente utilizados em citometria de fluxo para insetos, o que é o mais recomendado para formigas; avaliamos a eficácia da citometria de fluxo na análise de diferenças no conteúdo de DNA entre colônias de diferentes locais, ou seja, testamos a aplicabilidade da citometria de fluxo em estudos envolvendo genética de populações (Capítulo 2). Também expandimos o banco de dados de tamanho de genoma para Formicidae; estabelecemos um protocolo de determinação de composição de bases AT/GC através de citometria de fluxo em formigas; examinamos os padrões de distribuição e variação do tamanho do genoma e da composição de bases entre os táxons; fornecemos uma perspectiva filogenética sobre a evolução do tamanho de genoma na família e reconstruímos o estado ancestral desse traço (Capítulo 3). *Drosophila melanogaster* foi um padrão interno melhor do que *Scaptotrigona xanthotricha*, uma vez que a maioria das formigas estudadas possui tamanho do genoma (valor 1C) correspondente ao de *S. xanthotricha*, o que resulta em uma sobreposição de picos nos histogramas; como *D. melanogaster* tem um tamanho de genoma menor isso não acontece, sendo o padrão interno mais apropriado para formigas. O tampão Galbraith foi mais eficaz para

Myrmicinae e Pseudomyrmecinae, e LB01 para Ectatomminae e Ponerinae. O tampão OTTO também funcionou, mas com um coeficiente de variação maior que o considerado aceitável (<5%). No capítulo 2, o tamanho do genoma estimado variou entre as populações das quatro espécies estudadas essa variação pode estar relacionada ao estresse na colonização de novos ambientes. A citometria de fluxo mostrou ser um método eficaz de quantificação de DNA em formigas e aplicável em estudos de genética de populações. O coeficiente de variação entre os dias não foi significativamente diferente, o que reflete a eficácia do método e a estabilidade do equipamento. Novas estimativas de tamanho do genoma foram apresentadas para 100 espécies de formigas, 92 das quais correspondendo a novas espécies e o tamanho médio do genoma da família Formicidae foi de 0,38 pg. A relação AT/GC obtida para a família foi AT = 62,40% e GC = 37,60%. No capítulo 3, a família Formicidae foi recuperada como um clado com alto valor de probabilidade posterior na análise filogenética. O tamanho do genoma ancestral reconstruído para Formicidae foi de 0,38 pg de acordo com o método de Inferência Bayesiana e 0,37 pg de acordo com os métodos ML e Parcimônia e diferenças significativas no tamanho de genoma foram observadas entre as subfamílias amostradas. Os resultados deste estudo sugerem que a evolução do DNA C-valor em Formicidae ocorreu devido à perda e acumulação de regiões não codificadoras, principalmente elementos transponíveis e, em alguns casos específicos, por duplicação completa do genoma. No entanto, os processos por trás dessas expansões e retrações do genoma ainda precisam ser entendidos, principalmente através de estudos de diversificação de espécies, a fim de verificar se essas mudanças no conteúdo de DNA estão relacionadas ao processo de colonização da espécie, como sugerido no nível intraespecífico.

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ABSTRACT

MOURA, Mariana Neves, D.Sc., Universidade Federal de Viçosa, March, 2018**. Diversity and evolution of genome size: a perspective on Formicidae and Bromeliaceae.** Adviser: Maykon Passos Cristiano. Co-adviser: Danon Clemes Cardoso.

Genome size, also named DNA content, DNA amount or DNA C–value, has been described as a trait that 'uniquely lies at the intersection of phenotype and genotype' and the genome size of eukaryotes varies over five orders of magnitude, with a distribution skewed toward small values, around 2 picograms (pg). In Formicidae the method for estimating genome size is still less widespread, which makes it difficult to compare studies and to understand the evolution of this trait. In this study, we assemble and investigate the information about the genome sizes in ants, compiling the DNA content estimated so far. We also analyzed the method applied, the type of tissue used and the internal standard. All values were placed in a phylogenetic tree and the means of the subfamilies were then compared statistically (Chapter 1). We standardize a protocol of flow cytometry (FCM) analyses in ants; assay, among the internal standards currently used in flow cytometry for insects, which is the most recommended for ants; evaluate the efficacy of flow cytometry for analysis of differences in DNA content between colonies of different locations, that is, test the applicability of flow cytometry in studies involving population genetics (Chapter 2). We also expand the GS database of Formicidae; establish a protocol of base composition determination through flow cytometry in ants; examine the patterns of distribution and variation of genome size and base composition among taxa; and provide a phylogenetic perspective on GS evolution in the family and reconstruct the ancestral state of this character (Chapter 3). *Drosophila melanogaster* was a better internal standard than *Scaptotrigona xanthotricha*, since the majority of the studied ants have genome size (1C-value) corresponding to that of *S. xanthotricha*, which results in an overlapping of peaks on the histograms; as *D. melanogaster* has a tinier genome size, this does not happen being the most appropriate internal standard to studies with ants. The Galbraith buffer was most effective for Myrmicinae and Pseudomyrmecinae, and LB01 for Ectatomminae and Ponerinae. The OTTO buffer also worked, but with a coefficient of variation greater than that considered acceptable (<5%). The genome size estimated varied among populations of the four

species studied and this variation is more likely to be related to the stress in the colonization of new environments. Flow cytometry proved to be an effective method of DNA quantification in ants and to the application on population genetic studies. The coefficient of variation between days was not significantly different that reflect the effectiveness and stability of the method.. New genome size estimates were present for 100 species of ants, 92 of which corresponding to new taxa, and the mean genome size of Formicidae was 0,38 pg. The AT/GC ratio obtained for the Family was AT = 62,40% and GC = 37,60%. Formicidae family was recovered as monophyletic with high value of posterior probability on the phylogenetic analysis. The reconstructed ancestral genome size for Formicidae was 0,38 pg according the Bayesian Inference and 0,37 pg according to ML and parsimony methods and significant differences in GS were observed between the subfamilies sampled. The results of this study suggest that the evolution of GS in Formicidae was due to the loss and accumulation of non-coding regions, mainly transposable elements, and in some specific cases by whole genome duplication. However, the processes behind these genome enlargements and retractions still need to be understood, mainly through species diversification studies, in order to verify if these changes in DNA content are related to the colonization process of the species, thus as suggested at the intraspecific level.

SUMÁRIO

INTRODUÇÃO GERAL

A delimitação de espécies é fundamental no estudo dos organismos e é premissa básica em estudos de conservação e evolução. Existe um consenso quanto à necessidade de que o conceito de espécies delimite linhagens evolutivamente distintas, mas o critério utilizado difere segundo a percepção de importância das várias características das populações (Bacon et al., 2012). A delimitação de gêneros deve seguir a mesma lógica, apesar dos problemas neste nível taxonômico serem mais raros. Com o advento da biologia molecular, o conceito de linhagens, principalmente de espécies, se tornou um estudo multidisciplinar, englobando a utilização de caracteres morfológicos e moleculares dos organismos. A criação de bancos de dados genéticos por meio do sequenciamento de DNA tem proporcionado o conhecimento das informações genéticas de diferentes táxons, contribuindo para estudos sobre taxonomia, evolução e ecologia. Essa nova abordagem, que consiste na identificação de espécies usando um código de barras genético, é conhecida por taxonomia molecular (Bueno-Silva, 2012). No entanto, apesar de ser uma ferramenta muito útil, a obtenção de sequências de DNA de amostras biológicas é laborioso e com um custo elevado (Sandberg et al., 2009).

Uma alternativa a este problema seria a estimativa do tamanho de genoma do organismo (DNA C-valor) por meio de técnicas baseadas na obtenção da massa de DNA em unidades absolutas, como em picogramas (pg) ou mega pares de bases (Mbp). Um dos principais métodos utilizados para estimar o tamanho do genoma é a citometria de fluxo (Moscone et al., 2003), que envolve a análise das propriedades ópticas, principalmente fluorescência, de núcleos em suspensão (Dolezel et al., 2007). É considerada uma técnica rápida, acurada e com repetibilidade, visto que o

conteúdo total de DNA de uma quantidade representativa de núcleos pode ser mensurado em um curto período de tempo (Dolezel et al., 2007). Baseia-se no pressuposto da existência de uma relação linear entre quantidade de DNA e fluorescência emitida pelos núcleos da amostra de interesse e do padrão interno de referência, que possui um conteúdo de DNA conhecido (Zoldo et al., 1998).

A Citometria de fluxo foi originalmente utilizada para identificar e caracterizar células cancerígenas através do seu conteúdo de DNA e agora se tornou cada vez mais útil no contexto de ecologia, biologia evolutiva e sistemática, especialmente nos últimos 15 anos. A metodologia envolve a seleção de tecidos adequados, isolamento dos núcleos celulares, coloração com fluorocromos específicos de DNA e leitura da amostra em um citômetro de fluxo (Kron et al., 2007). Segundo Galbraith et al. (1983) uma grande diversidade de fluorocromos é empregada para quantificação do tamanho de genoma, e estão principalmente divididos em dois grupos: os corantes intercalantes de DNA, incluindo iodeto de propídio (IP) e brometo de etídio, e os base-específicos, incluindo mitramicina, cromomicina (CMA), olivomicina, 4,6-Diamidino-2-fenilindol (DAPI) e Hoechst.

O conteúdo de DNA haploide (C-valor, em picogramas) está disponível para 6.222 espécies de animais (3.793 vertebrados e 2.429 não vertebrados), e os insetos representam quase 21,6% desse total (Gregory, 2018). As formigas representam o grupo mais numeroso dentre os insetos, e a distribuição do tamanho de genoma ainda é pouco conhecida principalmente devido a grande riqueza e diversidade de espécies. São organismos particularmente interessantes pois formam níveis avançados de sociedade: a eussocialidade. As formigas, algumas vespas e abelhas, são considerados insetos eussociais, compondo a ordem Hymenoptera, porém apenas as

formigas apresentam eussocialidade em todas as espécies do grupo (Hölldobler & Wilson, 1990). As formigas estão incluídas em uma única família, Formicidae, com aproximadamente 13.369 espécies descritas até este ano, distribuídas por todas as regiões do planeta, exceto nas regiões polares (Bolton, 2018). Representam um importante grupo de insetos para investigar a relação entre as linhagens genealógicas e os padrões de distribuição das espécies, devido à ocorrência onipresente nos diferentes habitats dos mais diversos ecossistemas (Goodisman et al., 2008; Cristiano, 2013).

Atualmente a família Formicidae é considerada um grupo monofilético e está dividida em 17 subfamílias existentes e mais 3 subfamílias extintas (Bolton, 2018). Em termos locais e mundiais a subfamília Myrmicinae é considerada a maior e mais diversificada subfamília de formigas (Françoso & Brandão, 1993; Brandão, 1999). Esta é uma subfamília importante pelo grande número, variabilidade de espécies e por abranger as espécies com maior grau de complexidade social dentro a ordem Hymenoptera (Jaffe, 2004). Dentro de Myrmicinae, a tribo Attini se destaca por compreender as formigas cultivadoras de fungos (cerca de 250 espécies; Mehdiabadi & Schultz, 2010; Branstetter et al., 2017), comportamento complexo que surgiu apenas quatro vezes na história evolutiva (Mueller et al., 2005). Essas espécies possuem uma relação mutualística com fungos basidiomicetos, onde estes são cultivados como fonte de alimentação e recebem em retorno proteção contra patógenos e competidores (Weber, 1972; Hölldobler & Wilson, 1990). Nesse grupo encontram-se formigas de grande importância econômica, como as conhecidas formigas cortadeiras, *Atta* e *Acromyrmex*, endêmicas do continente americano (Forel, 1895; Mikheyev et al., 2008). Essas formigas são especializadas em utilizar diversas

espécies de vegetais para suprir o fungo cultivado, causando sérios prejuízos à agricultura (Weber, 1966). Entretanto, apesar de causar danos aos sistemas agrícolas, as formigas cortadeiras desempenham papéis importantes na manutenção dos ecossistemas tropicais da América, participando especialmente da ciclagem de compostos vegetais (Wirth et al., 2003).

Muitas das questões atuais sobre a variação do tamanho do genoma exigem dados adicionais provenientes de uma grande distribuição taxonômica e, apesar da utilidade das estimativas de tamanho do genoma, pouco se sabe sobre a distribuição dos valores do conteúdo de DNA haploide dentro da família Formicidae e sobre os mecanismos responsáveis pela variação entre organismos, principalmente entre espécies estreitamente relacionadas. Segundo Boomsma et al. (2017) nos últimos sete anos, 20 genomas de formiga completamente sequenciados foram publicados, colocando as formigas entre as famílias de insetos genômicamente mais conhecidas. No entanto, uma comparação em larga escala sobre a diversidade genômica em toda família ainda é desconhecida.

O principal objetivo da presente tese de doutorado foi estudar o tamanho de genoma em formigas utilizando uma grande diversidade de espécies. A tese está estruturada em três capítulos cujo tema norteador é o tamanho de genoma, porém cada capítulo apresenta uma abordagem especifica sobre o assunto. No primeiro capítulo foi realizado uma revisão de literatura a respeito do que se conhece do tamanho de genoma para família Formicidae. Dados do *Animal Genome Size Database* (Gregory, 2018) e de outros artigos foram compilados com o intuito de verificar o que foi publicado até o momento sobre variação do tamanho de genoma entre as subfamílias, gêneros e espécies. Ainda, foi realizado um levantamento do

número cromossômico dessas espécies e de quais são as hipóteses relacionadas às variações encontradas nos tamanho do genoma. O segundo capítulo está centrado no estabelecimento de um protocolo para obtenção do tamanho de genoma em formigas por meio da citometria de fluxo (FCM). Foram testados os tampões de lise para suspensão dos núcleos mais utilizados na ordem Hymenoptera, especialmente em formigas, e os dois padrões internos de referência mais utilizados, *Drosophila melanogaster* e *Scaptotrigona xantotricha*, com o intuito de verificar qual o mais recomendado para formigas. Além disso, avaliou-se a aplicabilidade da citometria de fluxo em genética de populações, na medida em que dados de tamanho de genoma foram utilizados para analisar diferenças entre colônias da mesma espécie provenientes de diferentes localidades. O terceiro e último capítulo teve por objetivo expandir a base de dados de tamanho de genoma para a família Formicidae para averiguar a amplitude de variação desse traço e estabelecer um protocolo para determinação da composição de bases AT/GC em formigas. Além disso, uma análise evolutiva do tamanho de genoma em Formicidae foi conduzida por meio da reconstrução do genoma ancestral sob uma perspectiva filogenética. Os dados obtidos neste estudo contribuirão significativamente para o delineamento de espécies e populações, para o entendimento da variação e a evolução do tamanho de genoma..

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Capítulo I

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GENOME SIZE IN ANTS: RETROSPECT AND PROSPECT

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ABSTRACT

Genome size is very useful in studies regarding taxonomy, evolution, and reproductive biology in many animal groups, including insects. Herein, we review the information about genome size in ants, compiling the DNA content estimated so far. We also analyzed methods applied, types of tissues used, and internal standards included in each study. All values were placed in a phylogenetic tree and the means of the subfamilies were then compared statistically. The compiled data resulted in 86 specimens of ants, comprising 69 different species. This number represents 0.52% of the total number of 13,369 ant species described, covering only 40 from 333 valid extant genera. The average genome size for the Formicidae family was 0.36 pg (± 0.13). Most of the estimates were obtained through flow cytometry (83.5%), commonly using brain tissues, with *Drosophila melanogaster* as internal standard (76%). We propose that the differences in DNA content found between ant species may be related to the different amounts of heterochromatin in the chromosomes as it is not related with chromosome number or body size. The genome size estimations

currently available in the literature for ants has highlighted the scarcity of estimates for this hyper–diverse family. Furthermore, we conclude that the standardization of the techniques used and a large–scale study of the ant genome size are urgently required, given the importance of this group and the need for improvement in our knowledge of ant evolution.

Keywords: C-value, DNA content, Genetic diversity, Genome, Evolution, Phylogeny.

Introduction

Ants comprise a monophyletic group with approximately 13,369 valid species distributed throughout the planet, with exception of extreme northern and southern latitudes (Bolton, 2018). They are one of the largest groups among insects in species diversity and biomass and together with some wasps and bees, are known as eusocial insects, being part of the order Hymenoptera (Hölldobler and Wilson, 1990; Ardila–Garcia et al., 2010). They represent an important group of insects to investigate the relationship between the genealogical lineages and the distribution patterns of species, due to their occurrence in different habitats of the most diverse ecosystems (Goodisman et al., 2008). Currently, the family Formicidae is divided into 17 extant and 3 extinct subfamilies, spanning 333 valid extant genera and 154 extinct genera (Bolton, 2018). The subfamily Myrmicinae is the largest and most diverse subfamily worldwide, covering about 47% of all species of the family (Françoso and Brandão, 1993; Brandão, 1999).

Genome size, also named DNA content, DNA amount, or DNA C–value, has been described as a trait that ‘uniquely lies at the intersection of phenotype and

genotype', and the genome size of eukaryotes varies over five orders of magnitude, with a distribution skewed toward small values, around 2 picograms (pg) (Oliver et al., 2007). This variation does not seem to be correlated with the complexity of the organism or with the number of genes in eukaryotes, leading to what is called the "C–value paradox" (Moore, 1984; Gregory, 2001, 2005a; Eddy, 2012). It has been questioned, for example, why similar organisms with similar amounts of coding sequence have different amounts of DNA. While changes in gene sequences are often slow and gradual, changes in genome size can be rapid and abrupt as a consequence of chromosomal rearrangements or duplications (Alberts et al., 2007).

The main methods used to estimate the total nuclear genome size are image cytometry, flow cytometry (FCM), and complete genome sequencing (Gregory, 2005b). Image cytometry was the first method used to determine genome size estimates. Basically, it operates by statically imaging a large number of cells stained with specific staining chemicals, or fluorochromes, using optical microscopy (Torresan et al., 1994; Basiji et al., 2007). In contrast, flow cytometry evaluates the relative fluorescence intensity of suspended nuclei, also stained with specific fluorochromes, and presents the data in a typical histogram with a higher peak relative to the nuclei in the G0/G1 phase of cell cycle, and a lower peak, relative to the nuclei in G2 phase (Price et al., 2000; Dolezel and Bartos, 2005). The complete genome sequencing method, on the other hand, provides the complete DNA sequence of the genome of an organism at a single time with the precise order of the nucleotides and an estimate of the genome size after its assembly (Klug et al., 2014). A fourth less common technique known as biochemical analysis (BCA) was used during the early stages of genome size study. It involves 'the chemical extraction and

quantification of DNA combined with cell counts to give an average DNA amount per nucleus or the reassociation kinetics, in which the DNA molecule was denatured and then the time taken for the strands to reanneal used to calculate the amount of DNA present' (Gregory, 2005b). Among the methods, flow cytometry has been shown to be the least expensive technique when compared to other molecular tools and provides rapid generation of accurate results (Merkel et al., 1987; Doležel et al., 2007).

According to Gregory (2018), haploid DNA contents (C–values, in picograms) are currently available for 6,222 species of animals (3,793 vertebrates and 2,429 invertebrates), with insects representing 21.6% of this total. The first DNA content estimation of an ant specie was conducted by Li and Heinz (2000), in which biochemical analysis (BCA) was used to quantify the genome of *Solenopsis invicta.* Subsequently, Johnston et al. (2004) also estimated the genome size of *S. invicta* but using flow cytometry. In 2008, Tsutsui et al. (2008) carried out the first comprehensive study regarding the evolution of the genome size in ants, reporting genome size estimates for 40 species from nine distinct subfamilies. This was the last inclusion of a large number of ant species estimates to the genome size database that was followed by the study of Ardila–Garcia et al. (2010), which added a further 29 species. These two studies raised different questions about genome size, being the first a study of genome size evolution in Formicidae family and the second a study of correlation between genome size with parasitism or eusociality in the order Hymenoptera as a whole. Also, they applied distinct methodologies in genome size estimation: in Tsutsui et al. (2008) the DNA content was estimated by using only flow cytometry, while Ardila–Garcia et al. (2010) also performed the FIAD method

(Feulgen image analysis densitometry) to estimate the DNA content, and contrasted the results from both techniques.

Later, others studies explored the DNA content of ants, however in some cases covering only one species through complete genome sequencing (*e.g.* Nygaard et al., 2011) or, in other cases, considering specifically an ant genus through flow cytometry. The genome size of the genus *Mycetophylax* (*sensu* Klingenberg and Brandão, 2009) was estimated by Cardoso et al. (2012) that explored the data placing them in a phylogenetic context, also correlating with chromosome number of fungus–growing ants; and Aguiar et al. (2016) that evaluated three *Camponotus* species, exploring their correlation with the karyotype of the studied species.

Despite of the importance of genome size, little is know about the ecological e evolutionary consequences of DNA amount on ants. Yet, the biological significance and evolution of the genome size diversity in other groups has received much more attention over the last decades (Dufresne and Jeffery, 2011; Alfsnes et al., 2017; Pellicer et al., 2018). The diversity of genome size in plants has been proved to influence several phenotypic features of cells and ultimately the organisms, for instance plant with higher genomes are adapted to xeric and higher elevation environments (*e.g.* Bottini et al., 2000). Here, we review the information about the genome size of ants, compiling the DNA content estimated so far in order to provide insights into the distribution, evolution and possible consequences of ant genome size diversity. We have also investigated and verified the need of a re–evaluation in the genome size data (DNA C–value) for ants, as well the technique used in the estimation of the DNA content in respect of methodological issues such as internal standard and tissues used in the analysis. The basic information about ant genomes

analyzed here may improve our knowledge about the evolution and diversification concerning this diverse group of insects and serve as a baseline and guidance for future genome size studies.

Materials and Methods

In this review, we compiled the haploid genome size estimates for ants and others insect groups from the Animal Genome Size Database (Gregory, 2018) and from the literature by means of searching the publication databases Scopus® and Web Science Knowledge™, using the terms “genome size”, “DNA amount”, “C–value” and “ants”. In addition, we analyzed the method applied, the type of tissue used to obtain the total content of DNA and the internal standard.

To analyze the genome size variation over Formicidae subfamilies we compiled in a Table all the values available in the literature, expressed in picograms of DNA (pg) and mega base pairs (Mbp), and manually placed them in the phylogenetic tree proposed by Moreau and Bell (2013), collapsing branches with equal names and separating the subfamilies by color. General linear models were built to check for differences between the average genome sizes of the sampled subfamilies. The differences in genome size average for each subfamily were assessed by variance analysis of the GLM. When the p-value of ANOVA was significant ($p < 0.05$), a contrast analysis at 5% level was then performed through "coms.R" to check which was different. The statistical analysis was performed in R v2.15.1 software (R Core Team, 2013) and GLM was submitted to residual analysis to evaluate adequacy of the error distribution (Crawley, 2013).

Results and Discussion

Overview: number of estimates, methods, tissues and internal standards used

The compiled data resulted in 86 specimens of ants whose genome size had been estimated, comprising 69 different species (Table 1). This number represents 0.52% of the total number of 13,369 ant species accepted until now, covering only 40 genera from 333 accepted (Bolton, 2018).

From 17 existing subfamilies, we only found estimates for nine, with Myrmicinae having the largest number of species evaluated (32 spp.) (Figure 1). This may reflect the diversity of this subfamily which is the most diverse within Formicidae, followed by Formicinae and Dolichoderinae, that together presents 20 spp. with DNA content estimates available. These three subfamilies together represent 65% of DNA content estimates of Formicidae.

The two main methods used to estimate DNA content in ants were FCM and FIAD. A third method, biochemical analysis (BCA), was used in a pioneering work for this type of study and generated the quantification of genome size only for *Solenopsis invicta*. This species has the genome size estimated by all three methods listed above, a different value was obtained in each estimate: 0.60 pg with BCA (Li and Heinz, 2000), 0.47 pg with FIAD (Ardila-Garcia et al., 2010) and 0.77 pg with flow cytometry (Johnston et al., 2004). All genome sizes are obtained through a known genome value, this being the internal standard. As internal standard, *Drosophila melanogaster* with 0.18 pg, *Scaptotrigona xantotricha* with 0.43 pg and *Tenebrio molitor* with 0.52 pg are commonly used in genome size estimates for order Hymenoptera as a whole. Most of the estimates were obtained using *Drosophila melanogaster* as internal standard (76%), while FCM was the most common method

used (83.5%). Generally, brain tissue is used to estimate nuclear genome size, but cells (hemocytes) obtained through hemolymph smears have also been tested (Ardila-Garcia et al., 2010).

As observed in the work of Ardila-Garcia et al. (2010), in species which had the genome measured by both FIAD and FCM, such as *Odontomachus brunneus*, *Pseudomyrmex gracilis*, and *Solenopsis invicta*, estimates using the first method tended to be smaller. However, it is difficult to say that this difference is solely due to the technique itself, since both the tissue and the internal standard used during the analysis were different.

The nuclear DNA content of some ants has also been measured using a fourth method which utilized complete genome sequencing techniques, in species such as *Acromyrmex echinatior* (Nygaard et al., 2011), *Atta cephalotes* (Suen et al., 2011), *Camponotus floridanus* (Bonasio et al., 2010), *Harpegnathos saltator* (Bonasio et al., 2010), *Linepithema humile* (Smith et al., 2011), *Pogonomyrmex barbatus* (Smith et al., 2011) and *Solenopsis invicta* (Wurm et al., 2011) (Table 1). The genome size of *Acromyrmex echinatior* was 313 Mbp (or 0.32 pg considering 1 pg = 978 Mbp; (Doležel et al., 2003)) obtained with complete genome sequencing (Nygaard et al., 2011) and 335 Mbp (0.36 pg) by FCM (Sïrvio et al., 2006). This difference can be attributed to the loss of repetitive regions and some chromosomal regions, such as telomeres, through genome sequencing techniques (Gregory, 2005b). The same was observed in *Atta cephalotes*, whose genome size was estimated by complete genome sequencing to be 290 Mbp (approximately 0.30 pg) (Suen et al., 2011) and by FCM was 303.18 Mbp (approximately 0.31 pg) **(**Tsutsui et al., 2008). The differences were greater in *Solenopsis invicta*, whose genome size was obtained with all four different

techniques (BCA, FIAD, FCM, and Genome Sequencing): 606 Mbp (0.62 pg) (Li and Heinz, 2000) with BCA, 459 Mbp (0.47 pg) (Ardila-Garcia et al., 2010) with FIAD, 753 Mbp (0.77 pg) (Johnston et al., 2004) with FCM and 482 Mbp (0.49 pg) (Wurm et al., 2011) with genome sequencing. Values obtained with FIAD and genome sequencing were most similar. So, considering the loss of certain regions of DNA by the complete genome sequencing and the difficulties in using other techniques such as BCA and FIAD (mainly due to the low number of repetitions available to estimate de DNA amount) the use of FCM has proven to be the most efficient methodology to obtain the total DNA content of a species.

Genome size evolution

DNA C–value of insects reported range from 0.07 pg (*Clunio tsushimensis* – Diptera) to 16.93 pg (*Podisma pedestris* – Orthoptera) and of the 1344 estimates found, 1224 (91%) were comprised of values between 0.07 to 2.00 pg (Gregory, 2018). From 27 orders of insects, 24 currently have estimates of genome size, with Diptera accounting for the largest number of measurements (386 specimens, 29% of the total), followed by Coleoptera (278 specimens, 21% of the total) and Hymenoptera (240 specimens, 18% of the total). The average genome size for the Formicidae family (Hymenoptera) was 0.36 pg (± 0.13), with values ranging from 0.18 pg (the smallest value, found in Dolichoderinae and in Formicinae) to 0.77 pg in *Solenopsis invicta* (Myrmicinae) (Table 1; Figure 2), thus always being less than 1 pg. This is in accordance with a pattern already observed for others eukaryotes that most of the distribution of genome size is skewed towards smaller values (Oliver et al., 2007), since it is evident that the number of species declines as the genome doubles in size.

As can be seen in Figure 2 the variation of genome size among species of a subfamily is similar to the variation found between subfamilies. Significant differences in GS were observed between the subfamilies sampled (ANOVA, *p-value* < 0.01). Through contrast analysis, most of the subfamilies grouped statistically (group average = 0.34 pg, *p-value* > 0.05) except for Ponerinae whose average was different from the others (average = 0.47, *p-value* < 0.01). Ectatomminae (*Ectatomma tuberculatum*, 0.71 pg) and Myrmeciinae (*Myrmecia varians*, 0.28 pg) were not considered in the analysis because only one value for each was available, so it was not possible to calculate a mean for the comparison test (Figure 2). Differences in the genome size were also observed between genera within the sampled subfamilies and mainly between species of the same genus, as observed in *Atta spp*. (*e.g. Atta cephalotes* = 0.31 pg and *Atta texana* = 0.27 pg), *Camponotus spp.* (*e.g. Camponotus floridanus* = 0.23 pg and *Camponotus pennsylvanicus* = 0.33 pg) and *Odontomachus spp.* (*e.g. Odontomachus brunneus* = 0.33 pg and *Odontomachus chelifer* = 0.54 pg) (Table 1, Figure 2). These differences in genome size among closely related species have been associated in several studies with the amount of heterochromatin in the chromosomes (Lopes et al., 2009; Tavares et al., 2010; Cardoso et al., 2012), transposable elements (Kidwell, 2002; Vieira et al., 2002) and other repetitive genome sequences (Gregory and Hebert, 1999; Petrov, 2001). In some species, as *Ectatomma tuberculatum* and *Apterostigma dentigerum* this differences in genome size was also been related with whole genome duplication events given the large genome size of this both species when compared with the others of Formicidae (0.71 pg and 0.65 pg, respectively) (Tsutsui et al., 2008).

The correlation between genome size and chromosome number has been reported in some studies for ants, for example, Cardoso et al. (2012) with fungus–growing ants. In their study, they found a relationship between these two characteristics being *Sericomyrmex amabilis* the species with the highest number of chromosomes and also the largest genome size. In contrast, the other two species of the genus with the lowest number of chromosomes also had a smaller genome size. Ardila–Garcia and Gregory (2009) also found this positive correlation among species of damselflies, but not in dragonflies (Insecta: Odonata). Lack of correlation between genome size and chromosome number has been shown in the highly eusocial stingless bees of the tribe Meliponini (Hymenoptera: Apidae) (Tavares et al., 2012). Yet, body size was correlated with genome size among dragonflies and damselflies (Ardila–Garcia and Gregory, 2009), but not among stingless bees (Tavares et al., 2010) or ants (Tsutsui et al., 2008). These contradictory observations remain the issue whether genome size is shaped by neutral or natural selection.

However, it has been proven that changes in genome size are related to the addition and deletion of heterochromatin and that species with low amounts of heterochromatin also have lower DNA content per haploid nucleus, likewise the reverse is also true (Tavares et al., 2017). Therefore, we propose that the differences in DNA content among ant species may also be related to the different amounts of heterochromatin in the chromosomes as it is not related with chromosome number or body size. Nevertheless, this can only be confirmed after a detailed study of chromosomal structure and chromosome counts across genera and subfamilies.

Conclusions and Perspectives

The compilation of the genome size data currently available in the literature for ants has highlighted the scarcity of estimates for this hyper–diverse family (with only 0.52% of known species having been estimated). Little is known about the methodologies employed and the lack of standardization of the works makes it problematic to compare the different estimates (Ardila–Garcia et al., 2010; Doležel and Greilhuber, 2010), especially regarding which buffer, tissue and internal standard were used. Also, the mechanisms involved in the evolution of the genome in ants are still unknown, especially those related to the total amount of heterochromatin in chromosomes and their correlation with genome size; the whole–genome duplication events, which could explain the large variation of the genome of some species, such as *Ectatoma tuberculatum* and *Apterostigma dentigerum* (Tsutsui et al., 2008); and polyploidy events as in *Solenopsis invicta* males (Glancey et al., 1976; Lorite and Palomeque, 2010). This review makes clear the importance and ease of use of FCM to estimate the genome size of species and the possibility of obtaining robust results, since a large number of nuclei are analyzed to determine the DNA content thus making sampling more efficient. Therefore, the standardization of the techniques used and a large–scale study of the ant genome size are urgently required, given the ecological and economic importance of this group and the need for improvement in our knowledge of ant evolution by utilizing cheaper molecular tools of study.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 - Overview of the genome size data available in literature for Formicidae species

Species	Subfamily	1C-value (pg)	1C-value (Mbp)	Method	Cell type	Standard	References
Amblyopone pallipes	Amplyoponinae	0.34	332.52	FCM	BR	DM	Tsutsui et al., 2008
Amblyopone pallipes	Amplyoponinae	0.37	361.86	FCM	BR	DM	Ardila-Garcia et al., 2010
Dolichoderus mariae	Dolichoderinae	0.18	176.04	FCM	BR	DM	Ardila-Garcia et al., 2010
Dolichoderus taschenbergi	Dolichoderinae	0.23	224.94	FCM	BR	DM	Ardila-Garcia et al., 2010
Dormyrmex bicolor	Dolichoderinae	0.25	244.5	FCM	BR	DM	Tsutsui et al., 2008
Dorymyrmex bureni	Dolichoderinae	0.18	176.04	FIAD	HE	TM	Ardila-Garcia et al., 2010
Forelius pruinosus	Dolichoderinae	0.22	215.16	FIAD	HE	TM	Ardila-Garcia et al., 2010
Linepithema humile	Dolichoderinae	0.26	254.28	FCM	BR	DM	Tsutsui et al., 2008
Linepithema humile	Dolichoderinae	0.26	250.8	Genome sequencing	NS	NS	Smith et al., 2011
Liometopum occidentale	Dolichoderinae	0.29	283.62	FCM	BR	DM	Tsutsui et al., 2008
Tapinoma sessile	Dolichoderinae	0.37	361.86	FCM	BR	DM	Ardila-Garcia et al., 2010
Tapinoma sessile A	Dolichoderinae	0.38	371.64	FCM	BR	DM	Tsutsui et al., 2008
Tapinoma sessile B	Dolichoderinae	0.61	596.58	FCM	BR	DM	Tsutsui et al., 2008
Cerapachys edentata	Dorylinae	0.22	215.16	FCM	BR	DM	Tsutsui et al., 2008
Eciton burchelli	Dorylinae	0.27	264.06	FCM	BR	DM	Tsutsui et al., 2008
Labidus coecus	Dorylinae	0.37	361.86	FCM	BR	DM	Tsutsui et al., 2008
Ectatomma tuberculatum	Ectatomminae	0.71	694.38	FCM	BR	DM	Tsutsui et al., 2008
Camponotus castaneus	Formicinae	0.31	303.18	FCM	BR	DM	Tsutsui et al., 2008
Camponotus crassus	Formicinae	0.29	283.62	FCM	BR	SX	Aguiar et al., 2016
Camponotus floridanus	Formicinae	0.23	224.94	FIAD	HE	TM	Ardila-Garcia et al., 2010
Camponotus floridanus	Formicinae	0.245	240	Genome sequencing	NS	NS	Bonasio et al., 2010
Camponotus pennsylvanicus	Formicinae	0.33	322.74	FCM	BR	DM	Tsutsui et al., 2008
Camponotus renggeri	Formicinae	0.29	283.62	FCM	BR	SX	Aguiar et al., 2016
Camponotus rufipes	Formicinae	0.29	283.62	FCM	BR	SX	Aguiar et al., 2016

Species	Subfamily	1C-value (pg)	1C-value (Mbp)	Method	Cell type	Standard	References
Formica pallidifulva	Formicinae	0.39	381.42	FCM	BR	DM	Tsutsui et al., 2008
Lasius (Acanthomyops) latipes	Formicinae	0.27	264.06	FCM	BR	DM	Ardila-Garcia et al., 2010
Lasius alienus	Formicinae	0.31	303.18	FCM	BR	DM	Tsutsui et al., 2008
Lasius minutus	Formicinae	0.23	224.94	FCM	BR	DM	Ardila-Garcia et al., 2010
Paratrechina longicornis	Formicinae	0.18	176.04	FIAD	HE	TM	Ardila-Garcia et al., 2010
Prenolepis imparis	Formicinae	0.30	293.4	FCM	BR	DM	Tsutsui et al., 2008
Myrmecia varians	Myrmeciinae	0.28	273.84	FCM	BR	DM	Tsutsui et al., 2008
Acromyrmex echinatior	Myrmicinae	0.36	335	FCM	BR	CRBC	Sïrvio et al., 2006
Acromyrmex echinatior	Myrmicinae	0.32	313	Genome sequencing	NS	NS	Nygaard et al., 2011
Aphaenogaster (rudis-texana group N16)	Myrmicinae	0.43	420.54	FCM	BR	DM	Ardila-Garcia et al., 2010
Aphaenogaster (rudis-texana group N17)	Myrmicinae	0.46	449.88	FCM	BR	DM	Ardila-Garcia et al., 2010
Aphaenogaster (rudis-texana group N22b)	Myrmicinae	0.44	430.32	FCM	BR	DM	Ardila-Garcia et al., 2010
Aphaenogaster fulva	Myrmicinae	0.42	410.76	FCM	BR	DM	Ardila-Garcia et al., 2010
Aphaenogaster treatae	Myrmicinae	0.50	489	FCM	BR	DM	Ardila-Garcia et al., 2010
Apterostigma dentigerum	Myrmicinae	0.65	635.7	FCM	BR	DM	Tsutsui et al., 2008
Atta cephalotes	Myrmicinae	0.31	303.18	FCM	BR	DM	Tsutsui et al., 2008
Atta cephalotes	Myrmicinae	0.30	290	Genome sequencing	NS	NS	Suen et al., 2011
Atta columbica	Myrmicinae	0.31	303.18	FCM	BR	DM	Tsutsui et al., 2008
Atta texana	Myrmicinae	0.27	264.06	FCM	BR	DM	Ardila-Garcia et al., 2010
Crematogaster hespera	Myrmicinae	0.28	273.84	FCM	BR	DM	Tsutsui et al., 2008
Eurhopalothrix procera	Myrmicinae	0.39	381.42	FCM	BR	DM	Tsutsui et al., 2008
Messor andrei	Myrmicinae	0.26	254.28	FCM	BR	DM	Tsutsui et al., 2008
Monomorium viride	Myrmicinae	0.50	489	FIAD	HE	TM	Ardila-Garcia et al., 2010
Mycetophylax conformis	Myrmicinae	0.32	312.96	FCM	BR	SX	Cardoso et al., 2012
Mycetophylax morschi	Myrmicinae	0.32	312.96	FCM	BR	SX	Cardoso et al., 2012
Mycetophylax simplex	Myrmicinae	0.39	381.42	FCM	BR	SX	Cardoso et al., 2012

Species	Subfamily	1C-value (pg)	1C-value (Mbp)	Method	Cell type	Standard	References
Myrmecina americana A	Myrmicinae	0.26	254.28	FCM	BR	DM	Tsutsui et al., 2008
Myrmecina americana B	Myrmicinae	0.31	303.18	FCM	BR	DM	Tsutsui et al., 2008
Pheidole dentata	Myrmicinae	0.24	234.72	FIAD	HE	TM	Ardila-Garcia et al., 2010
Pheidole floridana	Myrmicinae	0.21	205.38	FIAD	HE	TM	Ardila-Garcia et al., 2010
Pheidole hyatti	Myrmicinae	0.33	322.74	FCM	BR	DM	Tsutsui et al., 2008
Pogonomyrmex badius	Myrmicinae	0.27	264.06	FCM	BR	DM	Tsutsui et al., 2008
Pogonomyrmex barbatus	Myrmicinae	0.24	235	Genome sequencing	NS	NS	Smith et al., 2011
Pogonomyrmex californicus	Myrmicinae	0.25	244.5	FCM	BR	DM	Tsutsui et al., 2008
Pogonomyrmex coarctatus	Myrmicinae	0.29	283.62	FCM	BR	DM	Tsutsui et al., 2008
Pyramica rostrata	Myrmicinae	0.28	273.84	FCM	BR	DM	Tsutsui et al., 2008
Sericomyrmex amabilis	Myrmicinae	0.45	440.1	FCM	BR	DM	Tsutsui et al., 2008
Solenopsis invicta	Myrmicinae	0.62	606.36	BCA	BR	NS	Li and Heinz 2000
Solenopsis invicta	Myrmicinae	0.77	753.06	FCM	BR	DM	Johnston et al., 2004
Solenopsis invicta	Myrmicinae	0.47	459.66	FIAD	HE	TM	Ardila-Garcia et al., 2010
Solenopsis invicta	Myrmicinae	0.49	482	Genome sequencing	NS	NS	Wurm et al., 2011
Solenopsis molesta	Myrmicinae	0.38	371.64	FCM	BR	DM	Ardila-Garcia et al., 2010
Solenopsis xyloni	Myrmicinae	0.48	469.44	FCM	BR	DM	Tsutsui et al., 2008
Temnothorax ambiguus	Myrmicinae	0.31	303.18	FCM	BR	DM	Ardila-Garcia et al., 2010
Temnothorax texanus	Myrmicinae	0.32	312.96	FCM	BR	DM	Ardila-Garcia et al., 2010
Tetramorium caespitum	Myrmicinae	0.26	254.28	FCM	BR	DM	Tsutsui et al., 2008
Tetramorium caespitum	Myrmicinae	0.27	264.06	FCM	BR	DM	Ardila-Garcia et al., 2010
Trachymyrmex septentrionalis	Myrmicinae	0.25	244.5	FIAD	HE	TM	Ardila-Garcia et al., 2010
Dinoponera australis	Ponerinae	0.57	557.46	FCM	BR	DM	Tsutsui et al., 2008
Harpegnathos saltator	Ponerinae	0.34	330	Genome sequencing	NS	NS	Bonasio et al., 2010
Odontomachus bauri	Ponerinae	0.49	479.22	FCM	BR	DM	Tsutsui et al., 2008
Odontomachus brunneus	Ponerinae	0.33	322.74	FIAD	HE	TM	Ardila-Garcia et al., 2010

Species	Subfamily	1C-value (pg)	1C-value (Mbp)	Method	Cell type	Standard	References
Odontomachus brunneus	Ponerinae	0.44	430.32	FCM	BR	DM	Tsutsui et al., 2008
Odontomachus cephalotes	Ponerinae	0.43	420.54	FCM	BR	DM	Tsutsui et al., 2008
Odontomachus chelifer	Ponerinae	0.54	528.12	FCM	BR	DM	Tsutsui et al., 2008
Odontomachus clarus	Ponerinae	0.42	410.76	FCM	BR	DM	Tsutsui et al., 2008
Odontomachus haematodus	Ponerinae	0.51	498.78	FCM	BR	DM	Tsutsui et al., 2008
Ponera pennsylvanica	Ponerinae	0.55	537.9	FCM	BR	DM	Ardila-Garcia et al., 2010
Ponera pennsylvanica	Ponerinae	0.60	586.8	FCM	BR	DM	Tsutsui et al., 2008
Pseudomyrmex ejectus	Pseudomyrmicinae	0.29	283.62	FIAD	HE	TM	Ardila-Garcia et al., 2010
Pseudomyrmex gracilis	Pseudomyrmicinae	0.35	342.3	FCM, FIAD	BR, HE	DM, TM	Ardila-Garcia et al., 2010
Pseudomyrmex gracilis	Pseudomyrmicinae	0.40	391.2	FCM	BR	DM	Tsutsui et al., 2008

Legend. Method: FCM = Flow cytometry, FIAD = Feulgen image analysis densitometry; Cell type: BR = Brain tissue, HE = Haemocyte; Standard: DM = Drosophila melanogaster, CRBC = Chicken Red Blood Cells, SX = Scaptotrigona xantotricha, TM = Tenebrio molitor, NS = not specified.

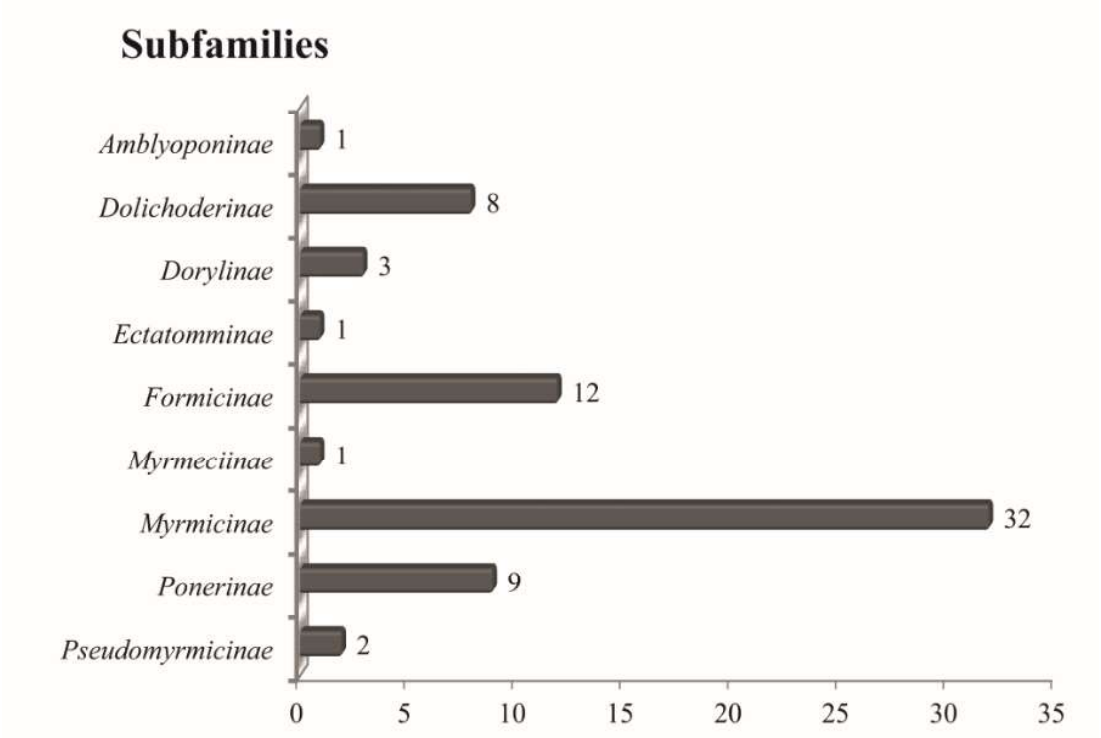

Figure 1 Number of published species presenting estimates of genome size per Formicidae subfamily. The list of species are presented in Table 1.

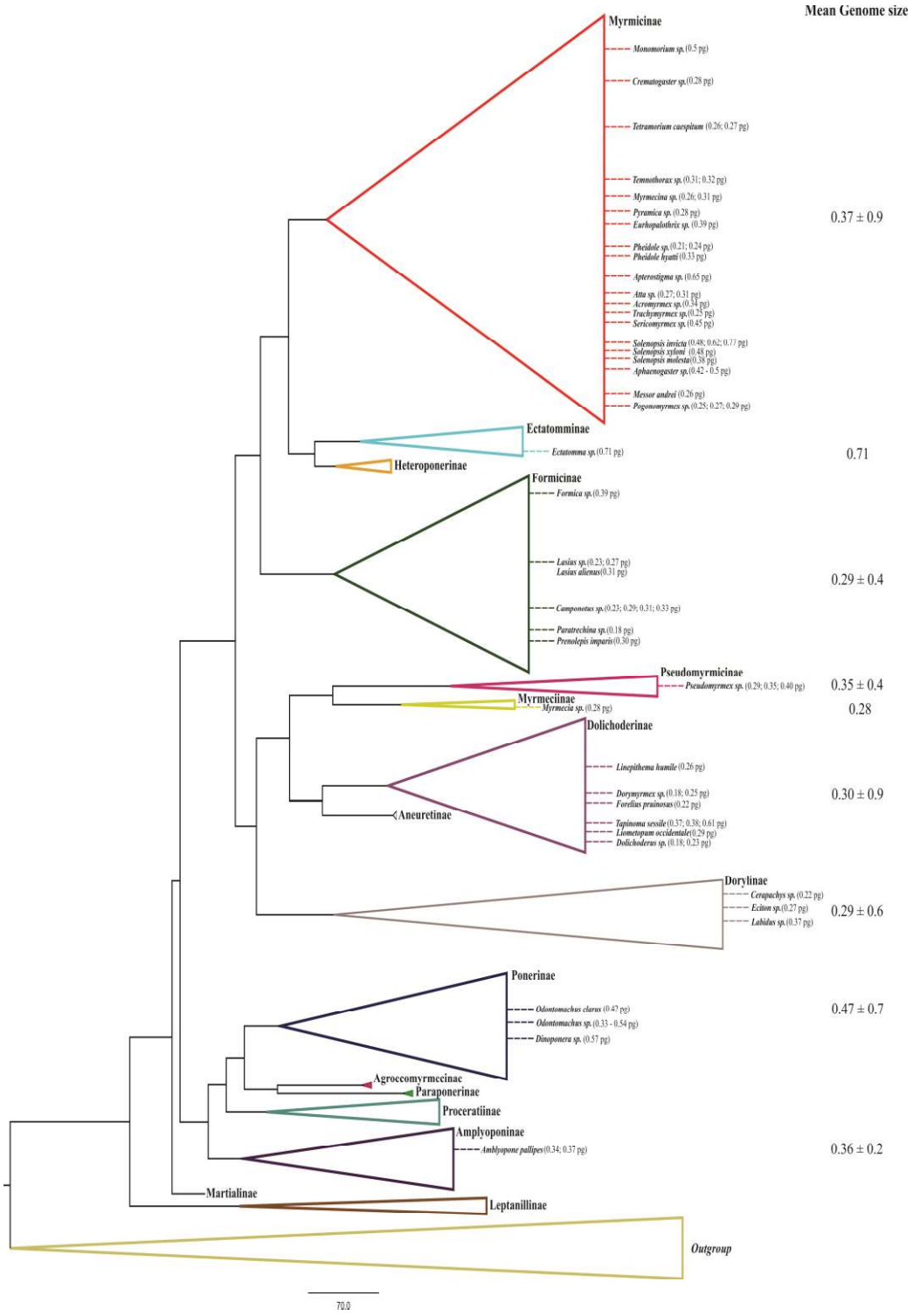

Figure 2 Phylogeny of the extant Formicidae. Phylogenetic tree redrawn from Moreau and Bell (2013). The figure highlights the subfamilies containing species with estimated genome size. Aside of each terminal on the tree the genome size is shown in picograms (pg) of DNA and also the mean genome size per Formicidae subfamilies.

Capítulo II

Artigo escrito de acordo com as normas da revista: Cytometry Part A

Title: Estimation of nuclear DNA content in Formicidae by flow cytometry: standardization and application in population genetics

Running headline: Nuclear DNA content estimation in Formicidae

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Abstract

The DNA C-values for the family Formicidae range from 0.18 pg to 0.77 pg, but, to date, genome size estimates exist for only 0.52% of the total number of 13,369 ant species, and these estimates only cover 40 from 333 valid extant genera. In addition, a number of flow cytometry methods are currently used to estimate genome size in ants, which makes it difficult to compare studies and to understand the evolution of this trait. Genome size estimates are important, but there is very little available information about ant genome size. Therefore, the aims of this study were: 1) standardize a protocol for flow cytometry (FCM) analyses of ants; 2) determine

which of the internal standards currently used in flow cytometry for insects are the most suitable for ants; and 3) evaluate the efficacy of flow cytometry when it is used to analyze the differences in DNA content between colonies from different locations. We investigated the applicability of flow cytometry in studies involving population genetics by testing the three buffers generally used in flow cytometry (Galbraith, LB01, and OTTO's), and two commonly used internal standards: the bee *Scaptotrigona xanthotricha* and the fruit fly, *Drosophila melanogaster*. The differences in the genome sizes of different populations of four species from the Myrmicinae subfamily were also assessed. These were *Mycetophylax conformis*, *Mycetophylax morschi*, *Mycetophylax simplex*, and *Trachymyrmex holmgreni*. Furthermore, cytometer measurements were taken on different days and the coefficient of variation between them was calculated in order to test the efficacy and reliability of the equipment. *Drosophila melanogaster* was a better internal standard than *S. xanthotricha* because the majority of the studied ants had genome sizes (1C-value) that corresponded to that of *S. xanthotricha*, which resulted in overlapping peaks in the histograms. In contrast, *D. melanogaster* has a smaller genome, so this overlapping did not occur, which means that it is the most appropriate internal standard for studies on ants. The Galbraith buffer was most effective for Myrmicinae and Pseudomyrmecinae, and LB01 was more suitable for Ectatomminae and Ponerinae. The OTTO's buffer also worked, but it had an unacceptably large coefficient of variation (< 5%). The genome size estimates varied among the populations of the four species studied and this variation was probably related to colony stress caused by the colonization of new environments. Flow cytometry proved to be an effective DNA quantification method for ants and could be used in population genetics studies. The coefficient of variation between days was not

significantly different, which further confirms the effectiveness and stability of the method.

Keywords**:** Fungus-farming ants; Genome size; Evolution; Variation.

Introduction

The genome size (GS, DNA content, or DNA C-value) of an organism is the total amount of DNA contained in a haploid chromosome set (**1**) and is measured in picograms (pg) or number of base pairs (bp) (**2**). It is an important trait because it corresponds to the DNA bulk, which is a unit of measurement that is considered to be one of the most fundamental properties of any organism (**3**). A commonly used technique for genome size estimates is flow cytometry (FCM), which has been established as a quick, efficient, and reproducible method (**4**).

The haploid DNA content of insects ranges from 0.07 pg (*Clunio tsushimensis*, Diptera) to 16.93 pg (*Podisma pedestris*, Orthoptera), and GS estimates have been obtained for 24 out of the 27 insect orders. Diptera accounts for the largest number of measurements with 386 specimens (29% of the total), followed by Coleoptera with 278 specimens (21%) and Hymenoptera with 240 specimens (18%) (**5**, Moura et al., in preparation). In Formicidae, the DNA content has been estimated from 79 specimens covering 67 different species. This represents only 0.41% of the total number of species described (**6–12,** Moura et al. in preparation) and most belong to the diverse Myrmicinae subfamily. The genome size values range from 0.18 pg in *Dorymyrmex bureni* (Dolichoderinae subfamily) and *Paratrechina longicornis* (Formicinae subfamily) (**10**) to 0.77 pg in the *Solenopsis invicta* (Myrmicinae subfamily) (**7**).

To date, there have been very few studies on ant genome sizes and the FCM analysis methodologies are still superficially understood because previous studies used different procedures to obtain their estimates (Moura et al., in preparation). Furthermore, there have been few studies on genome size variation between closely

related species and between populations of the same species, which is an important approach that has been used to analyze environmental effects on genome size and the correlations between genome size and physiological characteristics (**1, 13–16**).

Currently, the monophyletic Formicidae family is divided into 17 existing subfamilies plus four extinct families (**17**). Myrmicinae is the largest and most diverse subfamily (**18**) and contains about 230 species of fungus-growing ants (**19**). These fungus-growing ants have a unique mutualistic relationship with fungi, which is a complex behavior that has emerged only four times in the evolutionary history of the group (**20**). Cardoso et al. (**11**) demonstrated that, although the genome size of fungus-growing ants tends to be a constant characteristic within a species (**21,22**), there are clear differences in genome sizes among closely related species in the genus *Mycetophylax* (*sensu* **23**). This variation has been shown to be related to the differences in chromosome number and heterochromatin content (**11,24**) because *M. simplex* has a large genome size and chromosome number, and high heterochromatin levels. However, no study on intraspecific variation has been undertaken so far.

Genome size analyzes and estimates are important areas of research and there have been very few previous studies that have investigated ant genome sizes and estimates. Therefore, the aims of this study were 1) to determine which of the internal standards and lysis buffers currently used in insect FCM analyses are most appropriate for ants; 2) to standardize a protocol for obtaining adequate nuclei suspensions for ant FCM analyses; and 3) to evaluate the efficacy of FCM when it is used to analyze the differences in DNA content between colonies from different locations in order to determine whether flow cytometry could be used in studies involving population genetics.

Materials and Methods

Colony sampling

Four species of fungus-growing ants were sampled from different localities and analyzed using FCM. The four species were *Mycetophylax conformis* (Mayr, 1884), *Mycetophylax morschi* (Emery, 1888), *Mycetophylax simplex* (Emery, 1888), and *Trachymyrmex holmgreni* Wheeler, 1925. The species were chosen because they are currently being used in several population studies that are in progress in the laboratory of Laboratório de Genética Evolutiva e de Populações of Universidade Federal de Ouro Preto, which facilitated colony monitoring. Furthermore, a previous study on *Mycetophylax* species had shown that there were genome size variations between species, but intraspecific variation was not evaluated. The samples were collected during field expeditions in the Brazilian states of Bahia (BA), Minas Gerais (MG), Rio de Janeiro (RJ), Santa Catarina (SC), and Rio Grande do Sul (RS). Excavation was carried out according to protocols developed by Cardoso et al. (**25**) and 36 colonies were collected in total (Table 1). The whole colony was collected, transported to the laboratory, and kept under laboratory conditions. In some field expeditions, another four species of others Formicidae subfamily were also collected. These were *Ectatomma brunneum* Smith, 1858; *Neoponera marginata* (Roger, 1861); *Pseudomyrmex gracilis* (Fabricius, 1804), and *Pseudomyrmex schuppi* (Forel, 1901). Exemplary samples of each species collected were subsequently stored in absolute alcohol, assembled and sent for identification by Dr. Rodrigo Feitosa, at Universidade Federal do Paraná. The genome size data for the *Mycetophylax* species estimated in previous studies were retrieved from Cardoso et al. (**11**).

Internal standards and lysis buffers

The two internal standards most commonly used for the order Hymenoptera, and especially for ants, are *Drosophila melanogaster* (1C = 175 Mb = 0.18 pg) and *Scaptrotrigona xantotricha* female (1C = 430 Mb= 0.44 pg) (e.g. **9–12**). These standards were tested so that we could determine the best internal reference standard for estimating the nuclear DNA content in ants. About the buffer, the three most common nuclear isolation buffers were tested, being two most used for order Hymenoptera (OTTO I/OTTO II and Galbraith buffers). Further, we tested the LB01 buffer that is commonly used in plant estimates (Table 2) (**26,27**). The selection of the buffers was based on the results reported by Loureiro et al. (**27**), who found that these three buffers, due to their different chemical compositions, worked in different ways depending on the analyzed species.

Tissue and sample preparation, and flow cytometry (FCM) analyses

To determine the amount of DNA we used heads of adult ant workers of the target species. First, both internal standards and buffers cited above were tested on each species in individual experiments. Also, the two internal standards were first tested together to confirm each value of genome size. Full heads were cut with a blade, placed in a 1.5 mL microtube, and immersed in 100 μL of the buffer. Some experiments were carried out using pupae ganglia of the target species in order to verify if there were differences in genome size estimates between adults and pupae tissue. With the aid of a pistil for grinding samples, "up and down" movements were made into the microtube to detach the cells from the tissue and release the cells nuclei. Then 600 μL of the buffer was added to the microtubes. An additional centrifugation step followed by cell resuspension was included when the OTTO's

buffer was used (**32**). The nuclei suspension in each sample was filtered through a 40 μm nylon mesh and stained with 6.5 μL of propidium iodide solution at a concentration of 1.0 mg/mL. Exactly 3.5 μL of 10 mg/mL RNAse was also added. The samples were then stored in the cold (4°C) and in the dark and analyzed for up to 1 hour after preparation.

The analysis was performed on a FACSCalibur (Becton Dickinson) cytometer at the Universidade Federal de Ouro Preto. It was equipped with a laser source (488 nm) and the histograms were obtained using Cell Quest software. For each sample, at least 10,000 nuclei were analyzed regarding their relative fluorescence intensity and the cytometer was configured to run at low speed during the experiments. Three independent replications were analyzed and histograms with a coefficient of variation (CV) above 5% were rejected. The histograms were analyzed using Flowing 2.5.1 software (http://www.flowingsoftware.com). The genome size of each sample was calculated using the 1C-values for *D. melanogaster* or the *S. xantotricha* female, and the values were obtained according to the Doležel and Bartos (**26**) equation. The calculation was based on three samples per species (replicates). The values were then converted to megabase pairs (1 pg = 978 Mbp) (**2**).

Statistical analyses

General linear models were built to check for differences between the average genome sizes for the sampled colonies of the four Myrmicinae species shown in Table 3. The differences in genome size average for each species were assessed by variance analysis of the General Linear Model (ANOVA). When the p-value of the ANOVA was significant ($p < 0.05$), a contrast analysis at the 5% level was then performed using "coms.R" to check which was different. Furthermore, the

differences between the average genome sizes acquired using the different buffers were tested, as well as the difference in genome size between pupae and adult workers.

To evaluate the efficacy and reliability of the flow cytometer and to validate the genome size estimates, the coefficient of variation (CV) between days of measurements were calculate by using samples from the same colonies. Loureiro et al. (**27**) found variations between days of measurements and between different operators, so in the present study only the effects of different days of measurement were tested because the operator was always the same. All the sampled colony estimates for the four Myrmicinae species were used in the statistical analysis. The CV was calculated by dividing the standard deviation (SD) by the mean genome size of the species analyzed on each measurement day. The differences between the means of the coefficients of variation for each day were assessed by variance analysis (ANOVA) of the general linear models (GLM).

All statistical analysis was performed by R v2.15.1 software (**33**) and a residual analysis of the GLM was undertaken so that the error distribution could be evaluated (**34**).

Results

The most appropriate internal standard to use during the FCM analysis of ants was determined by testing two internal standards commonly used with insects. *Drosophila melanogaster* (Figure 1-b) proved to be a better internal standard for ants than the *Scaptotrigona xanthotricha* female (Figure 1-c) because the majority of the studied ants had a genome size (1C-value) ranging from 0.30 pg to 0.42 pg (Table 3), which corresponds approximately to that of *S. xanthotricha* (1C = 0.44 pg). This leads to peak overlaps, as can be observed when *S. xantotricha* was used as the internal standard during the *Mycetophylax simplex* analysis (1C = 0.40 pg, Figure 1-a) (Figure 1-e). However, *D. melanogaster* (1C = 0.18 pg) has a smaller genome size (Figure 1-d), so peak overlaps did not occur. The *D. melanogaster* results show that it could potentially be used to analyze species with a large range of genome size values.

The histograms used to infer the genome size (GS) of each specimen showed peaks corresponding to the G_0/G_1 and G_2 nuclei of the target species and the internal standard (Figures 1-d,e). All the histograms used to calculate the genome size had a high resolution and their coefficients of variation were always less than 5%, which is considered ideal for GS determination using FCM (**35,36**).

The three buffers commonly used in FCM analysis were tested on all the ants shown in Table 3. The Galbraith buffer was most effective when the Myrmicinae and Pseudomyrmecinae species were analyzed. It had good resolution and the variation coefficients were always less than 5%, as can be seen in *Mycetophylax* species histograms shown in Figure 1. The OTTO's buffer (Otto I/Otto II) also worked, but it had a coefficient of variation that was often unacceptably large (i.e. greater than 5%).

Therefore, the genome size data obtained using the OTTO's buffer were discarded. The LB01 buffer also worked for these two subfamilies, but the Galbraith buffer was preferentially used in the experiments. Nevertheless, there was no difference in the genome size averages obtained using the Galbraith and LB01 buffers ($p > 0.05$). However, the Galbraith and OTTO's buffers worked did not work when species belonging to the Ponerinae and Ectatomminae subfamilies were analyzed, which meant that their histograms did not appear in the analysis results. In these cases, only the LB01 buffer worked, and it had an excellent resolution and a coefficient of variation less than 5%.

The haploid genome size of *Mycetophylax conformis* range from 0.27 to 0.35 pg and colonies from the Brazilian states of Bahia and Rio de Janeiro were statistically analyzed. No significant differences were found between populations from the same locality ($p > 0.05$), but the mean genome sizes for the two states were significantly different ($p < 0.01$) (Figure 2-a). The average genome size of the Bahia populations was approximately 0.28 pg (Table 3, SD = 0.004), with individual values ranging between 0.27 pg and 0.29 pg, whereas the mean for the populations from Rio de Janeiro was approximately 0.33 pg (Table 3, SD = 0.013), with individual values ranging between 0.32 pg and 0.35 pg. Cardoso et al. (**11**) reported a similar value for this species when the samples were collected in Rio de Janeiro State ($p > 0.05$).

The haploid genome sizes for *Mycetophylax morschi* ranged from 0.32 to 0.38 pg. The colonies from the Brazilian states of Bahia, Rio de Janeiro, Santa Catarina (Araranguá) and Rio Grande do Sul (Torres) were statistically analyzed. The ANOVA results showed that the means for the four states were significantly different ($p < 0.01$), but there was no significant difference between populations from the

same locality ($p > 0.05$). Through the contrast analysis, the genome sizes of the populations from Bahia (average 0.35 pg, standard deviation 0.008) and Rio de Janeiro (average 0.37 pg, standard deviation 0.013) grouped statistically as well as the populations of Araranguá (SC) (average 0.33 pg, standard deviation 0.0001) and Torres (RS) (average 0.33 pg, standard deviation 0.008) (Table 3, Figure 2-b). The mean 1C-values for the *M. morschi* ants from Torres and Araranguá were similar ($p > 0.05$) to the values reported by Cardoso et al. (**11**). However, in Cardoso et al. (**11**), the mean value also included colonies from Rio de Janeiro state, which had a different average 1C-value to the value obtained in this study, which meant that the differences in values for this one state may have been masked when the overall mean value was calculated.

The *Mycetophylax simplex* haploid genome size values ranged from 0.37 pg to 0.42 pg with an average of 0.40 pg (standard deviation of 0.023 pg) when the colonies from Araranguá (SC) were analyzed. As the colonies sampled were from the same locality, a statistical analysis was performed to determine any differences in genome size between the colonies. There was no significant difference ($p > 0.05$) in the C-value DNA between the analyzed colonies (Table 3, Figure 2-c). Furthermore, there was no significant difference ($p > 0.05$) between the average estimate in this study and the value measured by Cardoso et al. (**11**) ($p > 0.05$, Table 3).

The efficacy of the FACSCalibur flow cytometer measurements was tested by repeating the same experiments on different days and calculating the coefficient of variation (CV) for the genome size of each species on different measurement days. There were no significant differences between days ($p > 0.05$), which confirmed the accuracy and reliability of the equipment. The CVs for each measurement day are

plotted in Figure 3. In addition, there were no significant differences between the genome sizes of adult workers and pupae ($p > 0.05$). Therefore, pupae measurements were also included in the mean genome size calculations for the species shown in Table 3.

Discussion

The standardization of the methodology used in studies involving the FCM analysis of ants was urgently required because few studies have used the same approach, which has made it difficult to compare existing estimates (Moura et al. in preparation). Most of the genome size estimates for Formicidae were obtained by using *Drosophila melanogaster* as the internal standard (**9,10**) but *Scaptotrigona xantotricha* female was also used by (**11)** and **(12**), mainly to analyze the order Hymenoptera and especially in studies with stingless bees (**37–39**). In the present study, it was demonstrated that *D. melanogaster* is a better internal standard for ants than *S. xantotricha*. The main reason is due to the small 1C-value of *D. melanogaster*, which avoids potential overlapping of the target species peaks with the internal standard peaks. When this happens, it is difficult to accurately estimate the C-value. The small *D. melanogaster* 1C-value potentially means that it could be used to analyze a wide range of species with large genome size distributions.

Early studies used Feulgen densitometry to estimate the genome size of *D. melanogaster*, which is considered a model organism in biology (**40**). They generated a 1C-value estimate of 0.18 pg (**41,42**). Due the wide importance and use of *D. melanogaster* in studies on genetics, cytology, and developmental biology (**41**), several later studies included estimates of genome size for this organism that were based on FCM and other techniques in an attempt to unravel the composition of its

genome (**43**–**47**). One of these studies was that of Adams et al. (**43**) estimated the genome size of *D. melanogaster* using a whole genome sequencing technique, which gave a haploid value of 0.18 pg (2C-value = 0.36 pg.). In addition to these it's important to highlight the work of Bennett et al. (**45**), who undertook a comparative study using other commonly used internal reference standards in flow cytometry, estimated and reaffirmed the amount of DNA for this organism (1C = 0.18 pg, 2C = 0.36 pg).

In contrast, only Lopes et al. (**37**) and Tavares et al. (**39**) have estimated genome size of *Scapitotrigona xantotricha*, with a difference in the values for males and females since. This was probably because bees, like ants, have a haplodiploid sex-determination system with haploid males and diploid females (workers and queens). As ant workers are a diploid organism, is necessary to use only female bees to avoid linearity problems in the measurements. Thus, it is needed to sex the individuals (pupae or adults) to perform the analysis because the differences between sexes may bias the results. This is not the case with *D. melanogaster* because both males and females are diploid, which overcomes the potential bias issue. Therefore, the stability of the *D. melanogaster* nuclear DNA content and the convenience of this organism, reaffirmed by the several studies cited above, it is possible to reinforce that this is the most appropriate internal standard for genome size studies on ants. It also fits the desired guidelines for the FCM analysis of plants established by Doležel and Bartos (**26**).

This study is the first to test different lysis buffers used to obtain nuclei suspensions, which can then be used to measure genome size in ants. In plants, this type of comparative study is already widespread, showing that there is not only a better

buffer, but rather that it is necessary to observe the specificity of each organism studied when choosing the most suitable buffer so that histograms with a lower coefficient of variation are generated and there is little background debris (**4,26,27**). Two of the three buffers were clearly more efficient at extracting nuclei from the ants. These were Galbraith buffer for Myrmicinae and Pseudomyrmecinae, and LB01 buffer for Ponerinae and Ectatomminae. The Galbraith buffer has previously been used on ants by Tsutsui et al. (**9**) and Ardila-Garcia et al. (**10**), and the OTTO's buffers were used by Cardoso et al. (**11**) and Aguiar et al. (**12**). However, none of these studies tested alternative buffers and did not discuss the reason for using such buffers in the experiments because there had been no previous comparative buffer studies on ants. Nevertheless, the genome size values measured by Cardoso et al. (**11**) for species in the *Mycetophylax* genus using pupae and the OTTO's buffer were the same of those obtained in this study using Galbraith buffer and the heads of adult workers collected from colonies found in the same locality.

These results corroborate Loureiro et al. (**27**) who demonstrated that each buffer contains reagents that act differently (**26**) and that these differences need to be taken into account when choosing a buffer for a specific analyzed organism. In ants, the presence of cuticular hydrocarbons distinguishes one colony from another, and is used as a biological reference for these social insects (**48,49**). These hydrocarbons can vary in composition between species (**50**). The thickness of the exoskeleton and presence/size/composition of glands in the heads of adults also vary, which makes the analysis of the buffer fundamental to obtaining intact nuclei from the worker ant cells used in the FCM analysis

The genome size of adult ant workers and pupae were compared for the first time in this study. The results showed that there were no significant differences between them. This is a very important step forward in the FCM analysis of ants because sampling adult individuals is less labor-intensive than collecting the entire colony. Previously it was usually necessary to obtain blood, which depends on the colony life cycle and the season of the year. The use of adults in FCM analyses also excludes the need to test for false polyploidy specimens and species because ants are holometabolous, which means that the possibility of finding polyploidy tissues is much higher in the blood than in the adult. Therefore, because it is rare to find many cells undergoing the division process when adult worker ants are used, the appearance of multiple peaks during the analysis must correspond to true polyploidy.

Doležel and Bartos (**26**) demonstrated that one of the difficulties in using FCM to analyze plants was related to the requirement for fresh-live tissue and the same is confirmed for the FCM analysis of ants. This means that the procedures need to be performed on living individuals because some fixatives alter the stoichiometry between the DNA and propidium iodate, which can make the analysis doubtful and inaccurate. Furthermore, some fixatives can prevent the release of the cell nuclei, which means that the FCM analysis can be inaccurate.

The genome sizes estimated in this study after using FCM were accurate and could feasibly be used in future studies on population genetics. The different colonies of the four Myrmicinae species had their nuclear DNA contents evaluated and the results showed that there were differences between populations of the same species that had come from different localities. This confirmed the results reported by Nardon et al. (**15**) who studied the influence that the colonization of new

environments had on the genome size of Diptera species. They found that genome size varied between different populations of the same *Drosophila* species and that populations from the probable origin region have smaller genomes than more recent populations. Based on this, they also suggested the hypothesis that new colonization events are followed by an increase in genome size caused by the effect of stress during this process, and that this was probably caused the accumulation of transposable elements, as found in maize (**51**) and as proposed by Chênais et al. (**52**).

The four Myrmicinae species studied in the present work belong to the outstanding group of fungus-growing ants endemic from the New World (**19**). In a recent study, Branstetter et al. (**53**) suggested that the diversification of this group probably occurred when they spread from Middle America to South America and that the occupation of inhospitable habitats followed by isolation was fundamental to the domestication of the cultivated fungus and the survival of the group. Following this line, and based on the finding by Nardon et al. (**15**), it was expected that the genome size of the studied Myrmicinae populations would increase from north to south Brazil, i.e., northern populations would have a smaller genome size than the southern populations, assuming ancient to recent colonization. The data on the genome size produced by this study reflected this hypothesis. *Mycetophylax conformis* populations from Bahia (northeastern Brazil) had smaller average genome sizes than populations from Rio de Janeiro (southeastern Brazil). The same was observed for *Trachmyrmex holmgreni*, where populations from Minas Gerais (southeastern Brazil) and Araranguá (SC, southern Brazil) had smaller genome sizes than Balneário Gaivota (SC, southern Brazil) and Torres (RS, southern Brazil). Although they are also from the southern region of Brazil, Balneário Gaivota and Torres are located

more to the south than Araranguá, which may suggest that recent colonization going to southern Atlantic coast.

Genome size estimates for *Mycetophylax morschi* also varied between different localities, but had a pattern that differed from what was expected. The Rio de Janeiro and Bahia (southeastern and northeastern Brazil, respectively) populations had larger genome sizes than those in the south (Santa Catarina and Torres-RS). This study is the first to report *M. morschi* in the state of Bahia. The populations are very similar in behavior to those collected in the state of Rio de Janeiro, but somewhat different from the southern colonies. Furthermore, Cardoso et al. (**24**) showed that some populations from Rio de Janeiro had a chromosomal number equal to 2n = 30 (n = 15), while the populations from Santa Catarina had a chromosome number of 2n = 26 (n = 13). Therefore, these results suggest that colonies from Bahia and Rio de Janeiro may be different species. However, this can only be confirmed by accurate studies on the phylogeography, cytogenetics, morphology, and behavior of the populations combined with the genome size data reported in this study.

In summary, this study has established a new standardized procedure for quantifying genome size in ants. The results suggest that *D. melanogaster* is the most appropriate internal reference standard, the needs of search for the optimal lysis buffer for obtaining nuclei suspensions and the use of adult workers as a tissue source. The analysis of genome size is a very important approach in the evolutionary study of species, and that genome size data can be used in population genetics studies. The genome size estimated in this study varied among populations of the same species and this variation is more likely to be related to the stress hinted during the colonization of new environments. Finally, FCM proved to be an effective DNA

quantification method because the coefficient of variation between measurement days was not significantly different. This demonstrated the effectiveness of the method and the stability of the equipment.

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Table 1 – Colonies sampled during field expeditions with Locality, Collection Date and Geographical Coordinates.

Voucher	Colonies	Locality	Collection Date	Geographical coordinates
MYCO0192	*Mycetophylax conformis* - Colony 1	Uruçuca - Bahia	October/2015	14°29'60"S; 39°2'7"W
MYCO0187	*Mycetophylax conformis* - Colony 2	Uruçuca - Bahia	October/2016	14°29'60"S; 39°2'7"W
MYCO0151	*Mycetophylax conformis* - Colony 1	Cabo frio - RJ	07/September/2016	22°54'37.5"S; 42°02'37.8"W
MYCO0154	*Mycetophylax conformis* - Colony 2	Peró - RJ	07/September/2016	22°50'29.8"S; 41°58'59.2"W
MYCO0149	*Mycetophylax conformis* - Colony 3	Arraial do Cabo - RJ	05/September/2016	22°57'42.8"S; 42°02'38.9"W
MYMO0193	*Mycetophylax morschi* - Colony 1	Itacaré - Bahia	October/2015	-
MYMO0191	*Mycetophylax morschi* - Colony 2	Pontal - Bahia	October/2015	-
MYMO0148	*Mycetophylax morschi* - Colony 1	Cabo frio - RJ	07/September/2016	22°54'37.5"S; 42°02'37.8"W
MYMO0158	*Mycetophylax morschi* - Colony 2	Cabo frio - RJ	06/September/2016	22°54'37.6"S; 42°02'34.4"W
MYMO0247	*Mycetophylax morschi* - Colony 1	Araranguá - SC	-	28°56'57"S; 49°22'15"W
MYMO0188	*Mycetophylax morschi* - Colony 2	Araranguá - SC	2015	28°56'08"S; 49°21'29"W
MYMO0184	*Mycetophylax morschi* - Colony 1	Torres - RS	2016	29°22'00"S; 49°44'45.4"W
MYMO0183	*Mycetophylax morschi* - Colony 2	Torres - RS	2016	29°22'00"S; 49°44'45.4"W
MYSI0182	*Mycetophylax simplex* - Colony 1	Araranguá - SC	2015	-
MYSI0146	*Mycetophylax simplex* - Colony 2	Araranguá - SC	14/August/2016	28°55'43"S; 49°21'18"W
MYSI0144	*Mycetophylax simplex* - Colony 3	Araranguá - SC	13/August/2016	28°56'57"S; 49°22'152"W
TRHO0197	*Trachymyrmex holmgreni* - Colony 1	Cachoeira do campo - MG	01/July/2016	20°21'56.24"S; 43°38'28.52"W
TRSP0098	*Trachymyrmex holmgreni* - Colony 2	Cachoeira do campo - MG	08/October/2015	20°21'23"S; 43°39'27"W
TRTH0489	*Trachymyrmex holmgreni* - Colony 3	Cachoeira do campo - MG	21/September/2017	20°21'23"S; 43°39'27"W
TRTH0489	*Trachymyrmex holmgreni* - Colony 4	Cachoeira do campo - MG	21/September/2017	20°21'23"S; 43°39'27"W
THMC0018	*Trachymyrmex holmgreni* - Colony 1	Araranguá - SC	30/March/2016	28°56'08"S;49°21'29"W
THMC0066	*Trachymyrmex holmgreni* - Colony 2	Araranguá - SC	05/November/2016	28°56'08"S; 49°21'29"W
TRHO0404	*Trachymyrmex holmgreni* - Colony 3	Araranguá - SC	25/May/2017	28°56'08"S; 49°21'29"W
THMC0059	*Trachymyrmex holmgreni* - Colony 4	Araranguá - SC	02/November/2016	28°56'08"S; 49°21'29"W
THMC0064	*Trachymyrmex holmgreni* - Colony 5	Araranguá - SC	05/November/2016	28°56'08"S; 49°21'29"W

Voucher	Colonies	Locality	Collection Date	Geographical coordinates
THBG0041	*Trachymyrmex holmgreni* - Colony 1	Balneário Gaivota - SC	02/April/2016	29°11'42.61"S; 49°36'30.85"W
THBG0044	*Trachymyrmex holmgreni* - Colony 2	Balneário Gaivota - SC	02/April/2016	29°11'42.61"S; 49°36'30.85"W
THBG0049	*Trachymyrmex holmgreni* - Colony 3	Balneário Gaivota - SC	03/November/2016	29°12'53.5"S; 49°37'37.9"W
THBG0054	*Trachymyrmex holmgreni* - Colony 4	Balneário Gaivota - SC	03/November/2016	29°12'53.0"S; 49°37'39.0"W
THBG0052	*Trachymyrmex holmgreni* - Colony 5	Balneário Gaivota - SC	03/November/2016	29°12'53.2"S; 49°37'39.3"W
THBG0032	*Trachymyrmex holmgreni* - Colony 6	Balneário Gaivota - SC	02/April/2016	29°11'42.09"S; 49°36'30.37"W
THBG0031	*Trachymyrmex holmgreni* - Colony 7	Balneário Gaivota - SC	02/April/2017	29°11'42.03"S; 49°36'30.75"W
THTO0023	*Trachymyrmex holmgreni* - Colony 1	Torres - RS	01/April/2016	29°22'00.14"S; 49°44'45.11"W
THTO0020	*Trachymyrmex holmgreni* - Colony 2	Torres - RS	01/April/2016	29°22'00.30"S; 49°44'45.15"W
THTO0070	*Trachymyrmex holmgreni* - Colony 3	Torres - RS	04/November/2016	29°24'07.4"S; 49°46'33.1"W
THTO0071	*Trachymyrmex holmgreni* - Colony 4	Torres - RS	04/November/2016	29°24'07.3"S; 49°46'32.9"W
GNSP0167	*Ectatoma brunneum*	Cabo frio - RJ	07/September/2016	22°54'45.7"S; 42°02'42.4"W
POSP0201	*Neoponera marginata*	Cachoeira do campo - MG	01/July/2016	20°21'40.6"S; 43°38'26.3"W
POSP0164	*Pseudomyrmex gracilis*	Cachoeira do campo - MG	01/July/2016	20°21'40.6"S; 43°38'26.3"W
POSP0202	*Pseudomyrmex shuppi*	Cachoeira do campo - MG	01/July/2016	20°21'40.6"S; 43°38'26.3"W

Table 2 – Three nuclear isolation buffers tested in this study

Buffer	Composition	References
Galbraith	45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100, pH 7.0	(**28**)
LB01	15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine.4HCl, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, 15 mM β-mercaptoethanol, pH 7.5	(**29**)
OTTO's	OTTO I: 100 mM citric acid monohydrate, 0.5% (v/v) Tween 20 (pH 2–3) OTTO II: 400 mM $Na_2PO_4.12H_2O$ (pH 8–9)	(**30**) (**31**)

Legend: MOPS, 4-morpholinepropane sulfonate; DTT, dithiothreitol; Tris, tris-(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

Table 3 – Mean genome size estimates of the four Myrmicinae species studied in this work plus the four other species of Fomicidae family using *Drosophila melanogaste*r as internal standard and genome size data of the *Mycetophylax* species estimated in previous studies (**11**).

Species	Locality	Mean genome size 1C (pg-Mbp)	Standard Deviation (SD-pg)	Buffer	References
Mycetophylax conformis	Bahia	0.28 - 273.84	0.005	Galbraith	This sudy
Mycetophylax conformis	Rio de Janeiro	0.33 - 322.74	0.013	Galbraith	This sudy
Mycetophylax conformis	Rio de Janeiro	0.32 - 312.96	-	Otto's	(**11**)
Mycetophylax morschi	Bahia	0.35 - 342.30	0.008	Galbraith	This sudy
Mycetophylax morschi	Rio de Janeiro	0.37 - 361.86	0.013	Galbraith	This sudy
Mycetophylax morschi	Araranguá (SC)	0.33 - 322.74	0.0001	Galbraith	This sudy
Mycetophylax morschi	Torres (RS)	0.33 - 322.74	0.008	Galbraith	This sudy
Mycetophylax morschi	Santa Catarina and Rio de Janeiro	0.32 - 312.96	-	Otto's	(**11**)
Mycetophylax simplex	Araranguá (SC)	0.40 - 391.20	0.023	Galbraith	This sudy
Mycetophylax simplex	Santa Catarina and Rio de Janeiro	0.39 - 381.42	-	Otto's	(**11**)
Trachymyrmex holmgreni	Cachoeira do Campo (MG)	0.32 - 312.96	0.019	Galbraith	This sudy
Trachymyrmex holmgreni	Araranguá (SC)	0.31 - 303.18	0.013	Galbraith	This sudy
Trachymyrmex holmgreni	Balneário Gaivota (SC)	0.35 - 342.30	0.017	Galbraith	This sudy
Trachymyrmex holmgreni	Torres (RS)	0.35 - 342.30	0.009	Galbraith	This sudy
Ectatoma brunneum	Rio de Janeiro	0.40 - 391.20	0.010	LB01	This sudy
Neoponera marginata	Cachoeira do Campo (MG)	0.65 - 635.70	0.005	LB01	This sudy
Pseudomyrmex gracilis	Cachoeira do Campo (MG)	0.41 - 400.98	0.010	Galbraith	This sudy
Pseudomyrmex schuppi	Cachoeira do Campo (MG)	0.38 - 371.64	0.016	Galbraith	This sudy

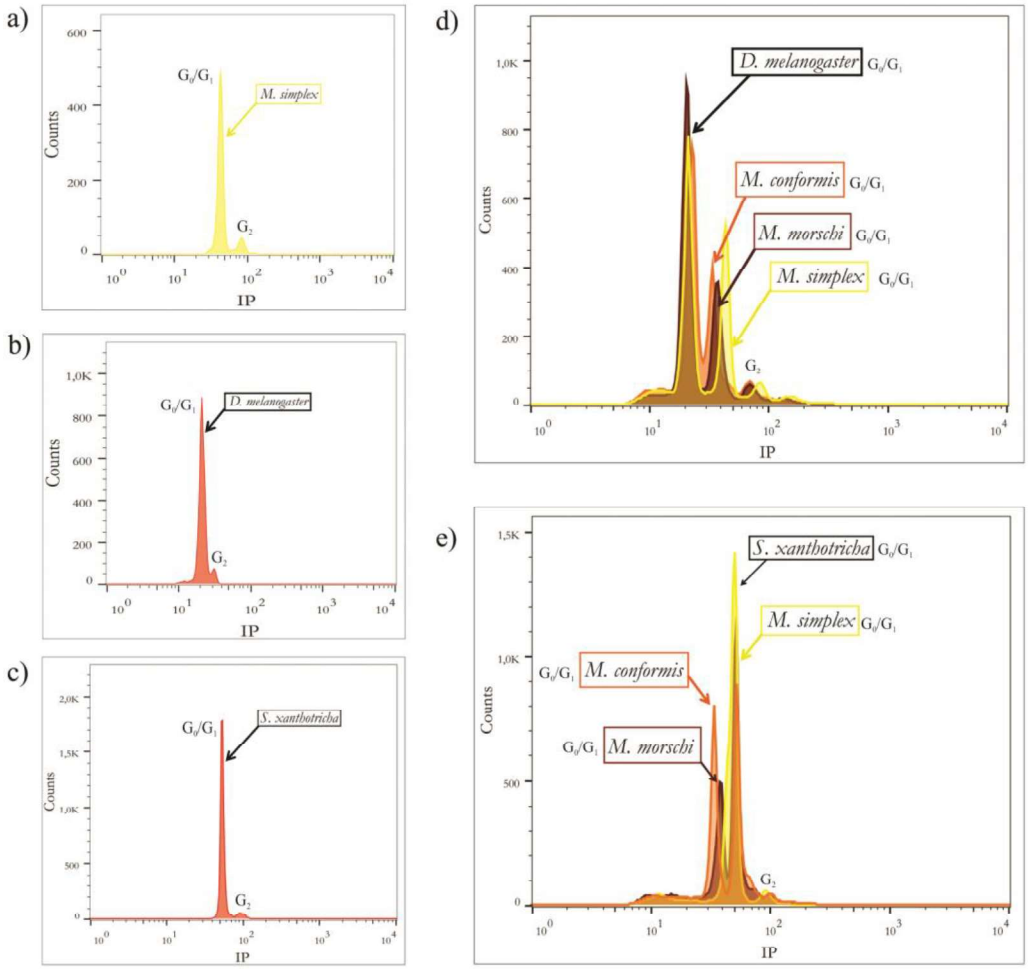


Figure 1. Genome size DNA histograms of *Mycetophylax* species and the internal standards stained with propidium iodide. Nuclei suspensions were obtained with Galbraith buffer. a) *M. simplex*; b) *Drosophila melanogaster*; c) *Scaptotrigona xanthotricha* female; d) *M. morschi*, *M. conformis* and *M. simplex* tested with *D. melanogaster* as internal standard; e) Same species tested with *S. xanthotricha* female as internal standard. The x axis corresponds to an arbitrary scale of fluorescence intensity (proportional to the size of the genome), and the y axis represents the number of nuclei with that fluorescence intensity.

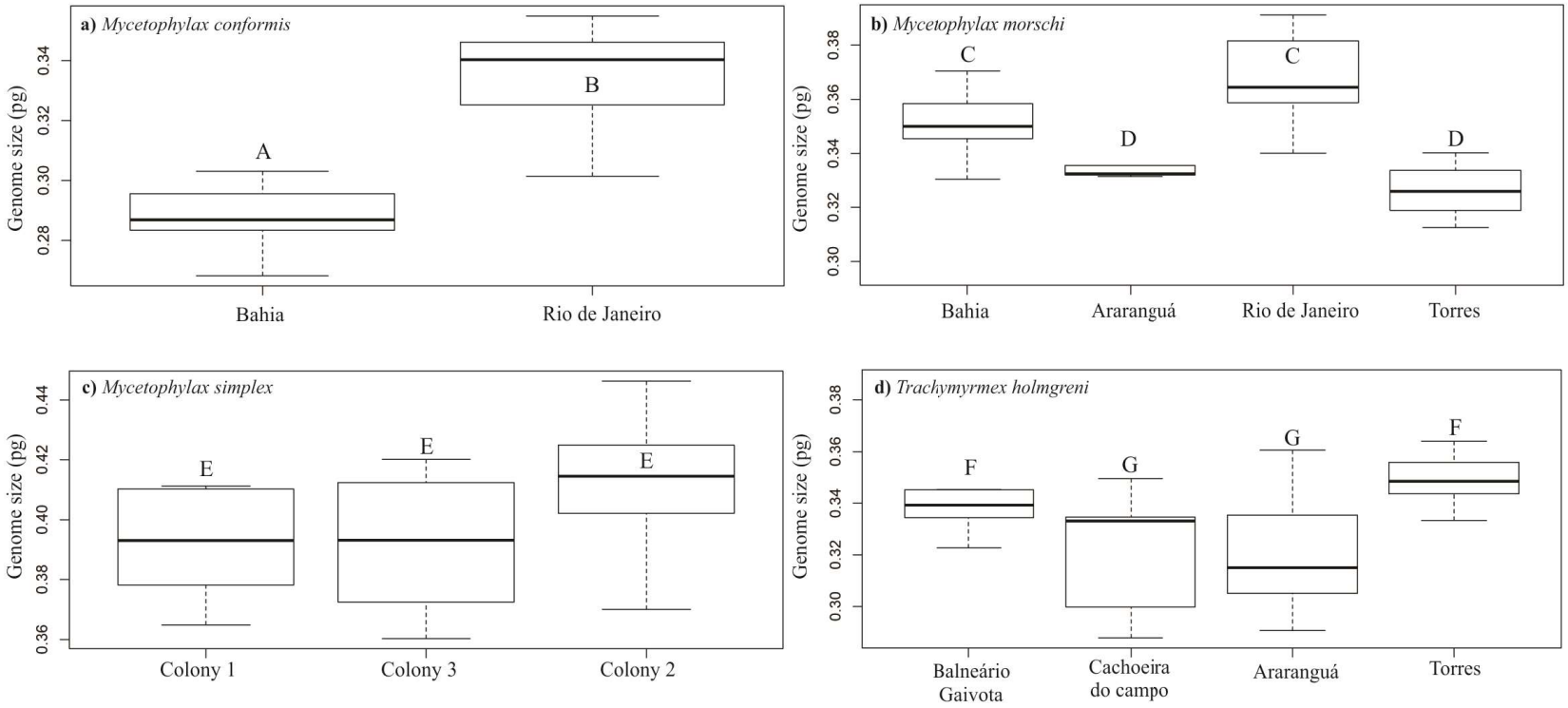


Figure 2. Box plots showing the average and the range of genome sizes (GS) encountered in a) *Mycetophylax conformis*, b) *Mycetophylax morschi,* c) *Mycetophylax simplex* and d) *Trachymyrmex holmgreni*. The x axis corresponds to the locality where the colonies were sampled and, for *Mycetophylax simplex*, the colonies that were sampled. The y axis shows the 1C-values in picograms (pg) of DNA. Letters A-G above or inside the boxplots indicate the different averages.

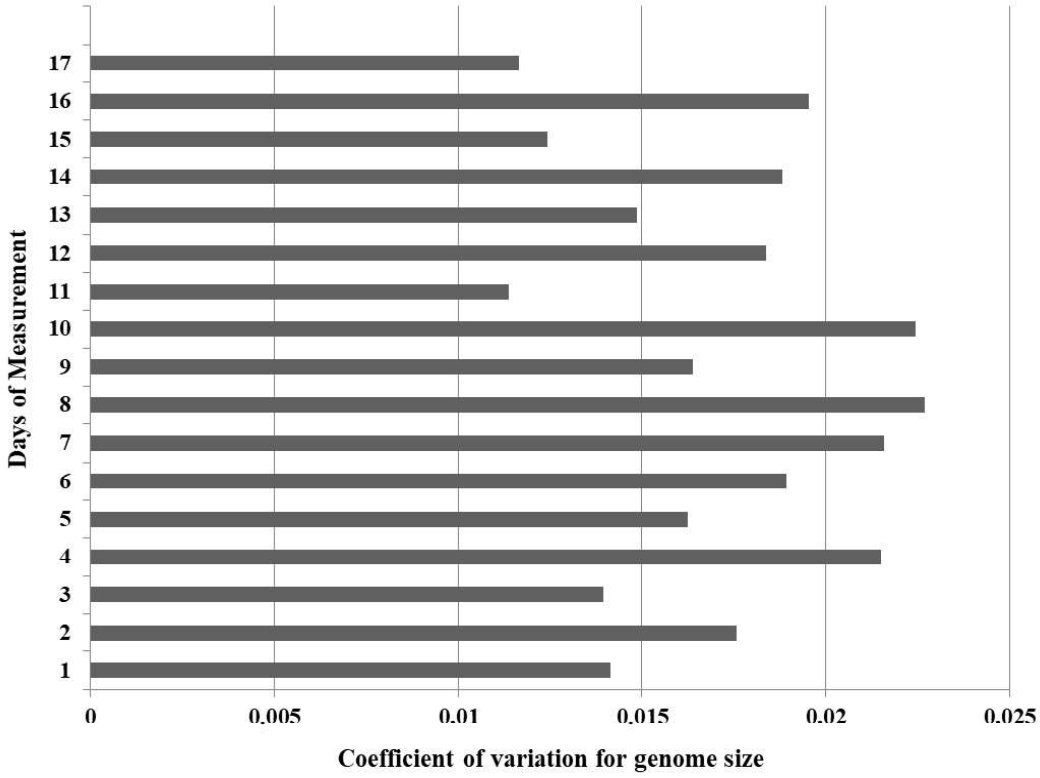
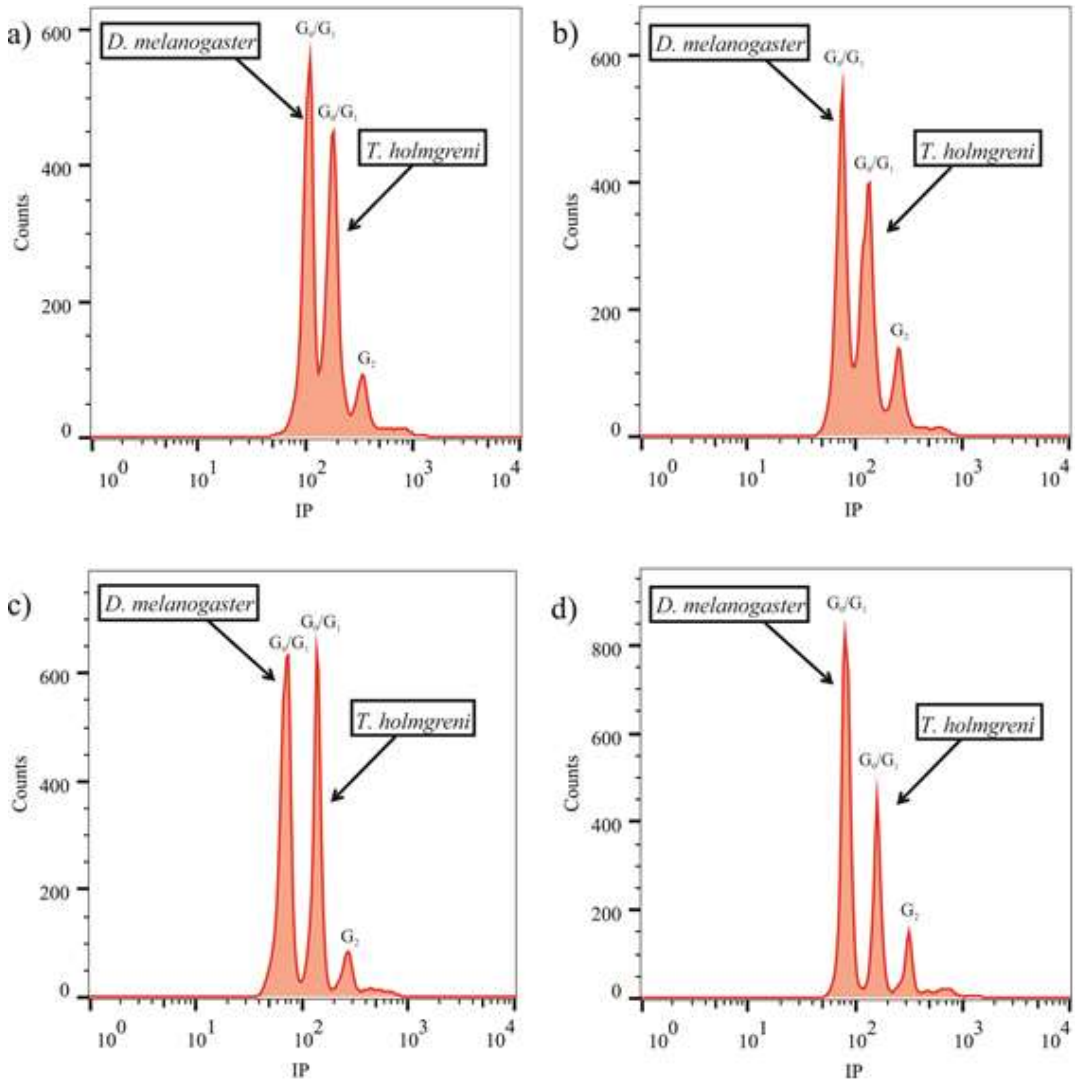


Figure 3. Coefficient of variation (CV) of genome size calculated per day of measurement on the flow Cytometer using the sampled colonies of the four Myrmicinae species with *D. melanogaster* as internal standard and Galbraith's buffer.



Appendix 1 - Genome size DNA histograms of *Trachymyrmex holmgreni* colonies with *D. melanogaster* as internal standard and Galbraith's buffer stained with propidium iodide. a) Populations from Cachoeira do Campo – MG (1C = 0.32 pg); b) Populations from Araranguá – SC (1C = 0.31 pg); c) Populations from Balneário Gaivota – SC (1C = 0.35 pg); d) populations from Torres – RS (1C = 0.35 pg).

Capítulo III

Artigo escrito de acordo com as normas da revista: Insect Molecular Biology

Genome size estimates of Formicidae species by Flow Cytometry: evolution at light of ancestral state reconstruction and base composition

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Abstract

Genome size is a fundamental characteristic of an organism and varies more than 200,000-fold in eukaryotes. There is still a lack of knowledge about which are the mechanisms that drives the change and variation of this trait among organisms. In ants the patterns regarding this issue are a bit overrated given the small set of species studied. So, the aim of the present study were expand the GS database of Formicidae family; establish a protocol of base composition determination through flow cytometry in ants; examine the patterns of distribution and variation of genome size and base composition among taxa; and provide a phylogenetic perspective on GS evolution in the family and reconstruct the ancestral state of this character. New genome size estimates were present for 99 species of ants, 91 of which corresponding to new taxa, and the mean genome size of Formicidae family was 0.38 pg. The AT/GC ratio obtained for the Family was AT = 62.40% and GC = 37.60%. Formicidae family was recovered as monophyletic with high value of posterior probability on the phylogenetic analysis. The reconstructed ancestral genome size for

Formicidae was 0.38 pg according to the MCMC method and 0.37 pg according to ML and parsimony methods and significant differences in GS were observed between the subfamilies sampled. The results of this study suggest that the evolution of GS in Formicidae was due to the loss and accumulation of non-coding regions, mainly transposable elements, and in some specific cases by whole genome duplication. However, the processes behind these genome enlargements and retractions still need to be understood, mainly through species diversification studies, in order to verify if these changes in DNA content are related to the colonization process of the species, thus as suggested at the intraspecific level.

Keywords: AT/GC ratio, Character reconstruction, DNA content, Evolution, Phylogeny, Transposable Elements, Whole genome duplication.

1. INTRODUCTION

The genome size (GS) of an organisms is a fundamental characteristic whose variation and evolution still remain as a puzzle in genome biology (Gregory, 2005; Bennett & Leitch, 2011; Kang *et al.*, 2014). This is a trait that in eukaryotes varies more than 200,000-fold (Gregory, 2001) without an apparent correlation with either the complexity of the organism or the number of genes (Petrov, 2011). Such lack of correlation has been called as “C-value enigma” in exchange of "C–value paradox" (Thomas, 1971), as the current understanding of genome has knock down the idea that they are simple linear collections of genes, making obsolete the old interpretation of GS variation as ‘paradoxical’ (Gregory, 2001).

Genome size (also known as DNA content, DNA amount or DNA C–value) are widely disseminated and studied in plants and the main works on the variation of GS between species, references on standardization of procedures for GS quantification are particularly concentrate on these organisms (e.g. Bennett *et al.*, 2003; Dolezel & Bartos, 2005; Gregory, 2005; Dolezel, 2007; Bennett & Leitch, 2011, 2012; Vu *et al.*, 2015; Hidalgo *et al.*, 2017). For instance, “The Plant DNA C-values Database” (http://data.kew.org/cvalues/) currently contains data for 8,510 plant species. Yet, the haploid DNA contents (C–values, in picograms) are currently available for 6,222 species (3,793 vertebrates and 2,429 non–vertebrates), with insects representing almost 21.6% of this total (Gregory, 2018).

An open question regarding this issue still is which are the main mechanisms that drive changes in the genome size, refleting the observed variation among organisms, especially between species. In some studies, genome size variation between closely related species were associated to chromosome number (Cardoso *et al.*, 2012;

Ardila–Garcia & Gregory, 2009), although other studies did not show any correlation (Gregory, 2001; Tavares *et al.*, 2012). On the other hand, mutations, recombination, and accumulation of non-conding DNA are considered the main mechanisms for genome size change and are considered the driving forces for species diversification (El-Shehawi & Elseehy, 2017).

In general, the increase of genome size in many organisms are more related to polyploidy events (Adams & Wendel, 2005), amount of heterochromatin (Lopes *et al.*, 2009; Tavares *et al.*, 2010; Cardoso *et al.*, 2012), amplification of non-coding repetitive DNA (Kidwell, 2002; Vieira *et al.*, 2002) and other repetitive genome sequences (Gregory & Hebert, 1999; Petrov, 2001). Moreover, a positive correlation between genome size and base composition, mainly the G + C content, has been found within several groups of vertebrates (e.g., Vinogradov & Borkin, 1993; Vinogradov, 1994; Vinogradov, 1998), bacteria (Guo *et al.*, 2009; Nishida, 2012; Zhang & Gao, 2017) and in some monocot plants (Li & Du, 2014). However, studies regarding base composition and the effect on genome size variation in other groups are still lacking (Li & Du, 2014).

In a recent work, Alfsnes *et al.* (2017) study the patterns of genome size variation among organisms at different levels of taxonomic relatedness considering the two major arthropod groups: crustaceans (Subphylum: Crustacea) and insects (Class: Insecta) based on openly available data. They found that the main causes of expansion of genome size are proliferation of non-coding elements and/or duplication events. However for other groups of organisms, such as ants, these patterns are a bit expeculative since there are only two studies involving a larger number of species in an attempt to elucidate the GS variations within the family and

the mechanisms of genome evolution, being that of Tsutsui *et al.* (2008) and Ardilla-Garcia *et al.* (2010). In a recent work, Moura *et al.* (in preparation – A) estabilish a protocol to obtain adequate nuclei suspension for flow cytometry (FCM) analyses in ants and suggest that the variation of genome size among populations of the same species are more likely to be related to the stress hinted during the colonization of new environments, and the increase in GS may be correlated wih the accumulation of transposable elements.

Thus, in the present study we first aim to expand the GS database of Formicidae family, specifically within subfamilies, in an attempt to verify the amplitude of variation of this trait; second we establish a protocol of base composition determination through flow cytometry in ants in order to examine the patterns of distribution and variation of genome size and base composition among taxa. We firther correlate and provide a phylogenetic perspective on GS evolution in the family by reconstructing ancestral states.

2. MATERIAL AND METHODS

2.1 GENOME SIZE ESTIMATIONS

2.1.1 Colony sampling

Colonies of different species were collected in several field expeditions. The colonies were detected visually, both by observation of the individuals and by the identification of the nest entrance. When it was possible, the entire colony was collected following the protocols of Cardoso *et al.* (2011), transported and kept under laboratory conditions. In other cases, several individuals from the same colony were collected and transported to the laboratory for immediate realization of the

experiments. The samples were collected in a wide diversity of environments, in the Brazilian states of Tocantins (TO), Bahia (BA), Minas Gerais (MG), Rio de Janeiro (RJ), Santa Catarina (SC) and Rio Grande do Sul (RS) and in the whole individuals belonging to 174 colonies were collected (Table 1). Exemplary samples of each species collected were subsequently stored in absolute alcohol, assembled and sent for identification by Dr. Rodrigo Feitosa, at Universidade Federal do Paraná, and Msc. Júlio Chaul, at Universidade Federal de Viçosa. Specimens that have not yet been identified at the species level were identified at the maximum hierarchical level of genus and are described in the present study followed by "sp". These specimens were photographed to assist in later identification and are arranged in Appendix 1. All vouchers are deposited in the collection of the Laboratório de Genética Evolutiva e de Populações at Universidade Federal de Ouro Preto.

Table 1 – Species sampled during field expeditions with Subfamily, Locality, Collection Date and Geographical Coordinates.

Voucher	Specie	Subfamily	Locality	Collection Date	Geographical Coordinates
ACAB0121	*Acromyrmex ambiguus*	Myrmicinae	BR, Santa Catarina, Araranguá	14/August/2016	28°55'43.92"S;49°21'18.45"W
ACBA0227	*Acromyrmex balzani* - Colony 1	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	04/November/2016	-
ACBA0228	*Acromyrmex balzani* - Colony 2	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	04/November/2016	-
ACCR0127	*Acromyrmex coronatus*	Myrmicinae	BR, Minas Ferais, Viçosa	2014	20°45'17.52"S;42°52'42.88"W
ACCO0263	*Acromyrmex coronatus*	Myrmicinae	BR, Minas Gerais, Poços de Caldas - Cachoeira Véu da Noiva	10/February/2017	21°46'42.24"S;46°36'41.58"W
ACCR0205	*Acromyrmex crassispinus*	Myrmicinae	BR, Minas Gerais, Ouro Preto - UFOP	24/August/2016	20°23'53.00"S;43°30'35.75"W
ACDS0125	*Acromyrmex desciger*	Myrmicinae	BR, Minas Ferais, Viçosa	2014	20°45'17.52"S;42°52'42.88"W
ACNG0123	*Acromyrmex niger*	Myrmicinae	BR, Minas Ferais, Viçosa	2014	20°45'17.52"S;42°52'42.88"W
ACNI0453	*Acromyrmex nigrosetosus* - Colony 1	Myrmicinae	BR, Minas Gerais, Paraopeba - Fazenda Itapoã	18/July/2017	19°16'19.5"S;44°29'19.5"W
ACNI0454	*Acromyrmex nigrosetosus* - Colony 2	Myrmicinae	BR, Minas Gerais, Paraopeba - Fazenda Itapoã	18/July/2017	19°16'11.3"S;44°28'18.2"W
ACNI0455	*Acromyrmex nigrosetosus* - Colony 3	Myrmicinae	BR, Minas Gerais, Paraopeba - Fazenda Itapoã	18/July/2017	19°16'15.7"S;44°29'24.3"W
ACNI0458	*Acromyrmex nigrosetosus* - Colony 4	Myrmicinae	BR, Minas Gerais, Paraopeba - Fazenda Itapoã	18/July/2017	19°16'17.0"S;44°29'18.7"W
ACNI0459	*Acromyrmex nigrosetosus* - Colony 5	Myrmicinae	BR, Minas Gerais, Paraopeba - Fazenda Itapoã	18/July/2017	19°16'04.0"S;44°28'20.1"W
ACSP0365	*Acromyrmex rugosus*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'41.2"S;42°35'31.07"W
ACST0118	*Acromyrmex striatus* - Colony 1	Myrmicinae	BR, Santa Catarina, Araranguá	13/August/2016	28°55'43.92"S;49°21'18.45"W
ACST0119	*Acromyrmex striatus* - Colony 2	Myrmicinae	BR, Santa Catarina, Araranguá	13/August/2016	28°56'57.37"S;49°22'15.12W
ACST0120	*Acromyrmex striatus* - Colony 3	Myrmicinae	BR, Santa Catarina, Araranguá	14/August/2016	28°56'57.37"S;49°22'15.12W
ACSP0434	*Acromyrmex subterraneus brunneus*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°47'02.71"S;42°35'13.57"W
ACSM0122	*Acromyrmex subterraneus molestans*	Myrmicinae	BR, Minas Ferais, Viçosa	2014	20°45'17.52"S;42°52'42.88"W

Voucher	Specie	Subfamily	Locality	Collection Date	Geographical Coordinates
ACSB0479	*Acromyrmex subterraneus molestans*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	19/October/2017	19°45'46.97"S;42°37'52.41"W
ACSS0126	*Acromyrmex subterraneus subterraneus*	Myrmicinae	BR, Minas Ferais, Viçosa	2014	20°45'17.52"S;42°52'42.88"W
ACSS0469	*Acromyrmex subterraneus subterraneus*	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
APSP0204	*Apterostigma sp.*1	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	02/August/2016	20°21'40.6"S;43°38'26.3"W
APSP0209	*Apterostigma sp.*2	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	27/September/2016	20°21'40.6"S;43°38'26.3"W
Semid0392	*Apterostigma sp.*3	Myrmicinae	BR, Minas Gerais, Ouro Branco - Monumento Natural do Itatiaia	21/February/2017	20°29'53.72"S;43°35'41.52"W
APSP0464	*Apterostigma sp.*4	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
ATSP0128	*Atta laevigata*	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	26/July/2016	20°21'40.6"S;43°38'26.3"W
ATLA0456	*Atta laevigata*	Myrmicinae	BR, Minas Gerais, Paraopeba - Fazenda Itapoã	18/July/2017	19°18'13.2"S;44°31'26.6"W
ATSP0135	*Atta robusta* - Colony 1	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	07/September/2016	22°53'36.3"S;42°01'38.1"W
ATSP0136	*Atta robusta* - Colony 2	Myrmicinae	BR, Rio de Janeiro, Arraial do Cabo	08/September/2016	22°57'52.2"S;42°02'19.2"W
ATSR0124	*Atta sexdens rubropilosa*	Myrmicinae	BR, Minas Ferais, Viçosa	2012	20°45'17.52"S;42°52'42.88"W
SemId0233	*Atta sexdens rubropilosa*	Myrmicinae	BR, Tocantins, Araguaína - UFT	19/November/2016	-
SemId0239	*Atta sexdens rubropilosa*	Myrmicinae	BR, Tocantins, Araguaína - UFT	19/November/2016	-
ATSP0348	*Atta sexdens rubropilosa*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	25/April/2017	19°46'19.744"S;42°35'26.753"W
ATSE0452	*Atta sexdens rubropilosa*	Myrmicinae	BR, Minas Gerais, Ouro Preto - UFOP	29/June/2017	20°23'53.00"S;43°30'35.75"W
AZSP0339	*Azteca chartifex spiriti*	Dolichoderinae	BR, Minas Ferais, Marliéria - PERD	25/April/2017	19°46'38.856"S;42°35'14.352"W
AZMU0311	*Azteca muelleri*	Dolichoderinae	BR, Minas Ferais, Viçosa	22/March/2017	20°45'17.52"S;42°52'42.88"W
SemId0225	*Brachymyrmex coactus*	Myrmicinae	BR, Minas Gerais, Ouro Preto - PEIT - Parque Est. Do Itacolomi	26/October/2016	20°26'32.01"S;43°27'43.39"W
SemId0226	*Brachymyrmex coactus*	Myrmicinae	BR, Minas Gerais, Ouro Preto - PEIT - Parque Est. Do Itacolomi	26/October/2016	20°26'32.01"S;43°27'43.39"W
BRSP0169	*Brachymyrmex sp.*1	Formicinae	BR, Rio de Janeiro, Arraial do Cabo	05/September/2016	22°57'42.8"S;42°02'38.9"W
SemId0162	*Brachymyrmex sp.*2	Formicinae	BR, Rio de Janeiro, Rio das Ostras	07/September/2016	22°31'59.6"S;41°55'49.4"W

Voucher	Specie	Subfamily	Locality	Collection Date	Geographical Coordinates
Semid0403	*Brachymyrmex sp.*3	Formicinae	BR, Santa Catarina, Araranguá	25/May/2017	-
BRSP0447	*Brachymyrmex sp.*4	Formicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'21.2"S;43°30'39.0"W
CASP0242	*Camponotus blandus*	Formicinae	BR, Tocantins, Araguaína	20/November/2016	-
CASP0229	*Camponotus bonariensis*	Formicinae	BR, Santa Catarina, Araranguá	02/November/2016	29º57'20"S;49º22'39"O
CASP0230	*Camponotus bonariensis*	Formicinae	BR, Santa Catarina, Araranguá	02/November/2016	29º57'20"S;49º22'39"O
CASP0203	*Camponotus crassus*	Formicinae	BR, Minas Gerais, Cachoeira do Campo	02/August/2016	20°21'40.6"S;43°38'26.3"W
CASP0244	*Camponotus renggeri*	Formicinae	BR, Tocantins, Araguaína	20/November/2016	-
CASP0405	*Camponotus sp.*	Formicinae	BR, Santa Catarina, Jaguaruna	26/May/2017	-
CEAT0132	*Cephalotes atrattus*	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	26/July/2016	20°21'56.24"S;43°38'28.52"W
CESP0236	*Cephalotes depressus*	Myrmicinae	BR, Tocantins, Araguaína - UFT	19/November/2016	-
CESP0237	*Cephalotes depressus*	Myrmicinae	BR, Tocantins, Araguaína - UFT	19/November/2016	-
CEPU0490	*Cephalotes pusillus*	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	21/September/2017	20°21'23"S;43°39'27"W
CEPU0200	*Cephalotes pusillus*	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	02/August/2016	20°21'56.24"S;43°38'28.52"W
CESP0349	*Cephalotes pusillus*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'41.2"S;42°35'31.07"W
CESP0378	*Cephalotes pusillus*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'41.2"S;42°35'31.07"W
CRSP0210	*Crematogaster goeldii*	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	27/September/2016	20°21'40.6"S;43°38'26.3"W
CRNI0463	*Crematogaster nigropilosa*	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
CRSP0295	*Crematogaster nigropilosa*	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
CRSP0297	*Crematogaster sp.*2	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
CRSP0170	*Crematogaster torosa*	Myrmicinae	BR, Rio de Janeiro, Arraial do Cabo	05/September/2016	22°57'46.6"S;42°02'34.7"W
CYSP0470	*Cyphomyrmex sp.*1	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
CISP0296	*Cyphomyrmex sp.*2	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
CYSP0443	*Cyphomyrmex sp.*3	Myrmicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'21.2"S;43°30'39.0"W
CYSP0306	*Cyphomyrmex pr. minutus*	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W

Voucher	Specie	Subfamily	Locality	Collection Date	Geographical Coordinates
CYSP0161	*Cyphomyrmex transversus*	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	09/September/2016	22°53'45.5"S;42°01'41.6"W
CYSP0343	*Cyphomyrmex transversus*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	25/April/2017	19°46'19.74"S;42°35'26.75"W
DOBI0460	*Dolichoderus bispinosus*	Formicinae	BR, Minas Gerias, Paraopeba - FLONA de Paraopeba	28/July/2017	19°16'54"S44°24'32"O
DOSP0208	*Dorymyrmex sp.*1	Dolichoderinae	BR, Rio de Janeiro, Cabo Frio	06/September/2016	22°54'29.7"S;42°02'15.4"W
PASP0241	*Dorymyrmex sp.*2	Dolichoderinae	BR, Tocantins, Araguaína - UFT	19/November/2016	-
Semid0351	*Dorymyrmex brunneus*	Dolichoderinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'41.2"S;42°35'31.07"W
ECSP0488	*Eciton burchelli*	Dorylinae	BR, Minas Ferais, Marliéria - PERD	18/October/2017	19°45'49.33"S;42°37'29.31"W
GNSP0137	*Ectatomma brunneum*	Ectatomminae	BR, Rio de Janeiro, Cabo Frio	06/September/2016	22°54'34.8"S;42°02'16.3"W
GNSP0206	*Ectatomma brunneum*	Ectatomminae	BR, Rio de Janeiro, Cabo Frio	07/September/2016	22°50'23.8"S;41°58'56.2"W
GNSP0167	*Ectatomma brunneum*	Ectatomminae	BR, Rio de Janeiro, Cabo Frio	07/September/2016	22°54'45.7"S;42°02'42.4"W
ECED0301	*Ectatomma edentatum*	Ectatomminae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
GNSP0307	*Gnamptogenys sp.*	Ectatomminae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
GNST0462	*Gnamptogenys striatula*	Ectatomminae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
HYSP0466	*Hylomyrma reitteri*	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
HYSP0304	*Hypoponera sp.*1	Ponerinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
HYSP0308	*Hypoponera sp.*2	Ponerinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
HYSP0409	*Hypoponera sp.*3	Ponerinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
HYSP0444	*Hypoponera sp.*4	Ponerinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'21.2"S;43°30'39.0"W
HYSP0467	*Hypoponera sp.*5	Ponerinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
DOSP0231	*Linepithema micans*	Dolichoderinae	BR, Santa Catarina, Araranguá	02/November/2016	29º57'20"S;49º22'39"O
MEIN0196	*Megalomyrmex incisus*	Myrmicinae	Bahia	October /2015	14°29'60"S;39°2'7"W
CYSP0356	*Mycetarotes paralellus*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'21.828"S;42°38'2.810"W
CYSP0357	*Mycetarotes paralellus*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'21.828"S;42°38'2.810"W

Voucher	Specie	Subfamily	Locality	Collection Date	Geographical Coordinates
CYSP0358	*Mycetarotes paralellus*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'21.828"S;42°38'2.810"W
MCSP0300	*Mycetarotes sp.*	Myrmicinae	BR, Minas Ferais, Viçosa	22/March/2017	20°45'17.52"S;42°52'42.88"W
MYCO0192	*Mycetophylax conformis*	Myrmicinae	Bahia	October /2015	14°29'60"S;39°2'7"W
MYCO0187	*Mycetophylax conformis*	Myrmicinae	Bahia	October /2015	14°29'60"S;39°2'7"W
MYCO0151	*Mycetophylax conformis*	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	07/September/2016	22°54'37.5"S;42°02'37.8"W
MYCO0154	*Mycetophylax conformis*	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	07/September/2016	22°50'29.8"S;41°58'59.2"W
MYCO0149	*Mycetophylax conformis*	Myrmicinae	BR, Rio de Janeiro, Arraial do Cabo	05/September/2016	22°57'42.8"S;42°02'38.9"W
MYMO0184	*Mycetophylax morschi*	Myrmicinae	BR, Rio Grande do Sul, Torres	04/November/2016	29°22'00"S;49°44'45.4"W
MYMO0183	*Mycetophylax morschi*	Myrmicinae	BR, Rio Grande do Sul, Torres	04/November/2016	29°22'00"S;49°44'45.4"W
MYMO0148	*Mycetophylax morschi*	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	07/September/2016	22°54'37.5"S;42°02'37.8"W
MYMO0158	*Mycetophylax morschi*	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	06/September/2016	22°54'37.6"S;42°02'34.4"W
MYMO0193	*Mycetophylax morschi*	Myrmicinae	Bahia	October/2015	-
MYMO0191	*Mycetophylax morschi*	Myrmicinae	Bahia	October /2015	-
MYMO0247	*Mycetophylax morschi*	Myrmicinae	BR, Santa Catarina, Araranguá	14/August/2016	28°56'57"S;49°22'15"W
MYMO0188	*Mycetophylax morschi*	Myrmicinae	BR, Santa Catarina, Araranguá	2015	28°56'08"S;49°21'29"W
MYSI0182	*Mycetophylax simplex*	Myrmicinae	BR, Santa Catarina, Araranguá	2015	28°56'08"S;49°21'29"W
MYSI0146	*Mycetophylax simplex*	Myrmicinae	BR, Santa Catarina, Araranguá	14/August/2016	28°55'43"S;49°21'18"W
MYSI0144	*Mycetophylax simplex*	Myrmicinae	BR, Santa Catarina, Araranguá	13/August/2016	28°56'57"S;49°22'152"W
CYSP0344	*Mycocepurus goeldii*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	25/April/2017	19°45'39.31"S;42°34'57.25"W
CYSP0353	*Mycocepurus goeldii*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'41.2"S;42°35'31.07"W
MYSP0173	*Myrmicocrypta sp.*1 (JSC - 048 _Sosa Calvo)	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	09/September/2016	22°54'29.7"S;42°02'15.0"W
MYSP0172	*Myrmicocrypta sp.*1 (JSC - 048 _Sosa Calvo)	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	09/September/2016	22°54'29.8"S;42°02'14.7"W
MYSP0465	*Myrmicocrypta sp.*2	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
NESP0471	*Neocerapachys sp.*	Dorylinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
POSP0201	*Neoponera marginata*	Ponerinae	BR, Minas Gerais, Cachoeira do Campo	02/August/2016	20°21'40.6"S;43°38'26.3"W
POSP0212	*Neoponera marginata*	Ponerinae	BR, Minas gerais, Belo Horizonte	22/September/2016	20°03'33.68"S;45°56'46.36"W

Voucher	Specie	Subfamily	Locality	Collection Date	Geographical Coordinates
ODSP0243	*Odontomachus bauri*	Ponerinae	BR, Tocantins, Araguaína	20/November/2016	-
ODME0461	*Odontomachus meinerti*	Ponerinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
PAST0310	*Pachycondyla estriata*	Ponerinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
PHSP0298	*Pheidole oxyops*	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
PHSP0235	*Pheidole radoszkowskii*	Myrmicinae	BR, Tocantins, Araguaína - UFT	19/November/2016	-
PHSP0299	*Pheidole sp.*2	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
POSP0406	*Pheidole sp.*3	Myrmicinae	BR, Santa Catarina, Araranguá	25/May/2017	-
PLPU0163	*Platythyrea punctata*	Ponerinae	Alemanha	-	-
POSP0355	*Pogonomyrmex naegelii*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'41.2"S;42°35'31.07"W
POSP0164	*Pseudomyrmex gracilis*	Pseudomyrmecinae	BR, Minas Gerais, Cachoeira do Campo	26/July/2016	20°21'40.6"S;43°38'26.3"W
POSP0202	*Pseudomyrmex shuppi*	Pseudomyrmecinae	BR, Minas Gerais, Cachoeira do Campo	02/August/2016	20°21'40.6"S;43°38'26.3"W
POSP0213	*Pseudomyrmex termitarius*	Pseudomyrmecinae	BR, Minas Gerais, Cachoeira do Campo	27/September/2016	20°21'40.6"S;43°38'26.3"W
POSP0354	*Pseudomyrmex termitarius*	Pseudomyrmecinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'41.2"S;42°35'31.07"W
PSTE0491	*Pseudomyrmex termitarius*	Pseudomyrmecinae	BR, Minas Gerais, Cachoeira do Campo	21/September/2017	20°21'23"S;43°39'27"W
SESP0483	*Sericomyrmex sp.*1	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	18/October/2017	19°45'49.33"S;42°37'29.31"W
SESP0485	*Sericomyrmex sp.*2	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	18/October/2017	19°45'49.33"S;42°37'29.31"W
SESP0484	*Sericomyrmex sp.*3	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	18/October/2017	19°45'49.33"S;42°37'29.31"W
SESP0486	*Sericomyrmex sp.*4	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	18/October/2017	19°45'49.33"S;42°37'29.31"W
SOSP0341	*Solenopsis saevissima*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	24/April/2017	19°46'45.76"S;42°35'25.51"W
SOSP0340	*Solenopsis saevissima*	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	24/April/2017	19°46'45.76"S;42°35'25.51"W
Semid0350	*Solenopsis sp.*1	Myrmicinae	BR, Minas Ferais, Marliéria - PERD	26/April/2017	19°46'41.2"S;42°35'31.07"W
SOSP0445	*Solenopsis sp.*2	Myrmicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'21.2"S;43°30'39.0"W
SOSP0449	*Solenopsis sp.*3	Myrmicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'25.1"S;43°30'38.4"W
STDE0315	*Strumigenys denticulata*	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W

Voucher	Specie	Subfamily	Locality	Collection Date	Geographical Coordinates
STDE0446	*Strumigenys denticulata*	Myrmicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'21.2"S;43°30'39.0"W
STSP0302	*Strumygenys sp.*1 (ufv 06)	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
STSP0294	*Strumygenys sp.*1 (ufv 06)	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W
STSP0468	*Strumygenys sp.*1 (ufv 06)	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	23/August/2017	20°48'35.5"S;42°51'31.07"W
TAME0451	*Tapinoma melanocephalum*	Dolichoderinae	BR, Minas Gerais, Ouro Preto - UFOP	29/June/2017	20°23'53.00"S;43°30'35.75"W
TRHO0088	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Araranguá	10/February/2015	29º57'20"S;49º22'39"W
THTO0072	*Trachymyrmex holmgreni*	Myrmicinae	BR, Rio Grande do Sul, Torres	04/November/2016	29°24'07.3"S;49°46'32.9"W
THMC0018	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Araranguá	30/March/2016	28°56'08"S;49°21'29"W
THMC0066	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Araranguá	05/November/2016	28°56'08"S;49°21'29"W
TRHO0404	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Araranguá	25/May/2017	28°56'08"S;49°21'29"W
THMC0059	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Araranguá	02/November/2016	28°56'08"S;49°21'29"W
THMC0064	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Araranguá	05/November/2016	28°56'08"S;49°21'29"W
THBG0041	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	02/April/2016	29°11'42.61"S;49°36'30.85"W
THBG0044	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	02/April/2016	29°11'42.61"S;49°36'30.85"W
THBG0049	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	03/November/2016	29°12'53.5"S;49°37'37.9"W
THBG0054	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	03/November/2016	29°12'53.0"S;49°37'39.0"W
THBG0052	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	03/November/2016	29°12'53.2"S;49°37'39.3"W
THBG0032	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	02/April/2016	29°11'42.09"S;49°36'30.37"W
THBG0031	*Trachymyrmex holmgreni*	Myrmicinae	BR, Santa Catarina, Balneário Gaivota	02/April/2017	29°11'42.03"S;49°36'30.75"W
THTO0023	*Trachymyrmex holmgreni*	Myrmicinae	BR, Rio Grande do Sul, Torres	01/April/2016	29°22'00.14"S;49°44'45.11"W
THTO0020	*Trachymyrmex holmgreni*	Myrmicinae	BR, Rio Grande do Sul, Torres	01/April/2016	29°22'00.30"S;49°44'45.15"W
THTO0070	*Trachymyrmex holmgreni*	Myrmicinae	BR, Rio Grande do Sul, Torres	04/November/2016	29°24'07.4"S;49°46'33.1"W
THTO0071	*Trachymyrmex holmgreni*	Myrmicinae	BR, Rio Grande do Sul, Torres	04/November/2016	29°24'07.3"S;49°46'32.9"W
TRHO0197	*Trachymyrmex holmgreni*	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	01/July/2016	20°21'56.24"S;43°38'28.52"W
TRSP0098	*Trachymyrmex holmgreni*	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	08/October/2015	20°21'23"S;43°39'27"W
TRTH0489	*Trachymyrmex holmgreni*	Myrmicinae	BR, Minas Gerais, Cachoeira do Campo	21/September/2017	20°21'23"S;43°39'27"W

Voucher	Specie	Subfamily	Locality	Collection Date	Geographical Coordinates
TRHO0147	*Trachymyrmex iheringi*	Myrmicinae	BR, Santa Catarina, Araranguá	14/August/2016	28°56'57.37"S;49°22'15.12W
SemId0234	*Trachymyrmex urichii*	Myrmicinae	BR, Tocantins, Araguaína - UFT	19/November/2016	-
WAAU0166	*Wasmannia auropunctata*	Myrmicinae	BR, Santa Catarina, Araranguá	14/August/2016	-
SemID0168	*Wasmannia auropunctata*	Myrmicinae	BR, Rio de Janeiro, Cabo Frio	09/September/2016	22°53'37.0"S;42°01'35.9"W
WASP0232	*Wasmannia auropunctata*	Myrmicinae	BR, Santa Catarina, Araranguá	05/November/2016	29º57'20"S;49º22'39"O
WALU0442	*Wasmannia lutzi*	Myrmicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'23.4"S;43°30'36.5"W
WALU0448	*Wasmannia lutzi*	Myrmicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'25.1"S;43°30'38.4"W
WALU0450	*Wasmannia lutzi*	Myrmicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'28.9"S;43°30'37.8"W
WALU0457	*Wasmannia lutzi*	Myrmicinae	BR, Minas Gerais, Ouro Preto - Fazenda da Brígida	19/June/2017	20°21'23.4"S;43°30'36.5"W
WASP0305	*Wasmannia sp.*	Myrmicinae	BR, Minas Ferais, Viçosa - EPTEA Mata do Paraíso	22/March/2017	20°48'35.5"S;42°51'31.07"W

2.1.2 Flow cytometry (FCM) analyses

Total DNA content determination

The Flow cytometry experiments were performed following the protocol established in Moura *et al.* (in preparation - A). The nuclear DNA content of the target species was measured using *Drosophila melanogaster* as the internal standard (1C = 0.18 pg; Moura *et al.*, in preparation - A). Galbraith lises buffer was used for Dolichoderinae, Formicinae, Myrmicinae, and Pseudomyrmecinae subfamilies; LB01 buffer was used for Dorylinae, Ectatomminae and Ponerinae subfamilies (see Moura *et al.*, in preparation - A). Briefly, heads of adult ant workers and the internal standard were cut with a cutting blade and immersed in 100-300 μL of the buffer in a 1.5 mL microtube, and grinded to release the cells nuclei. Then, 600 μL of the buffer were added to the microtube, filtered through a 40 μm nylon mesh and stained with the addition of 6.5 μL of propidium iodide solution and 3.5 μl of RNAse. The samples were stored in the cold (4 ° C) and in the dark and analyzed for up to 1 hour after preparation.

The analysis was performed on a FACSCalibur (Becton Dickinson) cytometer at Universidade Federal de Ouro Preto, equipped with a laser source (488 nm) and the histograms obtained by the Cell Quest software. For each sample, at least 10,000 nuclei were analyzed regarding their relative fluorescence intensity. Three independent replications (i.e. three individuals per specimen) were conducted and histograms with a coefficient of variation (CV) above 5% were rejected. Histograms were analyzed using the Flowing 2.5.1 software (http://www.flowingsoftware.com). The genome size of each specimen was calculated using the 1C-value of *Drosophila*

melanogaster and the values were obtained according to the Doležel and Bartos (2005) equation. A general average was presented per species sampled.

Additional GS data for 79 specimens belonging to 67 ant species were extracted from the Animal Genome Size Database (Gregory, 2018) of previously published studies (Aguiar *et al.*, 2016; Ardila-Garcia *et al.*, 2010; Cardoso *et al.*, 2012; Johnston *et al.*, 2004; Li and Heinz, 2000; Sirvo *et al.*, 2006; Tsutsui *et al.*, 2008) and two GS-values for the outgroups species *Apis mellifera* and *Chalybion californicum* of the phylogenetic tree.

Determination of genomic AT and GC bases composition

The mean AT/GC ratio was calculated for some species following the established by Schwencke *et al.* (1998) for plants. First de total nuclear content of these species was determinate according to the procedure described above and subsequently the 4,6-Diamidino-2-fenilindol (DAPI) was used in the staining specificaly AT-rich regions of the genome. *D. melanogaster* was also used as internal standard in the estimates since its bases composition is already determined (AT = 59%, GC = 41%; Ahuja & Neale, 2005; Adams *et al.*, 2000; Danilevskaya *et al.*, 1991; Soares, 2012).

The analysis was performed on a FACSCanto II (Becton Dickinson) cytometer at Universidade Federal de Juiz de Fora, equipped with a UV lamp (388 nm), and at least 10,000 nuclei were analyzed for each sample. Three independent replications were conducted and histograms with a coefficient of variation (CV) above 5% were rejected. Histograms were analyzed as aforementined. The AT composition of the target species was determined using the formula described by Godelle *et al.* (1993): $AT\ (\%)_{\text{sample}} = AT\ (\%)_{\text{Internal standard}}\ X\ (R_{DAPI} / R_{IP})^{1/3}$, where R_{IP} = ratio of sample

fluorescence intensity relative to the standard using the IP (propidium iodide) fluorochrome, and the R_{DAPI} the DAPI ratio. The composition of GC bases was determined as GC (%) = 100%-AT (%), as suggested by Bogunic *et al.* (2003).

2.2 PHYLOGENETIC ANALYSIS

2.2.1 Taxon sampling and Phylogenetic analyses

A total of 83 Formicidae species were used on the phylogenetic analysis, including one taxa of Amblyoponinae subfamily, eight of Dolichoderinae, two of Dorylinae, four of Ectatomminae, eight of Formicinae, 45 of Myrmicinae, 10 of Ponerinae and four of Pseudomyrmecinae. Three species were included as outgroups: *Apis mellifera* (Apidae), *Chalybion californicum* (Sphecidae) and *Mischocyttarus flavitarsis* (Vespidae). All of the molecular operational taxonomic units were obtained from GenBank (Supporting Information - Appendix 2) and the long wavelength rhodopsin (*LW-Rh*) and wingless (*Wg*) genes were choosed for the analysis. The selection of taxa and genes was due the matching with the genome size data. Therefore, subfamilies and species that do not have estimates of genome size were not included in phylogenetic analysis.

The long wavelength rhodopsin (*LW-Rh*) and wingless (*Wg*) nuclear genes were aligned separately using the Muscle algorithm (Edgar, 2004) provided in MEGA 7.0 (Kumar *et al.*, 2016). The intron of the gene *LW-Rh* was excluded from the alignment and the aligned sequences of both genes were manually concatenated for further analyses. In order to select the substitution model of DNA evolution that fits best to each potential partition under Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC), the software PartitionFinder2 (Lanfear et al. 2014, 2017) was used. The models of evolution estimated for each gene codon position was presented in Table 2. Taking into account the estimated parameters, Bayesian analysis was conducted for phylogenetic inference using MrBayes 3.2.6 (Ronquist *et al.*, 2012). Trees were searched with two independent runs, with four Markov chains

each (one cold and three heated). Each chain was run for 50 million generations and sampled every 5,000 generations. The convergence of the cold chains was checked using the program Tracer 1.6 (Drummond & Rambaut, 2007), and a traditional burn-in on the first 25% of the trees was performed before using the remaining topologies to build a final majority rule consensus tree with its respective branch lengths, which was viewed using FigTree v.1.3 (Raumbaut, 2008).

Table 2 – Models of evolution estimated for each gene and codon position with PartitionFinder2. The models listed were employed for eah data partition in Bayesian analysis.

Gene	Codon Position	Substitution Model
LW-Rh	1 st base	GTR+I+G
	2 nd base	GTR+I+G
	3 nd base	SYM+I+G
WG	1 st base	K80+I+G
	2 nd base	K80+I+G
	3 nd base	GTR+I+G

2.3 ANCESTRAL GENOME SIZE

All 1C values estimated in this study and the 1C-values extracted from the Animal Genome Size Database (Gregory, 2018) were plotted on the phylogenetic tree. To estimate the ancestral genome size throughout the phylogeny, three different reconstruction methods were used: maximum parsimony (MP) analysis in Mesquite 3.04 (Maddison & Maddison, 2011); the maximum likelihood (ML) reconstruction method implemented in Stable Traits (Elliot, 2014); and a Bayesian Inference (BI) via Markov chain Monte Carlo (MCMC) in BayesTraits 3.0 (Pagel *et al.*, 2017) with the "continuous random walk" model. Also, was verified whether the GS evolved

according to a Brownian motion model of evolution throughout the phylogeny in BayesTraits and the ancestral genome was calculated both assuming a Brownian motion model along the phylogeny and using a model with the correction of the parameters δ, κ e λ in the phylogeny, as described in Pagel *et al.* (1997, 2004).

2.4 STATISTICAL ANALYSES

In an attempt to analyze the genome size variation over Formicidae subfamilies the mean genome size per subfamily was calculated and ploted in the phylogenetic tree generated in this study with collapsed branches. General linear models were built to check for differences between the average genome sizes of the sampled subfamilies. The differences in genome size average for each subfamily were assessed by variance analysis of the GLM. When the p-value of ANOVA was significant ($p < 0.05$), a contrast analysis at 5% level was then performed through "coms.R" to check which was different. The statistical analysis was performed in R v2.15.1 software (R Core Team, 2013) and GLM was submitted to residual analysis to evaluate adequacy of the error distribution (Crawley, 2013).

3. RESULTS

3.1 GENOME SIZE ESTIMATIONS

In this study we present new genome size estimates for 99 species (Table 1), 91 of which corresponding to new taxa that have not been previously studied. To calculate the following means and percentage, the 79 estimates from literature that corresponds to 67 species were also used. The number of estimates was increased by more than one hundred percent and now represents 1.19% of the total of 13,364 valid species (Bolton, 2017). Of the 333 accepted genera (Bolton, 2017) there are now estimates of genome size for 56 of them, being *Acromyrmex* the genus with the largest number of species estimated (13 in total). From 17 existing Formicidae subfamilies, there are estimates for nine, being Myrmicinae the one with the largest number of measurements (100) and species (90 different species) followed by Formicinae with a total of 22 estimates corresponding to 20 different species, and Dolichoderinae with 18 estimates from 16 species.

The mean genome size of Formicidae family was 0.38 pg. The lowest 1C-value was found in *Dolichoderus mariae*, *Dorymyrmex bureni* and *Paratrechina longicornis*, with 0.18 pg and the highest value was found in *Apterostigma sp.3*, with 0.81 pg. Overall, the estimated 1C-values varied between subfamilies, ranging from 0.18 to 0.61 pg in Dolichoderinae (average 0.29 pg), 0.22 to 0.37 pg in Dorylinae (average 0.30 pg), 0.32 to 0.71 pg in Ectatomminae (average 0.45 pg), 0.18 to 0.39 pg in Formicinae (average 0.31 pg), 0.21 to 0.81 pg in Myrmicinae (average 0.39 pg), 0.25 to 0.63 pg in Ponerinae (average 0.47 pg), and 0.29 to 0.41 pg in Pseudomyrmecinae (average 0.37 pg). Amblyoponinae subfamily comprised only two values with a mean genome size of 0.36 pg and Myrmeciinae only one value, being equal to 0.28

pg. From the total of 179 values estimated, 84% range from 0.25 to 0.50 pg (Table 3; Figure 2).

Table 3 – Genome sizes estimated for all studied specimens, the mean calculated for each species in pictograms (pg) and Mega-base pairs (Mbp), standard deviation and applied methodology.

Species	Subfamilia	Mean 1C-value (pg)	SD	Mean 1C-value (Mbp)	References	Method	Cell type	Std sp
Acromyrmex ambiguus	Myrmicinae	0.33	0.01	322.74	This study	FCM	BR	DM
Acromyrmex balzani	Myrmicinae	0.37	0.00	361.86	This study	FCM	BR	DM
Acromyrmex coronatus	Myrmicinae	0.34	0.00	332.52	This study	FCM	BR	DM
Acromyrmex crassispinus	Myrmicinae	0.34	0.01	332.52	This study	FCM	BR	DM
Acromyrmex desciger	Myrmicinae	0.33	0.00	322.74	This study	FCM	BR	DM
Acromyrmex echinatior	Myrmicinae	0.36	-	335	Sirvo *et al.,* (2006)	FCM	BR	CRBC
Acromyrmex niger	Myrmicinae	0.36	0.00	352.08	This study	FCM	BR	DM
Acromyrmex cf. nigrosetosus	Myrmicinae	0.35	0.00	342.3	This study	FCM	BR	DM
Acromyrmex rugosus	Myrmicinae	0.35	0.00	342.3	This study	FCM	BR	DM
Acromyrmex striatus	Myrmicinae	0.35	0.00	342.3	This study	FCM	BR	DM
Acromyrmex subterraneus brunneus	Myrmicinae	0.34	0.00	332.52	This study	FCM	BR	DM
Acromyrmex subterraneus molestans	Myrmicinae	0.34	0.01	332.52	This study	FCM	BR	DM
Acromyrmex subterraneus subterraneus	Myrmicinae	0.35	0.01	342.3	This study	FCM	BR	DM
Amblyopone pallipes	Amblyoponinae	0.34	-	335.5	Tsutsui *et al.,* (2008)	FCM	BR	DM
Amblyopone pallipes	Amblyoponinae	0.37	-	361.86	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Aphaenogaster fulva	Myrmicinae	0.42	-	410.76	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Aphaenogaster (rudis-texana group N16)	Myrmicinae	0.43	-	420.54	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Aphaenogaster (rudis-texana group N17)	Myrmicinae	0.46	-	449.88	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Aphaenogaster (rudis-texana group N22b)	Myrmicinae	0.44	-	430.32	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Aphaenogaster treatae	Myrmicinae	0.50	-	489	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM

Species	Subfamilia	Mean 1C-value (pg)	SD	Mean 1C-value (Mbp)	References	Method	Cell type	Std sp
Apterostigma dentigerum	Myrmicinae	0.65	-	636.4	Tsutsui *et al.,* (2008)	FCM	BR	DM
Apterostigma sp.1	Myrmicinae	0.74	0.00	723.72	This study	FCM	BR	DM
Apterostigma sp.2	Myrmicinae	0.69	0.01	674.82	This study	FCM	BR	DM
Apterostigma sp.3	Myrmicinae	0.81	0.01	792.18	This study	FCM	BR	DM
Apterostigma sp.4	Myrmicinae	0.63	0.00	616.14	This study	FCM	BR	DM
Atta cephalotes	Myrmicinae	0.31	-	300.1	Tsutsui *et al.,* (2008)	FCM	BR	DM
Atta columbica	Myrmicinae	0.31	-	298.8	Tsutsui *et al.,* (2008)	FCM	BR	DM
Atta laevigata	Myrmicinae	0.33	0.01	322.74	This study	FCM	BR	DM
Atta robusta	Myrmicinae	0.34	0.00	332.52	This study	FCM	BR	DM
Atta sexdens rubropilosa	Myrmicinae	0.33	0.01	322.74	This study	FCM	BR	DM
Atta texana	Myrmicinae	0.27	-	264.06	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Azteca chartifex spiriti	Dolichoderinae	0.36	0.00	352.08	This study	FCM	BR	DM
Azteca muelleri	Dolichoderinae	0.37	0.00	361.86	This study	FCM	BR	DM
Brachymyrmex coactus	Formicinae	0.39	0.01	381.42	This study	FCM	BR	DM
Brachymyrmex sp.1	Formicinae	0.34	0.00	332.52	This study	FCM	BR	DM
Brachymyrmex sp.2	Formicinae	0.36	0.01	352.08	This study	FCM	BR	DM
Brachymyrmex sp.3	Formicinae	0.35	0.00	342.3	This study	FCM	BR	DM
Brachymyrmex sp.4	Formicinae	0.33	0.00	322.74	This study	FCM	BR	DM
Camponotus blandus	Formicinae	0.29	0.01	283.62	This study	FCM	BR	DM
Camponotus bonariensis	Formicinae	0.36	0.01	352.08	This study	FCM	BR	DM
Camponotus castaneus	Formicinae	0.31	-	304.2	Tsutsui *et al.,* (2008)	FCM	BR	DM
Camponotus crassus	Formicinae	0.29	-	286	Aguiar *et al.,* (2016)	FCM	BR	SX
Camponotus crassus	Formicinae	0.29	0.01	283.62	This study	FCM	BR	DM
Camponotus floridanus	Formicinae	0.23	-	224.94	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Camponotus pennsylvanicus	Formicinae	0.33	-	322.8	Tsutsui *et al.,* (2008)	FCM	BR	DM

Species	Subfamilia	Mean 1C-value (pg)	SD	Mean 1C-value (Mbp)	References	Method	Cell type	Std sp
Camponotus renggeri	Formicinae	0.29	-	286	Aguiar *et al.,* (2016)	FCM	BR	SX
Camponotus renggeri	Formicinae	0.32	0.01	312.96	This study	FCM	BR	DM
Camponotus rufipes	Formicinae	0.29	-	286	Aguiar *et al.,* (2016)	FCM	BR	SX
Camponotus sp.	Formicinae	0.31	0.01	303.18	This study	FCM	BR	DM
Cephalotes atrattus	Myrmicinae	0.40	0.02	391.2	This study	FCM	BR	DM
Cephalotes depressus	Myrmicinae	0.53	0.01	518.34	This study	FCM	BR	DM
Cephalotes pusillus	Myrmicinae	0.38	0.01	371.64	This study	FCM	BR	DM
Cerapachys edentata	Dorylinae	0.22	-	210.7	Tsutsui *et al.,* (2008)	FCM	BR	DM
Crematogaster goeldii	Myrmicinae	0.41	0.02	400.98	This study	FCM	BR	DM
Crematogaster hespera	Myrmicinae	0.28	-	275.9	Tsutsui *et al.,* (2008)	FCM	BR	DM
Crematogaster sp.2	Myrmicinae	0.40	0.01	391.2	This study	FCM	BR	DM
Crematogaster nigropilosa	Myrmicinae	0.39	0.00	381.42	This study	FCM	BR	DM
Crematogaster torosa	Myrmicinae	0.38	0.01	371.64	This study	FCM	BR	DM
Cyphomyrmex sp.1	Myrmicinae	0.42	0.01	410.76	This study	FCM	BR	DM
Cyphomyrmex sp.2	Myrmicinae	0.28	0.02	273.84	This study	FCM	BR	DM
Cyphomyrmex sp.3	Myrmicinae	0.32	0.00	312.96	This study	FCM	BR	DM
Cyphomyrmex pr. minutus	Myrmicinae	0.33	0.00	322.74	This study	FCM	BR	DM
Cyphomyrmex transversus	Myrmicinae	0.50	0.01	489	This study	FCM	BR	DM
Dinoponera australis	Ponerinae	0.57	-	554.7	Tsutsui *et al.,* (2008)	FCM	BR	DM
Dolichoderus bispinosus	Dolichoderinae	0.27	0.00	264.06	This study	FCM	BR	DM
Dolichoderus mariae	Dolichoderinae	0.18	-	176.04	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Dolichoderus taschenbergi	Dolichoderinae	0.23	-	224.94	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Dormyrmex bicolor	Dolichoderinae	0.25	-	249.0	Tsutsui *et al.,* (2008)	FCM	BR	DM
Dorymyrmex brunneus	Dolichoderinae	0.26	0.01	254.28	This study	FCM	BR	DM

Species	Subfamilia	Mean 1C-value (pg)	SD	Mean 1C-value (Mbp)	References	Method	Cell type	Std sp
Dorymyrmex bureni	Dolichoderinae	0.18	-	176.04	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Dorymyrmex sp.1	Dolichoderinae	0.23	0.01	224.94	This study	FCM	BR	DM
Dorymyrmex sp.2	Dolichoderinae	0.27	0.00	264.06	This study	FCM	BR	DM
Eciton burchelli	Dorylinae	0.27	-	263.9	Tsutsui *et al.,* (2008)	FCM	BR	DM
Eciton burchelli	Dorylinae	0.29	0.00	283.62	This study	FCM	BR	DM
Ectatomma brunneum	Ectatomminae	0.38	0.01	371.64	This study	FCM	BR	DM
Ectatomma edentatum	Ectatomminae	0.36	0.00	352.08	This study	FCM	BR	DM
Ectatomma tuberculatum	Ectatomminae	0.71	-	690.4	Tsutsui *et al.,* (2008)	FCM	BR	DM
Eurhopalothrix procera	Myrmicinae	0.39	-	377.2	Tsutsui *et al.,* (2008)	FCM	BR	DM
Forelius pruinosus	Dolichoderinae	0.22	-	215.16	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Formica pallidifulva	Formicinae	0.39	-	385.1	Tsutsui *et al.,* (2008)	FCM	BR	DM
Gnamptogenys sp.	Ectatomminae	0.32	0.02	312.96	This study	FCM	BR	DM
Gnamptogenys striatula	Ectatomminae	0.50	0.01	489	This study	FCM	BR	DM
Hylomyrma reitteri	Myrmicinae	0.45	0.01	440.1	This study	FCM	BR	DM
Hypoponera sp.1	Ponerinae	0.40	0.01	391.2	This study	FCM	BR	DM
Hypoponera sp.2	Ponerinae	0.50	0.00	489	This study	FCM	BR	DM
Hypoponera sp.3	Ponerinae	0.39	0.01	381.42	This study	FCM	BR	DM
Hypoponera sp.4	Ponerinae	0.47	0.01	459.66	This study	FCM	BR	DM
Hypoponera sp.5	Ponerinae	0.36	0.02	352.08	This study	FCM	BR	DM
Labidus coecus	Dorylinae	0.37	-	365.8	Tsutsui *et al.,* (2008)	FCM	BR	DM
Lasius latipes	Formicinae	0.27	-	264.06	Ardila-Garcia et al., (2010)	FCM	BR	DM
Lasius alienus	Formicinae	0.31	-	307.7	Tsutsui et al., (2008)	FCM	BR	DM
Lasius minutus	Formicinae	0.23	-	224.94	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Linepithema humile	Dolichoderinae	0.26	-	250.8	Tsutsui *et al.,* (2008)	FCM	BR	DM

Species	Subfamilia	Mean 1C-value (pg)	SD	Mean 1C-value (Mbp)	References	Method	Cell type	Std sp
Linepithema micans	Dolichoderinae	0.29	0.01	283.62	This study	FCM	BR	DM
Liometopum occidentale	Dolichoderinae	0.29	-	282.0	Tsutsui *et al.,* (2008)	FCM	BR	DM
Megalomyrmex incisus	Myrmicinae	0.46	0.05	449.88	This study	FCM	BR	DM
Messor andrei	Myrmicinae	0.26	-	253.5	Tsutsui *et al.,* (2008)	FCM	BR	DM
Monomorium viride	Myrmicinae	0.50	-	489	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Mycetarotes paralellus	Myrmicinae	0.38	0.01	371.64	This study	FCM	BR	DM
Mycetarotes sp.	Myrmicinae	0.48	0.01	469.44	This study	FCM	BR	DM
Mycetophilax conformis	Myrmicinae	0.31	0.01	303.18	This study	FCM	BR	DM
Mycetophylax conformis	Myrmicinae	0.32	-	312.96	Cardoso *et al.,* (2013)	FCM	BR	ST
Mycetophilax morshii	Myrmicinae	0.34	0.01	332.52	This study	FCM	BR	DM
Mycetophylax morschi	Myrmicinae	0.32	-	312.96	Cardoso *et al.,* (2013)	FCM	BR	ST
Mycetophilax simplex	Myrmicinae	0.41	0.01	400.98	This study	FCM	BR	DM
Mycetophylax simplex	Myrmicinae	0.39	-	381.42	Cardoso *et al.,* (2013)	FCM	BR	ST
Mycocepurus goeldii	Myrmicinae	0.42	0.00	410.76	This study	FCM	BR	DM
Myrmecia varians	Myrmeciinae	0.28	-	269.5	Tsutsui *et al.,* (2008)	FCM	BR	DM
Myrmecina americana A	Myrmicinae	0.26	-	250.7	Tsutsui *et al.,* (2008)	FCM	BR	DM
Myrmecina americana B	Myrmicinae	0.31	-	302.9	Tsutsui *et al.,* (2008)	FCM	BR	DM
Myrmicocrypta sp.1 (JSC - 048 _Sosa Calvo)	Myrmicinae	0.48	0.01	469.44	This study	FCM	BR	DM
Myrmicocrypta sp.2	Myrmicinae	0.39	0.00	381.42	This study	FCM	BR	DM
Neocerapachys sp.	Dorylinae	0.36	0.00	352.08	This study	FCM	BR	DM
Neoponera marginata	Ponerinae	0.63	0.01	616.14	This study	FCM	BR	DM
Odontomachus bauri	Ponerinae	0.49	-	477.3	Tsutsui *et al.,* (2008)	FCM	BR	DM
Odontomachus bauri	Ponerinae	0.38	0.01	371.64	This study	FCM	BR	DM
Odontomachus brunneus	Ponerinae	0.33	-	322.74	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Odontomachus brunneus	Ponerinae	0.44	-	429.8	Tsutsui *et al.,* (2008)	FCM	BR	DM

Species	Subfamilia	Mean 1C-value (pg)	SD	Mean 1C-value (Mbp)	References	Method	Cell type	Std sp
Odontomachus cephalotes	Ponerinae	0.43	-	425.0	Tsutsui *et al.,* (2008)	FCM	BR	DM
Odontomachus chelifer	Ponerinae	0.54	-	523.2	Tsutsui *et al.,* (2008)	FCM	BR	DM
Odontomachus clarus	Ponerinae	0.42	-	414.0	Tsutsui *et al.,* (2008)	FCM	BR	DM
Odontomachus haematodus	Ponerinae	0.51	-	496.5	Tsutsui *et al.,* (2008)	FCM	BR	DM
Odontomachus meinerti	Ponerinae	0.55	0.00	537.9	This study	FCM	BR	DM
Pachycondyla estriata	Ponerinae	0.51	0.01	498.78	This study	FCM	BR	DM
Paratrechina longicornis	Formicinae	0.18	-	176.04	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Pheidole dentata	Myrmicinae	0.24	-	234.72	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Pheidole floridana	Myrmicinae	0.21	-	205.38	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Pheidole hyatti	Myrmicinae	0.33	-	326.7	Tsutsui *et al.,* (2008)	FCM	BR	DM
Pheidole oxyops	Myrmicinae	0.37	0.00	361.86	This study	FCM	BR	DM
Pheidole radoszkowskii	Myrmicinae	0.28	0.02	273.84	This study	FCM	BR	DM
Pheidole sp.2	Myrmicinae	0.37	0.01	361.86	This study	FCM	BR	DM
Pheidole sp.3	Myrmicinae	0.36	0.00	352.08	This study	FCM	BR	DM
Platythyrea punctata	Ponerinae	0.25	0.01	244.5	This study	FCM	BR	DM
Pogonomyrmex badius	Myrmicinae	0.27	-	262.8	Tsutsui *et al.,* (2008)	FCM	BR	DM
Pogonomyrmex californicus	Myrmicinae	0.25	-	249.5	Tsutsui *et al.,* (2008)	FCM	BR	DM
Pogonomyrmex coarctatus	Myrmicinae	0.29	-	282.9	Tsutsui *et al.,* (2008)	FCM	BR	DM
Pogonomyrmex naegelii	Myrmicinae	0.36	0.02	352.08	This study	FCM	BR	DM
Ponera pennsylvanica	Ponerinae	0.55	-	537.9	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Ponera pennsylvanica	Ponerinae	0.60	-	591.9	Tsutsui *et al.,* (2008)	FCM	BR	DM
Prenolepis imparis	Formicinae	0.30	-	296.2	Tsutsui *et al.,* (2008)	FCM	BR	DM
Pseudomyrmex ejectus	Pseudomyrmecinae	0.29	-	283.62	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM

Species	Subfamilia	Mean 1C-value (pg)	SD	Mean 1C-value (Mbp)	References	Method	Cell type	Std sp
Pseudomyrmex gracilis	Pseudomyrmecinae	0.35	-	342.3	Ardila-Garcia *et al.,* (2010)	FCM. FIA	BR. HE	DM. TM
Pseudomyrmex gracilis	Pseudomyrmecinae	0.40	-	387.0	Tsutsui *et al.,* (2008)	FCM	BR	DM
Pseudomyrmex gracilis	Pseudomyrmecinae	0.41	0.01	400.98	This study	FCM	BR	DM
Pseudomyrmex shuppi	Pseudomyrmecinae	0.38	0.02	371.64	This study	FCM	BR	DM
Pseudomyrmex termitarius	Pseudomyrmecinae	0.39	0.01	381.42	This study	FCM	BR	DM
Pyramica rostrata	Myrmicinae	0.28	-	278.6	Tsutsui *et al.,* (2008)	FCM	BR	DM
Sericomyrmex amabilis	Myrmicinae	0.45	-	440.7	Tsutsui *et al.,* (2008)	FCM	BR	DM
Sericomyrmex sp.1	Myrmicinae	0.42	0.03	410.76	This study	FCM	BR	DM
Sericomyrmex sp.2	Myrmicinae	0.42	0.01	410.76	This study	FCM	BR	DM
Sericomyrmex sp.3	Myrmicinae	0.42	0.01	410.76	This study	FCM	BR	DM
Sericomyrmex sp.4	Myrmicinae	0.39	0.00	381.42	This study	FCM	BR	DM
Solenopsis invicta	Myrmicinae	0.62	-	606.36	Li and Heinz (2000)	BCA	BR	NS
Solenopsis invicta	Myrmicinae	0.77	-	753.06	Johnston *et al.,* (2004)	FCM	BR	DM
Solenopsis invicta	Myrmicinae	0.48	-	469.44	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Solenopsis molesta	Myrmicinae	0.38	-	371.64	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Solenopsis saevissima	Myrmicinae	0.50	0.01	489	This study	FCM	BR	DM
Solenopsis sp.1	Myrmicinae	0.45	0.01	440.1	This study	FCM	BR	DM
Solenopsis sp.2	Myrmicinae	0.39	0.00	381.42	This study	FCM	BR	DM
Solenopsis sp.3	Myrmicinae	0.41	0.01	400.98	This study	FCM	BR	DM
Solenopsis xyloni	Myrmicinae	0.48	-	472.3	Tsutsui *et al.,* (2008)	FCM	BR	DM
Strumigenys denticulata	Myrmicinae	0.32	0.00	312.96	This study	FCM	BR	DM
Strumygenys sp.1 (ufv 06)	Myrmicinae	0.32	0.00	312.96	This study	FCM	BR	DM
Tapinoma melanocephalum	Dolichoderinae	0.28	0.01	273.84	This study	FCM	BR	DM
Tapinoma sessile	Dolichoderinae	0.37	-	361.86	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM

Species	Subfamilia	Mean 1C-value (pg)	SD	Mean 1C-value (Mbp)	References	Method	Cell type	Std sp
Tapinoma sessile A	Dolichoderinae	0.38	-	374.4	Tsutsui *et al.,* (2008)	FCM	BR	DM
Tapinoma sessile B	Dolichoderinae	0.61	-	593.1	Tsutsui *et al.,* (2008)	FCM	BR	DM
Temnothorax ambiguus	Myrmicinae	0.31	-	303.18	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Temnothorax texanus	Myrmicinae	0.32	-	312.96	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Tetramorium caespitum	Myrmicinae	0.26	-	256.4	Tsutsui *et al.,* (2008)	FCM	BR	DM
Tetramorium caespitum	Myrmicinae	0.27	-	264.06	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Trachmyrmex urichii	Myrmicinae	0.47	0.01	459.66	This study	FCM	BR	DM
Trachymyrmex holmgreni	Myrmicinae	0.33	0.01	322.74	This study	FCM	BR	DM
Trachmyrmex ihering	Myrmicinae	0.40	0.01	391.2	This study	FCM	BR	DM
Trachymyrmex septentrionalis	Myrmicinae	0.25	-	244.5	Ardila-Garcia *et al.,* (2010)	FIA	HE	TM
Wasmannia auropunctata	Myrmicinae	0.36	0.01	352.08	This study	FCM	BR	DM
Wasmannia lutzi	Myrmicinae	0.37	0.00	361.86	This study	FCM	BR	DM
Wasmannia sp.	Myrmicinae	0.38	0.00	371.64	This study	FCM	BR	DM
Apis mellifera	Apidae - outgroup	0.24	-	234.72	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM
Chalybion californicum	Sphecidae - outgroup	0.54	-	528.12	Ardila-Garcia *et al.,* (2010)	FCM	BR	DM

The base composition values in Myrmicinae oscillated from AT% = 59.17, in *Pheidole sp.2* to 67% in *Mycetophylax simplex,* and the mean AT was 62.63%. In Ectatomminae and Pseudomyrmecinae the values found were similar, equal to AT = 60.22% and 60.44%, respectively. It was not possible to compare the AT and GC means of the subfamilies because only Myrmicinae was represented by more than one species. The highest AT values, as in *M. simplex* (67%) and *Atta sexdens rubropilosa* (64.82%) were not accompanied by the larger genome sizes, since the first one had 0.41 pg and the second 0.33 pg and the highest GS value was found in *Apterostigma sp. 3*, with 0.81 pg and AT = 62.18%. The values of genome size (pg) and base composition (AT and GC %) estimated for the species in the present study are summarized in Table 4.

Table 4 – Percentage of AT and GC bases of the species with genome size estimated in the present study.

Species	Subfamily	Mean 1C-value	AT (%) sample	GC (%) sample
Acromyrmex cf. nigrosetosus	Myrmicinae	0.35	63.75	36.25
Acromyrmex rugosus	Myrmicinae	0.35	63.23	36.77
Acromyrmex subterraneus brunneus	Myrmicinae	0.34	63.38	36.62
Acromyrmex subterraneus subterraneus	Myrmicinae	0.35	63.72	36.28
Apterostigma sp.3	Myrmicinae	0.81	62.18	37.82
Atta sexdens rubropilosa	Myrmicinae	0.33	64.82	35.18
Cephalotes pusillus	Myrmicinae	0.38	62.59	37.41
Cyphomyrmex transversus	Myrmicinae	0.50	62.03	37.97
Ectatomma brunneum	Ectatomminae	0.38	60.22	39.78
Megalomyrmex incisus	Myrmicinae	0.46	61.27	38.73
Mycetarotes sp.	Myrmicinae	0.48	61.54	38.46
Mycetarotes paralellus	Myrmicinae	0.38	61.80	38.20
Mycetophylax conformis	Myrmicinae	0.31	62.92	37.08
Mycetophylax morshii	Myrmicinae	0.34	61.82	38.18
Mycetophylax simplex	Myrmicinae	0.41	67.00	33.00
Myrmicocrypta sp.1	Myrmicinae	0.48	61.45	38.55
Pheidole sp.2	Myrmicinae	0.37	59.17	40.83
Pseudomyrmex termitarius	Pseudomyrmecinae	0.39	60.44	39.56
Trachymyrmex holmgreni	Myrmicinae	0.33	62.58	37.42
Trachmyrmex ihering	Myrmicinae	0.40	62.14	37.86

The simultaneous analyses of the target species and *D. melanogaster* (internal standard) nuclei suspensions provided histograms with fluorescence peaks corresponding to the mean DNA content of the G0/G1 and G_2 nuclei of both organisms, stained with PI (Figure 1, a-c) and, for some species, DAPI (Figure 1, d-f). The G_0/G_1 peaks of all specimens included in this study can be clearly discriminated, and their variation coefficients were always less than 5%, which is considered appropriate for GS determination using FCM (Cardoso *et al.*, 2012). Six representative histograms, three for genome size and three for base composition, are shown in Figure 1.

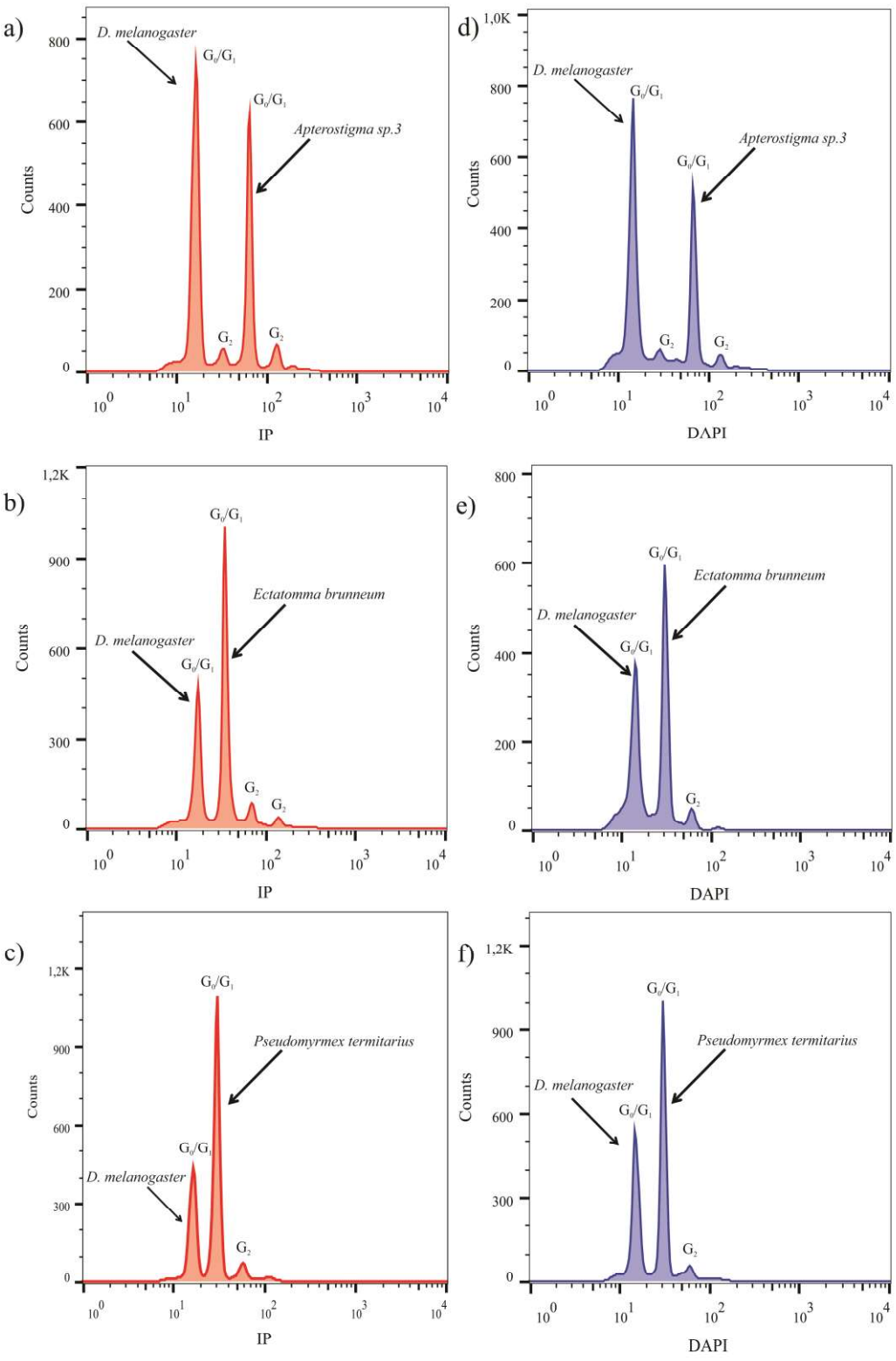

Figure 1 - Fluorescence intensity histograms obtained in three different species with *D. melanogaster* as internal standard stained with propidium iodide (a-c) and DAPI (d-f). The x axis corresponds to a scale of fluorescence intensity and the y axis represents the number of nuclei with that fluorescence intensity.

3.2 PHYLOGENETIC ANALYSIS

The alignment length of 871 bp was obtained for the *LW-Rh* and *WG* nuclear regions using 83 sequences from species belonging to Formicidae and sequences from three accessions of outgroups species, which included 500 variable sites (57.4%). Figure 2 shows the Bayesian consensus phylogenetic tree based on the *LW-Rh* and *WG* genes. Formicidae family was recovered as monophyletic with high value of posterior probability (PP) (node 1 (n1), PP = 1). The subfamily Amblyoponinae, represented by only one species, was recovered as a sister group to all other ants, as well as Ponerinae with all the species grouped into one clade (node 2 (n2), PP = 1). The node 3 (n3), which had a PP = 0.93, comprises the remaining subfamilies sampled, having Dorylinae (node 4 (n4), PP = 1) the sister group of the others. The node 5 (n5) with a high posterior probability value (PP = 0.95) was divided into two clades: the first (node 6 (n6), PP = 0.69) containing the subfamilies Formicidae (node 7 (n7), PP = 0.97), Ectatomminae (node 8 (n8), PP = 1) and Pseudomyrmicinae (node 9 (n9), PP = 1); and the second clade (node 10 (n10), PP = 0.71) containing the other two subfamilies, Dolichoderinae (node 11 (n11), PP = 1) and Myrmicinae (node 12 (n12), PP = 1). Within Myrmicinae, a last monophyletic clade stands out (node 13 (n13), PP = 1), being composed by the restricted group of Fungus-growing ants.

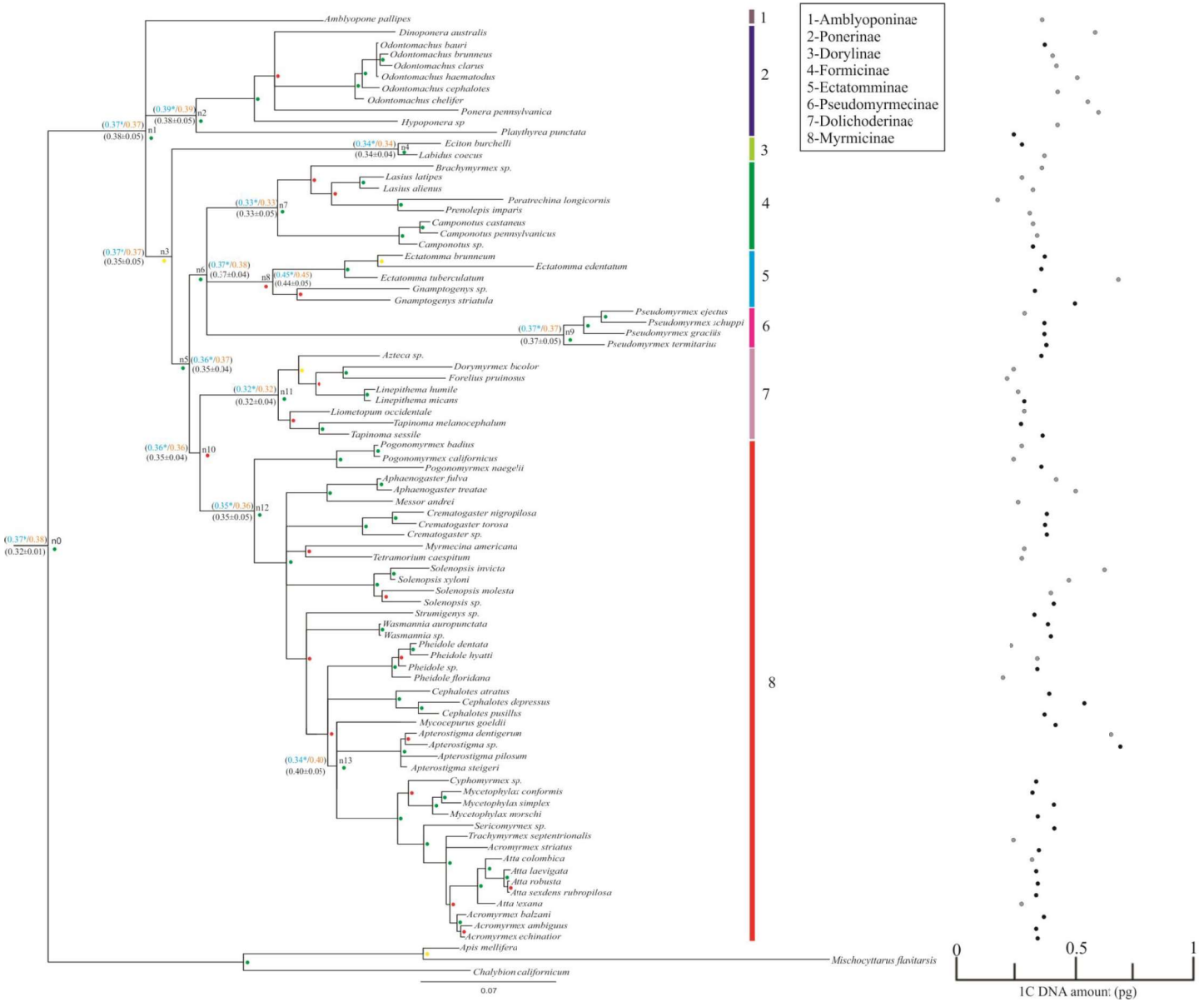

Figure 2 – Bayesian consensus tree resulting from the *LW-Rh* and *WG* alignment (871 bp length). Colored dots on the branches indicate the values of posterior probability (PP): green dots represent values between 1 and 0.95; yellow dots represent values between 0.94 and 0.9; and red dots represent values smaller or equal to 0.89. The nodes are indicated with numbers. Values above and below the branches represent the ancestral genome size (GS; 1C-values in pg) at particular nodes: in blue is the value generated by the maximum likelihood (ML) (asterisks are related to confidence interval [IC] values shown in Supporting Information - Appendix 3); orange is the value generated by the maximum parsimony (MP); black, given below the branches, is the value generated by Bayesian inference (BI). Genome size data (1C-values) obtained in the present work (●) or taken from Literature (●).

3.3 ANCESTRAL GENOME SIZE

In the present study, we verified whether the genome size evolved according to a Brownian motion model of evolution along the phylogeny, and the test revealed that it is indeed sufficient according to the available data (p-value > 0.05). This could be verified by computing the phylogeny correction parameters, δ (Delta), κ (Kappa) and λ (Lambda), since the values found were very close to 1, being consistent with the constant-variance model (sometimes called Brownian motion) and being a correct representation of the data (Pagel *et al.*, 2016). However, to reaffirm this result and leave no possible bias in the reconstruction analysis of the ancestral genome, the values found with Maximum Likelihood and Bayesian Inference were calculated both assuming a Brownian motion model in the phylogeny and with the correction of the parameters, showing no difference between the results generated.

Besides that, the genome size values of the ancestor nodes were calculated using three different methods, also resulting in no significant difference among them. The ancestral Formicidae (n1, Figure 3) GS reconstructed using MP was 0.37 pg, using ML was 0.37 pg (0.20–0.55, 95% highest posterior density), and based on BI was 0.38 ± 0.05 pg. The values obtained with the three methods varied little or nothing among themselves, as observed, for example, for node 5 (MP = 0.37, ML = 0.36, and MCMC = 0.35 ± 0.04), node 8 (MP = 0.45, ML = 0.45, and MCMC = 0.44 ± 0.05) and n9 (MP = 0.37, ML = 0.37, and MCMC = 0.37 ± 0.05). The only node that varied a little more was the node 13 that presented a lower value with Maximum Likelihood (ML = 0.34 pg; 0.24-0.43, 95% highest posterior density) than with MP (0.40 pg) and BI (0.40 pg ±0.05), but the value found with the other two methods is within the confidence interval of the first.

3.4 STATISTICAL ANALYSES

Significant differences in GS were observed between the subfamilies sampled (ANOVA, p-value < 0.01). Through contrast analysis, the subfamilies Dolichoderinae, Dorylinae, Formicinae, Amblyoponinae and Pseudomyrmecinae grouped statistically (group average = 0.31 pg, p-value > 0.05) as well as Myrmicinae and Ectatomminae subfamilies (group average = 0.39 pg, p-value > 0.05) (Figure 3). Only the mean of Ponerinae differs from all the others (aveage = 0.47, p-value < 0.01). Myrmeciinae (0.28 pg) was not considered in the analysis because only one genome size value was available.

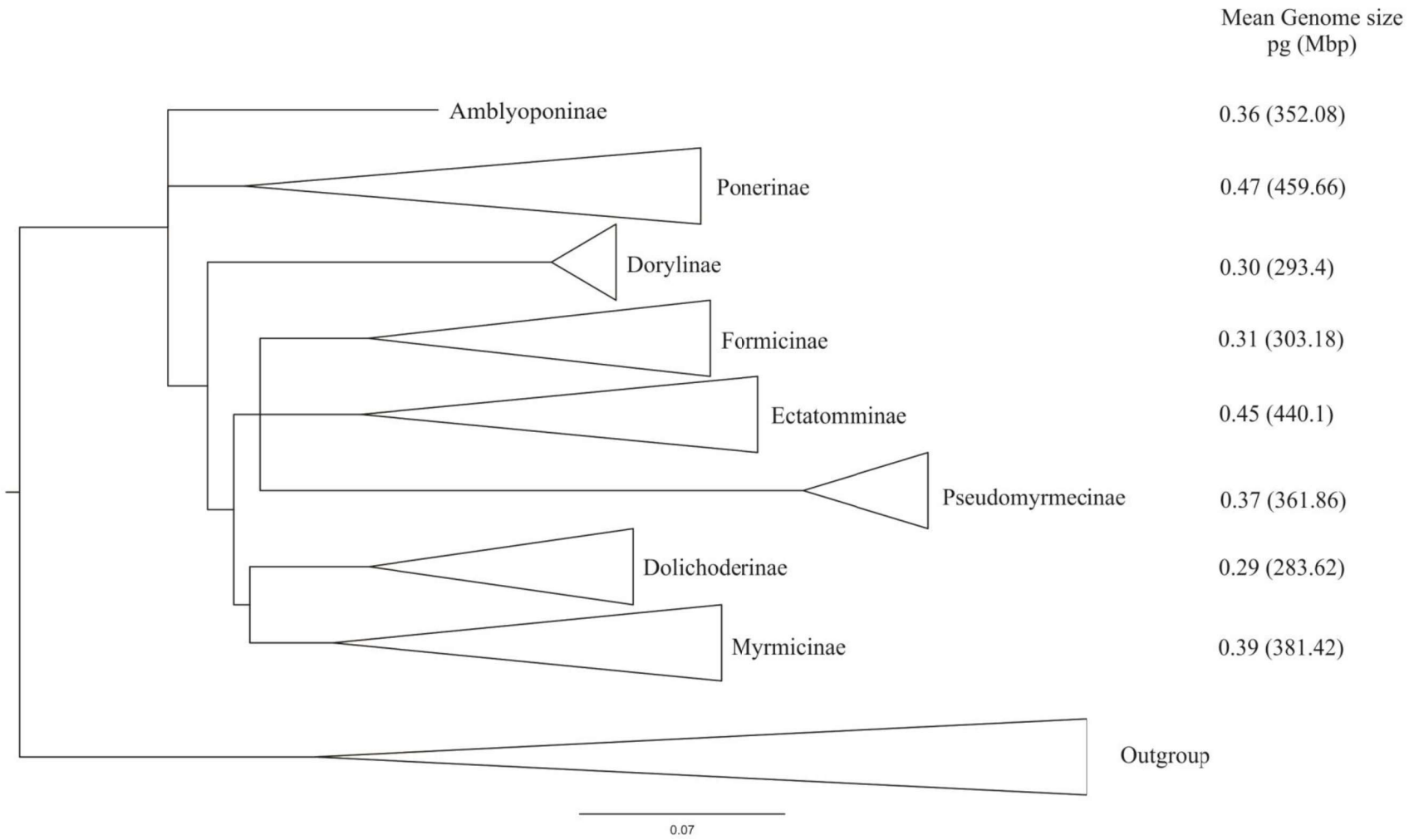

Figure 3 – Mean genome size estimated for Formicidae subfamilies. The phylogenetic tree generated in the present study was redrawn, with collapsed branches corresponding to species of the same subfamily.

4. DISCUSSION

The genome size values reported here (Table 3) for some species that have previously been estimated by other researchers with flow cytometry (and available in Gregory, 2018) were found to be very close. For example, the published values of *Eciton burchelli* was 0.27 pg in Tsutsui *et al.* (2008) and in the present study was 0.29 pg; for *Camponotus renggeri* was 0.29 pg in Aguiar *et al.* (2016) and 0.32 pg in the present study; and for *Camponotus crassus* the value found was 0.29 pg in Aguiar *et al.* (2016) and in the present study. The mean GS values for the Formicidae subfamilies were also similar to those found in the study by Tsutsui *et al.* (2008); however, the amplitude of variation was higher in the present study that covered a greater number of species. In general, all subfamilies sampled with a larger number of species (normally more than five species) present a large range of GS variation, being in Myrmicinae the most prominent (0.21 pg to 0.81 pg).

This amplitude of variation within a subfamily reflects mainly the large variation in genome size found between genera, and in a few cases between species of the same genus. This is the opposite of that found in the order Lepidoptera (Gregory & Hebert, 2003), where more variation between subfamilies was observed than within a subfamily. It is also the opposite of that suggested by Tsutsui *et al.* (2008) for ants, and the main reason arise in the number of species sampled. In the mean GS value found for Ectatomminae subfamily, which in Tsutsui *et al.* (2008) was 0.71 pg and in the present study was 0.45 pg. The explanation for this is that the only species addressed by the author showed a possible whole nuclear genome duplication, and this is not the pattern

for the other species of the genus, which was confirmed by the genome size estimate of two other species in this study that presented approximately half of the estimated value found in Tsutsui *et al.* (2008) (0.38 pg in *Ectatomma brunneum* and 0.36 pg in *Ectatomma edentatum*; Table 3).

Genomic expansion through whole genome duplication seems to have happened in *Apterostigma* linage, as also suggested by Tsutsui et al. (2008) based on *Apterostigma dentigerum* (0.65 pg). Two morphospecies *Apterostigma sp.2* and *sp.4*, presented GS very similar to the already published genome being 0.69 pg and 0.63 pg, respectively. Yet *Apterostigma sp.1* and *sp.3* presented higher values, equal to 0.74 pg and 0.81 pg, respectively. All these values are, in fact, almost twice as high as those estimated for other species of Myrmicinae subfamily, especially within the group of fungus-growing ants such as *Mycocepurus goeldii*, whose GS was 0.42 pg and the genus *Cyphomyrmex*, whose average GS was 0.37 pg.

Tsutsui *et al.* (2008) suggested that genome expansion by whole-genome duplication in *Ectatomma* and *Apterostigma* would have occurred in the ancestor of each genus, being in *Ectatomma* potentially 80-90 million years ago and *Apterostigma* more recently. However, this seems not to be the case, since the estimated values for the two other species of the genus *Ectatomma*, indicating that the process of genome expansion may have occurred at species level in this genus. Yet, for *Apterostigma* this seems likely since all five species with genome size estimated so far, presents apparently duplicate values. The results found in the present study also corroborate those of Cardoso *et al.* (2012) who found no evidence of whole-genome duplication in the Neoattini genera,

such as *Mycetophylax* (mean genome size = 0.35 pg), *Cyphomyrmex* (mean genome size = 0.37 pg), *Trachymyrmex* (mean genome size = 0.36 pg), *Sericomyrmex* (mean genome size = 0.42 pg), *Acromyrmex* (mean genome size = 0.34 pg) and *Atta* (mean genome size = 0.32 pg) and suggest that this phenomena is related only to the Paleoattini clade, that includes *Apterostigma*.

Base composition through flow cytometry was measured for the first time in Formicidae species. The mean values obtained for both the family (AT = 62.40%, GC = 37.60%) and the subfamilies Myrmicinae (AT = 62.63%, GC = 37.37%), Ectatomminae (AT = 60.22%, GC = 39.78%) and Pseudomyrmecinae (AT = 60.44%, GC = 39.56%) are very similar to the values reported for bees (*Scaptotrigona xantotricha*, AT = 61.32% and GC = 38.68%; *Trigona hyalinata*, AT = 62.40% and GC = 37.60%; *Partamona rustica* AT = 62.82% and GC = 37.18%) (Soares, 2012). Lorite & Palomeque (2010) suggested that large genome size values found in *Ectatomma* and *Apterostigma* were actually related to the difference in the amount of heterochromatin, since the chromosome number of *Ectatomma tuberculatum* was n = 18 (Barros *et al.*, 2008) and of a species of the genus *Apterostigma* was n = 10 – 12 (Murakami *et al.*, 1998), not considered high chromosome numbers. However, this theory falls apart with the analysis of the AT /GC ratio of the species in Table 4, where no correlation between genome size and total amout of AT or GC is observed.

The Bayesian consensus phylogenetic tree based on the *LW-Rh* and *WG* genes recovered Formicidae as monophyletic family with high value of posterior probability (PP) (n1, PP = 1). The relationship between subfamilies and species are also consistent with that

found in other studies, such as the work by Moreau *et al.* (2006, 2013), Ward *et al.* (2015) and Branstetter *et al.* (2017), although it comprise a subset of species and subfamilies. Several genera have also been recovered as monophyletic: *Odontomachus*, *Lasius*, *Camponotus*, *Ectatomma*, *Gnamptogenys*, *Pseudomyrmex*, *Linepithema*, *Pogonomyrmex*, *Aphaenogaster*, *Crematogaster*, *Solenopsis*, *Pheidole*, *Chepalotes*, *Apterostigma*, *Mycetohpylax* (*sensu* Klingenberg & Brandão, 2009), *Atta* and *Acromyrmex*, except for *A. striatus* that emerged as a sister group of the other leafcutter ants as demonstrated in Cristiano *et al.* (2013). However, several studies have already demonstrated paraphyletism in the genera *Aphaenogaster* (Brady *et al.*, 2006; Moreau, 2008, 2013), *Camponotus* (Brady *et al.* 2000, 2006; Moreau *et al.*, 2013), and *Odontomachus* (Moreau *et al.*, 2013) and the results obtained in this work may be a bias of the number of species sampled.

The reconstructed ancestral genome size for Formicidae was 0.38 pg according to the BI method and 0.37 pg according to ML and parsimony methods. This value is congruent with the overall mean genome size for the family, also reflecting the distribution of the data (see Figure 2). Despite this relatively small ancestral genome (smaller than 1 pg), smaller genomes than this can be visualized along phylogenetic tree, but the change in values does not follow the evolution of the subfamilies. This means that the evolution of the genome did not occur in a linear way, being smaller in the more basal subfamilies, such as Amblyoponinae, and higher in the most derived ones, such as Dolichoderinae, Ectatomminae and Myrmicinae. In contrast, both expansions and retractions of the genome occur in all subfamilies.

The reconstructed ancestral genome size of all subfamilies (n2 to n13, Figure 2) comprise similar values to those of Formicidae, equal to 0.37/0.38 pg, being higher only in Ectatomminae (n8: 0.45 pg with ML and MP, 0.44 pg with BI) and the clade of fungus-growing ants (n13: 0.40 pg with BI and MP; 0.34 pg with ML). Considering this, there is an interesting pattern to be pointed out: five of the nine sampled subfamilies (Dolichoderinae, Dorylinae, Formicinae, Myrmicinae and Poneriae) present at one extreme of genome size variation, values that are exactly half the value of the ancestral genome (i.e. 0.18 pg) and in the other extreme values that are practically twice the others, above 0.60 pg, indicating gain and loss of DNA amount per genera in the subfamilies sampled. On the other hand, on Ectatomminae were found values close to the ancestral genome (0.36 pg in *Ectatomma edentatum*), intermediate values (0.50 pg in *Gnamptogenys striatula*) and duplicate values as in *Ectatomma tuberculatum* (0.71 pg), indicating only increases in genome size in the subfamily. Amblyoponinae and Pseudomyrmicinae presentes the values around the ancestral genome, except by *Pseudomyrmex ejectus* which had the smallest genome size (0.29 pg) but was estimated through another methodological process, with image cytometry, using blood smear and *Tenebrio molitor* as internal standard.

When considering only the species collected and estimated in the present study the observed pattern is even more homogeneous. Of the 99 estimated values, 91% are between 0.25 pg and 0.50 pg, with only one value being lower than the minimum limit (0.23 pg in *Dorymyrmex* sp.1); only three values are intermediate between 0.50 pg and 0.60 pg (*Pachycondyla estriata* = 0.51 pg *Cephalotes depressus* = 0.53 pg and *Odontomachus meinerti* = 0.55 pg); and five values are above 0.60 pg, being reported

for the species of *Apterostigma* mentioned previously and *Neoponera marginata* (0.63 pg).

Increasing and decreasing in genome size are usually related to chromosomal alterations that can be numeric (euploidy and/or aneuploidy) or structural (deletion, inversion, duplication, translocation) (Moura et al, *in press*). These changes are considered key factors in the evolution of genomes in plants (e.g. Campos *et al.*, 2011; Lepers-Andrzejewski *et al.*, 2011; Szadkowsk *et al.*, 2011). However, these types of modifications in addition to changing the genome size are usually associated with deleterious phenotypic effects (Gregory, 2005) and are not considered the main mechanism that leads the GS changes in animal species. Instead, for some animal species it has already been demonstrated that genome size is strongly related to the abundance of transposable elements (TEs) and for human has been shown that nearly 45% of the genome is composed of transposable elements and their inactive remnants (Gregory, 2005; International Human Genome Sequencing Consortium, 2001)

Moura *et al.* (in preparation - A) suggested that the diferences in genome size found beetwen populations of the same species of ants could be related to the stress of colonization of new enviroments and the increase in GS may be correlated with the accumulation of transposable elements (TEs). Considering all the values discussed above in relation to the ancestral genome size of Formicidae and the base composition rate of the species showed on Table 4, everything leads to the understanding that some processes are more involved in the evolution of the genome for the family, as the

accumulation of noncoding elements, like the TEs, and whole genome duplication process.

Some authors suggest that the movement and accumulation of TEs have exerted a strong influence on the evolution of their hosts and that the accumulation of these elements in the genome is a gradual process (Alfsnes *et al.*, 2017; Feschotte, 2008; Brookfield, 2005). This accumulation of TEs explains, for example, the difference in genome size of many species and genera in relation to the ancestral genome of Formicidae and of the subfamilies themselves, especially those having a genome size greater than 0.38 pg but not greater than 0.60 pg, where the process of whole genome duplication would fit better. In addition, it also explains the decrease of the genome in relation to the ancestral, suggesting that the loss of DNA amount occurs in these non-coding regions, since no function or trait is lost in species with smaller genome sizes (e.g. below 0.25 pg).

In conclusion, the results obtained in this study improve the knowledge concerning Formicidae genome size, as well as the base composition of some species and patterns of GS evolution through phylogeny from an ancestral genome. Flow cytometry procedures were established for determination of the AT/GC ratio of the genome, a tool still less widespread for insects. The flow cytometry data reported here also contribute to the understanding of GS diversity and range of variation in Formicidae, knowledge hitherto skewed given the number of species analyzed in earlier studies. The results of this study suggest that the evolution of GS in Formicidae was due to the loss and accumulation of non-coding regions, mainly transposable elements, and in some specific cases by whole

genome duplication. However, the processes behind these genome enlargements and retractions still need to be understood, mainly through species diversification studies, in order to verify if these changes in DNA content are related to the colonization process of the species, thus as suggested at the intraspecific level (Moura *et al.*, in preparation – A). Furthermore, the genome base composition needs to be estimate in a greater number of species in order to verify the range of variation of AT/GC ratio in the subfamilies and mainly to understand if the composition differs in those species with smaller genome size, especially those with estimated GS less than 0.25 pg. Still, this work serves as a guide for future whole genome sequencing projects so they can cover the species in all the limits of variation in genome size.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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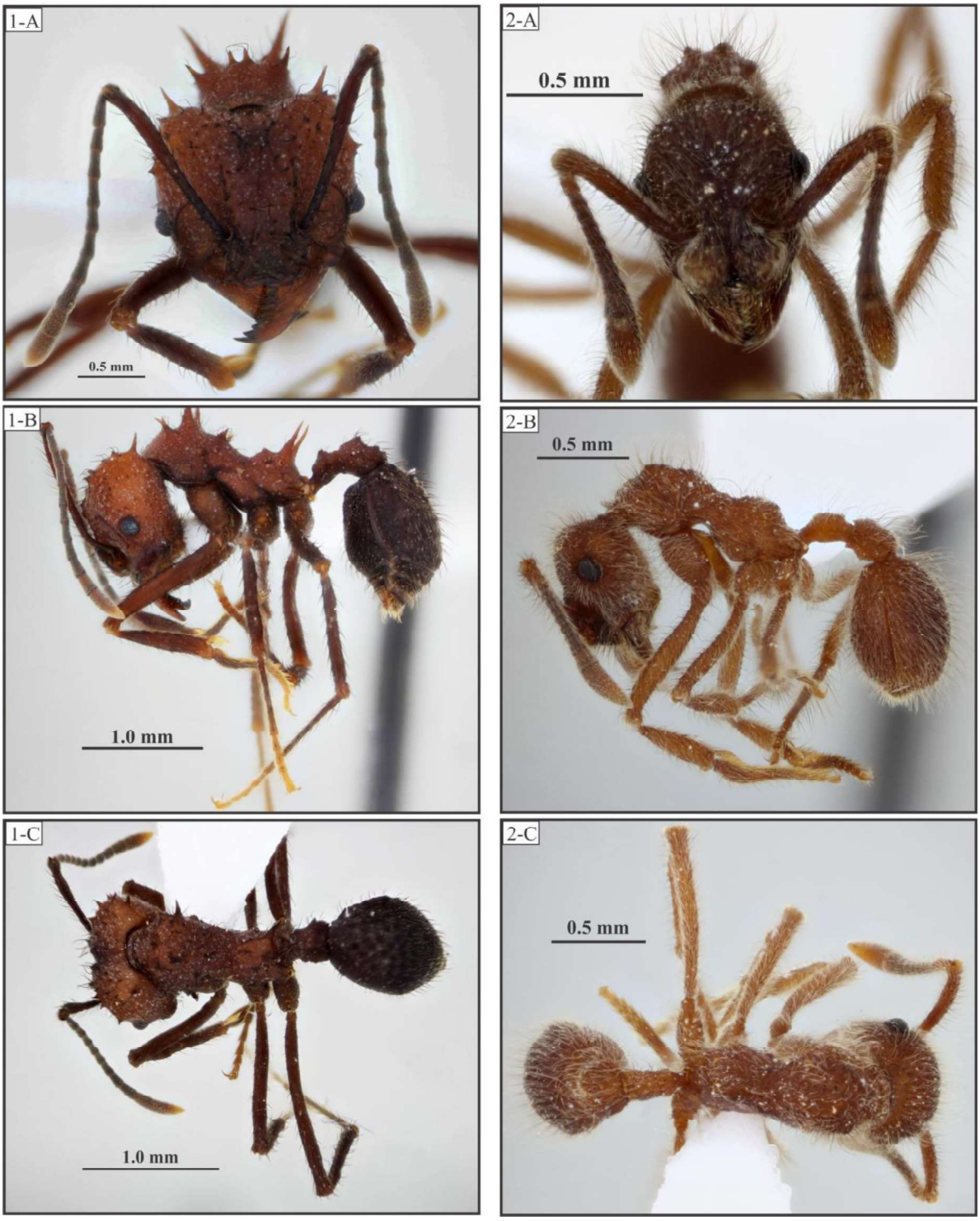
SUPPORTING INFORMATION – APPENDIX 1. Unidentified species. In all pictures: A) Head View; B) Profile View; C) Dorsal View. **Species: 1)** *Acromyrmex cf. nigrosetosus*; **2)** *Apterostigma sp.1*; **3)** *Apterostigma sp.2*; **4)** *Apterostigma sp.3*; **5)** *Apterostigma sp.4*; **6)** *Brachymyrmex sp.1*; **7)** *Brachymyrmex sp.2*; **8)** *Brachymyrmex sp.3*; **9)** *Camponotus sp.*; **10)** *Crematogaster sp.2*; **11)** *Cyphomyrmex sp.2*; **12)** *Cyphomyrmex sp.3*; **13)** *Gnamptogenys sp.*; **14)** *Hypoponera sp.1*; **15**) *Hypoponera sp.2*; **16)** *Hypoponera sp.3*; **17)** *Hypoponera sp.5*; **18)** *Mycetarotes sp.*; **19)** *Myrmicocrypta sp.1* (JSC - 048 _Sosa Calvo); **20)** *Myrmicocrypta sp.2*; **21)** *Pheidole sp.2*; **22)** *Pheidole sp.3*; **23)** *Sericomyrmex sp.1*; **24**) *Sericomyrmex sp.2*; **25)** *Sericomyrmex sp.3*; **26)** *Sericomyrmex sp.4*; **27)** *Solenopsis sp.1*; **28)** *Solenopsis sp.2*; **29)** *Strumygenys* (ufv 06); **30)** *Wasmannia sp*.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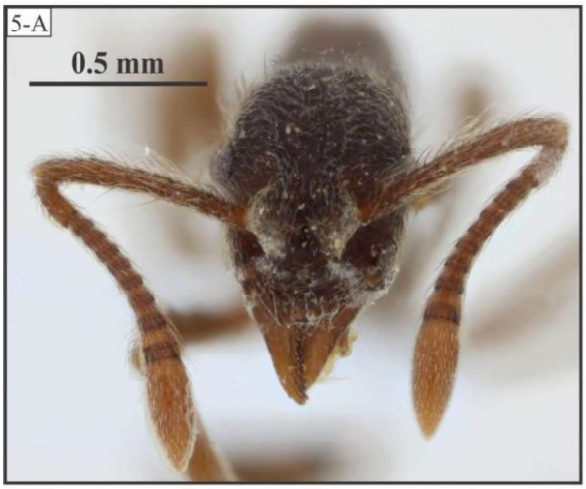
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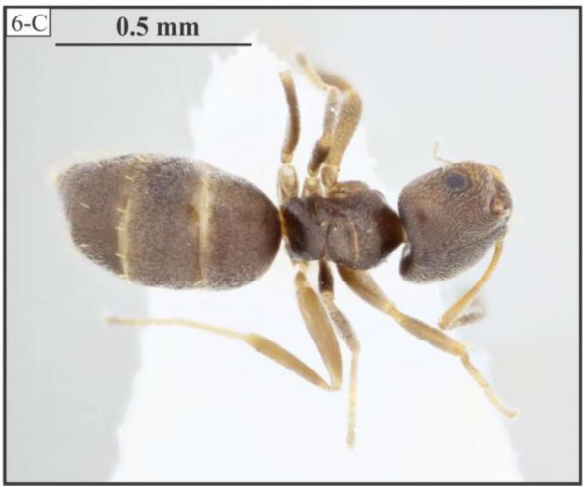
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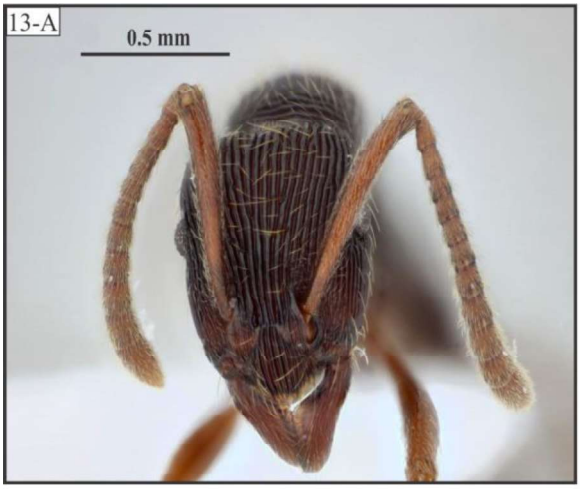
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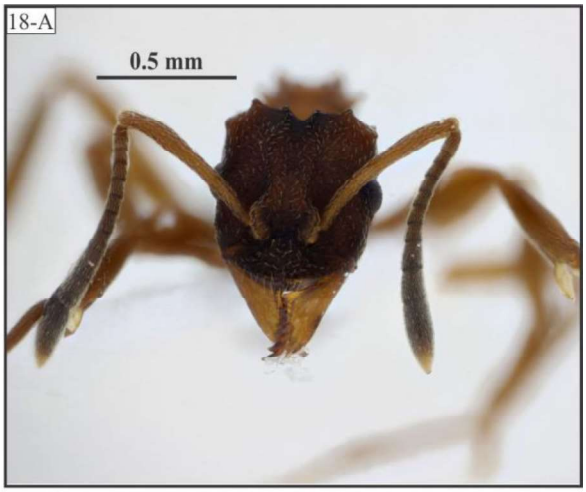
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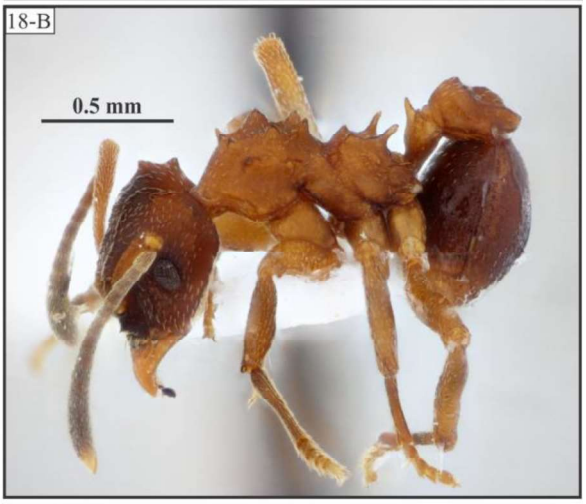
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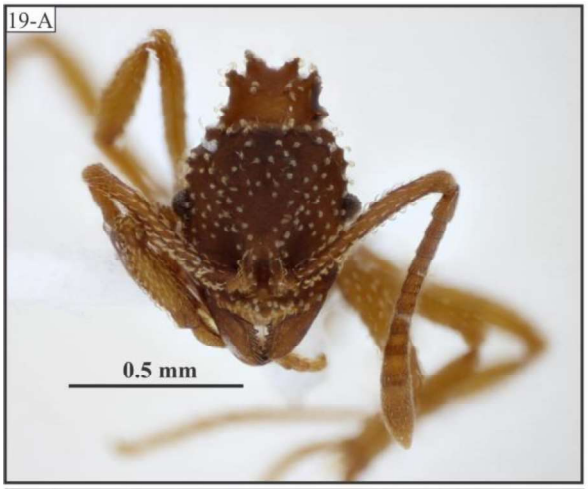
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21-A
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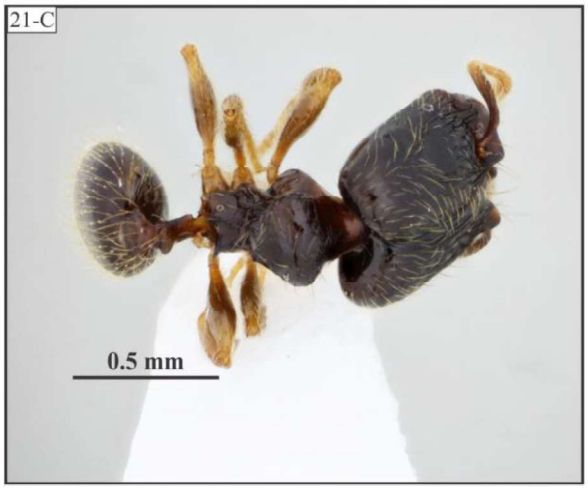
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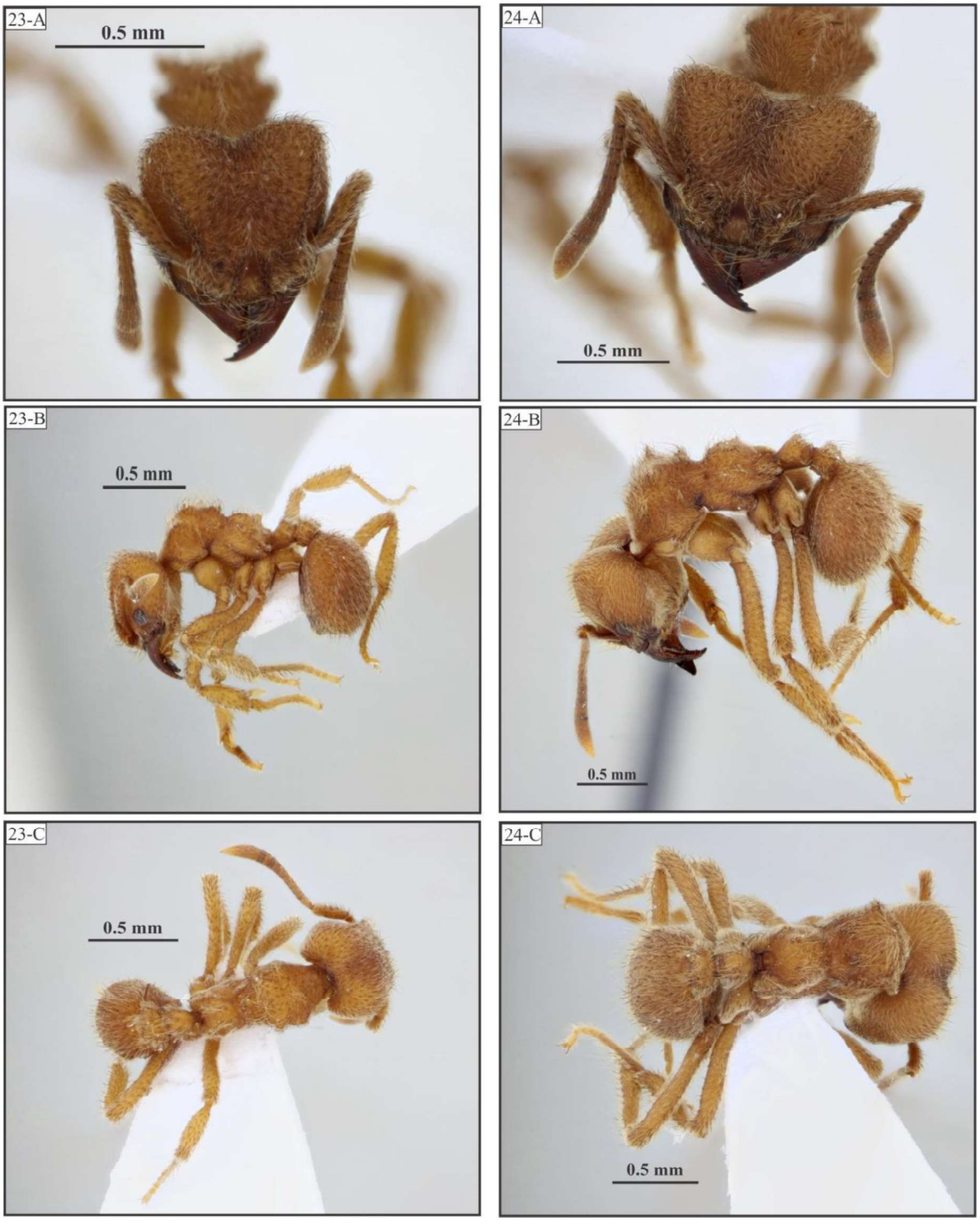
22-C
0.5 mm


23-A
0.5 mm
24-A
0.5 mm
23-B
0.5 mm
24-B
0.5 mm
23-C
0.5 mm
24-C
0.5 mm


25-A
0.5 mm


26-A
0.5 mm


25-B
0.5 mm


26-B
0.5 mm


25-C
0.5 mm


26-C
0.5 mm


27-A
0.5 mm

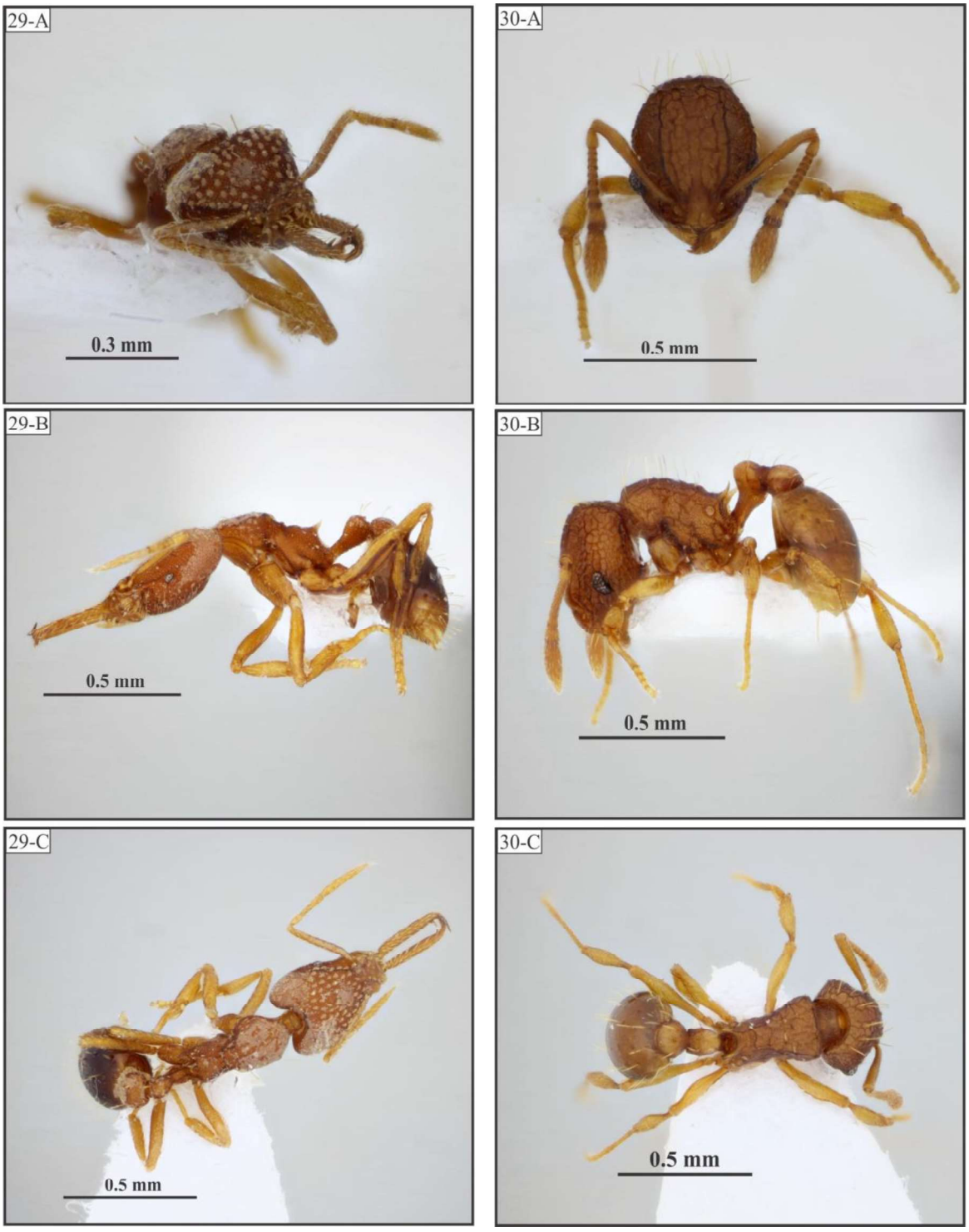

28-A
0.3 mm


27-B
0.5 mm


28-B
0.3 mm


27-C
0.5 mm


28-C
0.3 mm


29-A
0.3 mm
30-A
0.5 mm
29-B
0.5 mm
30-B
0.5 mm
29-C
0.5 mm
30-C
0.5 mm

SUPPORTING INFORMATION - APPENDIX 2. Sequences used in the phylogenetic analysis with their accession number in Genbank.

Species	Subfamily	Genbank number		
		LW exon 1	LW exon 2	WG
Acromyrmex ambiguus	Myrmicinae	JX198244.1	JX198244.1	JX198232.1
Acromyrmex balzani	Myrmicinae	JX198246.1	JX198246.1	JX198234.1
Acromyrmex echinatior	Myrmicinae	KC478104.1	KC478104.1	KC478095.1
Acromyrmex striatus	Myrmicinae	JX198253.1	JX198253.1	JX198236.1
Amblyopone pallipes	Amblyoponinae	AY703755.1	AY703755.1	DQ353119.1
Aphaenogaster fulva	Myrmicinae	KJ920625.1	KJ920625.1	KJ920659.1
Aphaenogaster treatae	Myrmicinae	KJ920642.1	KJ920642.1	KJ920677.1
Apterostigma dentigerum	Myrmicinae	EU204515.1	EU204272.1	EU204196.1
Apterostigma pilosum	Myrmicinae	EU204514.1	EU204271.1	KC964658.1
Apterostigma sp.	Myrmicinae	DQ353241.1	DQ353241.1	DQ353017.1 .
Apterostigma steigeri	Myrmicinae	KC964636.1	KC964636.1	KC964659.1
Atta colombica	Myrmicinae	KC478103.1	KC478103.1	KC478099.1
Atta laevigata	Myrmicinae	EU204481.1	EU204238.1	EU204161.1
Atta robusta	Myrmicinae	JX198254.1	JX198254.1	JX198242.1
Atta sexdens rubropilosa	Myrmicinae	KC478106.1	KC478106.1	KC478101.1
Atta texana	Myrmicinae	KJ861348.1	KJ861348.1	EU204206.1
Azteca sp.	Dolichoderinae	JQ868315.1	JQ868315.1	DQ353056.1
Brachymyrmex sp.	Formicinae	KT443491.1	KT443491.1	KT443261.1
Camponotus castaneus	Formicinae	KP860349.1	KP860349.1	KP730223.1
Camponotus pennsylvanicus	Formicinae	KJ920610.1	KJ920610.1	KJ920644.1
Camponotus sp.	Formicinae	KT443498.1	KT443498.1	KT443268.1
Cephalotes atratus	Myrmicinae	EU204480.1	EU204237.1	EU204160.1
Cephalotes depressus	Myrmicinae	KC335916.1	KC335833.1	KC335598.1
Cephalotes pusillus	Myrmicinae	KC335973.1	KC335890.1	KC335654.1
Crematogaster nigropilosa	Myrmicinae	JQ326703.1	JQ326703.1	JQ326349.1
Crematogaster sp.	Myrmicinae	JQ326687.1	JQ326687.1	JQ326430.1
Crematogaster torosa	Myrmicinae	JQ326674.1	JQ326674.1	JQ326320.1
Cyphomyrmex sp.	Myrmicinae	DQ353251.1	DQ353251.1	-
Dinoponera australis	Ponerinae	JN675416.1	JN675416.1	JN419133.1
Dorymyrmex bicolor	Dolichoderinae	EF013570.1	EF013570.1	EF013698.1
Eciton burchelli	Dorylinae	-	-	AY233615.1
Ectatomma brunneum	Ectatomminae	DQ353219.1	DQ353219.1	KF612870.1
Ectatomma edentatum	Ectatomminae	-	-	KF612864.1
Ectatomma tuberculatum	Ectatomminae	-	-	KF612899.1
Forelius pruinosus	Dolichoderinae	EF013574.1	EF013574.1	EF013702.1
Gnamptogenys sp.	Ectatomminae	DQ353201.1	DQ353201.1	KF612906.1

Species	Subfamily	Genbank number		
		LW exon 1	LW exon 2	WG
Gnamptogenys striatula	Ectatomminae	EF013575.1	EF013575.1	EF013703.1
Hypoponera sp.	Ponerinae	KP001411.1	KP001355.1	KM579198.1
Labidus coecus	Dorylinae	-	-	AY233621.1
Lasius latipes	Formicinae	DQ353208.1	DQ353208.1	DQ353091.1
Lasius alienus	Formicinae	KC820381.1	KC820381.1	DQ353096.1
Linepithema humile	Dolichoderinae	EF013583.1	EF013583.1	AY233596.1
Linepithema micans	Dolichoderinae	FJ161845.1	FJ161845.1	-
Liometopum occidentale	Dolichoderinae	KR829556.1	KR829556.1	AY233598.1
Messor andrei	Myrmicinae	EF013589.1	EF013589.1	EF013717.1
Mycetophylax conformis	Myrmicinae	EU204486.1	EU204243.1	EU204166.1
Mycetophylax morschi	Myrmicinae	KC964624.1	KC964624.1	KC964648.1
Mycetophylax simplex	Myrmicinae	KC964627.1	KC964627.1	KP939228.1
Mycocepurus goeldii	Myrmicinae	-	-	JN055090.1
Myrmecina americana	Myrmicinae	KJ861425.1	KJ861425.1	KJ861853.1
Odontomachus bauri	Ponerinae	EU155447.1	EU155447.1	EU155466.1
Odontomachus brunneus	Ponerinae	EU155448.1	EU155448.1	EU155467.1
Odontomachus cephalotes	Ponerinae	KU504964.1	KU504964.1	EU155468.1
Odontomachus chelifer	Ponerinae	KU504965.1	KU504965.1	EU155469.1
Odontomachus clarus	Ponerinae	EU155451.1	EU155451.1	EU155470.1
Odontomachus haematodus	Ponerinae	KU504970.1	KU504970.1	EU155472.1
Paratrechina longicornis	Formicinae	KT443526.1	KT443526.1	KP232870.1
Pheidole dentata	Myrmicinae	EF518964.1	EF518964.1	-
Pheidole floridana	Myrmicinae	EF518974.1	EF518974.1	-
Pheidole hyatti	Myrmicinae	EF013616.1	EF013616.1	EF013744.1
Pheidole sp.	Myrmicinae	KM597348.1	KM597348.1	KM579268.1
Platythyrea punctata	Ponerinae	EF013620.1	EF013620.1	DQ353133.1
Pogonomyrmex badius	Myrmicinae	FJ824492.1	FJ824492.1	KU318947.1
Pogonomyrmex californicus	Myrmicinae	JX908270.1	JX908270.1	JX908203.1
Pogonomyrmex naegelii	Myrmicinae	KU318919.1	KU318919.1	KU318970.1
Ponera pennsylvanica	Ponerinae	JN675464.1	JN675464.1	JN419185.1
Prenolepis imparis	Formicinae	EF013627.1	EF013627.1	DQ353081.1
Pseudomyrmex ejectus	Pseudomyrmecinae	KR829372.1	KR829372.1	KR828999.1
Pseudomyrmex gracilis	Pseudomyrmecinae	FJ436876.1	FJ436876.1	AY703663.1
Pseudomyrmex schuppi	Pseudomyrmecinae	KR829467.1	KR829467.1	KR829094.1
Pseudomyrmex termitarius	Pseudomyrmecinae	KR829480.1	KR829480.1	AY703683.1
Sericomyrmex sp.	Myrmicinae	DQ353205.1	DQ353205.1	KY828497.1
Solenopsis invicta	Myrmicinae	-	-	DQ353039.1
Solenopsis molesta	Myrmicinae	EF013642.1	EF013642.1	EF013770.1

Species	Subfamily	Genbank number		
		LW exon 1	LW exon 2	WG
Solenopsis sp.	Myrmicinae	KJ861476.1	KJ861476.1	KJ861905.1
Solenopsis xyloni	Myrmicinae	EF013643.1	EF013643.1	JQ742896.1
Strumigenys sp.	Myrmicinae	KM597389.1	KM597389.1	KC567805.1
Tapinoma melanocephalum	Dolichoderinae	KM597399.1	KM597399.1	FJ940042.1
Tapinoma sessile	Dolichoderinae	FJ161860.1	FJ161860.1	EF013774.1
Tetramorium caespitum	Myrmicinae	EF013652.1	EF013652.1	EF013780.1
Trachymyrmex septentrionalis	Myrmicinae	EU204503.1	EU204260.1	EU204184.1
Wasmannia auropunctata	Myrmicinae	EF013658.1	EF013658.1	EU204163.1
Wasmannia sp.	Myrmicinae	DQ353186.1	DQ353186.1	EU204209.1
Apis mellifera	Apidae - *outgroup*	-	-	AY703618.1
Chalybion californicum	Sphecidae - *outgroup*	EF013561.1	EF013561.1	JN374869.1
Mischocyttarus flavitarsis	Vespidae -*outgroup*	AY703754.1	AY703754.1	-

SUPPORTING INFORMATION - APPENDIX 3. Confidence Interval (IC) of the values generated by the Maximum Likelihood method in StableTraits.

Tree Node	Brownian model value	95%CI_Low	95%CI_High
n0	0.37	0.08	0.67
n1	0.37	0.20	0.55
n2	0.39	0.19	0.59
n3	0.37	0.21	0.53
n4	0.34	0.24	0.45
n5	0.36	0.22	0.51
n6	0.37	0.22	0.53
n7	0.33	0.16	0.51
n8	0.45	0.28	0.61
n9	0.37	0.24	0.50
n10	0.36	0.21	0.51
n11	0.32	0.19	0.45
n12	0.35	0.20	0.50
n13	0.34	0.24	0.43

CONSIDERAÇÕES FINAIS

- ➢ *Drosophila melanogaster* mostrou ser o padrão interno de referência mais apropriado para estudos de tamanho de genoma em formigas;
- ➢ A análise de diferentes tampões de lise é um passo fundamental para obtenção de núcleos intactos para citometria de fluxo em formigas;
- ➢ Não foram encontradas diferenças significativas entre os tamanhos de genoma de pupas e adultos testados, indicando que o protocolo estabelecido para citometria de fluxo em formigas adultas é adequado e possível para ser aplicada em larga escala;
- ➢ O uso de individos adultos contribui para a possiblidade de aumentar o conhecimento do tamanho do genoma em formigas, já que não é necessário mais a coleta de larvas ou pupas para a análise;
- ➢ O tamanho de genoma é uma abordagem importante em estudos evolutivos, e é recomendada para estudos de genética de populações, já que diferenças no conteúdo de DNA total foram observadas entre populações da mesma espécie;
- ➢ É mais provável que essa variação no tamanho de genoma entre populações da mesma espécie esteja relacionada com o estresse provocado pela colonização de novos ambientes, através do acúmulo de elementos transponíveis como já encontrado em estudos com plantas e populações de *D. melanogaster*;
- ➢ A citometria de fluxo provou ser um método eficaz para quantificação de DNA, já que o coeficiente de variação testado entre dias diferentes de mensuração não foi significativamente diferente, demonstrando a eficácia do método e estabilidade do equipamento;
- ➢ A média do tamanho de genoma para família Formicidae foi de 0,38 pg e a média de composição de bases AT/GC foi AT = 62,40% e GC = 37,60%;

- ➢ Em geral todas as subfamílias amostradas com um grande número de espécies apresentaram grande variação no tamanho de genoma, sendo em Myrmicinae a mais proeminente (de 0,21 pg a 0,81 pg);

- ➢ O tamanho de genoma ancestral em Formicidae foi estimado em 0,38 por meio do método de Inferência Bayesiana e 0,37 pg com Parcimônia e Máxima Verossimilhança;

- ➢ Os resultados encontrados no terceito capítulo contribuem para o aumento do conhecimento sobre o tamanho de genoma em formigas, assim como composição de bases AT/GC e evolução do caráter a partir de um genoma ancestral;

- ➢ Esses resultados também sugerem que a evolução do tamanho de genoma em Formicidae ocorreu pela perda e acúmulo de regiões não codificadoras do DNA, principalmente elementos transponíveis, como é sugerido para outros grupos de animais;

- ➢ Os processos envolvidos nessas contrações e expansões do genoma ainda precisam ser compreendidos, principalmente a partir de estudos que contemplem a diversificação das subfamílias.

- ➢ Ainda, este estudo serve como um guia para futuros trabalhos de sequenciamento de genoma completo.

Apêndice I

Artigo apresentado à banca examinadora, como parte das exigências do Programa de Pós-graduação em Ecologia, como exame de qualificação.
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Reconstruction of ancestral genome size in Pitcairnioideae (Bromeliaceae): what can genome size tell us about the evolutionary history of its five genera?

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We expand the genome size (GS) database for Bromeliaceae, specifically for subfamily Pitcairnioideae, and verify whether GS can provide information on the diversification of the five genera in this subfamily. We also provide a phylogenetic perspective on GS evolution in the subfamily and reconstruct the ancestral state for this character. We show that the evolutionary path of GS from the origin of angiosperms to the origin of Pitcairnioideae agrees with the proportional model of GS evolution. Furthermore, we propose that the high phenotypic diversity that is found across Bromeliaceae and that is well represented in Pitcairnioideae is both correlated with high rates of GS evolution of the species and associated with a short period of diversification. The paper also highlights the value of flow cytometry as a rapid and reliable technique for generating GS data which can be analysed in conjunction with other molecular and morphological data to help elucidate patterns of evolution and phylogenetic relationships within this family.

ADDITIONAL KEYWORDS: character reconstruction – DNA content – evolution – phylogeny – species delimitation.

INTRODUCTION

Taxon delimitation is a fundamental step for conservation, studying evolution and undertaking systematic studies of an organism (Sites & Marshall, 2003, 2004). There is a consensus that species should correspond to independent evolutionary lineages (Wilson, 1978; Duminil & Michele, 2009; Padial *et al.*, 2010). The use of different types of characters (e.g. morphological, physiological, molecular or karyotypic) in a given taxon can help to provide an accurate classification (Dayrat, 2005; Schlick-Steiner *et al.*, 2010; Cristiano, Cardoso & Fernandes-Salomão, 2013). Alternatively, using the same character in a large number of taxa provides opportunities for studying the evolution of that character. The evolution of a particular character may even help in understanding the evolutionary patterns and processes underpinning phylogenetic relationships among existing organisms (Le Quesne, 1974; Maddison & Maddison, 1989).

Bromeliaceae, a predominantly Neotropical family (Benzing, 2000), comprising 73 genera and nearly 3600 species (Givnish *et al.*, 2011; Butcher & Gouda, cont. updated), are considered to be a natural group. The monophyly of Bromeliaceae has been consistently confirmed in several studies (Stevenson & Loconte, 1995; Chase *et al.*, 1995, 2000; Givnish *et al.*, 2011). Givnish *et al.* (2007) suggested that a recent diversification of modern lineages of Bromeliaceae occurred *c.* 19 Mya. These authors inferred that such lineages first appeared in the Guayana Shield, northern South America, and then spread centripetally throughout the New World. In terms of chromosome number, most species present the basic number $x = 25$, with few exceptions (Gitaí, Horres & Benko-Iseppon, 2005; Ceita *et al.*, 2008; Gitaí *et al.*, 2014;

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Nunes & Clarindo, 2014). Available data also suggest that species of Bromeliaceae have small genome sizes (GSs), as their 2C-values range from 0.6 pg to 2.52 pg ($\bar{x}$ = 1.18 ± 0.37) (Angiosperm DNA C-values Database; Bennett & Leitch, 2012; 1 pg = 978 Mbp).

The subfamilies and genera of Bromeliaceae have frequently been questioned and changed over the last few decades (Grant, 1992, 1993, 1996; Gouda, 1994; Givnish *et al.*, 2004, 2007), as most genera have poor morphological delimitation and low molecular differentiation (Escobedo-Sarti *et al.*, 2013). Pitcairnioideae, one of these subfamilies, comprise the genera *Pitcairnia* L'Hér. (405 species), *Fosterella* L.B.Sm. (31 species), *Dyckia* Schult. & Schult.f. (168 species), *Encholirium* Mart. ex Schult. & Schult.f. (29 species) and *Deuterocohnia* Mez (18 species) (Butcher and Gouda, cont. updated; BFG, 2015; Forzza, 2017). The first two genera exhibit mesic morphological and physiological characteristics, whereas the other three exhibit xeric morphological and physiological characteristics and belong to a monophyletic group informally known as 'the xeric clade' of Pitcairnioideae (Givnish *et al.*, 2011; Santos-Silva *et al.*, 2013; Schütz *et al.*, 2016).

The relationships between the genera of Pitcairnioideae have been questioned and studied in recent years. Rex *et al.* (2009) presented a multilocus plastid DNA phylogenetic analysis of *Fosterella*, showing it to be monophyletic, but infrageneric relationships in other genera of the subfamily remained unclear. Similar results were reported by Schütz (2011) in a taxonomic revision based on plastid and nuclear DNA data for *Deuterocohnia*. The results showed low genetic variability in the genus, resulting in low-resolution trees and networks. A phylogenetic analysis by Saraiva, Mantovani & Forzza (2015), based on morphological characters alone, supported the monophyly of *Pitcairnia s.l.* and agreed with previous findings (Terry, Brown & Olmstead, 1997; Givnish *et al.*, 2004, 2007, 2011; Horres *et al.*, 2007), but their analysis did not resolve all species relationships within the genus. These relationships also remained unresolved in the molecular phylogenetic analysis of Schütz *et al.* (2016), as did the delimitation of *Dyckia* and *Encholirium*. M. N. Moura *et al.* (unpubl. data) conducted a more comprehensive molecular and morphological phylogenetic analysis of *Encholirium*, which supports the idea of the genus being paraphyletic and corroborates the hypothesis regarding the recent divergence of Pitcairnioideae (the crown age of Pitcairnioideae was 11.8 Mya according to Givnish *et al.*, 2011).

To study relationships between species that have recently diversified, DNA sequences, commonly used in phylogenetic studies, may not have had enough time to accumulate detectable differences to resolve phylogenetic relationships (Givnish *et al.*, 2011). On the other hand, abrupt changes in nuclear DNA content can occur over short periods of time (from one generation to another) as a result of numerical (euploidy and/or aneuploidy) and/or structural (deletion, inversion, duplication and/or translocation) alterations. These changes are common in many lineages of plants and are considered to be key factors in the evolution of the genomes of several species (e.g. Bennett & Smith, 1976; Kunkel, 1990; Soltis & Soltis, 2009; Campos *et al.*, 2011; Lee, Chang & Chung, 2011; Lepers-Andrzejewski *et al.*, 2011; Szadkowski *et al.*, 2011; Chester *et al.*, 2012).

According to Doležel, Greilhuber & Suda (2007), flow cytometry (FCM) is a fast and reliable method to estimate GS in plants. Information on nuclear GS in Bromeliaceae has toadied in studies regarding their taxonomy, evolution, genetic diversity and reproductive biology (Ebert & Till, 1997; Ramírez-Morillo & Brown, 2001; Sgorbati *et al.*, 2004; Favoreto *et al.*, 2012). Nevetheless, given the diversity of the family and ongoing taxonomic problems, as outlined above, there is still insufficient GS data available (< 3% for species of Bromeliaceae and < 7% for species of Pitcairnioideae), and hence our understanding of related evolutionary processes is poorly understood (Gitaí *et al.*, 2014).

In the present study, we aim to expand the GS database for Bromeliaceae, specifically Pitcairnioideae, and examine whether GS data can help to provide insights into the diversification of the five genera in the subfamily. We also provide a phylogenetic perspective on GS evolution in the subfamily and reconstruct the ancestral state of this character.

MATERIAL AND METHODS

Genome size estimations

Taxon sampling and flow cytometry

FCM analyses were carried out at the Laboratório de Genética da Universidade Federal de Juiz de Fora (UFJF). Leaf samples of 53 species of the five genera of Pitcairnioideae were collected from the living collection at the Jardim Botânico do Rio de Janeiro: one *Deuterocohnia* sp., 17 *Dyckia* spp., 16 *Encholirium* spp., one *Fosterella* sp. and 18 *Pitcairnia* spp. Vouchers have been deposited in herbarium RB (Jardim Botânico do Rio de Janeiro) and herbarium SPF (Universidade de São Paulo) under the numbers presented in Table 1. The nuclear DNA content of three samples of each studied species was measured using the DNA 2C-value of *Pisum sativum* L. as an internal standard (9.09 pg; Doležel *et al.*, 1998). To obtain a suspension of nuclei, 1.0 cm^2 leaf tissue of the standard and each sample were simultaneously chopped in 1.0 mL cold LB01

Table 1. Genome sizes estimated for all studied specimens, the mean calculated for each species, standard deviation and herbarium voucher

Species	2C (pg)	Reference	Voucher
Deuterocohnia longipetala (Baker) Mez	0.74	Bennett and Leitch (2011)	–
Deuterocohnia lorentziana (Mez) M.A.Spencer & L.B.Sm.	1.73 ± 0.021	Gitaí *et al.* (2014)	Leg. Nr. 130007†
Deuterocohnia meziana Kuntze ex Mez	1.20 ± 0.02	This study	RB 382284*
Deuterocohnia schreiteri A.Cast.	0.8	Bennett and Leitch (2011)	–
Dyckia brevifolia Baker	2.01 ± 0.08	This study	RB 571588*
Dyckia choristaminea Mez	1.78 ± 0.02	This study	RB 434135*
Dyckia consimilis Mez	2.17 ± 0.01	This study	RB 673612*
Dyckia distachya Hassler	1.86 ± 0.06	This study	RB 593320*
Dyckia estevesii Rauh	1.6	Bennett and Leitch (2011)	–
Dyckia floribunda Griseb.	1.58	Bennett and Leitch (2011	–
Dyckia granmogulensis Rauh	2.18 ± 0.01	This study	RB 458314*
Dyckia ibiramensis Reitz	2.13 ± 0.08	This study	RB 571595*
Dyckia maritima Baker	2.07 ± 0.06	This study	RB 1230313*
Dyckia marnier-lapostollei L.B.Sm.	1.95 ± 0.02	This study	RB 547282*
Dyckia minarum Mez	1.88 ± 0.05	This study	RB 377972*
Dyckia monticola L.B.Sm. & Reitz	2.08 ± 0.00	This study	RB 329151*
Dyckia pseudococcinea L.B.Sm.	2.10 ± 0.04	This study	RB 723296*
Dyckia pulquinensis Wittm.	2.38 ± 0.45	This study	RB 588007*
Dyckia reitzii L.B.Sm.	1.66 ± 0.00	This study	RB 583483*
Dyckia saxatilis Mez	1.88 ± 0.12	This study	RB 462377*
Dyckia sthreliana H.Büneker & R.Pontes	1.80 ± 0.03	This study	RB 595146*
Dyckia tenebrosa Leme & H.Luther	2.13 ± 0.01	This study	RB 484461*
Dyckia tuberosa (Vell.) Beer	1.82 ± 0.08	This study	RB 594245*
Encholirium agavoides Forzza & Zappi	2.11 ± 0.01	This study	RB 504154*
Encholirium biflorum (Mez) Forzza	1.40 ± 0.04	This study	RB 583280*
Encholirium ctenophyllum Forzza & Zappi	2.90 ± 0.06	This study	RB 504157*
Encholirium diamantinum Forzza	1.92 ± 0.04	This study	RB 458335*
Encholirium cf. *diamantinum* Forzza	2.05 ± 0.06	This study	RB 637204*
Encholirium gracile L.B.Sm.	1.56 ± 0.02	This study	RB 570910*
Encholirium heloisae (L.B.Sm.) Forzza & Wand.	1.94 ± 0.11	This study	RB 563567*
Encholirium horridum L.B.Sm.	1.57 ± 0.02	This study	RB 462618*
Encholirium irwinii L.B.Sm.	1.74	Bennett & Leitch (2011)	–
Encholirium irwinii L.B.Sm.	1.94 ± 0.03	This study	SPF 131942§
Encholirium luxor L.B.Sm. & R.W.Read	2.02 ± 0.03	This study	RB 391473*
Encholirium magalhaesii L.B.Sm.	1.94 ± 0.01	This study	RB 670779*
Encholirium pedicellatum (Mez) Rauh	1.69 ± 0.02	This study	RB 670694*
Encholirium pulchrum Forzza, Leme & O.B.C.Ribeiro	1.83 ± 0.01	This study	RB 504202*
Encholirium scrutor (L.B.Sm.) Rauh	1.96 ± 0.04	This study	RB 563568*
Encholirium spectabile Mart. ex Schult. & Schult.f.	2.16 ± 0.01	This study	RB 331722*
Encholirium subsecundum (Baker) Mez	2.02 ± 0.13	This study	RB 747925*
Fosterella penduliflora (C.H.Wright) L.B.Sm.	1.86	Bennett & Leitch (2011)	–
Fosterella villosula (Harms) L.B.Sm.	1.86	Bennett & Leitch (2011)	–
Fosterella windischii L.B.Sm. & R.W.Read	0.91 ± 0.01	This study	RB 452098*
Pitcairnia albiflos Herb.	1.39 ± 0.05	This study	RB 498556*
Pitcairnia andreana Linden	1.3	Bennett & Leitch (2011)	–
Pitcairnia angustifolia Aiton	1.06	Bennett & Leitch (2011)	–
Pitcairnia aphelandriflora Lem.	1.24	Bennett & Leitch (2011)	–
Pitcairnia atrorubens (Beer) Baker	1.2	Bennett & Leitch (2011)	–

Table 1. *Continued*

Species	2C (pg)	Reference	Voucher
Pitcairnia atrorubens (Beer) Baker	1.29 ± 0.013	Gitaí *et al.* (2014_	Leg. Nr. 16095†
Pitcairnia aureobrunnea Rauh	1.12	Bennett & Leitch (2011)	–
Pitcairnia azouryi Martinelli & Forzza	1.37 ± 0.10	This study	RB 484459*
Pitcairnia barbatostigma Leme & A.P.Fontana	1.26 ± 0.02	This study	RB 462404*
Pitcairnia bradei Markgr.	1.62 ± 0.09	This study	RB 586285*
Pitcairnia burchellii Mez	1.41 ± 0.02	This study	RB 526713*
Pitcairnia burle-marxii Braga & Sucre	1.43 ± 0.13	This study	RB 509698*
Pitcairnia cardenasii L.B.Sm.	1.02	Bennett & Leitch (2011)	–
Pitcairnia chiapensis Miranda	1.22	Bennett & Leitch (2011)	–
Pitcairnia chiapensis Miranda	1.31 ± 0.005	Gitaí *et al.* (2014)	Leg. Nr. 19495†
Pitcairnia decidua L.B.Sm.	1.78 ± 0.32	This study	RB 534853*
Pitcairnia feliciana (A.Chev.) Harms & Mildbr.	0.6	Bennett & Leitch (2011)	–
Pitcairnia flammea Lindl.	1.28	Bennett & Leitch (2011)	–
Pitcairnia flammea Lindl.	1.44	Nunes *et al.* (2013)	CESJ 5569‡
Pitcairnia flammea Lindl.	1.69 ± 0.14	This study	RB 597781*
Pitcairnia glauca Leme & A.P.Fontana	1.31 ± 0.06	This study	RB 565871*
Pitcairnia glaziovii Baker	1.56 ± 0.07	This study	RB 488168*
Pitcairnia grafii Rauh	1.34	Bennett & Leitch (2011)	–
Pitcairnia halophila L.B.Sm	1.08	Bennett & Leitch (2011)	–
Pitcairnia heerdeae E.Gross & Rauh	1.18	Bennett & Leitch (2011)	–
Pitcairnia heterophylla (Lindl.) Beer	0.88	Bennett & Leitch (2011)	–
Pitcairnia hitchcockiana L.B.Sm.	1.28	Bennett & Leitch (2011)	–
Pitcairnia irwiniana L.B.Sm.	1.51 ± 0.07	This study	RB 547217*
Pitcairnia macrochlamys Mez	1.2	Bennett & Leitch (2011)	–
Pitcairnia macrochlamys Mez	1.25 ± 0.014	Gitaí *et al.* (2014	Leg. Nr. 12596†
Pitcairnia micotrinensis Read	1.1	Bennett & Leitch (2011)	–
Pitcairnia nortefluminensis Leme	1.50 ± 0.05	This study	RB 511495*
Pitcairnia palmoides Mez & Sodiro	1.18	Bennett & Leitch (2011)	–
Pitcairnia paraguayensis L.B.Sm.	1.36	Bennett & Leitch (2011)	–
Pitcairnia patentiflora L.B.Sm.	1.09 ± 0.01	This study	RB 549440*
Pitcairnia piepenbringii Rauh & E.Gross	1.2	Bennett & Leitch (2011)	–
Pitcairnia poeppigiana Mez	1.2	Bennett & Leitch (2011)	–
Pitcairnia pomacochae Rauh	1.24	Bennett & Leitch (2011)	–
Pitcairnia prolifera Rauh	0.84	Bennett & Leitch (2011)	–
Pitcairnia rectiflora Rauh	1.2	Bennett & Leitch (2011)	–
Pitcairnia riparia Mez	1.14	Bennett & Leitch (2011)	–
Pitcairnia rubiginosa Baker	1.29 ± 0.01	This study	RB 547129*
Pitcairnia sceptrigera Mez	1.2	Bennett & Leitch (2011)	–
Pitcairnia sceptrigera Mez	1.2	Gitaí *et al.* (2014	Leg. Nr. F009†
Pitcairnia schultzei Harms	1.32	Bennett & Leitch (2011)	–
Pitcairnia spicata (Lam.) Mez	1.22	Bennett & Leitch (2011)	–
Pitcairnia staminea Lodd.	1.64 ± 0.05	This study	RB 427244*
Pitcairnia suaveolens Lindl.	1.20 ± 0.01	This study	RB 554600*
Pitcairnia tabuliformis Linden	1.1	Bennett & Leitch (2011)	–
Pitcairnia uaupensis Baker	1.57 ± 0.01	This study	RB 547093*
Pitcairnia ulei L.B.Sm.	1.54 ± 0.13	This study	RB 547266*
Pitcairnia venezuelana L.B.Sm. & Steyerm.	1.36	Bennett & Leitch (2011)	–
Pitcairnia villetaensis Rauh	1.26	Bennett & Leitch (2011)	–
Pitcairnia yaupibajaensis Rauh	1.12	Bennett & Leitch (2011)	–
Tillandsia usneoides (L.) L.	2.52	Zonneveld *et al.* (2005)	–

*Voucher of species of the present study, deposited in the herbarium of the Jardim Botânico do Rio de Janeiro - Herbarium RB.
†Cultivated, Palmengarten Frankfurt.
‡Herbarium CESJ of Universidade Federal de Juiz de Fora, Minas Gerais, Brazil.
§Herbarium SPF of Universidade de São Paulo.

buffer (Doležel, Binarová & Lucretti, 1989) with the aid of a cutting blade. The suspension of nuclei was subsequently filtered through 30-µm nylon mesh. The solution was supplemented with 5.0 µL of RNAse at a concentration of 100 µg mL^{-1} and then stained with 50 µL propidium iodide (PI) solution at a concentration of 1 mg mL^{-1}. The samples were stored in the dark and analysed within 1 h of preparation.

For GS estimates, the suspension was analysed using a FacsCalibur (Becton Dickinson) flow cytometer equipped with a laser source (488 nm). From each sample, 10 000 PI-stained nuclei were analysed for their relative fluorescence intensity. Three independent replications were performed, and histograms with a coefficient of variation > 5% were rejected. The nuclear GS average (pg) of each sample was measured in accordance with the formula given by Doležel & Bartos (2005). Histograms were analysed using Flowing 2.5.1 software (http://www.flowingsoftware.com).

Additional GS data for 40 species were extracted from the Plant DNA C-values Database (http://data.kew.org/cvalues/) and previously published data (Gitaí *et al.*, 2005, 2014; Zonneveld, Leitch & Bennett, 2005; Bennett & Leitch, 2011; Nunes *et al.*, 2013): three *Deuterocohnia* spp., two *Dyckia* spp., one *Encholirium* sp., two *Fosterella* spp. and 31 *Pitcairnia* spp., with five duplicate values, and one value for the outgroup species *Tillandsia usneoides* (L.) L. in the phylogenetic tree.

Statistical analyses

To check for differences between the average GS measurements of the sampled genera, statistical calculations were performed using R 2.15.1 (R Core Team, 2013). Because the variables did not fulfil the normality assumption (Shapiro–Wilk test), differences in 2C-values between pairs of genera were assessed using the non-parametric Wilcoxon rank sum test.

Sequence alignment and phylogeny

Taxon sampling

Ninety-seven samples were analysed, including 14 taxa of *Encholirium*, 24 of *Dyckia*, 11 of *Deuterocohnia*, 22 of *Pitcairnia* and 23 of *Fosterella*. Two species were included as outgroups: *Catopsis nitida* (Hook.) Griseb. and *Tillandsia usneoides*, both belonging to Tillandsioideae. Some of the molecular operational taxonomic units were obtained from M. N. Moura *et al.* (unpubl. data), and 84 sequences were obtained from GenBank (Supporting Information Appendix S1).

Phylogenetic analyses

The *matK* sequences were aligned using the Muscle algorithm (Edgar, 2004) provided in MEGA 5.0 (Tamura *et al.*, 2011). We performed the analysis using Bayesian inference (BI). To infer the best nucleotide substitution model, we used the program MrModelTest 2.3 (Nylander, 2004) with the Akaike information criterion, and the substitution model GTR + I + G was selected. The trees were queried using the software MrBayes 3.2.2 (Ronquist & Huelsenbeck, 2003) with two independent runs and four Markov chains each, one cold and three heated. Each chain was run for 50 million generations and sampled every 5000 generations. The convergence of the cold chains was checked using the program Tracer 1.6 (Drummond & Rambaut, 2007), and a burn-in on the first 25% of the trees was performed before using the remaining topologies to build a consensus topology with its respective branch lengths; this topology was then visualized using the FigTree 1.3 software program (Raumbaut, 2008).

Ancestral genome size

We plotted all 2C-values estimated in this study plus the 2C-values extracted from the Plant DNA C-values Database (http://data.kew.org/cvalues/) on the plastid phylogenetic tree. We used three different methods to estimate ancestral GS across the phylogenetic tree, aiming to ensure the consistency of the generated data: maximum parsimony (MP) analysis using Mesquite 3.04 (Maddison & Maddison, 2011); maximum likelihood (ML) reconstruction implemented in Stable Traits (Elliot, 2014); and BI using Markov chain Monte Carlo (MCMC) in BayesTraits 2.0 (Pagel, Meade & Barker, 2004) using the 'continuous random walk' model. We also verified whether the variables evolved according to a Brownian model of evolution across the phylogenetic tree using BayesTraits 2.0 (Pagel *et al.*, 2004).

RESULTS

Genome size estimations

We present new GS estimates for 53 species belonging to the five genera of Pitcairnioideae (Table 1), 51 of which correspond to taxa that have not been previously studied, plus 45 species from the literature. From 405 *Pitcairnia* spp., we now have 2C-value estimates for 48 species (*c.* 12% of the total); for *Fosterella*, only three out of the 31 species have GS estimates (9.7%); for *Deuterocohnia* 22% (four species out of 18); for *Dyckia* 11% (19 species out of 168) and for *Encholirium* we have estimates of GS for 16 species (55% of 29) (Gouda et al., 2015, cont. updated; BFG, 2015; Forzza, 2017). We have therefore expanded the genome database for Pitcairnioideae by 15% and this now enables us investigate whether GS data can be used as a

diagnostic character to distinguish between the five genera in this subfamily.

The lowest 2C-value was found in *Pitcairnia feliciana* (A.Chev.) Harms & Mildbr. (0.6 pg) and the highest value was found in *Encholirium ctenophyllum* Forzza & Zappi (2.90 pg). Overall, the estimated 2C-values varied between genera, ranging from 0.74 to 1.73 pg in *Deuterocohnia* (average 1.11 pg), 1.58 to 2.38 pg in *Dyckia* (average 1.95 pg), 1.4 to 2.9 pg in *Encholirium* (average 1.93 pg), 0.91 to 1.86 pg in *Fosterella* (average 1.54 pg) and 0.6 to 1.78 pg in *Pitcairnia* (average 1.27 pg). From the 98 values estimated, 77.5% range from 1.0 to 2.0 pg (Table 1).

FCM histograms used to infer the GS of each specimen showed peaks corresponding to the G_0/G_1 nuclei of the targeted species and the G_0/G_1 and G_2 nuclei of the internal standard used here (Fig. 1). The G_0/G_1 peaks of all specimens included in this study can be clearly discriminated, and their coefficients of variation were always < 5%, which is considered suitable for GS determination using FCM (Cardoso, Martinelli & Latado, 2012).

We used the Wilcoxon rank sum test to compare the average GS values of the five genera of Pitcairnioideae (Fig. 2). Significant differences in GS were observed between *Deuterocohnia* and *Dyckia* ($W = 3$, $P < 0.01$), *Deuterocohnia* and *Encholirium* ($W = 5$, $P < 0.01$), *Dyckia* and *Pitcairnia* ($W = 1150$, $P < 0.01$), and *Encholirium* and *Pitcairnia* ($W = 1035.5$, $P < 0.01$). All the other relationships tested presented P-values > 5%.

Phylogenetic analysis

The alignment length of 824 bp was obtained for the *matK* plastid region using 94 sequences from species belonging to Pitcairnioideae and sequences from three accessions of two species as outgroups, which included 152 variable sites (18.45%). Figure 3 shows the Bayesian consensus phylogenetic tree based on the *matK* gene. The majority of the species of the xeric clade (*Deuterocohnia*, *Dyckia* and *Encholirium*) were grouped into one clade with a high posterior probability (PP) (n2, PP = 1). In this clade, another containing all species of *Dyckia* and *Encholirium* was recovered (n3, PP = 0.95) in a polytomy.

The species of the mesic genera, *Fosterella* and *Pitcairnia*, were grouped into one clade (n6, PP = 0.79), but in *Pitcairnia* the species grouped into two clades: n9, which had a PP = 1 and contained the most species ($N = 13$); and n15, which had a PP = 1 and contained nine species. All *Fosterella* spp. formed a single clade with a high probability value (n7, PP = 1).

Deuterocohnia spp. were divided in the phylogenetic tree: some species [*Deuterocohnia brevispicata* Rauh & L. Hrom., *Deuterocohnia meziana* Kuntze ex Mez and *Deuterocohnia scapigera* (Rauh & L.Hrom.) M.A.Spencer & L.B.Sm.] appear in a polytomy with the other species of the xeric clade. The other species [*Deuterocohnia brevifolia* (Griseb.) M.A.Spencer & L.B.Sm., *Deuterocohnia longipetala* Mez, *Deuterocohnia schreiteri* A.Cast. and *Deuterocohnia glandulosa* E.Gross] form a sister group of the remaining Pitcairnioideae (n18, PP = 1).

Ancestral genome size

Many of the methods commonly used to reconstruct the ancestral state of a character assume a Brownian model of character evolution across the phylogenetic tree (Webster & Purvis, 2002). However, when certain evolutionary processes are involved in the evolution of the characters analysed, the Brownian model may not be adequate (Pagel, 1998; Freckleton & Harvey, 2006). In the present study, we verified whether the variables evolved according to a Brownian model of evolution, and the test revealed that a Brownian model is indeed sufficient according to the available data ($P > 0.05$). To further check this we chose to analyse the data using three methods, but we found no significant difference between them.

The ancestral GS for Pitcairnioideae (n1, Fig. 3) reconstructed using MP was 1.29 pg, using ML was 1.24 pg (0.75–1.71, 95% highest posterior density) and based on BI was 1.21 ± 0.15 pg. Across the phylogenetic tree, both increases and decreases in GS were reconstructed relative to the ancestral value. The values obtained with the three methods varied little among themselves, as observed, for example, for node 2 (MP = 1.50, ML = 1.49 and MCMC = 1.49 ± 0.16), node 3 (MP = 1.84, ML = 1.81 and MCMC = 1.84 ± 0.04) and node 7 (MP = 1.50, ML = 1.50 and MCMC = 1.52 ± 0.23).

DISCUSSION

The GS values reported here (Table 1) for species that have previously been estimated by other researchers (and available in the Plant DNA C-values database) were found to be close. For example, the published values of *Dyckia estevesii* Rauh and *Dyckia floribunda* Griseb. are 1.60 and 1.58 pg, respectively, and that of *Encholirium irwinii* L.B.Sm. is 1.74 pg (Ebert & Till, 1997).

No significant differences were observed between the GS of species classified as (1) *Dyckia* and *Encholirium*, (2) *Dyckia*, *Encholirium* and *Fosterella*, (3) *Deuterocohnia* and *Pitcairnia*, (4) *Deuterocohnia* and *Fosterella* or (5) *Pitcairnia* and *Fosterella*. This may be due to the low number of *Fosterella* samples analysed, and because the variation was higher in *Deuterocohnia* than in the other three genera.

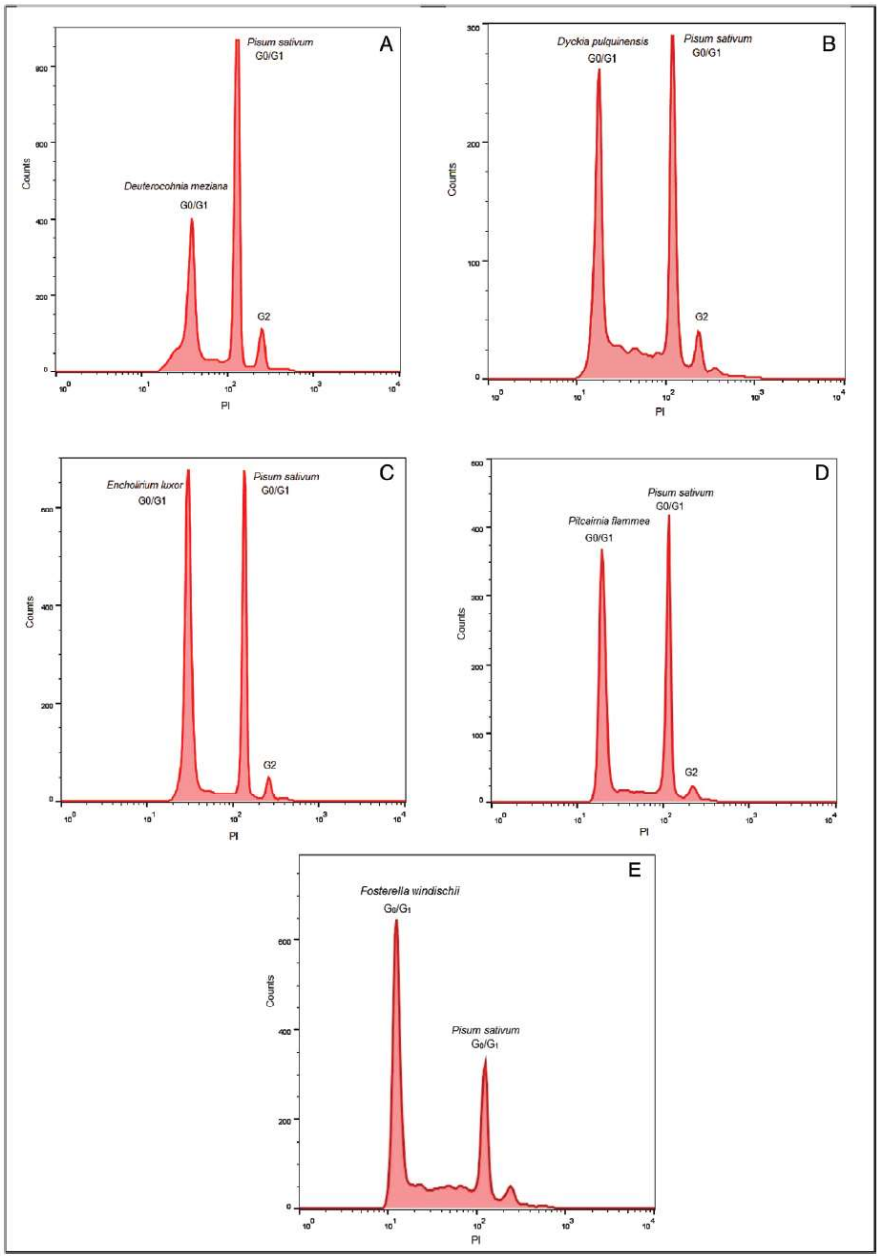

Figure 1. Fluorescence intensity histograms obtained in a) *Deuterocohnia meziana* Kuntze ex Mez, b) *Dyckia pulquinensis* Wittm., c) *Encholirium luxor* L.B.Sm. & R.W.Read, d) *Fosterella windischii* L.B.Sm. & R.W.Read, and e) *Pitcairnia flammea*

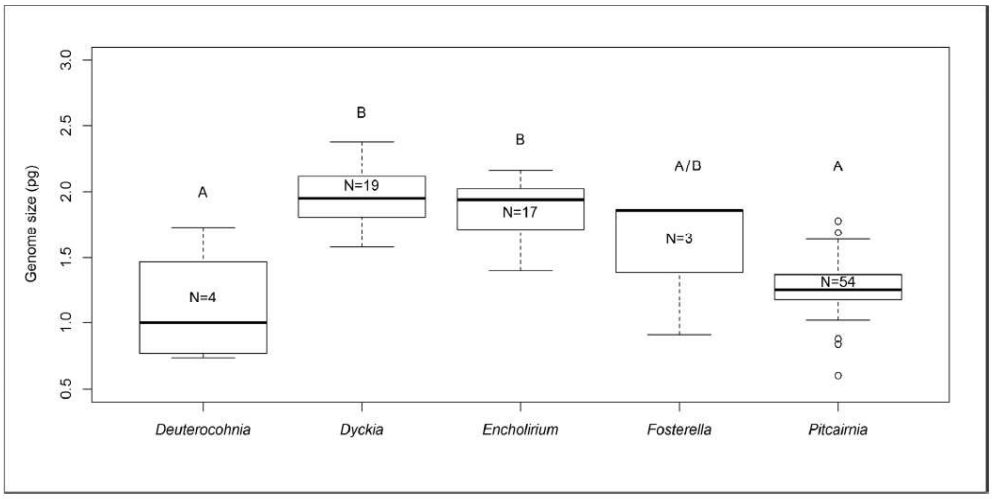

Figure 2. Box plots showing the range of genome sizes (GSs) encountered in *Deuterocohnia*, *Dyckia*, *Encholirium*, *Fosterella* and *Pitcairnia*. The *x*-axis shows the genera and the *y*-axis shows GS (2C-values). Numbers in boxes represent the number of species sampled within each genus. Letters A and B above the boxplots indicate the different averages.

Considering the problems of species delimitation in the genera belonging to the xeric clade (*Deuterocohnia* and *Dyckia* plus *Encholirium*) (Schütz, 2011; Santos-Silva *et al.*, 2013; Krapp *et al.*, 2014; Schütz *et al.*, 2016; M. N. Moura *et al.*, unpubl. data), differences in GS might be expected if the morphological characteristics that differentiate the genera had evolved rapidly from a sudden change in GS, possibly as a result of numerical and structural chromosomal alterations. In fact, despite the small sample available for *Deuterocohnia*, it appears that changes in GS may have occurred during its divergence (average = 1.00 pg) from *Dyckia* and *Encholirium* (average = 1.95 and 1.93 pg/2C, respectively), given the significant differences in GS observed between these lineages (Fig. 2). It is possible that changes in GS may have been abrupt and were accompanied by morphological and anatomical changes in these two groups of the xeric clades (*Deuterocohnia* and *Dyckia* plus *Encholirium*) shortly after their separation. Alternatively, the changes in GS could have accumulated slowly in both groups (Santos-Silva *et al.*, 2013). In the mesic genera (*Pitcairnia* and *Fosterella*), a similar scenario may perhaps be envisaged, because despite the broadly similar average 2C-values in *Pitcairnia* and *Fosterella* (1.27 and 1.54 pg, respectively), apomorphies have been identified in each of these genera (Santos-Silva *et al.*, 2013; Saraiva *et al.*, 2015).

Our molecular phylogenetic results highlighted a low level of nucleotide divergence between the species of Pitcairnioideae and support the hypothesis that the genera of the xeric clade have diverged recently. Nevertheless, the results did not resolve *Deuterocohnia* as monophyletic, as recovered by Givnish *et al.* (2011) and Santos-Silva *et al.* (2013). Instead, these results corroborate those of Schütz (2011), Schütz *et al.* (2016) and M. N. Moura *et al.* (unpubl. data), reinforcing the possible division of *Deuterocohnia*; some species appear in a polytomy with the other taxa of the xeric clade, whereas the other species form a group that is distinct from all the remaining species of the subfamily (Fig. 3). This separation is also apparent in the GS data for the *Deuterocohnia* spp. as the average GS for the species that appear in the polytomy with *Dyckia* and *Encholirium* is 1.2 pg/2C, whereas the average GS for the species that form a distinct clade is about half of this value, 0.77 pg/2C. In the study by Schütz (2011), *Deuterocohnia lorentziana* (Mez) M.A.Spencer & L.B.Sm. was recovered in the clade containing *Deuterocohnia longipetala* and *Deuterocohnia glandulosa*, and in Gitaí *et al.* (2014) this species was reported with a GS of 1.73 pg/2C

Lindl. The x axis corresponds to an arbitrary scale of fluorescence intensity (proportional to the size of the genome), and the y axis represents the number of nuclei with that fluorescence intensity.

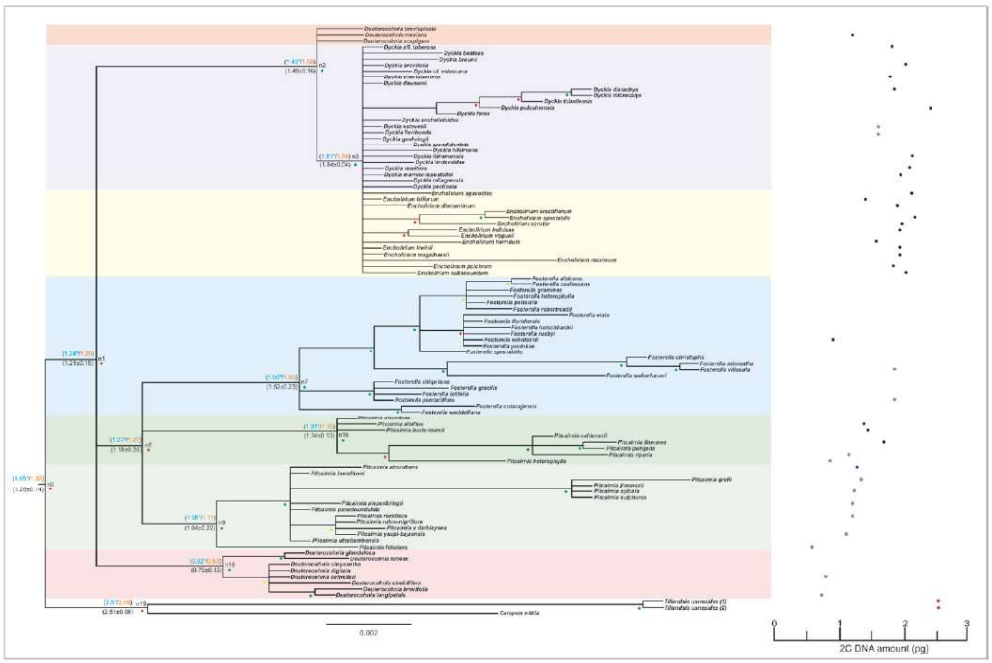

Figure 3. Bayesian consensus tree resulting from the *matK* alignment (824 bp length). Coloured dots on the branches indicate the posterior probability (PP) values: green dots represent values between 1 and 0.95; yellow dots represent values between 0.94 and 0.90; and red dots represent values smaller or equal to 0.89. The nodes are indicated with numbers. Values above and below the branches represent the ancestral genome size (GS; 2C-values in pg) at particular nodes: in blue is the value generated by the maximum likelihood (ML) method using StableTraits (asterisks are related to confidence interval values shown in Supporting Information, Appendix S2); orange is the value generated by the maximum parsimony (MP) method using Mesquite; black, given below the branches, is the value generated by Bayesian inference (BI) using BayesTraits. Different genera are indicated by coloured boxes. Light pink and Dark pink: *Deuterocohnia*; Purple: *Dyckia*; Yellow: *Encholirium*; Blue: *Fosterella*; Dark green and Light green: *Pitcairnia*. Genome size data (2C-values) obtained in this study (●) or taken from Bennett & Leitch (2011) (◉), Gitaí *et al.* (2014) (●) and Zonneveld *et al.* (2005) (●) are presented on the right of the phylogenetic tree.

(Table 1), notably higher than the 2C-values reported for the other two species of the same clade (i.e. 0.74 and 0.80 pg/2C, respectively). However, polyploidy has been observed for this species in Gitaí *et al.* (2005) since the chromosome number reported was $2n = 50$ and $2n = 100$, and this may explain the large GS variation observed in the species in this clade. For *Dyckia* and *Encholirium* the chromosome number is usually $2n = 50$ (Gitaí *et al.*, 2005, 2014).

Pitcairnia spp. formed two clades, as reported by Schütz *et al.* (2016) and Rex *et al.* (2009). In some studies using molecular (Givnish et al., 2004, 2007, 2011) and morphological (Saraiva *et al.*, 2015) datasets, this genus has been shown to be monophyletic. In the study by Schütz *et al.* (2016), which comprises the most recent and complete phylogenetic analysis of Pitcairnioideae, monophyly of *Pitcairnia* was recovered only using nuclear DNA sequences. When plastid data were used, the results were the same as those found in the present study. Therefore, the delimitation of the genus remains controversial, despite *Pitcairnia* being recovered as monophyletic using a morphological dataset (Saraiva *et al.*, 2015). The occurrence of cytotype diversity (i.e. different ploidies within a species) is apparent for two species of *Pitcairnia* for which GS is presented in this study (Table 1; Gitaí *et al.*, 2014): *Pitcairnia flammea* Lindl. (2C = 1.69 pg, this study; $2n = 50/2n \approx 100$, Gitaí *et al.*, 2014) and *Pitcairnia sceptrigera* Mez (2C = 1.2 pg; $2n = 50/2n \approx 100$, Gitaí *et al.*, 2014). Without

chromosome counts for the plants used to estimate GS it is not possible to determine whether the 2C-values reported are for the diploid or tetraploid cytotype of the species. For the other species presented in Table 1 that have karyotype data available, the diploid chromosome number is $2n = 50$ (Gitaí *et al.*, 2014).

All species of *Fosterella*, the other mesic genus, grouped into one clade and GS was estimated for only three species (Table 1): *Fosterella penduliflora* (C.H.Wright) L.B.Sm. (2C = 1.86 pg, Bennett & Leitch, 2011), *Fosterella villosula* (Harms) L.B.Sm. (2C = 1.86 pg, Bennett & Leitch, 2011) and *Fosterella windischii* L.B.Sm. & R.W.Read (2C = 0.91 pg, this study). Polyploidy has also been reported for the first two species ($2n = 100$ and $2n = 150$, respectively, Gitaí *et al.*, 2014) and no karyotype data are available for the third.

The reconstructed ancestral GS for Pitcairnioideae was 1.21 pg/2C according to the MCMC method, 1.24 pg according to the ML method and 1.29 pg according to the parsimony method. These values are smaller than the reconstructed ancestral GS of 1.45 pg/2C for all angiosperms (Puttick, Clark & Donoghue, 2015) and also smaller than 1.99 pg/2C estimated for the ancestral spermatophyte (Puttick *et al.*, 2015). Overall, our analysis of GS evolution in Pitcairnioideae reported here fits the model of proportional GS evolution proposed by Oliver *et al.* (2007) which predicts that 'the rate of GS evolution is proportional to the GS', i.e. if a given taxon has an ancestor with a large GS, the descendants of the ancestor can have a small or large GS, giving rise to a large variation in GS. By contrast, if the GS of the ancestor is small, the descendants of the ancestor are likely to have small genomes, which results in only limited variance in GS. In this sense, we do not expect drastic changes in GS in a group that has arisen from ancestors with small GS, as shown in Figure 3.

GS values have also been reported for other subfamilies of Bromeliaceae (Brocchinioideae, Bromelioideae, Puyoideae and Tillandsioideae) (Ebert & Till, 1997; Ramírez-Morillo & Brown, 2001; Zonneveld *et al.*, 2005; Favoreto *et al.*, 2012; Gitaí *et al.*, 2014) and the 2C-values range from 0.64 pg [*Orthophytum saxicola* (Ule) L.B.Sm., Bromelioideae; Ramírez-Morillo & Brown, 2001] to 2.52 pg [*Tillandsia usneoides* (L.) L., Tillandsioideae; Zonneveld *et al.*, 2005] (Gitaí *et al.*, 2014). A notably higher 2C-value was reported by Favoreto *et al.* (2012) for *Tillandsia loliacea* Mart. ex Schult. & Schult.f. (3.34 pg), but most 2C-values fall between 1.0 and 2.0 pg (*c.* 70% of estimates) as observed in the present study for Pitcairnioideae. This indicates that the ancestral Bromeliaceae may also have had a small GS with the variation of 2C-values in the family tending to be skewed towards smaller values (Oliver *et al.*, 2007).

Bromeliaceae are the most species-rich angiosperm family, almost exclusively native to the New World (Givnish *et al.*, 2011) and, although most 2C-values reported for the family show little variation, we found in this study with Pitcairnioideae some examples of larger variation in genera (*Deuterocohnia* and *Pitcairnia*; Table 1). This variation could be related to chromosomal numerical or structural changes, as reported by Gitaí et al. (2005, 2014). Kraaijeveld (2010) suggested that allopatric populations of organisms with small GS may have a higher rate of divergence than those with larger GS. He attributed this phenomenon to the assumption that in a small genome, the probability of phenotypic changes caused by mutations is larger than in a large genome, which has accumulated more non-coding DNA and repetitive DNA. In addition, Puttick *et al.* (2015) reported that the ability to alter GS (i.e. the rate of change of GS over time) exhibits the strongest correlation with species diversification rather than the size of the genome itself. In relation to the data presented here, we can perhaps assume that the high phenotypic diversity observed across Bromeliaceae and that is well represented in Pitcairnioideae may indeed be correlated with high rates of GS evolution of the species, given the short period of species diversification that is estimated for the family.

In summary, the results obtained in this study improve our knowledge concerning GS in Pitcairnioideae and will contribute to a better understanding of the natural history of this subfamily and Bromeliaceae as a whole. In addition, the FCM data reported here contribute to our knowledge of GS diversity in Bromeliaceae, which is still poorly known given the large numbers of species in this family. The paper also highlights the value of FCM as a rapid and reliable technique for generating GS data, which can be analysed in conjunction with other molecular and morphological data to help elucidate patterns of evolution and phylogenetic relationships in this family.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Appendix S1. Sequences used in the phylogenetic analysis with their specimen voucher and GenBank accession number

Appendix S2. Confidence interval of the values generated by the maximum likelihood method in StableTraits.

SUPPORTING INFORMATION - APPENDIX 1. Sequences used in the phylogenetic analysis with their specimen voucher and their accession number in Genbank

Species	**Specimen voucher**	**Genbank number *mat*K**
Deuterocohnia brevifolia (Griseb.) M.A. Spencer & L.B.Sm.	Balfanz 075	EU681895.1*
Deuterocohnia brevispicata Rauh & L.Hromadnik	Hromadnik 5213	EU681889.1*
Deuterocohnia chrysantha (Phil.) Mez	Zizka 8156	KJ188794.1*
Deuterocohnia digitata L.B.Sm.	Rauh 64142	KJ188802.1*
Deuterocohnia glandulosa E. Gross	Hromadnik 5167	EU681893.1*
Deuterocohnia longipetala (Baker) Mez	-	AF162231.2*
Deuterocohnia lotteae (Rauh) M.A. Spencer & L.B.Sm.	-	AF162232.2*
Deuterocohnia meziana Kuntze ex Mez	Forzza 2253	KX378385
Deuterocohnia scapigera (Rauh & L. Hrom.) M.A. Spencer & L.B.Sm.	Hromadnik 5275	EU681888.1*
Deuterocohnia schreiteri A. Cast.	Schuetz 06104	KJ188809.1*
Deuterocohnia strobilifera Mez	Schuetz 06072	KJ188807.1*
Dyckia beateae E. Gross & Rauh	Leme 1961	KF784675.1*
Dyckia braunii Rauh	BONN:4339	KF784639.1*
Dyckia brevifolia Baker	KAS:001	KF784652.1*
Dyckia choristaminea Mez	HEID:HD 130018	KF784624.1*
Dyckia dawsonii L.B.Sm.	-	AF162234.2*
Dyckia distachya Hassl.	B:26179	KF784710.1*
Dyckia encholirioides (Gaudich.) Mez	Schulte 280408-3	EU681883.1*

Species	**Specimen voucher**	**Genbank number *mat*K**
Dyckia estevesii Rauh	Heid 602151-602159	EU681886*
Dyckia ferox Mez	-	AF162235.2*
Dyckia floribunda Griseb.	WU:115/90	KF784647.1*
Dyckia goehringii E.Gross & Rauh	Rauh 37622	EU681884.1*
Dyckia grandidentata P.J. Braun & Esteves	Leme 6840	KF784712.1*
Dyckia hilaireana Mez	MSBG 1996-0638A	AF539966.1*
Dyckia ibiramensis Reitz	F.Santos-Silva 196	KX378386
Dyckia lindevaldae Rauh	HEID:HD 600180	KF784622.1*
Dyckia maritima Baker	WU:Genf	KF784697.1*
Dyckia marnier-lapostollei L.B.Sm.	HEID:HD 603051	KF784629.1*
Dyckia microcalyx Baker	HEID:HD 102970	KF784612.1*
Dyckia milagrensis Leme	Leme s.n.	KF784680.1*
Dyckia pectinata Smith & Reitz	Leme 6490	KF784714.1*
Dyckia pulquinensis Wittm.	Leme 2415	KF784722.1*
Dyckia tobatiensis Hassl.	WU:0004624	KF784621.1*
Dyckia tuberosa (Vell.) Beer	Leme 6837	KF784724.1*
Dyckia velascana Mez	WU:B 13/93	KF784690.1*
Encholirium agavoides Forzza & Zappi	R.C. Forzza 5475	KU756168
Encholirium biflorum (Mez) Forzza	M. N. Moura 74	KU756169
Encholirium diamantinum Forzza	M. N. Moura 55	KU756179

Species	**Specimen voucher**	**Genbank number *mat*K**
Encholirium erectiflorum L.B.Sm	HEID s.n.	KF784707.1*
Encholirium heloisae (L.B.Sm.) Forzza & Wand.	M. N. Moura 73	KU756170
Encholirium horridum L.B.Sm.	Schindhelm s.n.	EU681887.1*
Encholirium irwinii L.B.Sm.	M. N. Moura 09	KU756171
Encholirium magalhaesii L.B.Sm.	BONN:4344	KF784706
Encholirium maximum Forzza & Leme	R.C. Forzza 7476	KU756172
Encholirium pulchrum Forzza, Leme & O.B.C.Ribeiro	M. N. Moura 72	KU756173
Encholirium scrutor (L.B.Sm.) Rauh	R.C. Forzza 4856	KU756174
Encholirium spectabile Mart. ex Schult. & Schult.f.	G. Martinelli 15058	KU756177
Encholirium subsecundum (Baker) Mez	F.Santos-Silva 22	KX378389
Encholirium vogelli Rauh	M. N. Moura 48	KU756178
Fosterella albicans (Griseb.) L.B.Sm.	Vasquez 4626b	EU681838.1*
Fosterella caulescens Rauh	Rauh 40579a	EU681845.1*
Fosterella chiquitana Ibisch, R.Vásquez & E.Gross	FR:Rex & Schulte 301002-3	EU681864.1*
Fosterella christophii Ibisch, R.Vásquez & J.Peters	-	EU681863.1*
Fosterella cotacajensis M.Kessler, Ibisch & E.Gross	LPB:Kessler 9620b	EU681876.1*
Fosterella elata H.Luther	-	AF162240.1*
Fosterella floridensis Ibisch, R.Vásquez & E.Gross	SEL:Ibisch 02.0001	EU681878.1*
Fosterella gracilis (Rusby) L.B.Sm.	FR:Rex & Schulte 281002-6	EU681923.1*
Fosterella graminea (L.B.Sm.) L.B.Sm.	SEL:Mueller 216	EU681917.1*

Species	**Specimen voucher**	**Genbank number *mat*K**
Fosterella hatschbachii L.B.Sm. & Read	Leme 7100	HQ167790.1*
Fosterella heterophylla Rauh	Vasquez 3661	EU681849.1*
Fosterella latifolia Ibisch, R.Vásquez & E.Gross	Ibisch 98.0098	EU681873.1*
Fosterella micrantha (Lindl.) L.B.Sm.	Welz 3124	EU681860.1*
Fosterella penduliflora (C.H.Wright) L.B.Sm.	Vasquez 3817	EU681866.1*
Fosterella petiolata (Mez) L.B.Sm.	Peters 06.0115	HQ167778.1*
Fosterella robertreadii Ibisch & J. Peters	Friesen 18606	EU681847.1*
Fosterella rusbyi (Mez) L.B.Sm.	Rex & Schulte 251002-3	EU681842.1*
Fosterella spectabilis H.Luther	B:BGB 290-08-00-84	EU681852.1*
Fosterella villosula (Harms) L.B.Sm.	Vasquez 4623	EU681861.1*
Fosterella weberbaueri (Mez) L.B.Sm.	Kroemer 7286	EU681857.1*
Fosterella weddelliana (Brongn. ex Baker) L.B.Sm.	Mijagawa s.n.	EU681879.1*
Fosterella windischii L.B.Sm. & Read	Ibisch 03.0016	EU681851.1*
Fosterella yuvinkae Ibisch, R.Vásquez, E.Gross & S.Reichle	Reichle SR1	EU681850.1*
Pitcairnia abundans L.B.Sm.	HEID: 130636	KJ188819.1*
Pitcairnia albiflos Herb.	FRP s.n.	EU681896.1*
Pitcairnia atrorubens (Beer) Baker	Schulte 280408-1	EU681837.1*
Pitcairnia breedlovei L.B.Sm.	Rauh 52598	EU681890.1*
Pitcairnia burle-marxii R. Braga & Sucre	MSBG 1980-1647A	AF539973.1*
Pitcairnia calderonii Standl. & L.B.Sm.	HEID:G.Noller s.n.	KJ188829.1*

Species	**Specimen voucher**	**Genbank number *mat*K**
Pitcairnia feliciana (A.Chev.) Harms & Mildbraed	WU: s.n.	KF784703.1*
Pitcairnia flammea Lindl.	Forzza 2718	JN202267.1*
Pitcairnia grafii Rauh	Graf 6468	EU681891.1*
Pitcairnia heterophylla (Lindl.) Beer	Senghas 11230	KJ188816.1*
Pitcairnia jimenezii L.B.Sm.	Ariza-Julia s.n.	KJ188825.1*
Pitcairnia piepenbringii Rauh & E. Gross	HEID:HD 602406	KF784669.1*
Pitcairnia pseudoundulata Rauh	Rauh 40159	KJ188837.1*
Pitcairnia pungens Kunth	HEID:HD 130648	KF784668.1*
Pitcairnia rectiflora Rauh	Rauh 53673	KJ188838.1*
Pitcairnia riparia Mez	Senghas s.n.	KJ188821.1*
Pitcairnia rubro-nigriflora Rauh	Rauh 53676	EU681892.1*
Pitcairnia spicata (Lam.) Mez	Ariza-Julia s.n.	KJ188828.1*
Pitcairnia sulphurea Andrews	HEID:104601	KJ188840.1*
Pitcairnia utcubambensis Rauh	Rauh 53566	KJ188826.1*
Pitcairnia x darblayana Sallier	HEID: 132641	KJ188841.1*
Pitcairnia yaupi-bajaensis Rauh	Rauh 25718	KJ188824.1*
Tillandsia usneoides (L.) L.	Palim s.n.	AY614122.1*
Tillandsia usneoides (L.) L.	Rauh s.n.	KJ188793.1*
Catopsis nitida (Hooker) Griseb.	Rauh s.n.	KJ188793.1*

Species with * were acquired from GenBank and species without * were sequenced in Moura *et al.* (unpubl. data)

SUPPORTING INFORMATION - APPENDIX 2. Confidence Interval (IC) of the values generated by the Maximum Likelihood method in StableTraits.

Tree Node	Brownian model value	95%CI_Low	95%CI_High
n0	1.65	0.507	2.882
n1	1.24	0.752	1.712
n2	1.49	1.047	1.953
n3	1.81	1.705	2.007
n6	1.23	0.727	1.730
n7	1.5	0.934	1.992
n9	1.08	0.593	1.596
n15	1.31	0.911	1.699
n18	0.82	0.573	1.067
n19	2.5	2.303	2.711