UNIVERSIDADE FEDERAL DE VIÇOSA

MARINA SOUZA DA CUNHA

DIVERSIDADE CITOGENÉTICA EM APIDAE (HYMENOPTERA) COM FOCO NA EVOLUÇÃO CROMOSSÔMICA DA TRIBO MELIPONINI

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

Orientador: Denilce Meneses Lopes

Coorientadores: Lucio Antônio de Oliveira Campos
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Assentimento:

Marina Souza da Cunha
Autora

Denilce Meneses Lopes
Orientadora

Aos que me fizeram acreditar que a vida vale a pena ser vivida.

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A crítica válida presta um favor ao cientista.

Carl Sagan

RESUMO

CUNHA, Marina Souza, D.Sc., Universidade Federal de Viçosa, fevereiro de 2021. **Diversidade citogenética em Apidae (Hymenoptera) com foco na evolução cromossômica da tribo Meliponini**. Orientadora: Denilce Meneses Lopes. Coorientadores: Lucio Antônio de Oliveira Campos e Tânia Maria Fernandes Salomão.

A família Apidae engloba cerca de 20.000 espécies descritas no mundo. Destas, em torno de 200 espécies já foram cariotipadas e sabe-se o tamanho de genoma de apenas 70. A maioria destes estudos são focados na tribo Meliponini, conhecidas como abelhas sem ferrão. Nessa tribo, o número cromossômico varia de n=8 até n=17 na região neotropical, sendo possível reconhecer três grupos: n=9, n=15 e n=17. Os objetivos desta tese foram: (i) fazer uma revisão dos estudos citogenéticos publicados com abelhas e construir um domínio online para que os dados fiquem disponíveis permanentemente; (ii) isolar sequências altamente repetitivas em duas espécies do gênero *Melipona* para entender os padrões de crescimento e acumulação de heterocromatina neste gênero; (iii) entender como o número cromossômico e o tamanho do genoma influenciaram a evolução cariotípica das abelhas sem ferrão; (iv) identificar os rearranjos cromossômicos que ocorreram durante a evolução cariotípica da tribo Meliponini através da citogenética molecular. Como resultados, (i) o site www.bees.ufop.br foi criado, possibilitando fácil acesso a pesquisadores interessados em grupos específicos, bem como na identificação de padrões gerais para toda a família Apidae, mostrando os avanços da citogenética de abelhas no último século. (ii) O isolamento das sequências altamente repetitivas em espécies do gênero *Melipona*, através da técnica de c_0t-1, indicaram a independência do crescimento da heterocromatina nos subgêneros *Michmelia* e *Melikerria* e, ainda, possibilitou a inferência da origem dos cromossomos B por hibridização interespecífica em *Melipona quinquefasciata*. Um possível cenário para o crescimento da heterocromatina neste gênero foi hipotetizado. (iii) Através da coleta de representantes dos três grupos de Meliponini neotropical foi possível abranger a variação de número diploide de n=8 até n=17, e uma variação do tamanho de genoma de 1C=0,31 pg até 1C=0,92 pg. Esses dados foram combinados à filogenia existente da tribo e foram usados para inferir a importância das fusões Robertsonianas que levaram à diminuição do número cromossômico durante a evolução do clado Meliponini neotropical. (iv) Os marcadores microssatélites confirmaram a importância das fusões Robertsonianas na evolução do clado Meliponini neotropical a partir de um possível ancestral n=15 na separação do grupo 1 e n=17 na separação dos grupos 2 e 3. Conclui-se, também, que o aumento do número de regiões 18S rDNA e a diminuição do

número cromossômico se deu de maneira independente entre os gêneros, e que o microssatélite $(TTAGG)_6$ constitui a sequência telomérica das abelhas sem ferrão.

Palavras-chave: Abelhas. C_0t-1. Caracterização da cromatina. FISH. Sequências repetitivas. Tamanho de genoma nuclear.

ABSTRACT

CUNHA, Marina Souza, D.Sc., Universidade Federal de Viçosa, February, 2021. **Cytogenetic diversity in Apidae (Hymenoptera) focusing on the chromosomal evolution in the Meliponini tribe**. Adviser: Denilce Meneses Lopes. Co-advisers: Lucio Antônio de Oliveira Campos and Tânia Maria Fernandes Salomão.

The Apidae family encompasses around 20,000 described species worldwide. Of which, about 200 species have been studied with cytogenetic techniques, and the genome sizes are known for only 70. Most of these studies are focused on the Meliponini tribe, commonly referred as stingless bees. In this tribe, chromosome number vary from n=8 to n=17 in the neotropical region, with the recognition of three groups: n=9, n=15, and n=17. The goals of this theses were: (i) to revise the cytogenetic studies published with bees and create an online platform to display the data permanently; (ii) to isolate highly repetitive sequences in two *Melipona* species in order to understand the heterochromatin growth accumulation patterns in this genus; (iii) to understand how chromosome number and genome size influenced the karyotypic evolution of the stingless bees; (iv) to identify the chromosomal rearrangements that occurred during the karyotypic evolution of the Meliponini tribe trough molecular cytogenetics. The results were: (i) the website www.bees.ufop.br was created allowing access to researchers interested in specific bee groups, or in general patterns of the Apidae family, showing the advances in the field of bee cytogenetics in the last century. (ii) The highly repetitive sequences, isolated through c_0t-1 technique in the genus *Melipona,* indicated the independent heterochromatin growth between *Michmelia* and *Melikerria* subgenera and, also, the interspecific hybridization hypotheses of the origin of *Melipona quinquefasciata* B chromosomes. A possible scenario that led to this heterochromatin growth in the genus was hypothesized. (iii) The sampling of representative species of the three neotropical Meliponini groups covered the diploid number variation from n=8 to n=17 and genome size variation from 1C=0.31 pg to 1C=0.92 pg. These data were combined to the existent phylogenetic tree of the tribe and were useful to infer the importance of the Robertsonian fusions that resulted in the decrease in chromosome number during the karyotype evolution of the neotropical Meliponini clade. (iv) The microsatellite markers confirmed the importance of the Robertsonian fusions in the Meliponini karyotype evolution from a putative ancestral of n=15 in the split of group 1 and n=17 in the split of groups 2 and 3. The decrease in the diploid number and the increase in the 18S rDNA occurred independently between genera, and the microsatellite $(TTAGG)_6$ constitute the telomeric sequence of the stingless bees.

Keywords: Bees. C_0t-1. Chromatin characterization. FISH. Repetitive sequences. Nuclear genome size.

SUMÁRIO

1 INTRODUÇÃO GERAL

Abelhas constituem um grupo importante de insetos, cuja diversidade está estimada entre 18.000 e 20.000 espécies distribuídas nos cinco continentes, exceção à Antártica (Michener, 2007). Este táxon é representado por apenas uma família, Apidae Latreille, 1802, a qual se subdivide em sete subfamílias, Andreninae, Apinae, Collectinae, Halictinae, Megachilinae, Melittinae e Stenotritinae (Melo & Gonçalves, 2005).

As abelhas têm grande importância na polinização de plantas silvestres e cultivadas, sendo que diversas espécies produzem mel e outros produtos que são utilizados pelo homem, como geleia real, pólen, cera e própolis (revisado em Michener, 2007). Os resultados do colapso das colônias de abelhas *Apis mellifera*, ocorrido na última década, têm sido sentidos em diversos países ao redor do mundo, e também foram observados em espécies nativas de abelhas (Potts *et al.*, 2016; Woodcock *et al.*, 2017). Vários fatores têm sido apontados como causadores deste declínio, como perda e fragmentação de habitat, doenças, parasitas, poluição, mudanças climáticas e principalmente agentes tóxicos, como metais e inseticidas (Goulson *et al.*, 2015; Di Pasquale *et al.*, 2016; Chicas-Mosier *et al.*, 2017; Klein *et al.*, 2017; Woodcock *et al.*, 2017).

Tendo em vista a importância da abundância e biodiversidade de insetos na polinização de diversas culturas alimentícias (revisado em Garibaldi *et al.*, 2014), esforços de conservação devem ser voltados também para a manutenção da diversidade de espécies nativas de abelhas (Mallinger & Gratton, 2015), uma vez que a introdução da espécie *Apis mellifera* nem sempre substitui o papel das abelhas nativas na polinização de algumas culturas (Garibaldi *et al.*, 2014).

As abelhas pertencentes à tribo Meliponini, conhecidas popularmente como abelhas sem ferrão, ocorrem em todas as regiões tropicais e subtropicais da terra (Camargo & Pedro, 1992). Na região neotropical são descritas 417 espécies nesta tribo (Camargo & Pedro, 2013), entretanto, o número real dessa diversidade é difícil de estimar devido a grande quantidade de espécies crípticas e a falta de estudos sistemáticos sobre taxonomia deste grupo (Michener, 2007).

A elaboração de estratégias efetivas de conservação precisa levar em consideração os processos ecológicos e evolutivos que levaram a diversificação das espécies, fazendo com que os estudos de variabilidade genética, tanto interespecífica quanto intraespecífica, sejam de suma importância (Koffler *et al.*, 2017; Lopez-Uribe *et al.*, 2017). A diversidade genética em insetos sociais tem uma relevância ainda maior, especialmente em abelhas, onde uma baixa

diversidade aumenta os riscos de endogamia e pode levar à produção de machos diploides com efeitos desastrosos para as colônias (revisado em Rúa *et al.*, 2013). Neste contexto, a citogenética é uma ferramenta que permite acessar a variabilidade genética das espécies e, ao mesmo tempo, inferir sobre os processos evolutivos que moldaram a diversificação e especiação dos táxons (Gokhman & Kuznetsova, 2006).

A última revisão citogenética disponível sobre Meliponini, feita por Tavares *et al.* (2017), mostra que das mais de 400 espécies descritas, apenas 104 já foram estudadas com alguma técnica citogenética. Nesta tribo, o número diploide varia de n = 8 a n = 20 e, apesar do número cromossômico normalmente se manter constante entre as espécies de um mesmo gênero, as características citogenéticas como morfologia dos cromossomos, quantidade, distribuição e composição da heterocromatina seguem padrões distintos (revisado em Tavares *et al.*, 2017). Uma compilação dos dados citogenéticos incluindo diferentes ramos da família Apidae nunca foi realizada.

A primeira explicação para a variação cromossômica observada na tribo Meliponini foi proposta por Kerr & Silveira (1972), que sugeriram a poliploidia (duplicação completa do genoma) como principal evento responsável pela variação do número diploide. Na época, poucas espécies haviam sido cariotipadas e os dados indicavam que o número cromossômico poderia ter dobrado por eventos de poliploidia em algumas linhagens. Já a teoria da interação mínima (MIT), proposta por Imai (1988, 1994), propõe que rearranjos do tipo fissão cêntrica teriam sido os eventos mais importantes durante a evolução das espécies, este tipo de rearranjo resulta em cromossomos de menor tamanho e consequentemente diminui as possíveis interações deletérias entre os mesmos no núcleo. Outra consequência do aumento do número cromossômico seria o concomitante aumento da heterocromatina, necessário para estabilizar os novos telômeros. Essa teoria tem sido a mais usada para explicar a evolução cromossômica em toda a ordem Hymenoptera (Hoshiba & Imai, 1993).

Tavares *et al.* (2017) reconheceram 3 grupos dentro de Meliponini neotropical baseado no número cromossômico: n = 9 presente nas espécies de *Melipona*; n = 15 presente em diferentes gêneros não relacionados filogeneticamente, indicando que este número surgiu independentemente várias vezes ao longo da diversificação da tribo; e n = 17 indicado como o número mais comum presente na maioria das espécies estudadas até o momento. Devido este fato, estes autores propuseram um número cromossômico elevado como possível número ancestral deste grupo (n = 17 - 20) e eventos de fusão cêntrica explicariam a existência de espécies com menor número de cromossomos. Essa hipótese foi corroborada recentemente por Travenzoli *et al.* (2019) que, através de meta-análises utilizando uma abordagem

filogenética molecular, propuseram um número cromossômico ancestral de n = 18 para a tribo Meliponini e n = 17 para o clado Meliponini neotropical, constituindo uma hipótese alternativa à MIT para explicar a evolução das abelhas sem ferrão.

Assim, a busca de uma teoria que envolva diferentes tipos de rearranjos citogenéticos justifica a realização de novos estudos abrangendo diferentes técnicas citogenéticas, em grande número de espécies sendo algumas ainda não estudadas, a fim de contribuir com o conhecimento sobre a evolução cromossômica em abelhas.

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2 OBJETIVO GERAL

Esta tese teve como objetivo principal estudar a diversidade citogenética em Apidae, com foco na tribo Meliponini, a fim de contribuir com o conhecimento sobre a evolução cromossômica em abelhas.

2.1 OBJETIVOS ESPECÍFICOS

Capítulo 1: Criar uma database online (www.bees.ufop.br) com o objetivo de disponibilizar os dados citogenéticos da família Apidae de maneira online e atualizada e descrever os avanços que foram feitos no campo da citogenética de abelhas no último século.

Capítulo 2: Entender os processos de acumulação de heterocromatina no gênero *Melipona* e propor uma hipótese sobre o possível cenário evolutivo que permitiu seu crescimento acentuado de modo independente em dois subgêneros.

Capítulo 3: Relacionar número cromossômico e tamanho do genoma para melhor compreender a evolução cariotípica das abelhas sem ferrão, com foco na evolução do clado Meliponini neotropical.

Capítulo 4: Identificar os rearranjos cromossômicos que ocorreram durante a evolução cromossômica das abelhas sem ferrão do clado Meliponini neotropical fazendo uso de técnicas de citogenética molecular.

3 CAPÍTULO 1

The Bee Chromosome database (Hymenoptera: Apidae)

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Abstract

The bee diversity (Apidae) estimative ranges from 18,000 to 20,000 species worldwide. Together, they show an impressive diversity in morphological, ecological, and behavioral traits, and there is still much to be understood about their taxonomy and systematics. Their chromosome count variability and genome biology are also astonishing. To date, around 200 bee species have already been karyotyped, with chromosome numbers varying from $n = 3$ to $n = 28$, and nuclear haploid genome sizes are available for approximately 70 species with a variation of 1C = 0.19 pg to 1C = 1.38 pg. The Bee Chromosome database was created (www.bees.ufop.br) to summarize the Apidae cytogenetic knowledge by assembling all the cytogenetic information published on bees. Considering the importance of cytogenetic studies for taxonomy, phylogeny, genetics, systematics, conservation, and evolution, the main goal of this database is to outline the advances in the field of bee cytogenetics over the last century.

Keywords: Chromosomal evolution, chromosome number, cytogenetics, karyotypic formula, nuclear genome size.

Introduction

Bees are an important insect group represented by one family, Apidae Latreille, 1802, which is comprised of seven subfamilies: Andreninae, Apinae, Collectinae, Halictinae, Megachilinae, Melittinae, and Stenotritinae (Melo and Gonçalves 2005). Together, they represent between 18,000 and 20,000 species described worldwide (Michener 2007). A variety of species are good honey producers, and their pollination services play an important role in the ecosystem and human agriculture (Michener 2007; Roubik et al. 2018). Their morphological, ecological, and behavioral diversities are remarkable, and there is still much to be understood about their taxonomy, systematics, and evolution (Moure et al. 2007; Sedivy et al. 2013; Kapheim et al. 2015; Shell and Rehan 2018).

Databases provide a virtual space to store data on specific topics that can later, with cumulative information, be used to visualize patterns and produce useful knowledge (Fayyad et al. 1996). A range of databases have been created to compile different aspects of genetic data: cytogenetic information (Berrar et al. 2001; Vulto-van Silfhout et al. 2013; D'Ambrosio et al. 2017; Cardoso et al. 2018a; Perkins et al. 2019; Degrandi et al. 2020), DNA sequences (Benson et al. 2013), eukaryotic repetitive elements (Jurka et al. 2005), and nuclear genome sizes (Gregory et al. 2007).

Due to the great importance of genome size information (reviewed in Gregory 2005), a number of databases were created to assemble the available data on plants, animals, and fungi; the Plant DNA C-values Database, the Animal Genome Size Database, and the Fungal Genome Size Database (Gregory et al. 2007). Despite the increasing number of entries added to these databases over the years, studies involving bees are scarce. Only one study sampled a few representatives of all Apidae subfamilies, together with other hymenopterans, to study potential genome size constraints related to lifestyle (Ardila-Garcia et al. 2010). Despite an apparent general trend of small genomes reported in parasitic and social species (Johnston et al. 2004, 2007; Koshikawa et al. 2008), Ardila-Garcia et al. (2010) found that neither parasitism leads to small genomes nor non-parasitism results in the evolution of larger genomes, although both parasitoids and eusocial species exhibit significantly smaller genomes than those of non-parasitoid solitary species.

In addition, few studies have focused on the Meliponini tribe (Apinae), attempting to relate nuclear genome size with differences in heterochromatic patterns, finding a positive correlation between genome size and heterochromatin content in stingless bees (Lopes et al. 2009; Tavares et al. 2010, 2012). Still, some intriguing questions remain to be addressed, such as what types of sequences could be responsible for the differences in nuclear genome size among species, as well as the relationships with chromatin composition and structure, and evolution in bees.

Another relationship that has historically been discussed is the correlation between chromosome number and eusociality, with a higher chromosome number reported among social species in comparison with the lower numbers found in parasitic/solitary species (Sherman 1979; Templeton 1979; Anderson 1984; Gokhman 2009). To address this question, Ross and colleagues (2015) compared sister clades with different lifestyles among Hymenoptera and did not find strong support for this hypothesis, arguing the importance of increased recombination rates for social lifestyle evolution. Further investigation is needed to elucidate the importance of sociality for the evolution of these high recombination frequencies (Wilfert et al. 2007; Howard and Thorne 2010; Sirvio et al. 2011; Ross et al. 2015).

The first bee karyotype was described in 1913 from *Osmia cornuta* (Latreille, 1805) by Armbruster (Kerr and Laidlaw 1956). Since then, around 200 bee species have been karyotyped. Taken together, these findings show that the chromosome number in bees varies from $n = 3$ in *Andrena togashii* Tadauchi and Hirashima, 1984 to $n = 28$ in *Hylaeus* sp.2 (Hoshiba and Imai 1993). A number of hypotheses have been proposed to explain this variation: (1) the polyploidy hypothesis proposes that numerical changes in the whole set of

chromosomes are the main cytogenetic events responsible for the chromosomal variability in bees (Kerr 1952; Kerr 1969; Kerr and Silveira 1972). This hypothesis was based on the cytogenetic data available at time, and suggests that the chromosome number has doubled in some lineages. However, with accumulating information on several species with intermediate chromosome numbers, the polyploid hypotheses could no longer explain the observed numerical bee chromosomal variation; (2) The Minimum Interaction Theory (MIT) postulates that the chromosome number increases over evolutionary time due to centric fissions to minimize deleterious interactions between chromosomes in the nuclei (Imai et al. 1986, 1988; Imai, 1991; Hoshiba and Imai 1993; Imai et al. 1994). This hypothesis was based on the heterochromatin and chromosome morphology patterns, and has been the main theory used to explain chromosome number variation in hymenopterans; (3) The role of Robertsonian rearrangements and the importance of fusion events in bee evolution (Tavares et al. 2017; Travenzoli et al. 2019). This theory was based on meta-analyses mainly on the Meliponini tribe (Apinae), and the lack of concordance of some stingless bee chromosomal features to the MIT, broadening the bee karyotype evolution to a handful of cytogenetic rearrangements, and recognizing that generalizations should be made with caution.

To date, only one compilation of the available cytogenetic information regarding all Apidae subfamilies has been performed. Furthermore, it unfortunately contains some taxonomic misclassifications (Ross et al. 2015). Thus, in an attempt to assemble all the cytogenetic information published on bees, the Bee Chromosome database was created (www.bees.ufop.br). Whenever possible, nuclear genome size estimates (in picogram - pg) were also recorded. Since information dating back to 1913 was collected, we attempted to changes in species classification (original classifications are shown under the column “notes”). Considering the importance of cytogenetic studies for taxonomy, phylogeny, genetics, systematics, conservation, and evolution (Gokhman and Kuznetsova 2006; Potter and Deakin 2018), the aim of this database is to outline the advances that have been made in the field of bee cytogenetics over the last century.

Cytogenetic data assembly

In order to assemble all cytogenetic information available on bees, we searched published papers for the following topics: haploid/diploid number, nuclear genome size, and classic/molecular cytogenetic techniques. Keywords were also added to the search, such as “bees”, “Apidae”, subfamilies, and tribe names, in order to achieve more relevant and specific results. The search was performed on some of the main platforms, namely ISI Web of

Knowledge, SCOPUS, and Google Scholar, as well as the references cited in any of the articles downloaded. All of the published manuscripts were downloaded and checked for the aforementioned information. Unpublished data, such as in academic theses and abstracts from conferences, were not recorded. The current species classification was based on the Moure catalog and on recent taxonomic papers that can be assessed online at http://moure.cria.org.br/catalogue?id=1 (Catalogue of bees) and http://www.itis.gov (Integrated Taxonomic Information System - ITIS).

The information was extracted manually from each manuscript and recorded in an Excel sheet using the following fields: species name (subfamily, tribe, genus, species), sample locality, haploid/diploid number, karyotype, nuclear haploid genome size (picograms - pg), classic and molecular techniques available, and the respective references. Under "notes", some observations were explained, such as the presence of B chromosomes, changes in species classification, or if subgenera or subspecies were cited in the original articles. In this Excel sheet, each cell of the table represents a value, where the row A1 is the column title. The script that accesses this table and organizes the information to be displayed on the website was written in the programming language Python.

The karyotypic formula was presented as it was provided in the original paper, where some entries have the "Levan classification" and others have the "Imai classification" of the chromosomes. Levan and colleagues (1964) proposed a chromosome classification based on the arm ratio between the long and short arms, and classified them as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a). On the other hand, Imai (1991) classified the chromosomes in metacentric ($\bar{M}$), acrocentric (A), or pseudo-acrocentric (A^M), with a variety of forms depending on the position of the heterochromatin on the karyotype (*e.g.* A^e, A^{Mc}, $\bar{M}^t$, $\bar{M}^{cc}$, $\bar{M}^i$). Evidence of incorrect chromosome number designation was not included in the dataset, but is discussed later in this text. Differences among populations of the same species were regarded as individual entries in the database. All this information can be accessed at the permanent domain www.bees.ufop.br.

Dataset on the Apidae subfamilies

By the time of the publication of this database (2020), there were 236 entries representing 56 genera across five Apidae subfamilies, whereas two subfamilies had no records (Melittinae and Stenotritinae). In the Andreninae subfamily, only *A. togashii* has been cytogenetically characterized so far, holding the record of the smallest bee chromosome number (n = 3) (Hoshiba and Imai 1993). Apinae has the largest number of entries (198),

followed by Halictinae (19), Megachilinae (11), Collectinae (6), and Andreninae (2) (Fig. 1). The discrepancy of entries observed in Apinae could be credited to the difficulties in collecting solitary species in contrast to the ease of spotting nests of social species, and the fact that many species are bred by beekeepers.

The dataset can be accessed for each subfamily, tribe, or genus, and each search result can be downloaded as a CSV file. The results are shown in the format of a table with all the cytogenetic information available in the columns (Fig. 2) or as a histogram of the frequency of the haploid number in each searched taxon (Fig. 3), in which diploid (2*n*) values were converted to haploid (*n*). The full dataset can be visualized under the name "Apidae family" as well as in the tab "Statistics" (Fig. 4). This database will be kept up to date with the addition of new records by the authors but also by researchers abroad that are encouraged to use the tab "Submit". To report potential errors or suggest improvements in the website, use the tab "Contact Us".

Only a few descriptions of chromosome number were not included in the database because of incorrect chromosome counting: three species reported by Owen (1983) were corrected years later by Owen et al. (1995). Several species described by Kerr (1972) were later corrected in other publications (reviewed in Tavares et al. 2017). Studies performed before the 1980s used the squashing technique to obtain chromosomes, in contrast to the air-drying technique used more recently (see Imai et al. 1988), which is considered a better technique to count and visualize the morphology of chromosomes (Gokhman and Kuznetsova 2006). Many species described in the dataset have not been addressed in other studies since the first karyotype description (*e.g.* Meliponini Afrotropical); hence, the use of such information in future papers should require some caution, especially regarding descriptions based on papers without the images of the chromosomes (*e.g.* Kerr 1952; Kerr and Araújo 1957; Kerr and Silveira 1972) or with poor quality of the images (*e.g.* Kumbkarni 1965; Kerr 1972).

There were 68 entries regarding nuclear genome size in the database, which varied from 1C = 0.19 pg in *Apis cerana* Fabricius, 1793 to 1C = 1.38 pg in *Melipona capixaba* Moure and Camargo, 1995. Several methodological challenges still need to be addressed in this field, such as the standardization of the techniques and measurements. For instance, estimates vary from 1C = 0.42 pg to 1C = 0.62 pg in *Bombus terrestris* (Linnaeus, 1758) depending on the technique used (reviewed in Stolle et al. 2011). Other important topics for further study include the variations in genome size observed among populations from different geographical locations (see Ardila-Garcia et al. 2010; Cardoso et al. 2018b), and

between males and females (see Tavares et al. 2012), as well as the constraints related to developmental complexity (see Gregory 2002).

Conclusions and perspectives

Until the 1990s, cytogenetic research was restricted to the description of chromosome numbers. Since then, advances in techniques (mainly molecular cytogenetics) have allowed for the development of more elaborate studies, testing different hypotheses regarding the chromosomal evolution in bees (*e.g.* Piccoli et al. 2018; Santos et al. 2018; Travenzoli et al. 2019; Cunha et al. 2020; Pereira et al. 2020). However, these studies focused on the Meliponini tribe, and are still far from encompassing the current diversity of bees.

Karyotype rearrangements and nuclear genome size changes can result in reproductive isolation, contributing to diversification and speciation (Schubert and Lysak 2011; Ferree and Prasad 2012; Cardoso et al. 2018b). Collecting this information in an online public database will facilitate access to researchers focused on specific groups, as well as allow for the visualization of general patterns for the Apidae family and highlight existing knowledge gaps.

Recently, similar efforts were made to organize the cytogenetic data available on ants: the Ant Chromosome database - ACdb (Cardoso et al. 2018a, www.ants.ufop.br). There are over 15,000 ant species described worldwide (Bolton et al. 2007), only 520 of which have been karyotyped (reviewed in Cardoso et al. 2018a). Together, we share a common goal to contribute to future studies involving systematics, evolution, and chromosome biology in the order Hymenoptera.

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Authors’ Contribution

All authors contributed to the study conception and design. Literature review and data collection were performed by MSC. LAOC and MSC checked species classification. The first

draft of the manuscript was written by MSC and DCC. All authors commented on previous versions and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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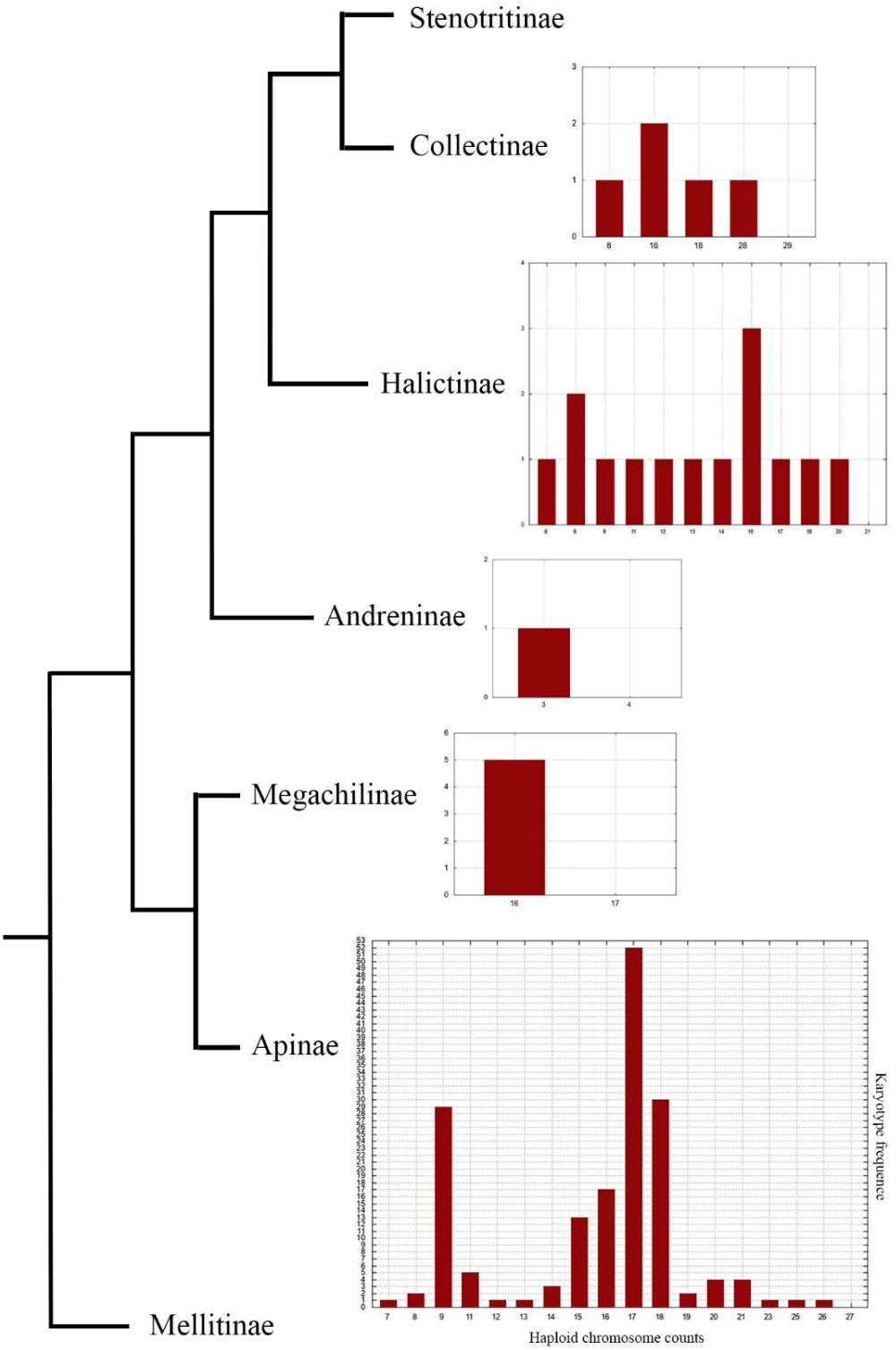
Figures

Fig. 1 Phylogenetic relationships between Apidae subfamilies redrawn from Danforth et al. (2013) and the respective variation on the haploid chromosome number (Mellitinae and Stenotritinae have no cytogenetic records)



Fonte: Autoral.

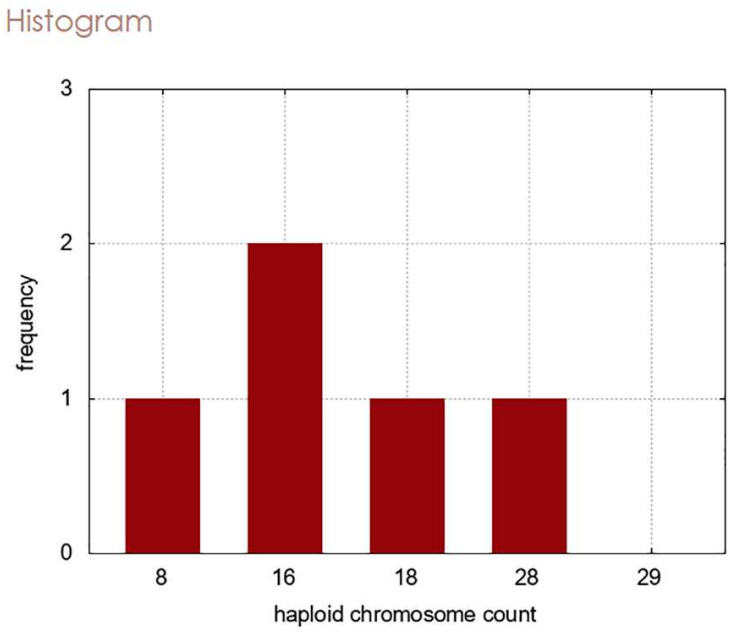
Fig. 2 Example of the data generated in the Bee Chromosome database as a table containing all the cytogenetic information available from the Collectinae subfamily

Cytogenetic data

Tribe	Species	Sample local	Country(ies)	Haploid(*n*)	Diploid(2*n*)	Karyotype	Notes	Genome size (pg)	Classic cytogenetic data	Molecular cytogenetic data	References
Colletini	*Colletes babai*	Rifu, Miyagi	Japan	16	32	k= 12AM + 4AMc			C-banding		Hoshiba and Imai 1993
Colletini	*Colletes esakii*	Daisen, Tottori	Japan	16		k= 6AM + 9AMc + 1Ai			C-banding		Hoshiba and Imai 1993
Hylaeini	*Hylaeus affinis*	Lake Placid, Florida	United States					0.64			Ardila-Garcia et al. 2010
Hylaeini	*Hylaeus nippon*	Matsue, Shimane	Japan	8		k= 1AMi + 4Mc + 3M			C-banding		Hoshiba and Imai 1993
Hylaeini	*Hylaeus sp. 1*	Rifu, Miyagi	Japan	18		k= 4Mc + 8M + 6Mcc			C-banding		Hoshiba and Imai 1993
Hylaeini	*Hylaeus sp. 2*	Rifu, Miyagi	Japan	28		k= 25AM + 1AMc + 1M + 1Mcc			C-banding		Hoshiba and Imai 1993

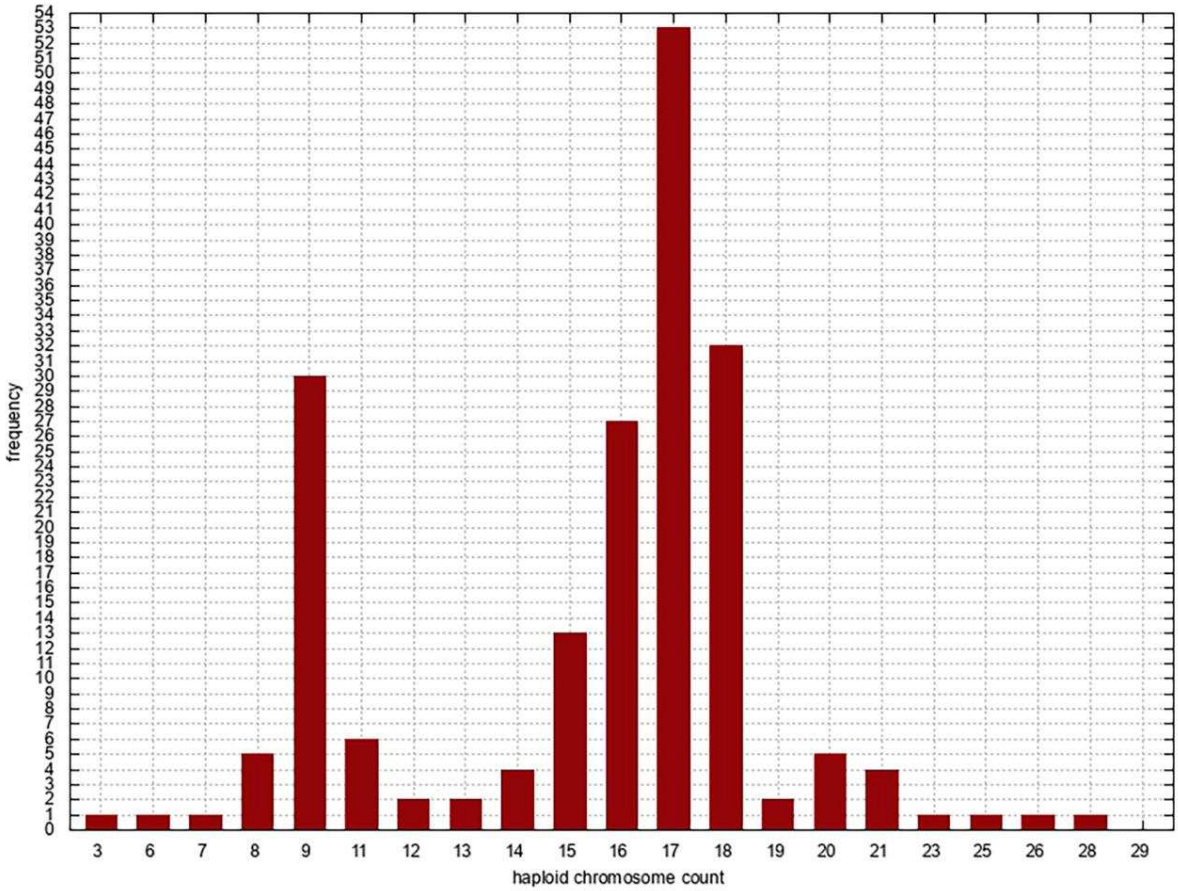
Fonte: Autoral.

Fig. 3 Example of the frequency histogram of the haploid chromosome count generated in the Bee Chromosome database for the Collectinae subfamily. Diploid (2*n*) values were converted to haploid (*n*) values



Fonte: Autoral.

Fig. 4 Frequency distribution of the haploid chromosome entries in the Bee Chromosome database in October 2020



Fonte: Autoral.

4 CAPÍTULO 2

Insights into the heterochromatin evolution in the genus *Melipona* (Apidae: Meliponini)

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Abstract

The species of the four *Melipona* subgenera can be subdivided into two cytogenetic groups: species belonging to Group I are characterized by having low heterochromatin content in their karyotypes (<50%) and species belonging to Group II by having high heterochromatin content (>50%). In order to study the patterns of heterochromatin accumulation in this genus, we isolated moderate to highly repetitive sequences in *M. (Michmelia) mondury* (Mmon probe) and *M. (Melikerria) fasciculata* (Mfas probe) obtained based on the renaturation kinetics of C_0t-1 DNA and used these fragments as probes to hybridize in thirteen species belonging to the four *Melipona* subgenera. The Mmon probe marked all chromosomes in all *Michmelia* species and additionally the secondary constriction of *M. (Eomelipona) puncticollis*. The Mfas probe showed positive markings only within *Melikerria.* These results indicate the independence of the heterochromatin growth between *Michmelia* and *Melikerria*. Absence of markings in the regular set of chromosomes in *M. (Melikerria) quinquefasciata* and positive markings in the Bs, with the Mfas probe, suggests an interspecific origin of these chromosomes. We also hypothesize about the possible scenario that led to the heterochromatin growth in the genus *Melipona*.

Keywords: C_0t-1, chromosomal evolution, Hymenoptera, molecular cytogenetics, repetitive sequences, stingless bees.

Introduction

After being considered as “junk DNA” for decades, the heterochromatin has gained proper attention for its structural role in the organization of the genome, and for its functional role in various essential biological processes for the functioning of the organism (reviewed in Grewal and Jia 2007; Diaz-Castillo 2017). This portion of the genome is composed by different classes of repetitive DNA, such as satellite DNAs and mobile elements (transposons and retrotransposons) (Moran and Morrish 2005). These sequences are known for their binding sites for several structural proteins, the production of regulatory non-coding RNAs, as well as for their direct participation in the regulation of gene expression (Ugarkovic 2005; Muotri et al. 2007; Palomeque and Lorite 2008; Ferree and Prasad 2012; Lower et al. 2018).

The C_0t-1 is a technique used to evidence fragments of repetitive DNA ranging from 50 to 500 bp (Alves-Silva et al. 2017). These moderate to highly repetitive sequences usually characterize the heterochromatin portion of the chromosomes and have been used to study heterochromatin composition and its evolution in some insect species (Cabral-de-Mello et al.

2011; Palacios-Gimenez et al. 2013, 2015; Xavier et al. 2014; Anjos et al. 2016). In bees, this technique was used only in *Melipona (Michmelia) scutellaris* to infer the independence of the heterochromatin growth between subgenera *Michmelia* and *Melikerria* (Piccoli et al. 2018).

The genus *Melipona* (Hymenoptera, Apidae) ranges from Sinaloa and Tamaulipas, Mexico, to Tucumán and Misiones, Argentina (Michner 2007). This genus has 76 valid species subdivided into four subgenera: *Eomelipona* (15 species), *Melipona* (13 species), *Melikerria* (10 species), and *Michmelia* (38 species) (Camargo and Pedro 2013). Out of this diversity, only 23 species were cytogenetically studied revealing an interesting feature that is unique to this genus: the separation of two groups based on the location and amount of heterochromatin present in the karyotype of its species (reviewed in Travenzoli et al. 2019). Low content Group I is composed of species that have less than 50% of heterochromatin distributed in the pericentromeric or subtelomeric regions in a few chromosome pairs, and representatives of this group are subgenera *Eomelipona*, *Melipona* and also *Melipona* (*Melikerria) quinquefasciata*; high heterochromatin Group II is composed of species that have more than 50% of heterochromatin occupying almost the entire interstitial region of the chromosomes, and representatives of this group are subgenus *Michmelia* and the rest of the studied species of *Melikerria* (Rocha and Pompolo 1998; Rocha et al. 2002; Tavares et al. 2010; Andrade-Souza et al. 2018; Cunha et al. 2018; Travenzoli et al. 2019).

In a phylogenetic point of view, the study of Ramírez et al. (2010) showed that *Melipona sensu strictu* appears as the base of the phylogeny, indicating that low heterochromatin content is the plesiomorphic condition in *Melipona* (Cunha et al. 208; Tranvenzoli et al. 2019). Three subgenera are monophyletic, *Melipona*, *Michmelia* and *Melikerria*, and only *Eomelipona* is polyphyletic, with a few species as sister clade of *Melikerria* and another group of species as sister clade of *Michmelia* (Ramírez et al. 2010). The *Eomelipona* relationships rise some doubts about the monophyletic nature of the high heterochromatin trait (as *Melikerria* and *Michmelia* are both monophyletic but not sister subgenera).

In order to improve our understanding of the heterochromatin accumulation processes in the genus *Melipona*, we isolated moderate to highly repetitive sequences from two species, *Melipona (Michmelia) mondury* and *Melipona (Melikerria) fasciculata*, to study the patterns of accumulation of these sequences in thirteen species belonging to the four subgenera described in this genus. In this paper we confirm the independence of the heterochromatin growth between *Michmelia* and *Melikerria* and also hypothesize about the possible scenario that led to this heterochromatin growth in the genus *Melipona*.

Material and Methods

Species sampling and chromosome preparation

Thirteen *Melipona* species were collected in different Brazilian regions (Table 1). The specimens were deposited in the scientific collection of the Apiário Central at Universidade Federal de Viçosa, Minas Gerais, Brazil. The mitotic chromosomes were obtained from cerebral ganglia of larvae in the final defecation stage (Imai et al. 1988). At least 10 metaphases of each species were analyzed to determine the described patterns.

Isolation of C_0t-1 DNA

Two species with known high heterochromatin content were chosen, *M. (Michmelia) mondury* and *M. (Melikerria) fasciculata*, to investigate the heterochromatin similarity between representatives of Groups I (low content) and II (high content) belonging to the four *Melipona* subgenera. Repetitive DNA-enriched samples from both species were obtained based on the renaturation kinetics of C_0t-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences) according to the protocol described by Alves-Silva et al. (2017) with the following modifications: DNA was extracted (100–500 ng/µl) according to Waldschimdt et al. (1997) and diluted in 0.3 M NaCl. For denaturation and fragmentation of the DNA, the DNA tube was autoclaved for 15 min at 121ºC (1.4 atm) using safe-lock tubes. Then, a sample was checked in an agarose gel 1% (the fragments should range from 100 to 1000 pb). After this checking point, the DNA tube was heated at 60 ºC for 15-150 min (using the formula: $t = [C_0tX \times 4.98]/C_0$ where *t* is the time of incubation, *X* is the fraction of C_0t (C_0t-1 = 1, C_0t-2 = 2, etc) and C_0 is the concentration of the initial DNA in $\mu g.\mu l^{-1}$) and placed on ice for 2 min. The DNA was transferred to water-bath 42 ºC and added preheated 10xS1 nuclease buffer and S1 nuclease to permit the digestion of single stranded DNA, and incubated for 1h. The amount of S1 nuclease and 10xS1 nuclease buffer is associated with the quantity of DNA and with the final total volume respectively. For each 1 µg of DNA, 1U of S1 nuclease is used, and the buffer represents 10% of the total volume (e.g. for 500 µg of DNA + 5.6 µl S1 nuclease (89 U/µ) + 50 µl of 10xS1 nuclease buffer + 444.4 µl of water, the final total volume is 500 µl). The DNA was precipitated by adding 0.1 vol. of 3 M sodium acetate and 1 vol. of 2-propanol, and centrifuged at 14.000 rpm for 20 min at 4 ºC. The supernatant was discarded and the DNA pellet was dried at room temperature. 100 µl of 70% ethanol was added and centrifuged again at 14.000 rpm for 10 min at 4 ºC. The supernatant was discarded carefully; the DNA pellet was dried at room temperature and dissolved in 50 µl

of TE buffer solution. A sample was checked in an agarose gel 0.8% (the fragments should range from 50 and 500 bp).

Fluorescence in situ hybridization

Fluorescence *in situ* Hybridization (FISH) followed the protocol described by Pinkel et al. (1986) using the C_0t-1 DNA fragments of both bee species as probes (*M. (Michmelia) mondury* probe is named as "Mmon" probe and *M. (Melikerria) fasciculata* is named as "Mfas" probe). These probes were labeled using Dig-Nick Translation Mix (Roche Applied Science) following the manufacturer's instructions and the signal was detected with anti-digoxigenin-rhodamine (Roche Applied Science). Chromosomes were counterstained with Fluoroshield with DAPI (Sigma). Digital images of the fluorescence techniques were captured in a BX53F Olympus microscope equipped with an MX10 Olympus camera, using the CellSens imaging software. Adobe® Photoshop® software were used for image processing.

Results

FISH pattern obtained after hybridization of the *M. mondury* C_0t-1 fragments (Mmon probe) in the thirteen analyzed species indicated positive markings in the heterochromatin portion of all chromosomes in the *Michmelia* species *M. flavolineata, M. fulva, M. lateralis, M. mondury, M. paraensis, M. rufiventris,* and *M. seminigra merrillae*, but also in one pair of chromosomes in the secondary constriction of *M. (Eomelipona) puncticollis* (Fig. 1). The results were negative with no markings in the other species: *M. (Eomelipona) bicolor*, *M. (Melikerria) fasciculata, M. (Melikerria) interrupta, M. (Melikerria) quinquefasciata,* and *M. (Melipona) quadrifasciata* (Fig. 1).

FISH pattern obtained after hybridization of the *M. fasciculata* C_0t-1 fragments (Mfas probe) in eleven species indicated positive markings in the heterochromatin portion of all chromosomes in the *Melikerria* species *M. fasciculata* and *M. interrupta*. Interestingly, *M. (Melikerria) quinquefasciata* had no markings in the regular set of chromosomes, but the heterochromatic B chromosomes were completely marked (Fig. 2). The results showed no markings in the other analyzed species: *M. (Eomelipona) bicolor*, *M. (Eomelipona) puncticollis, M. (Melipona) quadrifasciata, M. (Michmelia) flavolineata, M. (Michmelia) fulva, M. (Michmelia) lateralis, M. (Michmelia) mondury, M. (Michmelia) rufiventris,* and *M. (Michmelia) seminigra merrillae* (Fig. 2).

Eleven species had $2n = 18$ and two had $2n = 22$ (Table 1). Two or three B chromosomes were found in all *M. quinquefasciata* specimens (Figs. 1, 2). This is the first

description of the diploid number of *M. fulva* and *M. lateralis*. Both species belong to the *Michmelia* subgenus and share with other species of this subgenus the cytogenetic characteristics of Group II, i.e. high heterochromatin content in their karyotypes and the impossibility to determine the karyotypic formula due to the lack of visualization of the centromeres (Figs. 1, 2).

Discussion

We analyzed thirteen *Melipona* species belonging to the four subgenera (*Eomelipona*, *Melikerria*, *Melipona* and *Michmelia*) and the two cytogenetic groups (Group I and Group II) previously characterized in this genus (Table 1). Our results showed high specificity of the Mfas probe within *Melikerria* subgenus (Fig. 2), whereas the Mmon probe showed markings in all *Michmelia* species and additionally in the secondary constriction of *M. (Eomelipona) puncticollis* (Fig. 1). The secondary constriction is associated with the 18S rDNA in this species (Cunha et al. 2018), indicating that the heterochromatin is adjacent to the ribossomal region. Species with low heterochromatin content have the 18S rDNA cistrons in the interstitial position of one pair of chromosomes, whereas species with high content show these ribossomal cistrons in the terminal position also in one pair of chromosomes (reviewed in Cunha et al. 2018). Therefore, the restriction of euchromatin to the chromosome tips in high heterochromatin content species (Rocha et al. 2002) was probably due to interstitial and pericentromeric heterochromatin growth (Rocha & Pompolo, 2008), and possibly started its growth adjacent to the ribossomal region.

Due to the absence of shared heterochromatic sequences between *Michmelia* and *Melikerria*, the growth of heterochromatin probably occurred independently between the two subgenera, as previously indicated (Lopes et al. 2011; Cunha et al. 2018; Piccoli et al. 2018). C_0t-1 is an anonymous technique, combining all the heterochromatin sequences, therefore complementary studies are necessary to characterize the specific sequence observed in *M. (Eomelipona) puncticollis*, that is possibly shared with the ancestral of *Michmelia*, and to identify the repetitive elements that are specific to each subgenus and were responsible for the heterochromatin growth.

Although *M. quinquefasciata* belongs to the subgenus *Melikerria*, it did not show positive markings in the regular set of chromosomes with the Mfas probe not even in the heterochromatin regions, as it was observed in *M. (Melikerria) interrupta* (Fig. 2). Among the analyzed species of this subgenus, *M. quinquefasciata* is the only one with low heterochromatin content, the others being characterized by high heterochromatin content

(reviewed in Travenzoli et al. 2019) or high DNA content (Tavares et al. 2010). Thereby, the heterochromatin growth possibly did not occur early in *Melikerria* cladogenesis, since *M. quinquefasciata* constitute a basal species within the subgenus (Ramírez et al. 2010). The cytogenetic analysis of *Melipona beecheii*, another basal species within *Melikerria*, may help to elucidate this issue.

Interestingly, *M. quinquefasciata* has a genome size that is characteristic of group II species (Tavares et al. 2010), although heterochromatin content characteristic of group I. This discrepancy between low heterochromatin content in the karyotype and high DNA content in the genome can be attributed to the presence of heterochromatic B chromosomes in this species. Up to 4 Bs were already described from different populations (Silva et al. 2018). In the present study, 2 or 3 B chromosomes were found among the individuals and they were completely marked by the Mfas probe whereas it showed no markings with the Mmon probe (Figs. 1, 2). The presence of shared sequences between the heterochromatic B of *M. quinquefasciata* and the regular set of chromosomes of *M. fasciculata* and *M. interrupta*, but not with *Michmelia* species, suggests a probably interspecific origin of these chromosomes within *Melikerria*.

The first description of the karyotypes of *M. (Michmelia) fulva* and *M. (Michmelia) lateralis* showed $2n = 18$ in the former, the most common diploid number found in *Melipona* (reviewed in Travenzoli et al. 2019), and $2n = 22$ in the latter, a number that was until now described only in *M. (Michmelia) seminigra* subspecies (Francini et al. 2011; Andrade-Souza et al. 2018). The presence of positive markings with the Mmon probe in all *Michmelia* species regardless of the $2n = 18$ or $2n = 22$ indicate that the increase in the diploid number occurred after the growth of heterochromatin that characterizes this subgenus. A phylogenetic analysis showing the relationship between the species will help to elucidate if the increase of the $2n$ occurred only once in *Michmelia* or if it occurred independently in *M. lateralis* and *M. seminigra*. Both species co-occur in the Amazonas, being separated geographically by large rivers, however, possible hybrid colonies were reported in some regions (Garcia, M.V.B. unpublished data). Due to the shared chromosome number and the possibility of hybridization, they are likely sister-species.

Heterochromatin Evolution in the genus Melipona

Starting from a low heterochromatin ancestor, how did the heterochromatin become widespread among all chromosomes in the high content species? With this question in mind,

we hypothesize about the possible scenario that led to the heterochromatin growth in the genus *Melipona*.

We found a shared sequence between *Michmelia* heterochromatin adjacent to the ribossomal region of *M. (Eomelipona) puncticollis*. One satellite sequence in *Drosophila melanogaster*, associated with the rDNA region on the X chromosome, was linked to the regulation of the ribosomal gene expression through the interaction of proteins with both satellite and rDNA repeats (Battles et al. 2006). Satellite sequences observed in all chromosomes of other species are known for containing functional elements essential for the regulation of cellular processes, such as the cell cycle and chromosome segregation (Lorite et al. 2001, 2004; Shestakova et al. 2004; Tsoumani et al. 2013). In *Solenopsis* ants, the expansion of the centromeric heterochromatin in all chromosomes suggested that such expansion would bring advantages during the chromosomal segregation by serving as binding sites for spindle-associated proteins (Huang et al. 2016).

The presence of mobile genetic elements (transposons and retrotransposons) inside repetitive sequences is a known trait that could be responsible for their spreading to different chromosomal locations (Moran and Morrish 2005; Palomeque and Lorite 2008). In vertebrates, there are known examples of spreading of rDNA sequences to several chromosomes due to transposable elements (Vicari et al. 2008; Silva et al. 2013; Moraes et al. 2017), and the presence of dispersed mobile elements along the karyotypes (Schneider et al. 2013; Schemberger et al. 2014, 2016; Barbosa et al. 2015; Silva et al. 2017). Possibly, the presence of mobile elements led to the spreading of the heterochromatin among all chromosomes in the high content species.

The spreading of sequences associated with mobile elements has also been acknowledged to lead to genome size increasing (Kidwell 2002; Slotkin and Martienssen 2007; Satović and Plohl 2013; Ruiz-Ruano et al. 2016; Biscotti et al. 2018). The genome size is another trait that differentiates groups I and II in *Melipona*, with low content species having an average of 0.29 pg whereas high content species have an average of 0.98 pg (Tavares et al. 2010). The association of the increase in copy number of satellite sequences and the occurrence of chromosomic rearrangements have been an indicative of the importance of these sequences to chromosomal evolution, constituting potential barriers to gene flow, and may lead to reproductive isolation and speciation (Ugarkovic and Plohl 2002; Ferree and Prasad 2012; Cardoso et al. 2018).

Conclusion

The contribution of this work is the study of moderate and highly repetitive sequences in several species of the genus *Melipona* expanding the scarce existing knowledge about this type of DNA in bees. We also contributed to the better understanding of the heterochromatin evolution in *Melipona*, indicating the possible scenario that led to the heterochromatin growth that occurred independently in both *Michmelia* and *Melikerria*. Future studies will focus on the characterization of the unique sequences present on each subgenus.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by MSC. LAOC checked species identification. The first draft of the manuscript was written by MSC and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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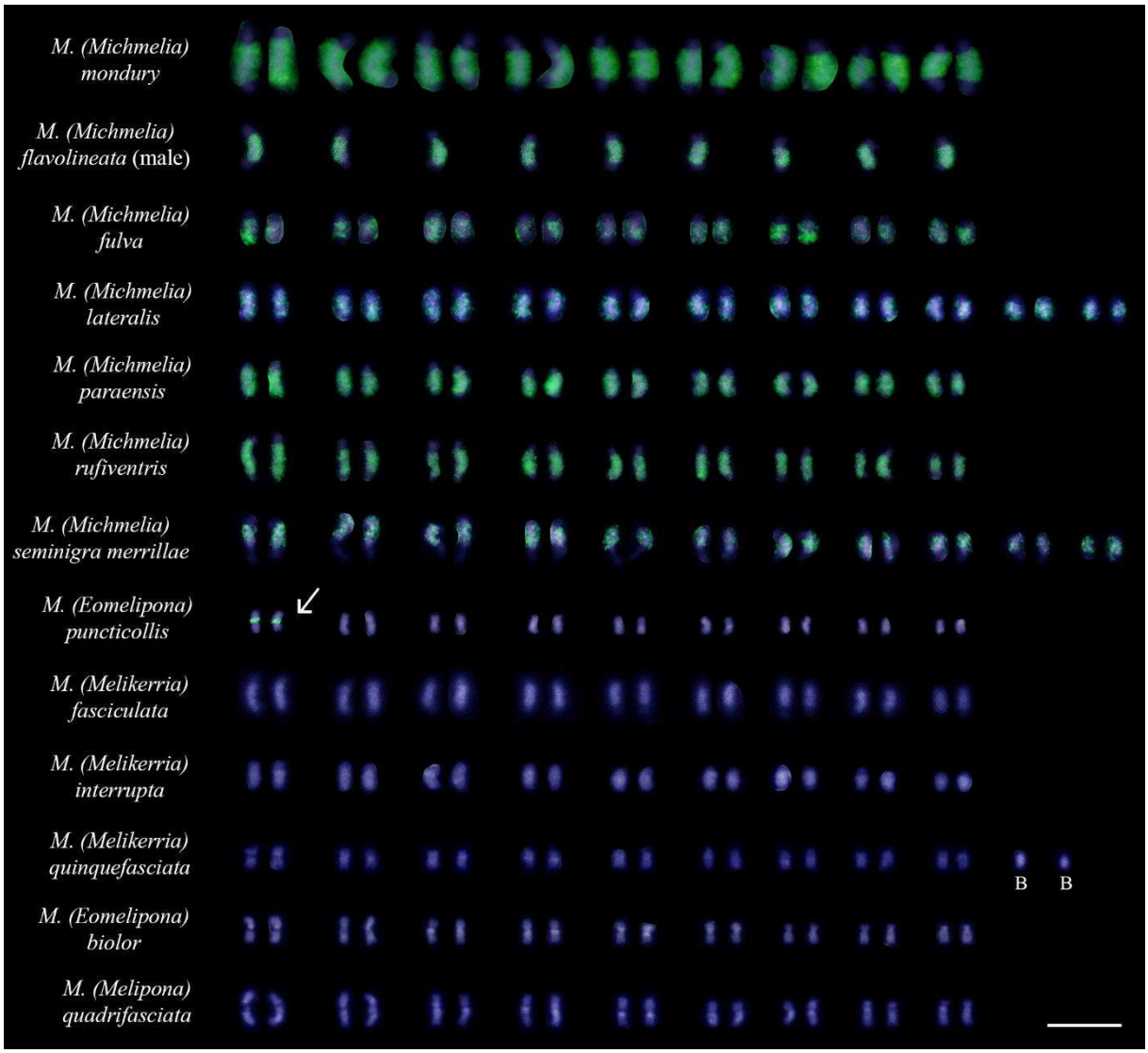
Table 1. Collection site of the *Melipona* species in different Brazilian regions. Species were assigned to subgenera based on the Moure's catalogue. Diploid number (2*n*) and heterochromatin content are also shown. Group I: low heterochromatin content in the karyotype, and Group II: high heterochromatin content in the karyotype.

Subgenera	*Species*	*2n*	*Heterochromatin content*	*Sample Locality*
Eomelipona	*M. bicolor*	18	Group I	Viçosa, Minas Gerais
	M. puncticollis	18	Group I	Altamira, Pará
Melikerria	*M. quinquefasciata*	18[a]	Group I	Viçosa, Minas Gerais
	M. fasciculata	18	Group II	São Luís, Maranhão
	M. interrupta	18	Group II	Iranduba, Amazonas
Melipona	*M. quadrifasciata*	18	Group I	Viçosa, Minas Gerais
Michmelia	*M. flavolineata*	18	Group II	Urbano Santos, Maranhão
	M. fulva	18	Group II	Presidente Figueiredo, Amazonas
	M. lateralis	22	Group II	Presidente Figueiredo, Amazonas
	M. mondury	18	Group II	Viçosa, Minas Gerais
	M. paraensis	18	Group II	Altamira, Pará
	M. rufiventris	18	Group II	Guimarânia, Minas Gerais
	M. seminigra merrillae	22	Group II	Iranduba, Amazonas

[a] 2 or 3 B chromosomes were found in *Melipona quinquefasciata*.

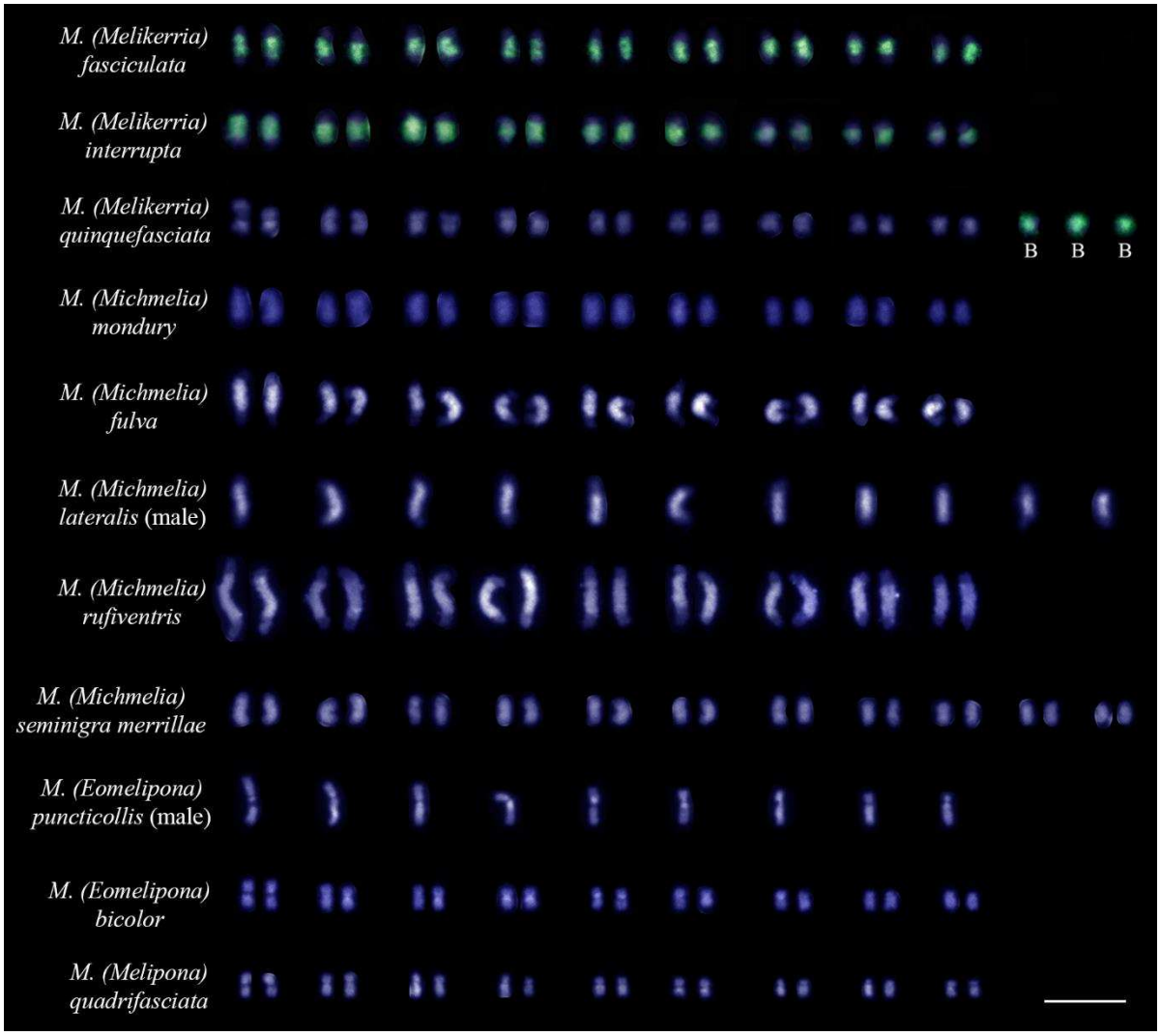
Figures

Fig. 1 Fluorescence *in situ* Hybridization pattern obtained after hybridization of the *Melipona (Michmelia) mondury* C_0t-1 fragments in other *Melipona* species. Chromosomes are shown in blue and the probe in green. The arrow indicates de markings only on the first chromosome pair of *M. (Eomelipona) puncticollis*. 'B' indicates B-Chromosomes. Scale bar = 5 μm



Fonte: Autoral.

Fig. 2 Fluorescence *in situ* Hybridization pattern obtained after hybridization of the *Melipona (Melikerria) fasciculata* C_0t-1 fragments in other *Melipona* species. Chromosomes are shown in blue and the probe in green. 'B' indicates B-Chromosomes. Scale bar = 5 μm



Fonte: Autoral.

5 CAPÍTULO 3

Robertsonian rearrangements in Neotropical Meliponini karyotype evolution (Hymenoptera: Apidae: Meliponini)

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Abstract

Genome changes, evidenced through karyotype or nuclear genome size data, can result in reproductive isolation, diversification, and speciation. The aim of this study was to understand how changes in the karyotype such as chromosome number and nuclear genome size accompanied the evolution of neotropical stingless bees, and to discuss these data in a phylogenetic context focusing on the karyotype evolution of this clade. We sampled 38 species representing the three Neotropical Meliponini groups; 35 for karyotype analyses and 16 for 1C value measurement. The chromosome number varied from 2*n*=16 to 2*n*=34, with distinct karyotypic formulae and the presence of a few polymorphisms, such as B chromosomes in one species and arm size differences between homologous chromosomes in two species. The mean 1C value varied from 0.31 pg to 0.92 pg. We associated empirical data on chromosome number and mean 1C value to highlight the importance of Robertsonian fusion rearrangements, leading to a decrease in chromosome number during the Neotropical Meliponini evolution. These data also allowed us to infer the independent heterochromatin amplification in several genera. Although less frequent, *Melipona* species with 2*n*=22 represent evidence of Robertsonian fissions. We also pointed out the importance of chromosomal rearrangements that did not alter chromosome number, such as inversions and heterochromatin amplification.

Keywords: Chromosome rearrangements, Cytogenetics, Flow cytometry, Genome size, Heterochromatin, Karyotypic formula, Stingless bees.

Introduction

The bees of the Meliponini tribe, commonly known as stingless bees, are distributed in tropical or subtropical areas (Michener, 2007) and constitute important pollinators for several angiosperms (Slaa *et al.,* 2006; Roubik *et al.,* 2018). Many species have also been used in meliponiculture for honey, pollen, resin, and cerumen production (Cortopassi-Laurino *et al.,* 2006; Roubik *et al.,* 2018). From a phylogenetic point of view, Meliponini is represented by three main clades: Afrotropical, Indo-Malay/Australasian, and Neotropical (Rasmussen and Cameron 2010). Because of its diversity, the Neotropical clade is subdivided into three groups; *Trigonisca s.l., Melipona s.l.,* and the remaining species are combined into a third group (Rasmussen and Cameron 2010). Altogether, this clade is represented by 417 species, and many are yet to be described (Camargo and Pedro 2013). Eighty of these neotropical species have been cytogenetically studied, showing a chromosome number variation from $2n = 16$ to $2n = 34$ as well as different patterns of heterochromatin accumulation (reviewed in Tavares *et al.,* 2017).

The minimum interaction theory (MIT) (Imai *et al.,* 1988) has been used to explain karyotype evolution in Hymenoptera, which includes Apidae (Hoshiba and Imai 1993; Pompolo and Campos 1995; Rocha *et al.,* 2003; Godoy *et al.,* 2013; Santos *et al.,* 2018). This theory predicts that the chromosome number increased from a low-numbered ancestral karyotype of $2n < 24$ due to centric fission events and concomitant heterochromatin amplification, which stabilizes the new telomeres (Imai *et al.,* 1988). Based on meta-analyses using a molecular phylogenetic approach, another hypothesis was recently postulated to explain karyotype evolution in the Meliponini tribe (Travenzoli *et al.,* 2019a). This alternative hypothesis suggests that fusion events decreased the chromosome number from a high-numbered ancestral karyotype of $2n = 34$ in the Neotropical Meliponini clade.

Besides the chromosome number, the nuclear genome size (1C value) provides valuable insights about genome and karyotype evolution (Gregory, 2005; Blommaert, 2020). Karyotype rearrangements involving duplication/amplification or deletion/reduction of chromosome portions (Schubert and Lysak 2011) can result in nuclear genome size changes and consequently in reproductive isolation, which prevents gene flow, leading to diversification and speciation (Ferree and Prasad 2012; Cardoso *et al.,* 2018). In bees, one study performed a broader sampling with focus on the possible constraints in 1C value regarding eusociality/parasitism (Ardila-Garcia *et al.,* 2010). Comparative studies with social stingless bee species have shown that variations in the nuclear genome size do not always correlate with changes in chromosome number, for example *Trigona pallens* and *Melipona*

fasciculata have close 1C values, 0.81 pg and 0.82 pg, respectively, and very different chromosome numbers, 2*n* = 34 and 2*n* = 18, respectively (Lopes *et al.*, 2009; Tavares *et al.*, 2012). On the order hand, the species heterochromatin content has been appointed as a karyotypic change correlated with nuclear genome size differences (Tavares *et al.*, 2010; Aguiar *et al.*, 2016; Moura *et al.*, 2019).

The aim of this study was to understand how changes in the karyotype such as chromosome number and nuclear genome size accompanied the evolution of neotropical stingless bees. We also discussed these data in a phylogenetic context focusing on the karyotype evolution of the Neotropical Meliponini clade.

Results

Karyotype analyses

The chromosome number determined for the 35 species varied from 2*n* = 16 to 2*n* = 34 (Figures 1, 2, 3). The karyotypic formulae also varied (Figure S1, Table 1). Chromosome arm ratios for each species are shown in the supporting information (Figures S2, S3, S4). Three species of the genus *Melipona* were classified as having low heterochromatin content and had their karyotypic formulae defined; the other seven *Melipona* species were classified as having high heterochromatin content. In these species, the karyotypic formulae were not determined due to the difficult for precise centromere visualization (Figure 1). More details will be provided in the discussion section. The heterochromatin results of the other species varied from presence in the centromeric position (*e.g. Cephalotrigona capitata*), presence in one chromosome arm (most frequent pattern), and absence in some chromosomes (*e.g.* half of the *Schwarziana quadripunctata* chromosomes).

Heterochromatic B chromosomes were found in every *Melipona quinquefasciata* specimen, with intraindividual variation of 2B or 3B per metaphase (Figure 1, Table 1). We identified differences between homologous chromosomes with respect to arm size in *Melipona fulva* (pair 1, Figure 1) and in *Schwarziana quadripunctata* (pair 1, Figure 3). In *M. fulva*, although the polymorphism is not in the ribossomal region, the 18S rDNA probe was used to confirm that the polymorphic chromosomes are homologues (Figure 4). All five analyzed individuals had this heteromorphic pair. In the case of *S. quadripunctata*, the polymorphism was confirmed by the morphometric data, and the analyses of individuals with and without this pattern. Five individuals were heteromorphic and two were homomorphic regarding this size difference.

Nuclear genome size measurement

Fresh larvae ganglia yielded a higher number of nuclei in relation to fixed adult ganglia. Based on this, two ganglia were used from fixed material to obtain at least 5,000 nuclei. Adequate flow cytometry (FCM) histograms were obtained from 10 crushes with the pestle for fresh larval ganglia and 12 crushes for fixed adult ganglia (Figure 5). Based on the external procedure, we checked two species with 1C values close to those of *S. xanthotricha*, *Duckeola ghilianii* and *Frieseomelitta* sp.1, using *M. fasciculata* as standard. For the other sampled species, *S. xanthotricha* was an adequate standard, even though *Plebeia* sp.1 and *Paratrigona lineata* also had close 1C values in relation to this standard. Thus, we suggest *S. xanthotricha* as an internal standard for future analyses of other bee species using fixed samples. The external flow cytometry procedure is fundamental to verify if the sample has the same DNA content in relation to the standard and, consequently, choose another standard if necessary.

The mean values obtained from fresh and fixed ganglia of the same species were statistically identical with $P > 5\%$ (P = 49%, r = 0.98). No significant difference was found between the 1C value of the same species measured with external and internal standard procedures with $P > 5\%$ (P = 41%, r = 0.95). Because of the non-significance of the statistical results and the low number of available individuals, the final mean 1C value was calculated using the values obtained from fresh and fixed ganglia, and the internal and external procedures. These results, as well as the available data for other Neotropical species, are summarized in Table 2 and Figure 6. The mean 1C value of the species clustered in group 1 had a variation of 1C = 0.35 pg - 0.49 pg, whereas the species in group 2 varied from 1C = 0.27 pg - 1.38 pg, and species in group 3 from 1C = 0.40 pg - 0.90 pg.

Species with 1C value at least 50% higher than its phylogenetic close relatives with the same chromosome number were observed in some genera, such as in *Melipona* (*Melikerria* and *Michmelia* subgenera), *Tetragonisca*, *Geotrigona,* and *Trigona* (Figure 6).

Discussion

We associated chromosome number with mean 1C value to infer the karyotypic changes in the Neotropical stingless bees' evolution. Based on the recent hypothesis of Robertsonian fusions to explain the karyotypic evolution of the Meliponini tribe (Travenzoli *et al.*, 2019a), such type of rearrangement leads to different degrees of loss of chromosomal segments (DNA sequences) of the short arm of the chromosomes (John and Freeman 1975). This could explain the lowest chromosome number/1C values found in *Leurotrigona muelleri*

(2*n* = 16/1C = 0.35 pg), and *Melipona* and *Eomelipona* subgenera (2*n* = 18/1C = 0.27 pg - 0.35 pg). This relation between fusion events and decrease in nuclear genome size has also been observed in other insects (Gregory *et al.*, 2003; Moura *et al.*, 2020).

The data reported by Travenzoli *et al.* (2019a) consisted of the sampling of two Group 1 species, one with 2*n* = 16 and one with 2*n* = 30, which resulted in equal weight of both chromosome numbers as the ancestral state of this group. Therefore, with the sampling of two new species from Group 1, we also suggest the possibility of 2*n* = 30 as the ancestral karyotype of Neotropical Meliponini. The ancestral state being probably 2*n* = 30 - 34 / 1C = 0.4 pg - 0.5 pg and, due to Robertsonian fusion events, the chromosome number decreased in the species with 2*n* = 16 from Group 1, 2*n* = 18 from Group 2, and 2*n* = 28-30 from Group 3, with different degrees of chromosomal loss.

Melipona species with 2*n* = 22 from the subgenus *Michmelia* most likely represent a case of Robertsonian fissions, as they possess 1C values that are intermediate to those observed in *Michmelia* species with 2*n* = 18 (Figure 6). As discussed by Perry *et al.* (2004), although chromosomal fissions are rare, sporadic observations are compatible with this mechanism in some animals. Until now, this seems to be the only cytogenetic evidence of a fission event in the Neotropical stingless bees. Although *M. lateralis* and *M. seminigra* belong to the same morphological subgroup inside subgenus *Michmelia* (Camargo and Pedro 2013), it is still not confirmed whether they are sister species and share a common ancestor with 2*n* = 22 or whether the increase in chromosome number evolved independently in these taxa (Ramírez *et al.*, 2010; Cunha *et al.*, 2020).

Our nuclear genome size data showed species with mean 1C values at least 50% higher than its close relatives with the same chromosome number. Genome size variations are usually credited to non-coding DNA, such as repetitive sequences and mobile elements, commonly present in the heterochromatin portion of the chromosomes (Moura *et al.*, 2019; Blommaert, 2020). Considering the phylogeny of the tribe (Rasmussen and Cameron 2010) and that the heterochromatin content influences the nuclear genome size (Tavares *et al.*, 2010; Aguiar *et al.*, 2016; Moura *et al.*, 2019), we suggest that the heterochromatin was independently amplified in a few Meliponini genera (Figure 6), as well-documented in the genus *Melipona* (*Michmelia* and *Melikerria* subgenera), and additionally in *Tetragonisca*, *Geotrigona*, and *Trigona*. This independent amplification led to the presence of unique heterochromatin composition in each genus (Lopes *et al.*, 2014; Pereira *et al.*, 2020), having shared sequences only among species from the same genus/subgenus (Cunha *et al.*, 2020; Pereira *et al.*, 2020).

The genus *Melipona* can be divided into two cytogenetic groups based on the heterochromatic patterns: group 1 classified as “low heterochromatin content” showing < 50% of heterochromatin in the karyotype, and group 2 classified as “high heterochromatin content” showing > 50% of heterochromatin in the karyotype (Rocha and Pompolo 1998; Rocha *et al.,* 2002). Therefore, the lack of definition of the karyotypic formula in seven *Melipona* species is shared with all *Michmelia* species and in almost all *Melikerria* species, and it is considered to be caused by the accentuated heterochromatin growth that hindered the centromeres, and occurred independently in these subgenera (Rocha and Pompolo 1998; Lopes *et al.,* 2008; Andrade-Souza *et al.,* 2018; Cunha *et al.,* 2018, 2020; Piccoli *et al.,* 2018; Travenzoli *et al.,* 2019b). Together with the heterochromatin content, the 1C value can also be used to distinguish the two *Melipona* groups: group 1 with 1C values between 0.2 pg - 0.4 pg, an average of 3.5 lower than group 2, which has 1C values between 0.7 pg - 1.4 pg, (Tavares *et al.*, 2010; Present study). *Melipona (Melikerria) quinquefasciata* is an exception, with low heterochromatin content in the karyotype (Figure 1) but a high mean 1C value (Tavares *et al.,* 2010). As up to four supernumerary chromosomes have been found in different populations of this species (Silva *et al.,* 2018), this discrepancy can be attributed to the presence of these heterochromatic extra chromosomes, which should be taken into account in genome size estimation studies.

The heterochromatin amplification in *Tetragonisca* and *Geotrigona* species was inferred based on their high 1C values (Table 2) and the presence of heterochromatic arms in all of their chromosomes (Rocha *et al.,* 2003; Barth *et al.,* 2011; Lopes *et al.,* 2014; Pereira *et al.,* 2020). Even though the *Trigona* species also have this heterochromatic arm, two monophyletic sister clades could be identified (Rasmussen and Camargo 2008; Rasmussen and Cameron 2010): Clade A, represented by *T. pallens* and *T. fulviventris,* has 1C values 1.5 higher than Clade B, which is represented by *T. hyalinata* and *T. spinipes* (Table 2). The distribution of heterochromatin in half of the *S. quadripunctata* chromosome pairs, the other half is completely euchromatic and smaller, is an intriguing pattern observed only in this species. This case remains a subject for future studies, to determine whether the heterochromatin was differently amplified in half of the chromosomes or whether other rearrangements, such as translocations, were responsible for chromatin reorganization in the karyotype.

A conserved chromosome number is observed inside most of the stingless bee genera, with exceptions in *Leurotrigona, Melipona,* and *Trigona* (reviewed in Tavares *et al.,* 2017). We report another variation inside *Geotrigona* genus. The two colonies of *Geotrigona*

subterranea analyzed in the present study from Passos, MG, and Lontra, MG, have 2*n* = 34 (Table 1), whereas one colony of *Geotrigona mombuca* described from Ribeirão Preto, SP, showed 2*n* = 30 (Rocha *et al.*, 2003). The sampling of other populations from both species, as well as of other *Geotrigona* species, will help determine the extent of this variation.

Another variation observed among species is regarding to the karyotypic formulae that is quite variable, from small differences within a genus or between close genera, such as the predominantly meta-submetacentric karyotype in both *Frieseomelitta* and *Duckeola* (Figure 3), to greater differences among others, like the predominance of metacentric chromosomes in *Cephalotrigona* whereas *Scaptotrigona* has mostly subtelocentrics (Figure 2). These examples highlight the importance of rearrangements that do not change the chromosome number, such as paracentric and pericentric inversions that promote karyotype diversification.

Polymorphisms regarding differences in arm size between homologous chromosomes have been reported only in *Melipona* species (Lopes *et al.*, 2008; Andrade-Souza *et al.*, 2018; Piccoli *et al.*, 2018; Travenzoli *et al.*, 2019b). We report two new cases, the first one also in a *Melipona* species, *M. fulva*, and the second one constituting the first record outside this genus, in *S. quadripunctata*, which represents the largest arm size difference reported. Unequal crossing over seems to be a more plausible explanation for such a difference, whereas slippage would cause only minor size differences between the homologous (Krebs *et al.*, 2017).

Conclusion

In the present study, we associated empirical data on chromosome number and nuclear genome size to highlight the importance of Robertsonian fusion rearrangements leading to the independent decrease of chromosome number and nuclear genome size in several Neotropical Meliponini genera. Until now, *Melipona* species with 2*n* = 22 represent the only evidence of a Robertsonian fission event in the Neotropical clade. We also pointed out the importance of chromosomal rearrangements that did not alter the chromosome number during the evolution of stingless bees, such as inversions and heterochromatin amplification. In addition, the nuclear genome size information could provide support in genome sequencing projects, guiding the necessary coverage for good assemblies, and the chromosome number data can help genome assembly by correlating the number of scaffolds with real chromosome number (Gregory, 2005; Deakin *et al.*, 2019; Blommaert, 2020).

Experimental procedures

Sampling

We collected 38 species belonging to the Meliponini tribe in different Brazilian regions (Table 1). These species are representative of the Neotropical Meliponini groups, which are, (1) *Trigonisca s.l.*, (2) *Melipona s.l.*, and (3) the remaining species (Rasmussen and Cameron 2010). Individuals were identified by Sílvia Regina de Menezes Pedro (Universidade de São Paulo, Ribeirão Preto, Brazil) and Fernando Amaral da Silveira (Universidade Federal de Minas Gerais, Minas Gerais, Brazil) and deposited in the scientific collection of the Apiário Central located at Universidade Federal de Viçosa, Minas Gerais, Brazil.

Few species were collected outside the current distribution range of the taxon; therefore, the "cf" was added until revision or an update can be performed. Another group clearly represents different species but with doubts about the specific epithet; therefore, the "sp." was added, and voucher specimens were sent for further taxonomic investigations.

Chromosome number and karyotype characterization

Thirty-five species were sampled for karyotype characterization, and some of them were cytogenetically described for the first time. Mitotic chromosomes were obtained from the cerebral ganglia of larvae or pre-pupae (Imai *et al.*, 1988). This protocol also allows visualization of the heterochromatin patterns without subsequent C-band treatment (Imai *et al.*, 1988). At least five metaphases of each species were captured using a photomicroscope BX 60 coupled with a Q-Color3 Olympus® image capture system. Chromosome number was determined, and morphometric data were measured using Image-Pro Plus® software. Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a) (Levan *et al.*, 1964). The 18S rDNA probe was performed on *M. fulva* through Fluorescence *in situ* Hybridization (FISH) technique (Pinkel *et al.*, 1986). The probe was labeled by an indirect method using digoxigenin-11-dUTP (Roche Applied Science), and the signal was detected with anti-digoxigenin-rhodamine (Roche Applied Science). Digital images of the fluorescence images were captured in a BX53F Olympus microscope equipped with an MX10 Olympus camera using CellSens imaging software.

Nuclear 1C value measurement

Sixteen species were sampled for 1C DNA content measurement by flow cytometry (FCM). Analyses were carried out using fixed adults from 15 species and fresh larvae from four species. Adaptation of the FCM procedure expands the possibility of measuring the 1C

value from fixed ganglia of insects (Desalle *et al.,* 2005; Gregory, 2005; Ardila-Garcia *et al.,* 2010; Hanrahan and Johnston 2011; Tavares *et al.,* 2012). Adults from 15 species were fixed in absolute ethanol and stored at -20°C for subsequent analyses, of which 12 were analyzed from fixed adults and three were analyzed from both fixed adults and fresh larvae (*Melipona amazonica, Melipona interrupta,* and *Melipona seminigra merrillae*). One species was analyzed using only fresh larvae (*Plebeia* sp.1) (Table 2). The sex of each adult individual was identified before the FCM, mainly because bees have a haplodiploid sex system: haploid males and diploid females.

We used the procedure described by Lopes *et al.* (2009) to prepare the nuclei suspension from the larvae fresh ganglia. For fixed samples, some modifications were performed. One or two ganglia from each sample and the reference standard were extracted and immediately placed in physiologic solution. They were separately placed in a 1.5 mL tube containing 100 µL of modified OTTO I buffer (Otto, 1990) containing double concentration of citric acid (0.2 M) (Otto, 2000) and 50 µg/mL RNAse (Sigma-Aldrich), pH 2.3. The ganglia were crushed 7, 10, 12, or 15 times with a pestle and 500 µL of the modified buffer was added. The suspensions were filtered through a 30 µm nylon mesh (Partec Gmbh®), 500 µL of the modified OTTO I buffer was added, and the suspensions were filtered again through a 30 µm nylon mesh (Partec Gmbh®). The tubes were centrifuged at 1100 rpm for 5 min, and the supernatant was discarded. The pellet was resuspended in 100 µL of modified OTTO I buffer and incubated for 5 min. OTTO II buffer (500 µL, Otto,1990) was added to each tube and supplemented with 75 µM propidium iodide (Sigma-Aldrich) and 50 µg/mL RNAse (Sigma-Aldrich), pH 7.8. The suspensions were filtered through a 20 µm nylon mesh (Partec Gmbh®) and incubated in the dark for at least 40 min.

As recommended for samples with unknown 1C values (Desalle *et al.,* 2005), FCM parameters were defined with the external standard procedure, *i.e.* each sample and standard separately (Doležel and Bartos 2005; Praça-Fontes *et al.,* 2011), such as the gain and the channel of the G_0/G_1 nuclei of each sample and of the standard. Based on this, an internal standard procedure was accomplished from nuclei suspensions prepared from simultaneous crushing and staining of each sample with the reference standard (Lopes *et al.,* 2009; Praça-Fontes *et al.,* 2011; Tavares *et al.,* 2012). Both external and internal standard procedures were performed on all samples. *Scaptotrigona xanthotricha* was used as the standard for most samples, and *Melipona fasciculata* was used for *Paratrigona* sp. and *Frieseomelitta* sp.1.

The nuclei suspensions were analyzed using a BD Accuri C6 flow cytometer (Accuri cytometers, Belgium) equipped with a 488 nm laser source. FL2 (585/640) and FL3 (670 LP)

filters were used to detect the fluorescence emitted by propidium iodide. The BD Csampler software (Accuri cytometers, Belgium) was used for histogram analyses to determine the nuclei count, G_0/G_1 peak channel, and the respective coefficient of variation (CV). The histograms were considered adequate with CV below 5% for G_0/G_1 peak and at least 5,000 nuclei. 1C value of each Meliponini species was measured according to the formula: (G_0/G_1 peak channel of the Meliponini species × 1C value in pg of the standard)/G_0/G_1 peak channel of the standard. Two to five individuals of each species were used for 1C value measurement (Table 2). We used a standard Student's *t*-test at 5% significance to determine the significant differences between the results from fixed and fresh ganglia as well as between external and internal FCM procedures. The correlation coefficient (r) was also calculated. These statistical analyses were performed using PAST 3.25 software (Hammer *et al.*, 2001).

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. Neotropical Meliponini sampling across different Brazilian regions. 2*n* chromosome number and karyotypic formula are shown. The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a). Species were assigned to groups based on the phylogeny proposed by Rasmussen and Cameron (2010).

Group	Species	Locality	2*n* chromosome number	Karyotypic formula
1	*Leurotrigona muelleri*	Passos, Minas Gerais	16	14m+ 2st
	Celetrigona longicornis	Nova Xavantina, Mato Grosso	30	18m+ 12sm
	Trigonisca sp.	Urbano Santos, Maranhão	30	4m+ 8sm+ 18st
2	*Melipona* sp.	Brasília, Distrito Federal	18	10m+ 6sm+ 2st
	Melipona quinquefasciata	Piumhi, Minas Gerais	18 + 3B [#]	8m+ 6sm+ 4st
	Melipona interrupta	Iranduba, Amazonas	18	-
	Melipona fasciculata	São Luís, Maranhão	18	-
	Melipona amazonica	Iranduba, Amazonas	18	10m+ 6sm+ 2st
	Melipona cf. *rufiventris*	Iranduba, Amazonas	18	-
	Melipona fulva	Presidente Figueiredo, Amazonas	18	-
	Melipona scutellaris	Nordeste	18	-
	Melipona lateralis	Presidente Figueiredo, Amazonas	22	-
	Melipona seminigra merrillae	Iranduba, Amazonas	22	-
3	*Paratrigona lineata*	Passos, Minas Gerais	-	-
	Paratrigona sp.	Rio Paranaíba, Minas Gerais	-	-
	Scaptotrigona sp.	Pará	34	10m+ 6sm+ 18st
	Scaptotrigona cf. *polysticta*	Presidente Figueiredo, Amazonas	34	8m+ 6sm+ 20st
	Scaptotrigona xanthotricha	Viçosa, Minas Gerais	34	10m+ 12sm+ 12st

Geotrigona subterranea	Passos, Minas Gerais; Lontra, Minas Gerais	34	14m+ 10sm+ 10st
Cephalotrigona capitata	Viçosa, Minas Gerais	34	30m+ 4a
Cephalotrigona femorata	Urbano Santos, Maranhão	34	24m+ 6sm+ 4st
Trigona hyalinata	Viçosa, Minas Gerais	34	26m+ 8sm
Trigona recursa	Januária, Minas Gerais	34	24m+ 8sm+ 2st
Frieseomelitta languida	Arcos, Minas Gerais	30	14m+ 14sm+ 2st
Frieseomelitta sp.1	Presidente Figueiredo, Amazonas	30	20m+ 10sm
Frieseomelitta sp.2	Iranduba, Amazonas	30	20m+ 10sm
Frieseomelitta sp.	Brasília, Distrito Federal	30	18m+ 10sm+ 2st
Frieseomelitta varia	Uberlândia, Minas Gerais	30	16m+ 12sm+ 2st
Duckeola ghilianii	Presidente Figueiredo, Amazonas	30	14m+ 12sm+ 4st
Lestrimelitta limao	Brazil	28	18m+ 10sm
Lestrimelitta sp.	Domingos Martins, Espírito Santo	28	18m+ 10sm
Lestrimelitta sulina	Lavras, Minas Gerais	-	-
Plebeia droryana	Santo Antônio do Jacinto, Minas Gerais	34	18m+ 16sm
Plebeia lucii	Viçosa, Minas Gerais	34	6m+ 6sm+ 22st
Plebeia sp.1	Presidente Figueiredo, Amazonas	34	6m+ 2sm+ 26st
Nannotrigona punctata	Altamira, Pará	34	12m+ 6sm+ 16st
Nannotrigona testaceicornis	Viçosa, Minas Gerais	34	10m+ 6sm+ 18st
Schwarziana quadripunctata	Viçosa, Minas Gerais	34	14m+ 8sm+ 12st

[#] B chromosomes were found in *Melipona quinquefasciata* (2 or 3).

Table 2. Mean haploid genome size estimates (1C value) available for Neotropical Meliponini. The values are shown in picograms (pg) and standard error (SE, whenever available). Number of fixed and fresh analyzed individuals are shown separately. Species were assigned to groups based on the phylogeny proposed by Rasmussen and Cameron (2010)

Group	Species	1C value (pg) ± SE	Number of fixed individuals	Number of fresh individuals	References
1	*Leurotrigona muelleri*	0.35 ± 0.057	3	0	a
	Leurotrigona muelleri	0.32 ± 0.003	0	3	b
	Celetrigona longicornis	0.49 ± 0.004	0	3	b
2	*Melipona (Melipona) subnitida*	0.27	0	3	c
	Melipona (Melipona) quadrifasciata	0.27 ± 0.002	0	3	b, c
	Melipona (Melipona) mandacaia	0.35 ± 0.004	0	3	b, c
2	*Melipona (Melikerria) quinquefasciata*	0.70 ± 0.011	0	3	b, c
	Melipona (Melikerria) interrupta	0.80 ± 0.057	3	2	a
	Melipona (Melikerria) fasciculata	0.82 ± 0.005	0	3	b
	Melipona (Melikerria) fasciculata	0.84 ± 0.076	3	0	a
	Melipona (Melikerria) grandis	0.95	0	3	c
2	*Melipona (Eomelipona) marginata*	0.28	0	3	c
	Melipona (Eomelipona) bicolor	0.28 ± 0.004	0	3	b, c
	Melipona (Eomelipona) asilvai	0.29 ± 0.003	0	3	b, c
	Melipona	0.31 ± 0.093	3	2	a

	(Eomelipona) amazonica				
2	*Melipona (Michmelia) crinita*	0.73	0	3	c
	Melipona (Michmelia) lateralis	0.87 ± 0.094	3	0	a
	Melipona (Michmelia) seminigra merrillae	0.92 ± 0.031	2	2	a
	Melipona (Michmelia) rufiventris	0.93	0	x	d
	Melipona (Michmelia) mondury	0.95	0	x	d
	Melipona (Michmelia) flavolineata	0.98 ± 0.023	0	3	b
	Melipona (Michmelia) scutellaris	1.08	0	3	c
	Melipona (Michmelia) fuscopilosa	1.10	0	3	c
	Melipona (Michmelia) eburnea	1.11	0	3	c
	Melipona (Michmelia) capixaba	1.38	0	3	c
3	*Paratrigona lineata*	0.49 ± 0.045	3	0	a
	Paratrigona sp.	0.44 ± 0.090	2	0	a
	Partamona helleri	0.55 ± 0.006	0	3	b
	Partamona rustica	0.59 ± 0.010	0	3	b
	Partamona chapadicola	0.63 ± 0.027	0	3	b
3	*Scaptotrigona xanthotricha*	0.44	0	x	d
	Scaptotrigona depilis	0.41 ± 0.008	0	3	b
	Scaptotrigona bipunctata	0.44 ± 0.005	0	3	b

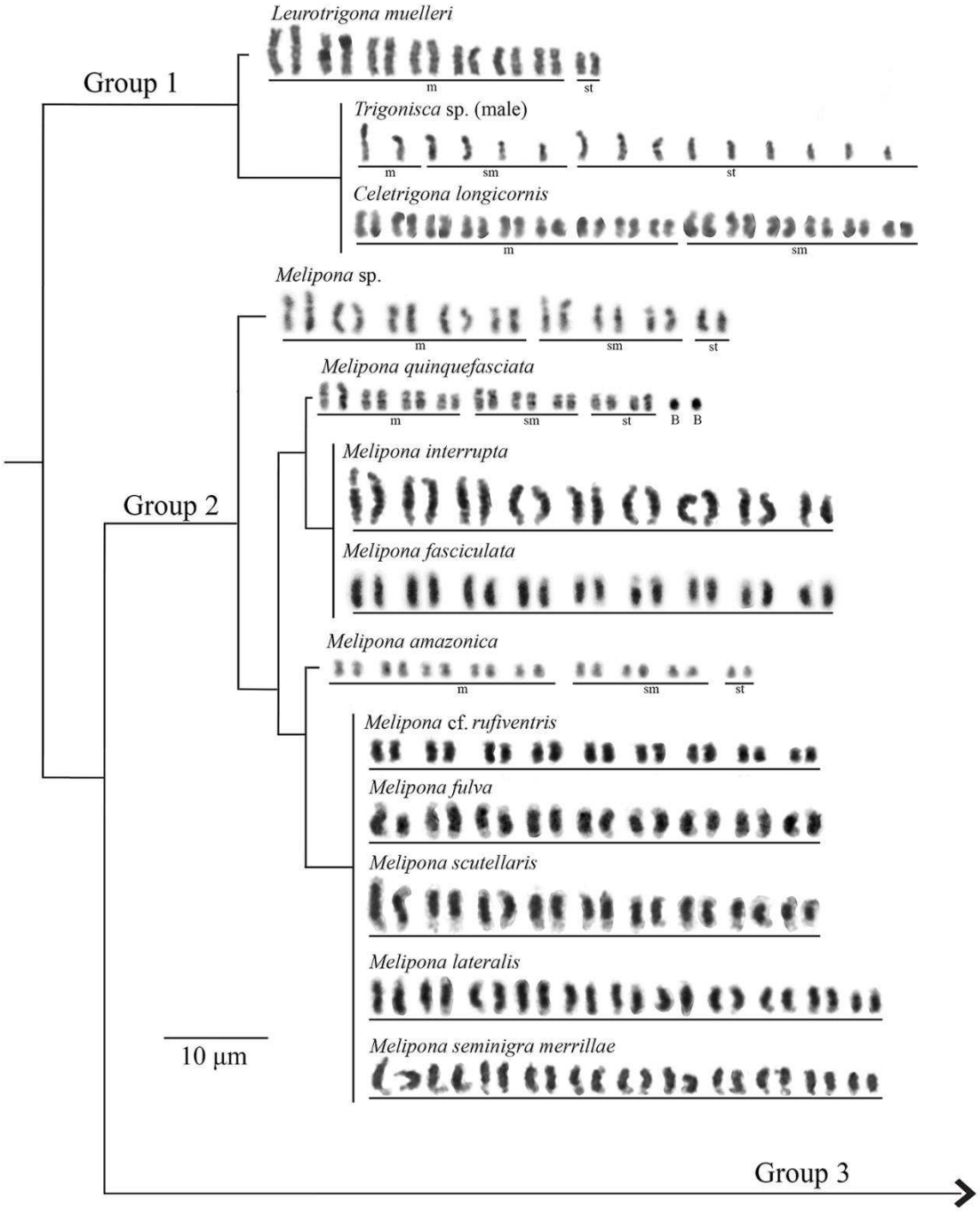
	Scaptotrigona tubiba	0.45 ± 0.006	0	3	b
	Scaptotrigona cf. *polysticta*	0.52 ± 0.099	3	0	a
	Geotrigona subterranea	0.83 ± 0.017	3	0	a
	Cephalotrigona sp.	0.55 ± 0.007	0	3	b
	Trigona spinipes	0.44 ± 0.010	0	3	b
	Trigona hyalinata	0.53 ± 0.005	3	0	a
	Trigona fulviventris	0.70 ± 0.013	0	3	b
	Trigona pallens	0.81 ± 0.027	0	3	b
3	*Tetragonisca angustula*	0.90 ± 0.015	0	3	b
	Frieseomelitta varia	0.48 ± 0.004	0	3	b
	Frieseomelitta sp.1	0.54 ± 0.041	3	0	a
	Duckeola ghilianii	0.48 ± 0.064	4	0	a
3	*Lestrimelitta sulina*	0.53 ± 0.113	3	0	a
	Plebeia sp.1	0.40 ± 0.00	0	2	a
	Plebeia lucii	0.43 ± 0.003	0	3	b
	Plebeia droryana	0.52 ± 0.007	0	3	b
	Nannotrigona testaceicornis	0.53 ± 0.009	0	3	b
	Schwarziana sp.	0.65 ± 0.008	0	3	b
	Schwarziana quadripunctata	0.67 ± 0.078	3	0	a

x = number of individuals not specified.

References: a Present Study; b Tavares *et al.*, 2012; c Tavares *et al.*, 2010; d Lopes *et al.*, 2009

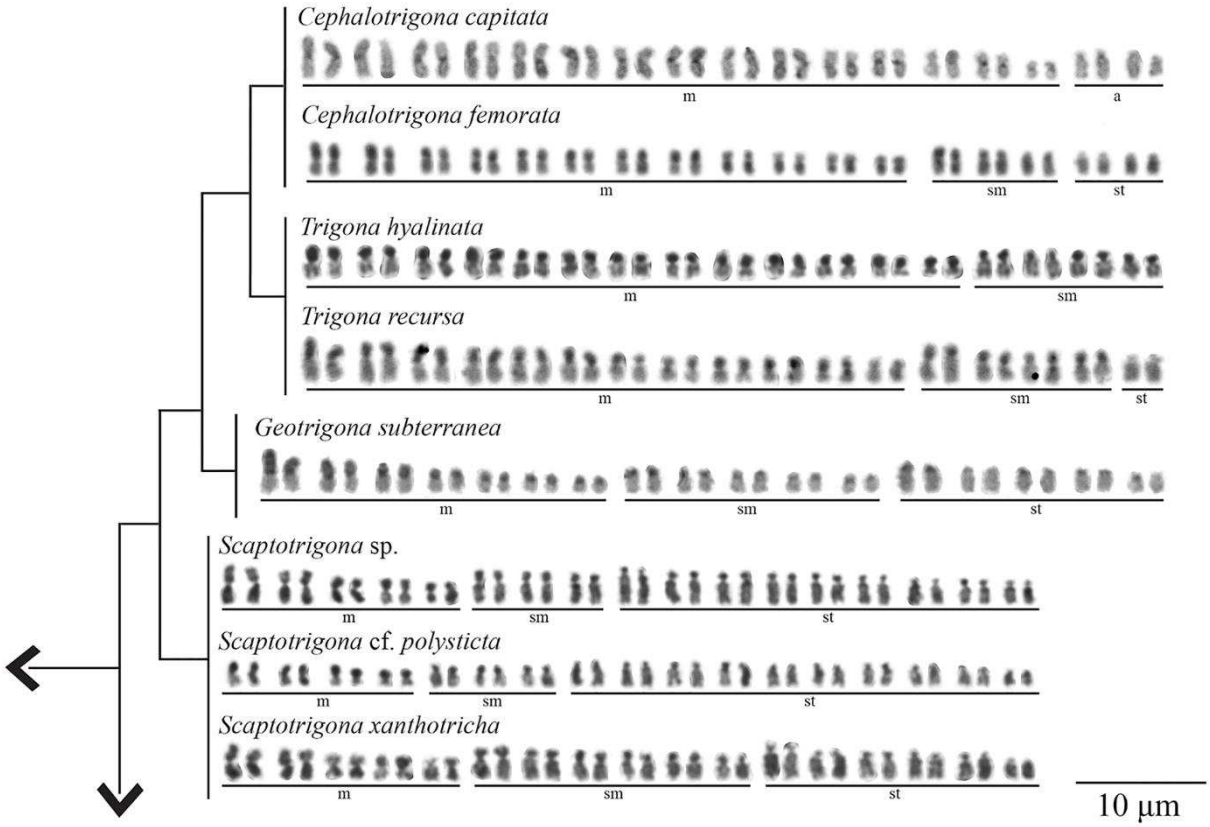
Figures

Figure 1. Karyotype of the Neotropical Meliponini species (part 1 of 3). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a). The clades are drawn based on the Rasmussen and Cameron (2010, Figure 3) phylogenetic Bayesian tree. Bar 10 µm



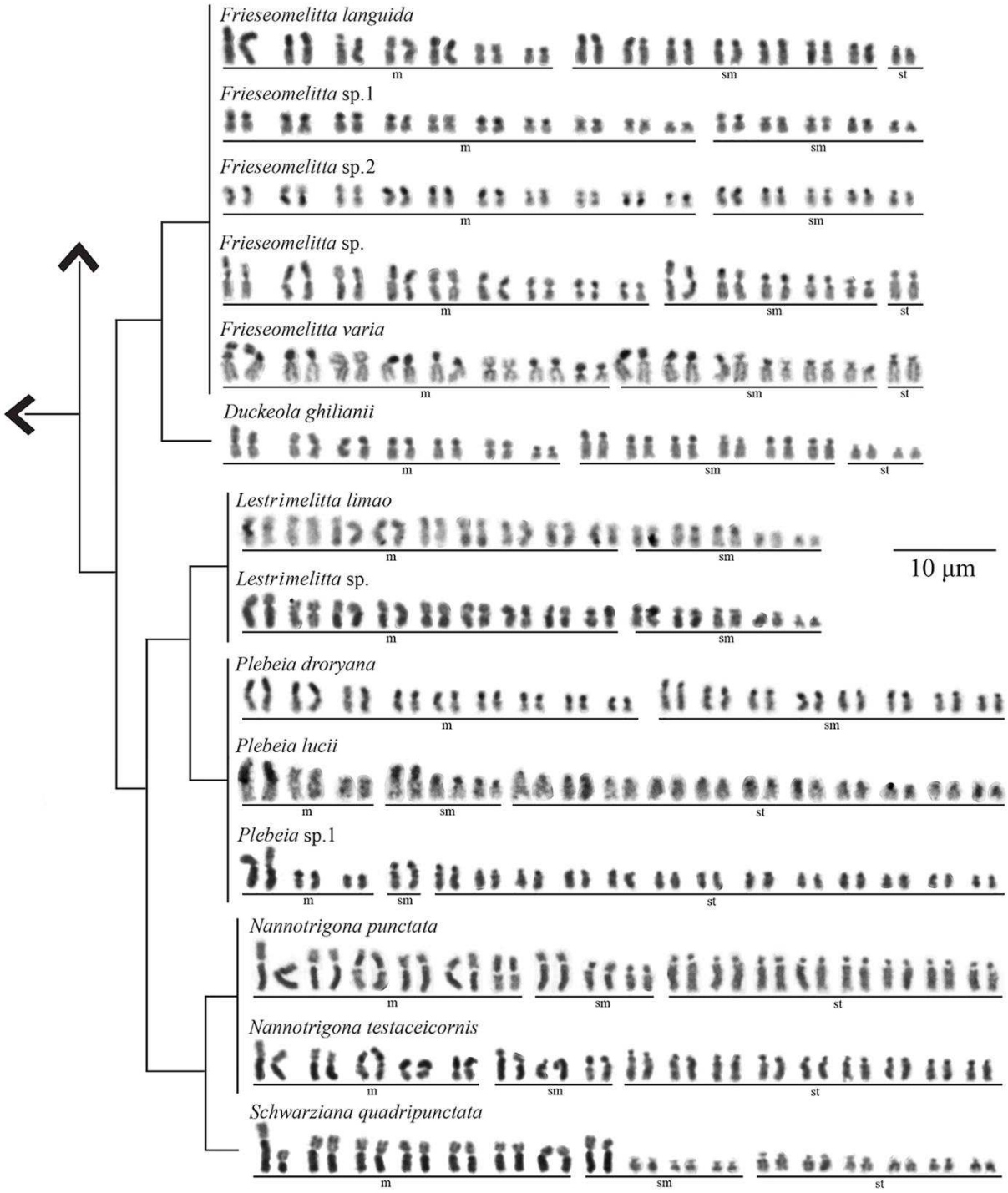
Fonte: Autoral.

Figure 2. Karyotype of the Neotropical Meliponini species (part 2 of 3). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a). The clades are drawn based on the Rasmussen and Cameron (2010, Figure 3) phylogenetic Bayesian tree. Bar 10 µm



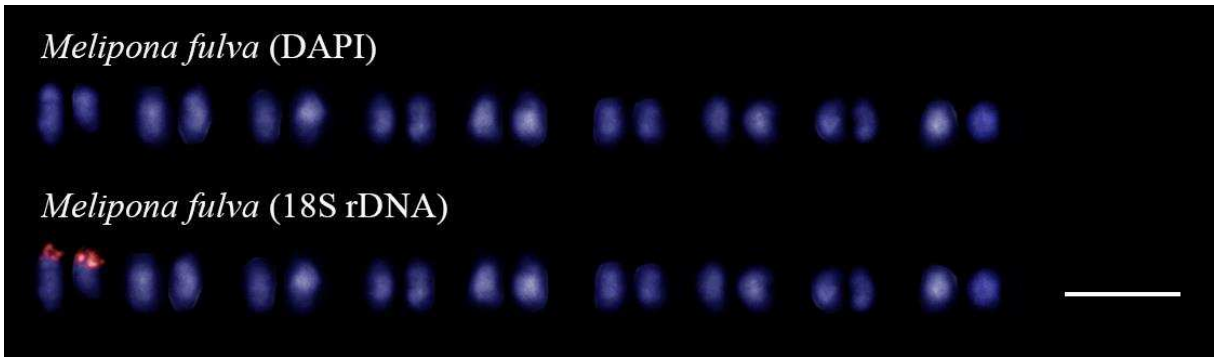
Fonte: Autoral.

Figure 3. Karyotype of the Neotropical Meliponini species (part 3 of 3). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a). The clades are drawn based on the Rasmussen and Cameron (2010, Figure 3) phylogenetic Bayesian tree. Bar 10 µm



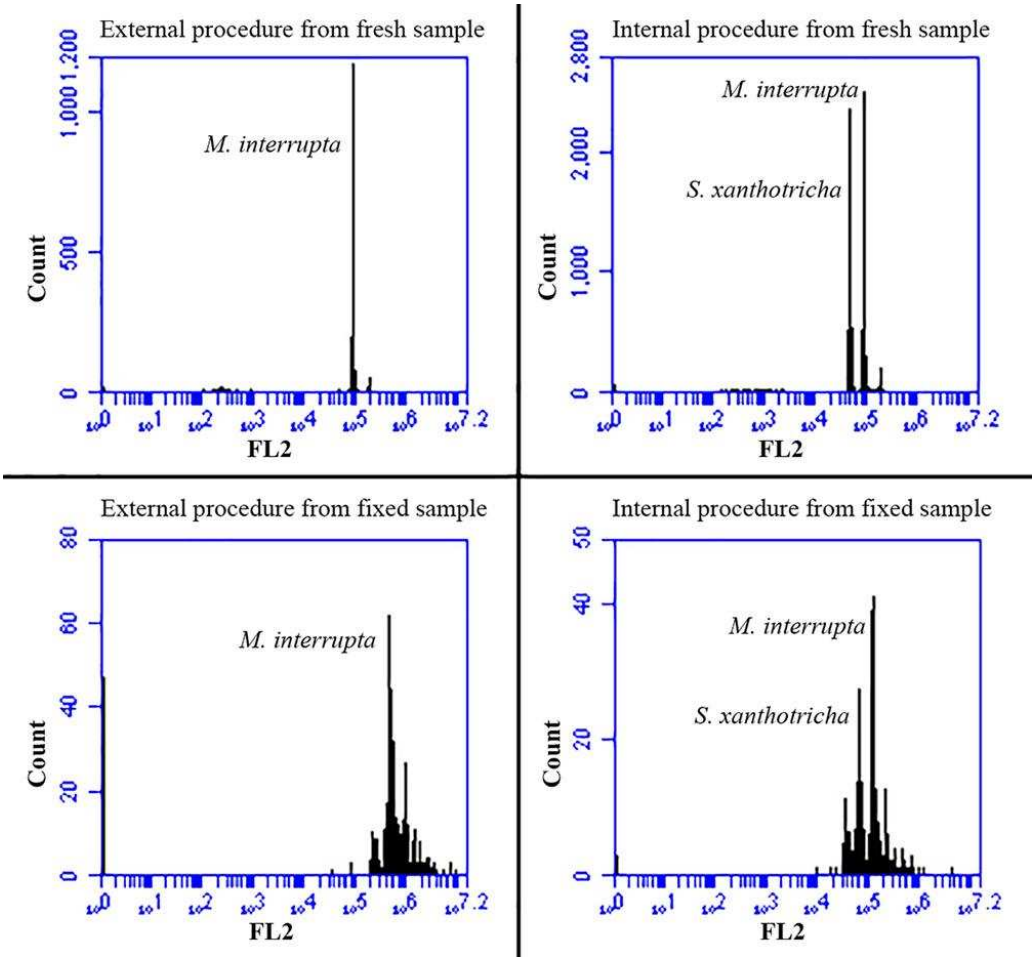
Fonte: Autoral.

Figure 4. Fluorescence *in situ* hybridization of the 18S rDNA probe in *Melipona fulva.* Bar 5 µm



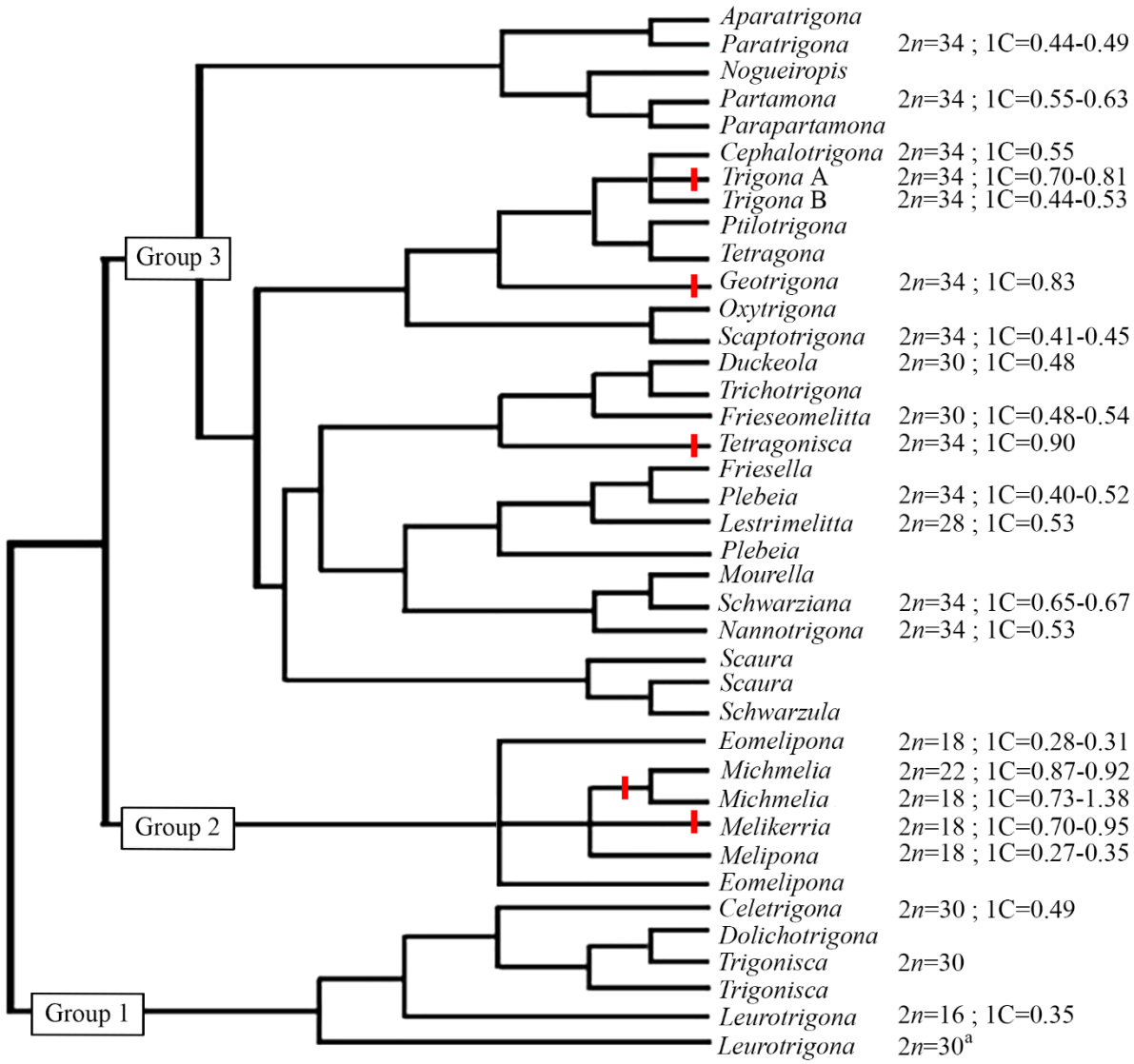
Fonte: Autoral.

Figure 5. Genome size DNA-histograms of *Melipona interrupta* by external and internal procedures showing the results from fresh and fixed samples. *Scaptotrigona xanthotricha* was used as standard in the internal procedures. FL2 – flow cytometry filter to detect propidium iodide fluorescence (585 – 640 nm)



Fonte: Autoral.

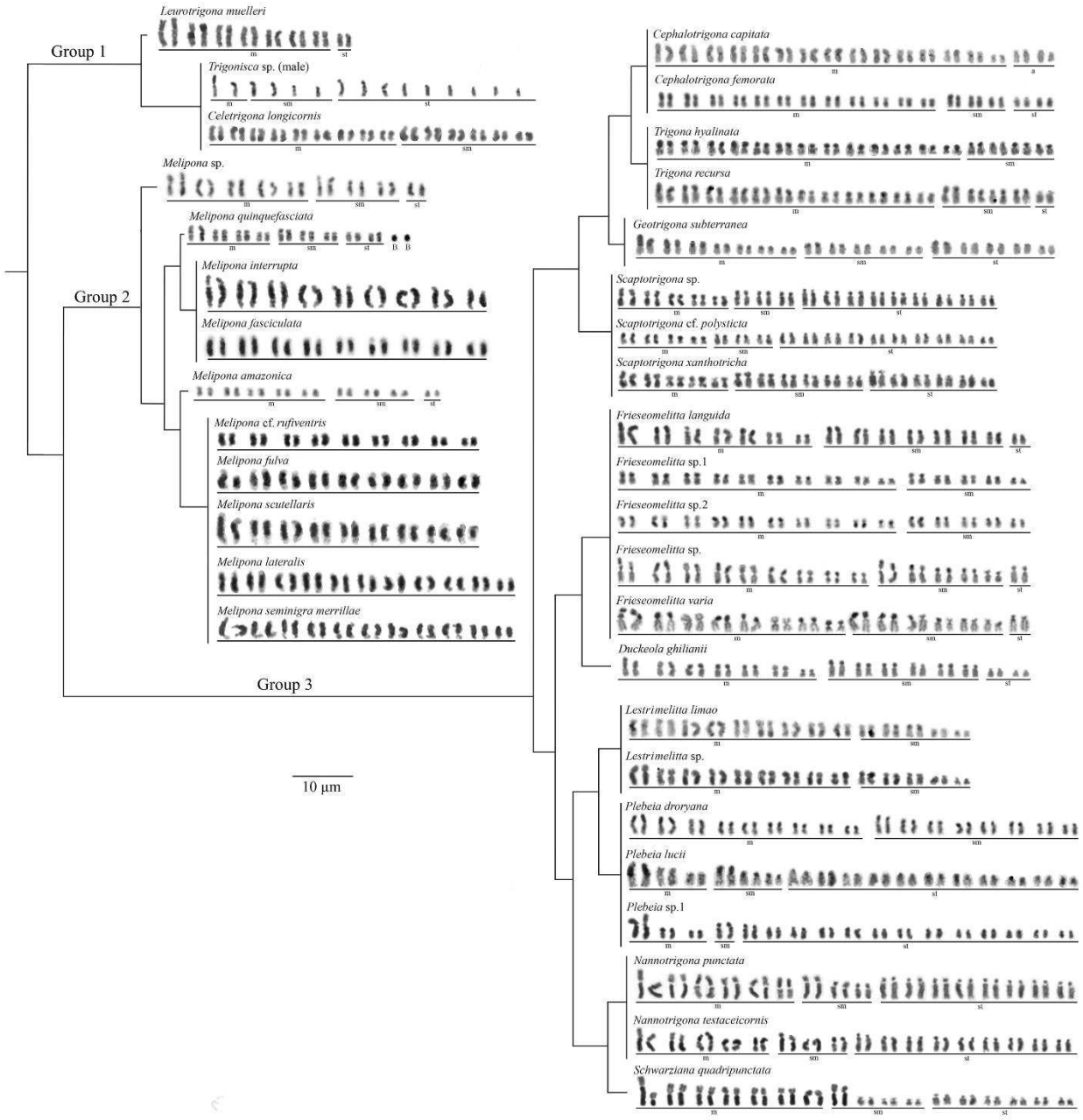
Figure 6. Redrawing of the Neotropical Meliponini phylogenetic Bayesian tree modified from Rasmussen and Cameron (2010, Figure 3). Diploid number (2*n*) and nuclear genome size variation (1C) were included in front of each genera. References of 1C values are shown in Table 2. The red marks show the presence of species with 1C value at least 50% higher than its phylogenetic close relatives. [a] Pompolo and Campos (1995)



Fonte: Autoral.

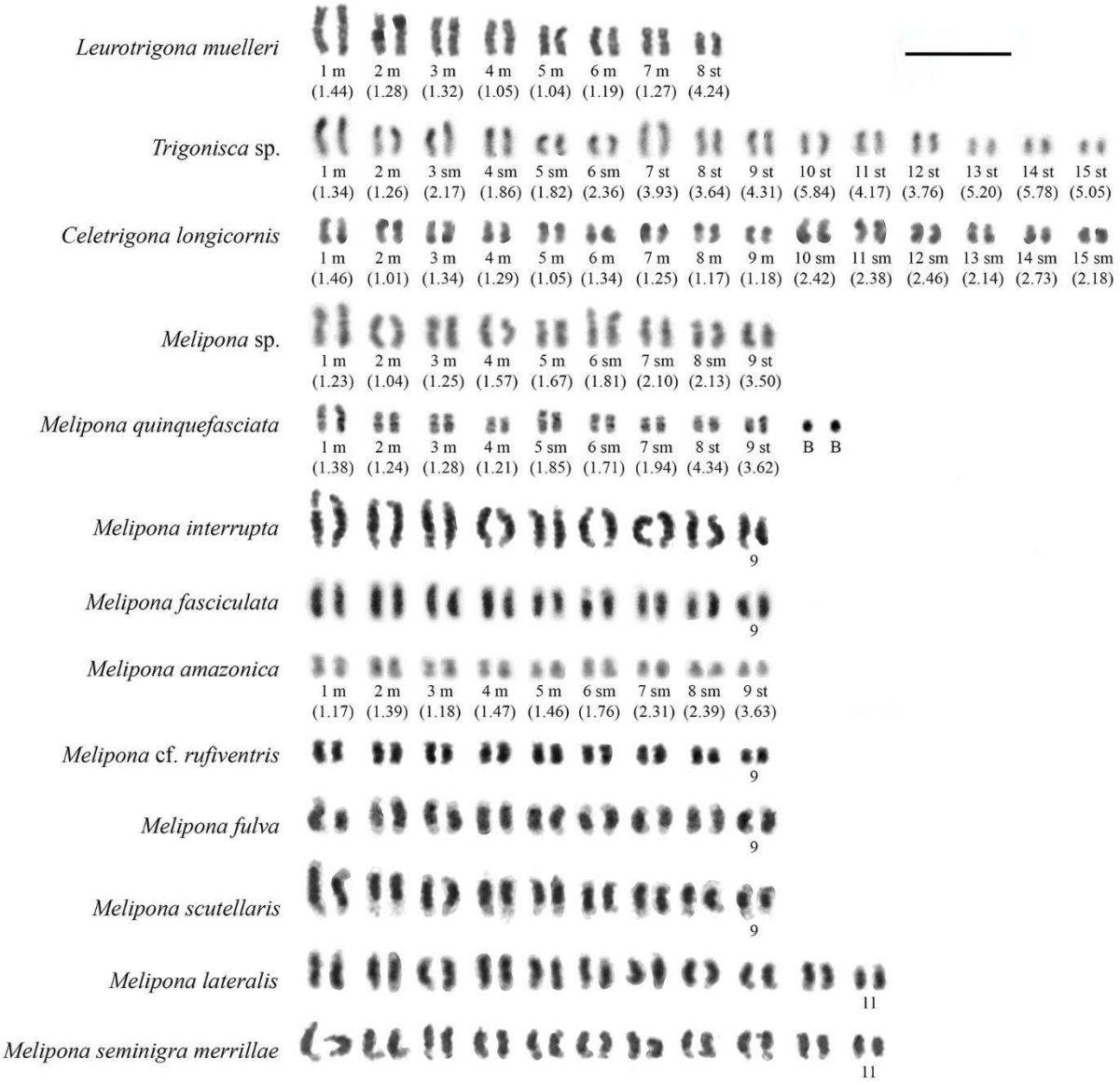
Supporting Information

Figure S1. Karyotype of the Neotropical Meliponini species. Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a). The clades are drawn based on the Rasmussen and Cameron (2010, figure 3) phylogenetic Bayesian tree. Bar 10 µm



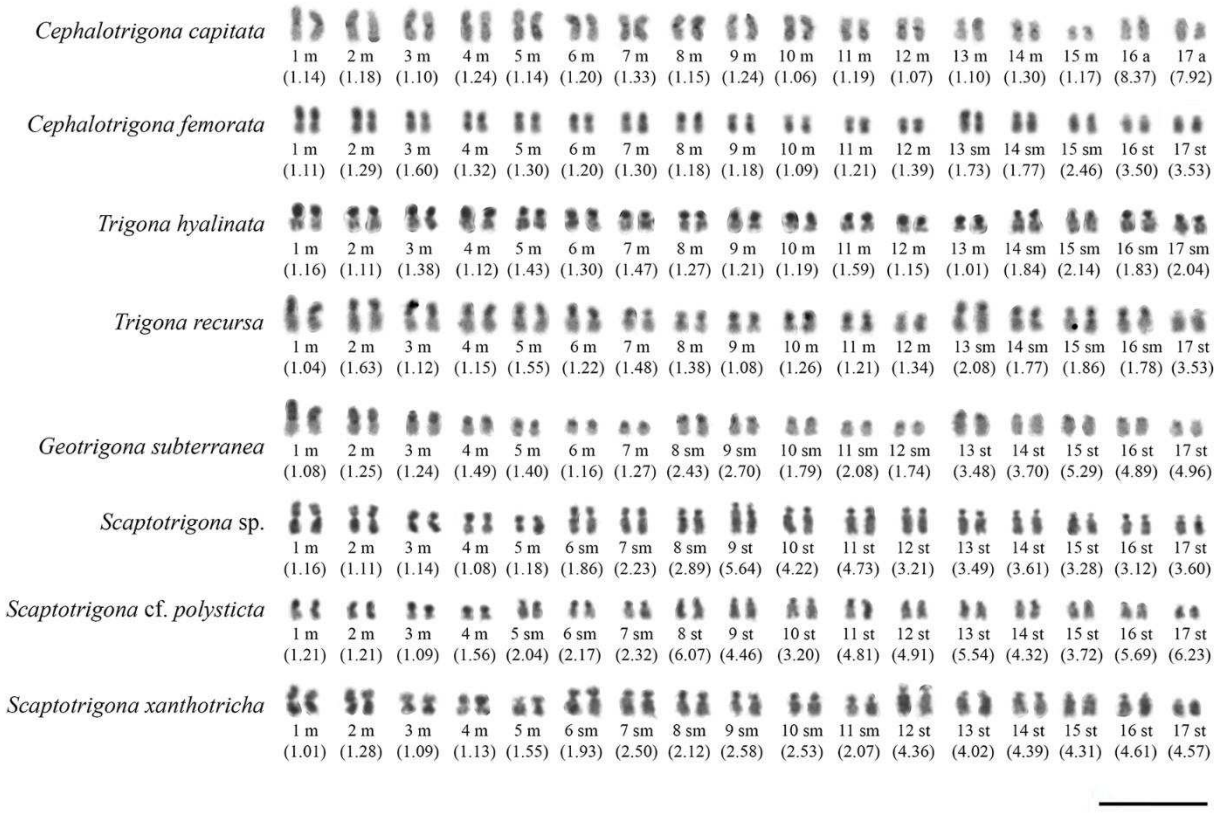
Fonte: Autoral.

Figure S2. Karyotype arm ratio measurements of each analyzed Neotropical Meliponini species (part 1 of 3). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a). Bar 10 µm



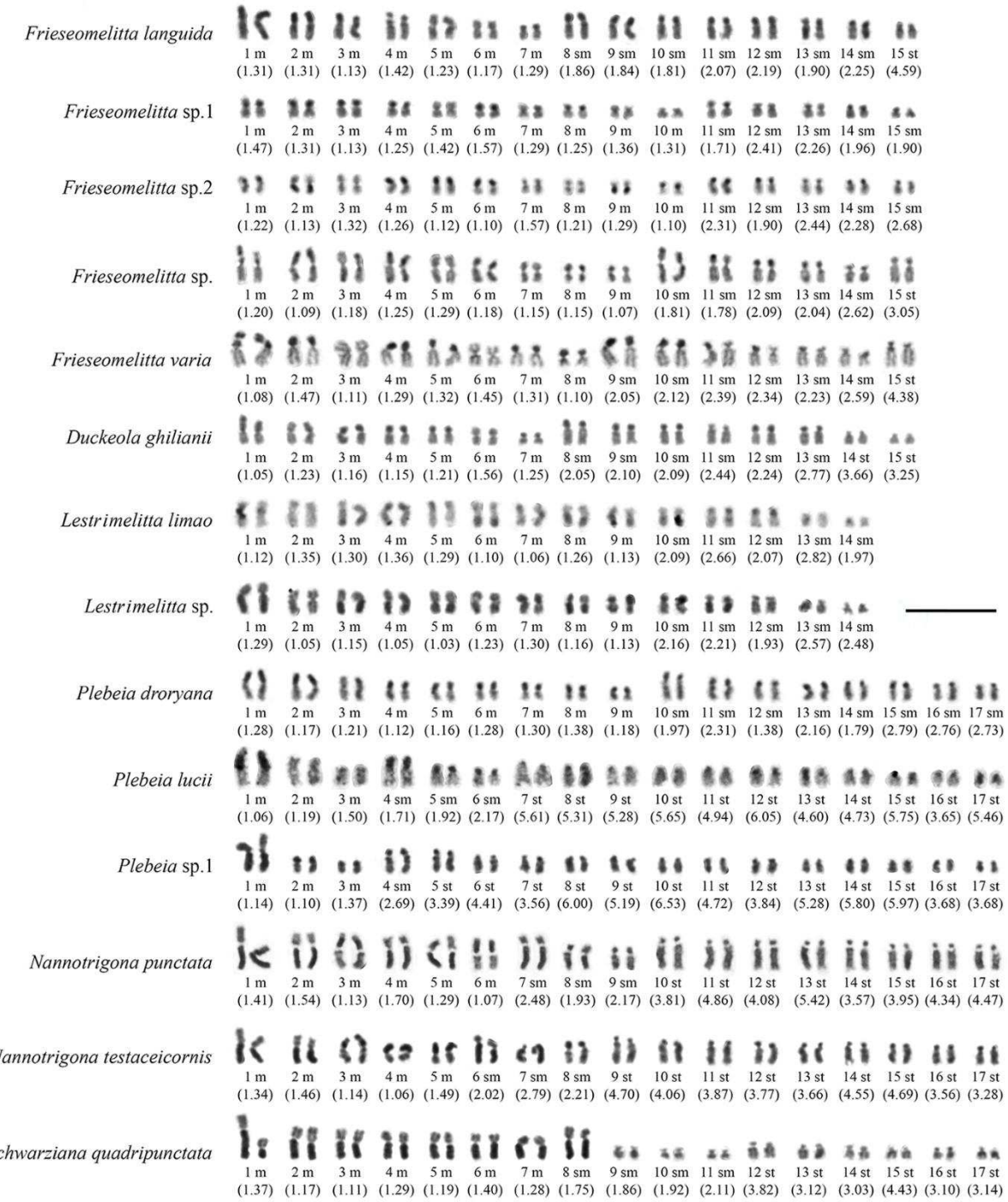
Fonte: Autoral.

Figure S3. Karyotype arm ratio measurements of each analyzed Neotropical Meliponini species (part 2 of 3). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a). Bar 10 µm



Fonte: Autoral.

Figure S4. Karyotype arm ratio measurements of each analyzed Neotropical Meliponini species (part 3 of 3). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a). Bar 10 µm



Fonte: Autoral.

6 CAPÍTULO 4

Microsatellite FISH patterns as a useful tool to understand the karyotypic evolution in Neotropical Meliponini (Hymenoptera: Apidae)

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Running title: Karyotypic evolution in Neotropical Meliponini

Article to be submitted.

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Abstract

Commonly known as stingless bees, the Neotropical Meliponini bees are phylogenetically subdivided in three clades, in which the chromosome numbers vary from *n*=8 to *n*=17. The goal of this study was to identify the major chromosomal rearrangements that occurred during the Neotropical Meliponini (Apidae) karyotypic evolution. 18S rDNA and five microsatellites were mapped on 33 species collected from different Brazilian regions. The 18S rDNA probe showed a variation from 2 to 12 marked chromosomes in different positions (terminal, subterminal or centromeric), including 2B chromosomes out of the 7B found in *Tetragonisca fiebrigi*. The microsatellite $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(TCAGG)_6$ probes formed clusters restricted to the euchromatic regions of the chromosomes, and were used to infer the Robertsonian fusion events that led to the decrease in the chromosome number during the evolution of the Neotropical Meliponini clade. $(TTAGG)_6$ constitute the telomeric sequence of the analyzed bees. The increase in the number of 18S sites could be explained due to ectopic recombination mediated or not by transposable elements. While the ancestral state of the three Neotropical clades is hard to infer, the putative ancestral karyotype probably had a single pair of 18S rDNA cistrons and was *n*=15 in the separation of clade 1, and *n*=17 in the split of clades 2 and 3. The decrease in the chromosome number and the increase in the 18S rDNA sites occurred independently between genera.

Keywords: 18S rDNA, chromosome evolution, cytotaxonomy, microsatellites, molecular cytogenetics, stingless bees.

Introduction

In the last decades, the molecular biology has flourished with new technologies contributing to advances in many areas, like the study of animal cytogenetics in cytotaxonomy (*e.g.* Barth et al. 2011; Palacios-Gimenez et al. 2015a; Santos et al. 2018), origin of different sex chromosome systems (*e.g.* Palacios-Gimenez et al. 2013, 2015b), and the evolution of supernumerary B chromosomes (*e.g.* Milani and Cabral-de-Mello 2014; Ruiz-Ruano et al. 2015; Milani et al. 2017). Thereby, the field of cytogenetics is of great importance in studies associated with genetic structure, phylogeny, and evolution (reviewed in Gokhman and Kuznetsova 2006).

Fluorescence *in situ* hybridization (FISH) has become one of the most important techniques in cytogenetics, allowing the localization of specific DNA sequences on the chromosomes (Trask 1991; Guerra 2004). In insects, the most common studied sequences are ribossomal DNA and microsatellite/satellite sequences (Huang et al. 2016; Ruiz-Ruano et al. 2017; Andrade-Souza et al. 2018; Menezes et al. 2019; Tranvezoli et al. 2019a; Teixeira et al. 2020; Pereira et al. 2020, 2021). Regardless of their coding function, theses sequences are important in the structure, regulation and evolutive adaptation of the organism genome (Shapiro and Von Sternberg 2005; Oliveira et al. 2006).

The microsatellite $(TTAGG)_n$ is considered the ancestral telomeric sequence of the class Insecta, though it was independently lost in several insect families (reviewed in Kuznetsova et al. 2020). In Hymenoptera, although the telomeric sequence in wasps are still unknown (Gokhman et al. 2014; Menezes et al. 2017), the canonic $(TTAGG)_n$ was already identified in several ant species (Meyne et al. 1990; Lorite et al. 2002; Pereira et al. 2018; Micolino et al. 2019a, 2020; Castro et al. 2020) and in a few bee species (Sahara et al. 1999; Travenzoli et al. 2019a).

The bees belonging to the Meliponini tribe (Apidae) arose around 80 million years ago, being restricted to tropical/subtropical areas of the globe. Three main clades compose this tribe: Afrotropical, Indo-Malay/Australasian and Neotropical, the latter being the most recent with around 30-40 million years (Rasmussen and Cameron 2010). Commonly known as stingless bees, these insects have great ecological and economical importance in the pollination of flowering plants, as well as in the production of honey (Heard 1999; Cortopassi-Laurino et al. 2006). Although 417 Meliponini species have been formally described in the Neotropical region (Camargo and Pedro 2013), this number is considered sub-estimated due to the presence of cryptic speciation and the lack of systematical taxonomic reviews in this group (Michener 2007).

The cytogenetic data available on Neotropical stingless bees show a chromosome number variation ranging from *n* = 8 to *n* = 17 (reviewed in Cunha et al. 2021). The Minimum Interaction Theory (MIT), initially proposed to explain ant karyotype evolution, predicts an ancestral with a low-numbered karyotype (n < 12) and, through a series of fission events, the chromosome number increased during evolutionary time (Imai et al. 1986; 1988). Based on certain heterochromatic patterns, it became the most acceptable theory to explain karyotypic variation in the whole order Hymenoptera (Hoshiba and Imai 1993; Pompolo and Campos 1995; Rocha et al. 2003; Godoy et al. 2013; Santos et al. 2018). However, recent data has pointed to an alternative direction, which from a high-numbered ancestral karyotype of *n* = 18 for the Meliponini tribe and *n* = 17 for the Neotropical clade, Robertsonian fusion events led to a decrease in chromosome number during the evolution of the stingless bees (Tranvezoli et al. 2019b). Additionally, high chromosome numbers are found in phylogenetically close tribes, *n* = 16 in Apini, *n* = 15-21 in Euglossini, and *n* = 12-26 in Bombini, as well as in other Meliponini branches, *n* = 14-18 in Meliponini Afrotropical and *n* = 18-20 in Meliponini Indo-Malay/Australasian (reviewed in Cunha et al. 2021).

With the popularization of the molecular cytogenetics, the data available for a few bee species (Rocha et al. 2002; Brito et al. 2005) has been growing in the last years, with the characterization of the chromosomic location of 18S ribossomal sites (Andrade-Souza et al. 2018; Cunha et al. 2018; Silva et al. 2018; Gonçalves et al. 2020; Pereira et al. 2021) and of distinct microsatellite sequences (Piccoli et al. 2018; Santos et al. 2018; Travenzoli et al. 2019a; Lopes et al. 2020). Given the importance of cytogenetics in highlighting the rearrangements involved in chromosomal changes and karyotype evolution of the species (Cristiano et al. 2013; Huang et al. 2016; Aguiar et al. 2017; Cardoso et al. 2018; Micolino et al. 2019b), the goal of this study was to identify and discuss the major chromosomal rearrangements that occurred during the Neotropical Meliponini karyotypic evolution.

Materials and Methods

Thirty-three species were collected from different Brazilian regions encompassing the three main clades of Neotropical Meliponini (Rasmussen and Cameron 2010): (1) *Trigonisca s.l.*, (2) *Melipona s.l.*, and (3) remaining Neotropical species (Table 1). The individuals were identified by Dr Sílvia Regina de Menezes Pedro (Universidade de São Paulo, Ribeirão Preto, Brazil) or by Dr Fernando Amaral da Silveira (Universidade Federal de Minas Gerais, Minas Gerais, Brazil) and deposited in the scientific collection of the Entomology Museum in the Universidade Federal de Viçosa, Minas Gerais, Brazil. Mitotic chromosomes were obtained

from cerebral ganglia of larvae or pre-pupae (Imai et al. 1988). The chromosomes were measured in the Image-Pro Plus® software and classified regarding to their arm ratios in metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a) (Levan et al. 1964).

The Fluorescence *in situ* Hybridization (FISH) technique followed Pinkel et al. (1986) using six repetitive DNA sequences as probes, the ribossomal 18S gene and five microsatellites. The 18S rDNA probe was obtained through the polymerase chain reaction (PCR) using the following primers: F 5'-TAATTCCAGCTCCAATAG-3' e R 5'-CCACCCATAGAATCAAGA-3'. The product was labeled with digoxigenin-11-dUTP (Roche Applied Science) and the signal was detected with anti-digoxigenin-rhodamine (Roche Applied Science). The microsatellite $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$, and $(TTAGG)_6$ probes were synthesized and labeled with Cy3 fluorochrome at the 5' end by Sigma (St. Louis, MO, USA). Digital images of the metaphases were obtained with the photomicroscope BX 53F Olympus using MX10 Olympus camera and CellSens Imaging software.

At least ten metaphases of each species were used to determine the FISH patterns. The idiogram of the karyotypes was done using Easy Idio software (Diniz and Melo 2006) and plotted in the phylogenetic tree proposed by Rasmussen and Cameron (2010).

Results

Chromosome numbers ranged from $n = 8$ to $n = 17$. The haploid numbers (n) and the number and location of the 18S rDNA sites of the 33 species are listed in Table 1. The 18S rDNA probe showed a variation from 2 to 12 marked chromosomes in different positions (terminal, subterminal or centromeric) mostly on the short arms, only *Tetragonisca fiebrigi* and *Schwarziana quadripunctata* had markings on the long arm in some chromosome pairs. Heteromorphims regarding size or position of this region between homologous chromosomes were observed in *Trigonisca* sp. (pair 6), *Melipona quinquefasciata* (pair 1), *Trigona recursa* (pair 14), *Duckeola ghilianii* (pair 1), *Frieseomelitta* sp. (pair 1), *Frieseomelitta* sp.1 (pair 1), *Plebeia lucii* (pair 1), *Plebeia phrynostoma* (pair 11), and *Nannotrigona testaceicornis* (pair 3). Polymorphisms outside the 18S region were also observed, such as in *Melipona fulva* (pair 1), *Scaptotrigona* sp. (pair 4), *Geotrigona subterranea* (pair 1), *Nannotrigona testaceicornis* (pair 7), and *S. quadripunctata* (pair 1). Representative species of this variability are shown in Fig. 1.

The microsatellite $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(TCAGG)_6$ probes formed clusters restricted to the euchromatic region of the chromosomes, although complete euchromatic chromosomes were not entirely marked (Figs. 2-5). All chromosomes had some region marked with these probes and they were marked on one chromosome arm or on both arms depending on the analyzed species. Microsatellite markings in only one chromosome arm was observed in the species with *n* = 15 from clade 1 and in all species with *n* = 17 from clade 3. Microsatellite markings in one arm of all chromosomes and additionally in the other arm was observed in the following species: *Leurotrigona muelleri* (both arms were marked in 7 out of the 8 chromosomes), *Melipona lateralis* and *Melipona seminigra pernigra* (6 out of the 11 chromosomes), remaining *Melipona* species (8 out of the 9 chromosomes), *Lestrimelitta* spp. (3 out of the 14 chromosomes), *Duckeola ghilianii*, *Frieseomelitta languida*, *Frieseomelitta* sp.1, and *Frieseomelitta* sp.2 (2 out of the 15 chromosomes). Some species also had markings in the 18S rDNA region with some of the microsatellites.

Exceptionally, *Frieseomelitta* sp. and *Frieseomelitta varia* (both with *n* = 15) showed microsatellite patterns differently from the other *Frieseomelitta*, with different microsatellite markings from each other and markings in both arms in a varied number of chromosomes depending on the analyzed probe, which also included interstitial telomeric sites (ITS) with the $(TTAGG)_6$ probe. In the remaining species, the telomeric probe $(TTAGG)_6$ was present only in the terminal region of the chromosomes, sometimes forming clusters (Fig. 6).

B chromosomes were found in *M. quinquefasciata* (up to 3) and in *T. fiebrigi* (up to 7) (Fig. 1). These supernumerary chromosomes are mostly heterochromatic and were marked in both extremities by the telomeric $(TTAGG)_6$ probe (Fig. 6). Out of the seven Bs found in *T. fiebrigi*, two were bearers of 18S rDNA and one had a small euchromatic region marked by the microsatellite $(TCAGG)_6$ (Fig. 7, Online Resource Fig. S19). The results were summarized on the idiogram of Fig. 7, except the telomeric probe that would mix with the other microsatellite patterns. One supplementary figure was provided as Supplementary Information for each species in order to facilitate the visualization of the microsatellite patterns within each taxon (Online Resource Figs. S01-S33).

Discussion

The Minimum Interaction Theory (MIT) predicts that, during evolution, chromosome number has a tendency to increase associated with a concomitant heterochromatin growth, to stabilize the new telomeres (Imai et al. 1988, 2001). However, some Meliponini species do not seem to fit this model, as we observe species with a high-numbered karyotype (*n* = 17)

and low heterochromatin content, such as *Cephalotrigona capitata* (Online Resource Fig. S15) and *Partamona helleri* (Martins et al. 2014). Recently, the hypothesis of chromosome fusions from a high-numbered ancestral karyotype (*n* = 18) was suggested through meta-analyzes using a molecular phylogenetic approach to explain the chromosomal evolution of the Meliponini tribe, indicating *n* = 17 as the putative ancestral haploid number of the Neotropical clade (Travenzoli et al. 2019b). Therefore, with the empirical cytogenetic data presented in this paper, we corroborate the importance of Robertsonian fusions in the stingless bees' karyotype evolution.

Microsatellite markings in only one chromosome arm is probably the ancestral condition, observed on *n* = 15 species from clade 1 and on *n* = 17 species from clade 3. The other species patterns could be interpreted as a series of Robertsonian fusions resulting in species with distinct chromosome numbers and microsatellite markings on both chromosome arms. In clade 1, the *n* = 8 of *L. muelleri* could be interpreted as a result of seven Robertsonian fusion events characterized by the presence of seven chromosome pairs with microsatellite markings in both arms as the evidence of the fusions from the plesiomorphic condition shared by *Celetrigona* and *Trigonisca, i.e. n* = 15 and microsatellite markings in only one chromosome arm (Fig. 7).

In clade 2, most of the *Melipona* species have *n* = 9, out of which eight chromosomes are marked in both arms with the microsatellites, suggesting the occurrence of eight Robertsonian fusion events through the presence of eight chromosome pairs with the microsatellites in both arms derived from the plesiomorphic condition shared by clades 2 and 3, *i.e. n* = 17 and microsatellite markings in only one arm (Fig. 7). Later in the evolution of the genus *Melipona*, Robertsonian fission events led to the increase in chromosome number and microsatellites distribution in *Melipona lateralis* and *Melipona seminigra pernigra* (both with *n* = 11 and only 6 chromosomes with microsatellites in both arms). These results indicate that after the fusion events that stablished the *n* = 9 in *Melipona*, subsequent fission events increased the chromosome number in both *M. lateralis* (Online Resource Fig. S10) and *M. seminigra pernigra* (Online Resource Fig. S11), as well as in the other *M. seminigra* subspecies (Francini et al. 2011; Andrade-Souza et al. 2018), probably reflecting the proximity of these taxon (Cunha et al. 2020).

In clade 3, the plesiomorphic condition of microsatellite markings in only one arm is shared by all species with *n* = 17. The *n* = 14 of *Lestrimelitta* spp. could be interpreted as a result of three Robertsonian fusions. The presence of three chromosome pairs with microsatellite markings in both arms supports this explanation. *Duckeola ghilianii*,

Frieseomelitta languida, *Frieseomelitta* sp.l, and *Frieseomelitta* sp.2, all share the *n* = 15 and microsatellite markings in both arms in two chromosome pairs, which suggest two Robertsonian fusions from the plesiomorphic condition shared by clades 2 and 3, *i.e. n* = 17 and microsatellite markings in only one chromosome arm (Fig. 7). Even though *Trigona braueri* was not analyzed in the present paper, the cytogenetic evidence of a fusion rearrangement in one chromosome pair led to the decrease from *n* = 17 to *n* = 16 in this species (Domingues et al. 2005; Cunha et al. 2021).

The unconformity with the other *Frieseomelitta* species observed in *Frieseomelitta* sp. and *Frieseomelitta varia* (*n* = 15 from clade 3) could depict an apomorphic trait of these species. Chromosome breakpoints could be associated to different kinds of rearrangements besides fusions and fissions, for example inversions, transpositions, and reciprocal translocations (Coghlan et al. 2005). Inversions are often associated with local adaptations and speciation (Kirkpatrick and Barton 2006; Hoffmann and Rieseberg 2008), and could have contributed to the diversity of markings observed in these *Frieseomelitta* spp. Carvalho and Costa (2011) argued about the importance of pericentromeric inversion rearrangements in the evolution of this genus, and we also indicate the presence of paracentromeric inversions, as we observe the prevalence of meta/submetacentric chromosomes in all *Frieseomelitta* species. Interestingly, $(TCAGG)_6$ markings in *Frieseomelitta* sp.1 and *Frieseomelitta* sp.2 are also characterized by these multiple markings, which could be associated with the presence of a series of DAPI negative portions in several chromosomes, a trait that is unique to the genus *Frieseomelitta* (Online Resource Figs. S21-25).

One chromosome pair bearer of the 18S rDNA cistrons is the most common pattern in the analyzed species, being kept in most species of clade 1, in all species of clade 2, and in more than half of the clade 3 species, probably constituting the plesiomorphic condition in Neotropical Meliponini (Fig. 7). The centromeric position found only in *L. muelleri* and in the low heterochromatin content *Melipona* species may be a consequence of the Robertsonian fusion events, whereas the high heterochromatin content *Melipona* species acquired the terminal position secondarily due to the accentuated heterochromatin growth (Cunha et al. 2020; Pereira et al. 2021).

The expansion in the number of ribossomal sites only occurred in species with terminal location of these genes, centromeric/interstitial positioning prevents or at least makes it difficult the recombination in these regions (Sochorová et al. 2018; Hirai 2020). The increase in the number of sites could be explained due to ectopic recombination mediated or

not by transposable elements (Silva et al. 2013; Menezes et al. 2019; Piscor et al. 2020; Hirai 2020).

Besides the diversification in the number of sites, we also observed the amplification of ribossomal copies in only one homologous chromosome in some species, a heteromorphism that could be generated through gene amplification caused by different sources, *i.e.* unequal crossover, gene conversion, gene duplication (Eickbush and Eickbush 2007; Hirai 2020). Another heteromorphism, detected only in *T. recursa*, refers to the different 18S rDNA position between the homologous of pair 14 (terminal x interstitial) that could be a consequence of a heterozygous inversion.

Polymorphisms outside the 18S region were also observed in the present paper. Some minor size differences between homologous chromosomes, such as in *Scaptotrigona* sp., *G. subterranea*, and *N. testaceicornis*, could be associated with heterochromatin amplification events on only one homologue, as it was suggested for some *Melipona* species (Lopes et al. 2008; Andrade-Souza et al. 2018; Travenzoli et al. 2019a). The major size differences observed in *M. fulva* and *S. quadripunctata* could be better explained through unequal crossing over (Schubert and Lysac 2011; Travenzoli et al. 2019a). The unequal sister chromatid exchange leads to deletion on one chromosome and duplication in the other, what could explain the change in the chromosome pair bearer of the 18S rDNA in *Melipona scutellaris* from pair 4 in a homomorphic colony (Fig. 1) to pair 1 in a heteromorphic colony (Piccoli et al. 2018) (the same pattern observed in the heteromorphic *M. fulva*). The same apply to *S. quadripunctata*, although this pair is not the bearer of the 18S region, and we observe individuals with and without the polymorphism (Online Resource Fig. 33).

In the present paper, we found up to 3 B chromosomes in *M. quinquefasciata* and up to 7 Bs in *T. fiebrigi*. Up to 4 supernumerary chromosomes have been reported in the former (Silva et al. 2018), and 2 B in the latter (Barth et al. 2011). Theories involving B chromosomes as parasites of the host genome predicts that the host would develop mechanisms to avoid accumulation of these elements in a coevolutionary arms race (Camacho et al. 2002). Usually Bs are small-sized and present in small numbers (reviewed in Camacho et al. 2004), such as the *M. quinquefasciata* ones. On the other hand, the *T. fiebrigi* B chromosomes are as big as the A-chromosomes and present in great number, suggesting that the host did not have time yet to develop mechanisms to avoid their spreading or, alternatively, these Bs could confer some adaptative advantage. Besides these two species, 1B have been found in *Melipona rufiventris* (Lopes et al. 2008), *Partamona cupira* (Marthe et al. 2010), and *Partamona rustica* (Tosta et al. 2014), and up to 7B in *Partamona helleri* (Martins

et al. 2014). Together with *P. helleri*, 7Bs in *T. fiebrigi* is the record found in the same individual of a stingless bee.

The Bs found in *M. quinquefasciata* are completely heterochromatic and were not marked by any microsatellite probe besides the telomeric (Online Resource Fig. S05). Among the seven Bs found in *T. fiebrigi*, besides the telomeric probe, one B were marked by the $(TCAGG)_6$ microsatellite and two were marked with the 18S rDNA probe (Online Resource Fig. S19). The small portions of euchromatin in these Bs show that they may contain potentially expressed genes, as already suggested in other species (Ruiz-Estevez et al. 2012; Banaei-Moghaddam et al. 2015; Valente et al. 2017). Consequently, they have the potential to confer some adaptative advantage that favors its presence and accumulation (Camacho et al. 2000; González-Sánchez et al. 2004; Montiel et al. 2014) or, at least, play some role in the B chromosome evolution (Ruiz-Ruano et al. 2015).

The $(TTAGG)_n$ is the telomeric sequence observed in all analyzed species (Fig. 6) and ITS were not observed among the chromosomes of most of them despite the evidences of Robertsonian fusions. In Robertsonian rearrangements, the extremities of the chromosome arms are lost generating adhesive ends that bind together in a fusion event causing the loss of telomeric repeats (Schubert et al. 1992; Slijepcevic 1998; Warchałowska-Sliwa et al. 2013, 2017). The exception observed in *Frieseomelitta* spp., the presence of $(TTAGG)_6$ in the DAPI negative regions of several chromosomes, could be associated with rearrangements other than fusions (Zattera et al. 2019, 2020), as inversions and translocations that served as hotspots for recombination events (reviewed in Bolzan 2017).

In a nutshell, the microsatellite FISH markings on clade 1 could be explained based on an ancestral karyotype of $n = 15$ for this clade, whereas the markings observed on clades 2 and 3 could be better explained based on an ancestral karyotype of $n = 17$ (Fig. 7). Based on this scenario, if $n = 15$ was the ancestral karyotype of the three Neotropical clades, fission events contributed to the increase in the chromosome number from 15 to 17 in the ancestor of clades 2 and 3, and the low sampling of clade 1 species in Tranvenzoli et al. (2019b) study could have underestimated the weight of $n = 15$ as the common ancestral karyotype. If the proposed by the previous authors are correct, and $n = 17$ is the ancestral karyotype of Neotropical Meliponini, evidences of the fusion events that were responsible for the decreasing of the chromosome number in the ancestor of clade 1 had already been erased by subsequent chromosomal rearrangements.

Conclusions

With the empirical cytogenetic data presented in this paper, we corroborate the recent proposition of Robertsonian fusion rearrangements to explain the karyotypic evolution in the stingless bees. While the ancestral state of the three Neotropical Meliponini clades is hard to infer, the putative ancestral karyotype probably had a single pair of 18S rDNA cistrons and was *n* = 15 in the separation of clade 1, and *n* = 17 in the split of clades 2 and 3. The decrease in the chromosome number and the increase in the 18S rDNA sites occurred independently between genera.

Disclosure of potential conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Marina Souza Cunha. The first draft of the manuscript was written by Marina Souza Cunha and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Table 1. Collection sites of the Neotropical Meliponini species in Brazil. The 33 species were assigned to the three clades proposed by Rasmussen and Cameron (2010). Haploid numbers (*n*) are shown with the respective number and location of chromosomes bearers of the 18S rDNA sites.

Clade	Species	Locality	n	18S rDNA
1	*Leurotrigona muelleri* (Fig. S01)	Passos, Minas Gerais	8	2 centromeric (pair 2)
	Celetrigona longicornis (Fig. S02)	Nova Xavantina, Mato Grosso	15	4 terminal (pairs 7, 11)
	Trigonisca sp. (Fig. S03)	Urbano Santos, Maranhão	15	2 terminal (pair 6)
2	*Melipona* sp. (Fig. S04)	Brasília, Distrito Federal	9	2 centromeric (pair 1)
	Melipona quinquefasciata (Fig. S05)	Piumhi, Minas Gerais	9 + 3B[#]	2 centromeric (pair 1)
	Melipona fasciculata (Fig. S06)	São Luís, Maranhão	9	2 subterminal (pair 1)
	Melipona fulva (Fig. S07)	Presidente Figueiredo, Amazonas	9	2 terminal (pair 1)
	Melipona scutellaris (Fig. S8)	Nordeste	9	2 terminal (pair 4)
	Melipona cf. *rufiventris* (Fig. S09)	Iranduba, Amazonas	9	2 terminal (pair 2)
	Melipona lateralis (Fig. S10)	Presidente Figueiredo, Amazonas	11	2 terminal (pair 2)
	Melipona seminigra pernigra (Fig. S11)	Altamira, Pará	11	2 terminal (pair 4)
3	*Scaptotrigona* sp. (Fig. S12)	Pará	17	6 terminal (pairs 1, 2, 5)
	Scaptotrigona xanthotricha (Fig. S13)	Viçosa, Minas Gerais	17	11 terminal (pairs 1, 2, 3, 4[‡], 5, 7)
	Geotrigona subterranea	Passos, Minas Gerais	17	4 subterminal

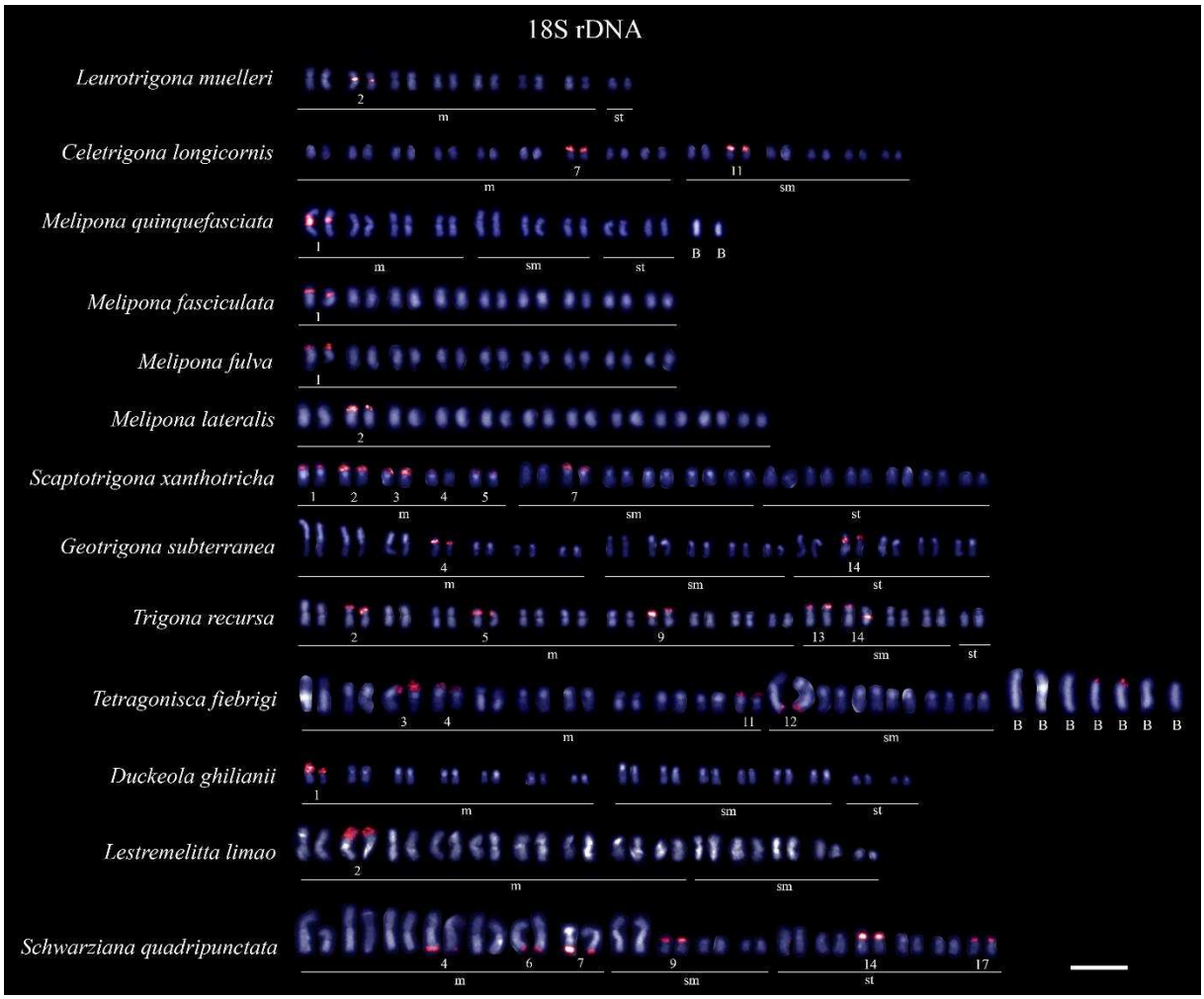
(Fig. S14)			(pairs 4, 14)
Cephalotrigona capitata (Fig. S15)	Viçosa, Minas Gerais	17	2 subterminal (pair 1)
Cephalotrigona femorata (Fig. S16)	Urbano Santos, Maranhão	17	2 subterminal (pair 2)
Trigona hyalinata (Fig. S17)	Viçosa, Minas Gerais	17	6 terminal (pairs 5, 9, 14)
Trigona recursa (Fig. S18)	Januária, Minas Gerais	17	10 terminal or subterminal (pairs 2, 5, 9, 13, 14)
Tetragonisca fiebrigi (Fig. S19)	Palotina, Paraná	17 + 7B[#]	8 terminal + 2B (pairs 3, 4, 11, 12 + 2B)
Duckeola ghilianii (Fig. S20)	Presidente Figueiredo, Amazonas	15	2 terminal (pair 1)
Frieseomelitta languida (Fig. S21)	Arcos, Minas Gerais	15	2 terminal (pair 3)
Frieseomelitta varia (Fig. S22)	Uberlândia, Minas Gerais	15	2 terminal (pair 1)
Frieseomelitta sp. (Fig. S23)	Brasília, Distrito Federal	15	2 subterminal (pair 1)
Frieseomelitta sp.1 (Fig. S24)	Presidente Figueiredo, Amazonas	15	4 terminal (pairs 1, 9)
Frieseomelitta sp.2 (Fig. S25)	Iranduba, Amazonas	15	2 terminal (pair 4)
Lestrimelitta limao (Fig. S26)	Brazil	14	2 terminal (pair 2)
Lestrimelitta sp. (Fig. S27)	Domingos Martins, Espírito Santo	14	2 terminal (pair 2)
Plebeia droryana (Fig. S28)	Santo Antônio do Jacinto, Minas Gerais	17	2 terminal (pair 4)
Plebeia lucii (Fig. S29)	Viçosa, Minas Gerais	17	2 terminal

			(pair 1)
Plebeia phrynostoma (Fig. S30)	Espírito Santo	17	2 terminal (pair 11)
Nannotrigona punctata (Fig. S31)	Altamira, Pará	17	2 terminal (pair 3)
Nannotrigona testaceicornis (Fig. S32)	Viçosa, Minas Gerais	17	4 terminal (pairs 3, 5)
Schwarziana quadripunctata (Fig. S33)	Viçosa, Minas Gerais	17	12 terminal (pairs 4, 6, 7, 9, 14, 17)

[#] B chromosomes were found in *Melipona quinquefasciata* (up to 3) and *Tetragonisca fiebrigi* (up to 7).
[‡] Only one of the homologs were marked with the 18S rDNA probe.

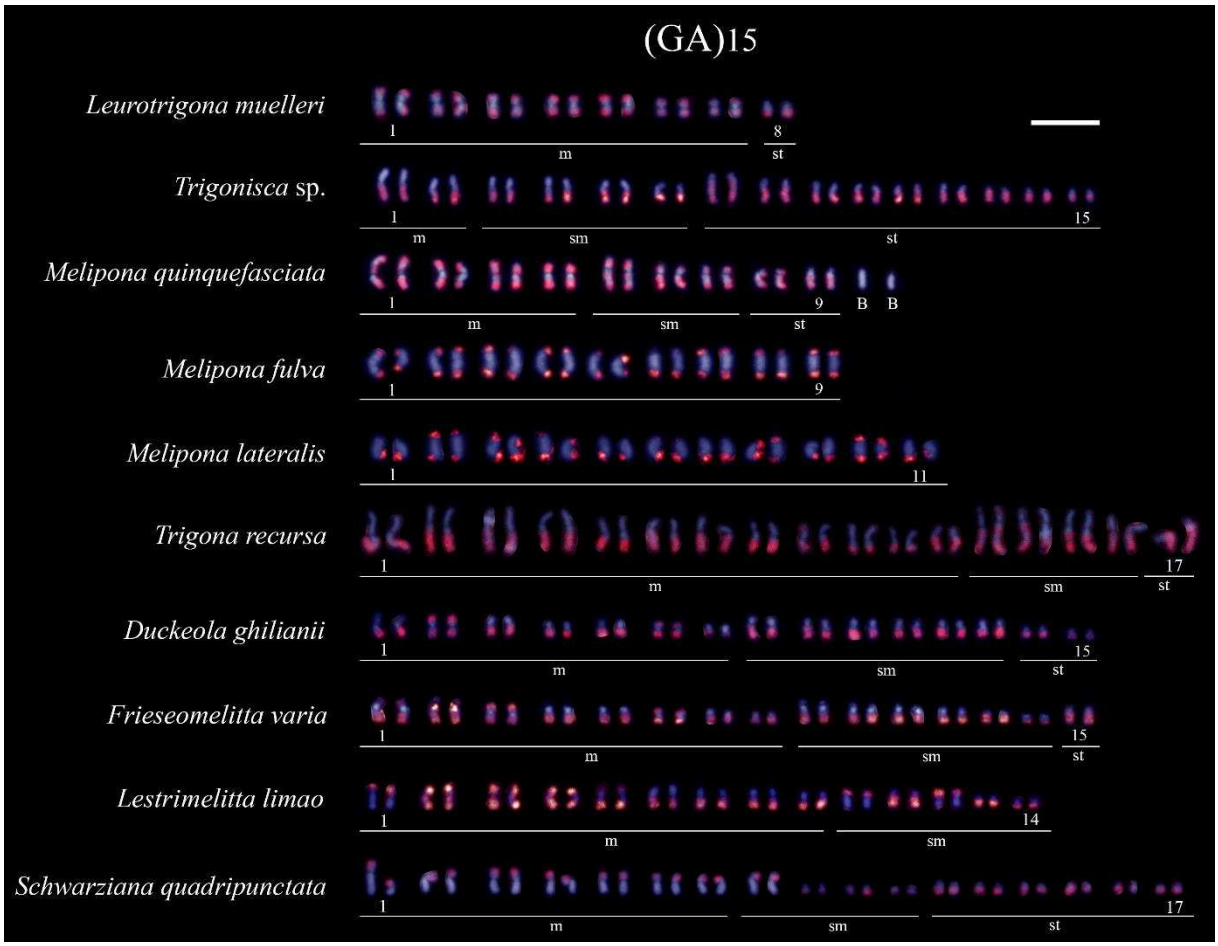
Figures

Fig. 1 Chromosome number and 18S rDNA variation in some of the analyzed species representing the three Neotropical Meliponini clades. Bar 5µm



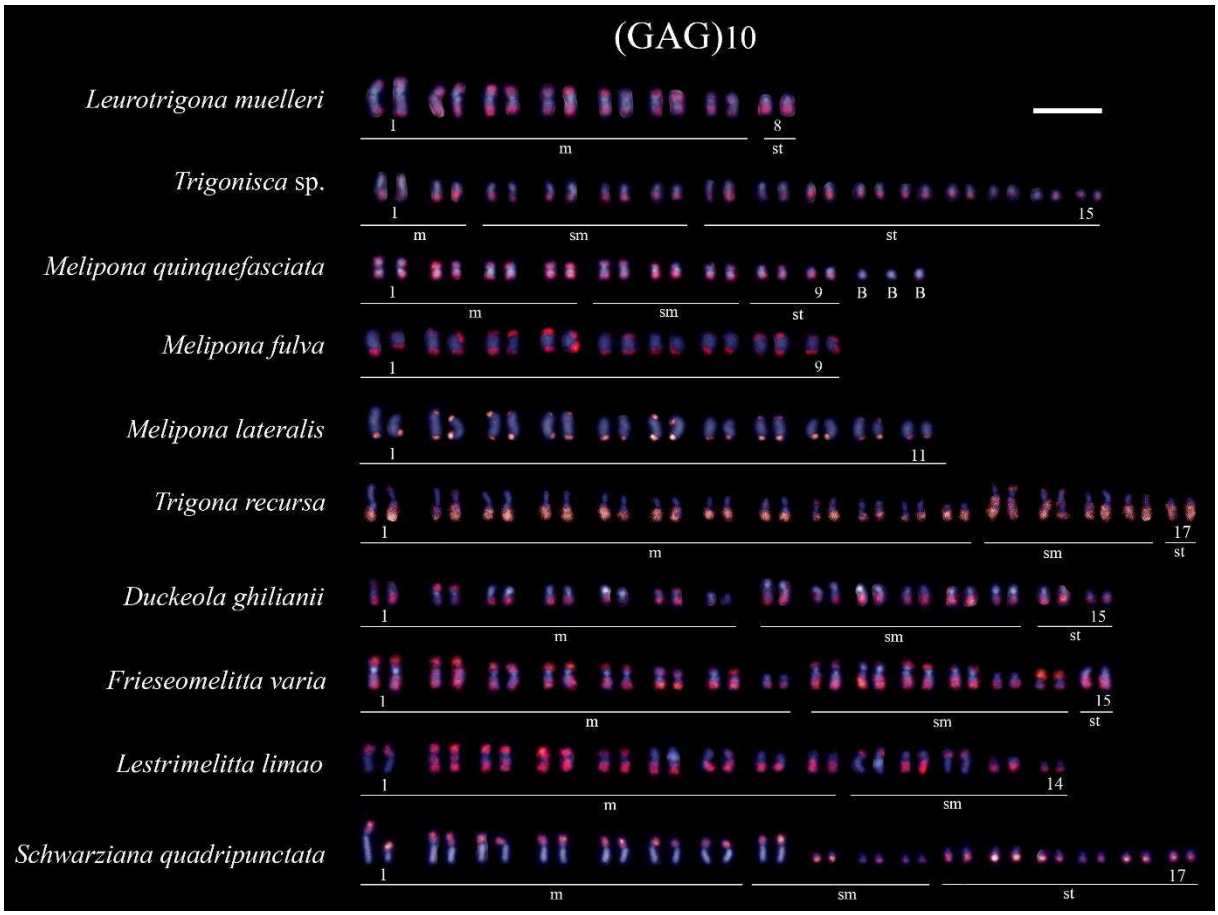
Fonte: Autoral.

Fig. 2 Chromosome mapping with $(GA)_{15}$ microsatellite in some of the analyzed species representing the three Neotropical Meliponini clades. Bar 5µm



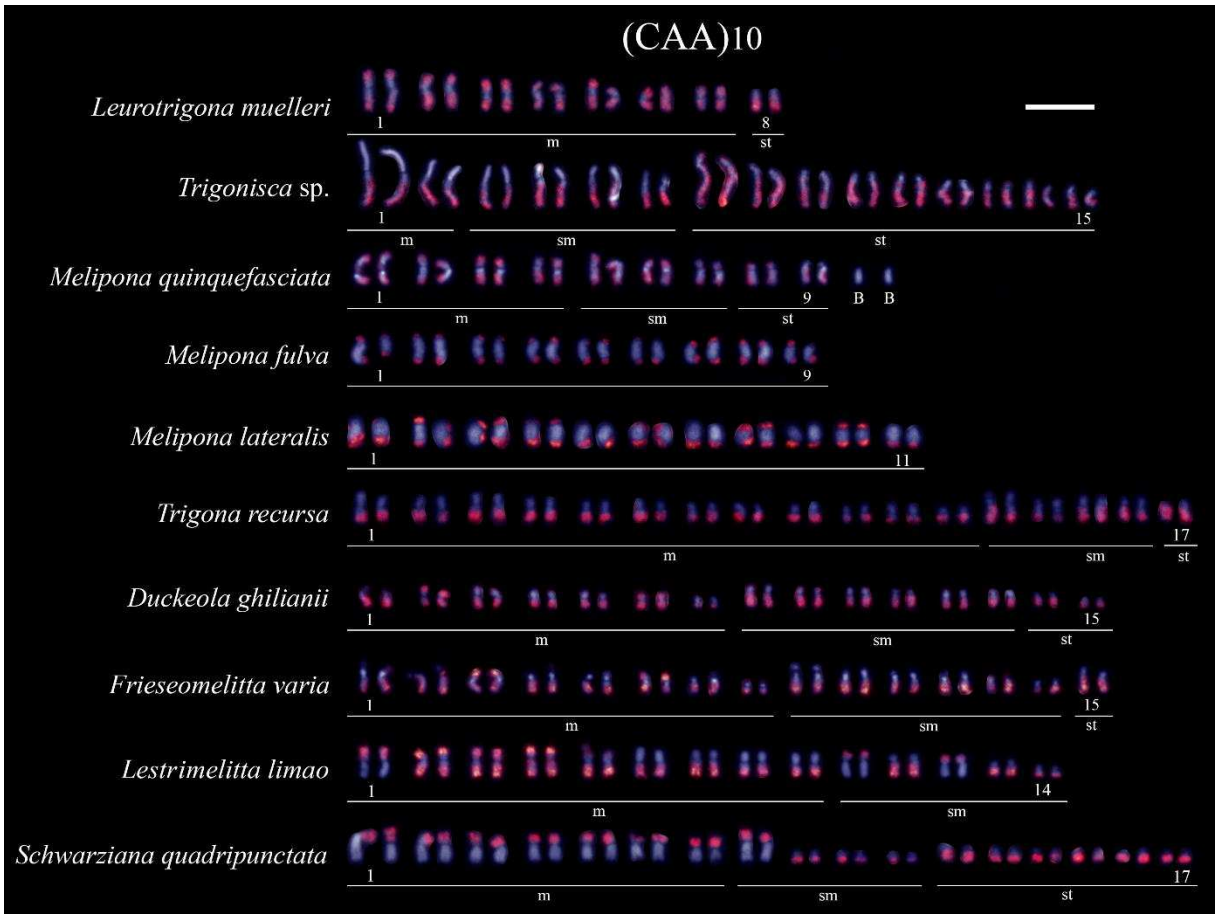
Fonte: Autoral.

Fig. 3 Chromosome mapping with $(GAG)_{10}$ microsatellite in some of the analyzed species representing the three Neotropical Meliponini clades. Bar 5µm



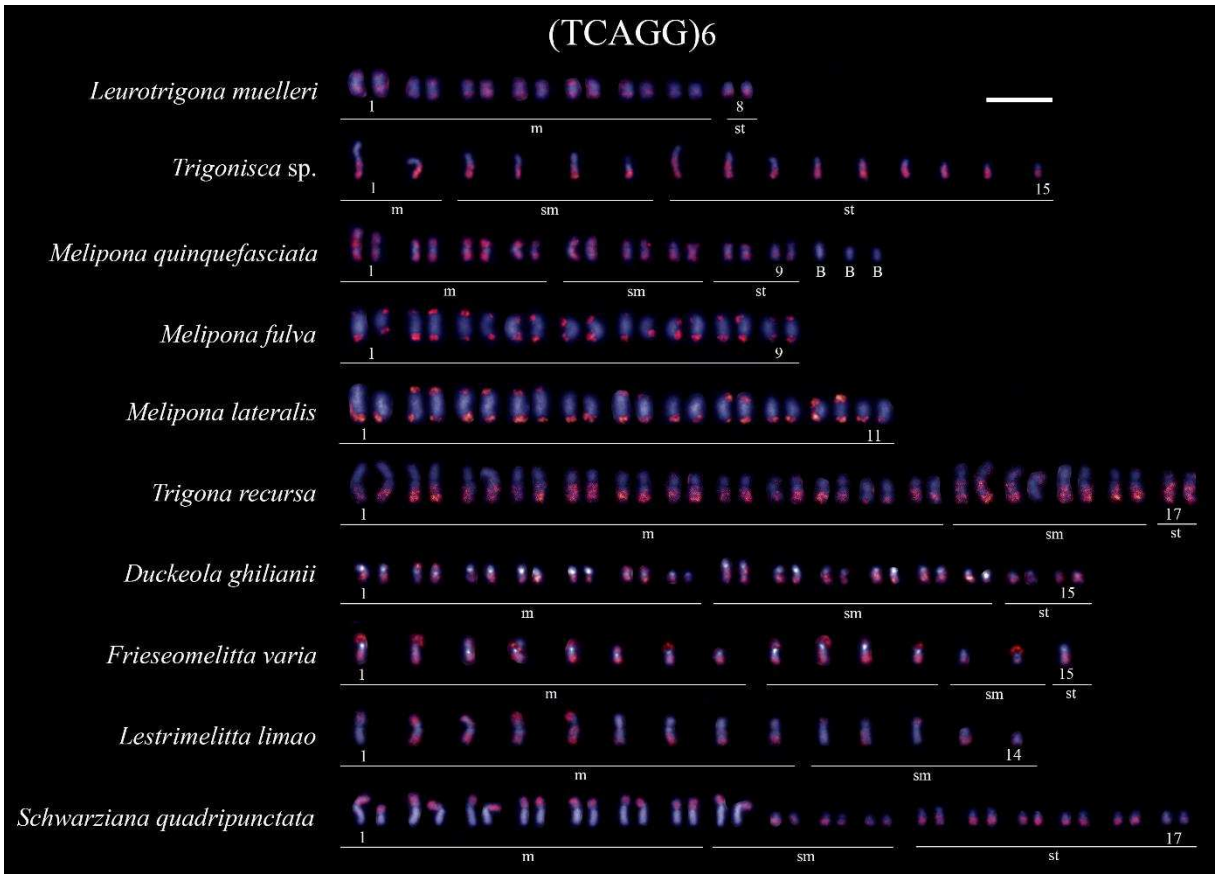
Fonte: Autoral.

Fig. 4 Chromosome mapping with $(CAA)_{10}$ microsatellite in some of the analyzed species representing the three Neotropical Meliponini clades. Bar 5µm



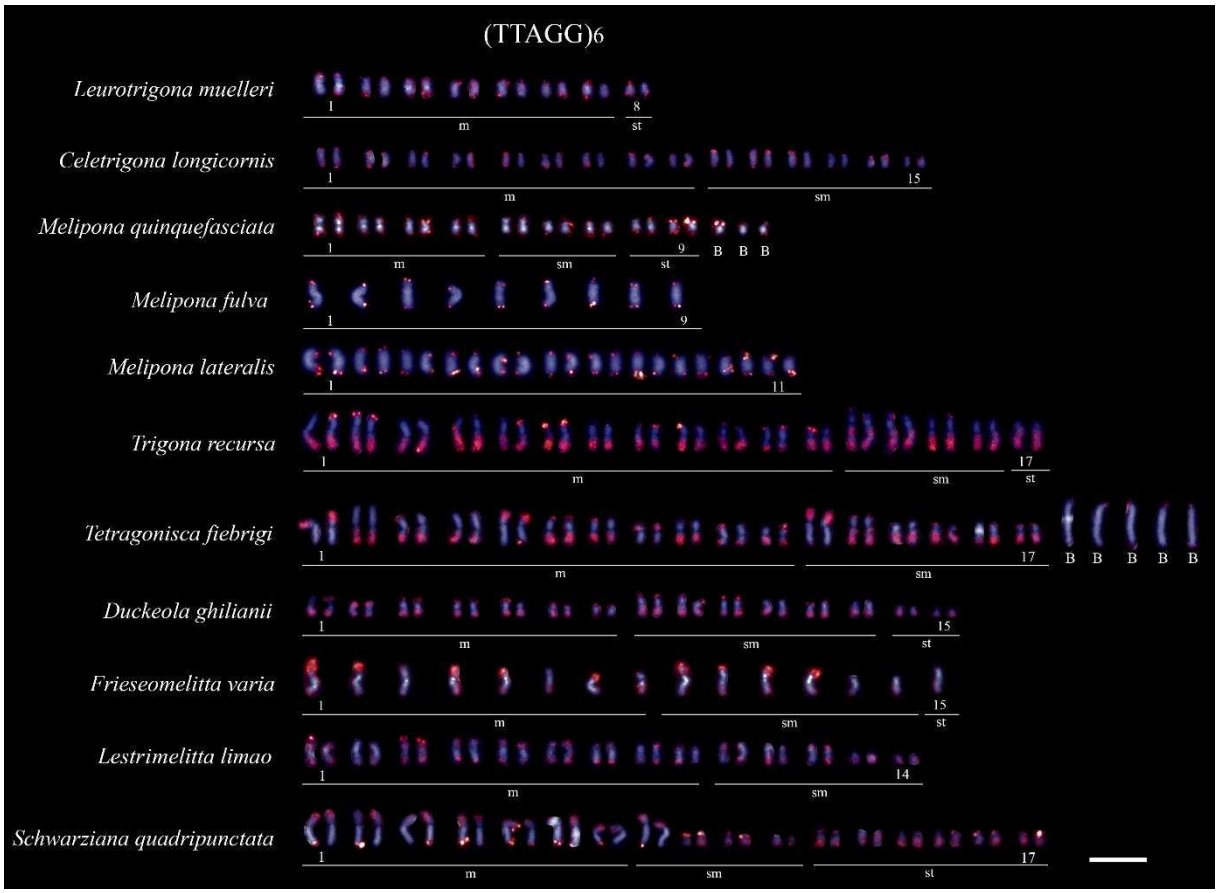
Fonte: Autoral.

Fig. 5 Chromosome mapping with $(TCAGG)_6$ microsatellite in some of the analyzed species representing the three Neotropical Meliponini clades. Bar 5µm



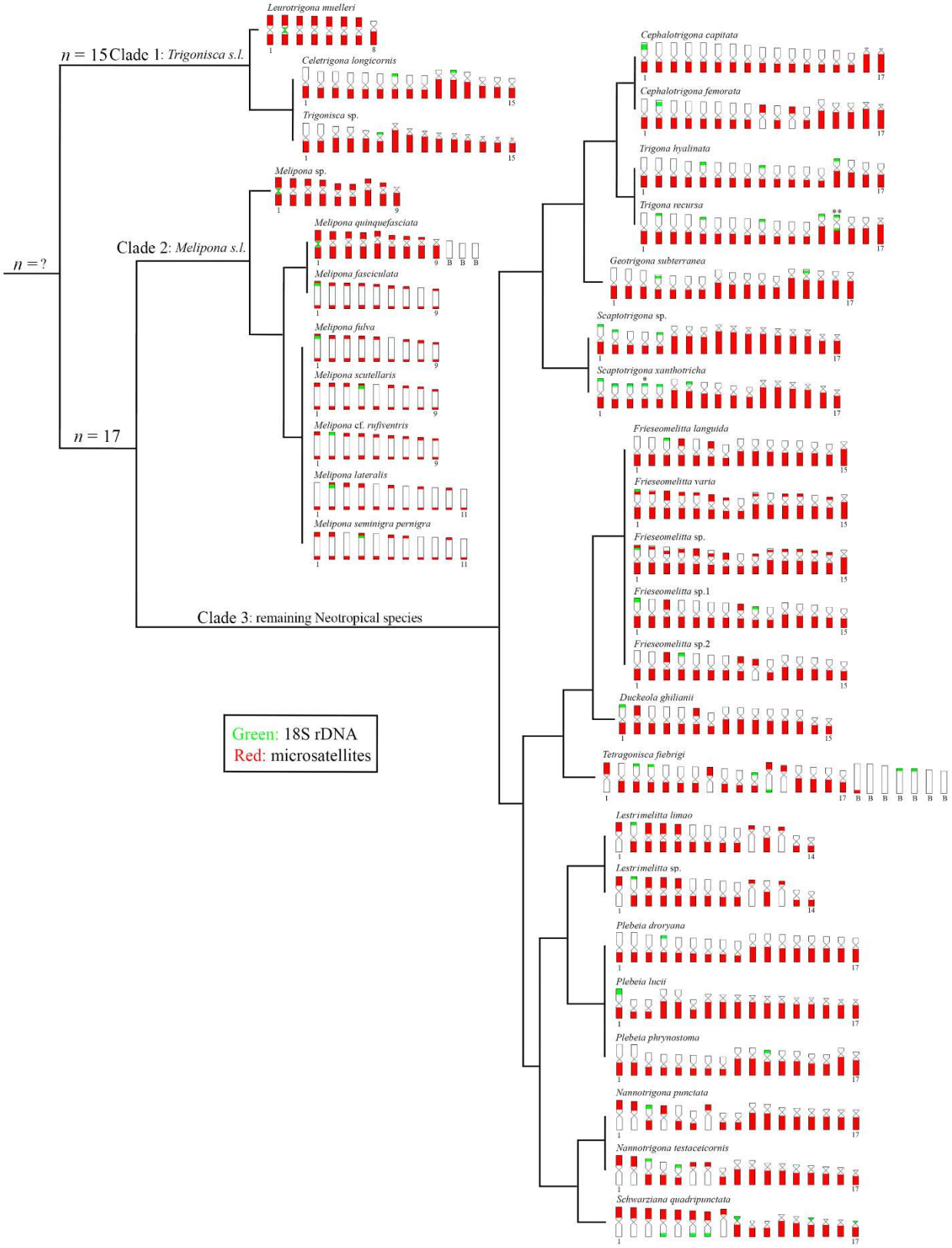
Fonte: Autoral.

Fig. 6 Chromosome mapping with $(TTAGG)_6$ telomeric probe in some of the analyzed species representing the three Neotropical Meliponini clades. Bar 5µm



Fonte: Autoral.

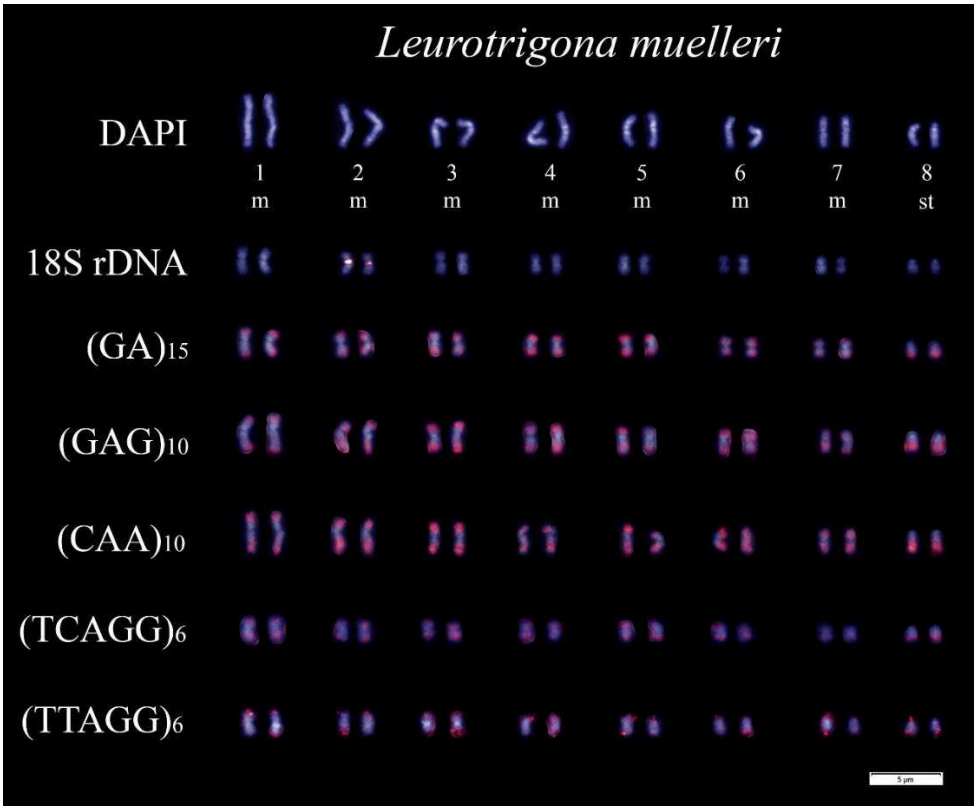
Fig. 7 Haploid idiogram of the karyotypes from the Neotropical Meliponini species. The 18S rDNA are shown in green and the microsatellites $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(TCAGG)_6$ are shown in red. The phylogenetic structure was redrawn from Rasmussen and Cameron (2010, figure 3). The $(TTAGG)_6$ telomeric probe was omitted from the idiogram. * indicate 18S rDNA markings in only one of the homologous chromosomes. ** indicate different position of the 18S rDNA markings between the homologous chromosomes (terminal x interstitial)



Fonte: Autoral.

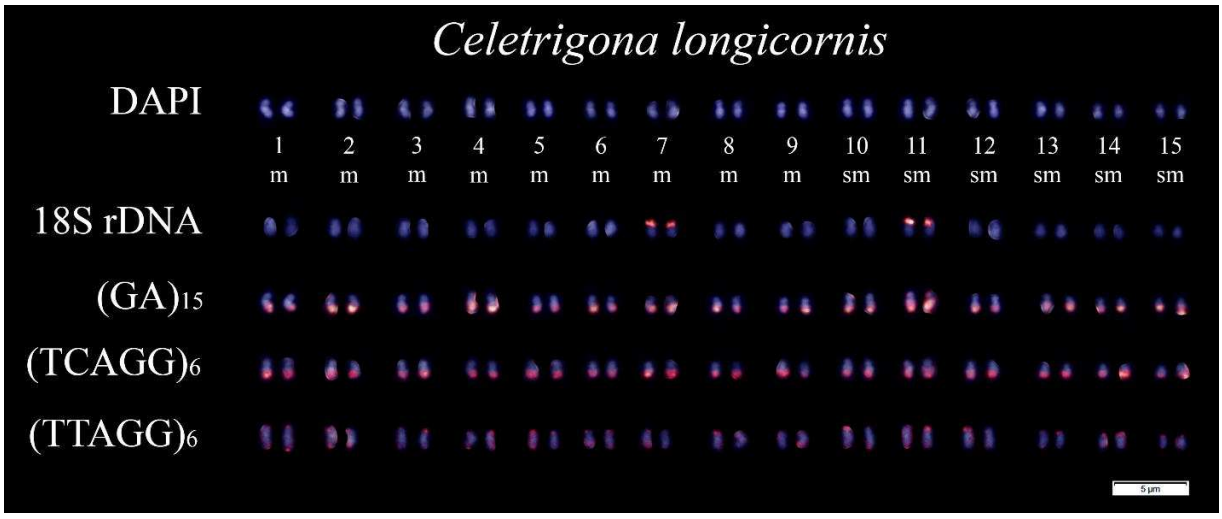
Supplementary Information

Online Resource Fig. S01 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Leurotrigona muelleri*



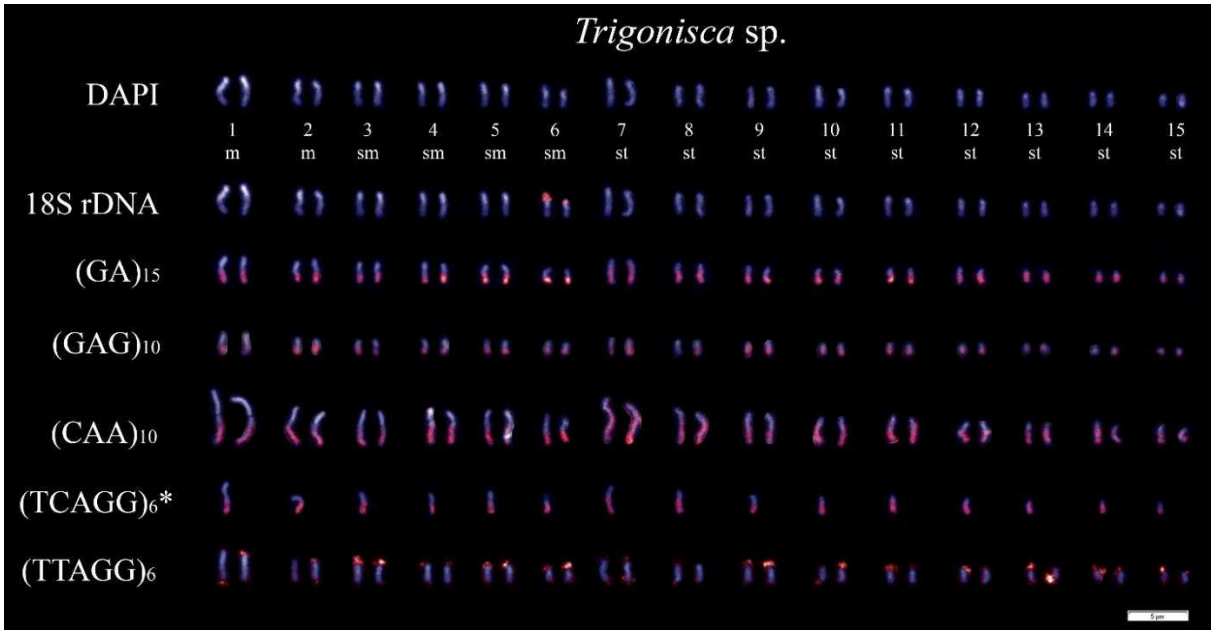
Fonte: Autoral.

Online Resource Fig. S02 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Celetrigona longicornis*



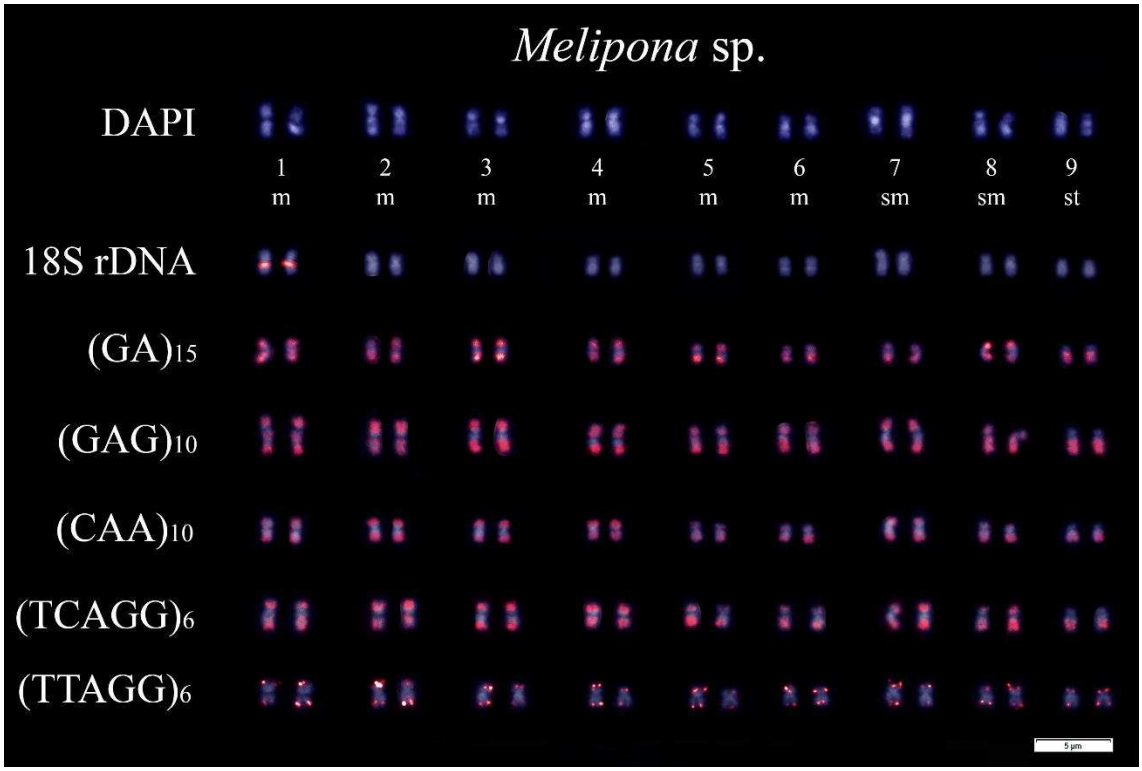
Fonte: Autoral.

Online Resource Fig. S03 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Trigonisca* sp. * indicate haploid male



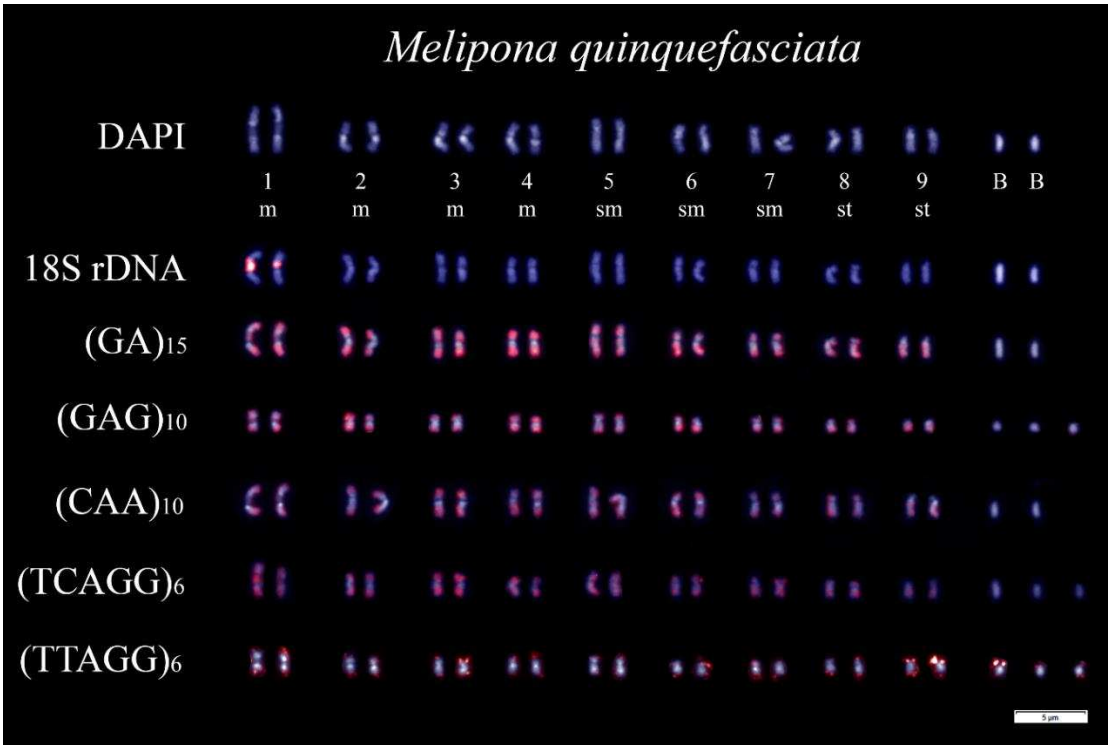
Fonte: Autoral.

Online Resource Fig. S04 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Melipona* sp.



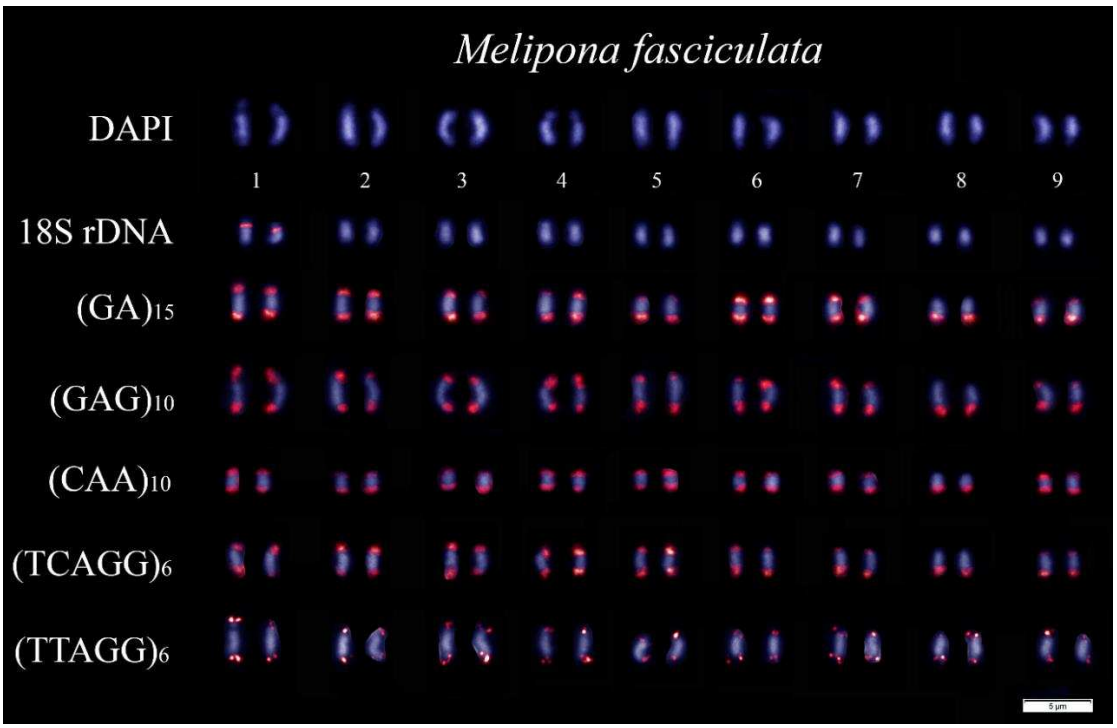
Fonte: Autoral.

Online Resource Fig. S05 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Melipona quinquefasciata*



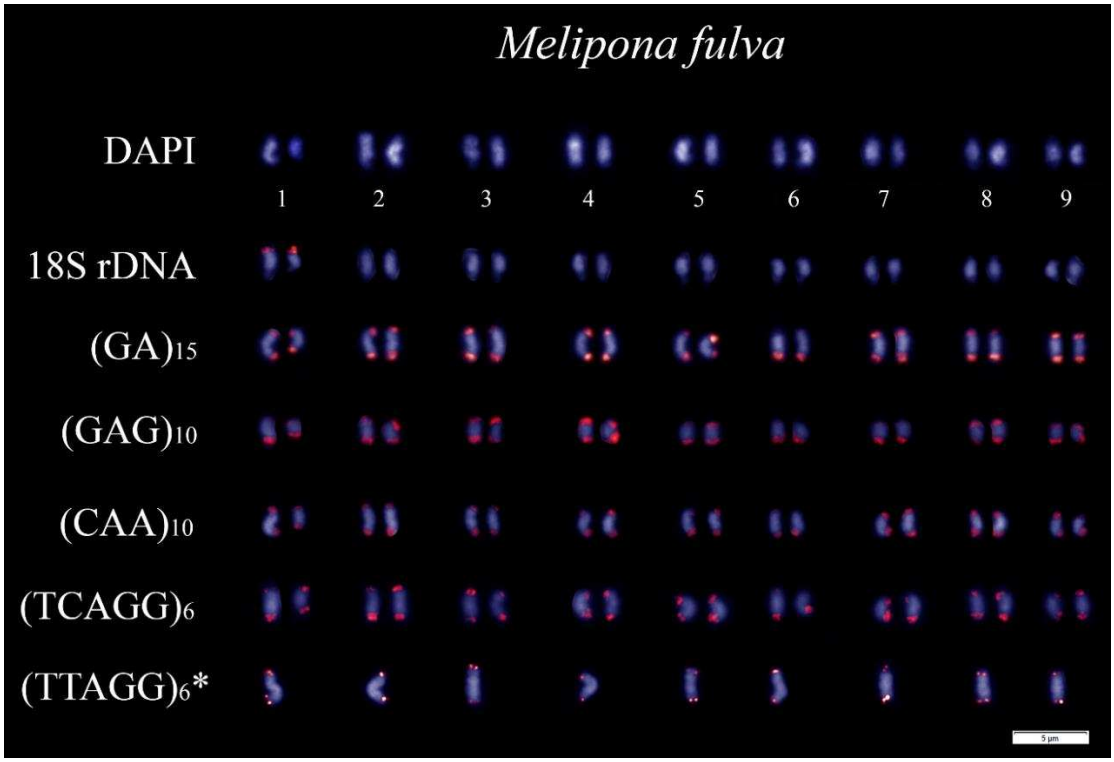
Fonte: Autoral.

Online Resource Fig. S06 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Melipona fasciculata*



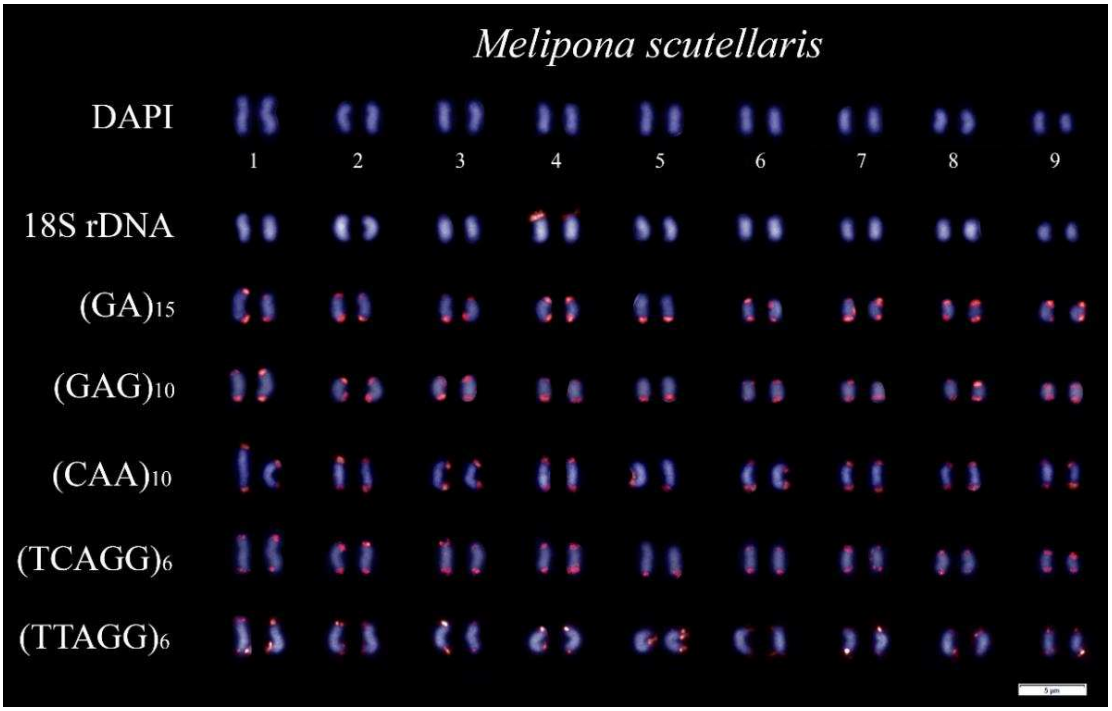
Fonte: Autoral.

Online Resource Fig. S07 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Melipona fulva*. * indicate haploid male



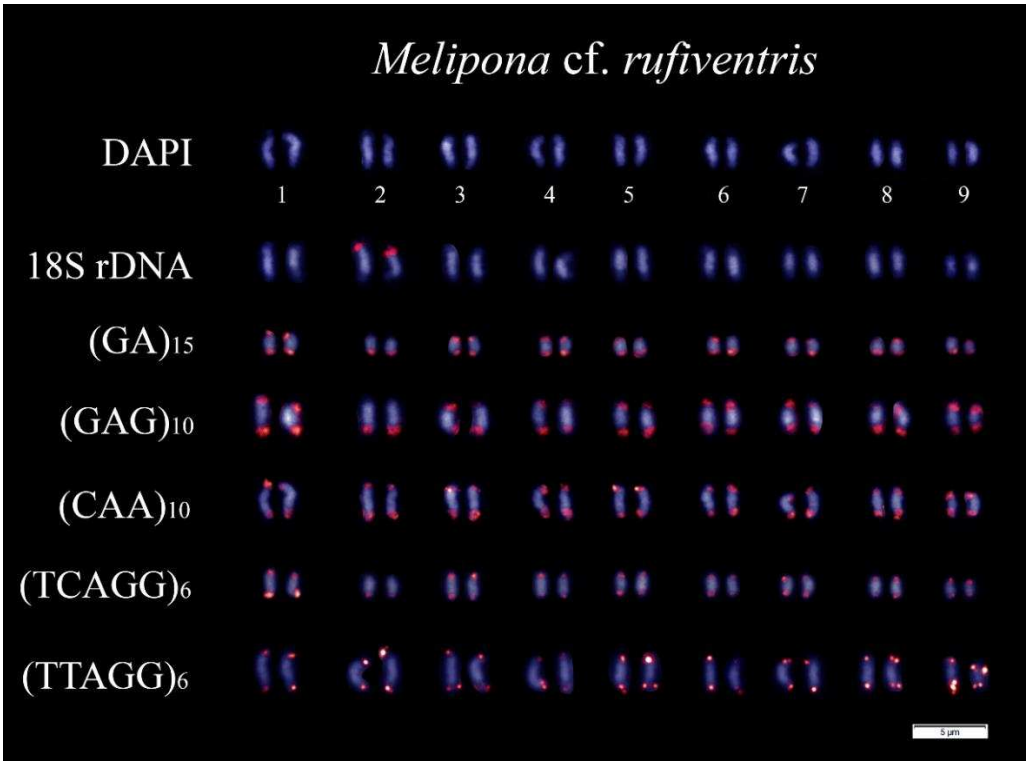
Fonte: Autoral.

Online Resource Fig. S08 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Melipona scutellaris*



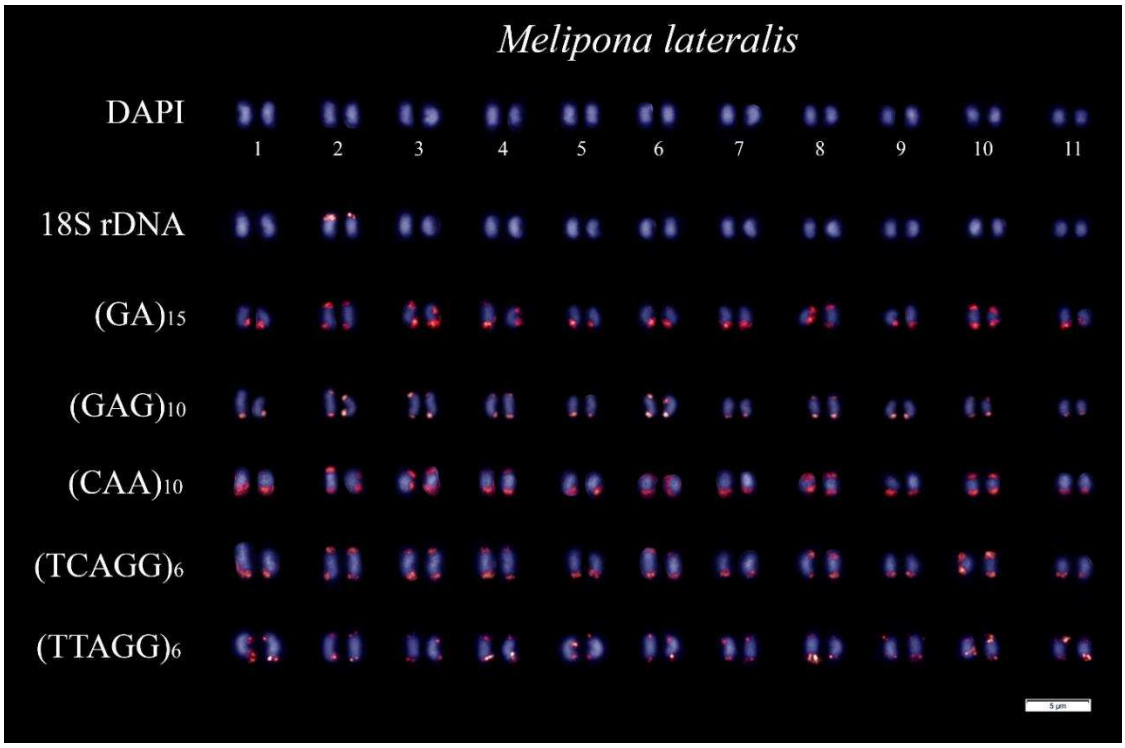
Fonte: Autoral.

Online Resource Fig. S09 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Melipona* cf. *rufiventris*



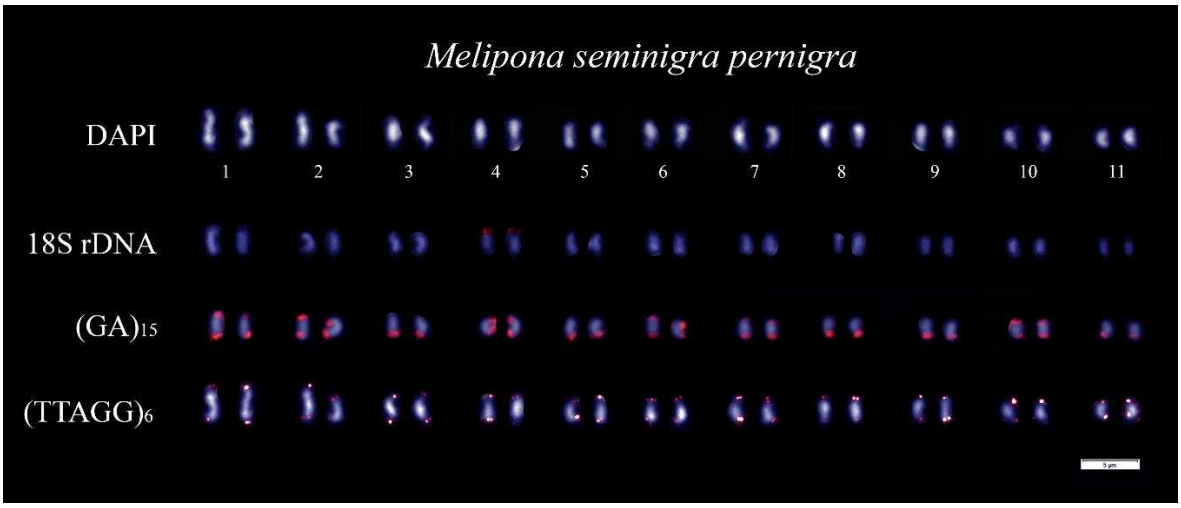
Fonte: Autoral.

Online Resource Fig. S10 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Melipona lateralis*



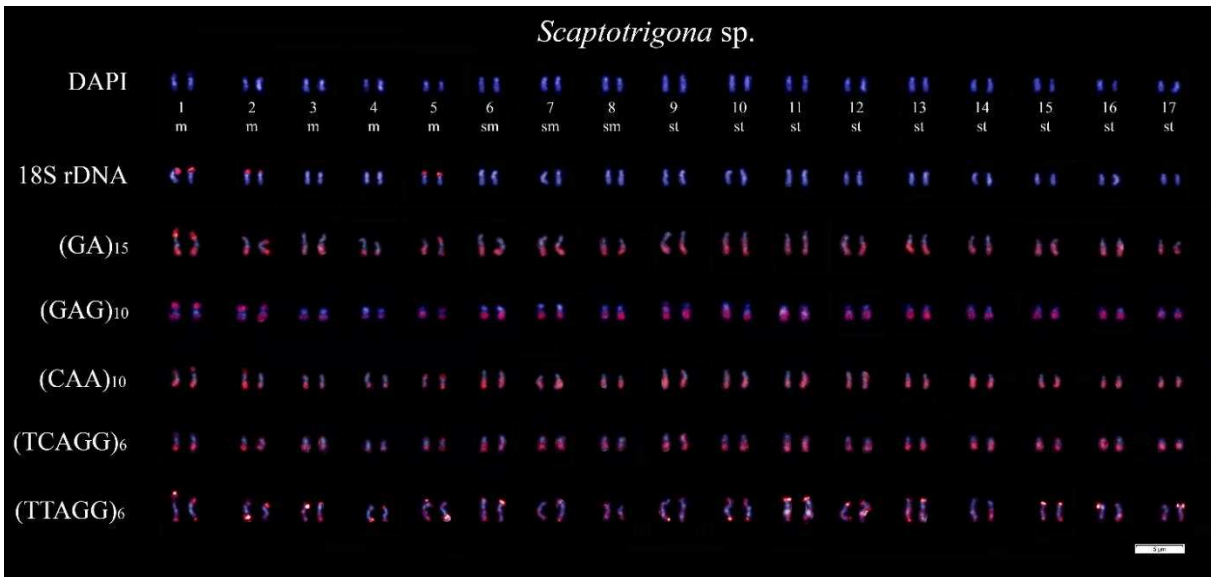
Fonte: Autoral.

Online Resource Fig. S11 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$ and $(TTAGG)_6$ microsatellites in *Melipona seminigra pernigra*



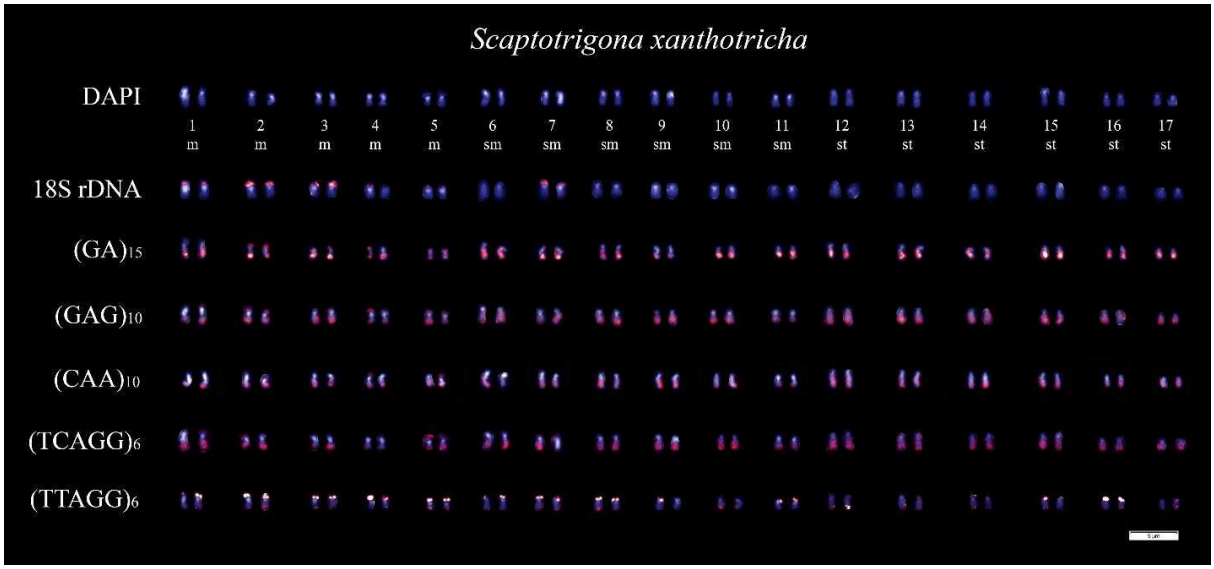
Fonte: Autoral.

Online Resource Fig. S12 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Scaptotrigona* sp.



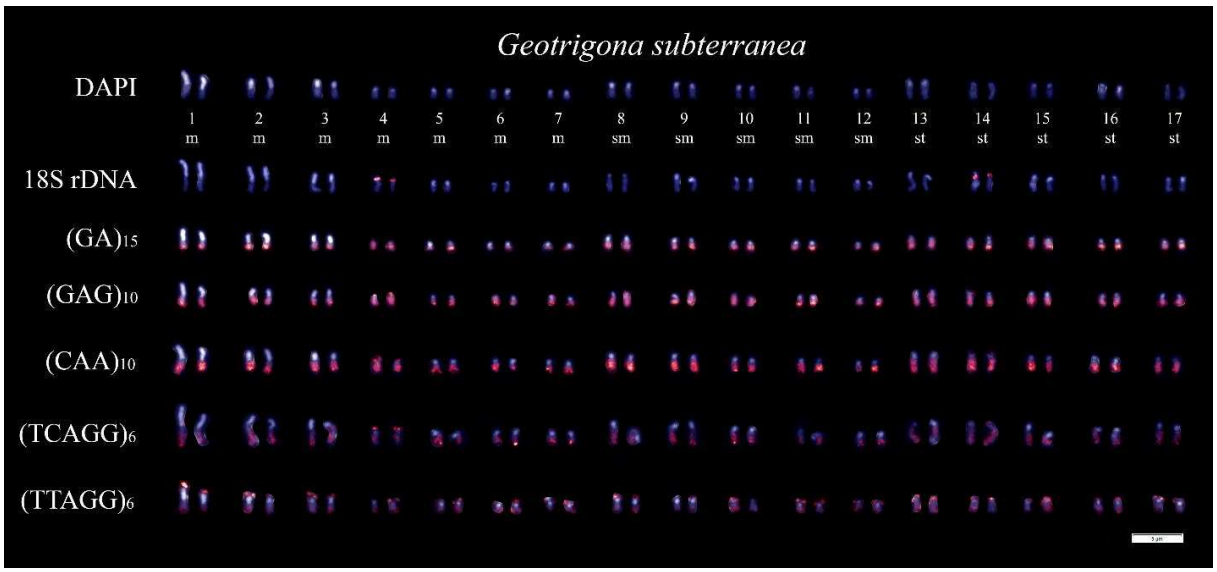
Fonte: Autoral.

Online Resource Fig. S13 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Scaptotrigona xanthotricha*



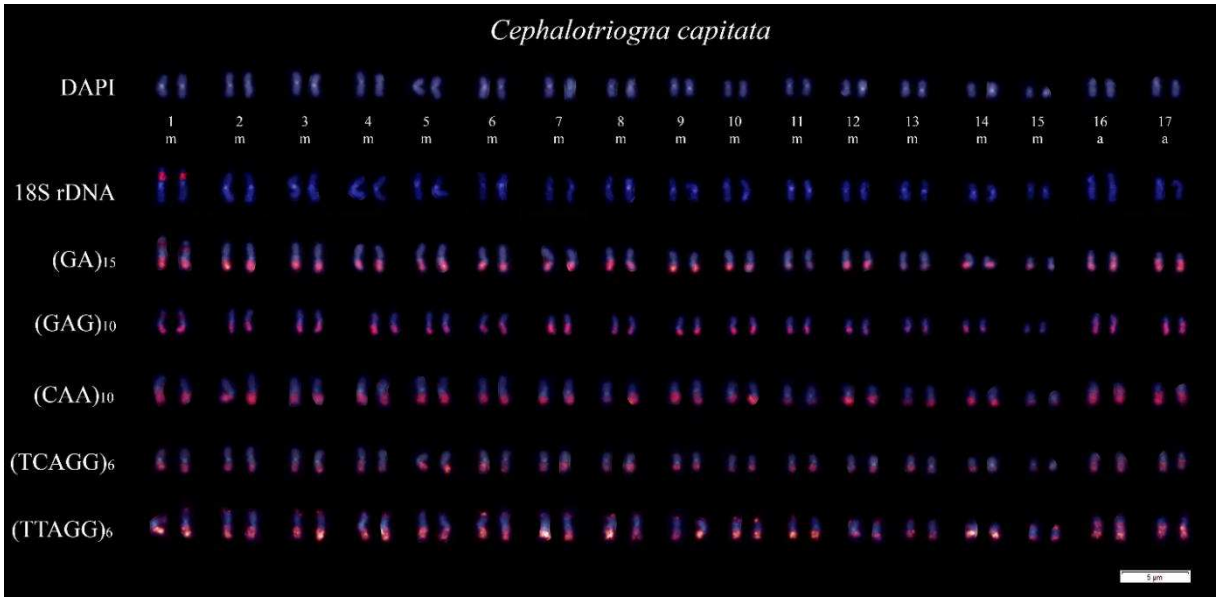
Fonte: Autoral.

Online Resource Fig. S14 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Geotrigona subterranea*



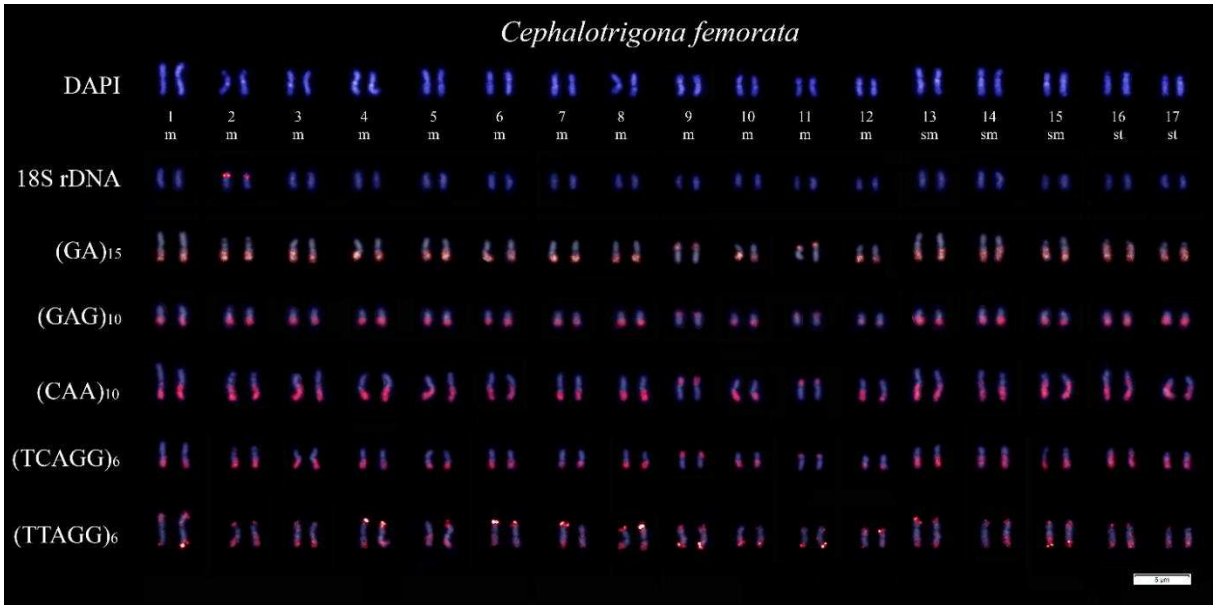
Fonte: Autoral.

Online Resource Fig. S15 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Cephalotrigona capitata*



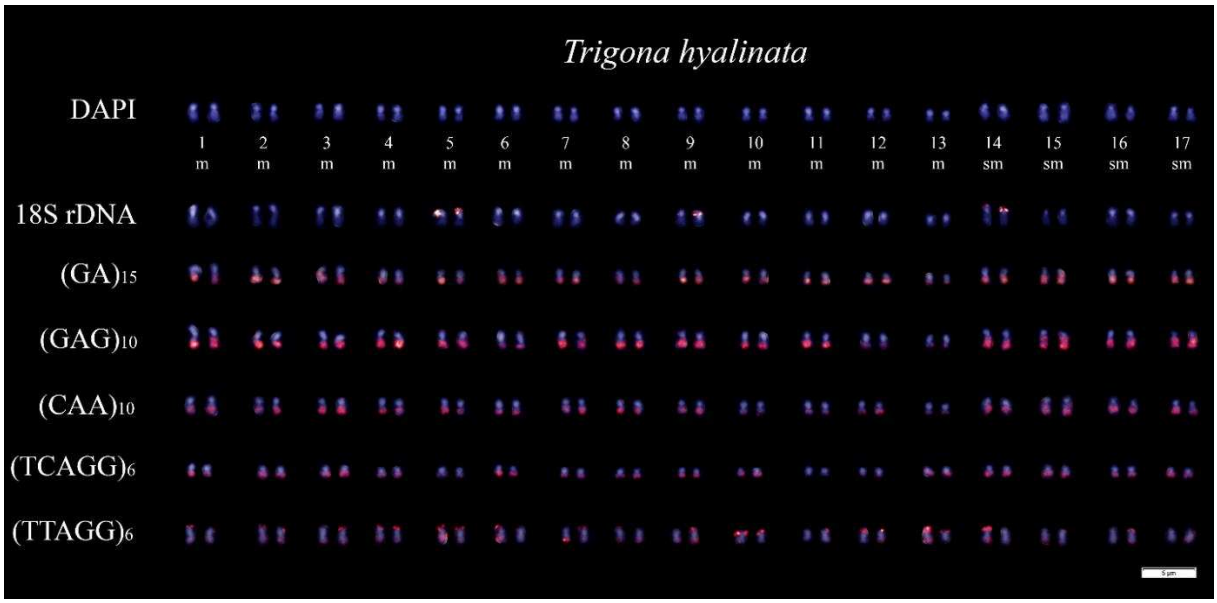
Fonte: Autoral.

Online Resource Fig. S16 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Cephalotrigona femorata*



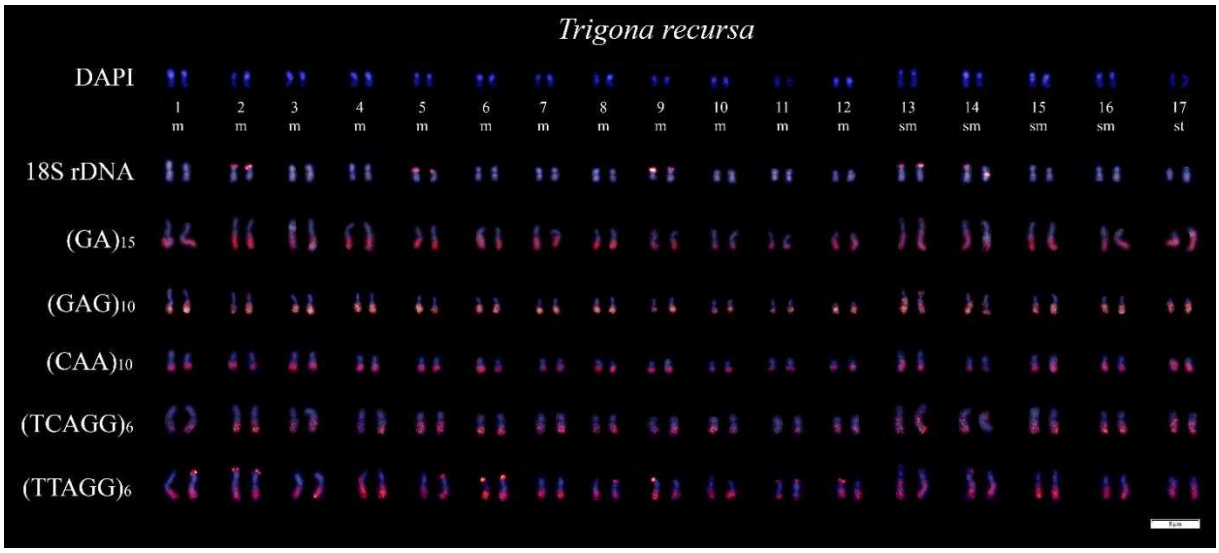
Fonte: Autoral.

Online Resource Fig. S17 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Trigona hyalinata*



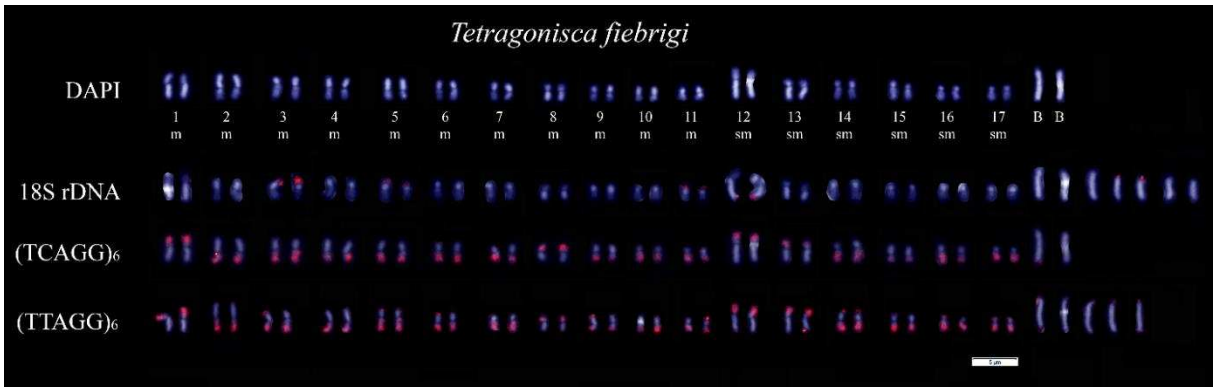
Fonte: Autoral.

Online Resource Fig. S18 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Trigona recursa*



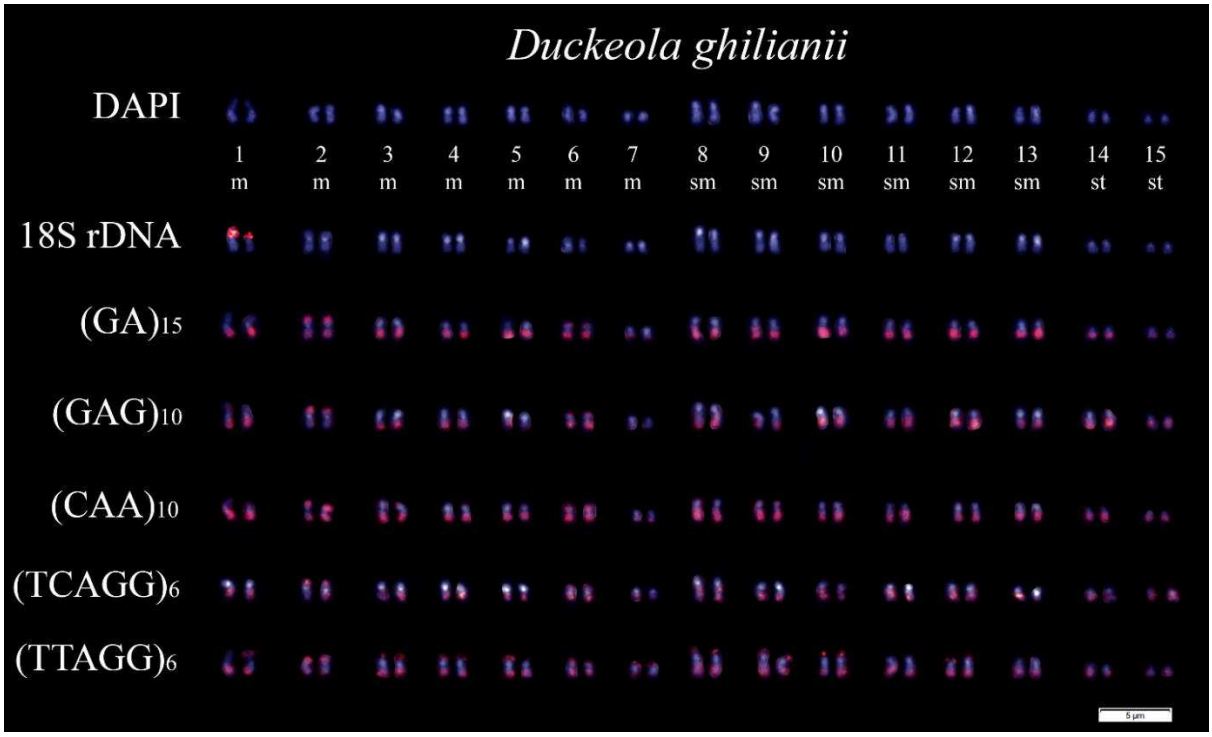
Fonte: Autoral.

Online Resource Fig. S19 Karyotype and chromosome mapping with 18S rDNA probe and $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Tetragonisca fiebrigi*



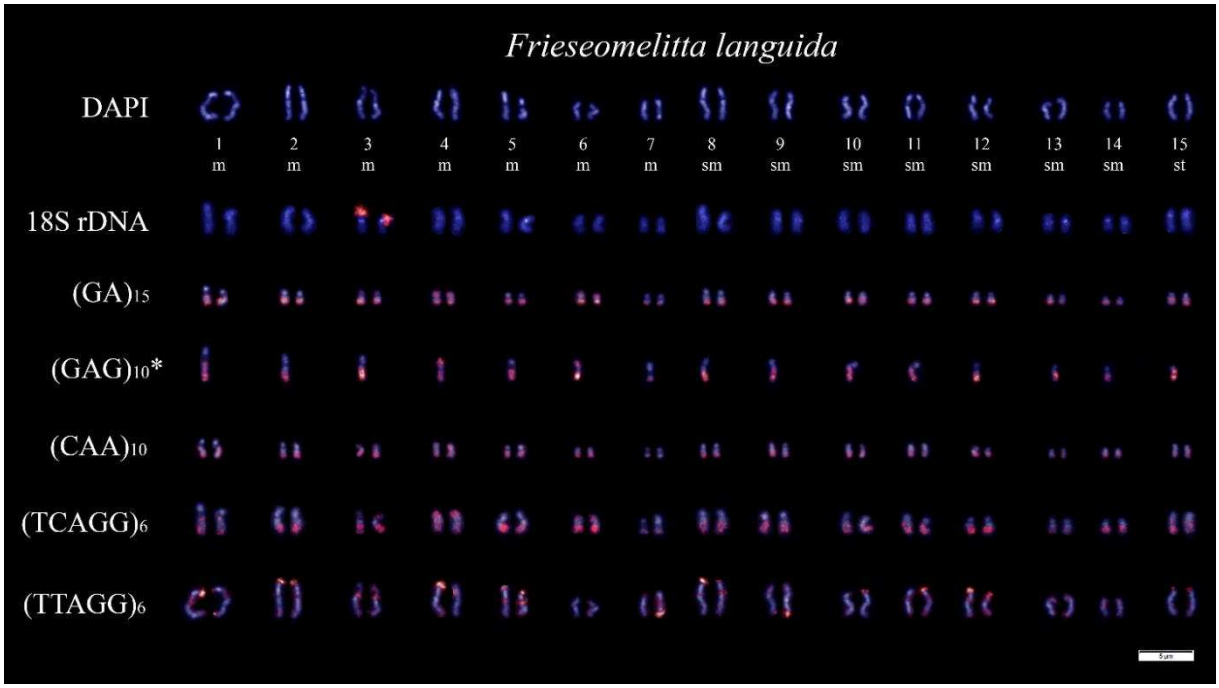
Fonte: Autoral.

Online Resource Fig. S20 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Duckeola ghilianii*



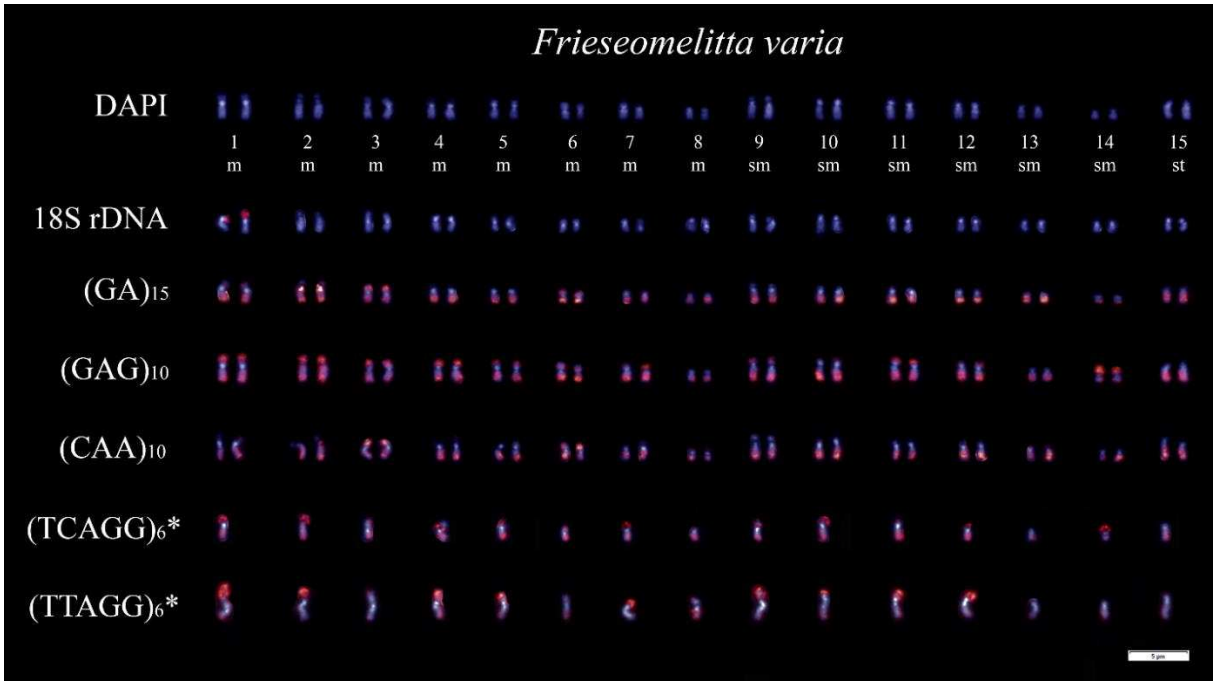
Fonte: Autoral.

Online Resource Fig. S21 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Frieseomelitta languida*. * indicate haploid male



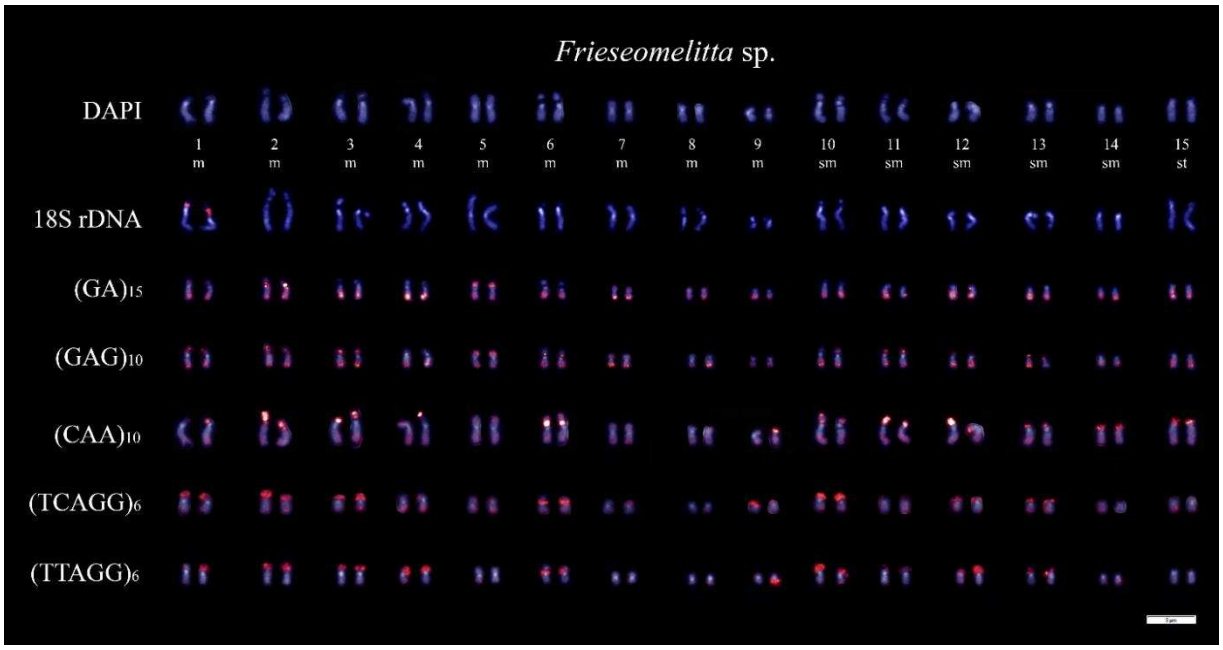
Fonte: Autoral.

Online Resource Fig. S22 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Frieseomelitta varia*. * indicate haploid male



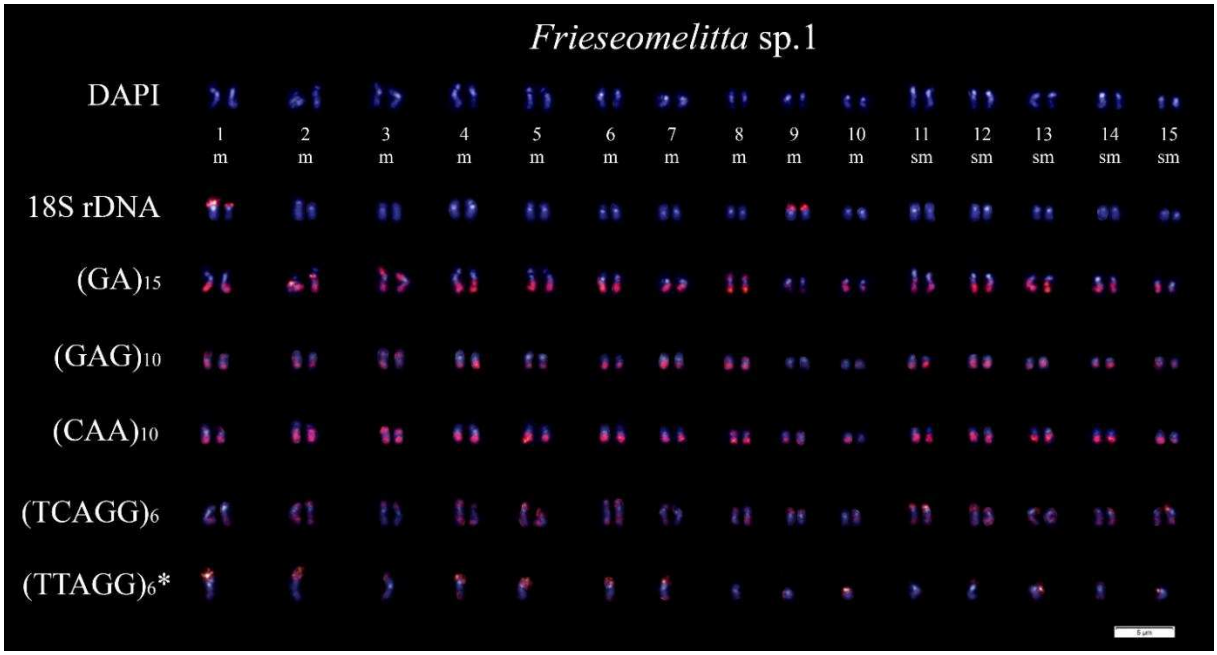
Fonte: Autoral.

Online Resource Fig. S23 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Frieseomelitta* sp.



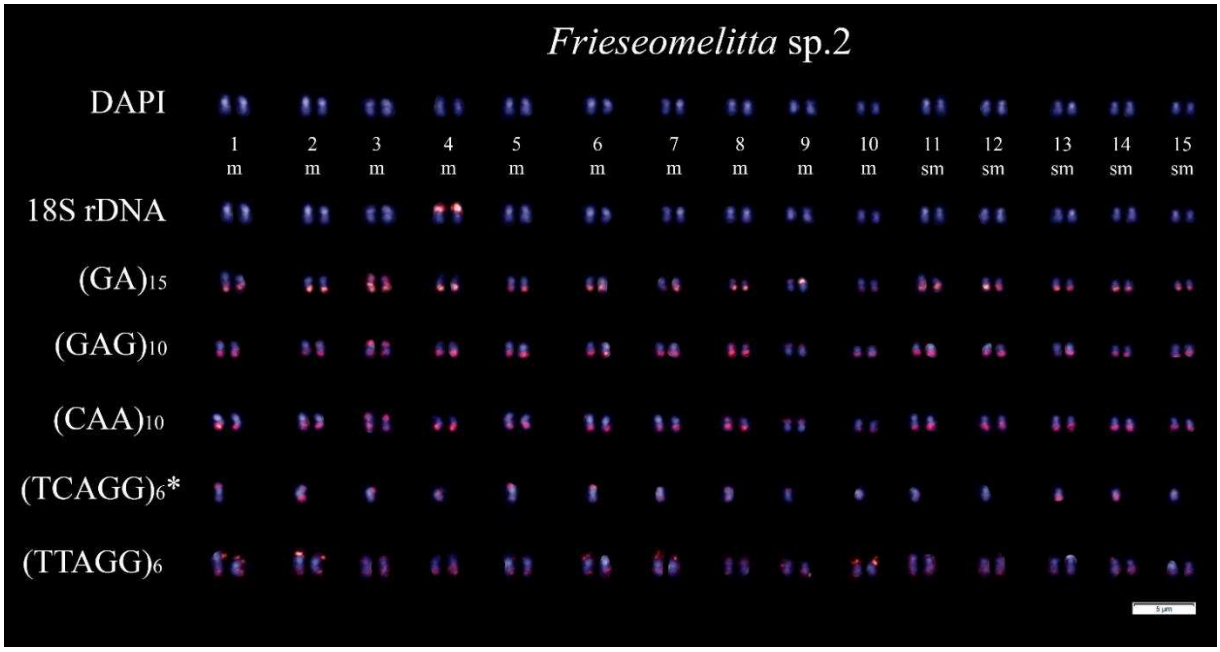
Fonte: Autoral.

Online Resource Fig. S24 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Frieseomelitta* sp.1. * indicate haploid male



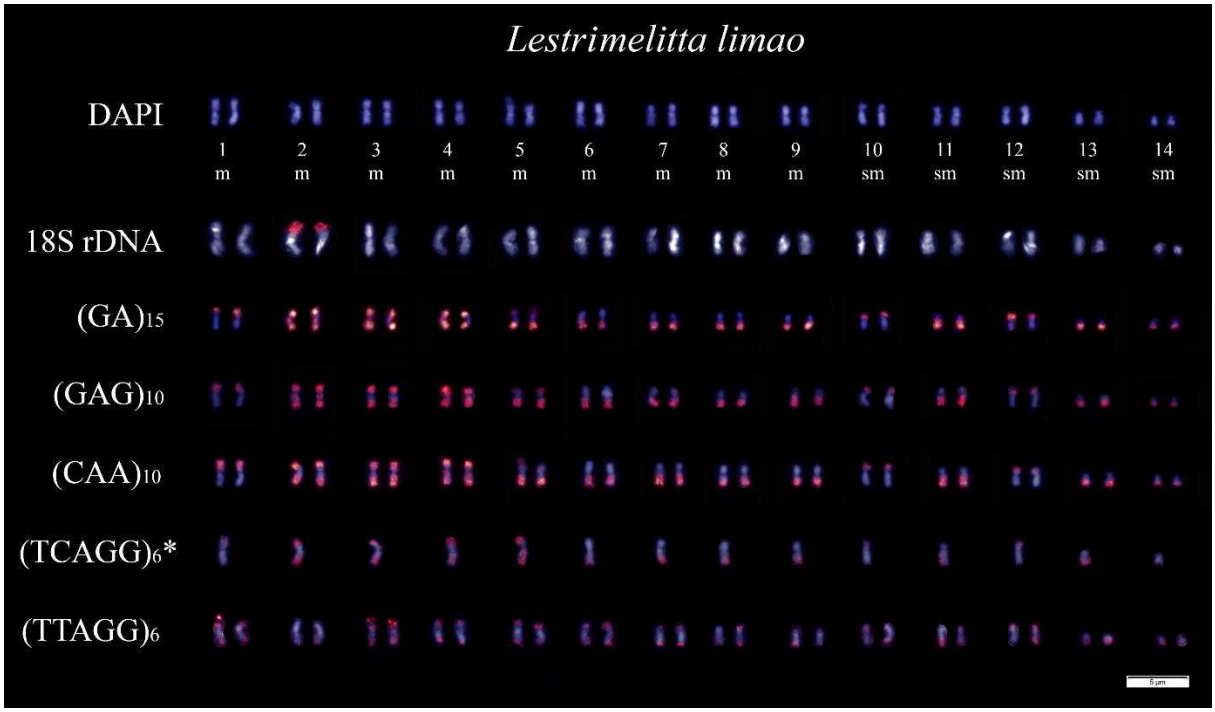
Fonte: Autoral.

Online Resource Fig. S25 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Frieseomelitta* sp.2. * indicate haploid male



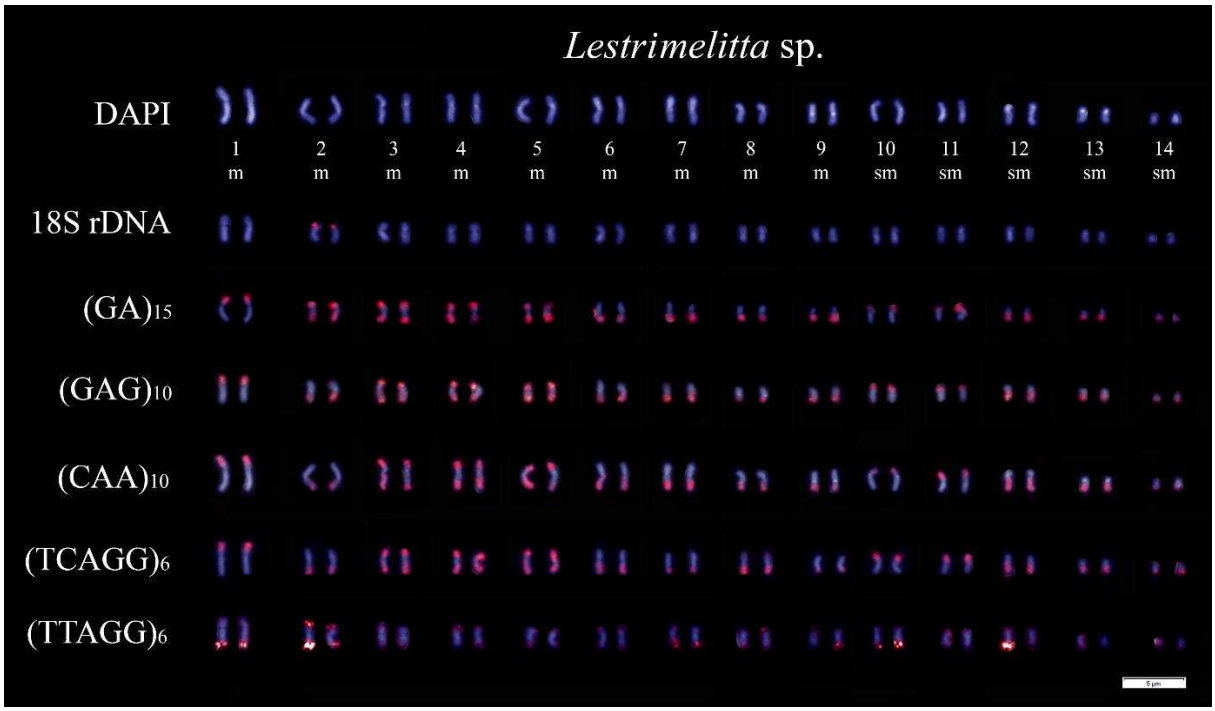
Fonte: Autoral.

Online Resource Fig. S26 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Lestrimelitta limao*. * indicate haploid male



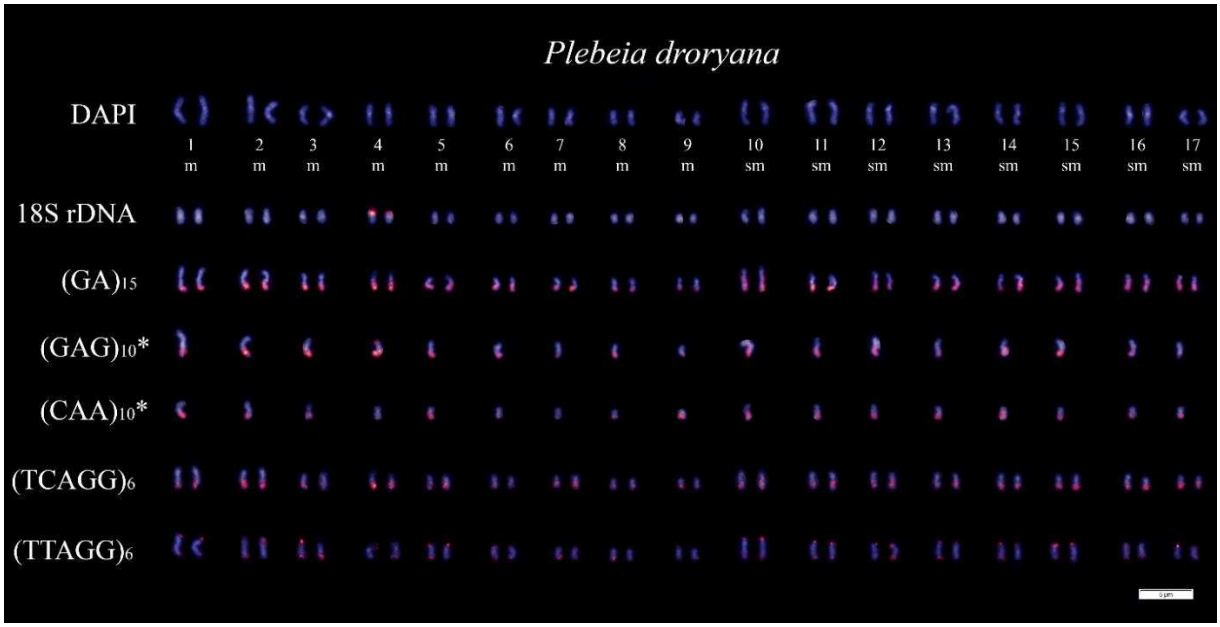
Fonte: Autoral.

Online Resource Fig. S27 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Lestrimelitta* sp.



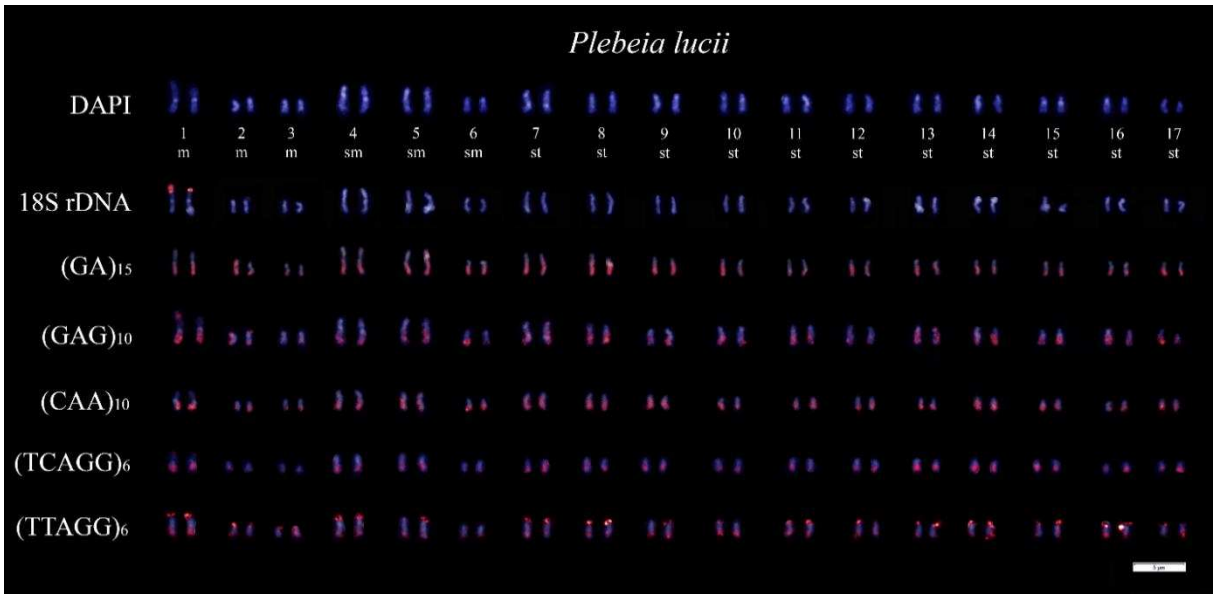
Fonte: Autoral.

Online Resource Fig. S28 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Plebeia droryana*. * indicate haploid male



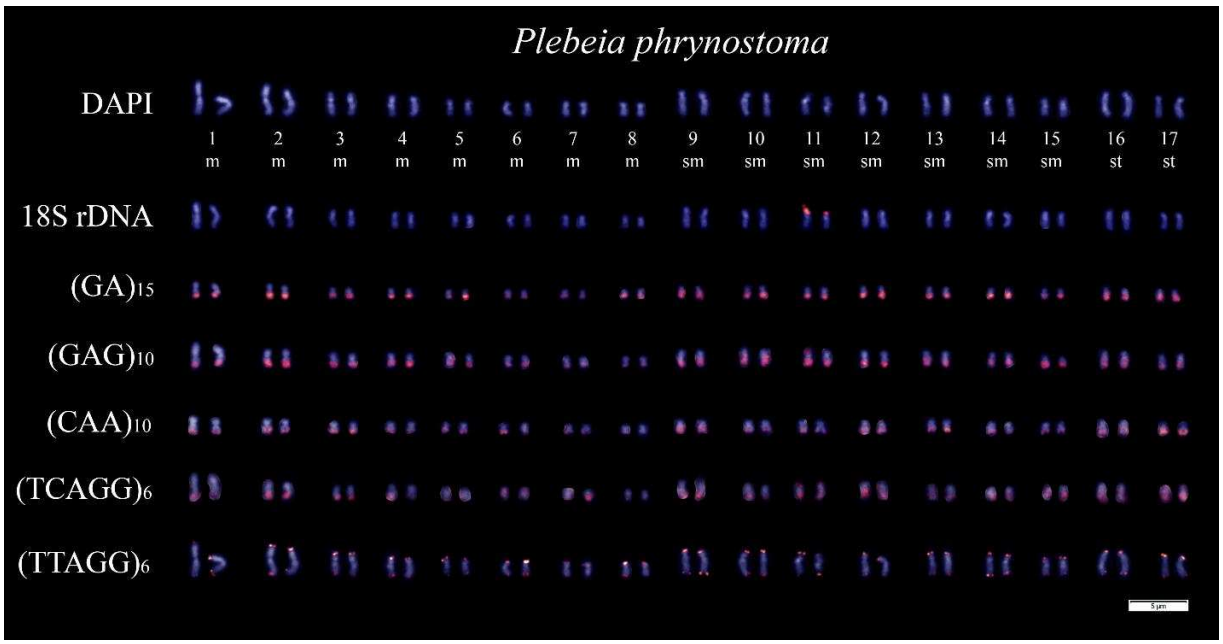
Fonte: Autoral.

Online Resource Fig. S29 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Plebeia lucii*



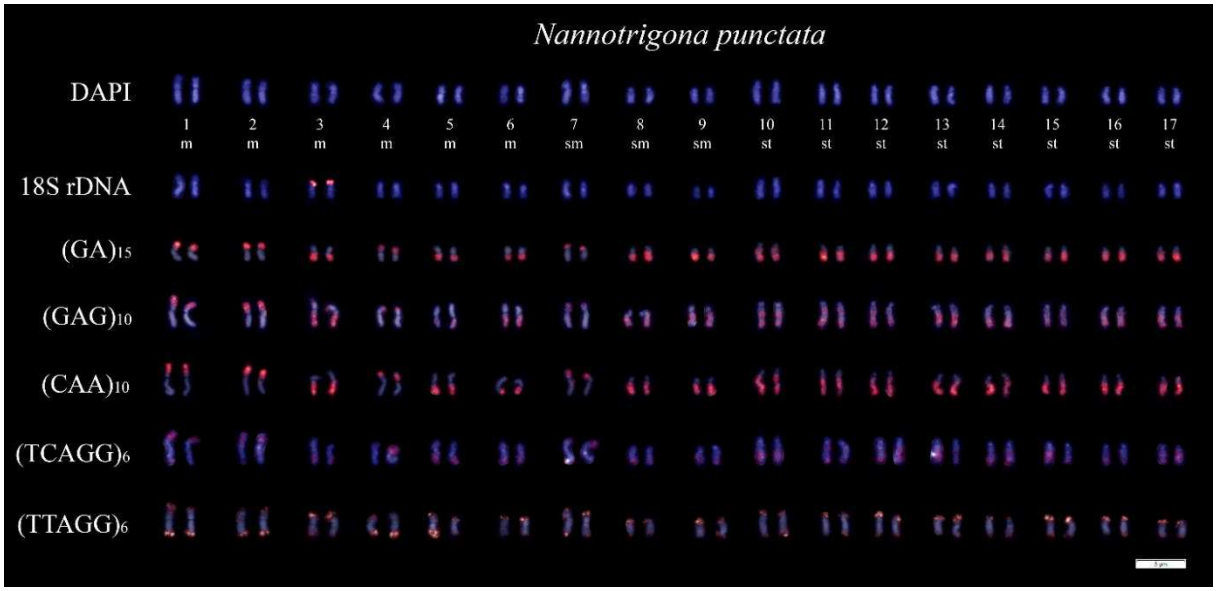
Fonte: Autoral.

Online Resource Fig. S30 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Plebeia phrynostoma*



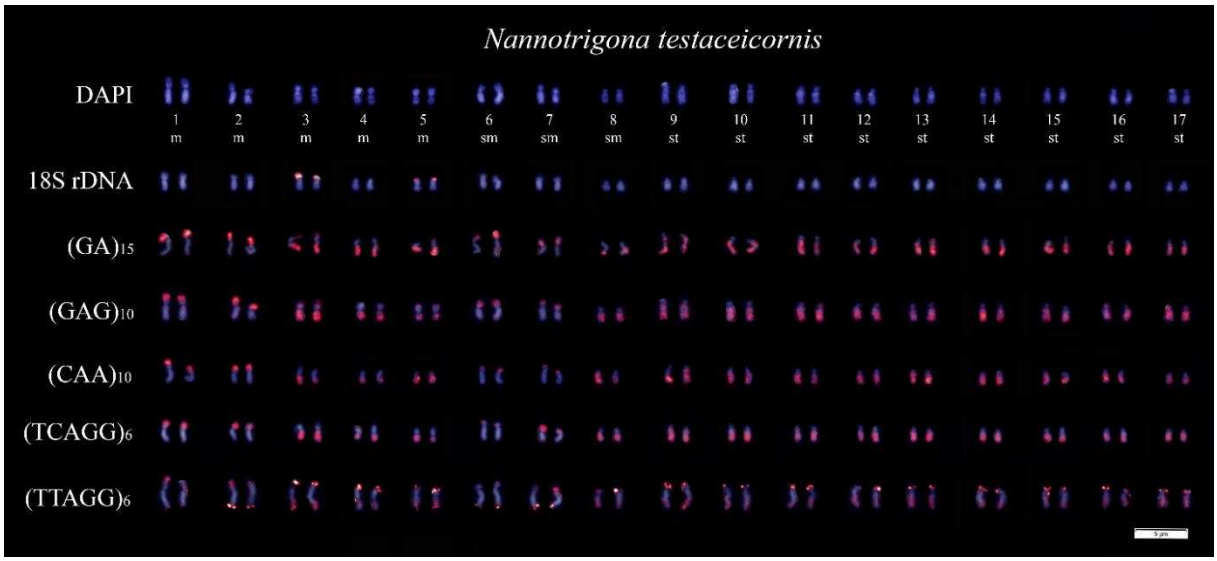
Fonte: Autoral.

Online Resource Fig. S31 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Nannotrigona punctata*



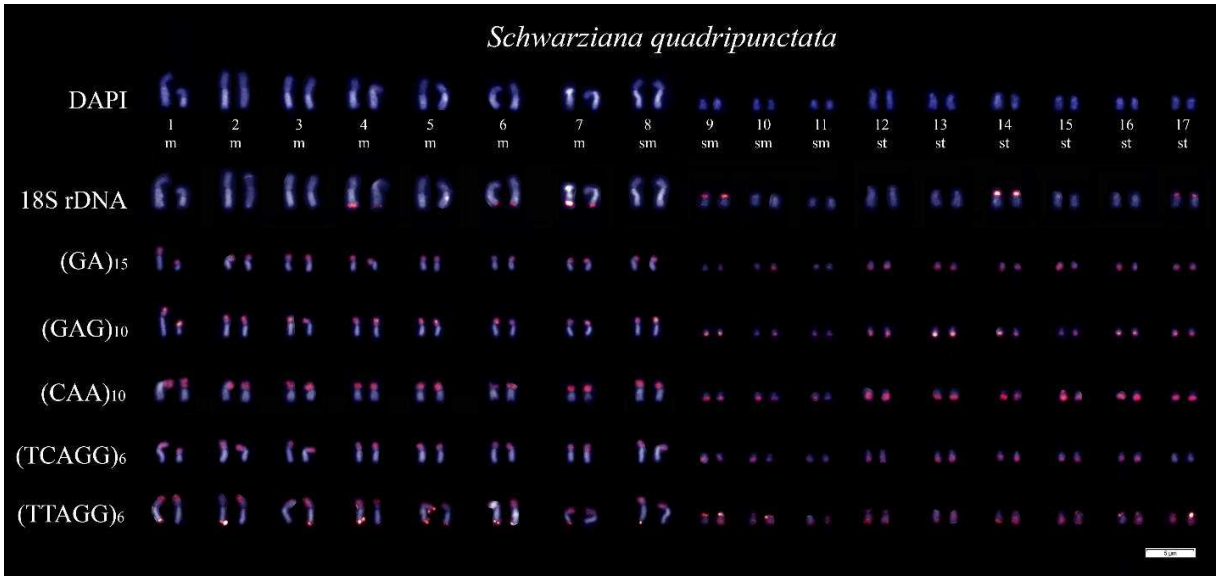
Fonte: Autoral.

Online Resource Fig. S32 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Nannotrigona testaceicornis*



Fonte: Autoral.

Online Resource Fig. S33 Karyotype and chromosome mapping with 18S rDNA probe and $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, $(TCAGG)_6$ and $(TTAGG)_6$ microsatellites in *Schwarziana quadripunctata*



Fonte: Autoral.

7 CONSIDERAÇÕES FINAIS

- O site www.bees.ufop.br disponibiliza, atualmente, dados citogenéticos de 56 gêneros de abelhas pertencentes a cinco subfamílias (Andreninae, Apinae, Collectinae, Halictinae e Megachilinae), sendo que duas subfamílias (Melittinae e Stenotritinae) ainda não possuem nenhum registro.

- O uso da técnica de C_0t-1 para produzir uma sonda com sequências altamente repetitivas que, posteriormente, foi hibridizada em espécies pertences aos quatro subgêneros de *Melipona*, permitiu novas inferências sobre o crescimento e acumulação da heterocromatina neste gênero.

- Uma ampla amostragem de espécies pertencentes aos três grupos de Meliponini neotropical foi utilizada para produzir dados empíricos em relação à número cromossômico, tamanho de genoma, padrões de distribuição de regiões ribossomais 18S rDNA e de microssatélites que corroboraram a importância das fusões Robertsonianas na evolução cariotípica da tribo.