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ORIGINAL RESEARCH ARTICLE

Cytotaxonomy and karyotype evolution in Neotropical Meliponini (Hymenoptera: Apidae) inferred by chromosomal mapping of 18S rDNA and five microsatellites

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ABSTRACT

The Neotropical Meliponini bees, commonly known as stingless bees, are phylogenetically subdivided into 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. In this way, we mapped 18S rDNA and five microsatellites in 33 stingless bee species collected from different Brazilian regions. The species belonged to 15 genera and showed six different chromosome numbers: $n = 8$, $n = 9$, $n = 11$, $n = 14$, $n = 15$, and $n = 17$. The 18S rDNA probe showed a variation from 2 to 12 marked chromosomes in different positions (terminal, subterminal, or centromeric), including 2 B chromosomes out of the 7 B found in *Tetragonisca fiebrigi*. The microsatellite $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(TCAGG)_6$ probes formed clusters on the euchromatic regions of the chromosomes and were useful in the identification of putative Robertsonian fusion events that led to the decrease in the chromosome number during the evolution of the Neotropical Meliponini clade. $(TTAGG)_6$ constituted the telomeric sequence of the analyzed species. The ancestral state of the three Neotropical Meliponini clades is difficult to infer, although, the putative ancestral karyotype probably had a single pair of 18S rDNA cistrons, and the decrease in chromosome number and increase in the 18S rDNA sites occurred independently between genera.

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18S rDNA; chromosome evolution; cytotaxonomy; microsatellites; molecular cytogenetics; stingless bees

Introduction

In recent decades, molecular biology has flourished with new technologies, contributing to advances in many areas, such as the study of animal cytogenetics, including cytotaxonomy (e.g., Barth et al., 2011; Palacios-Gimenez et al., 2015a; Santos et al., 2018), the origin of different sex chromosome systems (e.g., Palacios-Gimenez et al., 2013, 2015b), and evolution of supernumerary B chromosomes (e.g., Milani & Cabral-de-Mello, 2014; Ruiz-Ruano et al., 2015; Milani et al., 2017). Fluorescence *in situ* hybridization (FISH) has become one of the most important techniques in cytogenetics, allowing the localization of specific DNA sequences on chromosomes (Trask, 1991; Guerra, 2004). The most commonly studied sequences in insects are ribosomal DNA and microsatellite/-satellite sequences (Huang et al., 2016; Ruiz-Ruano et al., 2017; Andrade-Souza et al., 2018; Menezes et al., 2019; Travenzoli et al., 2019a; Teixeira et al., 2020; Pereira et al., 2020, 2021). Regardless of their coding function, these sequences are important for the structure, regulation, and evolutionary adaptation of the organism genome (Shapiro & Von Sternberg, 2005; Janssen et al., 2018).

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

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The cytogenetic data available on Neotropical stingless bees show a chromosome number variation ranging from $n = 8$ to $n = 17$, with the presence of three modal numbers: $n = 8$, $n = 15$, and $n = 17$ (reviewed in Cunha et al., 2021b). The Minimum Interaction Theory (MIT), initially proposed to explain ant karyotype evolution, predicts an ancestor with a low-numbered karyotype ($n < 12$) and, through a series of fission events, the chromosome number increases during the evolutionary time (Imai et al., 1986, 1988). Based on certain heterochromatic patterns, it became the most accepted theory to explain the karyotypic variation in the whole order Hymenoptera (Hoshiba & Imai, 1993; Pompolo & Campos, 1995; Rocha et al., 2003; Godoy et al., 2013). However, recent data have 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 may have led to a decrease in chromosome number during the evolution of the stingless bees (Tavares et al., 2017; Travenzoli et al., 2019b; Cunha et al., 2021c). Corroborating this high-numbered ancestral karyotype scenario in Neotropical Meliponini, high chromosome numbers are found in phylogenetically close tribes, $n = 16$ in Apini, $n = 15$-21 in Euglossini, and $n = 12$-26 in Bombini, and in other Meliponini branches, $n = 14$-18 in Meliponini Afrotropical and $n = 18$-20 in Meliponini Indo-Malay/Australasia (reviewed in Cunha et al., 2021b).

With the popularization of molecular cytogenetics and the characterization of the chromosomal location of 18S ribosomal sites and distinct microsatellite sequences, the amount of data on bee species has increased in recent years (Rocha et al., 2002; Brito et al., 2005; Andrade-Souza et al., 2018; Cunha et al., 2018; Piccoli et al., 2018; Santos et al., 2018; Silva et al., 2018; Travenzoli et al., 2019a; Gonçalves et al., 2020; Lopes et al., 2020; Pereira et al., 2021). 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., 2019; Cardoso & Cristiano, 2021), this study aimed to identify and discuss the major chromosomal rearrangements that occurred during the Neotropical Meliponini karyotype evolution using 18S rDNA and five microsatellites as chromosomal markers.

Materials and methods

Thirty-three species were collected from different Brazilian regions encompassing the three main clades of Neotropical Meliponini (Rasmussen & 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 the cerebral ganglia of larvae or pre-pupae (Imai et al., 1988). The karyotypic formula and chromosome measurements of 31 species are described elsewhere (Cunha et al., 2021c), and the karyotype of *Tetragonisca fiebrigi* and *Plebeia phrynostoma* were measured using Image-Pro Plus® software and the chromosomes were classified according 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 by Pinkel et al. (1986) uses six repetitive DNA sequences as probes, the ribosomal 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′ (Cunha et al., 2018). 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 53 F 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 drawn using Easy Idio software (Diniz & 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, with two marked chromosomes as the most common pattern observed in 23 species. The ribosomal markings were located in different positions, terminal, sub-terminal, or centromeric, although the terminal position was the most common chromosomal location observed in 25 species. They were mostly present on the short arms, only *Tetragonisca fiebrigi* and *Schwarziana quadripunctata* had ribosomal markings on the long arm in some

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* (Figure S1)	Passos, Minas Gerais	8	2 centromeric (pair 2)
	Celetrigona longicornis (Figure S2)	Nova Xavantina, Mato Grosso	15	4 terminal (pairs 7, 11)
	Trigonisca sp. (Figure S3)	Urbano Santos, Maranhão	15	2 terminal (pair 6)
2	*Melipona* sp. (Figure S4)	Brasília, Distrito Federal	9	2 centromeric (pair 1)
	Melipona quinquefasciata (Figure S5)	Piumhi, Minas Gerais	9 + 3B[#]	2 centromeric (pair 1)
	Melipona fasciculata (Figure S6)	São Luís, Maranhão	9	2 sub-terminal (pair 1)
	Melipona fulva (Figure S7)	Presidente Figueiredo, Amazonas	9	2 terminal (pair 1)
	Melipona scutellaris (Figure S8)	Nordeste	9	2 terminal (pair 4)
	Melipona cf. *rufiventris* (Figure S9)	Iranduba, Amazonas	9	2 terminal (pair 2)
	Melipona lateralis (Figure S10)	Presidente Figueiredo, Amazonas	11	2 terminal (pair 2)
	Melipona seminigra pernigra (Figure S11)	Altamira, Pará	11	2 terminal (pair 4)
3	*Scaptotrigona* sp. (Figure S12)	Pará	17	6 terminal (pairs 1, 2, 5)
	Scaptotrigona xanthotricha (Figure S13)	Viçosa, Minas Gerais	17	11 terminal (pairs 1, 2, 3, 4[‡], 5, 7)
	Geotrigona subterranea (Figure S14)	Passos, Minas Gerais	17	4 sub-terminal (pairs 4, 14)
	Cephalotrigona capitata (Figure S15)	Viçosa, Minas Gerais	17	2 sub-terminal (pair 1)
	Cephalotrigona femorata (Figure S16)	Urbano Santos, Maranhão	17	2 sub-terminal (pair 2)
	Trigona hyalinata (Figure S17)	Viçosa, Minas Gerais	17	6 terminal (pairs 5, 9, 14)
	Trigona recursa (Figure S18)	Januária, Minas Gerais	17	10 terminal or sub-terminal (pairs 2, 5, 9, 13, 14)
	Tetragonisca fiebrigi (Figure S19)	Palotina, Paraná	17 + 7B[#]	8 terminal + 2B (pairs 3, 4, 11, 12 + 2B)
	Duckeola ghilianii (Figure S20)	Presidente Figueiredo, Amazonas	15	2 terminal (pair 1)
	Frieseomelitta languida (Figure S21)	Arcos, Minas Gerais	15	2 terminal (pair 3)
	Frieseomelitta varia (Figure S22)	Uberlândia, Minas Gerais	15	2 terminal (pair 1)
	Frieseomelitta sp. (Figure S23)	Brasília, Distrito Federal	15	2 sub-terminal (pair 1)
	Frieseomelitta sp.1 (Figure S24)	Presidente Figueiredo, Amazonas	15	4 terminal (pairs 1, 9)
	Frieseomelitta sp.2 (Figure S25)	Iranduba, Amazonas	15	2 terminal (pair 4)
	Lestrimelitta limao (Figure S26)	Brazil	14	2 terminal (pair 2)
	Lestrimelitta sp. (Figure S27)	Domingos Martins, Espírito Santo	14	2 terminal (pair 2)
	Plebeia droryana (Figure S28)	Santo Antônio do Jacinto, Minas Gerais	17	2 terminal (pair 4)
	Plebeia lucii (Figure S29)	Viçosa, Minas Gerais	17	2 terminal (pair 1)
	Plebeia phrynostoma (Figure S30)	Espírito Santo	17	2 terminal (pair 11)
	Nannotrigona punctata (Figure S31)	Altamira, Pará	17	2 terminal (pair 3)
	Nannotrigona testaceicornis (Figure S32)	Viçosa, Minas Gerais	17	4 terminal (pairs 3, 5)
	Schwarziana quadripunctata (Figure 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 homologous chromosomes was marked with the 18S rDNA probe..

chromosome pairs. Heteromorphisms regarding the rDNA cluster size or position 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 of 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 Figure 1.

The microsatellite $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(TCAGG)_6$ probes formed clusters on the euchromatic region of the chromosomes, although complete euchromatic chromosomes were not entirely marked (Figure 2, Online Resource Figures S1–S33). All chromosomes had regions marked with these probes and were marked on one chromosome arm or both arms, depending on the analyzed species. Microsatellite markings in only one chromosome arm were 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 the other arm were observed in the following species: *Leurotrigona muelleri* (both arms were marked in seven out of the eight 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 showed microsatellite markings in the 18S rDNA region with some of the probes.

The microsatellite patterns in *Frieseomelitta* sp. and *Frieseomelitta varia* were different from the other *Frieseomelitta* species. They had different microsatellite 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, occasionally forming clusters along one chromosome arm (Online Resource Figures S1–S33).

B chromosomes were found in *M. quinquefasciata* (up to 3) and in *T. fiebrigi* (up to 7) (Figure 1). These supernumerary chromosomes were mostly heterochromatic and were marked in both termini with the telomeric $(TTAGG)_6$ probe. Of the seven Bs found in *T. fiebrigi*, two were marked by the 18S rDNA probe, and one had a small euchromatic region marked by the

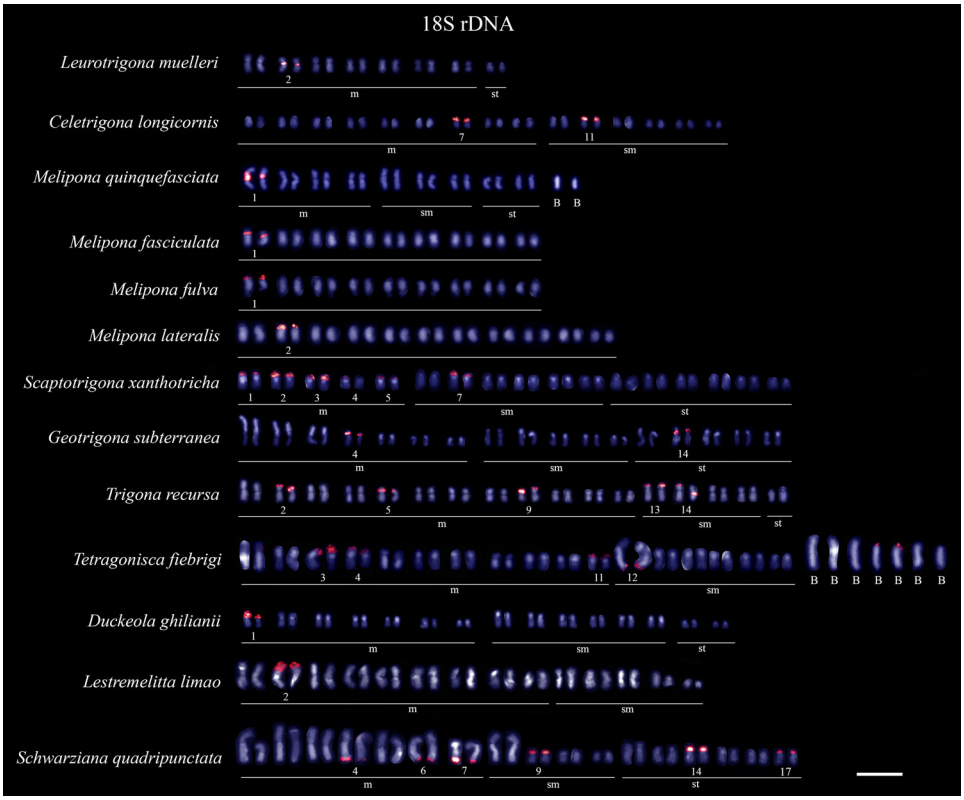

Figure 1. Variation in chromosome number ($2n = 16$ to $2n = 34$, plus B chromosomes) and 18S rDNA (2 to 12 marked chromosomes) in representative species from the three Neotropical Meliponini clades. Chromosomes were classified as metacentric (m), submetacentric (sm), and subtelocentric (st). Bar 5 μm.

microsatellite $(TCAGG)_6$ probe (Online Resource Figure S19). The results are summarized in the idiogram of Figure 3, except for the telomeric probe that would mix with the other microsatellite patterns. Supplementary information provides data on each species to facilitate visualization of the microsatellite patterns in each taxon (Online Resource Figures S1–S33).

Discussion

Chromosome numbers varying from $n = 8$ to $n = 17$ were within the observed range in the Neotropical stingless bee clade (reviewed in Cunha et al., 2021b). The Minimum Interaction Theory (MIT) predicts that during evolution, chromosome number has a tendency to increase associated with concomitant heterochromatin amplification to stabilize the new telomeres (Imai et al., 1988, 2001). However, some Meliponini species do not seem to fit this model, as we observed species with a high-numbered karyotype ($n = 17$) and low heterochromatin content, such as *Cephalotrigona capitata* (Online Resource Figure S15). Recently, the hypothesis of chromosome fusions from a high-numbered ancestral karyotype ($n = 18$) was suggested through meta-analyses using a molecular phylogenetic approach to explain the chromosomal evolution of the Meliponini tribe, indicating that $n = 17$ is the putative ancestral haploid number of the Neotropical clade (Travenzoli et al., 2019b). Therefore, with the empirical cytogenetic data presented in this study, we corroborate the importance of Robertsonian fusions in the karyotype evolution of stingless bees.

Microsatellite occurrence restricted to one chromosome arm is probably the ancestral condition as it presents a broad phylogenetic distribution, observed in the species with $n = 15$ from clade 1 and with $n = 17$ 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, $n = 8$ 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 evidence of fusions from the plesiomorphic condition shared by *Celetrigona* and *Trigonisca*, i.e., $n = 15$ and microsatellite markings in only one chromosome arm (Figure 3).

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 chromosome arm (Figure 3). Later in the evolution

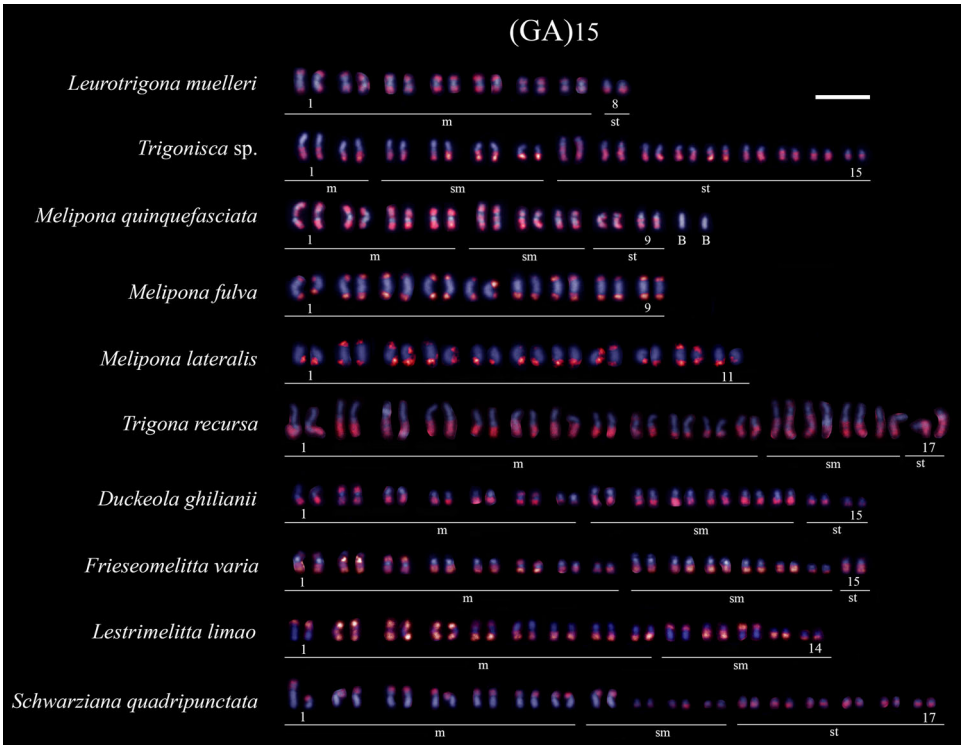

Figure 2. Chromosome mapping with $(GA)_{15}$ microsatellite probe showing one or both chromosome arms marked in representative species from the three Neotropical Meliponini clades. Chromosomes were classified as metacentric (m), submetacentric (sm), and subtelocentric (st). Bar 5 μm.

of the genus *Melipona*, Robertsonian fission events led to an increase in chromosome number in *Melipona lateralis* and *Melipona seminigra pernigra* (both with $n = 11$ and six chromosomes with microsatellites in both arms). These results indicate that after the fusion events that established $n = 9$ in *Melipona*, subsequent fission events increased the chromosome number in *M. lateralis* (Online Resource Figure S10), *M. seminigra pernigra* (Online Resource Figure S11), and in the other *M. seminigra* subspecies (Francini et al., 2011; Andrade-Souza et al., 2018), probably reflecting the proximity of these taxa (Cunha et al., 2020).

In clade 3, the plesiomorphic condition of microsatellite markings in only one arm was shared by all species with $n = 17$ (Figure 3). The $n = 14$ of *Lestrimelitta* spp. could be interpreted as a result of three Robertsonian fusions, and 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 have microsatellite markings in both arms in two chromosome pairs, suggesting two Robertsonian fusions. Although *Trigona braueri* was not analyzed in this study, the cytogenetic evidence of a fusion rearrangement in one chromosome pair was responsible for the decrease in the chromosome number in this species from $n = 17$ to $n = 16$ (Domingues et al., 2005; Cunha et al., 2021b).

An unconformity with the other *Frieseomelitta* species was observed in *Frieseomelitta* sp. and *Frieseomelitta varia* ($n = 15$ from clade 3), which indicated the apomorphic traits of these species. Chromosome breakpoints could be associated with different types of rearrangements in addition to fusions and fissions, such as inversions, transpositions, and reciprocal translocations (Coghlan et al., 2005). Inversions are often associated with local adaptations and speciation (Kirkpatrick & Barton, 2006; Hoffmann & Rieseberg, 2008) and could have contributed to the diversity of markings observed in these two *Frieseomelitta*. $(TCAGG)_6$ 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 regions on several chromosomes. This trait is unique to the genus *Frieseomelitta* (Online Resource Figures S21–S25).

One chromosome pair bearing of the 18S rDNA cistrons is the most common pattern observed in the analyzed species. It was observed in most species of clade 1, all species of clade 2, and more than half of species of clade 3, possibly constituting the plesiomorphic condition in the Neotropical Meliponini (Figure 3). Single 18S rDNA is also recognized as a putative ancestral pattern in Hymenoptera in general (Menezes et al., 2021; Teixeira et al., 2021). The centromeric position found only in *L. muelleri* and the low heterochromatin content *Melipona* species may be a consequence of the Robertsonian fusion events in these taxa, whereas the high heterochromatin content *Melipona* species acquired the

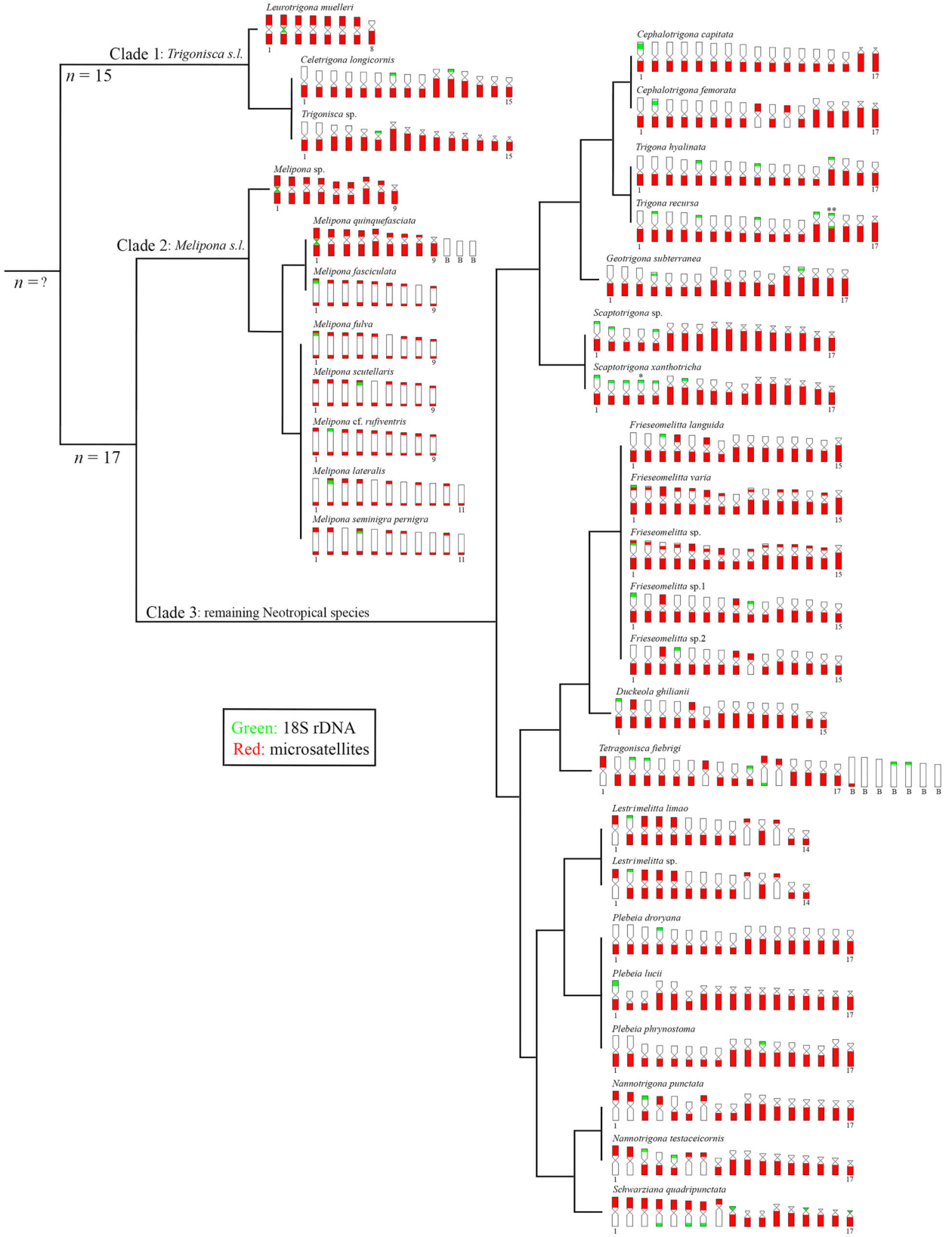

Figure 3. Haploid idiogram of the karyotypes from the Neotropical Meliponini species. The 18S rDNA are shown in green, and the microsatellites $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(TCAGG)_6$ are shown in red. The phylogenetic structure was redrawn from Rasmussen and Cameron (2010, Figure 3).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

terminal position secondarily due to the accentuated heterochromatin amplification (Cunha et al., 2020; Pereira et al., 2021).

The expansion in the number of ribosomal sites only occurred in species with the terminal location of these genes; centromeric/interstitial positioning

prevents or at least makes recombination in these regions difficult (Sochorová et al., 2018; Hirai, 2020). The increase in the number of sites could be explained by ectopic recombination mediated or not by transposable elements (Silva et al., 2013; Menezes et al., 2019; Piscor et al., 2020; Hirai, 2020). In addition to diversification in the number of sites, we also observed the amplification of ribosomal copies in only one homologous chromosome in some species. This heteromorphism could be generated through gene amplification caused by different sources, such as unequal crossover, gene conversion, and gene duplication (Eickbush & Eickbush, 2007; Schubert & Lysak, 2011; Teixeira et al., 2021). Another heteromorphism, detected only in *T. recursa*, refers to the different 18S rDNA positions between the homologous chromosomes of pair 14 (terminal x interstitial), which could be a consequence of a heterozygous inversion.

Polymorphisms outside the 18S region were also observed in this study. Some minor size differences between homologous chromosomes, such as in *Scaptotrigona* sp., *G. subterranea*, and *N. testaceicornis*, could be associated with heterochromatin amplification events in one homologue, as has been 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 by unequal crossing over (Cunha et al., 2021c). The unequal sister chromatid exchange leads to the deletion of one chromosome region and duplication in the other (Schubert & Lysak, 2011). This could explain the change in the chromosome pair bearer of the 18S rDNA in *Melipona scutellaris* from pair 4 in a homomorphic colony (Figure 1) to pair 1 in a heteromorphic colony (Piccoli et al., 2018). Therefore, without meiotic experiments to confirm the pairing of heteromorphic chromosomes, the 18S rDNA probe is a useful marker in mitotic metaphases to study this type of polymorphism in *Melipona* species.

We found up to 3 B chromosomes in *M. quinquefasciata* and up to 7 B in *T. fiebrigi*. Up to four supernumerary chromosomes have been reported in the former (Silva et al., 2018) and two in the latter (Barth et al., 2011). Theories involving B chromosomes as parasites of the host genome predict that the host would develop mechanisms to avoid the accumulation of these elements in a coevolutionary arms race (Camacho et al., 2002). In general, Bs are small and present in small numbers (reviewed in Camacho, 2004), such as those observed in *M. quinquefasciata*. In contrast, *T. fiebrigi* B chromosomes are as large as the A-chromosomes and present in great numbers, suggesting that the B invasion could have happened recently and the host genome did not have time to develop mechanisms to avoid their spread and suppress the B drive; alternatively, *T. fiebrigi* may have developed a certain tolerance for the negative B effects. To date, 7 B chromosomes is the highest number found in the same individual of a stingless bee, observed in *T. fiebrigi* (present study) and *Partamona helleri* (Martins et al., 2014).

The Bs found in *M. quinquefasciata* are completely heterochromatic and are not marked by any microsatellite besides the telomeric probe (Online Resource Figure S5). Among the seven Bs found in *T. fiebrigi*, in addition to the telomeric probe, one B was marked by the $(TCAGG)_6$ microsatellite, and two were marked with the 18S rDNA probe (Online Resource Figure S19). The small portions of euchromatin in these B chromosomes show that they may contain genes, as already suggested in other species (Ruiz-Estevez et al., 2012; Banaei-Moghaddam et al., 2015; Valente et al., 2017), favoring its presence and accumulation (Camacho et al., 2000; Gonzalez-Sanchez et al., 2004; Montiel et al., 2014) or, at least, playing a role in B chromosome evolution (Ruiz-Ruano et al., 2015, 2019; Ahmad et al., 2020).

The canonic (TTAGG)n is the telomeric sequence observed in stingless bees (Travenzoli et al., 2019a; present study) and, despite the evidence of Robertsonian fusions in this study, internal telomeric sequences (ITS) were not observed among the chromosomes of most species. 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; Schubert & Lysak, 2011; Warchałowska-Śliwa 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), such as inversions and translocations that served as hotspots for recombination events (reviewed in Bolzan, 2017).

Conclusions

In summary, 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 in clades 2 and 3 could be better explained based on an ancestral karyotype of $n = 17$ (Figure 3). 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. The low sampling of clade 1 species in the

Travenzoli et al. (2019b) study could have underestimated the weight of $n = 15$ as the putative ancestral karyotype. If $n = 17$ was the ancestral karyotype of Neotropical Meliponini, evidence of the fusion events responsible for the decrease in chromosome number in the ancestor of clade 1 had already been erased by subsequent chromosomal rearrangements. The ancestral state of the three Neotropical Meliponini clades is difficult to infer. Therefore, the putative ancestral karyotype probably had a single pair of 18S rDNA cistrons, and the decrease in chromosome number and increase in the 18S rDNA sites occurred independently between genera.

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