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Cytogenetic Analysis and Chromosomal Mapping of Repetitive DNA in *Melipona* Species (Hymenoptera, Meliponini)

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Keywords
Euchromatin · Fluorescence in situ hybridization · Heterochromatin · Molecular cytogenetics · Nucleotide constitution

Abstract
Stingless bees of the genus *Melipona* are subdivided into 4 subgenera called *Eomelipona*, *Melikerria*, *Melipona* sensu stricto, and *Michmelia* according to species morphology. Cytogenetically, the species of the genus *Melipona* show variation in the amount and distribution of heterochromatin along their chromosomes and can be separated into 2 groups: the first with low content of heterochromatin and the second with high content of heterochromatin. These heterochromatin patterns and the number of chromosomes are characteristics exclusive to *Melipona* karyotypes that distinguish them from the other genera of the Meliponini. To better understand the karyotype organization in *Melipona* and the relationship among the subgenera, we mapped repetitive sequences and analyzed previously reported cytogenetic data with the aim to identify cytogenetic markers to be used for investigating the phylogenetic relationships and chromosome evolution in the genus. In general, *Melipona* species have 2n = 18 chromosomes, and the species of each subgenus share the same characteristics in relation to heterochromatin regions, DAPI/CMA_3 fluorophores, and the number and distribution of 18S rDNA sites. Microsatellites were observed only in euchromatin regions, whereas the $(TTAGG)_6$ repeats were found at telomeric sites in both groups. Our data indicate that in addition to the chromosome number, the karyotypes in *Melipona* could be separated into 2 groups that are characterized by conserved cytogenetic features and patterns that generally are shared by species within each subgenus, which may reflect evolutionary constraints. Our results agree with the morphological separation of the *Melipona* into 4 subgenera, suggesting that they must be independent evolutionary lineages.

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Table 1. Species of *Melipona*, geographical coordinates, and collection sites in different states of Brazil

Species	GPS coordinates	Locality
M. asilvai Moure, 1971	15°38′2.99″S; 44°18′55.03″W	Pedra de Maria da Cruz, Minas Gerais
M. bicolor Lepeletier, 1836	20°45′19.74″S; 42°52′6.92″W	Viçosa, Minas Gerais
M. capixaba Moure & Camargo, 1994	20°21′34.68″S; 40°39′39.78″W	Domingos Martins, Espírito Santo
M. fasciculata Smith, 1854	03°44′22.53″S; 52°19′39.15″W	Altamira, Pará
M. flavolineata Friese, 1900	03°15′10.09″S; 43°18′5.12″W	Urbano Santos, Maranhão
M. fuliginosa Lepeletier, 1836	03°15′10.09″S; 43°18′5.12″W	Urbano Santos, Maranhão
M. mandacaia Smith, 1863	11°18′12.80″S; 41°51′22.14″W	Irecê, Bahia
M. mondury Smith, 1863	20°45′19.74″S; 42°52′6.92″W	Viçosa, Minas Gerais
M. paraensis Ducke, 1916	02°52′20.05″S; 52°15′56.23″W	Altamira, Pará
M. puncticollis Friese, 1902	02°52′20.05″S; 52°15′56.23″W	Altamira, Pará
M. quadrifasciata Lepeletier, 1836	20°45′19.74″S; 42°52′6.92″W	Viçosa, Minas Gerais
M. quinquefasciata Lepeletier, 1836	19°53′7.00″S; 43°39′51.93″W	Caeté and Viçosa, Minas Gerais
M. rufiventris Lepeletier, 1836	18°51′28.76″S; 46°41′12.27″W	Guimarânia, Minas Gerais
M. scutellaris Latreille, 1811	12°33′39.53″S; 41°23′40.64″W	Lençóis, Bahia
M. seminigra pernigra Moure & Kerr, 1950	02°52′20.05″S; 52°15′56.23″W	Altamira, Pará
M. subnitida Ducke, 1910	06°43′36.17″S; 36°46′17.89″W	Santana do Seridó, Rio Grande do Norte

The genus *Melipona* Illiger, 1806 comprises stingless bees belonging to the Meliponini tribe, which are widespread throughout the Neotropical region [Camargo and Pedro, 2013; Michener, 2013]. Based on species morphology, the genus is divided into 4 subgenera: *Eomelipona*, *Melikerria*, *Melipona* sensu stricto, and *Michmelia* [Camargo and Pedro, 2008], among which *Eomelipona* is considered polyphyletic and the others monophyletic [Ramírez et al., 2010; Rasmussen and Cameron, 2010]. Cytogenetically, *Melipona* is the most studied genus of the tribe and differs from the other Meliponini genera [reviewed in Tavares et al., 2017]. According to karyotype analysis, *Melipona* bees have a diploid chromosome number of 2n = 18 for females and n = 9 for males [reviewed in Tavares et al., 2017] and are subdivided into 2 groups according to heterochromatin content and distribution patterns [Rocha and Pompolo, 1998]. Species with a low proportion of heterochromatin observed in the pericentromeric region, such as *M. bicolor* Lepeletier, 1836 and *M. subnitida* Ducke, 1910 with 8% and 17% of heterochromatin regions, respectively, belong to Group I, whereas those with a high content of heterochromatin distributed along chromosomes, such as *M. crinita* Moure & Kerr, 1950 with 54% and *M. fuscopilosa* Moure & Kerr, 1950 with 73% of heterochromatin constitute Group II [Rocha et al., 2002]. It should be noted that the large proportion and dispersion of heterochromatin in Group II species obscures the visualization of centromeres and cytogenetic markers on the chromosomes, and thus impedes determination of the karyotype formula. Nevertheless, the distinct karyotype structure of *Melipona* species (heterochromatin patterns and bias in the regular chromosome number) suggests that the evolutionary history of this genus is different from that of the other Meliponini genera.

Mapping of microsatellite DNA by fluorescence in situ hybridization (FISH) is a valuable technique that has provided insights into genome structure and evolution of different taxa [Cuadrado and Jouve, 2011; Palacios-Gimenez and Cabral-de-Mello, 2015; Cunha et al., 2016; Peixoto et al., 2016]. Microsatellites, also known as short sequence repeats, are short tandem repeats of 2 to 7 nucleotides, which are widely distributed in genomes [Cuadrado and Jouve, 2011]. Microsatellites can be observed in heterochromatin [Cuadrado and Jouve, 2011], euchromatin [Cuadrado and Jouve, 2007], as well as in centromeric [Cuadrado and Jouve, 2007] and telomeric regions [Hatanaka et al., 2002]. They are considered as important polymorphic markers for population genetics studies [Goldstein and Schlötterer, 1999], mainly because there is evidence that each group of species underwent preferential accumulation of specific microsatellites in chromosomes [Tóth et al., 2000]. For example, in the grasshopper species *Abracris flavolineata* (De Geer, 1773), *Eyprepocnemis plorans* (Charpentier, 1825), and *Locusta migratoria* Linnaeus, 1758, microsatellites showed the same uneven and nonrandom distribution, with clear predominance of dinucleotide motifs dispersed in euchromatin regions [Ruiz-Ruano et al., 2015]. Therefore, microsatellite distribution patterns are considered important char-

Table 2. Cytogenetic data available for *Melipona* species

Species[a]	2n	Karyotype	Hetero-chromatin content	Genome size, pg	Number and patterns of bands/signals on chromosomes						
					CMA_3	18S/45S	Microsatellites				
							$(GA)_{15}$	$(GAG)_{10}$	$(CAA)_{10}$	$(CGG)_{10}$	$(TTAGG)_6$
Eomelipona											
M. asilvai[b]	18[c]	6m+10sm+2a*	Low[c]	0.29[d]	2 int[e]	2 int*/–	disp, eu*	disp, eu*	disp, eu*	disp, eu*	ter*
M. bicolor[b]	18[c]	6m+8sm+4a*	Low[c, u]	0.28[d]	2 int[e]	–/2 peri[u]	disp, eu*	disp, eu*	ter, eu*	–	ter*
M. marginata[b]	18[f]	–	Low[c]	0.28[d]	2 int[e]	2 int/–	–	–	–	–	–
M. puncticollis	18[g]	2m+14sm+2a[h]	Low[h]		2 int[h]	2 int[h]/–	disp, eu*	disp, eu*	ter, eu*	–	ter*
Melikerria											
M. fasciculata[b]	18[i]	–	High[j, u]	0.78[d]	2 int[j, u]	2 int, close to junction of eu- and heterochromatin*/2 int[u]	ter, eu*	ter, eu*	ter, eu*	ter, eu*	ter*
M. grandis	18[u]	–	High[u]		int[u]	–/2 int[u]	–	–	–	–	–
M. interrupta	18[k]	–	High[l]		2 int[l]	2 int, close to junction of eu- and heterochromatin[l]/–	ter, eu[l]	ter, eu[l]	ter, eu[l]	ter, eu[l]	ter[l]
M. quinquefasciata[b]	18[k]	10m+6sm+2a[m]	Low[m]	0.70[d]	2 int[m]	–/–	disp, eu*	disp, eu*	–	–	ter*
Melipona											
M. favosa	18[n]	2m+4sm+2a[n]	–		–	–/–	–	–	–	–	–
M. mandacaia	18[o]	2m+14sm+2a[o]	Low[o]	0.35[d]	2 peri[o]	–/–	disp, eu*	–	–	–	ter*
M. quadrifasciata[b]	18[f]	4m+12sm+2a[p]	Low[c, u]	0.27[d]	2 peri[e, u]	2 int*/2 peri[u]	disp, eu*	disp, eu*	ter, eu	–	ter*
M. subnitida	18[q]	4m+10sm+4a*	Low[e]	0.27[d]	2 peri[e]	–/–	disp, eu*	disp, eu*	ter, eu*	–	ter*
Michmelia											
M. capixaba[b]	18[c]	–	High[c]	1.38[d]	2 ter[e]	–/–	ter, eu*	ter, eu/disp on chromosomes*	ter, eu*	–	ter*
M. captiosa	18[c]	–	High[c]		–	–/–	–	–	–	–	–
M. crinita	18[e]	–	High[e, u]	0.73[d]	4 ter[e]	–/2 ter[u]	–	–	–	–	–
M. flavolineata	18[j]	–	High[j, u]		2 ter[j, u]	–/2 ter[u]	ter, eu*	ter, eu*	ter, eu*	–	ter*
M. fuscopilosa	18[c]	–	High[c, u]	1.10[d]	2 ter[e, u]	–/2 ter[u]	–	–	–	–	–
M. mondury[b]	18[v]	–	High[u, v]	0.95[r]	2 ter[u, v]	–/2 ter[u]	ter, eu*	ter, eu*	ter, eu*	ter, eu*	ter*
M. nebulosa	18[u]	–	High[u]		4 ter[u]	–/–	–	–	–	–	–
M. paraensis[b]	18[w]	–	High[h]		2 ter[h]	2 ter[h]/2 ter[u]	ter, eu*	–	ter, eu*	–	–
M. rufiventris[b]	18[f]	–	High[v]	0.93[r]	2 ter[r]	–/–	ter, eu*	ter, eu*	ter, eu*	ter, eu*	ter*
M. scutellaris	18[c]	–	High[c, u]	1.08[d]	2 ter[e]	–/2 ter[u]	ter, eu	ter, eu/disp on chromosomes*	ter, eu*	–	ter*
M. seminigra abunensis	22[u]	–	High[u]		2 ter[u]	–/2 ter[u]	–	–	–	–	–
M. seminigra merrillae	22[s]	–	High[s]	0.85[d]	–	–/2 ter[u]	–	–	–	–	–
M. seminigra pernigra	22[t]	–	High[h, u]		2 ter[h,u]	2 ter[h]/2 ter[u]	–	–	ter, eu*	–	ter*

a, acrocentric; disp, dispersed; eu, euchromatin; int, interstitial; m, metacentric; peri, pericentromeric; sm, submetacentric; ter, terminal.

[a] Subgenera are based on Camargo and Pedro [2013]. [b] Species with polymorphism. [c] Rocha and Pompolo, 1998. [d] Tavares et al., 2010. [e] Rocha et al., 2002. [f] Kerr, 1948. [g] Silva et al., 2013. [h] Cunha et al., 2018. [i] Kerr, 1952. [j] Lopes et al., 2011. [k] Kerr, 1969. [l] Travenzoli, 2018. [m] Rocha, 2002. [n] Hoshiba, 1988. [o] Rocha et al., 2003. [p] Silva et al., 2012. [q] Silveira, 1971. [r] Lopes et al., 2009. [s] Francini et al., 2011. [t] Silva et al., 2014. [u] Andrade-Souza et al., 2018. [v] Lopes et al., 2008. [w] Cassinela et al., 2013. * This study.

acteristics for understanding chromosome repatterning and genome organization and evolution [Ruiz-Ruano et al., 2015].

Thus, in order to analyze the chromosomal organization in *Melipona* and to determine the distribution of microsatellites (random or nonrandom) in the genomes of the different subgenera, we performed comparative cytogenetic analyses and physical mapping of repetitive sequences on the chromosomes of different *Melipona* species. We aimed to clarify whether microsatellite distribution is distinct among the subgenera and to identify new cytogenetic markers to contribute to the elucidation of the chromosome evolution in this group of bees.

Materials and Methods

We collected published data on *Melipona* cytogenetics, as chromosome number, karyotype formula, and distribution patterns of heterochromatin (DAPI/CMA_3 staining) and repetitive DNA elements, including rDNA.

For further molecular cytogenetic analyses, metaphase chromosomes were obtained as previously described by Imai et al. [1988] from the brain ganglia at the last larval instar of 16 *Melipona* species collected from 10 localities in Brazil (Table 1). Chromosomes were stained with Giemsa and, when possible, the karyotype formula was determined based on the arm ratio according to Levan et al. [1964]. Oligonucleotide probes $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(CGG)_{10}$, as well as the telomeric probe $(TTAGG)_6$ were directly labeled with Cy3 at the 5′ end (Sigma, St. Louis, MO, USA), and genetic mapping was performed by FISH according to Pinkel et al. [1986], with some modifications: metaphase chromosomes were denatured in 70% formamide/2× SSC at 75°C for 5 min; the probes were hybridized with chromosomes in 20 μL of hybridization mix (200 ng of labeled probe, 2× SSC, 50% formamide, and 10% dextrane sulfate). This hybridization mix was heated for 10 min at 85°C, and the slides were kept in a moist chamber at 37°C overnight. Then, the slides were washed in 4× SSC/Tween and dehydrated in an alcohol series. Finally, the chromosomes were counterstained with DAPI (DAPI Fluorshield, Sigma Aldrich) after FISH. In all analyses, 15 individuals were used, and 10 metaphases were examined on average per slide. Images were obtained under an Olympus BX53 microscope with an Olympus DP73F camera and analyzed using the CellSens Imaging software.

Results and Discussion

Diploid Number and Karyotype Formula

Among the 73 species described for the genus *Melipona*, 25 have already been analyzed cytogenetically (Table 2). The earliest karyotype description published in 1948 for *M. marginata* Lepeletier, 1836 revealed that the chromosome number for this species was 2n = 18. With the exception of *M. seminigra merrillae* Cockerell, 1919, *M. seminigra pernigra* Moure & Kerr, 1950, and *M. seminigra abunensis* Cockerell, 1912 which have 2n = 22, all other *Melipona* species have 2n = 18, indicating that this chromosome number is conserved in *Melipona* [Andrade-Souza et al., 2018; Cunha et al., 2018]. In general, bees show few variations in chromosome numbers within a particular genus; it concerns both social species such as *Bombus* Smith, 1869 (n = 18), *Frieseomelitta* Ihering, 1912 (n = 15), *Partamona* Schwarz, 1939 (n = 17), and *Trigona* Jurine, 1807 (n = 17) [Owen et al., 1995; reviewed in Tavares et al., 2017] as well as solitary species such as *Euglossa* (n = 21) [Fernandes et al., 2013]. The maintenance of constant chromosome numbers within bee genera suggests the existence of a mechanism preventing chromosomal changes such as robertsonian rearrangements and aneuploidy. Probably many chromosomal changes are little supported by these organisms. Another possibility is because in haplodiploid organisms genetic variations are transmitted slowly, large rearrangements are only observed among phylogenetically distant species. However, ants, which are also haplodiploid, exhibit significant variability in chromosome numbers among the species of the same genus as shown for *Dolichoderus* Lund, 1831 (2n = 10, 18, 20, 22, 28, and 38) [reviewed in Cardoso et al., 2018], *Mycetophylax* Emery, 1913 (2n = 13, 15, and 18) [Cardoso et al., 2014], and *Trachymyrmex* Forel, 1983 (2n = 12, 18, 20, and 22) [reviewed in Barros et al., 2018]. Thus, the haplodiploid sex determination system alone may not account for the low variability in chromosome numbers within so many genera of the Apidae family. The numerical conservation of bee karyotypes supports the idea of the "optimal karyotype" proposed by Bickham and Baker [1979], suggesting that chromosomal variations are a consequence of selective pressure and that with time, the rates of the chromosomal evolution would become slower, ultimately resulting in karyotype stability.

Although the chromosome number is conserved in *Melipona*, there are differences in the chromosome structure, as evidenced by the karyotype formulas of the species (Fig. 1; Table 2). Some chromosomal rearrangements alter the karyotype morphology of the group, increasing the number of metacentric and submetacentric chromosomes in some *Melipona* species or the number of telocentric chromosomes in others [Rocha and Pompolo, 1998; Rocha et al., 2002, 2003]. These observations are consistent with the recent hypothesis which is based on phylogenetic reconstruction and which states that re-

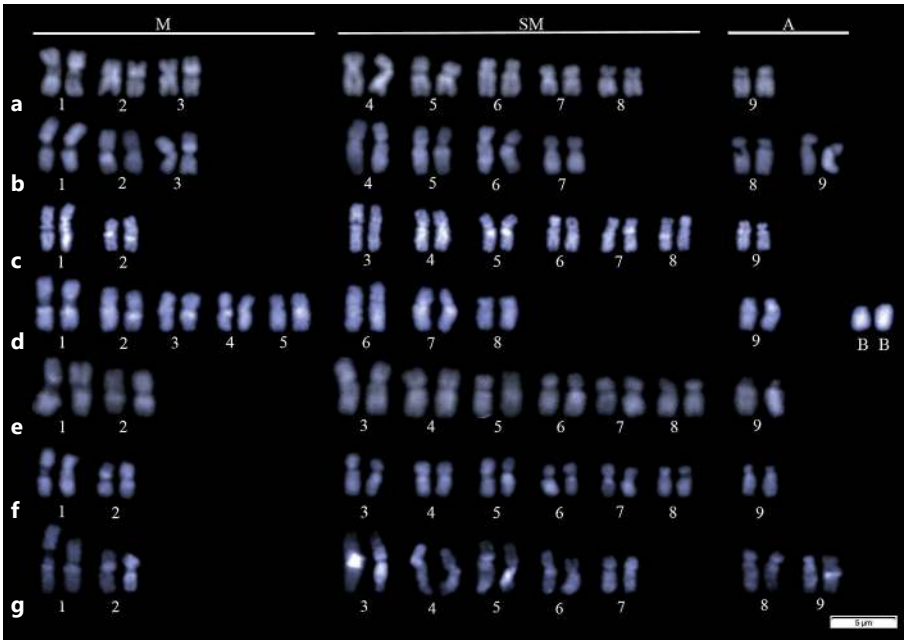

Fig. 1. Karyotypes of *Melipona* species. **a** *M. asilvai*. **b** *M. bicolor*. **c** *M. puncticollis*. **d** *M. quinquefasciata*. **e** *M. mandacaia*. **f** *M. quadrifasciata*. **g** *M. subnitida*. Chromosomes are stained with DAPI. M, metacentric; SM, submetacentric; A, acrocentric.

peated centric fusions may be responsible for the decrease of the chromosome number in *Melipona* [Travenzoli, 2018].

Chromosomal Polymorphism

As mentioned above, there are 2 types of chromosomal polymorphisms in *Melipona*, numerical and structural. Numerical polymorphisms are uncommon and have been observed only in *M. rufiventris* Lepeletier, 1836 and *M. quinquefasciata* Lepeletier, 1836 due to the presence of small accessory B chromosomes, which are heterochromatic and can vary from 1 to 4 in *M. quinquefasciata* [Rocha, 2002], whereas only 1 is present in *M. rufiventris* [Lopes et al., 2008]. The B chromosome of *M. rufiventris* is DAPI-positive ($DAPI^+$) but CMA_3-negative (CMA_3^-), which is similar to the heterochromatin composition of the A complement, suggesting that this chromosome may have originated by a fission of heterochromatic regions from the main genome [Lopes et al., 2008]. In *M. quinquefasciata*, it has been observed that although the species has a low heterochromatin content, the B chromosomes are mostly heterochromatic and have a molecular structure similar to that of chromatin in the Group II species [Rocha, 2002]. It is possible that the B chromosomes in *M. quinquefasciata* are due to amplification and subsequent cleavage of heterochromatin in chromosomes in a species with low heterochromatin content [Rocha, 2002].

In *Melipona*, structural polymorphisms are more frequent than numerical ones, and size variations between homologous chromosomes have been described. In *M. mondury* Smith 1863 and *M. rufiventris*, variations in heterochromatin content (C-bands) are related to the presence of heteromorphic chromosome pairs. Thus, it was suggested that heterochromatin duplications are responsible for the size difference between homologous chromosomes in a large metacentric pair [Lopes et al., 2008]. Already in *M. scutellaris* Latreille, 1811 [Piccoli et al., 2018], *M. asilvai* Moure, 1971, *M. bicolor*, *M. capixaba* Moure & Camargo, 1994, *M. crinita*, *M. fasciculata* Smith, 1854, *M. quadrifasciata* Lepeletier, 1836, *M. marginata,* and *M. seminigra*, polymorphisms were detected in GC-rich regions (CMA_3^+) with a higher rate than in the homologous regions of the other species [Rocha et al., 2002; Lopes et al., 2011; Andrade-Souza et al., 2018]. Such polymorphisms, which are generally observed in nucleolus organizer regions (NORs), may be related to distinct gene regulation in these chromosomes [Rocha et al., 2002], given small size differences between homologous chromosomes.

Among the analyzed species, *M. capixaba* and *M. flavolineata* presented a polymorphism in the first chromosome pair in 1 of the 2 colonies, which was found in all metaphase chromosomes of the examined individual bees (Fig. 2; Table 3). Both species had 1 colony with a homomorphic karyotype, in which the first pair was

Table 3. Analyzed *Melipona* species with and without chromosomal polymorphism

Species	Karyotype	Specimens, *n*		Metaphases, *n*	
		Male	Female	Male	Female
Melipona capixaba	No polymorphism	1	8	18	50
	With polymorphism	2	13	20	50
Melipona flavolineata	No polymorphism	1	16	17	50
	With polymorphism	1	9	11	50

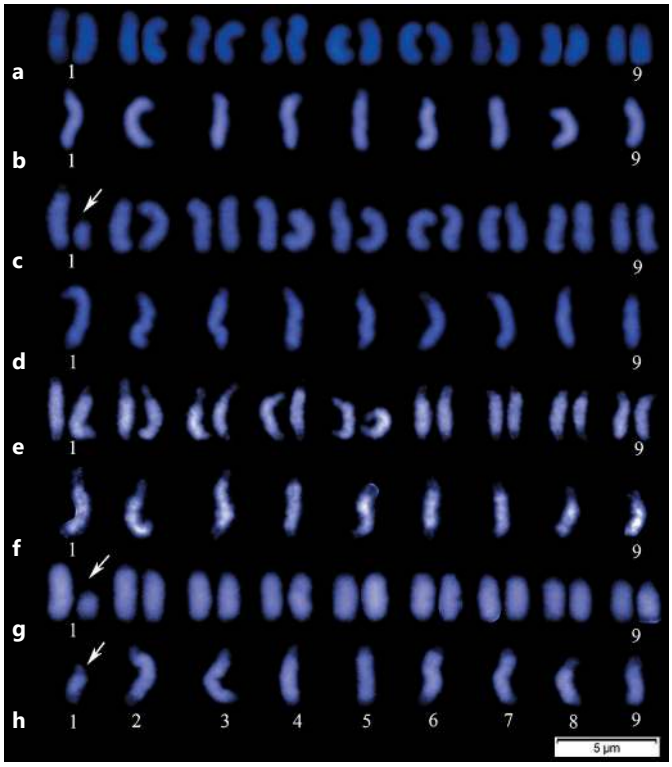

Fig. 2. Karyotypes of *Melipona capixaba* (**a–d**) and *Melipona flavolineata* (**e–h**). **a**, **b**, **e**, **f** Individuals without polymorphism. **a**, **e** Females (2n = 18). **b**, **f** Males (2n = 9). **c**, **d**, **g**, **h** Individuals with polymorphism in the first chromosome pair (arrows). **c**, **g** Females. **d**, **h** Males. Chromosomes are stained with DAPI.

formed by 2 large chromosomes, and another colony with a heteromorphic karyotype, in which one of the homologous chromosomes was twice the size of the other one (Fig. 2c, g, h). In *M. capixaba*, a smaller homologous chromosome was present only in females, in contrast to *M. flavolineata* in which both males and females had the smaller chromosome (Fig. 2c, g, h). Such structural chromosome polymorphism in *M. capixaba* and *M. flavolineata* has not been reported previously [Rocha and Pompolo, 1998; Rocha et al., 2002; Lopes et al., 2011]. Large size variations between homologous chromosomes of the first pair are not caused by differences in CMA_3^+ regions or rDNA but rather by duplications of regions due to slippage or uneven crossing-over.

Heterochromatic Patterns and Chromatin Composition

Although *Melipona* species have the same chromosome number, they demonstrate distinct patterns of heterochromatin content and distribution [Rocha et al., 2002]. Taking this into account, the genus is subdivided into 2 groups characterized by low and high heterochromatin amount, respectively (Fig. 3). In Group I, heterochromatin is observed in the pericentromeric region, whereas in Group II, it is dispersed along most chromosomes [Rocha and Pompolo, 1998; Andrade-Souza et al., 2018; Cunha et al., 2018]. Differences in genome size (DNA content) among *Melipona* species seem to conform to the group division [Tavares et al., 2010], indicating that the observed variation may be due to heterochromatin duplication or deletion. One exception is *M. quinquefasciata*, which, despite a low heterochromatin amount in the genome, has a high DNA content of 0.70 pg, while other Group I species have DNA contents between 0.27 and 0.35 pg. Further, the difference observed in *M. quinquefasciata* can be attributed to the presence of B chromosomes, and it has been suggested that the higher DNA amount compared to the other species from the same group with low heterochromatin content is likely due to these chromosomes [Tavares et al., 2010]. Among the species of Group I, *M. subnitida* has the karyotype with the highest proportion of heterochromatin (17%) [Rocha et al., 2002], possibly because of a large heterochromatic block present in the pericentromeric region of one of the chromosome pairs that was not observed in any other species with low heterochromatin content. All *Melipona* species, independent of the subgenus, have hetero-

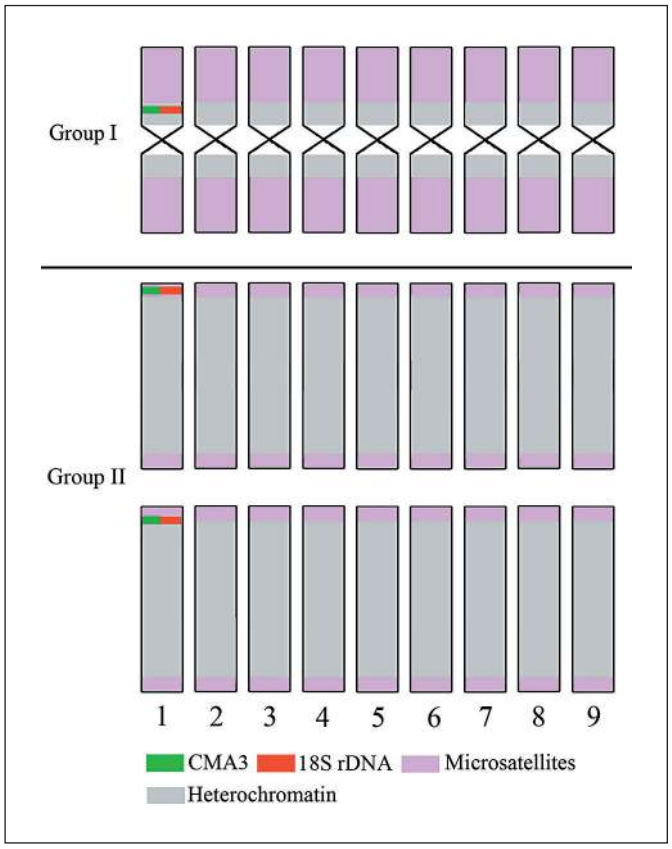

Fig. 3. Schematic illustration showing the location of bands/hybridization signals in the chromosomes of *Melipona* Group I and II species.

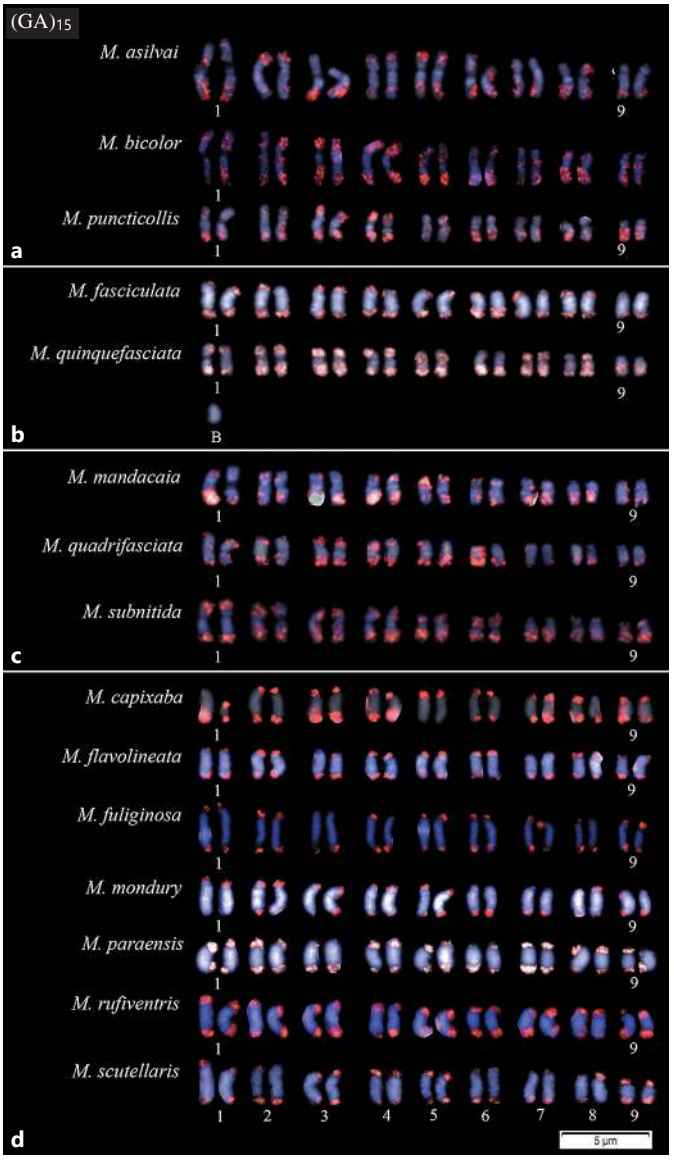

Fig. 4. FISH with the $(GA)_{15}$ probe in *Melipona* species belonging to the subgenera *Eomelipona* (**a**), *Melikerria* (**b**), *Melipona* sensu stricto (**c**), and *Michmelia* (**d**). Chromosomes are stained with DAPI, probe signals are in red.

chromatin rich in AT base pairs ($DAPI^+$), suggesting that in both Group I and II, heterochromatin regions have the same structure and nucleotide composition and possibly the same evolutionary history.

When we evaluated the heterochromatin organization from a taxonomic perspective, we verified that all species of the subgenera *Eomelipona* and *Melipona* sensu stricto had a low and those of *Michmelia* had a high heterochromatin content. In the subgenus *Melikerria*, 2 of the 3 cytogenetically analyzed species had a high heterochromatin content, and 1 had a low content [Rocha et al., 2002; Tavares et al., 2010; Lopes et al., 2011; Andrade-Souza et al., 2018; Travenzoli, 2018]. Previous studies found a correlation between heterochromatin content in *Melipona* species and the phylogenetic position of the genus and its subgenera, suggesting that the origin of heterochromatin and variations in its content occurred at different periods of the *Melipona* evolutionary history and that a low content of heterochromatin is possibly a plesiomorphy (shared ancestral trait) for both groups [Lopes et al., 2011; Andrade-Souza et al., 2018; Cunha et al., 2018; Piccoli et al., 2018].

In addition, the *Melipona* species cytogenetically analyzed here or in previous studies had 2 more evident markers with the base-specific fluorophore CMA_3 that stained 1 pair of chromosomes, which coincided with rDNA [Rocha et al., 2002; Cunha et al., 2018] (Table 2). Other Meliponini genera (*Partamona* Schwarz, 1939 and *Scaptotrigona* Moure, 1942) also showed correlations between $DAPI^+$ regions and heterochromatin and between CMA_3^+

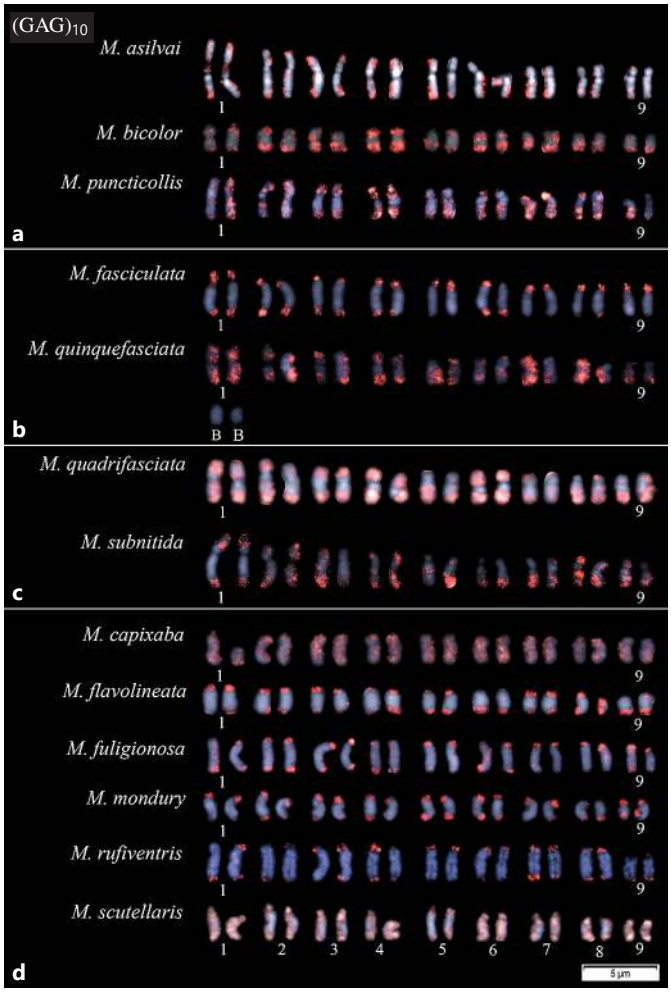

Fig. 5. FISH with the $(GAG)_{10}$ probe in *Melipona* species belonging to the subgenera *Eomelipona* (**a**), *Melikerria* (**b**), *Melipona* sensu stricto (**c**), and *Michmelia* (**d**). Chromosomes are stained with DAPI, probe signals are in red.

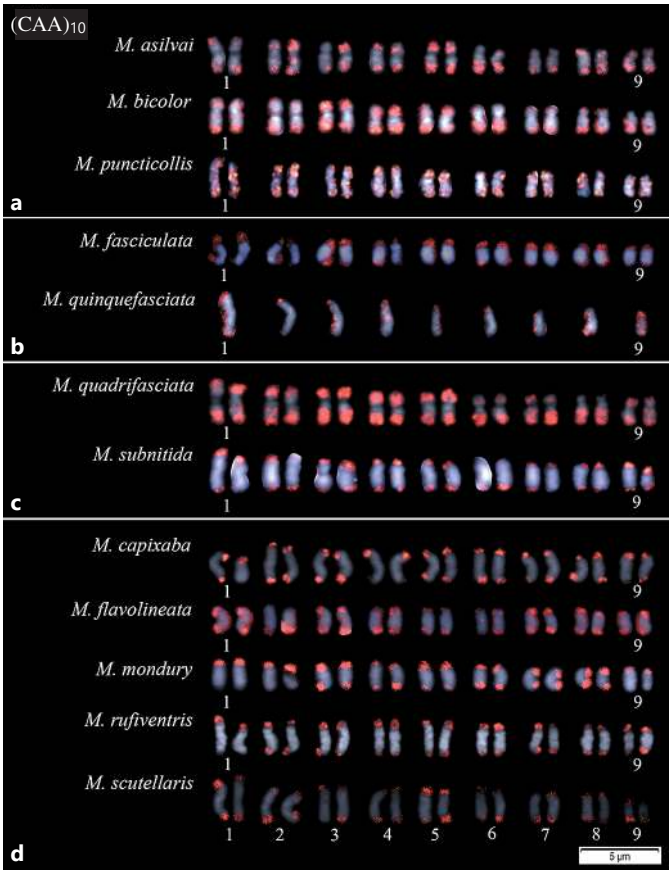

Fig. 6. FISH with the $(CAA)_{10}$ probe in *Melipona* species belonging to the subgenera *Eomelipona* (**a**), *Melikerria* (**b**), *Melipona* sensu stricto (**c**), and *Michmelia* (**d**). Chromosomes are stained with DAPI, probe signals are in red.

regions and 18S rDNA sites [Brito et al., 2005; Duarte et al., 2009]. However, despite similarity in the number of CMA_3-stained and rDNA sites, the location of these markings was different among the *Melipona* species.

Microsatellites were not located in heterochromatic regions and showed nonrandom distribution in the genome. The $(GA)_{15}$, $(GAG)_{10}$, $(CAA)_{10}$, and $(CGG)_{10}$ repeats coincided with and were confined to euchromatin regions in species with both high and low heterochromatin content. Thus, in Group I species, probe hybridization was observed along the chromosome arms, whereas in Group II species the markers were restricted to the terminal regions (Fig. 3–7). Similar results were obtained in other studies on Meliponini species such as *M. scutellaris* [Piccoli et al., 2018], *Melipona interrupta* Latreille, 1811 [unpubl. data], *Partamona chapadicola* Pedro & Camargo, 2003, *Partamona helleri* (Friese, 1900), *Partamona nhambiquara* Pedro & Camargo, 2003 [Lopes, pers. commun.], *Trigona spinipes* (Fabricius, 1793) [Ferreira et al., 2015], *Nannotrigona punctata* (Smith, 1854), and *Scaptotrigona bipunctata* (Lepeletier, 1836) [Novaes et al., 2015], indicating that microsatellites are genomic spacers in euchromatic regions and that other types of repeated sequences are present in heterochromatin of these species. We did not observe any hybridization of the probes used with the B chromosome of *M. quinquefasciata*, which has probably a heterochromatic origin, indicating potential homology between B chromosomes and heterochromatin regions of the A complement.

Our results show that the heterochromatic regions in *Melipona* can encompass other repetitive sequences such as transposons, which would justify their duplication and generalized expansion in Group II species, whose chro-

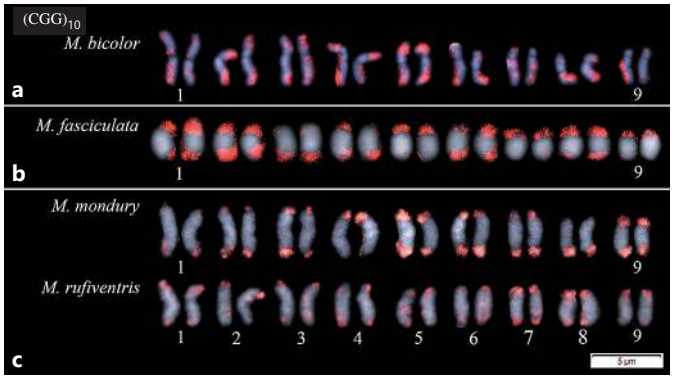

Fig. 7. FISH with the $(CGG)_{10}$ probe in *Melipona* species belonging to the subgenera *Eomelipona* (**a**), *Melikerria* (**b**), *Melipona* sensu stricto (**c**), and *Michmelia* (**d**). Chromosomes are stained with DAPI, probe signals are in red.

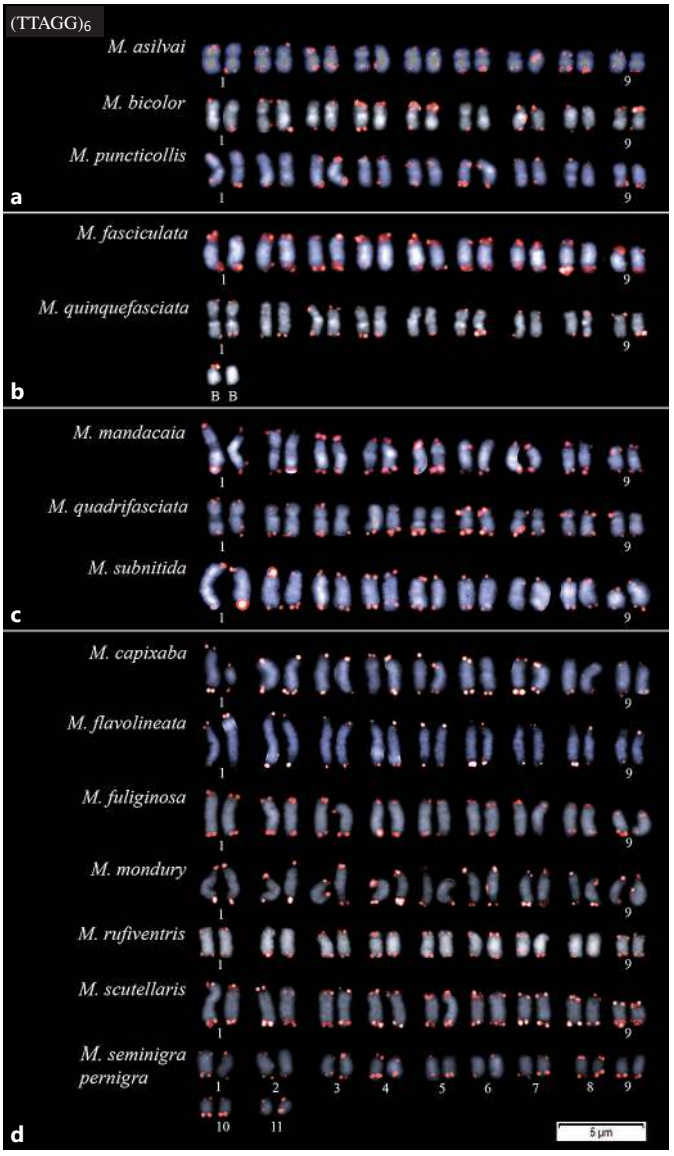

Fig. 8. FISH with the $(TTAGG)_6$ probe in *Melipona* species belonging to the subgenera *Eomelipona* (**a**), *Melikerria* (**b**), *Melipona* sensu stricto (**c**), and *Michmelia* (**d**). Chromosomes are stained with DAPI, probe signals are in red.

mosomes are formed by heterochromatin blocks as evidenced by C-banding. Transposing elements differ from other genomic sequences by the ability to move around the genome [Kazazian, 2004]. An important feature of transposons is frequent polymorphism due to insertions and variations in copy number, which can be observed both within and between species [Feschotte and Pritham, 2007; Lankenau and Volff, 2009].

Lopes et al. [2014] demonstrated that in *M. rufiventris* heterochromatin comprised sequences shared by all chromosomes, which were different from those in heterochromatin of *Tetragonisca fiebrigi*. Similar observations were reported for *M. scutellaris* [Piccoli et al., 2018], indicating relatedness between heterochromatin sequences of these species. In addition, these studies showed that the shared sequences were also present in Group II species of the same subgenus, but not among different *Melipona* subgenera. DNA sequencing should be performed to determine whether these sequences belong to satellite DNA or transposable elements.

An exception to the pattern of repetitive microsatellite markers in euchromatin was observed in *M. capixaba* and *M. scutellaris* in which the $(GAG)_{10}$ probe hybridized with both heterochromatic and euchromatic regions (Fig. 5d). The similarities in the marker distribution patterns between *M. scutellaris* and *M. capixaba* could be explained by close phylogenetic relatedness of these species [Cristiano et al., 2012].

The telomeric sequence probe $(TTAGG)_6$ marked telomeres on all chromosomes of *Melipona* species irrespective of heterochromatin content (Fig. 8), as well as on B chromosomes of *M. quinquefasciata* (Fig. 8b). In insects, 2 types of short telomeric repeats are observed: TTAGG [Sahara et al., 1999] and TCAGG [Mravinac et al., 2011]; the former is the most common in *Melipona* as well as in *Apis mellifera* Linnaeus, 1758 [Meyne et al., 1995; Sahara et al., 1999]. Telomeres are responsible for maintaining chromosomal integrity, and their location within chromosomal arms may suggest rearrangements that occurred during karyotype evolution of a taxon [Nanda et al., 2002; Bueno et al., 2013; Lanzone et al., 2015; Rovatsos et al., 2015]. Although it has been suggested that in *Melipona* repeated chromosomal fusions

are responsible for a lower chromosome number compared to the other Meliponini genera [Travenzoli, 2018], there was no interstitial hybridization of $(TTAGG)_6$ in chromosomes. Similar results were reported for ants, e.g. *Acromyrmex striatus* (Roger, 1863), where the absence of interstitial signals could indicate fusion and consequent telomere inactivation [Pereira et al., 2018]. Given that one of the prerequisites in robertsonian-type fusion events would be telomere loss or inactivation [Slijepcevic, 1998], the absence of interstitial sites in *Melipona* indicates that there was likely a loss rather than inactivation of telomeres.

The presence of telomeric sequences was also observed in the B chromosomes of *M. quinquefasciata*, which have the heterochromatin structure common for B chromosomes [Camacho, 2005], usually associated with the accumulation of repetitive sequences such as satellite DNA, rDNA, and transposable elements [Camacho et al., 2000]. The labeling of only $(TTAGG)_6$ repeats and the absence of $(GA)_{15}$ and $(GAG)_{10}$ probe hybridization demonstrates similarity between B chromosomes and complement A chromosomes in regard to their heterochromatic nature [Rocha, 2002].

Conclusion

The cytogenetic characteristics of *Melipona* species based on heterochromatin patterns, DAPI/CMA_3 staining, and rDNA sites confirm the division of *Melipona* into 2 groups, which have unique, generally conserved characteristics. Group I species have a low content of heterochromatin located in the pericentromeric region, and the first chromosome pair is CMA_3^+ in the pericentromeric region, coinciding with rDNA sites; this pattern is observed in *Eomelipona* and *Melipona* sensu stricto subgenera. Group II species have a high content of heterochromatin dispersed throughout chromosomes, 2 CMA_3^+ regions, and positivity for terminal or interstitial markers located close to the junction between euchromatin and heterochromatin of the first chromosome pair, both coinciding with rDNA; this pattern is observed in *Michmelia* and *Melikerria* subgenera with the exception of *M. quinquefasciata*, which seems to have evolved independently. The presence of microsatellite-like repetitive DNA sequences preferentially in euchromatin of both groups suggests that other families of repetitive DNA should be present in heterochromatin.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors declare that they have no potential conflict of interest.

Author Contributions

N.M.T. and D.M.L. conceived this research and designed experiments; N.M.T. and B.A.S. performed experiments and analyses. All authors wrote, read, and approved the final manuscript.

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