




Comparative cytogenetics in three *Melipona* species (Hymenoptera: Apidae) with two divergent heterochromatic patterns

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Abstract

The genus *Melipona* is subdivided into four subgenera based on morphological characteristics, and two groups based on cytogenetic patterns. The cytogenetic information on this genus is still scarce, therefore, the goal of this study was to characterize *Melipona paraensis*, *Melipona puncticollis*, and *Melipona seminigra pernigra* using the following techniques: C-banding, DAPI/CMA₃ fluorochromes, and FISH with an 18S rDNA probe. *Melipona paraensis* ($2n=18$) and *M. seminigra pernigra* ($2n=22$) were classified as high heterochromatin content species (Group II). Their euchromatin is restricted to the ends of the chromosomes and is CMA₃⁺; the 18S rDNA probe marked chromosome pair number 4. *Melipona puncticollis* ($2n=18$) is a low heterochromatin content species (Group I) with chromosome pair number 1 marked with CMA₃ and 18S rDNA. Low heterochromatin content is a putative ancestral karyotype in this genus and high content is not a monophyletic trait (*Melikerria* presents species with both patterns). Differences concerning the karyotypic characteristics can be observed among *Melipona* species, revealing cytogenetic rearrangements that occurred during the evolution of this genus. Studies in other species will allow us to better understand the processes that shaped the chromatin evolution in *Melipona*.

Keywords: Chromosomal evolution, DAPI/CMA₃ fluorochromes, Fluorescent *in situ* Hybridization (FISH), heterochromatin, Meliponini.

Received: October 26, 2017; Accepted: February 23, 2018.

Introduction

Species belonging to the Meliponini tribe are also known as stingless bees. These highly eusocial bees are of pantropical distribution and are important both economically and ecologically. They produce honey and propolis, pollinate a variety of cultivated and native crops, and play an important role as providers of ecosystem services (Kerr *et al.*, 1996; Heard, 1999; Cortopassi-Laurino *et al.*, 2006; Michener, 2007). In the Neotropics, Meliponini is composed of 33 genera with approximately 417 valid species (Camargo and Pedro, 2013). Among these genera, *Melipona* Illiger 1806 is the most species-rich genus in this tribe (Silveira *et al.*, 2002), represented by 73 described

species of which 43 occur in Brazil, and it is subdivided into four subgenera based on morphological characteristics: *Eomelipona*, *Melipona stricto sensu*, *Michmelia*, and *Melikerria* (Camargo and Pedro, 2013). It is important to highlight that a revision is needed, since *Eomelipona* is the only subgenus that was not recovered as a monophyletic clade in a molecular phylogenetic analysis (Ramírez *et al.*, 2010; Rasmussen and Cameron, 2010).

Cytogenetic studies on 22 *Melipona* species indicate a preserved autosome diploid number of $2n = 18$ chromosomes in most of the species studied so far, with *Melipona seminigra merillae* Cockerell, 1919 being the exception, showing $2n = 22$ chromosomes (reviewed in Tavares *et al.*, 2017). Despite the conservatism in the diploid number, the *Melipona* species have a divergent pattern regarding heterochromatin content, and defined through C-banding technique it can be subdivided into two groups: Group I is comprised of species with a low content of heterochro-

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matin, while Group II is comprised of species with a high heterochromatin content (Rocha and Pompolo, 1998; Rocha *et al.*, 2002, 2003; Lopes *et al.*, 2008, 2011). In this context, the subgenera *Eomelipona* and *Melipona stricto sensu* are comprised only of species with a low content of heterochromatin, *Michmelia* only of species with a high content, while *Melikerria* has species with both patterns. The cytogenetic data available on the genus *Melipona* re-

garding chromosome number, C-banding, CMA₃, and 18S rDNA patterns is revised in Table 1.

The goal of this study was to describe the cytogenetic characteristics (chromosomal number, heterochromatin content, DAPI/CMA₃ fluorochromes, and 18S rDNA patterns) of three *Melipona* species (*Melipona paraensis* Ducke, 1916, *Melipona puncticollis* Friese, 1902, and *Melipona seminigra pernigra* Friese, 1903), and to compile

Table 1 - Cytogenetic data available on 22 *Melipona* species regarding their chromosome number (karyotypic formula), C-banding (high or low content), CMA₃ and 18S rDNA patterns. Species were assigned to subgenera based on the Moure's catalogue.

Subgenus	Species	Chromosome Number	C-Banding	CMA ₃	18S rDNA	References
<i>Eomelipona</i>	<i>M. asilvai</i>	2n = 18	Low content	2 interstitial markings	2 interstitial markings *	Rocha and Pompolo, 1998; Rocha <i>et al.</i> , 2002; Rocha <i>et al.</i> , 2007
	<i>M. bicolor</i>	2n = 18	Low content	2 interstitial markings	-	Rocha and Pompolo, 1998
	<i>M. marginata</i>	2n = 18	Low content	2 interstitial markings	2 interstitial markings *	Rocha and Pompolo, 1998; Maffei <i>et al.</i> , 2001; Rocha <i>et al.</i> , 2007
	<i>M. puncticollis</i>	2n = 18 (2m+14sm+2a)	Low content	2 interstitial markings	2 interstitial markings	Present study
<i>Melikerria</i>	<i>M. fasciculata</i>	2n = 18	High content	Terminal marks on all chromosomes	2 terminal markings [†]	Rocha <i>et al.</i> , 2002; Lopes <i>et al.</i> , 2011
	<i>M. quinquefasciata</i>	2n = 18 [‡]	Low content	2 interstitial markings	-	Rocha <i>et al.</i> , 2007
<i>Melipona</i>	<i>M. favosa</i>	2n = 18 (12m+4sm+2a)	-	-	-	Hoshiba, 1988
	<i>M. mandacaia</i>	2n = 18 (2m+14sm+2a)	Low content	2 interstitial markings	-	Rocha <i>et al.</i> , 2003
	<i>M. quadrifasciata</i>	2n = 18 (4m+12sm+2a)	Low content	2 interstitial markings	-	Rocha and Pompolo, 1998
	<i>M. subnitida</i>	2n = 18	Low content	2 interstitial markings	-	Rocha <i>et al.</i> , 2002; Rocha <i>et al.</i> , 2007
	<i>M. capixaba</i>	2n = 18	High content	Terminal marks on all chromosomes	-	Rocha and Pompolo, 1998; Rocha <i>et al.</i> , 2002
<i>Michmelia</i>	<i>M. captiosa</i>	2n = 18	High content	-	-	Rocha and Pompolo, 1998
	<i>M. crinita</i>	2n = 18	High content	Terminal marks on all chromosomes	-	Rocha <i>et al.</i> , 2002
	<i>M. flavolineata</i>	2n = 18	High content	Terminal marks on all chromosomes	-	Lopes <i>et al.</i> , 2011
	<i>M. fuliginosa</i>	2n = 18	High content	Terminal marks on all chromosomes	-	Lopes <i>et al.</i> , 2011
	<i>M. fuscopilosa</i>	2n = 18	High content	Terminal marks on all chromosomes	-	Rocha and Pompolo, 1998; Rocha <i>et al.</i> , 2002
	<i>M. mondury</i>	2n = 18	High content	Terminal marks on all chromosomes	-	Lopes <i>et al.</i> , 2008
	<i>M. paraensis</i>	2n = 18	High content	Terminal marks on all chromosomes	2 terminal markings	Present study
	<i>M. rufiventris</i>	2n = 18 [‡]	High content	Terminal marks on all chromosomes	-	Lopes <i>et al.</i> , 2008
	<i>M. scutellaris</i>	2n = 18	High content	Terminal marks on all chromosomes	-	Rocha and Pompolo, 1998; Rocha <i>et al.</i> , 2002
	<i>M. seminigra merrillae</i>	2n = 22	Low content? [§]	-	-	Francini <i>et al.</i> , 2011
<i>M. seminigra pernigra</i>	2n = 22	High content	Terminal marks on all chromosomes	2 terminal markings	Present study	

* Ag-NOR data.

[†] *M. compressipes* in the paper of Rocha *et al.* (2002) is indeed *M. fasciculata* (Tavares *et al.*, 2017).

[‡] B chromosomes were reported in these two species.

[§] Reevaluated as high content. More details are given in the text.

the cytogenetic data available for this taxon in order to identify the chromosomal variation that is characteristic for each *Melipona* Group (I and II), as well as to understand the role of these regions in the evolution of chromosomes in the genus.

Material and Methods

The three *Melipona* species (*M. paraensis*, *M. puncticollis*, and *M. seminigra pernigra*) were collected in Altamira, state of Pará, Brazil. The specimens were identified by Sílvia Regina de Menezes Pedro (Universidade de São Paulo, Ribeirão Preto, Brazil), and deposited in the scientific collection of the Apiário Central at Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. Mitotic chromosomes were obtained from cerebral ganglia of larvae in the final defecation stage (Imai *et al.*, 1988). The conventional staining was done using Giemsa diluted in Sorensen buffer in a 1:30 proportion, and at least 25 larvae of each species were analyzed. The chromosomes were classified following the arm ratios given by Levan *et al.* (1964). Heterochromatin was visualized through C-banding (Sumner, 1972) and digital images of the metaphases were taken in a BX53F Olympus microscope equipped

with a DP73F Olympus camera, using CellSens imaging software.

Sequential staining with the fluorochromes 4'-6-diamidino-2-phenylindole (DAPI) and chromomycin A₃ (CMA₃) was performed following the method of Schweizer (1980). Fluorescent *in situ* Hybridization (FISH) followed the protocol described by Pinkel *et al.* (1986) using a ribosomal 18S rDNA probe isolated from *M. mondury* obtained through Polymerase Chain Reaction (PCR) using the following primers: 5'-TAATTCCAGCTCCAATAG-3' and 5'-CCACCCATAGAATCAAGA-3'. This 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. An average of 10 metaphases was analyzed to determine the cytogenetic patterns revealed by the different techniques used in this study.

Results

The diploid chromosome number of *M. paraensis* was defined as $2n = 18$ (Figure 1a). C-banding revealed that

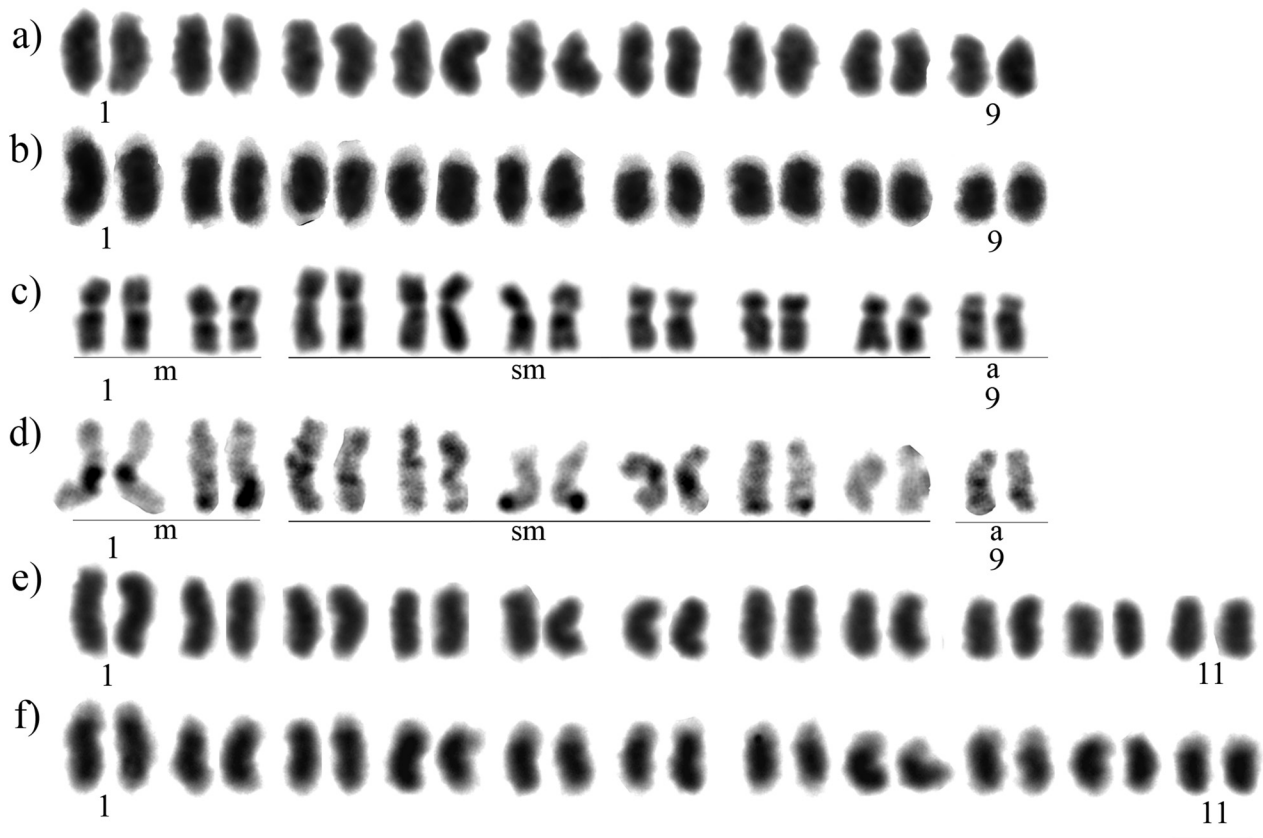


Figure 1 - Karyotypes of *Melipona paraensis* (a - Giemsa-stained, b - C-banding); *Melipona puncticollis* (c - Giemsa-stained, d - C-banding); and *Melipona seminigra pernigra* (e - Giemsa-stained, f - C-banding). Scale bar = 5 μ m.

the major part of each chromosome is comprised of heterochromatin. This hindered the visualization of centromeres, and hence it was not possible to define the karyotypic formula (Figure 1b). The DAPI/CMA₃ analysis indicated that the heterochromatin is DAPI⁺ (Figure 2a and Figure S1a-c), while CMA₃⁺ marked all the extremities of the chromosomes corresponding to the euchromatin region (Figure 2b). FISH with 18S rDNA probe marked chromosome pair number 4 in its terminal position (Figure 3a).

The diploid chromosome number of *M. puncticollis* was defined as $2n = 18$ and its karyotypic formula as $2n = 2m + 14sm + 2a$ (Figure 1c). C-banding indicated a low content of heterochromatin that is restricted to the pericentromeric region of chromosome pair numbers 1, 3, 6, 9, and the subtelomeric region of the long arms of chromosome pair numbers 2, 4, 5 and 7, while chromosome pair number 8 is completely euchromatic (Figure 1d). Sequential staining with DAPI/CMA₃ fluorochromes indicated strong DAPI⁺ bands corresponding to the heterochromatin region (Figure 2c), while CMA₃ marked the interstitial region of chromosome pair number 1 (Figure 2d). The same result was found with the 18S rDNA FISH probe (Figure 3b).

The diploid chromosome number of *M. seminigra pernigra* was defined as $2n = 22$ (Figure 1e). C-banding revealed that the majority of each chromosome is composed of heterochromatin, hindering the visualization of the centromeres, so it was not possible to define the karyotypic formula (Figure 1f). DAPI/CMA₃ analysis indicated that the heterochromatin is DAPI⁺ (Figure 2e and Figure S1d-f). CMA₃⁺ marked all the extremities of the chromosomes corresponding to the euchromatin region, and we could identify one chromosome pair that strongly stained with CMA₃ fluorochrome (Figure 2f). FISH with the 18S rDNA probe marked chromosome pair number 4 in its terminal position (Figure 3c).

Discussion

The three species analyzed in this study presented distinct chromosome numbers: *M. (Michmelia) paraensis* and *M. (Eomelipona) puncticollis* presented $2n = 18$ chromosomes, while *M. (Michmelia) seminigra pernigra* showed $2n = 22$ chromosomes. The autosomal chromosome number that prevails in the genus *Melipona* is $2n = 18$, but a few exceptions can be found, such as in *M. (Michmelia) seminigra merrillae* that also has $2n = 22$, and *Melipona (Melikerria) quinquefasciata* Lepeletier, 1836 and *Melipona (Michmelia) rufiventris* Lepeletier, 1836, both of which have $2n = 18$ autosomal chromosomes, but they present variation with respect to the number of B chromosomes found in different populations (reviewed in Tavares *et al.*, 2017).

Regardless of the conservatism in the diploid number, differences concerning the karyotypic formula and heterochromatin content could be observed among species, re-

vealing cytogenetic rearrangements that have occurred during the evolution of the genus. Changes in the karyotypic formula among species belonging to Group I indicate the occurrence of pericentric inversions that altered the number of metacentric and submetacentric chromosomes in this group: *Melipona favosa* Fabricius, 1798 ($2n = 12m + 4sm + 2a$) (Hoshiya, 1988), *Melipona mandacaia* Smith, 1863 ($2n = 2m + 14sm + 2a$) (Rocha *et al.*, 2003), *M. puncticollis* ($2n = 2m + 14sm + 2a$) (present study), and *Melipona quadrifasciata* Lepeletier, 1836 ($2n = 4m + 12sm + 2a$) (Silva *et al.*, 2012). In Group II species, the high heterochromatin content masks the position of the centromere, and therefore, makes it difficult to identify the morphology of the chromosomes to define the karyotypic formula of these species. This is a common trait among the *Melipona* species belonging to Group II, rather than an issue related to the quality of the metaphases (Rocha *et al.*, 2002; Lopes *et al.*, 2008, 2011).

In the species analyzed in this study, *M. puncticollis* is a low heterochromatin content species, while *M. paraensis* and *M. seminigra pernigra* are high heterochromatin content species (Figure 1). The first description of the C-banding pattern on *M. seminigra merrillae* indicated this subspecies as part of Group I, with low heterochromatin content (Francini *et al.*, 2011), but analyzing the images from that publication, the pattern seems to be very similar to the high heterochromatin content species, as it was not possible to visualize the centromeres, and they had heterochromatin as the predominant constituent of the chromosomes. Ongoing cytogenetic studies on this subspecies confirm that *M. seminigra merrillae*, as well as *M. seminigra pernigra* are high heterochromatin content subspecies belonging to Group II (unpublished data).

In eusocial bees, the heterochromatin is usually AT-rich (DAPI⁺) (Brito *et al.*, 2003; Rocha *et al.*, 2003; Lopes *et al.*, 2011; Godoy *et al.*, 2013), and this is a pattern shared by *Melipona* species with both low and high heterochromatin content (Figure 2). CMA₃⁺ positive bands are another characteristic used to distinguish Groups I and II in this genus (Table 1): Group I species have only one chromosome pair CMA₃⁺ marked in its interstitial position, and this chromosome pair is usually related to the nucleolar organizing region (NOR) (Rocha *et al.*, 2002), indicating that the NOR is CG-rich in this group; Group II species have CMA₃⁺ terminal markings on all of the chromosomes corresponding to the euchromatin, indicating that these regions are CG-rich, and in some cases it is possible to identify one pair with the brightest mark as associated with ribosomal cistrons, as for instance in *M. seminigra pernigra* (Figure 2F) and other high heterochromatic content species (Lopes *et al.*, 2008, 2011). It is interesting to note that the solitary bee *Melitoma segmentaria* Fabricius, 1804 has the opposite pattern, as the euchromatic portion of the chromosomes are CMA₃⁻ and the heterochromatic ones are CMA₃⁺ (Cristiano *et al.*, 2014). Other solitary bees,

such as *Euglossa townsendi* Cockerell, 1904 and *Euglossa carolina* Linnaeus, 1758), have the same cytogenetic diver-

gence as *Melipona* with regard to the heterochromatin content (species with low and high heterochromatin content),

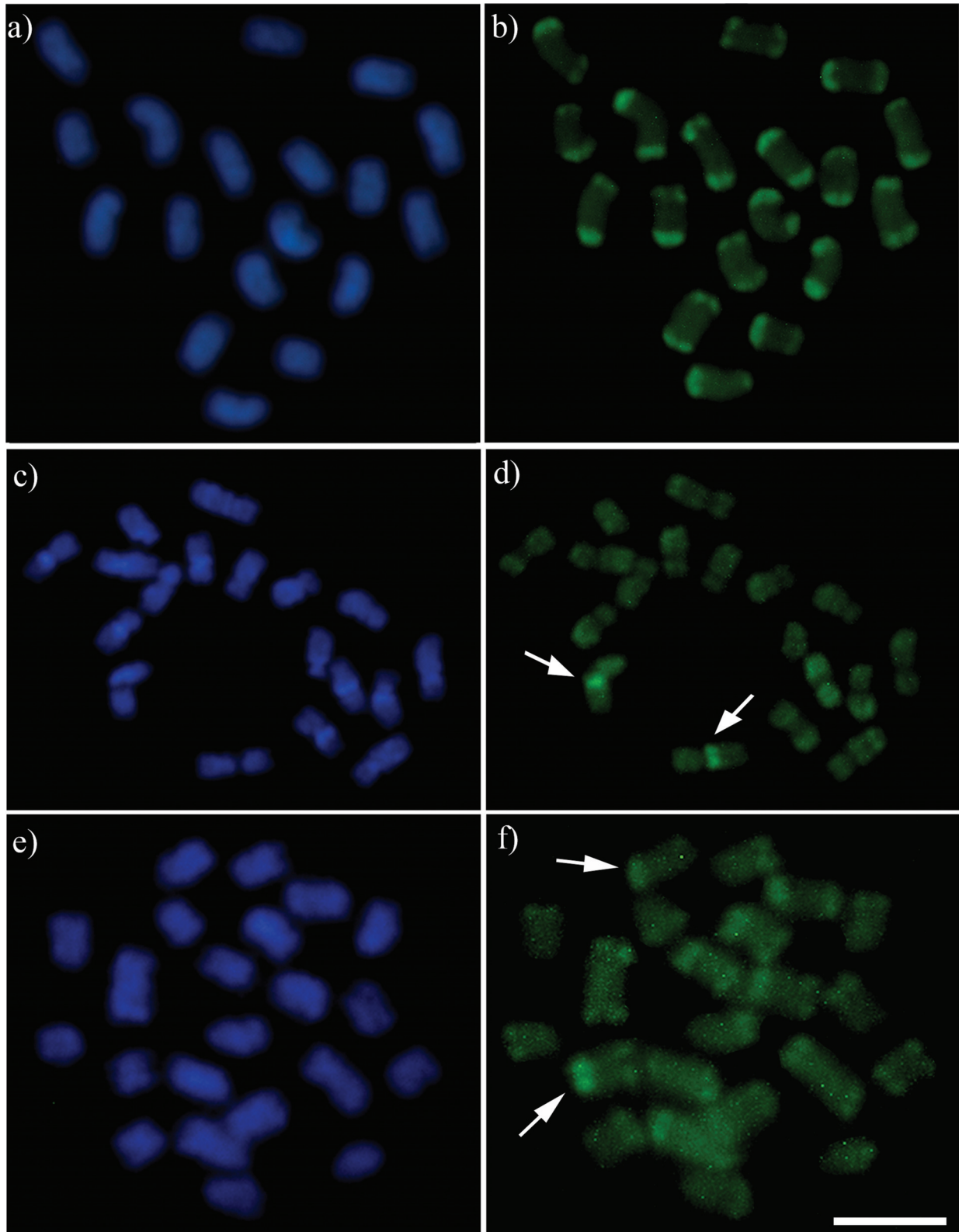


Figure 2 - Sequential staining with DAPI/CMA₃ fluorochromes: *Melipona paraensis* (a - DAPI, b - CMA₃); *Melipona puncticollis* (c - DAPI, d - CMA₃); and *Melipona seminigra pernigra* (e - DAPI, f - CMA₃). The arrows indicate the organizing region of the nucleoli. Scale bar = 5 μm.

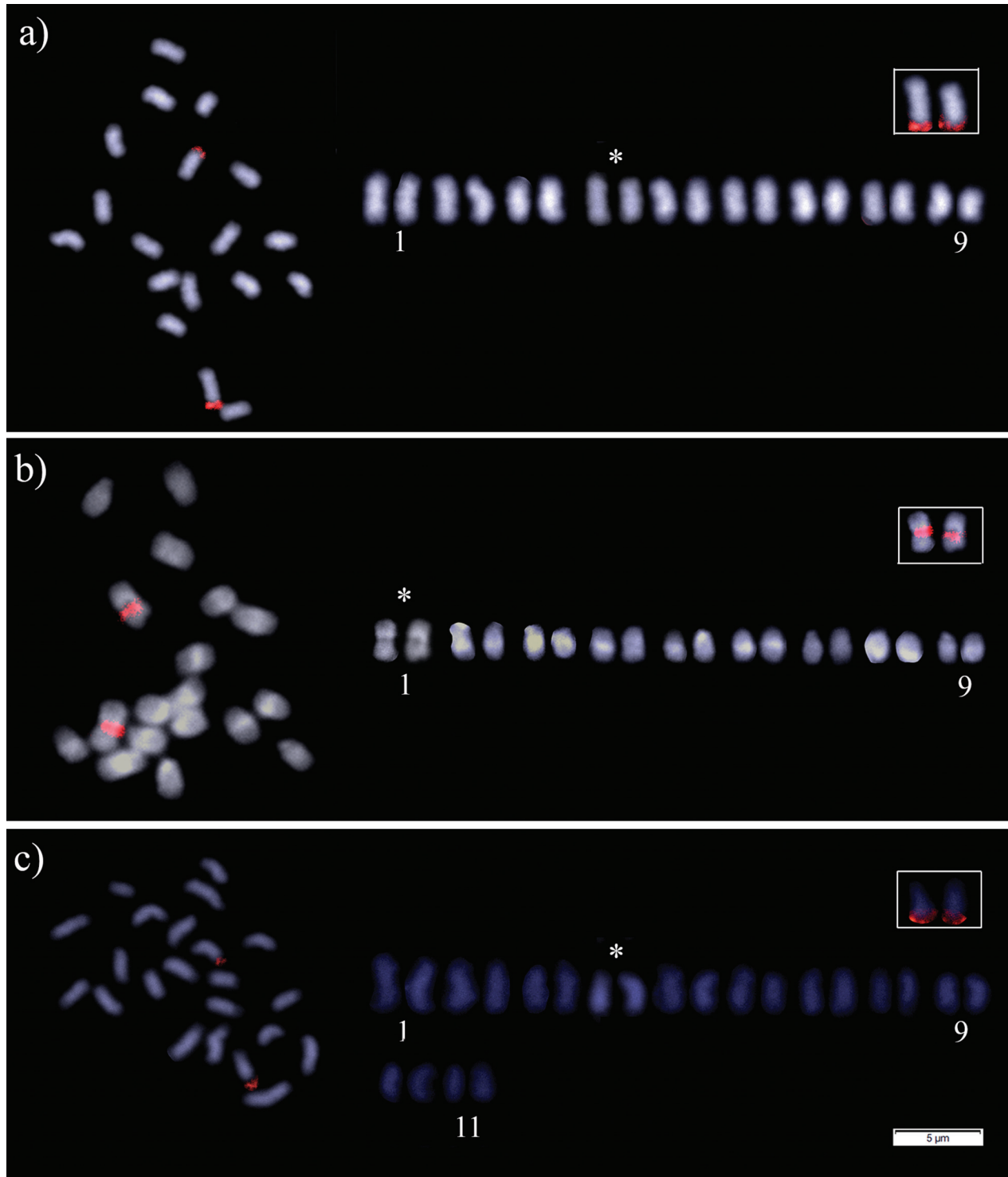


Figure 3 - Fluorescent *in situ* hybridization (FISH) pattern with 18S rDNA probe: metaphase cells and arranged karyotype of (a) *Melipona paraensis*; (b) *Melipona puncticollis*; and (c) *Melipona seminigra pernigra*. * denotes chromosome pair marked by the probe indicated in the box. Scale bar = 5 μ m.

but they have unique CMA₃ accumulation patterns, showing that the heterochromatin is heterogeneous with respect to its composition, with some blocks rich in AT and others rich in CG (Fernandes *et al.*, 2013), highlighting the diversity of the patterns observed among bees.

To this date, there is only one report that has used FISH to confirm the position of the NORs with an 18S rDNA FISH probe; this was done in *Melipona fasciculata* Smith, 1854 (Rocha *et al.*, 2002, revised in Tavares *et al.*, 2017). Together with our study, it seems that only one pair

of chromosomes labeled with this probe, which can be considered as a conserved characteristic in this genus (Figure 3). Studies applying ribosomal probes in bees are still scarce, but analyses combining Ag-NOR, CMA₃⁺ bands, and FISH 18S rDNA techniques have been used to identify NORs in different Meliponini species (Rocha *et al.*, 2002; Brito *et al.*, 2005; Duarte *et al.*, 2009; Krinski *et al.*, 2010; Lopes *et al.*, 2011; Godoy *et al.*, 2013; Miranda *et al.*, 2013). Based on these three different techniques it can be inferred that having only one pair of NORs is a conserved characteristic in *Melipona* (Table 1).

Regardless of the conservatism in the number of markings, the position of the 18S rDNA cistrons can be used to differentiate Groups I and II in *Melipona*, as they are interstitial in low content species and terminal in high content ones (Table 1). Independent of the technique applied, the literature indicates pair number 1 as the NOR bearer in this genus. For: *Melipona asilvai* Moure, 1971, *M. mandacaia*, and *Melipona marginata* Moure, 1992 this was inferred by Ag-NOR (Maffei *et al.*, 2001; Rocha *et al.*, 2002, 2003). For *Melipona bicolor* Lepeletier, 1836, *Melipona capixaba* Moure and Camargo, 1994, *Melipona mondury* Smith, 1863, *M. quadrifasciata*, *M. quinquefasciata*, *M. rufiventris*, and *Melipona subnitida* Ducke, 1910 the identification was done by CMA₃ fluorochrome (Rocha *et al.*, 2007; Lopes *et al.*, 2008), and for *M. fasciculata* it was identified by FISH with a ribosomal probe (Rocha *et al.*, 2002 revised in Tavares *et al.*, 2017). However, in our study only the low content species *M. puncticollis* had the first pair as the NOR bearer, while the high content species *M. paraensis* and *M. seminigra pernigra* had pair number 4 marked with the 18S rDNA probe (Figure 3), highlighting another distinct characteristic between Groups I and II. We argue that none of the cited studies above arranged the karyotype. Hence in metaphase cells, the terminal location of the probe in the high content species might have given the impression of a bigger chromosome (see Figure 3).

Despite being polyphyletic, basal *Eomelipona* species group together with *Melipona stricto sensu* (Ramírez *et al.*, 2010), and this clade is composed of species with low heterochromatin content (Table 1), indicating that this is the plesiomorphic characteristic of the genus, while high heterochromatin content appeared more than once during the evolution and diversification of this taxon, emerging in both *Melikerria* and *Michmelia* subgenera. As we could observe both heterochromatin patterns in *Melikerria* (Table 1), the classification of the *Melipona* species into low and high heterochromatin content species did not form natural groups and did not represent monophyletic clades in the phylogenetic analysis.

The current study aimed to describe three *Melipona* species with divergent patterns of heterochromatin accumulation, arguing that a karyotype with low heterochromatin content is a putative ancestral karyotype in this genus and that high heterochromatin content is not a monophy-

letic characteristic. We also contributed with new cytogenetic data on Groups I and II, highlighting the cytogenetic rearrangements that occurred during the chromosome evolution in this major stingless bee genus. Finally, we emphasize the importance of cytogenetic analyses to evidence the chromosomal rearrangements that occurred during the evolution of different species (Imai *et al.*, 1994; Menezes *et al.*, 2014). Studies in other species will allow us to better understand the processes that shaped chromatin evolution in *Melipona*.

Acknowledgments

The authors wish to thank NORTE ENERGIA and BIOTA for logistical support during field work done in Altamira, state of Pará, Brazil. Financial support is acknowledged from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Fundação Amazônia de Amparo a Estudos e Pesquisas do Pará (FAPESPA).

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Supplementary material

The following online material is available for this article:

Figure S1 – DAPI stained metaphases of *Melipona paraensis* *Melipona seminigra pernigra*.

Associate Editor: Yatio Yassuda-Yonenaga

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