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# Comparative molecular cytogenetics of *Melipona* Illiger species (Hymenoptera: Apidae)

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**Abstract**

Cytogenetic studies of *Melipona* are scarce with only 24 species analyzed cytogenetically. Of these, six species had the rDNA sites physically mapped and characterized by Fluorescent in situ hybridization (FISH). The aim of this study was to perform karyotype analyzes on *Melipona* species from different regions of Brazil, with a greater sampling representative of the Amazonian fauna and using conventional, fluorochrome staining and FISH with heterologous rDNA probes. The predominant chromosome number was 2n = 18, however, the subspecies *Melipona seminigra abunensis* Cockerell and *Melipona seminigra pernigra* Moure & Kerr showed 2n = 22 chromosomes. The karyotypes were symmetrical, however *Melipona bicolor* Smith, *Melipona quadrifasciata* Guérin, *Melipona flavolineata* Friese, *Melipona fuscopilosa* Moure & Kerr, *Melipona nebulosa* Camargo presented the first pair heteromorphic in length. $CMA_3^+$ blocks also exhibited heteromorphism of size and in almost all cases coincided with rDNA sites, except for *Melipona crinita* Moure & Kerr and *M. nebulosa*, which presented additional non-coincident $CMA_3^+$ blocks. The $CMA_3$/rDNA sites were terminal and interstitial in species with high heterochromatic content, and pericentromeric in the species with low heterochromatic content. In addition to describing cytogenetic features of cytotaxonomic importance, the reorganization of the genome in *Melipona* is also discussed.

## Introduction

*Melipona* is a Neotropical genus of stingless bees, that occurs from Mexico to Argentina (Michener, 2007) and with greater diversity in the Amazon basin (Silveira et al., 2002). Moure divided it into four subgenera, *Eomelipona*, *Melikerria*, *Melipona* and *Michmelia* (Moure et al., 2007). Michener (2007) did not recognize the subgenera for considering them very similar. Silveira et al. (2002) also recognize subgenera, but suggest some modifications in Moure's proposal. Molecular analyzes of mitochondrial and nuclear genes support this classification (Fernandes-Salomão et al., 2002; Ramirez et al., 2010; Rasmussen & Cameron, 2010). *Melipona* shows unique characteristics among the Meliponini, such as the genetic-alimentary process of caste determination and absence of morphologically distinct queen cells (Michener, 2007).

Although there are several studies involving *Melipona* biology, there is a paucity of molecular cytogenetic studies. So far, 23 out of 74 described *Melipona* species (Camargo and Pedro, 2013; Pedro, 2014), were included in cytogenetic studies (Tavares et al., 2017), however, with concentrated samplings in the south and southeast Brazil. The chromosome numbers n = 9 and 2n = 18, are conserved in this genus and were found in almost all species (Rocha et al., 2007; Tavares et al., 2017) except for *Melipona seminigra merrillae* Cockerell (2n = 22) (Francini et al., 2011). In *Melipona quinquefasciata* Lepeletier (2n = 18 to 2n = 22) (Rocha et al., 2007) and *Melipona rufiventris* Lepeletier (2n = 18 to 2n = 19) (Lopes et al., 2008) the differentiated chromosome number varied due to the presence of supernumerary chromosomes. In the first species 2n = 18 is the chromosome number of the regular complement, and there is a variable number of B chromosomes.

In the second species only one supernumerary chromosome was recorded.

Most of the previous cytogenetic analyzes basically included conventional staining with Giemsa, staining with the $CMA_3$/DAPI fluorochromes and C-banding. Only *Melipona compressipes* (Fabricius) (Rocha et al., 2002), *M. quinquefasciata*, *Melipona capixaba* Moure & Camargo, *Melipona quadrifasciata* Lepeletier, *Melipona scutellaris* Latreille and *Melipona bicolor* Smith (Rocha et al., 2007) were analyzed using fluorescence in situ hybridization (FISH) with probes for localization of rDNA sites. Characteristics such as conserved DNA sequences and large variation in the number of copies make ribosomal genes good cytological markers in the characterization of the chromosome set by in situ hybridization, making possible to make inferences about genetic variability, intra and interspecific divergence (Rafael et al., 2003; Sochorová et al., 2018). This technique has been used in studies of different insect orders, such as Lepidoptera (Nguyen et al., 2010), Diptera (Roy et al., 2005), Hymenoptera (Carabajal Paladino et al., 2013) and Hemiptera (Salanitro et al., 2017) using homologous probes. In the Coleoptera (Vitturi et al., 1999), Orthoptera (Cabrero & Camacho, 2008; Loreto et al., 2008), Lepidoptera (Vershinina et al., 2015) and Hymenoptera (Hirai et al., 1996) heterologous probes were also used. Among the bees, stingless bees are still poorly studied in this respect.

In this study a comparative karyotype analysis was performed using FISH with a 45S rDNA heterologous probe, conventional staining and fluorochrome staining, among *Melipona* species from different regions of Brazil, with the inclusion of scarcely studied species of the Amazonian fauna.

## Material and Methods

The analyzed samples were collected directly from bees' nests in their natural habitat or in colonies maintained by research institutions, such as CEPLAC (Research Center for Cacao Crops - Ilhéus/BA), Universidade Federal de Viçosa (MG), Embrapa (PA) as listed in Table 1. A minimum number of five individuals per species and 10 metaphases per slide were analyzed. Specimens of vouchers identified by Dr. Gabriel A. R. Melo were deposited in the Entomological Collection Pe. J. S. Moure of the Universidade Federal do Paraná (DZUP), Curitiba, Brazil.

Mitotic metaphases were obtained from cerebral ganglia of last-instar larvae, treated with colchicine (0.005%) for 20 min following Imai et al. (1988). The chromosomes were stained with Giemsa 3% in phosphate buffer, pH 6.8. The selected metaphases were photographed under an Olympus CX-41 microscope, equipped with a digital camera. The karyotypes were organized using the Adobe Photoshop CS4 program. The nomenclatura used was of Levan et al. (1964).

After air drying, the slides were stained with $CMA_3$ (Chromomycin $A_3$) (0.5 mg / ml) and counterstained with DAPI (4 ', 6-diamidino-2-phenylindole) (2 µg / ml) according to Guerra and Souza (2002). The images were analyzed using the Leica DMRA2 epifluorescence microscope, captured with the IM50 Software and overlaid using Adobe Photoshop CS4. The slides were decolorized and stored at -20°C for further in situ hybridization with 45S rDNA probe.

The rDNA sites were localized using heterologous 45S rDNA probe R2 *Arabidopsis thaliana* (Brassicaceae), a fragment of 6.5 kb containing copies of the 18S-rDNA unit

**Table 1** *Melipona* species analyzed and collection sites.

| Species | Locality | Geographic Coordinates |
|---|---|---|
| *M. (Eomelipona) bicolor* Smith | Viçosa-Minas Gerais | 42 W 52' 54", 20 S 45' 15" |
| *M. (Melikerria) fasciculata* Smith | Belém-Pará | 48 W 30' 15", 1 S 27' 21" |
| *M. (Melikerria) grandis* Guérin | Xapuri-Acre | 68 W 30' 15", 10 S 39' 06" |
| *M. (Melipona) quadrifasciata* Guérin | Viçosa-Minas Gerais | 42 W 52' 54", 20 S 45' 15" |
| *M. (Michmelia) fuscopilosa* Moure & Kerr | Xapuri-Acre | 68 W 30' 15", 10 S 39' 06" |
| *M. (Michmelia) seminigra pernigra* Moure & Kerr | Santarém-Pará | 54 W 42' 29", 2 S 26' 36" |
| *M. (Michmelia) seminigra abunensis* Cockerell | Rio Branco-Acre | 67 W 48' 35", 9 S 58' 29" |
| *M. (Michmelia) crinita* Moure & Kerr | Xapuri-Acre | 68 W 30' 15", 10 S 39' 06" |
| *M. (Michmelia) flavolineata* Friese | Belém-Pará | 48 W 30' 15", 1 S 27' 21" |
| *M. (Michmelia) mondury* Smith | Viçosa-Minais Gerais | 42 W 52' 54", 20 S 45' 15" |
| *M. (Michmelia) nebulosa* Camargo | Xapuri-Acre | 68 W 30' 15", 10 S 39' 06" |
| *M. (Michmelia) scutellaris* Latreille | Ilhéus-Bahia | 39 W 02' 57", 14 S 47' 21" |
| *M. (Michmelia) aff. flavolineata* | Capixaba-Acre | 67 W 40' 31", 10 S 34' 2" |
| *M. (Michmelia) aff. flavolineata* | Brasiléia-Acre | 68 W 44' 52", 11 S 00' 9" |
| *M. (Michmelia) aff. flavolineata* | Xapuri-Acre[1] | 68 W 30' 15", 10 S 39' 06" |
| *M. (Michmelia) aff. flavolineata* | Xapuri-Acre[2] | 68 W 30' 15", 10 S 39' 06" |

5.8-25S (Wanzenböck et al., 1997). The FISH procedures were done according to the protocol of Moscone et al. (1996) with small modifications, at 72% of stringency. The probes were labeled with cyanine 3 (Cy3-dUTP) by nick translation (Invitrogen). The hybridization mixture contained 50% v/v formamide, 5% w/v dextran sulfate and 2xSSC, 2-5 ng/ µL of the probe. The preparations were hybridized in situ overnight followed by stringency washing. The preparations were counterstained with DAPI (2 µg/ml) and mounted on Vectashield mount medium. The images were obtained using the Leica DRMA2 microscope.

## Results

The chromosome number found for most species analyzed was 2n = 18, except for *Melipona seminigra abunensis* Cockerel and *Melipona seminigra pernigra* Moure & Kerr, which presented 2n = 22 (Fig 1). The species *Melipona grandis* Guérin, *Melipona nebulosa* Camargo and *M. seminigra abunensis* and *M. seminigra pernigra* had the karyotypes determined for the first time.

The species had symmetrical karyotypes, with slight gradual decrease of size in the chromosomal pairs.


**Fig 1**. *Melipona* karyograms based on Giemsa staining. Species with 2n = 18: *Melipona bicolor* (A), *Melipona quadrifasciata* (B), *Melipona flavolineata* (C), *Melipona fuscopilosa* (D), *Melipona nebulosa* (E), *Melipona scutellaris* (F), *Melipona grandis* (G), *Melipona mondury* (H), *Melipona fasciculata* (I), *Melipona aff. flavolineata* (Capixaba-AC) (J), *M. aff. flavolineata* (Brasiléia-AC) (K), *M. aff. flavolineata* (Xapuri-AC) (L), *M. aff. flavolineata* (Xapuri-AC) (M), *Melipona crinita* (N), and with 2n = 22 *Melipona seminigra pernigra* (O) and *Melipona seminigra abunensis* (P). The chromosomes were arranged in descending order of size. Bar = 10 µm.

Nevertheless they differed substantially in other features. The species *M. bicolor*, *M. quadrifasciata*, *Melipona flavolineata* Friese, *Melipona fuscopilosa* Moure & Kerr, *M. nebulosa* have the first heteromorphic chromosome pair, with one of the chromosomes of the pair with a distinctly longer size compared to the other chromosomes (Fig 1A-E). In *M. scutellaris*, *M. seminigra abunensis* and *M. seminigra pernigra* the first pair is found in the homomorphic or heteromorphic condition (Fig 1F, O, P). The karyotypes of *M. flavolineata*, *M. fuscopilosa*, *M. nebulosa*, *M. scutellaris*, *M. grandis*, *Melipona mondury* Smith, *Melipona fasciculata* Smith, *M.* aff. *flavolineata*, *Melipona crinita* Moure and Kerr, *M. seminigra pernigra*, *M. seminigra abunensis* presented chromosomes with the euchromatin distribution restricted to the terminal regions and a high heterochromatic interstitial content, making it difficult to locate the centromere and determine the morphology of the chromosomes (Fig 1C-P). *M. bicolor* presented submetacentric, acrocentric chromosomes, and the first pair metacentric (Fig 1A). *M. quadrifasciata* showed submetacentric and acrocentric chromosomes, and the first heteromorphic pair composed of a longer metacentric and shorter submetacentric (Fig 1B), as previously described.

The $CMA_3$/DAPI fluorochrome staining revealed heteromorphisms in relation to the length of the $CMA_3^+$ blocks, differences in the chromosomal locations and in the number of karyotypic markings. *M. fasciculata* and *M. grandis* (Fig 2A, B) had interstitial markings, *M. quadrifasciata* and *M. bicolor* (Fig 3A, F), pericentromeric markings and in the other species (Fig 2C-H, 3B -E, H) markings occurred in the terminal regions. In most species a single chromosomal pair with $CMA_3^+$ regions has been observed. *M. nebulosa* and *M. crinita* (Fig 3G, H), however revealed a distinct pattern, showing four

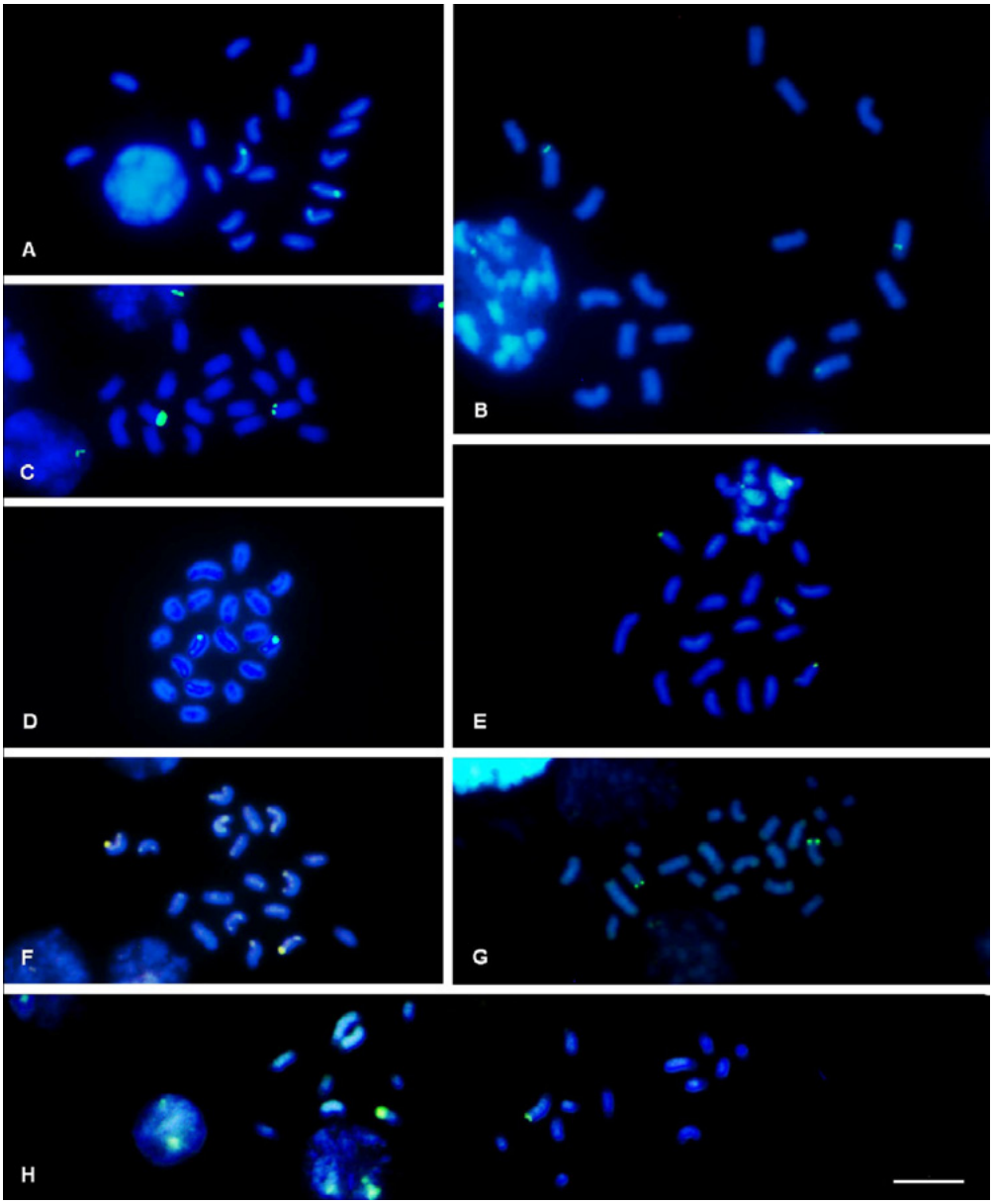


**Fig 2**. $CMA_3$/DAPI staining in metaphases of: *Melipona fasciculata* (A), *Melipona grandis* (B), *Melipona flavolineata* (C), *Melipona scutellaris* (D), *Melipona fuscopilosa* (E), *Melipona mondury* (F), *Melipona seminigra pernigra* (G) and *Melipona seminigra abunensis* (H). $CMA_3^+$ (Green bands). Bar = 10 μm.

$CMA_3^+$ blocks, located in the first and second chromosome pairs. In the first species the blocks were homomorphic and in the second species there was heteromorphism related to the location of the $CMA_3^+$ block between the two pairs. The DAPI evenly stained almost the entire chromosome, except the terminal regions that were weakly stained.

The signals of hybridizations with the rDNA probe coincided in number, size and chromosomal pair with the $CMA_3^+$ bands (Fig 4 and 5), except for *M. crinita* and *M. nebulosa* (Fig 5G, H) in which only two of the markings $CMA_3^+$ sites coincided with rDNA sites. Most of the samples had the rDNA band in the first pair except *M. flavolineata*, *M. scutellaris*, *M. crinita*, *M. seminigra abunensis* and *M. seminigra pernigra*, which was located in the second chromosomal pair and *M. fuscopilosa*, with marking located in the 6th chromosomal pair.

## Discussion

The chromosomal number 2n = 18 obtained in most of the species analyzed in this study is consistent with the predominant chromosome number in the genus *Melipona*. Except for the *M. seminigra* species in which the chromosome number 2n = 22 (Fig 1O, P) was similar to that found for the subspecies *M. seminigra merrillae* (Francini et al., 2011). Rocha and Pompolo (1998) reported the different chromosome number, 2n = 18, for *M. seminigra fuscopilosa* species. However, Camargo and Pedro (2013) propose the specific status for this form as *M. fuscopilosa*. Our results support this specific status by recording the differentiated chromosome number in the two subspecies maintained in *Melipona seminigra* Friese.

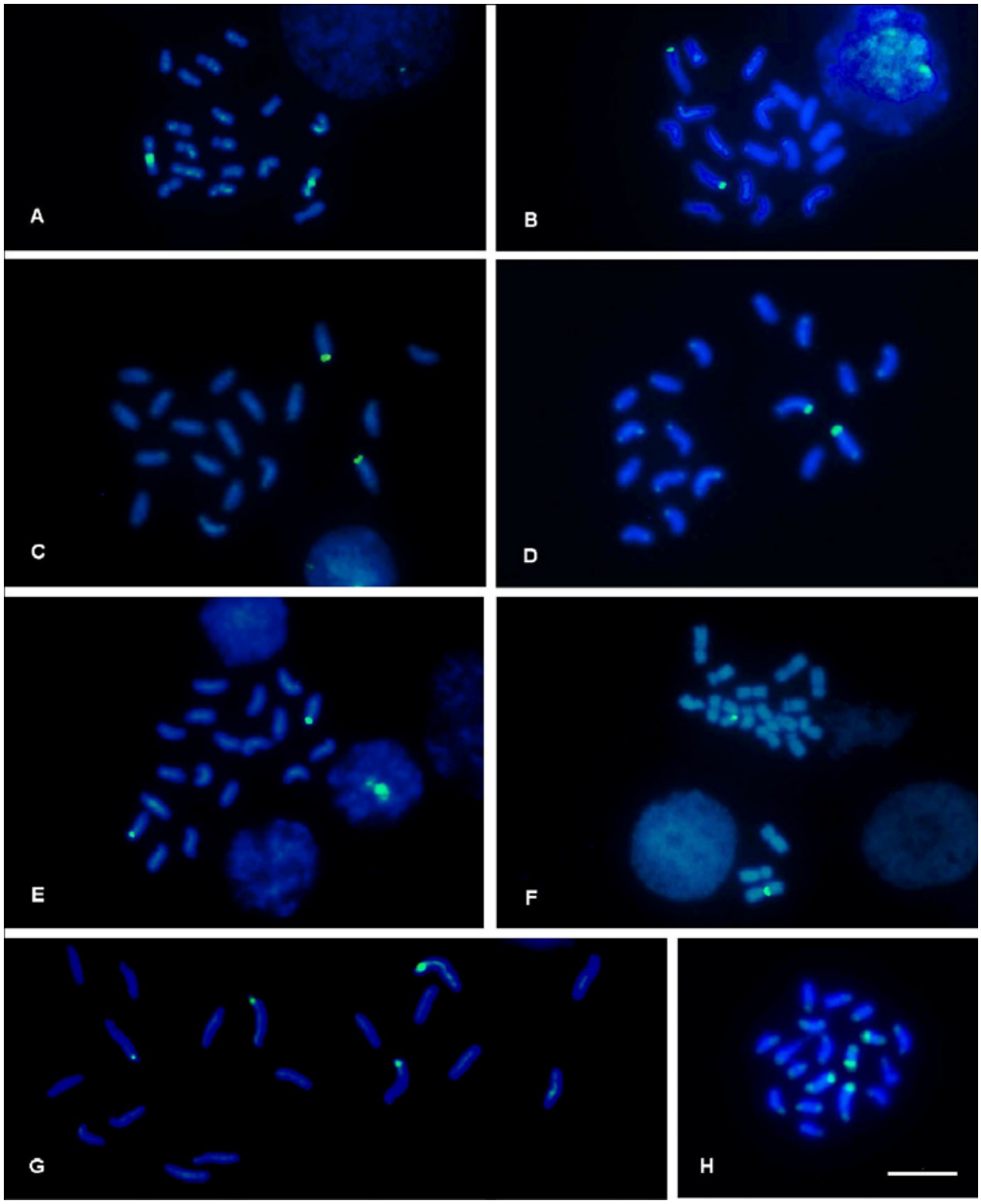


**Fig 3**. $CMA_3$/DAPI staining in metaphases of *Melipona quadrifasciata* (A), *Melipona. aff. flavolineata* (Xapuri-AC) (B), *M. aff. flavolineata* (Brasiléia-AC) (C), *M. aff. flavolineata* (Xapuri-AC) (D), *M. aff. flavolineata* (Capixaba-AC) (E), *Melipona bicolor* (F), *Melipona crinita* (G) and *Melipona nebulosa* (H). $CMA_3^+$ (Green bands). Bar = 10 μm.

The different chromosome number obtained for the subspecies of *M. seminigra* suggests that the increased chromosome number is a derived character within the *Michmelia* subgenus, and may have originated by chromosome fission (Francini et al., 2011; Tavares et al., 2017) with subsequent addition of heterochromatin, resulting in a similar size to the other chromosomes of the karyotype. Analysis of other species of this group, however, may reveal whether the chromosome number 2n = 22 is maintained or if there is variation between closely related species.

The heteromorphism in the first chromosome pair was observed in eight of the 13 species analyzed. This record had already been made in *M. bicolor* and *M. quadrifasciata* (Rocha & Pompolo, 1998), and *M. mondury* (Lopes et al., 2008), however, for *M. flavolineata* in the analysis of Lopes et al. (2011) this pair was homomorphic. This heteromorphic difference, according to Lopes et al. (2008) and Rocha and Pompolo (1998) are due to an additional amount of heterochromatin in the larger chromosome, indicating C-band heteromorphism. The great similarity as to the relative size

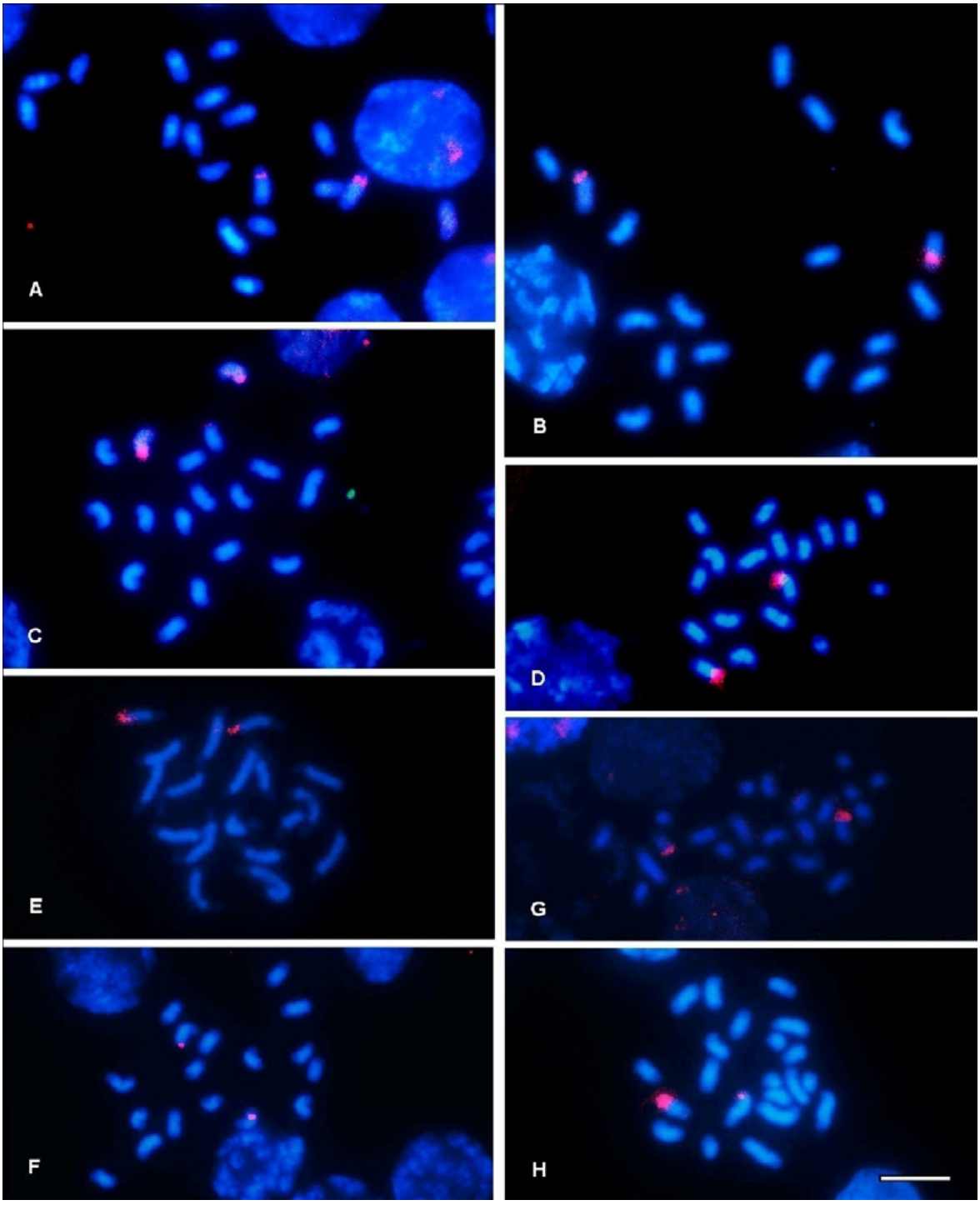


**Fig 4**. FISH with 45S rDNA in metaphases of: *Melipona fasciculata* (A), *Melipona grandis* (B), *Melipona flavolineata* (C), *Melipona scutellaris* (D), *Melipona fuscopilosa* (E), *Melipona mondury* (F), *M. seminigra pernigra* (G) and *M. seminigra abunensis* (H). Bar = 10 µm.

and morphology of this pair among the species suggests the homeology and consequently stability of this chromosomal pair in the karyotype evolution of *Melipona*. Cytogenetic confirmation of this hypothesis would require additional analyzes such as microdissection and chromosome painting by heterologous hybridization.

According to Rocha et al. (2002) species of group II, those with high interstitial heterochromatic content (*Michmelia* and *Melikerria* subgenera), form a natural group and these authors suggest that the higher content of heterochromatin is a derived character. However, due to the polyphyletic character of the subgenus *Eomelipona*, whose diagnostic morphological characters are symplesiomorphies (Silveira et al., 2002) and the fact that the representatives of the subgenus are separated in the molecular phylogeny of Ramirez et al. (2010), the consideration of group II as natural should be discussed and investigated with the cytogenetics of a larger group of species.

Interestingly, although there is a large interspecific difference in relation to heterochromatic content, the chromosome number is conserved in *Melipona*. These results suggest that there was conversion of euchromatin into heterochromatin or vice versa besides the heterochromatin addition. This transformation was proposed to explain this karyotype difference between *Mytilus* species (Mollusca) (Martínez-Expósito et al., 1997). In *Melipona*, the presence of the low eucromatic content in the species of the supposed group I, compared to those of the group II (sensu Rocha & Pompolo, 1998) reinforces this hypothesis of alteration in the content of euchromatin and heterochromatin.

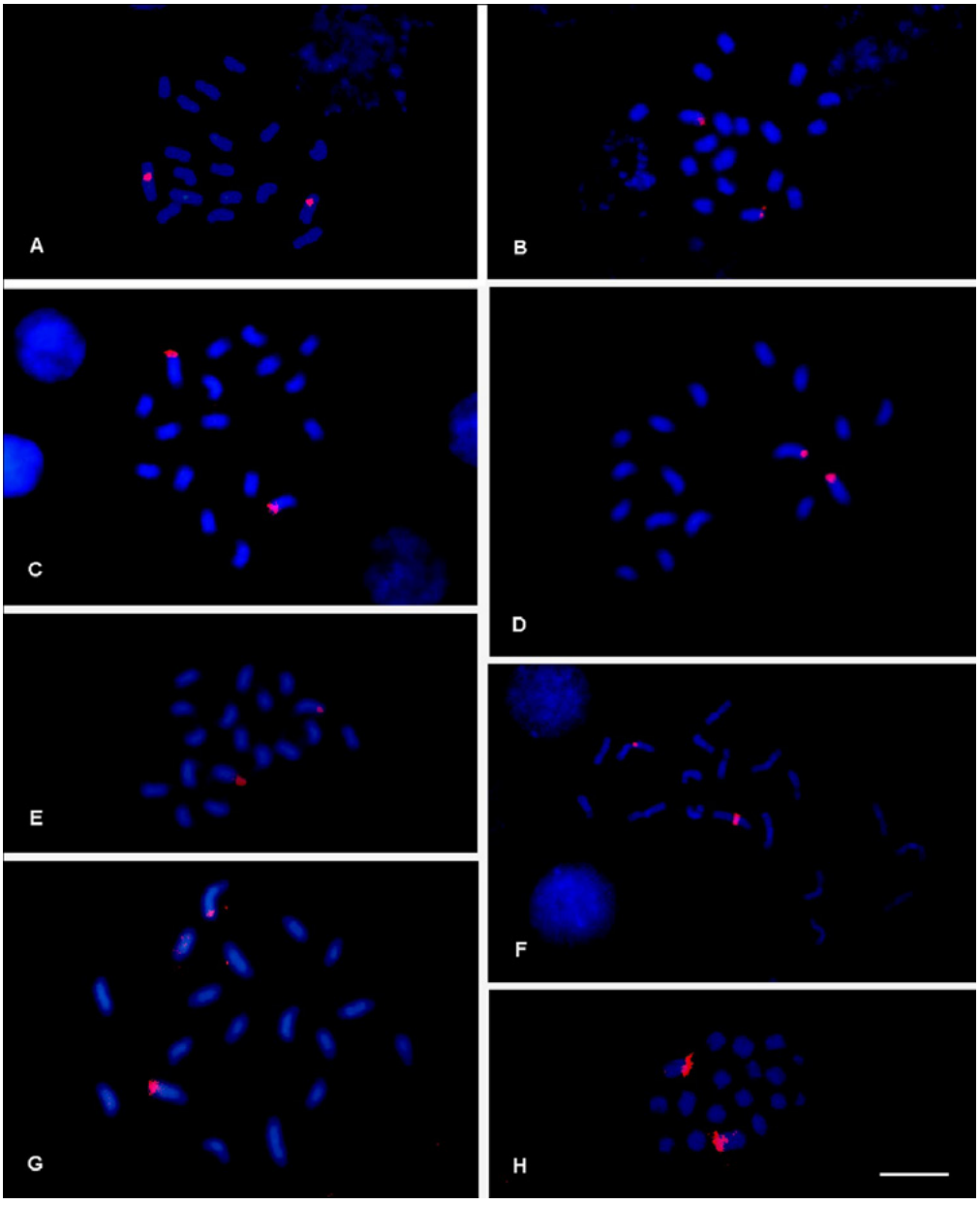


**Fig 5**. FISH with 45S rDNA in metaphases of *M. quadrifasciata* (A), *Melipona aff. flavolineata* (Xapuri-AC[1]) (B), *M. aff. flavolineata* (Brasiléia-AC) (C), *M. aff. flavolineata* (Xapuri-AC[2]) (D), *M. aff. flavolineata* (Capixaba-AC) (E), *Melipona bicolor* (F), *Melipona crinita* (G) and *Melipona nebulosa* (H). Bar = 10 μm.

The location of the $CMA_3^+$ blocks was also different among the species with low and high heterochromatic content analyzed. Among the former are *M. quadrifasciata* and *M. bicolor* analyzed in the present study (Fig 3A, F) and *Melipona asilvae* Moure, *Melipona marginata* Lepeletier, *Melipona subnitida* Ducke (Rocha et al., 2002) and *Melipona mandacaia* Smith (Rocha et al., 2003), which had markers on the pericentromeric regions coincident with the respective C-band regions (Rocha et al., 2002). The second group comprises the species that had the $CMA_3^+$ markings in the terminal regions or interstitial euchromatic regions, which stained weakly with DAPI. These characteristics were also observed in other species of this genus by Rocha et al. (2002) and Lopes et al. (2008). The interstitial marking in *M. fasciculata* was previously observed by Lopes et al. (2011).

*M. mondury* and *M. scutellaris*, exhibited moderate GC content, whose fluorescence was less intense, in the terminal regions of all chromosomes. Lopes et al. (2008) also observed this characteristic in *M. mondury* and *M. rufiventris*, however in that study it was more evident possibly due to the method of staining applied with $CMA_3$ and distamycin ($CMA_3$-DA), which confers higher contrast in the regions rich in GC. The registration of four $CMA_3^+$ markings in *M. nebulosa and M. crinita* is unprecedented in the genus and differs from Rocha et al. (2002), who recorded only one chromosomal pair with $CMA_3^+$ band in *M. crinita* from the same locality. In the phylogeny of Ramirez et al. (2010) these species are in separate groups, and in *M. flavolineata* that is in the same group of *M. crinita* only two $CMA_3^+$ sites were observed, suggesting that the presence of the two additional $CMA_3^+$ sites may have arisen independently in the respective groups.

The correlation between $CMA_3^+$ regions and rDNA sites observed here was also found in other stingless bee species, such as *Partamona peckolti* (Friese) and *Partamona cupira* (Smith) (Brito et al., 2003; Marthe et al.,2010) and *Melipona* (Rocha et al., 2002).

FISH analysis in the present study revealed that the additional $CMA_3^+$ bands in *M. crinita* and *M. nebulosa* do not represent additional rDNA sites (Fig 3G, H, and 5G, H). These bands may correspond to a satellite DNA region rich in GC base pairs resulting from translocation or chromosomal inversion, since they are located in the interstitial region. However, in the review on insect satellite DNA, AT content in Hymenoptera ranged from 46-72%, indicating that there is no predominance of GC content (Palomeque & Lorite, 2008). Confirmation of this information would reveal a different type of satellite DNA in these species. For clarification, FISH with specific satellite DNA probe would be recommended.

A characteristic present in all species analyzed was heteromorphism of size of the $CMA_3^+$ sites and rDNA sites. Different hypotheses attempt to explain this variation, including the terminal localization of rDNA sites that influence variation in the number of copies of the genes (Hanson et al., 1996), the occurrence of unequal crossing over and the differential amplification of intergenic sequences in NOR (nucleolar organizer regions) (Fernandes & Martins-Santos, 2006). The heteromorphism in these sites has been well documented in *Megoura viciae* (Aphididae) Buckton (Mandrioli et al., 1999), *Drosophila* (Roy et al., 2005), *Maxillaria* (Orchidaceae) (Cabral et al., 2006) and *Salvelinus* (Salmonidae) (Śiliwińska-Jewsiewicka et al., 2015).

Within the *Michmelia* subgenus we found differences regarding the location of clusters of ribosomal genes and their position on the chromosome, including closely related groups such as the species *M. flavolineata* from the state of Pará and *M.* aff. *flavolineata* from the Acre region. The chromosomal number 2n = 22 for *M. seminigra* species suggests a derived state in the subgenus. These characteristics together with the presence of additional $CMA_3^+$ sites in *M. crinita* and *M. nebulosa* suggest that the genome of this group undergoes a more accelerated restructuring process. Other analyzes including a larger number of species, especially the subgenus *Eomelipona*, *Melipona* and *Melikerria*, should be carried out in order to expand information on karyotype evolution in the entire genus *Melipona* and to correlate them with available phylogenetic proposals.

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**Authors' contribution**

V Andrade-Souza, MGC Costa and MA Costa conceived the study; V Andrade-Souza wrote the manuscript and analyzed the data; OMP Duarte, CCC Martins and IS Santos helped in the preparation of the slides.

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