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Original article

Repetitive DNAs in *Melipona scutellaris* (Hymenoptera: Apidae: Meliponidae): chromosomal distribution and test of multiple heterochromatin amplification in the genus

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Abstract – *Melipona* bees are remarkable due to the high contrast in heterochromatin amounts, making this group interesting for studying repetitive DNA amplification. Here, we performed the first efforts for the chromosomal localization of different repetitive DNAs in *M.* (*Michmelia*) *scutellaris* and tested for unique or multiple heterochromatin amplification in *Melipona* subgenera. Our data revealed enrichment of repetitive DNAs in chromosomal heterochromatic arms demonstrated by C_0t-DNA and DOP-PCR probe hybridization, although microsatellites and multigene families were located at terminal euchromatic regions. Analysis using C_0t-DNA probe from *M. scutellaris* showed positive hybridization only in *Michmelia* species, suggesting monophyletic amplification and sharing of heterochromatin sequences between species. However, the subgenus *Melikerria*, with a high amount of heterochromatin, probably underwent independent heterochromatin amplification or experienced sequence modification.

bee / FISH / heterochromatin / multigene families / repetitive DNAs

1. INTRODUCTION

Eukaryote genomes are composed of the following two types of repetitive DNAs: (i) in tandem arranged elements, including satellite DNAs, microsatellites, minisatellites, and several multigene families, and (ii) scattered elements, which are represented by transposons and retrotransposons (Charlesworth et al. 1994; Nowak 1994, López-Flores and Garrido-Ramos 2012; Martins 2007). The abundance, easy isolation, and facility in chromosome mapping, allowed the frequent use of these sequences as markers to understand chromosomal and genome organization and evolution in most diverse taxa, including some insect groups. However, few studies have been undertaken in bees (Brito et al. 2005; Rocha et al. 2002; Lopes et al. 2014).

Stingless bees (Meliponini) comprise a diverse group of highly eusocial insects mostly occurring in the New World tropics (Michener 2007, 2013; Camargo 2013). They have great ecological importance as pollinators of native and agricultural plants (Heard 1999; Pedro 2014). The genus *Melipona* comprises at least 50 species distributed from Mexico to Argentina (Michener 2007). *Melipona* species have been the focus of ecological, behavioral, phylogenetic, and genetic studies, but studies focused on their chromosomes are still scarce (Roubik 2006; Rocha et al. 2007; Ramirez et al. 2010; Tavares et al. 2017).

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Until now, *Melipona* karyotypes had been described for 24 species, displaying a very conserved chromosome number of $n = 9$ ($2n = 18$). This was observed in 23 species and only two *M. seminigra* subspecies showed $n = 11$, which probably originated from fissions in two chromosomes (revised by Tavares et al. 2017). Interestingly, the heterochromatin amounts and chromosomal distribution are highly divergent in *Melipona*. According to Rocha and Pompolo (1998), they can be divided into two groups when analyzing by C-banding. One group comprises species with low heterochromatic content (less than 50%) that is primarily distributed in pericentromeric regions or in the short chromosomal arms of their chromosomes (group I). The other group includes species with high heterochromatic content, which occupy almost the entire chromosomes (group II). The heterochromatin in *Melipona* was studied until now by C-banding and fluorochrome staining providing only general information about distribution and base pair enrichment (reviewed by Rocha et al. 2007).

To better understand the chromosomal distribution of repetitive sequences and their relationship with eu- or heterochromatin in the genus *Melipona*, we characterized the chromosomes from *M.* (*Michmelia*) *scutellaris*, a species with high heterochromatic content, using distinct probes for repetitive DNAs. Moreover, to test the hypothesis of multiple or unique rounds of heterochromatin amplification and dispersion in *Melipona*, we isolated the pool of repetitive DNAs (C_0t-DNA fraction) from *M. scutellaris* and used as probe against genomic DNA from five other species belonging to the distinct subgenera and displaying the distinct heterochromatin distribution.

2. MATERIALS AND METHODS

Melipona scutellaris larvae were obtained from apiary colonies at the Bioscience Institute at São Paulo State University, Rio Claro/SP. The mitotic chromosomes were obtained according to Imai et al. (1988) using the cerebral ganglia from post-defective larvae as the source. Slides were prepared by maceration followed by drying on a heat plate (45 °C). For conventional analyses, the slides were stained with 5% Giemsa for chromosome counting and heterochromatin detection was performed according to Sumner (1972).

Genomic DNA was extracted from adults using the phenol/chloroform method (Sambrook and Russel 2001). Multigene families were amplified by polymerase chain reaction (PCR) using specific primers for the 18S rDNA (Cabral-de-Mello et al. 2010) and U2 snDNA (Bueno et al. 2013). Two strategies were used to obtain the repetitive DNA pools: (i) the reassociation kinetics technique (C_0t) and (ii) DOP-PCR. The reassociation kinetics technique (C_0t) was performed according to Zwich et al. (1997) with modifications. Approximately 8 μg of DNA was fragmented with DNAse (Sigma-Aldrich) for 1 min. The generated fragments were checked by 1% agarose gel electrophoresis. The DNA samples were then denatured at 95 °C for 10 min and incubated on ice for 10 s. Then, the samples were subjected to a reassociation temperature of 65 °C for 10 min. After the samples were incubated at 37 °C for 8 min with S1 nuclease, the reaction was stopped by the addition of liquid nitrogen. The DNA was purified with phenol/chloroform (1:1, v/v) and the results were confirmed by electrophoresis on a 1% agarose gel. For amplification by DOP-PCR, a protocol proposed by Telenius et al. (1992) using the degenerate primer 5′-CCGA CTCGACNNNNNNATCTGG was utilized.

The repetitive DNA sequences were labeled with digoxigenin 11-dUTP or biotin 11-dATP by PCR or nick-translation. The seven microsatellite probes were directly labeled with biotin-14 dATP at the 5′-end during their synthesis (Sigma, St Louis, MO, USA). Fluorescent in situ hybridization (FISH) followed the protocol described by Pinkel et al. (1986), with modifications proposed by Cabral-de-Mello et al. (2010), and the probes were detected using anti-digoxigenin-rhodamine (Roche) or Alexa-fluor-488-conjugated streptavidin (Life Technologies). All the preparations were stained with DAPI and mounted with Vectashield mounting medium (Vector, Burlingame, CA, USA). FISH signals were observed using an Olympus BX61 microscope equipped with a fluorescent lamp.

To check whether the pool of repetitive DNAs obtained by C_0t-DNA from *M. scutellaris* is

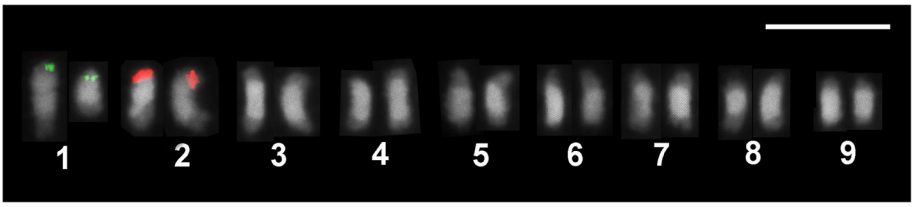

Figure 1. Fluorescence in situ hybridization using 18S rDNA (green) and U2 snDNA (red) as probes in *Melipona scutellaris* chromosomes. Note the hybridization signals are present in the terminal regions of the distinct chromosome pairs. The pair 1 is heteromorphic in size.

shared with other species from the same genus, we performed dot-blot analysis according to the protocol described by Anjos et al. (2016). The probe was tested against genomic DNA from species with low amount of heterochromatin *M.* (*Eomelipona*) *bicolor* and *M.* (*Melipona*) *quadrifasciata* and with high amount of heterochromatin *M.* (*Melikerria*) *fasciculata*, *M.* (*Michmelia*) *rufiventris*, and *M.* (*Michmelia*) *seminigra.* The genomic DNA of *M. (Michmelia*) *scutellaris* was used as positive control.

3. RESULTS AND DISCUSSION

The $2n = 18$ (female) observed in *M. scutellaris* (Figure 1) was previously reported by Rocha et al. (2007). This condition was also reported in 22 other species from the same genus, with variant diploid numbers only occurring in *M. seminigra* when considering the A complement. Among Meliponini species including *Melipona*, the diploid number conservation is recurrent in distinct genera, with slight variations (revised by Tavares et al. 2017).

The mapping of repetitive DNAs is scarce in bees, and FISH mapping using multigene families as a probe in *Melipona* is restricted to major rDNAs in a few species (Rocha et al. 2002, 2007). A common feature of the *Melipona* karyotypes is the heteromorphism in size for the chromosome 1, and in *M. scutellaris*, this chromosome carries 18S rRNA at the terminal region (Figure 1). rDNA loci restricted to one chromosomal pair were also observed in other species of *Melipona* through FISH. Similar data was observed through silver nitrate staining (Rocha et al. 2002; Rocha 2002). It could indicate a modal pattern in the genus, but a higher number of species should be studied. This pattern is also recurring in other Hymenoptera, such as parasitic and social wasps (Gokhman et al. 2014; Menezes et al. 2013) and ants (Imai et al. 2001). However, reports of multiple 18S rDNA signals were also

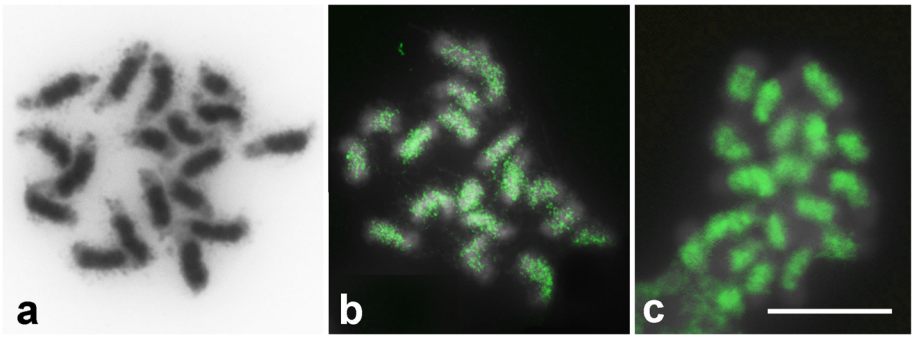

Figure 2. Mitotic metaphases of *Melipona scutellaris*. **a** Heterochromatin location revealed by C-banding. Note large amount of heterochromatin distributed along almost the entire chromosomes and euchromatin restrict to terminal of chromosomes. **b** Chromosomal mapping through fluorescence in situ hybridization (FISH) using as probe the C_0t-DNA fraction of *M. scutellatis*. **c** FISH with the DOP-PCR probe obtained from the genomic DNA of *M. scutellaris*. Note the spread marks along the heterochromatic regions.

described in Hymenoptera, as in *Partamona* (Meliponini) (Brito et al. 2005) and some parasitoid wasps (Paladino et al. 2013; Gokhman et al. 2014). The U2 snDNA was located in pair 2 at the terminal region (Figure 1). This is the first effort to locate this sequence in Hymenoptera. The presence of a unique chromosomal cluster of U2 snDNA was also observed in true bugs (Bardella et al. 2016) and the grasshoppers *Abracris flavolineata* and *Schistocerca gregaria* (Bueno et al. 2013; Camacho et al. 2015), although multiple clusters were also noticed in other species, like grasshoppers (Palacios-Gimenez et al. 2013; Castilho et al. 2017). The use of this probe in other *Melipona* and Hymenoptera will elucidate the organization and evolution patterns of this sequence, which could be a good tool for understanding chromosome evolution.

The heterochromatin was distributed along the chromosomal arms, except in the terminal regions (Figure 2a), which has been observed in specimens from other colonies and is putatively caused by high heterochromatin additions (Rocha and Pompolo 1998; Rocha et al. 2002; Rocha et al. 2007). According to Rocha et al. (2002), heterochromatin amplification could have a crucial role during chromosomal evolution in bees. The differences in the heterochromatin amounts and distribution are remarkable aspects of *Melipona*, and based in these differences, Rocha and Pompolo (1998) divided the genus into two groups (see introduction). Species with high heterochromatic contents also display larger genome sizes, such as *M. scutellaris*, whose genome is 1.08 pg (Tavares et al. 2010). The notion of the amplification of repetitive DNAs, which is associated with large heterochromatic blocks, is reinforced in this work by the analysis of the DOP-PCR and C_0t-DNA fraction probe hybridization that labeled the same regions corresponding to heterochromatin (Figure 2b, c).

In contrast to the mapping with the C_0t-DNA and DOP-PCR probes, seven microsatellite repeats revealed enrichment in the

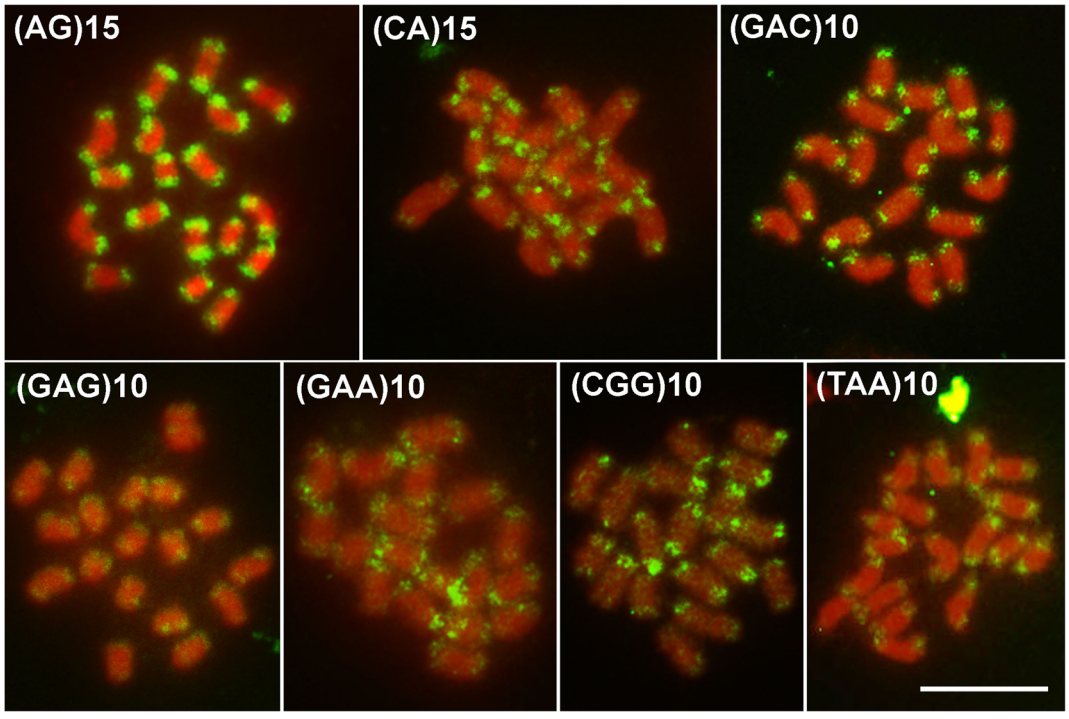

Figure 3. FISH with microsatellites probes in mitotic metaphases of *Melipona scutellaris*. Observe the stronger hybridization signals in the terminal euchromatic regions of chromosomes in comparison to heterochromatin. The microsatellite motifs are indicated directly in each image.

euchromatic regions, although faint scattered signals were also observed in the heterochromatin. No remarkable variability in density was observed in the distinct chromosomes (Figure 3). In other insects, euchromatic locations for some microsatellite repeats have been reported, such as in grasshoppers and crickets (Milani and Cabral-de-Mello 2014; Ruiz-Ruano et al. 2015; Palacios-Gimenez and Cabral-de-Mello 2015). In the Hymenoptera model species *Nasonia vitripennis*, the microsatellite composition, abundance, and density in distinct chromosomes were analyzed using genomic data. The data contrasted with observations in this study based on the density heterogeneity of microsatellites in the five chromosomes from this species. Moreover, considering the heat map for the chromosomal distribution of microsatellites, there are no common regions with higher microsatellite density (Pannebakker et al. 2010) as observed for the distal euchromatic regions in *M. scutellaris*. It has been documented that the genome size could correlate with microsatellite length and frequency. The presence of several microsatellites contributes to increased genome sizes in many organisms, although it is not a general rule (Hancock 1996, 2002; Butcher et al. 2000; Tóth et al. 2000; Warner and Noor 2000; Comeron 2001). Considering the enrichment for microsatellites in *M. scutellaris* euchromatin and that the genome increase is related to heterochromatin amplification, we could speculate that the microsatellite motifs mapped here play a minor role in genome size increase in this species. Moreover, the microsatellite mapping indicates the sharing of repetitive DNAs enriched in euchromatin (C-negative regions), similar to what was observed for *M. rufiventris* heterochromatin (Lopes et al. 2014). Putatively, the repetitive DNAs associated with heterochromatin amplification in *M. scutellaris* are satDNAs and transposable elements, which are generally isolated in C_0t-DNA fraction. However, as C_0t-DNA is anonymous,

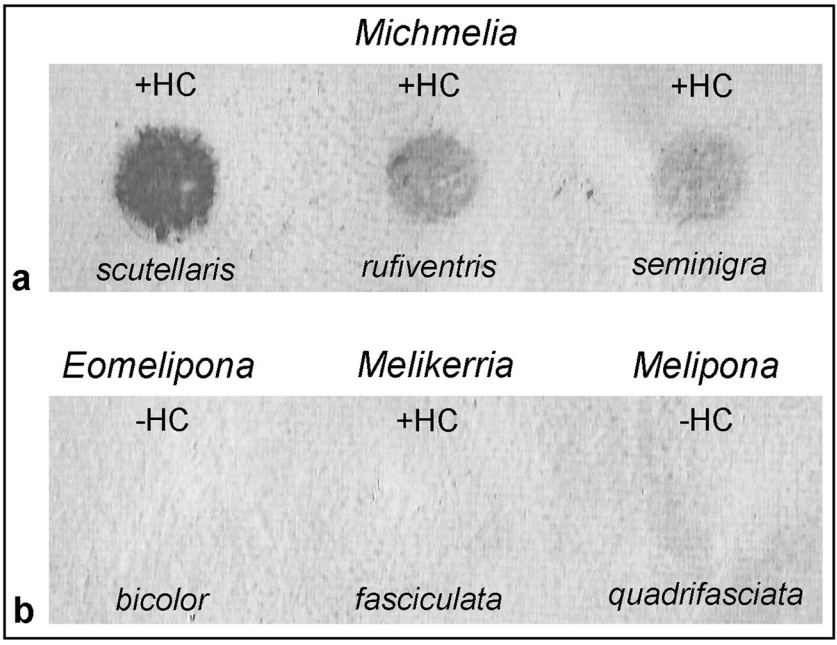

Figure 4. Dot-blot hybridization using *M. scutellaris* C_0t-DNA fraction (repetitive DNA enriched fraction) as a probe in species from the *Melipona* genus groups, group I and group II. **a** Note the positive signals in *M. rufiventris* and *M. seminigra*, which belong to the subgenus *Michmelia* and have high heterochromatin contents. **b** Note the absence of signals in species belonging to the other subgenus, including a species with high heterochromatic content, *M.* (*Melikerria*) *fasciculata*. Species with high heterochromatin amount are identified by (+HC) while species with low heterochromatin amount by (−HC).

specific sequences should be characterized to ascertain it.

Melipona species with high heterochromatin amounts are placed into two distinct subgenera (*Michmelia* and *Melikerria*). Based on phylogenetic data, Lopes et al. (2011) proposed that heterochromatin amplification occurred more than once in *Melipona*. Our data using dot-blot hybridization shed light on this question. Membrane hybridization using the C_0t-DNA fraction of *M. scutellaris* as a probe against the genomic DNA of some *Melipona* species (*M. rufiventris*, *M. seminigra*, *M. bicolor*, *M. fasciculate*, and *M. quadrifasciata*) only showed positive hybridization to *M. rufiventris* and *M. seminigra*, which are species belonging to the same subgenus (*Michmelia*) (Figure 4). These data support the presence of monophyletic heterochromatin amplification and conservation of sequence similarity among species of *Michmelia*. These findings also suggest more than one round of heterochromatin amplification, i.e., independent amplification of distinct repetitive DNAs, in distinct subgenera of *Melipona* or sequence diversification between subgenera. The occurrence of a polyphyletic subgenus (*Eomelipona*) and of species not assigned to a specific subgenus (Ramirez et al. 2010) is a challenge for a more conclusive hypothesis. The analysis of other species and the better understanding of taxonomy and phylogenetic relationships between *Melipona* subgenera could shed light on the hypothesis about heterochromatin amplification. Even though the pool of repetitive DNAs is shared between *Michmelia* species, the specific sequences responsible for heterochromatin amplification should be better investigated, using for example, genome-sequencing data. This is an interesting issue to be investigated in the future and will allow for a more conclusive hypothesis for the causes and the sequences involved in heterochromatin amplification in *Melipona*. These data together with the chromosomal mapping of repetitive DNAs studied here contribute to the improvement of the hitherto poor knowledge of chromosomal organization of repetitive DNAs among bees, mostly specifically in *Melipona* species.

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AUTHORS' CONTRIBUTION

DCCM conceived the study and designed the experiments; MCAP and VBB performed the experiments; MCAP, VBB, and DCCM interpreted the data and wrote the manuscript. All the authors read and approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

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

ADN répétitifs chez *Melipona scutellaris* (Hymenoptera: Apidae: Meliponidae): distribution chromosomique et test d'amplification multiple de l'hétérochromatine dans le genre

abeilles / FISH/ hétérochromatine / familles multigéniques / ADN répétitifs

Repetitive DNA in *Melipona scutellaris* (Hymenoptera: Apidae: Meliponidae): Verteilung über die Chromosomen und Test auf multiple Amplifikation von Heterochromatin innerhalb der Gattung

Biene / FISH/ Heterochromatin / Multigenfamilien / repetitive DNA

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