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Different Levels of Chromatin Condensation in *Partamona chapadicola* and *Partamona nhambiquara* (Hymenoptera, Apidae)

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Chromosome evolution · Cytogenetics · Fluorescence in situ hybridization · Heterochromatin · Stingless bees

Abstract
Studies in several organisms have contributed to the understanding of heterochromatin and its biological importance. In bees of the tribe Meliponini, the presence of chromosomes with totally heterochromatic arms has been attributed to the mechanism of karyotype evolution in which this group accumulated heterochromatin to maintain telomere stability after centric fission events. In the present study, the use of classical and molecular cytogenetic techniques as well as automated image analysis software for the description of the karyotypes of *Partamona chapadicola* and *P. nhambiquara* bee species revealed variability in the compaction and patterns of chromatin structure. Although both species have the same chromosome number as other species in the genus *Partamona* (2n = 34), C-banding and image analyses indicated the existence of chromosomes with 3 regions of different staining intensities, suggesting a chromatin structure with distinct patterns and characteristics. Repetitive DNA probes hybridized only in the euchromatic regions, whereas the regions with intermediate staining intensity did not show any hybridization signals. This suggests that these regions present features more similar to heterochromatin. Evidence of the existence of a chromatin class with intermediate condensation compared to euchromatin and heterochromatin indicates a potential mechanism for heterochromatin amplification and demonstrates the need for further studies on this topic. This previously unrecognized class of chromatin should be taken into account in the study of all Meliponini chromosomes.

The composition and organization of chromosomes have been widely studied in several groups of organisms [Guerra, 1988]. Heterochromatin is one of the most interesting and most important topics in the study of chromosomes. Since its discovery in 1928 [Heitz, 1928], much has been learned about heterochromatin; however, there is no consensus about its complete function or how its composition and location may be related to events that lead to karyotype evolution in specific groups of organisms. Constitutive heterochromatin is characterized by a high number of repetitive DNA sequences and gene inactivity [Grewal and Jia, 2007]. Alternatively, facultative heterochromatin can be transcribed, but the genes pres-

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ent there are silenced in specific cell types or during the development phase in which this region behaves in a truly heterochromatic manner [Grewal and Jia, 2007]. Normally, this gene inactivation in facultative heterochromatin occurs due to differential methylation patterns of histone H3, which is associated with DNA in those regions [Grewal and Jia, 2007].

The heterochromatin distribution patterns in the insect order Hymenoptera are related to the processes of chromosome evolution. The most accepted mechanism to explain karyotype evolution within the order was proposed by Imai et al. [1986; reviewed in Imai et al., 1988, 1994]. In those studies, Imai and colleagues postulated the Minimum Interaction Theory (MIT) that predicts how often changes occur in the karyotypes of Hymenoptera and other organisms in order to minimize the deleterious interactions between chromosomes. This minimization is hypothesized to occur through centric fission events that decrease the chromosome size, therefore reducing the likelihood of chromosomal rearrangements. However, according to the MIT, these centric fission events would generate instability in the break region, which would lead to the incorporation of heterochromatin at the newly generated chromosome end. As a consequence of this heterochromatin amplification, most of the chromosomes present in Hymenoptera would contain a euchromatic chromosome arm and a heterochromatic chromosome arm (a chromosome morphology referred to as pseudo-acrocentric or A^M). This morphology can be verified in several species of the tribe Meliponini [Rocha et al., 2003; Tavares et al., 2017]. However, some recent data are incompatible with MIT, indicating that for bees there must be other mechanisms complementary to those proposed by Imai and colleagues [Fernandes et al., 2013; Travenzoli et al., 2019a].

Some species of the tribe Meliponini have heterochromatin only in the pericentromeric chromosome regions. Images of C-banding show that in the chromosomes of many of these species regions with intermediate staining can be identified [Brito et al., 2003; Miranda et al., 2013]. However, this feature has not been explored in depth. One possible explanation for the intermediate staining is that these are regions of heterochromatin formation that is involved in more than just the stabilization of the telomeric region, as proposed in Imai et al. [1988]. Recent studies have shown that heterochromatin emerged at different times during the evolution of Meliponini species, indicating that its formation is a continuous and recurrent process [Cunha et al., 2018; Piccoli et al., 2018]. Recent developments in the field of cytogenetics include new techniques for studying chromosomes and new software for analyzing images, both leading to the investigation of chromosome banding patterns with improved depth and reliability. These new methods include chromosome measurements and determination of color intensity, allowing for better statistical analysis of these parameters [Krinski et al., 2012].

Among the 33 genera in the tribe Meliponini, the Neotropical genus *Partamona* Schwarz, 1939 comprises 33 species ranging from Mexico to southern Brazil [Camargo and Pedro, 2003; Moure et al., 2007]. However, cytogenetic studies have been published for only 8 of these species: *Partamona* aff. *nigrior* (Cockerell, 1925), *P. ailyae* Camargo, 1980, *P. cupira* (Smith, 1863), *P. helleri* (Friese, 1900), *P. mulatta* Moure in Camargo, 1980, *P. pearsoni* (Schwarz, 1938), *P. peckolti* (Friese, 1901), and *P. vicina* Camargo, 1980 [Tarelho, 1973; Brito, 1998; Brito-Ribon et al., 1999; Brito et al., 2003; Marthe et al., 2010].

Therefore, the aim of this work was to characterize the chromosomes of 2 more of these species, *Partamona chapadicola* Pedro & Camargo, 2003 and *P. nhambiquara* Pedro & Camargo, 2003, using classical and molecular cytogenetic techniques as well as image analysis tools for more precise studies of the chromosome banding patterns. Furthermore, we wanted to test the hypothesis that the different heterochromatic patterns present in *Partamona* species are due to the existence of a chromatin class with intermediate condensation compared to euchromatin and heterochromatin.

Materials and Methods

Post-defecant larvae of *P. chapadicola* and *P. nhambiquara* were collected and processed according to the methodology described by Imai et al. [1988] to obtain mitotic metaphase chromosomes. *P. chapadicola* were collected from 5 colonies in Urbano Santos, MA, Brazil (3°12′29″ S; 43°24′18″ W), and *P. nhambiquara* were collected in from 2 colonies Tangará da Serra, MT, Brazil (14°37′10″ S; 57°29′09″ W). Conventional staining was performed using 4% Giemsa for 20 min. The fluorochromes chromomycin A_3 (CMA_3) and 4′,6-diamidino-2-phenylindole (DAPI) were used as previously described [Schweizer, 1980]. For C-banding, the parameters established by Rocha and Pompolo [1998] were applied. The slides were then stained with 8% Giemsa in Sörensen buffer pH 6.8. The metaphase images were captured on an Olympus BX60 microscope using the Q3 capture program. The karyotypes were assembled and classified according to chromosome arm ratios [Levan et al., 1964] and to the chromosome classification proposed by Imai [1991] that relies on the heterochromatin distribution pattern detected by the C-banding technique.

The Image-Pro Plus 2D Image Analysis Software (version 6.3, Media Cybernetics 2009) was used to assemble karyotypes and make histograms. The intensity of each pixel along the longitudi-

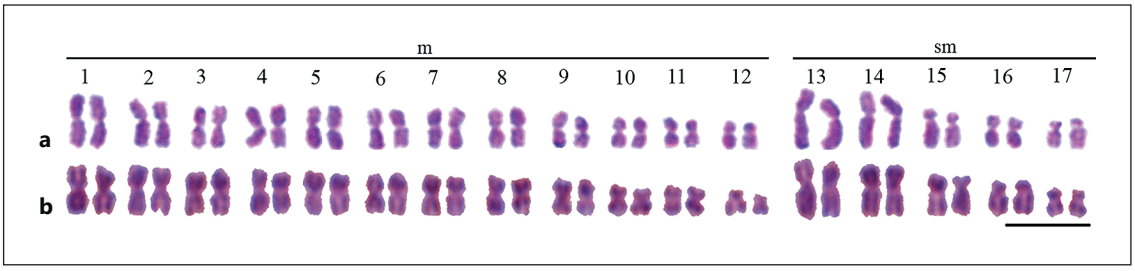

Fig. 1. Giemsa-stained karyotypes of *Partamona chapadicola* (**a**) and *P. nhambiquara* (**b**). Scale bar, 5 μm.

nal axis of the chromosome was measured and expressed using a scale of shades of gray varying from 0 (white) to 255 (black). For each region differentiated by C-banding, the average intensity of the gray tones was calculated from 20 measurements per region. In order to verify that the differences between the means of each chromosome region were statistically significant, we performed variance analyses and Tukey tests (at 1% probability) using the GENES software program [Cruz, 2006].

FISH experiments were conducted following the protocol of Cioffi et al. [2011]. The oligonucleotide probes with microsatellite sequences $(GAG)_{10}$ and $(GA)_{15}$ were commercially synthesized and labeled on the 5′ end with fluorochrome Cy3 (Sigma, St. Louis, MO, USA). For the 18S rDNA probe experiments, we followed the protocol of Pinkel et al. [1986], but with some modifications: metaphase chromosomes were denatured in 70% formamide, 2× SSC at 75°C for 5 min. The 18S rDNA was obtained by amplification using primers F1 (5′-GTCATATGCTTGTCTCAAAGA-3′) and 18SR1.1 (3′-TCTAATTTTTTCAAAGTAAACGC-5′), designed for the species *Melipona quinquefasciata* Lepeletier, 1836 [Pereira, 2006]. Labeling was performed by PCR using digoxigenin-11-dUTP-labeled nucleotides; anti-digoxigenin rhodamine was applied for signal detection (Roche Applied Science, Penzberg, Germany). The chromosomes were counterstained with DAPI and analyzed on an Olympus MX10 epifluorescence microscope, equipped with DP73 camera, using cellSens Imaging Software (Olympus, Tokyo, Japan).

Partamona helleri has 34 chromosomes with one euchromatic and another heterochromatic arm [Costa et al., 1992], not showing this intermediate pattern. Therefore, we used samples of this species collected in Viçosa, MG, Brazil (20°45′14″ S; 42°52′55″ W) to compare the distribution of microsatellites in *P. chapadicola* and *P. nhambiquara*, mainly checking the presence or absence of hybridization signals in the intermediate region.

Results and Discussion

The chromosome number of *P. chapadicola* and *P. nhambiquara* is 2n = 34 for females and n = 17 for males, and according to their arm ratios, both species have a karyotype formula of 24m + 10sm (Fig. 1). The same number was found in 9 other *Partamona* species that have been studied cytogenetically [Tarelho, 1973; Brito et al., 1997, 2003, 2005; Brito, 1998; Brito-Ribon et al., 1999; Marthe et al., 2010]. Some exceptions present *Partamona* species that have populations with supernumerary chromosomes including *P. helleri* and *P. cupira* [Costa et al., 1992; Brito et al., 1997; Brito, 1998; Marthe et al., 2010].

Based on the results of C-banding, the chromosomes in both species displayed a euchromatic arm and a heterochromatic arm, which is characteristic of pseudo-acrocentric (A^M) chromosomes according to the nomenclature proposed by Imai [1991] (Fig. 2). A preliminary analysis of the C-banding pattern in the chromosomes with A^M morphology indicated the existence of 3 distinct shades of coloration along some of the chromosomes in both species. We then used image analysis software to generate histograms to confirm that there were 3 regions with distinct staining intensities for specific chromosomes (Fig. 3).

Figure 3b shows a representative chromosome that can be divided into 3 distinct portions based on coloration. Region 1 represents the euchromatin region and has a lighter coloration with an average intensity of 104.04 on the tone scale. Region 2, in the central portion of the chromosome, corresponds to the heterochromatin region and is intensely stained with an average intensity of 232.19. Region 3 has an intermediate staining intensity between that of regions 1 and 2, with an average of 150.90. Comparison of the average intensities of these 3 regions by the Tukey test (1% probability) confirmed that these were statistically different with respect to their coloration intensity. Chromosomes with this "new class" of intermediately stained chromatin identified in the present work are not encountered in the nomenclature of Imai et al. [1986, 1988, 1994]. Therefore, to differentiate these from the standard pseudo-acrocentric (A^M) chromosomes, we will refer to this new class of chromosomes as A^{Mn} (new pseudo-acrocentric). Both of the *Partamona* species studied in the present work presented chromosomes with the A^{Mn} morphology. Thus, the

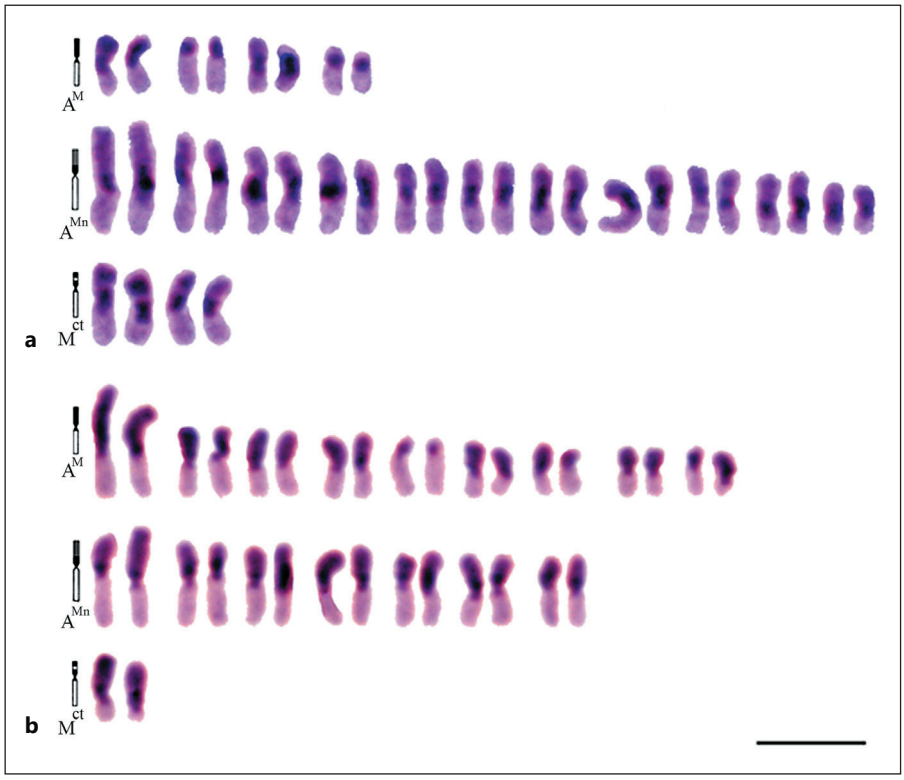

Fig. 2. C-banded karyotypes of *Partamona chapadicola* (**a**) and *P. nhambiquara* (**b**). Scale bar, 5 µm.

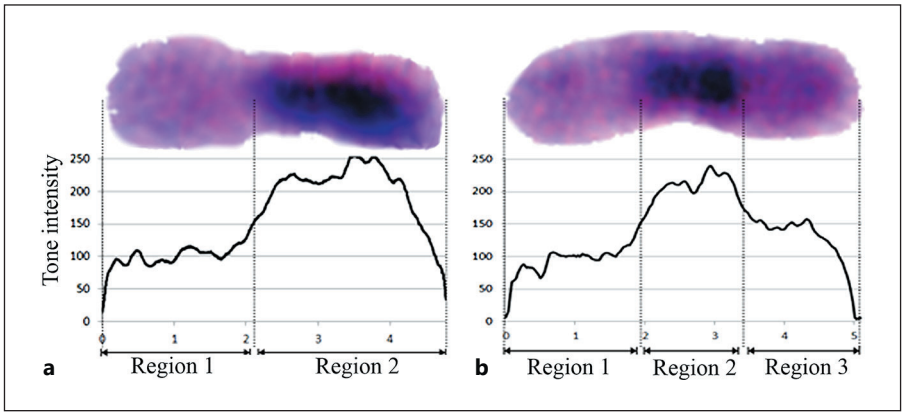

Fig. 3. Histograms of an A^m (**a**) and an A^{Mn} (**b**) chromosome found in *Partamona chapadicola* and *P. nhambiquara*. **a** In the A^M chromosome, 2 regions can be recognized according to the intensity of C-banding. **b** In the A^{Mn} chromosome, 3 distinct regions are present.

karyotype formulas of *P. chapadicola* and *P. nhambiquara* found in the present work are $2K = 8\ A^M + 22\ A^{Mn} + 4\ M^{ct}$ (metacentric chromosome with terminal and centromeric heterochromatin) and $2K = 18\ A^M + 14\ A^{Mn} + 2\ M^{ct}$, respectively (Fig. 2).

Staining with the DAPI fluorochrome marked most of the regions of heterochromatin determined by C-banding, indicating that those regions are rich in AT base pairs (Fig. 4a, c) in the 2 species. In Neotropical Meliponini species, heterochromatin generally is AT-rich [Brito, 1998; Brito et al., 2003; Lopes et al., 2008]. Here, 4 blocks at the end of some chromosomes were strongly marked by the fluorochrome CMA_3, indicating that these regions are rich in CG base pairs (Fig. 4b, d). Along with the increase in the number of cytogenetically characterized *Partamona* species, the pattern of heterochromatin composition has been shown to be constant for members of the tribe Meliponini. Most of the observed heterochromatin found is DAPI-positive, with only a few small CMA_3-positive blocks associated with nucleolus organizer regions (NORs) in some species [Brito et al., 1997; Brito, 1998; Maffei et al., 2001; Rocha et al., 2003, 2007; Lopes

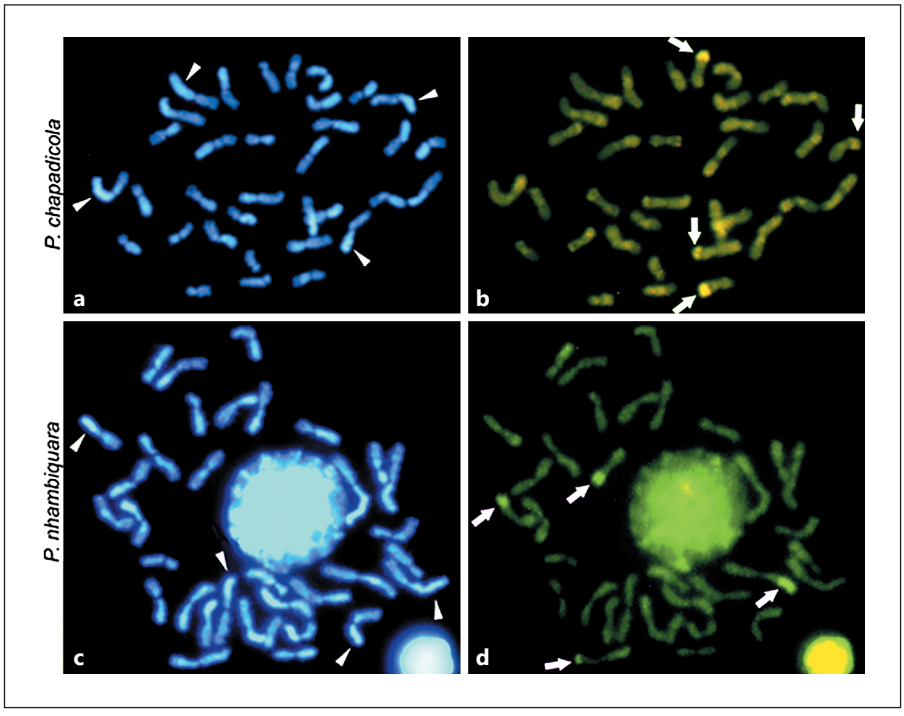

Fig. 4. Metaphases of *Partamona chapadicola* (**a**, **b**) and *P. nhambiquara* (**c**, **d**) stained with DAPI (**a**, **c**) and CMA_3 (**b**, **d**). Arrowheads and arrows indicate DAPI- and CMA_3-positive regions, respectively.

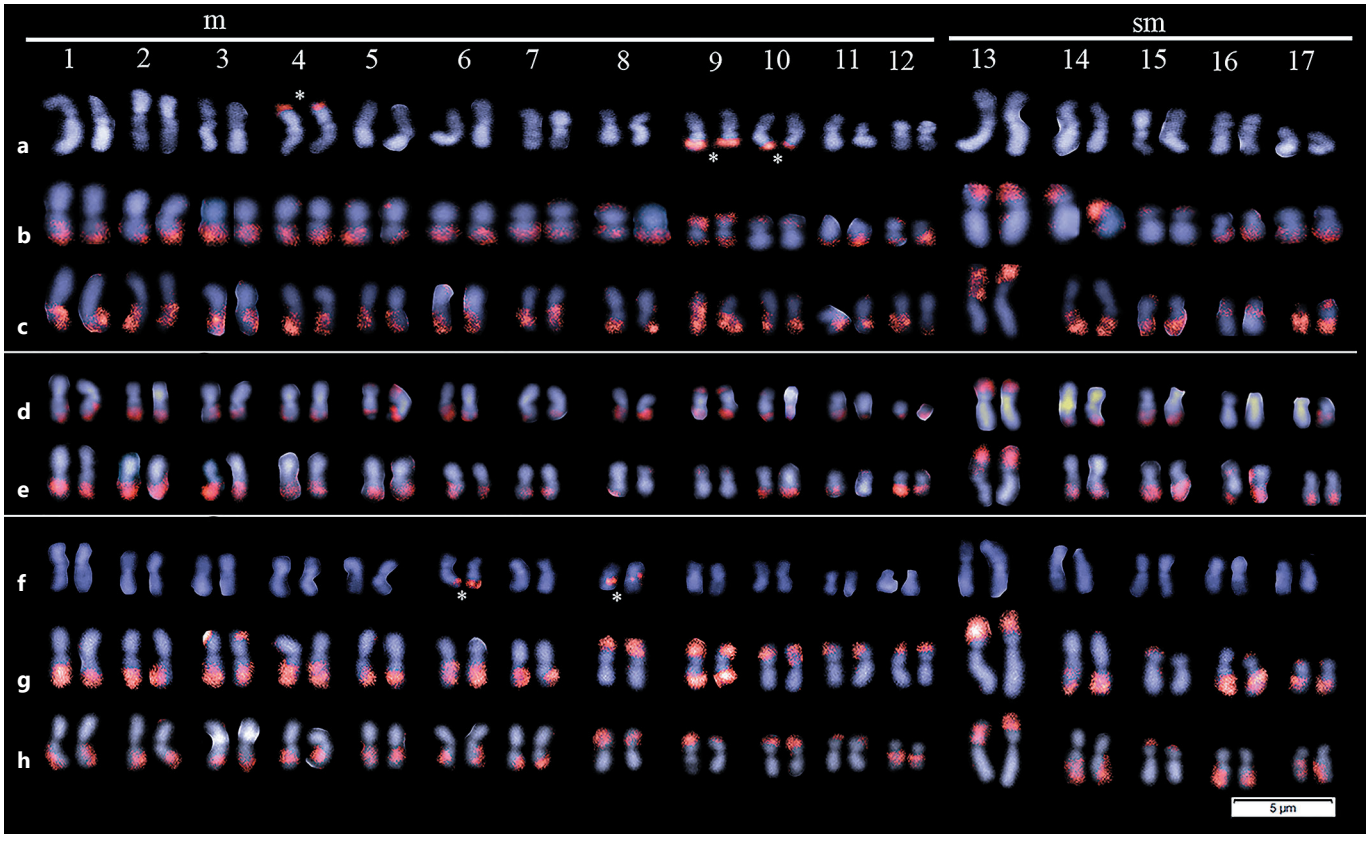

Fig. 5. Karyotypes of *Partamona chapadicola* (**a–c**), *P. helleri* (**d**, **e**), and *P. nhambiquara* (**f–h**) after FISH with 18S rDNA probe (**a**, **f**), and $GAG_{(10)}$ (**b**, **d**, **g**) and $GA_{(15)}$ (**c**, **e**, **h**) microsatellite probes. Asterisks indicate the chromosome pairs with 18S marking. Scale bar, 5 µm.

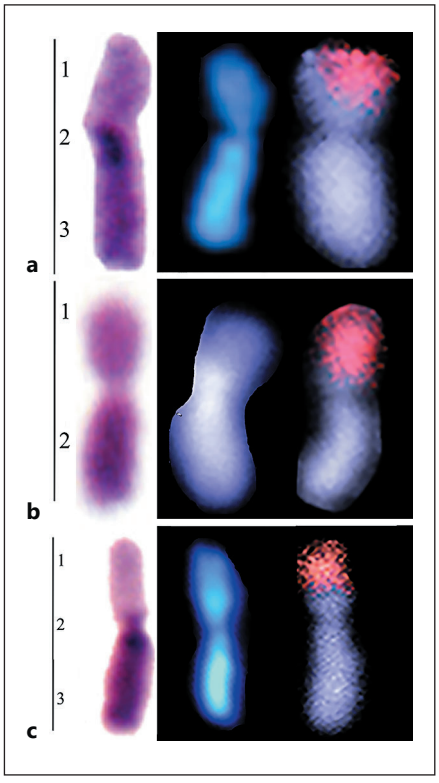

Fig. 6. Comparison of chromosomes after C-banding, DAPI staining, and FISH with a microsatellite probe in *Partamona chapadicola* (**a**), *P. helleri* (**b**), and *P. nhambiquara* (**c**).

et al., 2008; Duarte et al., 2009; Martins et al., 2009; Barth et al., 2011; Lopes et al., 2011].

The correlation between positive C-banding, bright CMA_3 bands, and NOR markers has been previously reported for the genus *Partamona* [Brito et al., 1997, 2005; Brito, 1998], as well as for other species of the tribe Meliponini [Maffei et al., 2001; Rocha et al., 2002, 2007; Duarte et al., 2009; Lopes et al., 2011] and for other organisms such as wasps [Menezes et al., 2011], Hemiptera [Criniti et al., 2009], mollusks [Petrović et al., 2009], and fish [Swarça et al., 2003]. However, the use of an 18S rDNA probe revealed signals in 6 chromosomes in *P. chapadicola* (Fig. 5a) and 4 signals in *P. nhambiquara* (Fig. 5f). The correlation between CMA_3 staining and rDNA was not observed in this work. In *P. chapadicola*, the number of signals was different, and in *P. nhambiquara*, the position of the 18S rDNA sites differs from the CMA_3 bands. The 18S rDNA was mostly located in the terminal region of the chromosomes, with the exception of *P. nhambiquara*, which showed one 18S rDNA signal in the interstitial region and another in the terminal region (Fig. 5a, f).

Figure 6 shows a particular chromosome of each of the 3 species submitted to C-banding, DAPI staining, and FISH with microsatellite $GA_{(15)}$. The euchromatin (region 1) is DAPI negative, the heterochromatin (region 2) is DAPI positive, together with the intermediate portion (region 3). Microsatellite hybridization was observed in the DAPI-negative portion corresponding to euchromatin. Similar results have been obtained in other Meliponini studies [Ferreira, 2015; Piccoli et al., 2018; Travenzoli et al., 2019b]. The microsatellite probes bound and marked the chromosomes in all species tested (Fig. 5). Thus, the intermediate region was DAPI positive with no labeling. These results indicate that the regions 2 and 3 possess similar characteristics, and probably both consist mainly of heterochromatin.

It was not possible to infer gene activity levels within these intermediate regions, which could have indicated whether these regions consist of constitutive heterochromatin, with little or no gene activity, or facultative heterochromatin, with genes that are silenced only at specific times. However, it is known that compaction of heterochromatin is related to histone H3 methylation, and certain studies have shown differences in the organization of these 2 types of heterochromatin [Grewal and Jia, 2007]. Thus, constitutive heterochromatin is organized in a regular matrix and is therefore more compact due to a lower possibility of methylation of the histone H3 lysine residues. Alternatively, facultative heterochromatin has a greater chance of methylation, conferring a less regular matrix and less compaction [Dillon, 2004; Grewal and Jia, 2007]. Theoretically, this difference could favor the alkaline treatment during the C-banding procedure, which would explain the intermediate staining (an intensity between that of euchromatin and constitutive heterochromatin regions) observed in region 3 of the A^{Mn} chromosomes.

Considering that heterochromatin in Meliponini arose at different times [Cunha et al., 2018; Piccoli et al., 2018; Pereira, 2018] and that chromatin with intermediate staining was observed only in species with centromeric heterochromatin, this "new class" of chromatin can indicate the beginning of the process of heterochromatinization in these species, and will result in the formation of completely heterochromatic arms as seen in high frequency in the Meliponini. Therefore, the verification of the existence of a new class of chromatin with intermediate staining intensity, between that of the euchromatin and heterochromatin, demonstrates a possible path of how karyotype evolution has taken place in the tribe Meliponini.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors declare that they have no potential conflict of interest in relation to the study described in this paper.

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Author Contributions

D.M.L. and L.A.O.C. conceived the study and designed the experiments. N.M.T. and A.F. performed the experiments and analyses. All authors participated in writing the paper, read, and approved the final manuscript.

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