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Comparative cytogenetic analysis reveals chromosomal variability in five stingless bees of the genus *Trigona* (Apidae, Apinae, Meliponini)

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Abstract – The stingless bee genus *Trigona* includes 32 species, exclusive to the New World, which are grouped into two clades (A and B) according to phylogenetic molecular data. Cytogenetic studies have been performed in only seven *Trigona* taxa, and molecular cytogenetic data are available for only one species. These studies have been important for the chromosomal characterization of the species; however, discussions focusing on the karyotype evolution of *Trigona* in a phylogenetic context are lacking. In this study, we characterized the karyotype, through classical and molecular cytogenetics, of five *Trigona* species: *T. pallens* and *T. williana*, from clade A, and *T. hypogea*, *T. aff. fuscipennis*, and *T. truculenta*, from clade B, in order to provide insights into the karyotype evolution in *Trigona* and investigate whether the analyzed cytogenetic markers may have a phylogenetic signal. All five *Trigona* species have $2n = 34$ chromosomes. Variations in the karyotype formula were observed in *T. truculenta* and *T. hypogea* compared with the other three species. Although heterochromatin distribution was restricted to one of the arms in most of the chromosomes of the five species, C-banding experiments highlighted a lower degree of heterochromatin compaction in *T. pallens* and *T. williana*. The microsatellite (GA)₁₅ was exclusively located in the euchromatic regions of the chromosomes in all five species. The number of pairs bearing rDNA genes varied among the species studied, and this cytogenetic trait seems to reflect the phylogeny, separating the species into two clades. This study showed interspecific variations to a greater or lesser degree among *Trigona* species, highlighting the intense chromosomal evolutionary dynamics in the genus.

karyotype / chromosomal evolution / heterochromatin / ribosomal genes / microsatellites

1. INTRODUCTION

Stingless bees (Meliponini) play important ecological and economic roles as pollinators of several wild and cultivated plants; therefore, they

are crucial for biodiversity conservation (Heard 1999; Slaa et al. 2006; Ramírez et al. 2018). However, intense stingless bee declines have been observed due to anthropogenic actions, such as fragmentation/loss of habitat, agricultural intensification, and introduction/spread of exotic competing bee species, which brings attention to the conservation efforts of these insects (Freitas et al. 2009; Ramírez et al. 2013). Several scientific areas may generate useful

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information for conservation strategies (Zayed 2009; Murray et al. 2009; Lozier and Zayed 2017; López-Uribe et al. 2017). Cytogenetics, for example, may be helpful because the existence of chromosomal polymorphisms between populations can be a barrier to species reintroduction since mating between individuals with different cytotypes can result in mortality or infertility in offspring (Robinson and Elder 1993; Mariano et al. 2008; Potter and Deakin 2018).

In this context, cytogenetic studies have already been performed on 32 genera and 108 taxa of stingless bees, with most of the data belonging to species from the Neotropical region; such studies have allowed a better understanding of chromosomal evolution in this group (reviewed by Cunha et al. 2021a). To date, it is known that chromosome numbers range from $n = 8$ to $n = 40$; however, most species have $n = 17$ chromosomes (Tavares et al. 2017; Cunha et al. 2021a). Through molecular and cytogenetic approaches, Travenzoli et al. (2019a) suggested that $n = 17$ is the ancestral chromosome number in Neotropical Meliponini, and chromosomal variations observed among the species possibly originated from Robertsonian rearrangements. Another notable cytogenetic characteristic of stingless bees is that amplifications of their heterochromatin seem to explain the increases in genome size observed among the species (Tavares et al. 2012; Cunha et al. 2021b).

Molecular cytogenetic studies have contributed to the knowledge of heterochromatin composition, showing that repetitive sequences that constitute it may have differentiated or arisen independently along the stingless bee evolution (Cunha et al. 2020; Lopes et al. 2020; Pereira et al. 2020, 2021a, b). In addition, molecular cytogenetic data show that microsatellites are predominantly located in euchromatin, although for some species they have also been reported in heterochromatin. Studies also display that $(TTAGG)_n$ motif constitutes the telomeres of these insects (Santos et al. 2018; Travenzoli et al. 2019b; Barbosa et al. 2021; Barboza and Costa 2021; Elizeu et al. 2021). Concerning the physical mapping of ribosomal genes, available data evidence that these genes may be located in the

terminal, pericentromeric, or interstitial regions of one or more chromosome pairs (Lopes et al. 2020; Pereira et al. 2021a, b; Barboza and Costa 2021; Elizeu et al. 2021). Together, these studies have provided a better understanding of the diversity and evolutionary patterns of the repetitive portion of the Meliponini genome.

Regarding the cytogenetic data available by genera, *Melipona* Illiger, 1806, *Frieseomelitta* Ihering, 1912, and *Partamona* Schwarz, 1939 concentrate the largest number of studied species, whereas in the other genera, relatively few studies have been performed, for example, in *Trigona* Jurine, 1807 (reviewed by Cunha et al. 2021a). This genus includes 32 stingless bee species that are exclusive to the New World, with a large Neotropical distribution, from Mexico to Argentina (Camargo and Pedro 2013; Pedro 2014; Rasmussen and Cameron 2007). The phylogenetic hypothesis based on molecular data and nest architecture characteristics indicates that *Trigona* is a monophyletic genus in which species are grouped into two main clades (Rasmussen and Cameron 2008).

From a cytogenetic point of view, seven *Trigona* taxa have been studied, mainly through classical methods, describing the number and morphology of chromosomes as well as heterochromatin patterns and/or distribution of regions rich in GC base pairs (reviewed by Cunha et al. 2021a). Most of the species studied present $2n = 34$ chromosomes, with the exception of *Trigona braueri* Friese, 1900 (as *Trigona fulviventris* Guérin, 1844), which has a chromosome number of $2n = 32$; however, the chromosome morphology varies among species (reviewed by Tavares et al. 2017; Cunha et al. 2021a). The presence of heterochromatin in one of the arms of most chromosomes and one euchromatic chromosome pair are common traits observed in the *Trigona* species studied (Rocha et al. 2003; Costa et al. 2004; Domingues et al. 2005). In addition, the number of GC-rich regions differs among species, being observed in one or several chromosome pairs (Costa et al. 2004; Domingues et al. 2005; Fernandes et al. 2013). Recently, two population studies performed in *Trigona spinipes* (Fabricius, 1793)

have physically mapped, through fluorescence in situ hybridization, ribosomal genes and microsatellite (GA)₁₅ and showed variations in the pattern of markings among populations (Barboza and Costa 2021; Tavares et al. 2021).

The cytogenetic studies conducted in *Trigona*, to date, have been important for the chromosomal characterization of the species. However, a cytogenetic approach based on phylogenetic relationships among species is still lacking. Therefore, in the present study, we characterized the karyotype, using classical and molecular cytogenetics, of five *Trigona* species, including representatives of the two phylogenetic clades: *T. pallens* (Fabricius, 1798) and *T. williana* Friese, 1900 from clade A, and *T. hypogea* Silvestri, 1902, *T. aff. fuscipennis* Friese, 1900, and *T. truculenta* Almeida, 1984 from clade B. Our goal was to provide insights into chromosomal rearrangements that shaped karyotype evolution in *Trigona* and investigate whether cytogenetic markers may have a phylogenetic signal.

2. MATERIALS AND METHODS

Colonies of *Trigona* species were collected in Brazil from the locations: Altamira, in the Pará state (3° 22' 18,99" S / 51° 56' 25,74" W) and Florestal, in the Minas Gerais state (21° 10' 40" S / 47° 48' 36" W) (Table 1). Adult vouchers were identified by Dr. Fernando Amaral da Silveira and deposited in the entomological collection of the Apiário Central located at Universidade Federal de Viçosa, Minas Gerais, Brazil and zoological collection at Universidade Federal de Minas Gerais, Minas Gerais, Brazil, under records UFMG-IHY-1403740, UFMG-IHY-1403739, UFMG-IHY-1403738, and UFMG-IHY-1403736.

Mitotic metaphases were obtained from cerebral ganglia of larvae after meconium elimination according to Imai et al. (1988). For analysis of chromosome number and morphology, metaphases were submitted to conventional staining with 4% Giemsa in Sørensen phosphate buffer (0.06 M, pH 6.8) for 15 min at room temperature. Chromosomes were arranged in order of decreasing size, measured and classified according to

the methodology proposed by Levan et al. (1964) that is based on the ratio of the chromosome arm lengths ($r = \text{long arm}/\text{short arm}$). The chromosomes were classified as m = metacentric ($r = 1-1.7$), sm = submetacentric ($r = 1.7-3$), st = subtelocentric ($r = 3-7$) and a = acrocentric ($r > 7$). Chromosomes were organized using Adobe Photoshop® CS6 and measured using Image Pro Plus®.

The heterochromatin distribution pattern was obtained using the C-banding technique according to Sumner (1972), with timing adaptations. The first procedure using the five species consisted of the following times: HCl treatment (6 min), incubation with Ba(OH)₂ (14 min), and 2xSSC solution (2 min). In addition, a second procedure was performed using *T. pallens*, *T. williana*, and *T. aff. fuscipennis* with longest times in HCl treatment (7 min), and incubation with Ba(OH)₂ (14 min, 30 seg). The metaphases submitted to Giemsa staining and C-banding technique were photographed using an epifluorescent microscope Olympus BX60 attached to an image system QColor Olympus®.

Two repetitive sequences were physically mapped on the chromosomes of *Trigona* species using fluorescence in situ hybridization (FISH) technique according to Pinkel et al. (1986): 18S ribosomal genes, denominated herein rDNA genes, and (GA)₁₅ microsatellite. The 18S rDNA gene probes were obtained by amplification via PCR employing the primers rDNA 18SF1 (5'-GTC ATA GCT TTG TCT CAA AGA-3') and 18SR1.1 (5'-CGC AAA TGA AAC TTT TTT AAT CT-3') designed for the bee *Melipona quinquefasciata* Lepelletier, 1836 (Pereira 2006) and isolated from total DNA of *T. spinipes*. 18S rDNA probes were labeled maintaining the conditions for PCR amplification (Pereira 2006) by the indirect method using digoxigenin-11-dUTP (Roche, Mannheim, Germany), and the FISH signals were indirectly detected with anti-digoxigenin-rhodamine (Roche Applied Science), following the manufacturer's protocol. In addition, the (GA)₁₅ microsatellite was directly labeled with Cyanine-3 (Cy3) in the 5' terminal during synthesis by Sigma (St. Louis, MO, USA).

Table 1 Cytogenetic data from the five *Trigona* species used in this study, organized in monophyletic clades according to Rasmussen and Cameron (2008). Diploid chromosome number (2n), diploid karyotypic formula, heterochromatin (Het) distribution, microsatellite (GA)₁₅ location, and 18S rDNA gene locations (position and chromosome pairs)

Clade	Species <i>Trigona</i>	Locality	2n	Karyotypic formula	Het pattern	(GA) ₁₅ pattern	rDNA location	
							Position	Chromosomes
A	<i>Trigona pallens</i>	Altamira, PA, Brazil	34	26 m + 6sm + 2st	M ^a , SM ^a , ST ^c	SE	Interst	11 th m
A	<i>Trigona williana</i>	Altamira, PA, Brazil	34	26 m + 6sm + 2st	M ^a , SM ^a , ST ^c	SE	Interst	11 th m
B	<i>Trigona</i> aff. <i>fuscipennis</i>	Florestal, MG, Brazil	34	26 m + 6sm + 2st	M ^a , SM ^a , ST ^c	SE	Term	1 st m, 3 rd m, 7 th m
B	<i>Trigona hypogea</i>	Altamira, PA, Brazil	34	24 m + 8sm + 2st	M ^a , SM ^a , ST ^c	SE	Term	8 th m, 12 th m
B	<i>Trigona truculenta</i>	Altamira, PA, Brazil	34	18 m + 14sm + 2st	M ^a , SM ^a , ST ^c	SE	Term	7 th m, 9 th m, 2 nd sm, 4 th sm

Brazilian states: MG – Minas Gerais; PA – Pará;

Chromosomal types according to Levan et al. (1964): m – metacentric; sm – submetacentric; st – subtelocentric;

(GA)₁₅ pattern: SE – Scattered in euchromatin;

Heterochromatin pattern: M^a, SM^a – heterochromatin located in one of the chromosome arms in metacentric and submetacentric chromosomes; ST^c – euchromatic subtelocentric pair; ST^c – heterochromatin located in centromeric region of subtelocentric pair;

rDNA gene location: Interst – interstitial region; Term – terminal region

The metaphases were observed and documented using a fluorescence microscope Olympus BX 53F coupled with an Olympus MX10 camera and the image software CellSens[®] with the filter WG (510–550 nm) for the Cy3, and rhodamine, and WU (330–385 nm) for the DAPI. At least 30 metaphases were analyzed for FISH technique.

3. RESULTS

All five *Trigona* species of this study had a diploid chromosome number of 2n = 34 (Figure 1). The karyotype formulas varied among species as follows: 2n = 26 m + 6sm + 2st in *T. pallens* (Figure 1a), *T. williana* (Figure 1b), and *T. aff. fuscipennis* (Figure 1c); 2n = 24 m + 8sm + 2st in *T. hypogea* (Figure 1d); and 2n = 18 m + 14sm + 2st in *T. truculenta* (Figure 1e).

After the C-banding procedure using the shortest incubation times in HCl and Ba(OH)₂, heterochromatin was detected in one of the arms of most chromosomes in all five *Trigona* species analyzed (Figure 2). However, in *T. pallens* and *T. williana*, the heterochromatic regions were less evident (Figure 2a, b) than in the other three *Trigona* spp. (Figure 2c–e). In addition, the heterochromatin pattern of the subtelocentric chromosome pair was an exception. This pair did not show any heterochromatic blocks in the *T. pallens*, *T. williana*, and *T. truculenta* (Figure 2a, b, d), whereas it presented discrete centromeric heterochromatic blocks in the *T. hypogea* and *T. aff. fuscipennis* (Figure 2c, e). The C-banding procedure using the longest incubation times in HCl and Ba(OH)₂ was performed to test whether the discrete heterochromatic pattern observed in the chromosomal arms of *T. pallens* and *T. williana* actually represented differences in relation

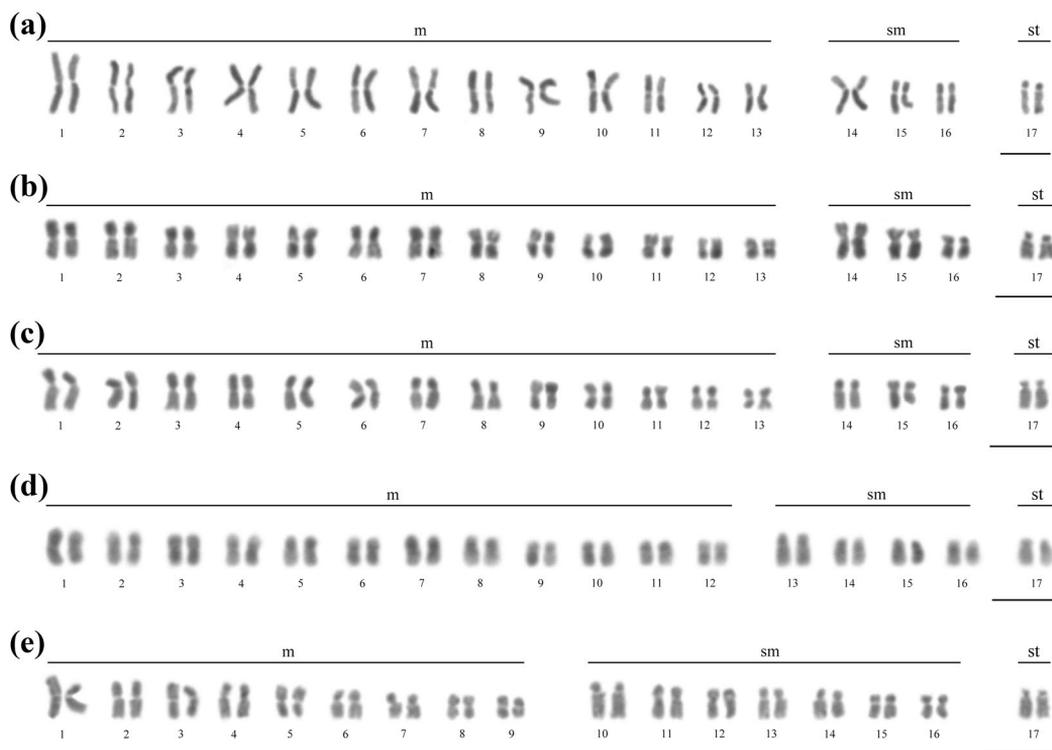


Figure 1. Female karyotypes of five *Trigona* species: (a) *T. pallens* ($2n=34$, $26\ m+6sm+2st$), (b) *T. williana* ($2n=34$, $26\ m+6sm+2st$), (c) *T. aff. fuscipennis* ($2n=34$, $2n=26\ m+6sm+2st$), (d) *T. hypogea* ($2n=34$, $24\ m+8sm+2st$), and (e) *T. truculenta* ($2n=34$, $2n=18\ m+14sm+2st$). Bars: 5 μm .

to those in other species. The results showed that heterochromatin was restricted to the centromeric regions of most chromosomes in *T. pallens* and *T. williana* (Figure 3a, b). However, in *T. aff. fuscipennis*, no differences were observed in the heterochromatin pattern throughout the chromosomal arm (Figure 3c).

Microsatellite (GA)₁₅ was located exclusively in the euchromatic regions of the chromosomes of the five *Trigona* species (Figure 4). However, this microsatellite was not present in the entire euchromatin of chromosomes, as the euchromatic arms of some chromosomes were not entirely labeled with the probe. In addition, the short arm of the euchromatic submetacentric pair did not show any hybridization signal with the (GA)₁₅ probe (Figure 4, arrowheads).

The distribution pattern of ribosomal genes varied among *Trigona* species. In *T. pallens* and

T. williana, the rDNA 18S clusters were located in the interstitial region of the long arm of the 11th metacentric pair (Figure 5a, b). In the other three species, these genes were located in the terminal region of more than one chromosome pair: in *T. aff. fuscipennis* in the 1st, 3rd, and 7th metacentric pairs (Figure 5c), whereas in *T. hypogea* they were located in the 8th and 12th metacentric pairs (Figure 5d) and in *T. truculenta* in the 7th and 9th metacentric pairs and the 2nd and 4th submetacentric pairs (Figure 5e).

4. DISCUSSION

All five *Trigona* species in this study presented the same diploid chromosome number ($2n=34$), similar to most other *Trigona* spp. karyotyped to date (reviewed by Tavares et al. 2017 and Cunha

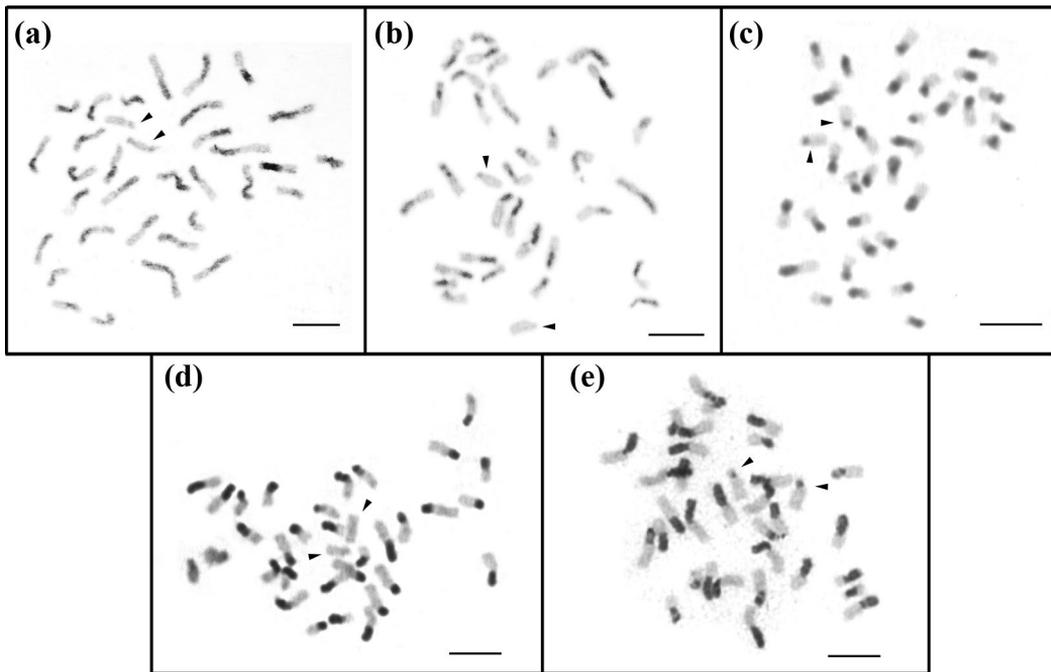


Figure 2. Female metaphases of five *Trigona* species submitted to C-banding using the shortest incubation times in HCl and Ba(OH)₂: (a) *T. pallens*, (b) *T. williana*, (c) *T. hypogea*, (d) *T. truculenta*, and (e) *T. aff. fuscipennis*. Dark regions represent heterochromatin. Arrowheads indicate the subtelo-centric pair. Bars: 5 µm.

et al. 2021a). The only exception is *Trigona braueri* Friese, 1900 (as *Trigona fulviventris* Guérin, 1844), which has a chromosome number of 2n=32 that may have originated by chromosomal fusion (Domingues et al. 2005). As already mentioned, the chromosome number 2n = 34 is the most common and considered the probable ancestral number in Neotropical Meliponini

(Travanzoli et al. 2019a). If true, it would indicate that most *Trigona* species maintained the ancestral chromosome number and reinforce the hypothesis that *T. braueri* has a derived karyotype due to chromosomal fusion. Conservation of chromosome number is a common cytogenetic trait in stingless bee genera (Tavares et al. 2017; Travanzoli et al. 2019a; Cunha et al. 2021a).

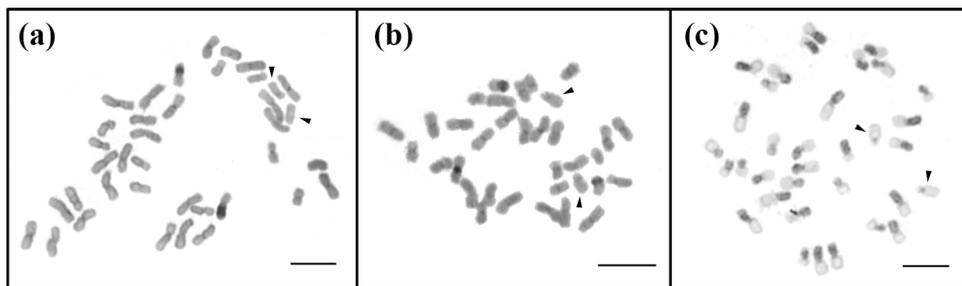


Figure 3. Female metaphases of three *Trigona* species submitted to C-banding using the longest incubation times in HCl and Ba(OH)₂: (a) *T. pallens*, (b) *T. williana*, and (c) *T. aff. fuscipennis*. Dark regions represent heterochromatin. Arrowheads indicate the subtelo-centric pair. Bars: 5 µm.

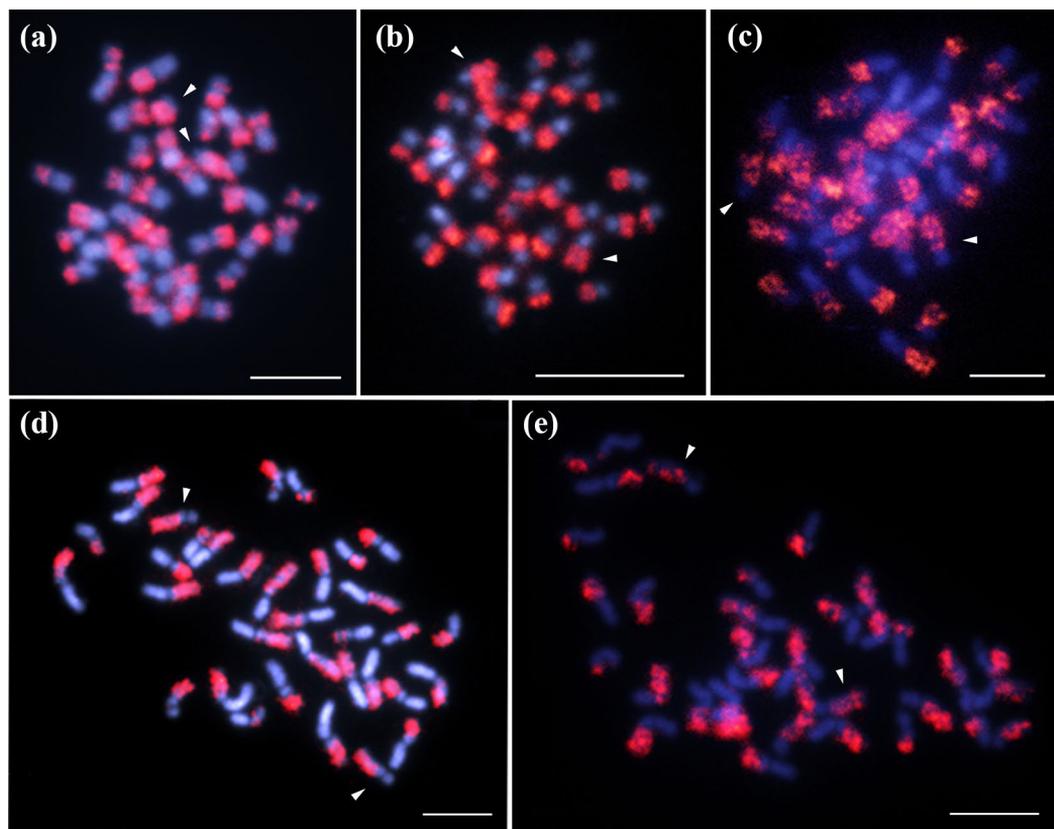


Figure 4. Female metaphases of *Trigona* species submitted to fluorescence in situ hybridization with microsatellite (GA)₁₅ probe (red regions): **(a)** *T. pallens*, **(b)** *T. hypogea*, **(c)** *T. truculenta*, **(d)** *T. williana*, and **(e)** *T. aff. fuscipennis*. Arrowheads indicate the subtelocentric pair. Bars: 5 μ m.

Although chromosome number is highly conserved in *Trigona*, variations in chromosome morphology were observed in the species studied. Previous cytogenetic studies performed in *Trigona* classified chromosomes based on the pattern of heterochromatin location, according to the methodology by Imai (1991), and showed that the karyotype formula varied across species in relation to the number of acrocentric/pseudoacrocentric chromosomes (reviewed by Cunha et al. 2021a). Other recent studies conducted on *T. spinipes* populations (Barboza and Costa 2021; Tavares et al. 2021), *Trigona recurva* Smith, 1863, and *Trigona hyalinata* (Lepeletier, 1836) (Cunha et al. 2021b) used Levan's classification, which is based on the ratio of the lengths

of the chromosomal arms (larger arm/smaller arm) (Levan et al. 1964).

Several studies in hymenopterans have highlighted the importance of using chromosomal measurements for classification since they provide accurate and objective comparisons among karyotypes of related taxa (Gokhman 2009; Barros et al. 2016; Tavares and Teixeira 2021); therefore, in this study, chromosomal measurements were performed in the five *Trigona* species. Differences in the number of metacentric/submetacentric pairs have been observed among species (this study; Barboza and Costa 2021; Tavares et al. 2021; Cunha et al. 2021b). These differences in chromosomal morphology among *Trigona* species can be explained by the

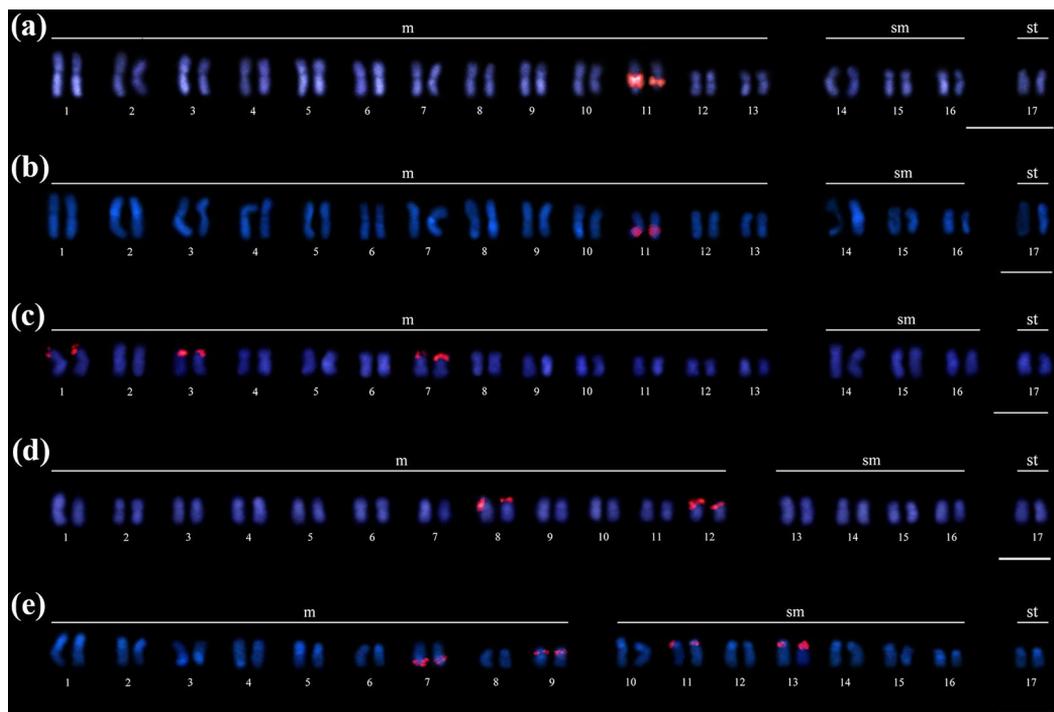


Figure 5. Female karyotypes of *Trigona* species submitted to fluorescence in situ hybridization with rDNA 18S probe (red regions): (a) *T. pallens*, (b) *T. williana*, (c) *T. aff. fuscipennis*, (d) *T. hypogea*, and (e) *T. truculenta*. Bars: 5 µm.

occurrence of duplications/deletions of heterochromatic segments as a result of unequal crossing over. The presence of heterochromatin in only one of the arms of most chromosomes of the *Trigona* species from this and previous studies (Rocha et al. 2003; Costa et al. 2004; Domingues et al. 2005; Tavares et al. 2021) supports this hypothesis. Alternatively, the occurrence of pericentric inversions could also explain chromosomal variations in *Trigona*. Inversions and heterochromatin amplifications/deletions seem to be important rearrangements involved in the origin of chromosomal variations in Meliponini (Cunha et al. 2021b).

Although heterochromatin distribution restricted to one of the arms is a common feature among *Trigona* spp. as well as other Meliponini genera (reviewed in Tavares et al. 2017; Santos et al. 2018; Lopes et al. 2020; Elizeu et al. 2021), the C-banding experiments performed in this study highlighted a lower degree of

heterochromatin compaction in *T. pallens* and *T. williana* (clade A) compared to that in the other *Trigona* species (clade B). In *T. pallens* and *T. williana*, the heterochromatic regions were lighter and when subjected to long incubation times in HCl and Ba(OH)₂, they were restricted to the centromeric regions. However, this is not a characteristic observed in all the studied species included in clade A. For example, *T. chanchamayoensis* Schwarz, 1948 presented very evident heterochromatic regions when long treatment incubation times were used for C-banding technique, similar to the heterochromatin pattern observed in *Trigona* species from clade B (this study; Costa et al. 2004; Tavares et al. 2021). These results indicate that the heterochromatin in *T. chanchamayoensis* and clade B species has a higher degree of compaction, becoming less accessible to the compounds used in the C-banding technique; consequently, they are less susceptible to degradation, as observed for *T. aff. fuscipennis*.

Noteworthy, two other species, *Trigona branneri* Cockerell, 1912 and *T. braueri*, seem to have less evident heterochromatin block patterns (Costa et al. 2004; Domingues et al. 2005); however, these species have not been included in the available phylogeny, and thus, their phylogenetic positions remain unknown.

Intermediate levels of heterochromatin compaction have been observed in chromosomes of stingless bee species of the *Partamona* genus, and it has been suggested that these regions may be associated with the initiation of heterochromatin amplification (Lopes et al. 2020). The less-condensed heterochromatin pattern observed in *T. pallens* and *T. williana* may also be associated with amplification processes in these chromosomal regions. This hypothesis is further supported by the fact that *T. pallens* has a larger genome size than other clade B species, maybe due to the amplification of heterochromatic segments (Tavares et al. 2012; Cunha et al. 2021b). Qualitative and quantitative changes in repetitive sequences (satellite DNA and transposable elements) can significantly contribute to the amplification of heterochromatin, as observed in the stingless bee genus *Melipona* (Pereira et al. 2021b), and may be involved in the different heterochromatic patterns observed in this study in *T. pallens* and *T. williana*. Studies performed on different Meliponini species have demonstrated the intense evolutionary dynamics of heterochromatin and its importance for karyotype modulation in stingless bees (Lopes et al. 2014, 2020; Piccoli et al. 2018; Cunha et al. 2020; Pereira et al. 2020, 2021a, b), highlighting that heterochromatin should be the focus of future investigations from an epigenetic and cytogenomic perspective.

The physical mapping of microsatellite (GA)₁₅ showed that this sequence was located exclusively in the euchromatic regions of the chromosomes in the five *Trigona* species studied, as observed in some populations of *T. spinipes* (Tavares et al. 2021), other stingless bees, such as *Melipona* (Piccoli et al. 2018; Travenzoli et al. 2019b; Barbosa et al. 2021) and *Partamona* (Lopes et al. 2020), and other hymenopterans (Barros et al. 2018; Micolino et al. 2022; Marchioro et al. 2020). However, it was possible

to observe differential euchromatin composition in the chromosomes of the studied species, since not all euchromatic regions were labeled with the (GA)₁₅ probe, like, for example, the short arm of the subtelocentric pair. Furthermore, a different (GA)₁₅ distribution pattern was observed in populations of *T. spinipes*, which presented this microsatellite in some heterochromatic regions, in addition to euchromatin (Barboza and Costa 2021; Tavares et al. 2021). The presence of (GA)₁₅ in a few heterochromatic regions, some of which coincided with ribosomal genes, has also been observed in the stingless bee *Friesella schrottkyi* (Friese 1900) (Elizeu et al. 2021). Other di- and trinucleotide microsatellites are located exclusively in euchromatin (Piccoli et al. 2018; Travenzoli et al. 2019b), heterochromatin (Barbosa et al. 2021), or in both chromosomal regions of stingless bee karyotypes (Santos et al. 2018). Different patterns of microsatellite accumulation are observed in the chromosomes of stingless bees, and an increase in the number of species studied will be useful to better understand the evolution of these repetitive sequences in the Meliponini genome.

Concerning 18S ribosomal gene location, the position and number of pairs bearing these genes varied among the *Trigona* species studied. In the clade A species *T. pallens* and *T. williana*, 18S rDNA genes were observed in the interstitial region of a single chromosome pair (this study). Similarly, *T. chanchamayoensis* presented interstitial GC-rich regions in only a single chromosome pair, which probably corresponds to rDNA genes (Costa et al. 2004; Fernandes et al. 2013), because these genes are usually GC-rich (reviewed by Teixeira et al. 2021). However, species from clade B, such as *T. hypogea*, *T. aff. fuscipennis*, *T. truculenta* (this study), and *T. spinipes* (Barboza and Costa 2021; Tavares et al. 2021) showed terminal rDNA clusters in two to five chromosome pairs. Menezes et al. (2021) suggested that a single chromosome pair bearing ribosomal genes is the ancestral pattern in aculeate Hymenoptera. In addition, these authors also proposed that centric fissions played a crucial role in the dispersion of rDNA clusters in the karyotype during evolution in Hymenoptera.

However, centric fissions are not associated with rDNA gene dispersion in *Trigona* because the studied species have the same chromosome number ($2n = 34$) (this study; reviewed by Cunha et al. 2021a); thus, other mechanisms should be involved in the origin of different number of rDNA-bearing pairs in this genus.

Recently, Hirai (2020) proposed that the chromosomal location of rDNA genes influences the dispersion of these sequences within a karyotype. According to Hirai's model, associations between rDNA genes and repetitive sequences of non-homologous chromosomes can occur during the formation of meiotic bouquet when these sequences are located in the subterminal/terminal regions of the chromosomes. These associations allow ectopic recombination between non-homologous chromosomes and, consequently, promote the dispersion of rDNA genes in the genome. However, intrachromosomal rDNA genes have little chance of interacting with other sequences during meiosis. The rDNA gene evolution in the karyotypes of ants (Teixeira et al. 2021), moths, butterflies (Nguyen et al. 2010), and plants (Roa and Guerra 2012) follow Hirai's model. The relationship between the chromosomal location of ribosomal genes and their dispersion in the genome was also observed in *Trigona* according to Hirai (2020) since species from clade A showed a single intrachromosomal rDNA site and species from clade B presented multiple rDNA sites in subterminal/terminal regions. Thus, considering an ancestor with a single intrachromosomal rDNA site, we hypothesized that a chromosomal inversion occurred in the ancestor of clade B, moving the rDNA genes to the terminal/subterminal region. This location would facilitate the association of rDNA genes with the heterochromatic sequences of other non-homologous chromosomes and the occurrence of ectopic recombination, leading to the dispersion of these genes in the karyotype, as observed in the *Trigona* species of clade B.

Regarding the karyotype characteristics studied in *Trigona*, to date, the number of chromosomal

pairs bearing ribosomal genes seems to reflect the phylogeny proposed by Rasmussen and Camargo (2008), separating the species into two clades. The other cytogenetic traits were shared to a greater (chromosome number and location of microsatellite $(GA)_{15}$) or lesser degree (karyotype formula and heterochromatin distribution) among species of the two clades. An increase in the number of species studied and population-level studies will be useful to confirm whether the number of rDNA gene sites in the karyotype has a phylogenetic signal.

In summary, the cytogenetic features analyzed in the five *Trigona* species in this study, together with previously published data, show interspecific variations to a greater or lesser degree, which highlights an intense chromosomal evolutionary dynamics in *Trigona*. In this study, different chromosomal rearrangements were suggested, such as heterochromatic segment amplifications/losses, inversions, and ectopic recombination involving rDNA genes, to explain the karyotypic differences observed among *Trigona* species. In addition, our data show that the number of chromosome pairs carrying ribosomal genes seems to be promising for separating *Trigona* species into clades according to their phylogeny. Using classical and molecular cytogenetic techniques, we provide new insights into the chromosomal evolution of *Trigona* and stingless bees. The cytogenetic information obtained in this study may be useful for future conservation strategies for *Trigona* species.

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AUTHOR CONTRIBUTIONS

RPF and DML conceived the study and designed the experiments. RPF and GAT performed the cytogenetic experiments. All authors analyzed the results, wrote the manuscript, read and approved the final version.

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AVAILABILITY OF DATA AND MATERIAL

Not applicable.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

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