Heterochromatin distribution and chromosomal mapping of microsatellite repeats in the genome of *Frieseomelitta* stingless bees (Hymenoptera: Apidae: Meliponini)

**Jádilla Mendes dos Santos**, Débora Diniz, Tecavita Ananda Santos Rodrigues, Marcelo de Bello Cioffi, Ana Maria Waldschmidt*

**Abstract**

The stingless bees of tribe Meliponini are efficient pollinators playing a key role in ecosystem services. *Frieseomelitta* Friese (Hymenoptera: Apidae: Meliponini) includes 16 Neotropical species, of which 6 are found in the state of Bahia, northeastern Brazil. In order to provide a refined cytotaxonomic analysis, we characterized the heterochromatin composition and variation among 6 *Frieseomelitta* species. All species shared a diploid number (2n) of 30 chromosomes. *Frieseomelitta dispar* Moure, *Frieseomelitta francoi* Moure, and *Frieseomelitta meadevaldoi* Cockerell (Hymenoptera: Apidae), presented GC-rich heterochromatic regions while *Frieseomelitta sp.n., Frieseomelitta varia* Lepelitier, and *Frieseomelitta doederleini* Friese (Hymenoptera: Apidae) were characterized by homogenous heterochromatin, without evidence of AT or GC-rich sites. The number and location of microsatellite repeats mapped by fluorescence in situ hybridization revealed interspecific variation. These data were useful to identify each species based on chromosomal markers, and represent important tools for clarifying the interspecific differentiation among *Frieseomelitta* species and for understanding the genome evolution in bees as a whole.

Key Words: Cytotaxonomy; fluorescent in situ hybridization; heterochromatin; microsatellites; stingless bees

**Resumo**


Palavras Chave: Citotaxonomia; hibridação fluorescente in situ; heterocromatina; microsatélites; abelhas sem ferrão

Cytogenetic reports are important to infer evolutionary processes and assess the genomic organization of species, thus being informative to taxonomy and systematics (Sumner 2003). Approximately 65 stingless bee species have been cytogenetically analyzed so far (Cristiano et al. 2014), but only 5 of them include the genus *Frieseomelitta*, in which only conventional techniques (Gierna staining, C-banding, and base-specific fluorochrome staining), have been applied (Rocha et al. 2003; Carvalho & Costa 2011). Even though the number of cytogenetic studies in meliponines is underrepresented, the chromosomal data reported so far demonstrate a high amount of heterochromatin present in most species (Rocha et al. 2003).

The richness of heterochromatic regions might act as a hotspot for chromosomal rearrangements (Sumner 2003). A refined analysis of

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Materials and Methods

The species *Frieseomelitta* sp.n.; *Frieseomelitta varia* Lepeletier; *Frieseomelitta dispar Moure*; *Frieseomelitta francoi* Moure; *Frieseomelitta doederleini* Friese; and *Frieseomelitta meadewaldoi* Cockerell, were sampled in distinct localities of southeastern and northeastern Brazil (Table 1). Adult specimens were collected from each sampled colony for taxonomic identification (by Profa. Dra. Favízia Freitas de Oliveira, Instituto de Biologia, Departamento de Zoologia, Universidade Federal do Bahia). Thirty post-defecating larvae were collected per colony for cytogenetic procedures.

The larvae were immersed in hypotonic 0.005% colchicine solution and the chromosomes were obtained from cerebral ganglia, as described by Imai et al. (1988). Afterwards, the metaphase spreads were stained with Giemsa diluted in Sörensen buffer (1:30).

The C-banding was carried out according to Sumner (1972), with slight changes in the treatment with barium hydroxide [5% Ba(OH)$_2$], as follows: 20 seconds for *F. francoi* and F. sp.n., 18 seconds for *F. doederleini*, and 25 seconds for *F. varia*, *F. dispar*, and *F. meadewaldoi*.

Base-specific fluorochrome staining using Chromomycin A3 (CMA3) and 4′-diamidino-2-phenylindole (DAPI) was used to detect GC- and AT-rich regions, respectively, while Distamycin A (DA) was added as counter stain (Schweizer 1980).

Fluorescence in situ hybridization was performed as reported by Pinkel et al. (1986) with a stringency of 77%. The microsatellites repeats used as FISH probes in this study were: (GA)$_r^w$, (GC)$_c^w$, (GAA)$_w$ (CAA)$_w$, and (GAG)$_w$. These probes were directly labeled with Cy by their synthesis as reported by Kubat et al. (2008). Two series of FISH experiments were performed; the first involved hybridization of all probes in the 6 bee species. The second series included only the probes lacking signals in 1 or more species along with a positive control to confirm whether the failure of hybridization was related to technical artifact or to the lack of homologies between chromosomal and probe sequences. The chromosomes were counter-stained with DAPI (0.2 mg per mL) in Vectashield Mounting Medium (Vector Laboratories, Burlingame, California, USA).

At least 30 metaphase spreads per individual were analyzed to confirm the 2n, karyotype structure, and FISH results. The chromosomes were analyzed and photographed using an Olympus BX-51 epifluorescence microscope equipped with image capture digital system (Image Pro Plus 6.1, Media Cybernetics, Rockville, Maryland, USA). The chromosomal pairs were organized based on the Theory of Minimum Interaction, the visualization of chromosomal regions more susceptible to rearrangements is relevant to point out potential changes in chromosome structure.

Results

All female specimens of *Frieseomelitta* sampled here shared 2n = 30 chromosomes, as reported in other congeneric species (Rocha et al. 2003; Carvalho & Costa 2011). Similarly, the constitutive heterochromatin was distributed over centromeric, pericentromeric, and telomeric regions in the 6 analyzed species, with conspicuous C-bands in 1 arm of most chromosomes (Fig. 1).

In contrast, 5 karyotype formulae were defined, allowing differentiating *F. varia* and *F. doederleini*, F. sp.n. from *F. meadewaldoi*, *F. doederleini*, and *F. dispar*, F. francoi (Fig. 1, Table 1).

The karyotypic pattern observed in *F. varia* and *F. doederleini* corroborates that reported by Rocha et al. (2003) because both are characterized by a high number of acrocentric and pseudoacrocentric chromosomes.

Table 1. Studied species of *Frieseomelitta*, collection sites in Brazil, diploid number (2n) and karyotypes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection site</th>
<th>Geographic coordinates</th>
<th>2n</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Frieseomelitta varia</em></td>
<td>Lontra – MG</td>
<td>15.9033°S, 44.3050°W</td>
<td>2n = 30</td>
<td>2K = 4M + 4A + 22A&quot;</td>
</tr>
<tr>
<td><em>Frieseomelitta</em> sp.n.</td>
<td>Jequê – BA</td>
<td>13.8575°S, 40.0836°W</td>
<td>2n = 30</td>
<td>2K = 4M + 4A + 23A&quot;</td>
</tr>
<tr>
<td><em>Frieseomelitta meadewaldoi</em></td>
<td>Nova Ibiá – BA</td>
<td>13.8100°S, 39.6255°W</td>
<td>2n = 30</td>
<td>2K = 6M + 2M' + 4A + 18A&quot;</td>
</tr>
<tr>
<td><em>Frieseomelitta dispar</em></td>
<td>Jequê – BA</td>
<td>13.8575°S, 40.0836°W</td>
<td>2n = 30</td>
<td>2K = 4M + 2M' + 4A + 20A&quot;</td>
</tr>
<tr>
<td><em>Frieseomelitta francoi</em></td>
<td>Santa Inês – BA</td>
<td>03.6666°S, 45.3800°W</td>
<td>2n = 30</td>
<td>2K = 4M + 2M' + 4A + 20A&quot;</td>
</tr>
<tr>
<td><em>Frieseomelitta doederleini</em></td>
<td>Nova Ibiá – BA</td>
<td>13.8100°S, 39.6255°W</td>
<td>2n = 30</td>
<td>2K = 2M + 2M' + 4A + 12A&quot;</td>
</tr>
</tbody>
</table>
The karyotype formula of *F. dispar* (2K = 4M + 2M<sup>t</sup> + 6A + 18A<sup>M</sup>) herein described differs from that previously reported (Carvalho & Costa 2011).

The karyotype formula of *F. francoi* (2K = 4M + 2M<sup>t</sup> + 4A + 20A<sup>M</sup>) corroborates the report by Carvalho and Costa (2011).

As for the heterochromatin composition, GC-rich sites (CMA<sub>3+</sub>/DAPI<sup>-</sup>) were exclusively observed at terminal heterochromatin on short arms of 5 chromosomal pairs in *F. dispar* (Fig. 2).

A GC-rich heteromorphic block was observed on short arms of a single chromosomal pair in *F. meadewaldoi*, since CMA<sub>3+</sub> signals were more conspicuous in one of the homologous chromosomes. The base-specific fluorochrome staining in *Frieseomelitta* sp.n., *F. meadewaldoi*, *F. varia*, and *F. doederleini* revealed no richness of either AT or GC segments.

The microsatellites presented a wide distribution in *Frieseomelitta* species, besides interspecific differences in both amount and location of FISH signals (Fig. 3).

The (GA)<sub>15</sub> probe revealed specific signals at terminal regions of some chromosomal pairs in *F. varia* (Fig. 3-1a). On the other hand, *F. dispar* was characterized by large blocks of this microsatellite, including 1 pair with FISH signals over the whole chromosome (Fig. 3-4a). This repeat also was abundant in the chromosomes of *F. francoi* (Fig. 3-5a), including some more conspicuous marks. In *F. doederleini* (Fig. 3-6a), it was accumulated at terminal region of all pairs. No fluorescence in situ hybridization signals were observed for the (GA)<sub>15</sub> probe in *F. sp.n.* and *F. meadewaldoi* (Fig. 3-2a, 3a).

Interspecific variation also was observed in relation to the (GC)<sub>15</sub> probe. In *F. varia* and *F. doederleini*, this microsatellite encompassed nearly all chromosomal pairs and entire chromosomes (Figs. 3-1b, -6b). In *F. sp.n.*, this repetitive DNA was dispersed, except for a single pair with signals through the whole chromosome (Fig. 3-2b). Only 2 pairs were positively labeled with this probe in *F. meadewaldoi* (Fig. 3-3b) while no hybridization was observed in *F. dispar* (Fig. 3-4b). In *F. fran-
coi, positive signals were detected at terminal regions of 1 chromosomal arm in nearly all pairs (Fig. 3-5b).

In the case of the (CAA)$_{10}$ probe, the FISH signals were reduced but widespread through the karyotype of F. varia, occupying the telomeres of all chromosomal and interstitial regions of some pairs (Fig. 3-1c). In F. sp.n., this microsatellite repeat was mapped at terminal region of 1 chromosomal arm of most chromosomes besides a chromosomal pair with signals through the whole chromosome extension (Fig. 3-2c). Two pairs were entirely marked in F. meadewaldoi with the same probe, while the other pairs were characterized by reduced signals at terminal regions (Fig. 3-3c). No hybridization signal was observed for this repetitive DNA in F. dispar (Fig. 3-4c). In F. francoi, punctiform signals were observed, except for 2 pairs with positive marks through the whole chromosomes (Fig. 3-5c). Instead, this microsatellite is widespread in the chromosomes of F. doederleini, once positive signals were detected in all pairs (Fig. 3-6c).

A few hybridization signals were observed for the (GAA)$_{10}$ probe in Frieseomelitta (Fig. 3). Reduced signals were observed at terminal and interstitial regions of F. varia (Fig. 3-1d). In F. sp.n., the FISH signals with this probe were located on a single arm of most chromosomes, through the entire extension of 1 pair and at terminal and interstitial regions of another pair (Fig. 3-2d). The signals in F. meadewaldoi were subtle and mainly restricted to terminal region of chromosomes (Fig. 3-3d). In F. dispar, the (GAA)$_{10}$ signals were more conspicuous, being observed in most chromosomes (Fig. 3-4d), but they were absent in both F. francoi (Fig. 3-5d) and F. doederleini (Fig. 3-6d).

The hybridization with (GAG)$_{10}$ probe revealed weak signals in studied species in relation to other microsatellite repeats. In F. varia, positive signals were scattered through most chromosomes (Fig. 4-1e). In Frieseomelitta sp.n., reduced terminal and interstitial signals were spread through all chromosomes (Fig. 4-2e). No signals were detected for F. meadewaldoi and F. dispar (Figs. 3-3e, 3-4e, respectively). In F. francoi, this microsatellite was detected in all chromosomes, except for a single pair (Fig. 3-5e). Positive signals were not detected in F. doederleini as well (Fig. 3-6e).

Discussion

The diploid number found for all species in this study support the conservative evolution of chromosomal numbers in Frieseomelitta, even though most trigonines have 2n = 34, except for Trigona fulviven-tris (Hymenoptera: Apidae) (2n = 32) (Rocha et al. 2003; Domingues et al. 2005; Miranda et al. 2013; Godoy et al. 2013).

The large number of acrocentric and pseudoacrocentric chromosomes found here corroborates the Theory of Minimum Interaction (Imai 1991; Imai et al. 1988, 1994) as the most parsimonious model of karyotype evolution in Frieseomelitta. This large number and the lack of large GC+/AT+ blocks in F. varia, F. sp.n., and F. doederleini suggest that these species might be closely related. Moreover, the karyotypic patterns also indicate that F. varia is highly differentiated from F. francoi and F. dispar, which is supported by morphological data that split these species into distinct clades (Camargo & Pedro 2003). Apparently, the M’ chromosome characterized by the presence of heterochromatin blocks in pericentricomeric telomeric region in 1 of the arms evolved from a rare case of pericentric inversion that constitutes a synapomorphy in F. dispar and F. francoi (Carvalho & Costa 2011). Therefore, cytogenetic data suggest that F. dispar and F. francoi, as well as F. meadewaldoi, are likely part of the same clade.

Although F. francoi shares the same karyotype formula and has been placed in the same clade of above-mentioned species according to C-banding (Carvalho & Costa 2011), the heterochromatin composi-
tion is different in this species. The presence of 4 pairs bearing GC+/AT segments in the samples of *F. francoi* differs from that previously reported (Carvalho & Costa 2011).

Population karyotype variation found in *F. dispar* might be related to isolation by distance, since samples of this species were collected about 200 km from the locality sampled by Carvalho and Costa (2011). It is important to point out that both studies used the same tissue samples and similar procedures.

The presence of a reduced number of pseudoacrocentric (A') chromosomes in *F. dispar* and *F. meadewaldoi*, when compared to the other congeneric species, could be related to the accumulation of terminal heterochromatin, placing this species as karyotypically derived within *Frieseomelitta*.

Polymorphism of GC+ bands found in *F. meadewaldoi* has been reported in bees of the genus *Plebeia* (Hymenoptera: Apidae) (Godoy et al. 2013), but it seems to represent an autapomorphy of *F. meadewaldoi* because these marks are absent in other congeneric species studied so far. Yet, the present data show that heterochromatin composition in *Frieseomelitta* is more variable than previously reported.

The microsatellite repeats used as probes for fluorescence in situ hybridization experiments have proved to be resolute markers in the analyzed species. So far, reports of molecular cytogenetics in stingless bees were restricted to chromosomal mapping of 18S and 5S rDNAs or chromosome painting with probes derived by microdissection (Mampil pumbu and Pompolo 2000; Rocha et al. 2002; Brito et al. 2005; Fernandes et al. 2011; Martins et al. 2013; Lopes et al. 2014).

Fluorescence in situ hybridization results indicate that the genome of *Frieseomelitta* species are mostly AT-rich because the \((GA)_{10}, (CAA)_{10}, (GAA)_{10}\), and \((GAG)_{10}\) probes revealed a high number of conspicuous signals on chromosomes of studied species. In fact, previous studies with fluorochrome staining (Rocha et al. 2003; Brito et al. 2003; Lopes et al. 2008) suggested that chromosomes of Meliponini are AT-rich, except for some terminal regions of pseudoacrocentric pairs (usually GC-rich), probably related to the presence of ribosomal genes. Similarly, Miranda et al. (2013) revealed richness of AT bases in *Cephalotrigona* Schwarz, 1940 (Hymenoptera: Apidae), which is in accordance with data presented here. In general, microsatellites are located within heterochromatin segments (Kubat et al. 2008). Albeit

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**Fig. 3.** Metaphase spreads of females of *Frieseomelitta* species after fluorescence in situ hybridization with microsatellite probes.
variable, the distribution pattern of microsatellite repeats in *Friesoemelitta* species was mostly coincident with heterochromatic regions. However, the distribution of microsatellites in some of the analyzed species encompassed the whole chromosome, including euchromatin. Most likely, this result is due to the presence of reduced segments of repetitive DNA that could not be observed by C-banding, as already reported in beetles of genus *Dichotomius* (Scarabaeidae) (Cabraal-de-Melo et al. 2011).

As revealed in the present study, most microsatellite repeats were widely dispersed over most chromosomal pairs, indicating variation in heterochromatin composition. This behavior also has been reported in other organisms, determining species-specific distribution patterns for each repetitive sequence (Kubat et al. 2008; Poltronieri et al. 2013). This scenario reveals unique evolutionary and recombination pathways in each taxon, thus generating cytotoxiconomic markers, even among species with apparent similar karyotypes (Kubat et al. 2008; Poltronieri et al. 2013; Lopes et al. 2014).

In particular, some studies have shown that accumulation of microsatellites is strongly related to heterochromatinization, playing a key role in the differentiation of single sex chromosome systems in plants, fish, and humans (Kubat et al. 2008; Poltronieri et al. 2013). Additionally, Bacolla and Wells (2004) stated that microsatellites (and heterochromatin) act as hotspots for chromosomal rearrangements such as deletion, duplication, transposition, and inversions. Thus, the presence of these microsatellite repeats in *Friesoemelitta* could drive structural chromosomal changes, accounting for their chromosomal differentiation and rate of karyotype evolution, suggesting divergent evolutionary pathways.

In conclusion, C-banding, base-specific fluorescent staining, and mapping of microsatellites repeats by FISH in *Friesoemelitta* all together facilitated evaluation of the composition and distribution of repetitive DNA in the genome of 6 species of *Friesoemelitta* and determination of interspecific variation. These analyses should be extended to other meliponines in order to infer the evolutionary mechanisms underlying the microstructural differentiation in karyotype of stingless bees.

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