Heterochromatin Distribution and Chromosomal Mapping of Microsatellite Repeats in the Genome of Frieseomelitta Stingless Bees (Hymenoptera: Apidae: Meliponini)

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

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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 meadewaldoi* Cockerell (Hymenoptera: Apidae), presented GC-rich heterochromatic regions while *Frieseomelitta* sp.n., *Frieseomelitta varia* Lepeltier, 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

As abelhas sem ferrão pertencentes à tribo Meliponini são polinizadores eficientes e desempenham papel fundamental nos serviços ecossistêmicos. *Frieseomelitta* Friese (Hymenoptera) possui 16 espécies neotropicais das quais 6 se encontram no estado da Bahia, nordeste do Brasil. Caracterizamos a composição e variação da heterocromatina de 6 espécies de *Frieseomelitta* com a finalidade de uma análise citotaxonômica mais refinada,. Todas as espécies compartilharam um número diploide (2n) de 30 cromossomos. *Frieseomelitta dispar* Moure, *Frieseomelitta francoi* Moure, e *Frieseomelitta meadewaldoi* Cockerell (Hymenoptera: Apidae), apresentaram regiões heterocromáticas ricas em GC, enquanto *Frieseomelitta* sp.n., *Frieseomelitta varia* Lepeltier, e *Frieseomelitta doederleini* Friese (Hymenoptera: Apidae) foram caracterizadas por heterocromatina mais homogênea, sem evidência de locais ricos em AT ou GC. O número e a localização das repetições de microssatélites mapeadas pela hibridação in situ fluorescente revelaram variação interespecífica. Estes dados foram úteis para identificar as espécies de *Frieseomelitta* com base em marcadores cromossômicos sendo importantes para a diferenciação interespecífica e para compreender a evolução do genoma em abelhas como um todo.

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

Hymenoptera includes bees, wasps, and ants, being regarded as the third largest order of insects (Michener 2007). Within this group, the family Apidae Latreille, stands out because of their remarkable species richness. This family is subdivided into 5 subfamilies, such as Apinae Latreille, which includes the tribe Meliponini (Moure 2015). This tribe is composed of social bees with reduced stingers and different colors, shape, and behavioral patterns, popularly known as stingless bees or meliponines (Nogueira-Neto 1997). These bees are efficient pollinators and thus essential to the conservation of several plant species and their associated fauna (Kerr et al. 2001). The genus *Frieseomelitta* (Hymenoptera: Apidae) comprises 16 valid species occurring in the Neotropical region, ranging from South America to Mexico (Camargo & Pedro 2003; Oliveira et al. 2011).

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 (Giemsa 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

heterochromatin distribution and composition is important to improve our knowledge of karyotype evolution dynamics. However, detailed studies on the heterochromatin composition in stingless bees are scarce, in part because there is no effective fluorescence in situ hybridization technique procedure for this group. Indeed, the few available reports using fluorescence in situ hybridization are based on the mapping of ribosomal probes (18S and 5S) or chromosomal painting with probes derived by microdissection (Mampumbu and Pompolo 2000; Rocha et al. 2002; Brito et al. 2005; Fernandes et al. 2011; Martins et al. 2013; Lopes et al. 2014). Alternatively, microsatellites probes already have been produced and successfully tested to evaluate heterochromatin composition in several groups of animals and plants (Kubat et al. 2008; Martins et al. 2013; Parise-Maltempi et al. 2013; Lopes et al. 2014). Cytogenetic data in *Frieseomelitta* are restricted to conventional analyses (Giemsa-staining and C-banding) and staining with base-specific fluorochromes (Rocha et al. 2003; Carvalho & Costa 2011). Once the accumulation of repetitive DNA sequences plays a key role in chromosomal evolution (Charlesworth et al. 2005), serving as hotspots for chromosomal rearrangements, the chromosomal mapping of these sequences is useful to infer evolutionary pathways for distinct animal groups. Because the karyotype evolution in *Frieseomelitta* is inferred 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.

Therefore, based on the lack of knowledge about karyotype evolution and genome organization of Meliponini, we carried out cytogenetic analyses in 6 species of *Frieseomelitta* using C-banding, base-specific fluorochrome staining and fluorescence in situ hybridization with microsatellite probes to provide a refined analysis of the heterochromatin composition and variation among these species. Additionally, determining basic karyotypic aspects of *Frieseomelitta* species will help us to better understand their evolutionary relationships.

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 da 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 Sorënsen 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 4'6-diamidino-2-phenylindole (DAPI) was used to detect GC- and AT-rich regions, respectively, while Distamycin A (DA) was added as counter stainer (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: $(GA)_{15}$, $(GC)_{15}$, $(GAA)_{10}$, $(CAA)_{10}$, and $(GAG)_{10}$. These probes were directly labeled with Cy^3 during 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 chromosomal spreads 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 heterochromatin location (Imai 1991), into: Metacentric (M), Metacentric with pericentromeric and telomeric C-bands (M^t), Acrocentric (A), and Pseudoacrocentric (A^M).

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. meadwaldoi*, *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.

Frieseomelitta sp.n. and *F. meadewaldoi* were cytogenetically analyzed for the first time in this study, being the first species characterized by the karyotype formula: $2K = 6M + 4A + 20A^M$, and the second by the presence of M^t chromosomes. As for the heterochromatin composition, GC-rich sites (CMA_{3+} / DAPI.) were exclusively observed at terminal heterochromatin on short arms of 5 chromosomal pairs in *F. dispar* (Fig. 2).

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

| Species	Collection site	Geographic coordinates	2n	Karyotype
Frieseomelitta varia	Lontra – MG	15.9033°S, 44.3050°W	2n = 30	$2K = 4M + 4A + 22A^M$
Frieseomelitta sp. n.	Jequié – BA	13.8575°S, 40.0836°W	2n = 30	$2K = 4M + 4A + 22A^M$
Frieseomelitta meadewaldoi	Nova Ibiá – BA	13.8100°S, 39.6255°W	2n = 30	$2K = 6M + 2M^t + 4A + 18A^M$
Frieseomelitta dispar	Jequié – BA	13.8575°S, 40.0836°W	2n = 30	$2K = 4M + 2M^t + 4A + 20A^M$
Frieseomelitta francoi	Santa Inês – BA	03.6666°S, 45.3800°W	2n = 30	$2K = 4M + 2M^t + 4A + 20A^M$
Frieseomelitta doederleini	Nova Ibiá – BA	13.8100°S, 39.6255°W	2n = 30	$2K = 2M + 2M^t + 4A + 12A^M$
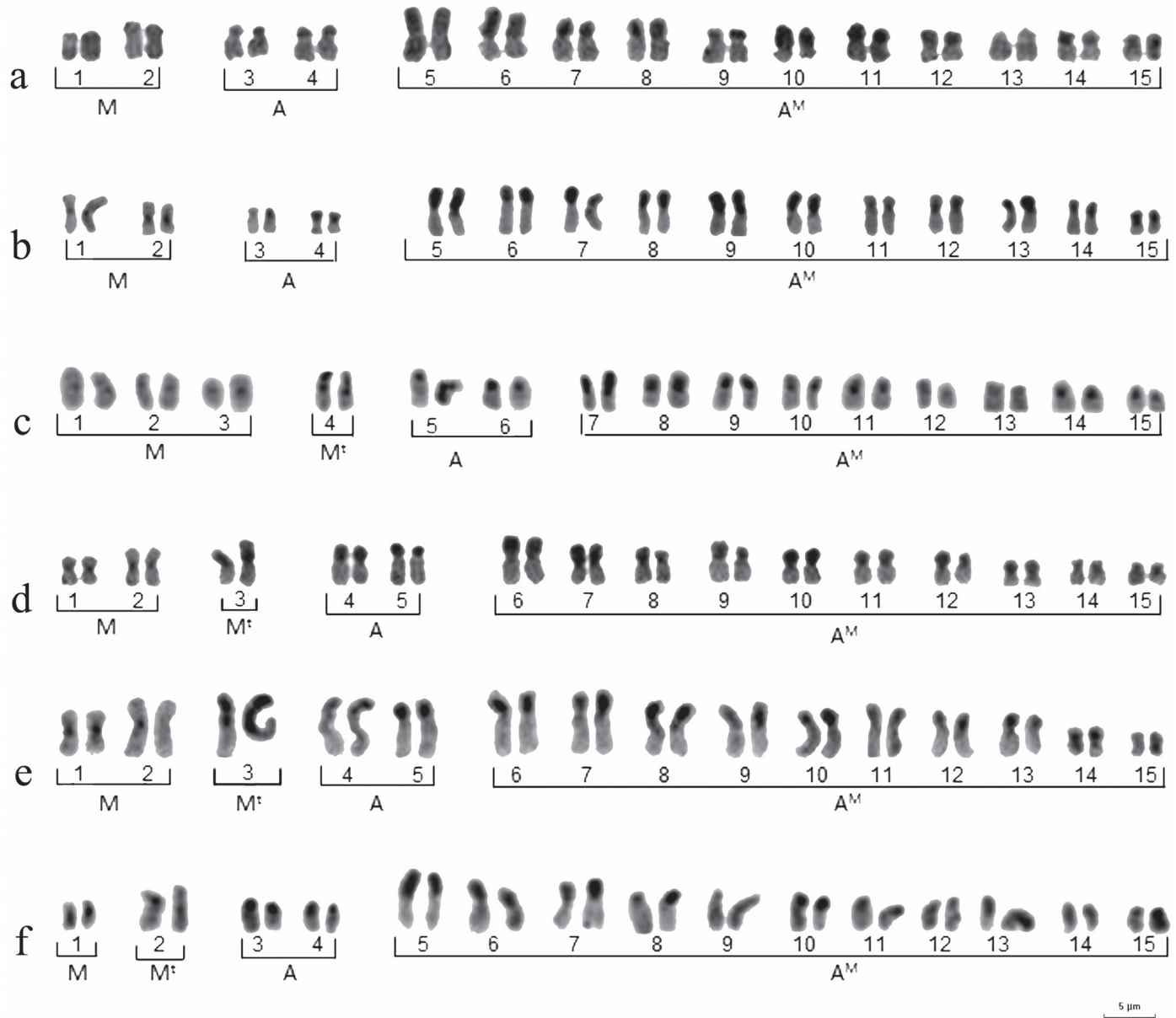
Fig. 1. C-banded karyotypes of females of *Frieseomelitta varia* (a), *Frieseomelitta doederleini* (b), *Frieseomelitta* sp. nov. (c), *Frieseomelitta meadewaldoi* (d), *Frieseomelitta dispar* (e), and *Frieseomelitta francoi* (f). (M = metacentric, A = acrocentric, M^T = metacentric with centromeric, and telomeric C-bands, A^M = pseudoacrocentric).

The karyotype formula of *F. dispar* ($2K = 4M + 2M^t + 6A + 18A^M$) herein described differs from that previously reported (Carvalho & Costa 2011).

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

As for the heterochromatin composition, GC-rich sites (CMA_{3+} / DAPI) 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_{3+} 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 $(GA)_{15}$ 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 $(GA)_{15}$ probe in *F.* sp.n. and *F. meadewaldoi* (Fig. 3-2a, 3a).

Interspecific variation also was observed in relation to the $(GC)_{15}$ 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-*
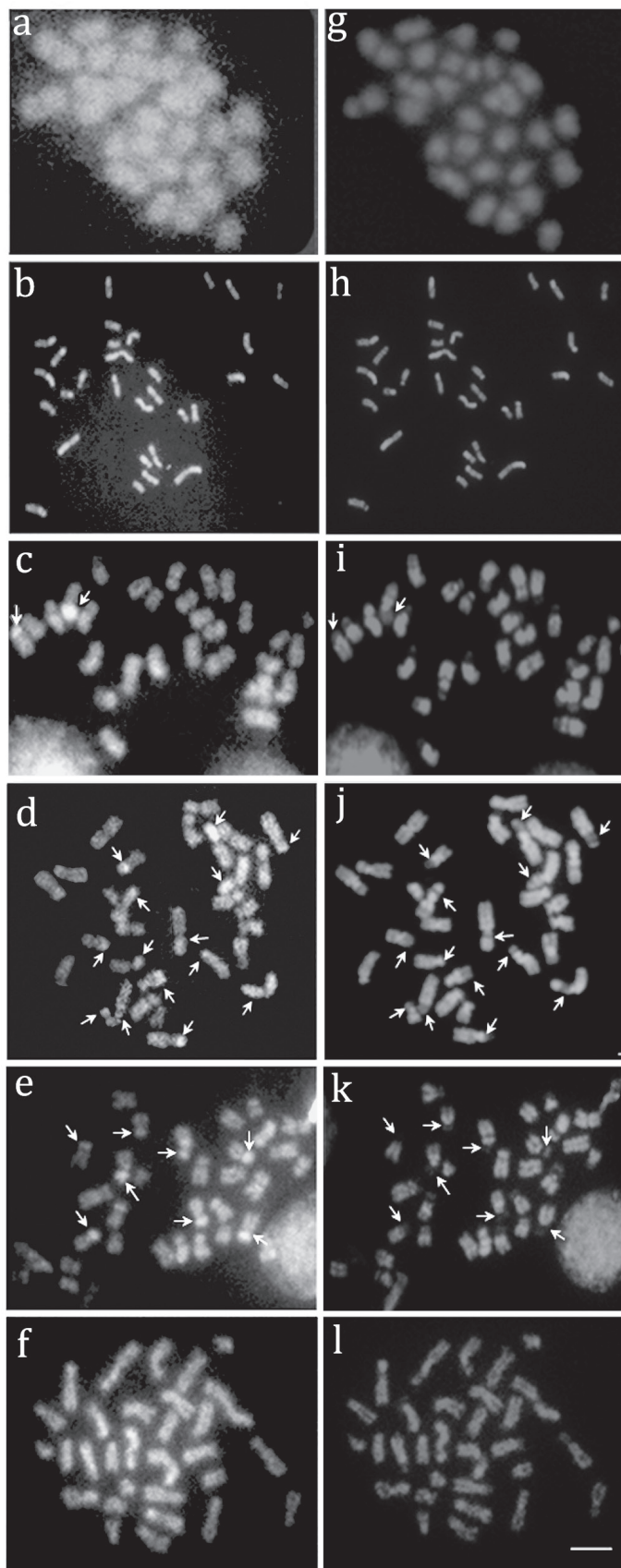
Fig. 2. Metaphase spreads of females of *Frieseomelitta varia* (a, g), *Frieseomelitta* sp. n. (b, h), *Frieseomelitta meadewaldoi* (c, i), *Frieseomelitta dispar* (d, j), *Frieseomelitta francoi* (e, k), and *Frieseomelitta doederleini* (f, l) after base-specific fluorochrome staining. The arrows indicate the GC-rich regions.

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 fulviventris* (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^t chromosome characterized by the presence of heterochromatin blocks in pericentromeric 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. meadwaldoi*, 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-
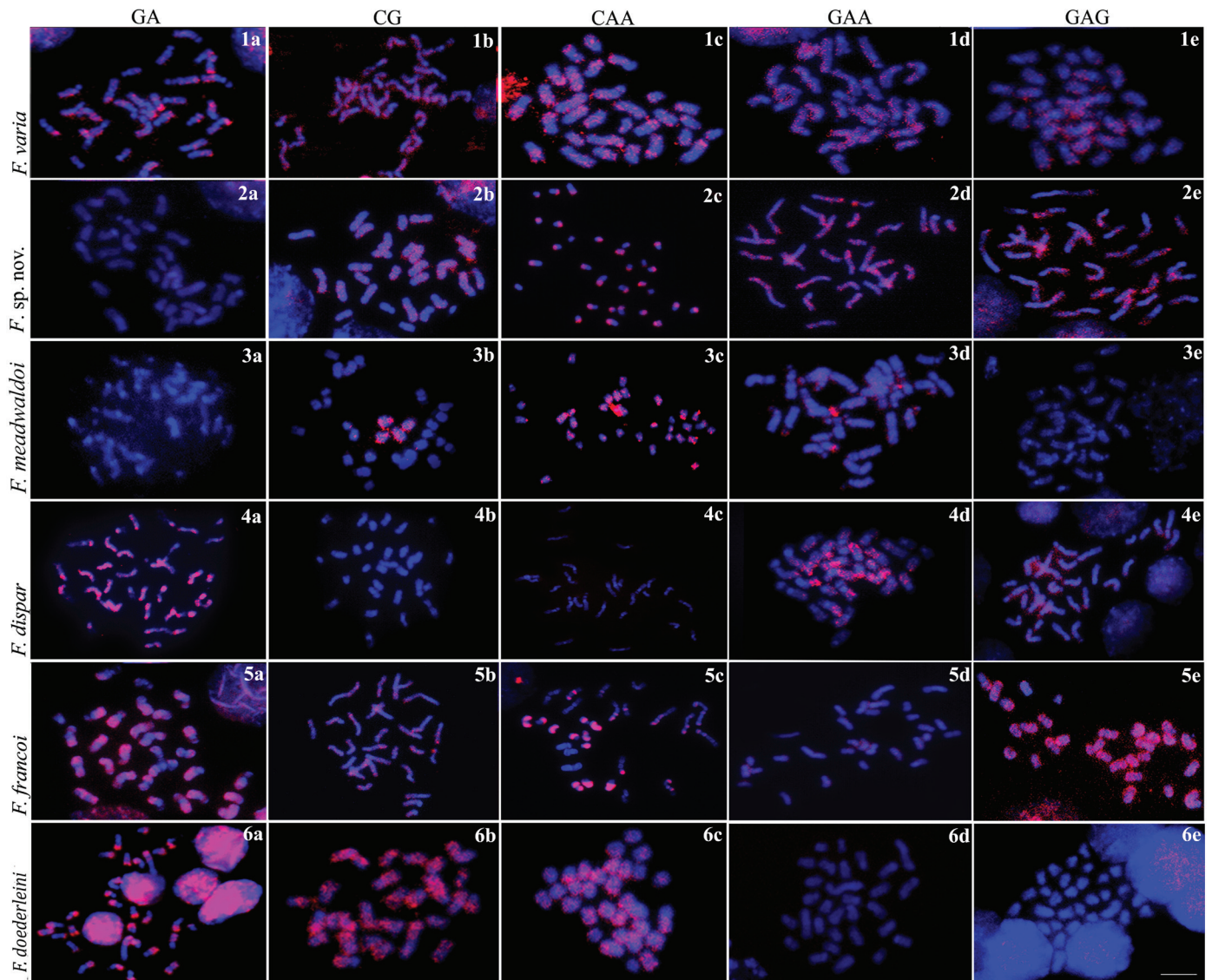
Fig. 3. Metaphase spreads of females of *Frieseomelitta* species after fluorescence in situ hybridization with microsatellite probes.

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^M) 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 (Mampumbu 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 that the genome of *Frieseomelitta* species are mostly AT-rich because the (GA)$_{15}$, (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

variable, the distribution pattern of microsatellite repeats in *Frieseomelitta* 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) (Cabral-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 cytotaxonomic 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 *Frieseomelitta* 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 fluorochrome staining, and mapping of microsatellites repeats by FISH in *Frieseomelitta* all together facilitated evaluation of the composition and distribution of repetitive DNA in the genome of 6 species of *Frieseomelitta* 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.

Acknowledgments

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