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Original article

Genome size variation in *Melipona* species (Hymenoptera: Apidae) and sub-grouping by their DNA content*

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Abstract – The stingless bees of the genus *Melipona* comprise a group with approximately 40 Neotropical species. Despite their ecological and economic importance, the size of the genomes of these species remains poorly known. Thus, the present study measured the DNA content of 15 *Melipona* species. The mean genome size (1C) of the females ranged from 0.27 pg to 1.38 pg, with increments of, approximately, 0.12 pg. It was possible to recognize two groups of species: the first presented relatively low DNA content (average = 0.29 pg), while the second showed high DNA content (average = 0.98 pg). This result corroborates the cytogenetic classification of these species into two groups, one of them comprising species with a low heterochromatin content (<50%), and the other species with high heterochromatin content (>50%). Amongst the groups with low and high DNA content, there was no significant correlation between the DNA content and the size of the bees. The data obtained may aid in the selection of species which are suitable for sequencing projects, besides providing an overview of the diversity in the genome size of the *Melipona* genus.

flow cytometry / genome size / Hymenoptera / *Melipona*

1. INTRODUCTION

The stingless bees of the genus *Melipona* (Illiger, 1806) comprise a group with approximately 40 Neotropical species (Michener, 2000) which are very diverse, abundant and both ecologically and economically important due to their role in the pollination of native and cultivated plants (Kerr et al., 1996; Heard, 1999).

Cytogenetic studies have shown that the majority of the species in this genus presents chromosomic number equal to $2n = 18$ (review in Rocha et al., 2003). However, the distribution and the amount of heterochromatin vary among the several species, which allowed the division of these bees into two groups. Group I comprises the species presenting a relatively low amount of heterochromatin (<50%) located in the pericentromeric region, while species in Group II present high heterochromatin level (>50%) distributed along almost all of the chromosome extension (Rocha and Pompolo, 1998; Rocha et al., 2002). According to Rocha and Pompolo (1998), this heterochromatin distribution pattern can contribute to the study of *Melipona* phylogeny.

Information regarding the size of the genome of the species in this genus can also provide data for comparative studies at several taxonomic levels (Finston et al., 1995; Gregory and Shorthouse, 2003; Tsutsui et al., 2008), and also for genome sequencing projects (Hardie et al., 2002; Gregory, 2005; Geraci et al., 2007). However such studies can not be applied to stingless bees species,

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because to date only two of them (*M. rufiventris* and *M. mondury*) have had their nuclear DNA content quantified (Lopes et al., 2009).

This paper aims at quantifying the DNA content of 15 species in the genus *Melipona* and also at verifying whether such data can be correlated to the cytogenetic classification of these species in two groups, based on their heterochromatin content.

2. MATERIALS AND METHODS

Samples of *Melipona quadrifasciata* (Viçosa/MG), *M. bicolor* (Viçosa/MG), *M. capixaba* (Domingos Martins/ES), *M. marginata* (Cunha/SP), *M. seminigra* (Nova Xavantina/MT), *M. mandacaia (*Uauá/BA), *M. asilvai* (São João do Sabugi/PB), *M. subnitida* (Santana do Seridó/PB), *M. scutellaris (*Pilões/PB), *M. compressipes* (Piripiri/PI), *M. quinquefasciata* (mountainous regions of the Ceará State), *M. grandis* (Xapuri/AC), *M. crinita* (Xapuri/AC), *M. fuscopilosa* (Xapuri/AC) and *M. eburnea* (Xapuri/AC) female pupae were analyzed.

2.1. Flow cytometry analysis

The flow cytometry (FCM) analyses were carried out at the Laboratory of Cytogenetics and Cytometry, Department of General Biology, Universidade Federal de Viçosa (UFV). The nuclear DNA content of the female larvae was measured using as internal standard the C DNA content value of a female of *Scaptotrigona xantotricha* which was confirmed against the international standard of *Drosophila melanogaster* as described by Lopes et al. (2009).

For preparation of FCM nuclei suspensions, brain ganglion nuclei of the standard and sample were excised in physiologic solution (0.155 mM NaCl). The materials were simultaneously crushed 10 times with a pestle in a tissue grinder (Kontes Glass Company®) with 100 μL OTTO-I lysis buffer (Otto, 1990) containing 0.1 M citric acid (Merck®), 0.5% Tween 20 (Merck®) and 50 μg mL^{-1} RNAse (Sigma-Aldrich®), pH = 2.3. The suspension was adjusted to 1.0 mL with the same buffer, filtered through 30 μm nylon mesh (Partec®) and centrifuged at 100 *g* in microcentrifuge tubes for 5 min.

The pellet was then incubated for 10 min in 100 μL OTTO-I lysis buffer and stained with 1.5 mL OTTO-I:OTTO-II (1:2) solution (30 min) (Loureiro et al., 2006a, b) supplemented with 75 μM propidium iodide (PI Sigma® – excitation/emission wavelengths: 480–575/550–740 nm, Shapiro, 2003) and 50 μg mL^{-1} RNAse (Sigma-Aldrich®), pH = 7.8. The nuclear suspension was filtered through 20 μm diameter mesh nylon filter (Partec®) and maintained in the dark for 5–40 min.

The suspension was analyzed with a Partec PAS® flow cytometer (Partec®) equipped with a Laser source (488 nm). PI fluorescence emitted from nuclei was collected through a RG 610 nm band-pass filter and converted to 1024 channels. The equipment was calibrated for linearity and aligned with microbeads and standard solutions according to the manufacturer's recommendations. FlowMax® software (Partec®) was used for data analyses. The standard nuclei peak was set to channel 100 and more than 10 000 nuclei were analyzed. Three independent replications were conducted and histograms with a coefficient of variation (CV) above 5% were rejected.

The mean genome size (pg) of each female bee sample was measured according to the formula adapted from Doležel and Bartos (2005) and subsequently converted to megabases pairs (1 pg = 978 Mbp) (Doležel et al., 2003). The 2C DNA content was converted to C-values and it will be presented here in picograms (pg) and megabases pairs.

2.2. Statistical analysis

We used a standard Student's t-test, with a *P*-value of $\leqslant 0.01$ to determine significant differences in genome size between species with high and low heterochromatin content and a Pearson correlation to examine relationships between genome size and the size of the bee estimated through the intertegular span and through the width of the head. In these analyses we also included the species *M. rufiventris* and *M. mondury* whose DNA content had been previously determined by our group (Lopes et al., 2009). These analyses were performed using GENES software (Cruz, 2009).

3. RESULTS

The mean genome size (1C) of the females belonging to the 15 species analyzed ranged from 0.27 pg for *M. quadrifasciata* to

Table I. Genome size estimates, chromosome number and relative heterochromatin content of different *Melipona* species.

Species	Mean genome size (1C) (pg – Mbp)	Chromosome number	Relative heterochromatin content*
1. *M. subnitida*[a]	0.27 – 264.06	2n = 18[1]	Low[2]
2. *M. quadrifasciata*[a]	0.27 – 264.06	2n = 18[1,3,4,5]	Low[5]
3. *M. marginata*[b]	0.28 – 273.84	2n = 18[3,4,5]	Low[5]
4. *M. bicolor*[b]	0.28 – 273.84	2n = 18[5]	Low[5]
5. *M. asilvai*[b]	0.29 – 283.62	2n = 18[5]	Low[5]
6. *M. mandacaia*[a]	0.35 – 342.3	2n = 18[2]	Low[6]
7. *M. quinquefasciata*[c]	0.70 – 684.6	2n = 20[7]	High[8]
8. *M. crinita*[d]	0.73 – 713.94	2n = 18[6]	High[2]
9. *M. compressipes*[c]	0.78 – 762.84	2n = 18[2]	High[2]
10. *M. seminigra*[d]	0.85 – 831.3	ND	ND
11. *M. rufiventris*[d]	0.93 – 909.54[10]	2n = 18[1,9]	High[9]
12. *M. mondury*[d]	0.95 – 929.1[10]	2n = 18[9]	High[9]
13. *M. grandis*[c]	0.95 – 929.1	ND	ND
14. *M. scutellaris*[d]	1.08 – 1056.24	2n = 18[5]	High[5]
15. *M. fuscopilosa*[d]	1.10 – 1075.8	2n = 18[5]	High[5]
16. *M. eburnea*[d]	1.11 – 1085.58	ND	ND
17. *M. capixaba*[d]	1.38 – 1349.64	2n = 18[5]	High[5]

[a] Subgenus *Melipona*, [b] Subgenus *Eomelipona*, [c] Subgenus *Melikerria*, [d] Subgenus *Michmelia*.
[1] Tarelho (1973); [2] Rocha et al. (2002); [3] Kerr (1948); [4] Kerr (1952); [5] Rocha and Pompolo (1998); [6] Rocha et al. (2003); [7] Pompolo (1994); [8] Pompolo, unpubl. data, [9] Lopes et al. (2008); [10] Lopes et al. (2009).
* Low heterochromatin content: <50%, high heterochromatin content: >50%. ND: cytogenetically non-determined.

1.38 pg for *M. capixaba* (Tab. I). It was possible to recognize two groups of species: the first presented relatively low DNA content (0.27–0.35 pg; average = 0.29 pg), while the second showed high DNA content (0.7–1.38 pg; average = 0.98 pg). The results of the *t* standard test showed statistically significant differences ($P \leqslant 0.01$) between the average sizes of the genome of these two groups of species.

Genome sizes were significantly correlated with the intertegular span (r = 0.585; $P \leq 0.05$) and with the width of the head (r = 0.542; $P \leq 0.05$) when all species were analyzed as a group. However, amongst the groups with low and high DNA content, there was no significant correlation between the DNA content and the size of the bees (Figs. 1a, b).

4. DISCUSSION

The haploid genome size variation observed in the present study provided a preliminary picture of the DNA content for the genus *Melipona*. Although there is no phylogenetic hypothesis proposed for the species from this genus, the data obtained in the present study corroborate the genetic relationships proposed for several *Melipona* species based on nuclear sequences (ITS-1) and mitochondrial restriction profiles (CO-I/CO-II) (Fernandes-Salomão et al., 2002, 2005). *M. quadrifasciata* and *M. mandacaia*, for example, are two species with low DNA content, and Fernandes-Salomão et al. (2002; 2005) have already demonstrated that a phylogenetic proximity exists between them. Fernandes-Salomão et al. (2002), based on the CO-I/CO-II restriction profiles, also proposed that considerable genetic proximity exists among *M. eburnea*, *M. crinita*, *M. rufiventris*, *M. scutellaris*, *M. capixaba*, and *M. seminigra*, species that possess high DNA content. Additionally, according to the morphological classification proposed by Moure (1992) ours results clearly

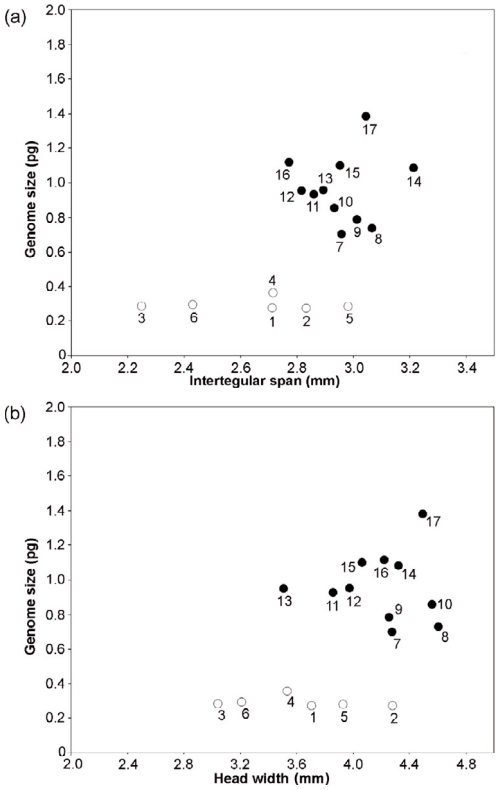

Figure 1. Relationships between genome size and intertegular span (a) and head width (b) in *Melipona* species with low (○) and high (●) heterochromatin content. (a) Intertegular span (r = –0.0123 and r = 0.0409, for species with low and high heterochromatin content, respectively). (b) Head width (r = –0.246 and r = –0.0275, for species with low and high heterochromatin content, respectively). The numbers 1–17 are the same as in Table I.

showed that species with low DNA content belong to the subgenera *Eomelipona* and *Melipona* while species with high DNA content belong to the subgenera *Melikerria* and *Michmelia.*

The recognition of these two groups also supports the proposal by Rocha et al. (2002) of dividing the genus *Melipona* into two groups, according to the heterochromatin amount. Thus, by bringing together the FCM measurements and cytogenetic data, we argue that the difference in the amount of DNA shown by the different species represents genome modifications which occurred through the addition or deletion of heterochromatin. According to Rocha et al. (2002), the addition of heterochromatin would be the main event in the karyotypic evolution of bees. Moreover, *M. marginata*, a species that possess great retention of ancestral characters (Camargo et al., 1967), presented low DNA content, which reinforces the claim that the genome with higher DNA content may be a derived characteristic.

Lopes et al. (2009) had previously observed that *M. rufiventris* and *M. mondury*, which present a greater amount of heterochromatin than *Scaptotrigona xantrotricha*, also have a greater quantity of DNA. Boulesteix et al. (2006) also proposed a relation between the greater heterochromatin amount in *Drosophila oreana's* chromosomes and the greater DNA content of this species in comparison to other species of the *melanogaster* subgroup. The genome size of species which presented high DNA content was significantly greater than the average of the genomes of other Hymenoptera which have already been measured (362 Mbp; C = 0.37 pg – Gregory, 2009). For instance, *Apis cerana* and *A. mellifera* have an estimated C value of 185.8 Mbp (0.19 pg) (Jordan and Brosemer, 1974) and 262 Mbp (0.27 pg) (The Honeybee Genome Sequencing Consortium, 2006), respectively. In spite of the high values found, compared to other species of Hymenoptera, all of the species analyzed present genomes smaller than 2.0 pg, a value proposed by Gregory (2002) as a standard for holometabolous insects.

Moreover, it was observed that the quantity of DNA in the species analyzed showed a range of values with increments of approximately 0.12 pg, i.e., half the smaller genome in the group. Among some invertebrates, the 1C DNA content of several congeneric species varies by multiples of the smallest genome in the group (Hughes-Schrader and Schrader, 1956; McLaren and Sévigny, 1989; Sella et al., 1993; Finston et al., 1995; Garagna et al., 1996; Rothfels et al., 1996; Gambi et al., 1997; Gregory et al., 2000). This variation pattern is particularly common in groups of invertebrates which present high taxonomic diversity (Gregory and Hebert, 1999), and it does not seem to be a result of polyploidy, since, as observed in the present and other studies, species

presenting different DNA values share a similar chromosomic number (Finston et al., 1995; Gregory, 2005; Gregory and Hebert, 1999).

The correlation between the size of the bees and the DNA content observed when species with high and low DNA content were analyzed together, as a single group, must be viewed with caution, since it is being established by the variables, once we are putting together groups genetically far different. Apparently this correlation reflects the taxonomic differences between the groups with low (*Eomelipona* and *Melipona*) and high (*Melikerria* and *Michmelia*) DNA content (Tab. I) and a more realistic relation among these characteristics is obtained when one considers the groups with high and low DNA content separately. In this case, it is easy to perceive that the variation in the DNA content, in both groups, is not related to the intertegular span or head width of the analyzed species (Fig. 1). These two parameters also seemed unrelated in oligochaetes (Gregory and Hebert, 2002) and in lepidopterans (Gregory and Hebert, 2003). However, positive relationships between DNA content and body size have been reported in aphids (Finston et al., 1995), copepods and flatworms (Gregory et al., 2000) and in dragonflies (Ardila-Garcia and Gregory, 2009), and a negative relationship was detected in damselflies (Ardila-Garcia and Gregory, 2009). So, further efforts regarding these relationships in insect are needed.

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Variation de la taille du génome chez les espèces de *Melipona* et classification en sous-groupes en fonction du contenu de leur ADN.

cytométrie de flux / taille du génome / Hymenoptera / *Melipona*

Zusammenfassung – Variation der Genomgrößen in *Melipona*-Arten (Hymenoptera: Apidae) und ihre Unterteilung anhand des DNA-Gehalts. Zu den stachellosen Bienen der neotropischen Gattung *Melipona* zählen etwa 40 Arten. Ungeachtet ihrer ökologischen Bedeutung sind jedoch Daten zum Umfang der Genome dieser Arten bisher nur spärlich vorhanden. Das Ziel dieser Studie ist es daher, den DNA-Gehalt von 15 Arten der Gattung *Melipona* zu quantifizieren, und die Eignung solcher Daten zur Bewertung einer zytogenetischen Klassifizierung dieser Arten in zwei Gruppen zu überprüfen, die auf der Grundlage ihres Heterochromatingehalts vorgeschlagen wurde. Der DNA-Gehalt des Zellkerns von weiblichen Larven wurde gegen einen internen Standard einer weiblichen *Scaptotrigona xantotricha* gemessen. Die mit Propidiumiodid versetzte Suspension wurde mit einem Durchflusszytometer mit einer Laserquelle (488 nm) analysiert. Die Fluoreszenz des PI wurde durch einen RG 610 Bandpassfilter geleitet und in 1024 Kanäle konvertiert. Der Standardpeak der Zellkerne wurde auf Kanal 100 eingestellt und mehr als 10 000 Zellkerne wurden analysiert. Drei unabhängige Wiederholungen wurden durchgeführt, wobei Histogramme mit Variationskoeffizienten von mehr als 5 % ausgeschlossen wurden. Die mittlere Genomgröße (1C) der weiblichen Tiere reichte von 0,27 pg bis 1,38 pg, mit Schritten von 0,12 pg (Tab. I). Es war möglich, zwei Gruppen von Arten zu erkennen: die erste hatte einen relativ niedrigen DNA-Gehalt, während die zweite einen hohen DNA-Gehalt aufwies. Diese Daten unterstützen die zytogenetische Klassifikation dieser Arten in zwei Gruppen, die auf der Basis ihres Heterochromatingehalts vorgenommen wurde. Innerhalb der Gruppen mit niedrigem und hohem DNA-Gehalt gab es keine signifikante Korrelation zwischen dem DNA-Gehalt und der Größe der Bienen (Abb. 1a und b). Die hier erhaltenen Daten können zur Auswahl von geeigneten Arten für Sequenzierungsprojekte beitragen, da die Größe des Genoms in solchen Projekten eine entscheidende Eigenschaft sein kann. Die Daten liefern außerdem einen Überblick über die Vielfalt der Genomgrößen in der Gattung *Melipona*.

Durchflusszytometrie / Genomgröße / Hymenoptera / *Melipona*

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