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Original article

Genome size estimation of three stingless bee species (Hymenoptera, Meliponinae) by flow cytometry*

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Abstract – The present study standardizes a reproducible flow cytometry (FCM) protocol for DNA content measurement of bee species and applied it to *Scaptotrigona* and *Melipona* species. The mean nuclear DNA content value of male and female *S. xantotricha* was 0.42 pg (410.8 Mbp) and 0.44 pg (430.3 Mbp), respectively, while the mean haploid genome size was determined to be 0.93 pg (909.5 Mbp) for *M. rufiventris* and 0.95 pg (929.1 Mbp) for *M. mondury*. The variation observed in this study, albeit in a preliminary way, may be related with the variation in the heterochromatin content in the chromosomes of *Scaptotrigona* and *Melipona* species. The results provide a starting point for comparative analysis on the patterns of genome size variation in the stingless bees.

flow cytometry / genome size / Hymenoptera / stingless bees

1. INTRODUCTION

The haploid genome sizes of insects ranges from 0.09 picogram (pg) (*Mayetiola destructor*) to 16.93 pg (*Podisma pedestris*), with an average of 1.29 pg ± 0.10 (Gregory, 2008). Most of the 602 insect C-values measured to date, however, belong to the orders Diptera, Coleoptera, Orthoptera and Hemiptera (Gregory, 2008). Specifically in Hymenoptera, one of the largest insect orders, with approximately 115.000 species described (La Salle and Gauld, 1993), the genome size has been measured in fifty two species: four bees (Jordan and Brosemer, 1974; Crain et al., 1976; Rasch, 1985; Petitpierre, 1996; Gadau et al., 2001; Wilfert et al., 2006; The Honeybee Genome Sequencing Consortium, 2006), six wasps (Rasch et al., 1975, 1977; Rasch, 1985; Johnston et al., 2004; Barcenas et al., 2008) and forty two ants (Li and Heinz, 2000; Johnston et al., 2004; Tsutsui et al., 2008).

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The knowledge of the genome size provides data for comparative studies in a variety of taxonomic levels and groups, for phylogenetic associations and for the design of sequencing projects. Due to this relevance, it is surprising the current lack of data related to the stingless bees' genome size, a group of high eusocial bees, particularly due to their abundance, diversity and important role in the pollination in native forest in Brazil.

Thus, considering that the genome size determination of a species may facilitate the design of further molecular studies, the aims of this research were to: (1) standardize a protocol to obtain adequate nuclei suspension for flow cytometry (FCM) analyses in bees and (2) measure the 2C DNA content of *Scaptotrigona xantotricha, Melipona rufiventris* and *M. mondury*, in order to provide a starting point for comparative analysis on the patterns of genome size variation in these stingless bees and its relationship to the evolution of sociality in this and in other insect groups.

Specifically, we choose *S. xanthotricha*, because their colonies produce haploid males all year long, fact that makes this species ideal for use as FCM internal standard. Otherwise, *M. rufiventris* and *M. mondury* were chosen because, due to morphological similarities, until recently they were considered a single species in Minas Gerais state (Melo, 2003) besides being objects of study in our laboratory.

2. MATERIALS AND METHODS

2.1. Biological material

Samples of *Scaptotrigona xanthotricha* female and male of post-defecating larvae (prepupal phase) and *Melipona rufiventris* and *M. mondury* female post-defecating larvae were collected from colonies from Viçosa/MG, Guimarânia/MG and Itamarandiba/MG regions, respectively. The FCM analyses were carried out at the Laboratory of Cytogenetics and Cytometry, Department of General Biology, Universidade Federal de Viçosa (UFV).

2.2. Experimental design

In order to determine a suitable internal standard for FCM measurements in bees and to avoid linearity problems, initially the C DNA content mean value of haploid male *S. xantotricha* was assessed using the adult female *Drosophila melanogaster* strain Iso-1 (internal standard – C = 0.18 pg – Bennett et al., 2003) obtained from University of California at Berkeley (CA, USA). The nuclear DNA content of the other female bees was measured using the internal standard as shown in Table I.

Table I. Internal standards used in the FCM measurements of *S. xantotricha, M. rufiventris* and *M. mondury* nuclei samples. The experimental design was applied in the order to prevent FCM linearity problems.

Sample species	Internal standard species
Male *S. xantotricha*	Female *D. melanogaster*
Female *S. xantotricha*	Male *S. xantotricha*
Female *M. rufiventris*	Female *S. xantotricha*
Female *M. mondury*	Female *S. xantotricha*

2.3. Flow cytometry analysis

Brain ganglion of the standard and sample were excised in a cooled physiologic solution (Arboreto®). Using a stereoscopic microscope, intact brain ganglions were carefully excised, transposed to a new drop of same solution and cleaned in order to avoid the contamination by other head tissues. 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 KGaA®), 0.5% Tween 20 (Merck KGaA®) and 50 μg mL^{-1} RNAse (Sigma-Aldrich®), at to pH = 2.3 to minimize enzymatic catalysis. The suspension was adjusted to 1.0 mL with the same buffer, filtered through 30 μm nylon mesh (Partec Gmbh®) and centrifuged at 100 *g* in Eppendorf 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 Gmbh®) and maintained in the dark for 5–40 min.

The suspension was analyzed with a Partec PAS® flow cytometer (Partec® Gmbh, Munster, Germany) 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 done and histograms with coefficient of variation (CV) above 5% were rejected.

The mean genome size of each female bee sample was measured according to formula adapted from Doležel and Bartos (2005):

$$\text{Sample 2C DNA content (pg)} = (\text{Mean } G_0/G_1 \text{ peak of the sample} \times \text{Standard 2C DNAcontent}) \times (\text{Mean } G_0/G_1 \text{ peak of the standard})^{-1}.$$

For male bee values, the sample 2C DNA content in the formula above was replaced to 1C DNA.

The mean genome sizes will be presented here as C-values in picograms (pg) and megabases pairs (1 pg = 978 Mbp) (Doležel et al., 2003).

3. RESULTS

In this study, the genome size of *S. xantotricha*, *M. mondury* and *M. rufiventris* was adequately measured by FCM. The analyses of the nuclei suspensions stained with PI generated histograms with peaks corresponding to the average relative DNA content of the G_0/G_1 nuclei of *S. xantotricha* (male and female), *M. rufiventris* (female), *M. mondury* (female) and the comparative internal standard (Fig. 1). The histograms showed good resolution levels (Fig. 1a–e) and CVs ranging from 2.87 to 4.14%.

The mean genome size values of male and female *S. xantotricha* was 410.8 Mbp (C = 0.42 pg) and 430.3 Mbp (C = 0.44 pg), respectively (Fig. 1a–b, Tab. II). The mean genome size of *M. rufiventris* and *M. mondury* females was 909.5 Mbp (C = 0.93 pg) and 929.1 Mbp (C = 0.95 pg), respectively (Fig. 1c–d, Tab. II). The similarity of these two genome sizes can be confirmed in the histogram of *M. rufiventris* and *M. mondury* nuclei suspension, where the G_0/G_1 peaks of both species were not distinguished (Fig. 1e).

4. DISCUSSION

The nuclei preparation employed in the present study resulted in suspensions with few artifacts such as fluorescent nonnuclear debris and nuclear aggregates. Crushing in a tissue grinder was also applied by Bennett et al. (2003) and Johnston et al. (2004), but these authors used Galbraith buffer (Galbraith et al., 1983) to generate the nuclei suspensions. In this study, the OTTO buffers (Otto, 1990) were applied for the same purpose. We opted for this buffer because, in our laboratory routine, histograms with high resolution and low CVs have been obtained, as confirmed by the results of this study. Also, Doležel and Bartoš (2005) and Loureiro et al. (2006a, b) recommended the OTTO buffers considering that the citric acid (OTTO-I) prevents differences in staining intensity of the nuclei. In addition, in agreement with Loureiro et al. (2006a, b), these buffers provided better results in species with low DNA content, such as bees.

Aron et al. (2003) applied the chopping procedure and detergent-trypsin buffer solution (Vindelov et al., 1983) for nuclei preparation from *Linepithema humile* adults and larvae. These authors obtained FCM histograms with CVs ranging from 4.2% to 7.3%. In the present study, the largest CV was 4.14%, value that is considered acceptable for FCM measurements (Galbraith et al., 2002), and indicates that the methods and buffers supplied adequate FCM nuclei suspensions.

Additionally, the methodology developed here suitably discriminated the G_0/G_1 nuclei of the haploid males and diploid females of *S. xantotricha*, which consequently strengthens the use of this species as internal standard for FCM analysis in bees, rather than distantly related organisms.

The small difference in the DNA content between males and females of *S. xantotricha* is unlikely to be measurement errors. Our measurements were repeated several times for both sexes simultaneously and demonstrated the same values differences. Additionally, the flow cytometry linearity was checked with standard microbeads and chicken red blood cell suspensions. The exact reasons of this difference, however, remain obscure and only additional studies could clarify this question.

FCM measurements also showed that the genome sizes of *M. rufiventris* and *M. mondury* differed by only 19.6 Mbp but were ~ 2.1-fold larger than that of *S. xantotricha* (Fig. 1c–d, Tab. II). The other few Apidae studied until now also show a smaller genome size than that of *M. rufiventris* and *M. mondury*. For example, *Apis cerana*, *A. mellifera* and *Bombus terrestris* possesses an estimated C-value of 185.8 Mbp (0.19 pg) (Jordan and Brosemer, 1974), 262 Mbp (0.268 pg) (The Honeybee Genome Sequencing Consortium, 2006) and 625 Mbp (0.639 pg) (Wilfert et al., 2006). The genome size of these two *Melipona* species is also larger than the mean

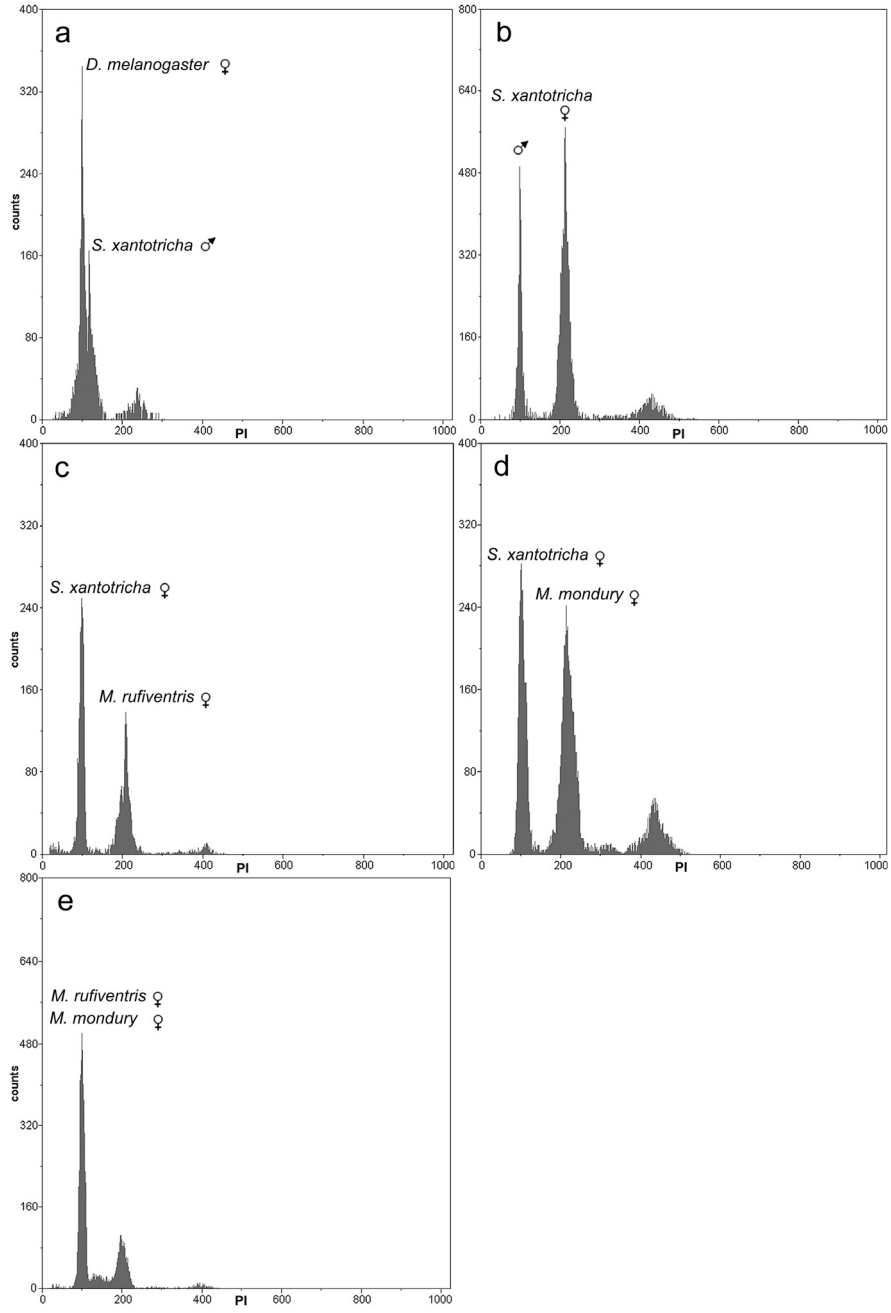

Figure 1. Genome size DNA-histograms of *S. xantotricha*, *M. rufiventris* and *M. mondury* through analysis of nuclear suspensions of cerebral ganglion tissue, stained with PI. (a) Female *D. melanogaster* (internal standard 2C = 0.36 pg, channel 100) and male *S. xantotricha* (1C = 0.42 pg, channel 117). (b) Male *S. xantotricha* (1C = 0.42 pg, channel 100) and female *S. xantotricha* (2C = 0.88 pg, channel 210). (c) Female *S. xantotricha* (2C = 0.88 pg, channel 100) and female *M. rufiventris* (2C = 1.85 pg, channel 210). (d) Female *S. xantotricha* (2C = 0.88 pg, channel 100) and female *M. mondury* (2C = 1.89 pg, channel 215). (e) Female *M. mondury* (channel 100) and female *M. rufiventris* (channel 100).

Table II. Genome estimation size of cerebral ganglion of *S. xantotricha, M. rufiventris* and *M. mondury*.

Species	Mean genome size (1C) (pg and Mbp)	Mean genome size/nucleus (G_1) (Mbp)
Male *S. xantotricha*	0.42–410.8	410.8
Female *S. xantotricha*	0.44–430.3	860.6
Female *M. rufiventris*	0.93–909.5	1819.0
Female *M. mondury*	0.95–929.1	1858.2

size estimated for the hymenopteran species (352 Mbp; C = 0.36 pg – Gregory, 2008) analyzed to date.

Cytogenetic studies have allowed the division of several *Melipona* species with the same number of chromosomes (2n = 18) in two groups based on their heterochromatin content. The first group comprises species with low heterochromatin content (*M. bicolor*, *M. quadrisfasciata*, *M. asilvai*, *M. marginata* and *M. subnitida*), while species in the second one (*M. capixaba*, *M. compressipes*, *M. crinita*, *M. seminigra*, *M. captiosa*, *M. scutellaris*, *M. rufiventris* and *M. mondury*) contain a high heterochromatin content (Rocha and Pompolo, 1998; Rocha et al., 2002; Lopes et al., 2008).

In this context, one mechanism that could give raise to the extra-DNA in *M. rufiventris* and *M. mondury* is the higher heterochromatin amount in the chromosomes of these species (Lopes et al., 2008) compared to *S. xantotricha* (Rocha et al., 2003). Boulesteix et al. (2006) have also mentioned that the large heterochromatin amount present in the chromosomes of *Drosophila orena* may be the main factor responsible for the greater increase in the genome size of this species in comparison to the others species of the *Drosophila melanogaster* subgroup.

Increases in genome size, however, have also been associated with transposable elements (TEs) (SanMiguel and Bennetzen, 1998; Vieira et al., 2002), repetitive sequences (Uozu et al., 1997; Ullmann et al., 2005), intron size (Moriyama et al., 1998) and microsatellite presence (Warner and Noor, 2000).

The variation among the genome sizes of the species analyzed herein may, therefore, reflect differences in their genomic structure. Nevertheless, no extrapolations should be made about the evolutive significance of the differences observed since we had estimated the genome size for only three stingless bees. Anyway, only a closer examination of genetic characteristics such as number of TEs, introns size and microsatellite size and number may elucidate the specific mechanisms by which their genomes expanded or contract. These studies should help us to understand the link between chromatin structure, genome size and evolution of the stingless bees.

Anyway, despite the high genomes sizes of *M. rufiventris* and *M. mondury* in comparison to the genome size of *S. xanthotricha* and other Hymenoptera species, our results still match the pattern verified for species that undergo complete metamorphosis, presenting genomes that are smaller than C = 2 pg (Gregory, 2002, 2005). Thus, our data represent a significant step towards a better understanding of the stingless bee genome.

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Estimation de la taille du génome de trois espèces d'abeilles sans aiguillon (Hymenoptera, Meliponinae) par cytométrie de flux.

Apoidea / abeille sans aiguillon / taille du génome / cytométrie de flux

Zusammenfassung – Schäztung der Genomgröße von drei Stachellosen Bienenarten (Hymenoptera, Meliponinae) mittels Durchflusszytometrie. Kenntnisse über Genomgrössen sind von Bedeutung für den Entwurf von Sequenzierungesprojekten und für vergleichende Studien

unterschiedlicher taxonomischer Gruppen. Nichtsdestoweniger ist die Genomgröße verschiedener Insektengruppen, einschliesslich der Stachellosen Biene nahe unbekannt, trotz ihrer ökologischen Bedeutung. Ziele dieser Untersuchung waren: (1) die Standardisierung eines Protokolls zur Gewinnung von Zellkernen für Durchflusszytometrieanalysen (FCM) bei Bienen, und (2) die Bestimmung des DNA-Gehalts für *Scaptotrigona xantotricha, Melipona rufiventris* und *M. mondury*, als Startpunkt für vergleichende Analysen der Variation der Genomgrössen Stachelloser Bienen. Das entwickelte Protokoll (Tab. I) erlaubte es, adequate Kernsuspensionen mit niedrigem Fluoreszenzhintergrund bedingt durch Kernfragmente und andere Organelle zu gewinnen, und es lieferte Histogramme mit Variationskoeffizienten zwischen 2,87 und 4,14 % (Abb. 1). Der mittlere DNA-Gehalt für Männchen von *S. xantotricha* lag bei 0,42 pg (410,8 Mbp), der von Weibchen betrug 0,44 pg (430,3 Mbp). Für *M. rufiventris* betrug die Größe des haploiden Genoms 0,93 pg (909,5 Mbp) und für *M. mondury* lag der Wert bei 0,95 pg (929,1 Mbp) (Tab. II). Diese Unterschiede im DNA-Gehalt könnten ihre Ursache in unterschiedlichen Heterochromatingehalten der Chromosomen von *Scaptotrigona* und *Melipona*-Arten haben. Die Ergebnisse stellen einenAusgangspunkt für vergleichende Untersuchungen zur Genomgröße und der entsprechenden Variationen bei Stachellosen Bienen dar.

Durchflusszytometrie / Genomgröße / Hymenoptera / Stachellose Bienen

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