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Estimation of Nuclear Genome Size of Three Species of *Camponotus* (Mayr, 1861) (Hymenoptera: Formicidae: Formicinae) and Their Cytogenetic Relationship

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Abstract

The chromosome variability among ant species is remarkable, and the processes generating such variation are still under discussion since polyploidy has been observed in some distinct taxa. The chromosome number of species belonging to the *Camponotus*, subgenera *Myrmothrix* and *Myrmobrachys*, are highly different, whereas, the first subgenus has double the number of chromosomes of the second. In order to test the hypothesis of chromosome number doubling through polyploidy, the genome sizes of *Camponotus* (*Myrmothrix*) *rufipes*, *Camponotus* (*Myrmothrix*) *renggeri* and *Camponotus* (*Myrmobrachys*) *crassus* were estimated by flow cytometry. The chromosome number of specimens from the nests studied was also defined. No significant variation was noted in the genome size among them. The mean haploid genome size value (1C) of workers for the three species was 286.16 Mpb (0.29 pg). The polyploidy hypothesis can be ruled out as an evolutionary step linking the karyotype variations among the three studied species since the genome size of *C. crassus* with 2n = 20 chromosomes was the same as that of *C. rufipes* and *C. renggeri* with 2n = 40. The lack of variation in the amount of DNA between the related species *C. rufipes* and *C. renggeri* also demonstrate that flow cytometry is not an adequate approach to distinguish them. Our results highlight the importance of combining distinct methods, DNA quantification, and cytogenetics from the same colony. Understanding the path of chromosome evolution of three species with distinct degrees of relatedness should provide further information in enriching our knowledge about the Minimum Interaction Theory.

Introduction

The genus *Camponotus* (Mayr, 1861) is widely distributed. This includes the so-called carpenter ants and, nowadays, is the richest ant genus in respect to the number of species, with 1099 described species in 46 subgenera (Bolton, 2014). Molecular phylogenetic studies indicate that this genus is paraphyletic (Brady et al., 2000), and the taxonomic relationship between different species of the genus *Camponotus* is even more critical. The Neotropical subgenus *Myrmothrix* is considered monophyletic (Brady et al., 2000; Hashmi, 1973), and it presents only 10 valid species (Bolton, 2014). However, due to the morphological variations within some species, this number is still under discussion.

Two species of subgenus *Myrmothrix*, *Camponotus rufipes* (Fabricius, 1775) and *Camponotus renggeri* Emery, 1894, have been the subject of much discussion, primarily due to their morphological similarities. Since the morphological techniques have limitations (Seifert, 2009; Schlick-Steiner et al., 2010), the use of integrative approach is very useful in distinguishing these species, as indicated by a recent study of Ronque et al. (2015).

Cytogenetics has drawn the attention of myrmecologists over recent years (Delabie et al., 2012), and it can be a useful tool in Integrative Taxonomy. Cytogenetic data are available for 71 taxa of *Camponotus* belonging to different countries in the world (revised in Mariano et al., 2003) showing that the chromosome number in *Camponotus* ranges from 2n =

18 to 2n = 50. Within the monophyletic Neotropical subgenus *Myrmothrix*, cytogenetic data are available only for *Camponotus punctatus* (Goñi et al., 1983), *C. atriceps*, *C. cingulatus* (Mariano et al., 2001), and *C. rufipes* (Goñi et al., 1983; Mariano et al., 2001), all presenting 2n = 40 chromosomes, and similar chromosome morphologies.

Cytogenetic studies on more than 500 species of ants allowed Imai et al. (1988; 1994) to develop the Minimum Interaction Theory (MIT) that explains how the chromosomes were modified in the course of evolution of Formicidae. This theory suggests a selective pressure that leads to a reduction in the size of the chromosomes, avoiding interactions that can reduce the fitness of individuals. Reduction in the size of chromosomes occurs mainly through centric fissions, although other types of chromosomal rearrangements that may not modify the size of the chromosomes, such as inversions and translocations, were also noted. One consequence of this process is the increase in the chromosome number. This theory suggests that the increase in terminal heterochromatin after centric fissions rearrangements is a part of the chromosomal evolution, which is correlated with chromosome stability (Imai et al., 1994).

The chromosome configuration of *Camponotus* (*Myrmobrachys*) *crassus* (Mayr, 1862) is in contrast with that of the species of *Camponotus* species inserted in the *Myrmothrix* subgenus (Mariano et al., 2001). The karyotype of *C.* (*Myrmobrachys*) *crassus* possesses one of the lowest diploid chromosome numbers described for the genus, with 2n = 20, all with metacentric morphology (Mariano et al., 2001). Since the *Myrmothrix* species have double the number of chromosomes (2n = 40) as that found in *C.* (*Myrmobrachys*) *crassus*, with all the chromosome centromeres positioned at the edges, the manner in which the chromosomes have modified can be superficially associated with the centric fission processes or chromosomal translocations. The use of the karyographic method by Mariano et al. (2003) indicated the presence of chromosomal rearrangements of the centric fission type in the karyotype evolution of *Camponotus* spp. However, there is a second possibility: through a single polyploidization event, followed by secondary chromosome modifications, the number of chromosomes can jump from 20 to 40 chromosomes. This is a real possibility since ants reproduce through arrhenotokous parthenogenesis, where fertilized eggs give rise to diploid females and unfertilized eggs originate haploid males (reviewed in Normark, 2003). Studies associating chromosome numbers and genome size to test the hypothesis of polyploidization in the genus were not yet evaluated. Karyotype-DNA content comparison is, therefore, a useful tool to understand the genome size evolution in ants.

Data concerning the genome size in Formicidae are restricted to 73 species (Tsutsui et al., 2008; Ardila-Garcia et al., 2010; Cardoso et al., 2012; Gregory, 2015). Three different techniques have been used to quantify the genome of ants as: flow cytometry (FCM), Feulgen image analysis densitometry (FIAD), and, more recently, through genomics (reviewed in Nygaard & Yannick, 2015). In general, ants have smaller genomes than other insects (Tsutsui et al., 2008). The genome size in Formicidae ranges from 210.7 Mb (0.22 pg) in *Cerapachys edentata*, to 690.4 Mb (0.71 pg) in *Ectatomma tuberculatum* (Tsutsui et al., 2008). The species *Apterostigma dentigerum* and *E. tuberculatum* showed DNA content which was twice higher than that of the most closely related species, however karyological information is not available, or is not related to the same nest. Studies concerning DNA content of the genus *Camponotus* are available for *C.* (*Camponotus*) *pennsylvanicus*, *C.* (*Tanaemyrmex*) *castaneus* (Tsutsui et al., 2008), and *C.* (*Myrmothrix*) *floridanus* (Ardila-Garcia et al., 2010; Bonasio et al., 2010), all from the USA (Table 1), and are not related with karyology.

Table 1 – Chromosome number and genome size values of the studied *Camponotus* species.

Camponotus spp.	Mean Genome size (1C) (pg – Mbp)	Chromosome number	Method	Reference
C. (*Myrmothrix*) *rufipes*	0.29 – 286.16	2n = 40[1,2]	FCM	Present study
C. (*Myrmothrix*) *renggeri*	0.29 – 286.16	2n = 40[2]	FCM	Present study
C. (*Myrmobrachys*) *crassus*	0.29 – 286.16	2n = 20[1,2]	FCM	Present study
C. (*Myrmothrix*) *floridanus*	0.23 – 224.94*	ND	FIAD	Ardila-Garcia et al. (2010)
C. (*Myrmothrix*) *floridanus*	0.245* – 240†	ND	Genome sequencing	Bonasio et al. (2010)
C. (*Tanaemyrmex*) *castaneus*	0.31 – 304.2	ND	FCM	Tsutsui et al. (2008)
C. (*Camponotus*) *pennsylvanicus*	0.33 – 322.8	ND	FCM	Tsutsui et al. (2008)

1: Mariano et al. (2001), 2: present study, ND: not defined.
FCM: flow cytometry; FIAD: Feulgen image analysis densitometry.
* Converted values from the available published data.
† Corresponding to more than 90% of the genome size sequenced.

Considering the lack of genome size and cytogenetics combined data in *Camponotus* spp., this study aimed to estimate the DNA content of *C. rufipes*, *C. renggeri* and *C. crassus* for a better understanding of the chromosome evolution of species of the genus *Camponotus*. The main basis of the MIT suggests small chromosome modifications usually with little variation in the amount of DNA. Therefore, the hypothesis of doubling the number of chromosomes among the species studied through polyploidization was tested, bringing further subjects to future discussions about the well accepted MIT in explaining chromosome variability among ant species.

Materials and Methods

Biological materials

Samples of *C. rufipes* and *C. crassus* were collected in Viçosa, state of Minas Gerais, Brazil (20°45'S, 42°51'W), and *C. renggeri* was collected in Nova Mutum, state of Mato Grosso, Brazil (13°49'S, 56°05'W). The colonies were maintained in plastic recipients at the Laboratório de Citogenética de Insetos, Departamento de Biologia Geral, Universidade Federal de Viçosa and fed with *Tenebrio molitor* (Coleoptera, Tenebrionidae) larvae and honey from bees in order to obtain pupae, which were utilized for the flow cytometer analysis.

The sampling authorization was provided by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (n° 34567-4), released by SISBio for Hilton J.A.C. de Aguiar. Adult specimens were identified by Dr. Jacques H. C. Delabie and were deposited as vouchers in the reference collection of the Laboratório de Mirmecologia, Centro de Pesquisas do Cacau (CEPEC/Brazil) #5755.

Genome size by FCM

The FCM analysis were performed at the Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV). The nuclear DNA content of three female pupae (workers and therefore diploid) of each species was measured by using an internal standard of the female pupae bee *Scaptotrigona xantotricha* (2C = 0.88 pg), as described by Lopes et al. (2009). For preparation of FCM nuclei suspensions, the cerebral ganglia nuclei of the standard and sample were excised in physiological solution (0.155mM NaCl). The materials were simultaneously crushed 10 times with a pestle in a tissue grinder (Kontes Glass Company®) with 100 µL of OTTO-I lysis buffer (Otto, 1990) containing 0.1 M citric acid (Merck®), 0.5% Tween 20 (Merck®), and 50 µgmL^{-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 100g in microcentrifuge tubes for 5 min.

The pellet was then incubated for 10 min in 100 µL of OTTO-I lysis buffer and stained with 1.5 mL of OTTO II solution (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 µgmL^{-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 at least 30 min.

The nuclear suspension was analyzed by using a Partec PAS® flow cytometer (Partec®) equipped with a laser source (488 nm). FlowMax® software (Partec®) was used for data analyses. The standard nuclei peak was set to channel 200 and more than 20.000 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 hymenopteran sample was measured according to the formula adapted from Doležel & Bartos (2005) and their values were subsequently converted to megabase pairs (1 pg = 978 Mbp) (Doležel et al., 2003). The 2C DNA content was converted to C-values, presented in this study here in picograms and megabase pairs.

Chromosome preparation

Mitotic metaphases were obtained from cerebral ganglia of the larvae from the same nests which were studied by FCM using the protocol of Imai et al. (1988) The metaphases were observed and photographed using a BX 60 microscope with a 100X objective, coupled with a Q-Color3 Olympus® image capture system to confirm the chromosome number of the same nests studied by means of FCM.

Results

In this study, the genome sizes of *C. rufipes*, *C. renggeri* and *C. crassus* were measured by FCM. The analyses of the nuclei suspensions generated histograms with peaks corresponding to the average relative DNA content of the G0/G1 nuclei (2C DNA amount), and a minor peak representing nuclei in G2 (4C DNA amount) of the three studied species, and *S. xantotricha*, the comparative internal standard (Fig. 1). The histograms revealed appropriate levels of resolution and CV ranging from 3.8% to 4.9%. The mean genome size (1C) values of *C. rufipes*, *C. renggeri* and *C. crassus* was approximately 286 Mbp (0.29 pg) (Table 1). The chromosome number observed were identical for *C. rufipes* and *C. renggeri* with 2n = 40 chromosomes (4sm + 34st + 2t). *C. crassus* presented 2n = 20 chromosomes (18m + 2sm) (Fig. 2).

Discussion

The three ant species *C. rufipes*, *C. renggeri* and *C. crassus* presented the same genome size and values that fell within the already known range for *Camponotus* species (Table 1) (Tsutsui et al., 2008; Ardila-Garcia et al., 2010). The DNA content data for the two species of subgenus *Myrmothrix* with double the chromosome number in comparison with

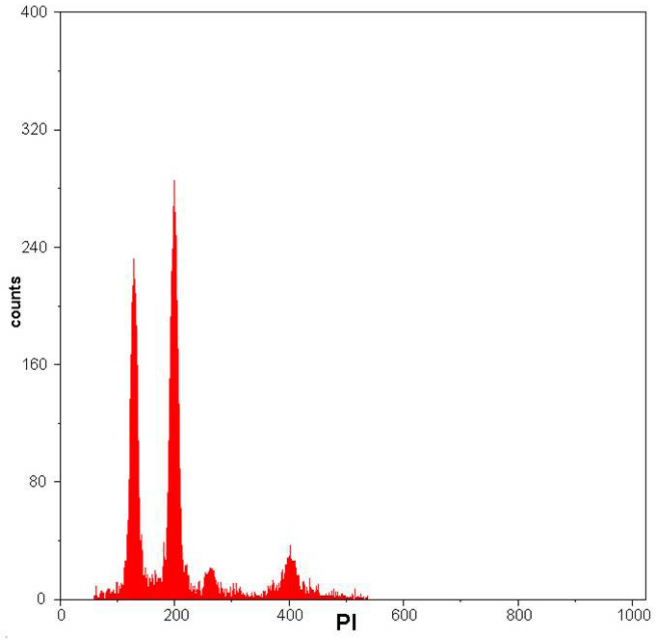

Fig 1. Histograms of relative nuclear DNA content from suspension obtained from cerebral ganglia cells after flow cytometric analysis with propidium iodide stained nuclei isolated of the ant *Camponotus* (*Myrmothrix*) *rufipes*. G0/G1 peak of unknown sample and the female bee *Scaptotrigona xantotricha* (2C = 0.88 pg), which served as internal reference standard, are clearly visible. 2C nuclear DNA content was determined based on the ratio of G0/G1 peak positions. The ants *C.* (*Myrmobrachys*) *crassus* and *C.* (*Myrmothrix*) *renggeri* presented indistinguishable results. Nuclei peak of female *C. rufipes* at channel 132 (2C = 0.58 pg, 1C = 0.29 pg) and nuclei peak of female *S. xantotricha* at channel 200 (internal standard 2C = 0.88 pg, 1C = 0.44 pg).

those of *C.* (*Myrmobrachys*) *crassus*, give support to the conclusions acquired by the karyographic method (Mariano et al., 2003). The present study rules out the possibility of polyploidy in the studied species of *Camponotus* subgenera *Myrmobrachys* and *Myrmothrix*, including species with different chromosome numbers (2n = 20 and 2n = 40). This is the first time the karyotype of *C. renggeri* is available. Further studies including chromosome banding and molecular cytogenetics are in preparation. The results are in accordance with the MIT, because we can observe the increase in the chromosome number of the species studied and the decrease in the chromosome size without alteration in the content of DNA.

The genome size investigation associated with the chromosome number of individuals from the species studied revealed its importance due to the possibility of intraspecific population variation as the presence of aneuploidy or B chromosomes. H. Aguiar observed one colony of *C. crassus* collected in Rio de Janeiro, Brazil, with the presence of supernumerary chromosomes (personal observation, July, 2012). This could influence its genome size. The ant colony collected from Minas Gerais in this study, presented 2n = 20, without supernumerary chromosomes, corroborating the previous cytogenetic surveys of Mariano et al. (2001) from the same locality.

Studies conducted in Formicidae indicated greater interspecific variations in the DNA content between subfamilies (58%) and genera (36%), and interspecific variations are less likely among species (3.8%) (Tsutsui et al., 2008). However, these authors suggested that this small variation may be due to the small number of species studied.

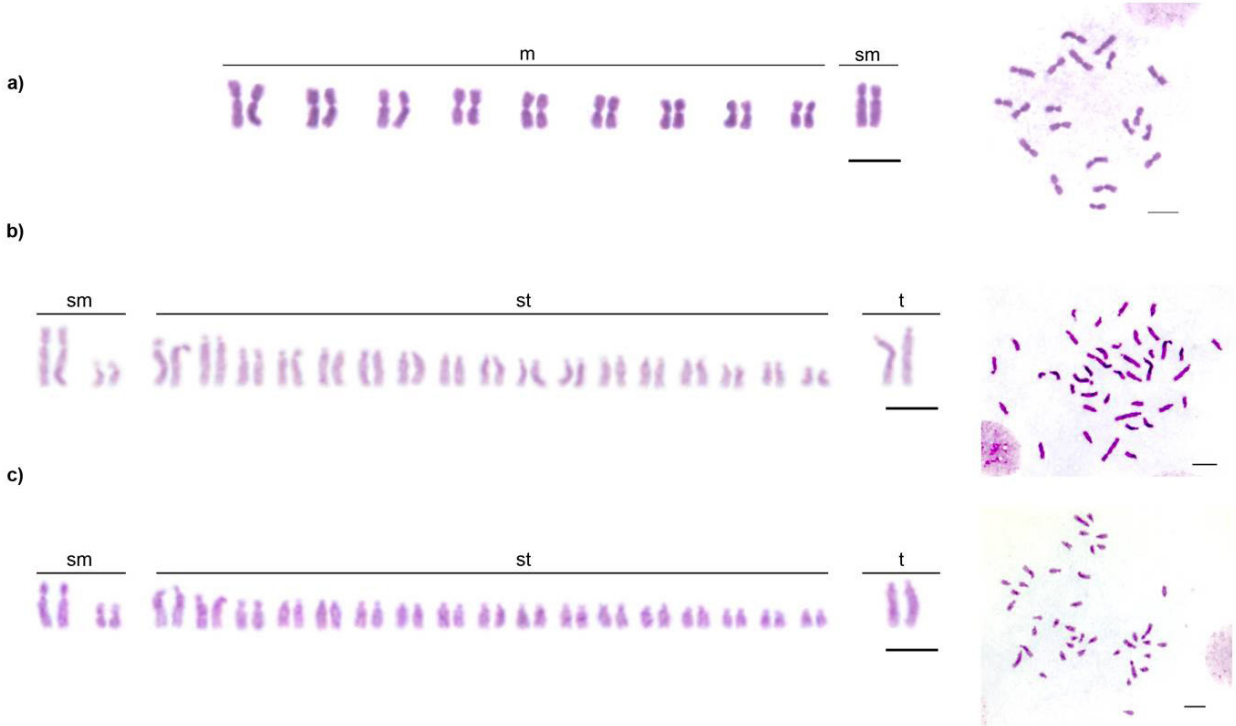

Fig 2. Metaphases and karyotypes of *Camponotus* species studied by means of flow citometry. a) *Camponotus* (*Myrmobrachys*) *crassus* (2n=20), b) *Camponotus* (*Myrmotrix*) *renggeri* (2n=40), c) *Camponotus* (*Myrmotrix*) *rufipes* (2n=40). m = metacentric; sm = submetacentric; st = subtelocentric; t = telocentric. Bars = 5 µm.

The fungus-growing ants, *Mycetophylax morschi* and *Mycetophylax conformis*, showed the same genome size and chromosome number. Only *Mycetophylax simplex* differed from their congeneric species, which may be due to the difference in the heterochromatin content (Cardoso et al., 2012). Although *M. morschi* shows variation in the chromosome number, differences in the DNA content within its variants were not observed (Cardoso et al., 2012). The stingless bees with high heterochromatin content *Melipona rufiventris* and *Melipona mondury* showed the same chromosome number, with little difference in the genome size; this observation suggests difference in the amount of heterochromatin (Lopes et al., 2009).

The recent study conducted by Ronque et al. (2015) based in an integrative approach showed very useful in understanding taxonomic issues of *C. rufipes* and *C. renggeri*. The lack of variation in the amount of DNA between these two *Camponotus* ants indicated that these species could not be distinguished based solely on genome size, highlighting the importance of additional approaches. On the other hand, *C.* (*Myrmobrachys*) *crassus* also showed a genome size of 286 Mbp, revealing that comparative investigation about the amount of DNA of phylogenetically distant *Camponotus* spp. may have limitations. The presence of supernumerary chromosomes could influence the DNA content, however, it can be ruled out for all three species in the present study, because the karyotype study was conducted for the same colonies analyzed by FCM.

Several ant species with the estimated genome size do not have basic cytogenetic data. The species *C. floridanus* has been taken for a model species in different fields of biological knowledge and, as a consequence, information about its genome size is already available (Ardila-Garcia et al., 2010; Bonasio et al., 2010). Unfortunately, there is no data regarding cytogenetics of this ant. Both morphological (Hashmi, 1973) and molecular observations (Brady et al., 2000) suggest that this species is closely related to *C. atriceps*, but there is no cytogenetic data available to support this finding. The *C. floridanus* chromosome number availability could enrich the discussion about the chromosome and also genome size evolution within this group.

Different methods were used to estimate the genome size of a species belonging to a few taxa. The study of the wasps *Aphidius ervi*, *Polistes fuscatus* and *Sceliphron caementarium* revealed differences with the data from FCM and FIAD (Ardila-Garcia et al., 2010). Genome size estimation using FIAD revealed lower values for the three species. The DNA content of the ant *Solenopsis invicta* also showed discrepancies probably due to the methodology employed, differences in the examined cell type, or other genetic differences between the studied populations (Wurm et al., 2010). The genome size of the main Neotropical malaria vector *Anopheles darlingi* obtained by FCM was similar in comparison with the value obtained by sequencing the genome after estimation and addition of repetitive portions as centromeres, telomeres and other portions of the genome rich in repetitive DNA sequences (Marinotti et al., 2013). The methodology employed to estimate the genome size of *C. floridanus* was FIAD, which may have underestimated the value (Ardila-Garcia et al., 2010). This value differs from that obtained by Bonasio et al. (2010) through partial genome sequencing. Both *C.* (*Tanaemyrmex*) *castaneus* and *C.* (*Camponotus*) *pennsylvanicus* do not belong to *Myrmothrix*, and the standardized FCM data obtained revealed values closer to those of the *Camponotus* species of the present study. These findings highlight the importance of method and the cell type standardization to study insect genome size (Ardila-Garcia et al., 2010), allowing comparisons for consistent evolutionary studies.

The present study represents the first data relating to the genome size and chromosome number of *Camponotus*, allowing more robust observations with respect to the evolution of genome size-karyotypes of these species. The estimation of the genome size of two related species *C. rufipes* and *C. renggeri*, and a third distant species *C. crassus*, have its importance in demonstrating the chromosome evolution, pointing that the transition between 2n = 20 and 2n = 40 did not involve genome duplication. However, further information from additional fields of knowledge is necessary for better understanding of the taxonomic relationship of *C. rufipes* and *C. renggeri*.

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