

ORIGINAL ARTICLE

Chromosomal mapping of repetitive DNAs in the beetle *Dichotomius geminatus* provides the first evidence for an association of 5S rRNA and histone H3 genes in insects, and repetitive DNA similarity between the B chromosome and A complement

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Chromosomal banding techniques and repetitive DNA mapping are useful tools in comparative analysis and in the elucidation of genome organization of several groups of eukaryotes. In this study, we contributed to the knowledge of Coleoptera genomes by reporting the chromosomal organization of repetitive DNA sequences, as well as the presence and characteristics of a B chromosome in two natural populations of *Dichotomius geminatus* (Coleoptera; Scarabaeidae) using classical, chromosomal banding and molecular cytogenetic techniques. As in other coleopteran species, the heterochromatin was mainly concentrated in pericentromeric regions and the B chromosome was composed almost entirely of heterochromatin. Physical mapping using double fluorescent *in situ* hybridization was performed for the first time in Coleoptera; using DNA probes for 5S and 18S ribosomal RNA (rRNA) and histone H3

genes, we showed that ribosomal 18S rDNAs are located in chromosomes 3 and 4, whereas 5S rRNA and histone H3 genes are colocalized in chromosomal pair 2 and show an apparently interspersed organization. Moreover, these genes are not present in the B chromosome, suggesting that the B chromosome did not originate from chromosomal pairs 2, 3 or 4. On the other hand, mapping of the C₀t-1 DNA fraction showed that the B chromosome is enriched in repetitive DNA elements, also present in the standard complement, indicating an intraspecific origin of this element in *D. geminatus*. These results will contribute to our understanding of genome organization and evolution of repetitive elements in Coleoptera and other insects regarding both A and B chromosomes.

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Introduction

Repetitive DNA elements make up a large portion of eukaryotic genomes and include tandem arrays and dispersed repeats. Tandem repeats comprise microsatellite, minisatellite and satellite DNAs (satDNA) (Charlesworth *et al.*, 1994) and multigenic families, such as histones and ribosomal RNAs (rRNAs) (Maxon *et al.*, 1983; Hadjiolov, 1985). Dispersed repeats are represented by transposons and retrotransposons (Charlesworth *et al.*, 1994). satDNA has been characterized as highly abundant and ubiquitous in eukaryotic genomes and is located in heterochromatic chromosomal compartments. These sequences are more variable than the sequences of multigenic families and, together with transposons and

retrotransposons, are responsible for the variations in the sizes of eukaryotic genomes (Cavalier-Smith, 1985; Elder and Turner, 1995). In particular, repetitive DNAs are of great importance to molecular cytogenetics and represent excellent chromosomal markers that are very useful in studies of species evolution, supernumerary chromosomes, sex chromosomes and for the identification of chromosomal rearrangements; these repetitive sequences are even used in applied genetics. Probes of repeated DNA elements, such as satDNA, rDNA, and to lesser extent, histones, have been used extensively for tracking historical and ongoing karyotype repatterning in fishes, mammals, mollusks, insects, plants and other groups.

Repeated DNA elements have found an application in studies involving supernumerary B chromosomes, which occur in addition to standard karyotypes and are found in ~15% of eukaryotic species. These elements are generally heterochromatic and are composed of repetitive DNA, mainly satDNA. However, B chromosomes can also harbor functional genes, such as rRNA genes (Camacho, 2005; Jones *et al.*, 2008). Among coleopterans, the presence of B chromosomes has been observed in

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several families, such as Buprestidae (Moura *et al.*, 2008), Cantharidae (James and Angus, 2007), Cicindelidae (Proença *et al.*, 2002) and Scarabaeidae (Angus *et al.*, 2007). In Scarabaeidae, analysis of B chromosomes has been restricted to polymorphism characterization using conventional staining, and there is little information about the genomic content of these elements.

Cytogenetic studies in Coleoptera that focus on repetitive sequences are scarce and are frequently restricted to chromosomal banding (C-banding), base-specific fluorochromes and, to a lesser extent, fluorescent *in situ* hybridization (FISH) using ribosomal DNA (rDNA) or satDNA as probes (Rožek *et al.*, 2004; Bione *et al.*, 2005a; Palomeque *et al.*, 2005). With the aim of contributing to the knowledge of coleopteran genomes, we investigated the organization of repeated DNA elements in the karyotype of *Dichotomius geminatus* and described the association of 5S rRNA and histone H3 genes, as well as the characteristics of a newly detected B chromosome.

Materials and methods

Animals, DNA samples and chromosome preparation

Samples of *D. geminatus* (Arrow 1913) individuals were collected in Igarassu (7°50'03"S:34°54'23"W) (43 males) and in Maracaípe (8°31'47"S:35° 01'71"W) (23 males), Pernambuco State, Brazil, using pitfall traps. The genomic DNA of individuals with 0B chromosomes was extracted using the phenol–chloroform procedure described by Sambrook and Russel (2001).

Meiotic chromosomes were obtained from testicular cells. All individuals from Igarassu provided usable preparations, although only eight animals from Maracaípe were useful to this study. The rest of the Maracaípe sample was not used because the individuals were not at the appropriate stage of sexual maturity. Slides for conventional chromosome analysis were stained with 2% Lacto-acetic orcein. Slides used for C-banding, silver nitrate staining and FISH analysis were prepared in 45% acetic acid and coverslips were removed after freezing the preparations by immersing in liquid nitrogen for a few seconds. C-banding was performed according to the method described by Sumner (1972) and silver nitrate staining was conducted according to Rufas *et al.* (1987).

Isolation of repetitive DNA

Partial sequences of 18S rRNA, 5S rRNA and histone H3 genes were obtained by PCR of genomic DNA from *Dichotomius semisquamosus*. Primer sets were designed on the basis of nucleotide sequences available for coleopterans and other insect species in the nucleotide database of the National Center for Biotechnology Information (NCBI), as follows: Sca18SF (5'CCC CGT AAT CGG AAT GAG TA), Sca18SR (5'GAG GTT TCC CGT GTT GAG TC), Sca5SF (5'AAC GAC CAT ACC ACG CTG AA), Sca5SR (5'AAG CGG TCC CCC ATC TAA GT), ScaH3F (5'GGC NMG NAC NAA RCA RAC) and ScaH3R (5'TGD ATR TCY TTN GGC ATD AT). PCR products were ligated into the plasmid pGEM-T (Promega, Madison, WI, USA), and the recombinant constructs were used to transform DH5 α *Escherichia coli*-competent cells. Positive clones were sequenced using an ABI Prism 3100 automatic DNA sequencer (Applied

Biosystems, Foster City, CA, USA) with a Dynamic Terminator Cycle Sequencing Kit (Applied Biosystems), as per the manufacturers' instructions. Nucleic acid sequences were subjected to BLAST (Altschul *et al.*, 1990) searches at the NCBI website (<http://www.ncbi.nlm.nih.gov/blast>) to check for similarities to other previously deposited sequences. The sequences were deposited in the NCBI database under the following accession numbers: GQ443313 (18S rRNA gene), GQ443312 (5S rRNA gene) and GQ443311 (histone H3 gene).

An enriched library with repetitive sequences of a 0B individual of *D. geminatus* was constructed on the basis of renaturation kinetics of C₀t-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences) (Zwick *et al.*, 1997, Ferreira and Martins, 2008). DNA samples (200 μ l of 100–500 ng μ l⁻¹ genomic DNA in 0.3 M NaCl) were autoclaved for 30 min at 1.4 atmospheres of pressure and 120 °C and the fragmented DNA was separated by electrophoresis with a 1% agarose gel. Expected DNA fragments should have ranged in size from 100 to 1000 bp. Samples of 50 μ l DNA fragments were denatured at 95 °C for 10 min, placed on ice for 10 s and transferred into a 65 °C water bath for reannealing. After 1 min of reannealing, the samples were incubated at 37 °C for 8 min with 1 U S1 nuclease to permit digestion of single-stranded DNA. The samples were immediately frozen in liquid nitrogen and DNA was extracted with phenol–chloroform. The DNA fragments obtained were used as probes for FISH in 0B and 1B individuals.

Fluorescence *in situ* hybridization

The plasmids containing the 18S rRNA and histone H3 genes, and the C₀t-1 DNA fraction were labeled by nick translation, using biotin-14-dATP (Invitrogen, San Diego, CA, USA). For simultaneous hybridization (double FISH), the 5S rRNA gene clone was labeled using digoxigenin-11-dUTP (Roche, Mannheim, Germany). Meiotic chromosome slides were incubated with RNase (100 μ g ml⁻¹) for 1.0 h and with pepsin (10 μ g ml⁻¹) for 20 min in a moist chamber at 37 °C. The slides were fixed at room temperature using 37% formaldehyde in phosphate buffer detergent solution and dehydrated in 70 and 100% ethanol for 5 min. The hybridization mixture (100 ng denatured probe, 50% formamide, 10 mg ml⁻¹ dextran sulfate, 2 \times SSC), in a final volume of 15 μ l, was dropped onto slides that were previously denatured using 70% formamide, 2 \times SSC for 40–60 s at 70 °C. In double FISH experiments, 15 μ l hybridization mixture of each probe was dropped onto the slides. The slides were covered with coverslips and incubated at 75 °C for 5 min. Hybridization was performed overnight at 37 °C in a moist chamber. The probes labeled with biotin were detected by avidin–FITC (fluorescein isothiocyanate) conjugate (Sigma, St Louis, MO, USA), and the digoxigenin-labeled probes were detected using anti-digoxigenin-Rhodamine (Roche). All preparations were counterstained with DAPI and mounted with Vectashield (Vector, Burlingame, CA, USA).

Results

Karyotype and chromosomal banding

The standard karyotype observed in *D. geminatus* was 2n = 18,Xy_p, with metacentric (1, 2, 5, 6 and 8),

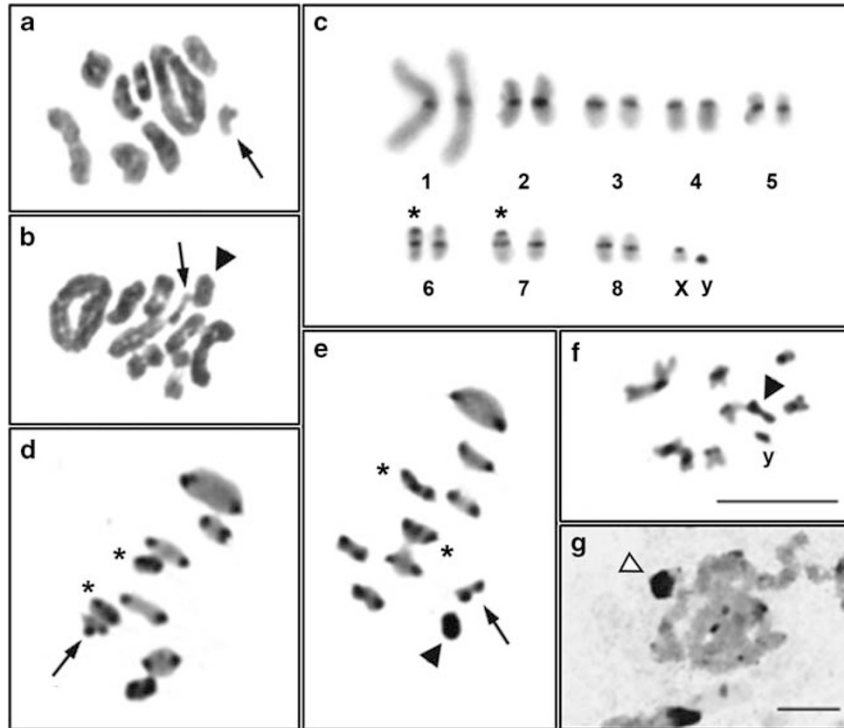


Figure 1 Male meiotic cells and karyotype of *Dichotomius geminatus*. Conventional staining of metaphase I chromosomes of 0B individuals (a) and 1B individuals (b); C-banded karyotype (c), metaphases I of 0B (d) and 1B (e) individuals and metaphases II of B-carrying individual (f). Silver nitrate staining in initial prophase (g). The arrows indicate the sex bivalents (Xy_p), full and empty arrowheads indicate the B chromosomes and the nucleolar organizer region (NOR), respectively, and the * (asterisk) indicates the chromosome pairs with additional heterochromatic blocks. Bar = 5 μ m.

submetacentric (3 and 7) and subacrocentric (4) autosomes, a subacrocentric X and a punctual y chromosome (Figure 1a). In addition, 9 individuals among the 43 analyzed from Igarassu and 2 of 8 from Maracáipe carried the 1B chromosome, corresponding to an average prevalence rate of 20.93 and 25.00%, respectively. For each individual bearing the B chromosome, at least 30 metaphases I were analyzed, and all of them presented 1 extra chromosome, indicating mitotic stability. The B chromosome has a condensation pattern similar to that of autosomes, but was easily recognized as a univalent element in metaphase I and was frequently observed outside the block formed by autosomes (Figure 1b).

Heterochromatic blocks were detected in the pericentromeric regions of all autosomes, in the small arm of the X chromosome and in almost the entire length of the y chromosome. In addition, 2 small chromosomal pairs (6 and 7) contained terminal blocks of heterochromatin in 1 homolog, forming heteromorphic pairs (Figures 1c and d). The B chromosome was completely heterochromatic (Figures 1e and f), and silver nitrate staining showed only one active nucleolar organizer region (Figure 1g).

Multigene family sequences and repetitive DNA mapping
 The nucleotide sequences obtained for 18S rRNA (822 bp), 5S rRNA (94 bp) and histone H3 (376 bp) genes were highly similar to sequences obtained from other organisms, found in NCBI databases. The 18S and 5S rDNAs had more than 90 and 85% identity, respectively,

with sequences from species belonging to distantly related taxa, including vertebrates. The histone H3 gene sequence had less similarity than the other sequences, with an average of 80–84% similarity with sequences from species belonging to the Porifera, Cnidaria, Echinodermata and Mollusca groups, as well as sequences obtained from some insects and vertebrates.

Fluorescent *in situ* hybridization with rDNA probes (18S and 5S) showed the presence of rDNA sites in distinct chromosomes. The 18S rDNA sites were located in the short arm of autosomal pairs 3 and 4, whereas hybridization of 5S showed 1 proximal site in pair 2 in most of the individuals analyzed (Figures 2a–c). In 3 specimens, pair 2 was heteromorphic for the presence of 5S rDNA sites, possessing only 1 site in 1 of the homologs (Figure 2c). The histone H3 cluster colocalized to the same region as the 5S rDNA site (Figures 2d and e). The C_0t-1 DNA fraction hybridization pattern was coincident with areas of heterochromatin, including the terminal blocks of two small pairs (Figure 3a).

In individuals with 1B chromosome, the hybridized probes of multigenic families (18S and 5S rDNAs and histone H3) showed the same pattern observed in 0B individuals, and no hybridization was seen in the B chromosome (Figures 2f–h). With regard to the C_0t-1 DNA fraction, the hybridization patterns of 1B individuals were similar to those of the A chromosomal complement observed in 0B individuals. Moreover, the B chromosome was entirely stained by C_0t-1 DNA hybridization (Figures 3b and c). All results of hybridized probes are schematized in Figure 3d.

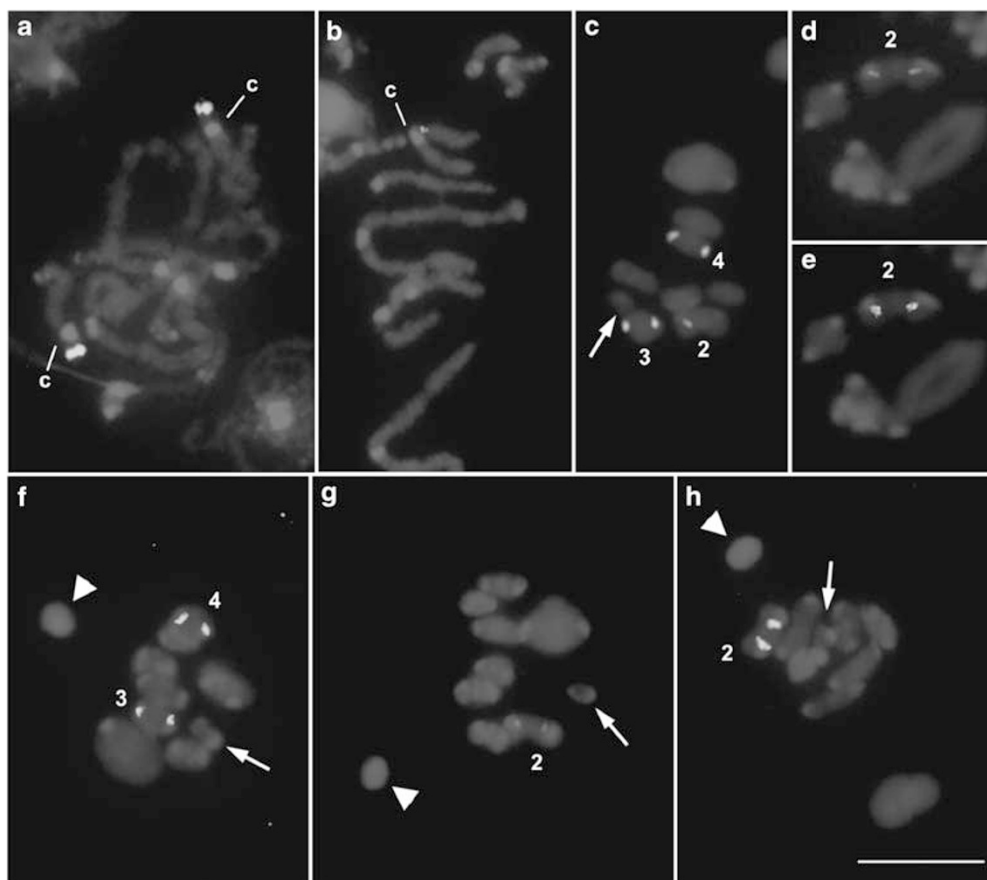


Figure 2 Fluorescent *in situ* hybridization (FISH) with 18S ribosomal RNA (rRNA), 5S rRNA and histone H3 gene probes in 0B and 1B individuals of *Dichotomius geminatus*. Pachytene chromosomes from 0B individuals hybridized using 18S (a) and 5S rDNAs (b); double FISH with 18S (green) and 5S (red) rDNAs in metaphase I chromosomes of 0B individuals (c); partial metaphase I chromosomes hybridized with 5S (d) and H3 (e) probes; metaphase I chromosomes showing the distribution pattern of 18S (f), 5S (g) and H3 (h) in 1B individuals. The absence of hybridization signals on the B chromosome (f–h) and the heterochromatin highlighted after DAPI staining (a–h) must be noted. The arrows indicate the sex bivalents (Xy_p), and arrowheads indicate the B chromosome. C = centromere. Bar = 5 μ m. A full color version of this figure is available at the *Heredity* journal online.

Discussion

Standard karyotype

The diploid number observed in *D. geminatus* differs from the most frequent and considered primitive to Scarabaeidae and Polyphaga suborder $2n=20$ (Smith and Virkki, 1978; Yadav and Pillai, 1979; Cabral-de-Mello *et al.*, 2008). The karyotype is $2n=18, Xy_p$ and the metacentric, submetacentric and subacrocentric chromosomes in this species are in concordance with the previous description by Cabral-de-Mello *et al.* (2008), and this karyotypic formula has been described for some other species of *Dichotomius* (Silva *et al.*, 2009).

The occurrence of heterochromatin mainly in the pericentromeric region of the autosomes in *D. geminatus* is a common feature among eukaryotes and suggests that repetitive DNA may be involved in centromeric function (Dawe, 2003). The hybridization of the C_0t-1 DNA fraction confirmed the heterochromatin distribution pattern, indicating the presence of highly and moderately repetitive sequences in these areas. In the Scarabaeidae family and in Coleoptera as a whole, the small blocks of heterochromatin in the pericentromeres represent a conspicuous pattern that has been described

in representative organisms from distinct and unrelated families (Moura *et al.*, 2003; Bione *et al.*, 2005a). On the other hand, some species had small additional blocks, as observed in *D. geminatus*, as was reported in Scarabaeidae (that is, *Bubas bison*) (Colomba *et al.*, 2006) and *Aphodius* representative species (Wilson and Angus, 2004). Moreover, in species belonging to the subfamily Scarabaeinae, large heterochromatic blocks were observed, as described for *Diabroctis mimas* and *Isocopriss inhiata* (Bione *et al.*, 2005b). These distinct patterns of heterochromatin distribution indicate that repetitive DNA sequences in Scarabaeidae are likely to show different dynamic processes of spreading governed by amplification and dispersion, through translocation of these elements, which is favored by chromocenter formation and ectopic heterochromatic associations.

Multigene family sequences and mapping

The different similarity indices seen in the comparative analysis of 5S rRNA, 18S rRNA and histone H3 genes against the NCBI database reflect differences in the evolutionary rates of these sequences in the distinct taxa.

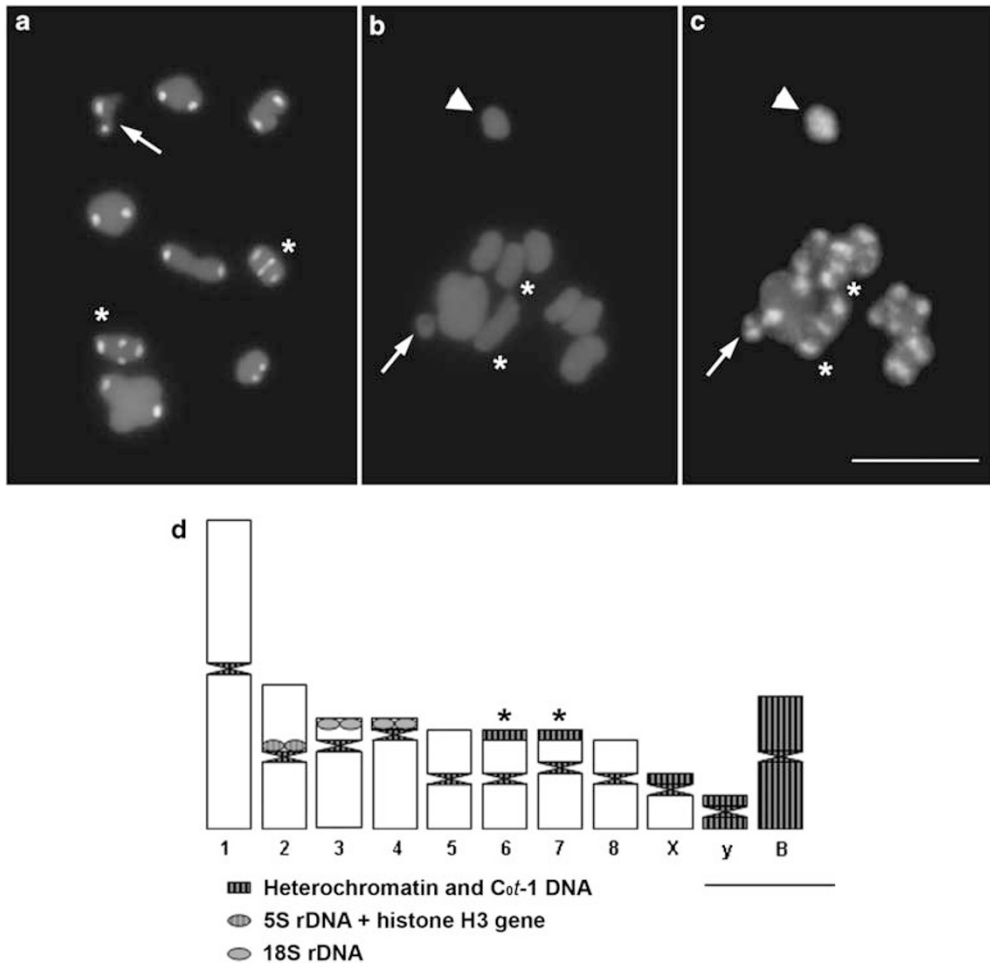


Figure 3 C₀t-1 DNA fraction hybridization in metaphase I chromosomes of 0B individuals (a) and 1B individuals (b,c). Ideogram (d) showing the hybridization patterns described in this work. The arrows indicate the sex bivalents (Xy_p), arrowheads indicate the B chromosome, and the * (asterisk) indicates the chromosome pairs with additional heterochromatic blocks. Bar = 5 μm. A full color version of this figure is available at the *Heredity* journal online.

However, despite these differences, an overall similarity index >80% from the three sequences was observed when compared with the NCBI database.

Chromosomal mapping of multigenic families is scarce in Coleoptera and is restricted to the description of 45S rDNA locations. The most common distribution pattern described for this insect order is the presence of one autosomal pair involved in nucleolar organization (reviewed in the study by Schneider *et al.*, 2007). These results were largely observed using silver nitrate staining, which detects only active nucleolar organizer regions. The presence of only one nucleolar organizer region site detected by silver nitrate staining in *D. geminatus* does not correspond to the real genome organization of 45S rDNA clusters (2 clusters of 18S rDNA detected by FISH). The presence of more than one 45S rDNA site was observed in other Scarabaeinae species, such as *Bubas bison* (Colomba *et al.*, 2006) and *D. mimas* (Bione *et al.*, 2005b), and in unrelated groups of Coleoptera, such as Cicindelidae and Scarabaeidae as a whole, indicating more than one dispersion event of rDNA sequences.

In this paper, 5S rDNA was mapped for the first time in Coleoptera using single and double FISH with 5S and

18S rDNAs as probes. Our results showed a single 5S rDNA site located in a different chromosome than the 18S rDNA sites. The presence of only one 5S rDNA site is common among eukaryotes, and the distinct chromosomal locations of 5S and 18S rDNA sites has been frequently reported for vertebrates (Mandrioli *et al.*, 2000; Sola *et al.*, 2000; Martins and Galetti, 2001). In protostome invertebrates, this kind of arrangement was described in some mollusks (López-Piñón *et al.*, 2005; Insua *et al.*, 2006; Huang *et al.*, 2007). On the other hand, some studies have shown a different scenario for rDNA location, with colocalization of these sequences in protostomes, as was reported for the Annelida, *Octodrilus complanatus* (Vitturi *et al.*, 2002), three mollusk species (Colomba *et al.*, 2002; Vitturi *et al.*, 2004; Wang and Guo, 2004) and in seven calanoid copepods-Crustacea (Drouin and Moniz de Sá, 1995), which presented an association of repeated 5S and 18S DNA sequences, as shown by Southern blotting.

With regard to mapping of the histone H3 sequence, there is no information related to coleopteran species and the limited available data in protostomes have frequently shown the presence of only one locus in the genome, similar to what has been described in *D. geminatus*.

Moreover, in this species, the histone cluster overlapped with 5S rDNA, showing an apparently interspersed organization of these sequences in the *D. geminatus* genome. The organization of histone and 5S rRNA genes has not been investigated in Coleoptera until now, and in protostomes, this kind of association was observed in two species of crustaceans (Drouin and Moniz de Sá, 1995; Barzotti *et al.*, 2000) and in one mussel species (Eirín-López *et al.*, 2004). Studies using Southern blot hybridization and/or fiber-FISH experiments will be necessary to clarify the precise organization of these multigene families, regarding the interspersed or syntenic organization in *D. geminatus* and other invertebrate genomes.

Although the association and/or interspersed of multigene families has been reported in protostome invertebrates (Drouin and Moniz de Sá, 1995; Barzotti *et al.*, 2000; Vitturi *et al.*, 2002; Eirín-López *et al.*, 2004; Vitturi *et al.*, 2004; Cabrero *et al.*, 2009), the significance of such association is still unclear. According to Kaplan *et al.* (1993), the association of these sequences might have a functional role in nuclear organization, whereas other researchers (Dover, 1986; Liu and Fredga, 1999) agree that this association is important for the maintenance of conserved and multiple arrays. On the other hand, specific association of 5S rRNA and histone H3 genes cannot be explained by an advantage in the co-transcription process, as these sequences are transcribed by different RNA polymerases, RNA polymerases III and II, respectively. Considering that the association of 5S rRNA and histone H3 genes was reported in crustaceans (Drouin and Moniz de Sá, 1995; Barzotti *et al.*, 2000) and in this study was detected for coleopterans, we could speculate that such association pattern could represent an ancient characteristic that has been maintained conserved in different arthropod groups.

The B chromosome

The presence of B chromosomes in Coleoptera has been reported in ~80 species, but these studies were focused on the presence or absence of this element, with no description of frequency in populations or in relation to its molecular content (Camacho, 2005; Angus *et al.*, 2007; Moura *et al.*, 2008). In the family Scarabaeidae, the presence of B chromosomes was described in representatives of the Cetoniinae and Scarabaeinae subfamilies (Angus *et al.*, 2007). This polymorphism in Scarabaeidae was found in more species belonging to Scarabaeinae than to the other subfamilies. According to Cabral-de-Mello *et al.* (2008), Scarabaeinae shows wide karyotypic variation, due to many different chromosomal rearrangements. In this group, the origin of the B chromosome can probably be related to the chromosomal rearrangements that occurred along with the chromosomal differentiation of the group. On the other hand, our results show that, at least in *D. geminatus*, the origin of the B chromosome is not related to the autosomal fusion process that occurred in this species. This information is corroborated by the absence of supernumerary elements in some other species from the genus that has the same macro chromosome pair, resulting from fusion, that was observed in *D. geminatus* ($2n = 18, Xy_p$) (Silva *et al.*, 2009).

There is a lack of information about the genomic characteristics of B chromosomes in Coleoptera and most

information is focused on the description of heterochromatin presence, with no data about the origin and DNA composition of this particular chromosomal element. In this paper, chromosomal banding and mapping of repetitive DNA sequences in *D. geminatus* allowed the most precise characterization of this polymorphism in a beetle species. The C_0t-1 DNA hybridized fraction, obtained from individuals with 0B chromosome, showed that this element is totally enriched in highly repetitive DNA and probably has an intraspecific origin, because of the presence of similar sequences in both the standard complement and the B chromosome. Moreover, the genome content similarity between the B chromosome and the A complement indicates that homogenization mechanisms can be occurring in the heterochromatin of *D. geminatus* or that this element is relatively new in this species and still share high sequence similarity with the A complement.

Although our results indicate an intraspecific origin of the B chromosome, it is difficult to propose the precise chromosomal A element involved in this process. The absence of ribosomal and histone H3 clusters in the B chromosome led us to suggest that the origin of this element is not related to chromosomal pairs 2, 3 or 4 (bivalents that harbor ribosomal and histone H3 clusters). This chromosome might be originated from one of the small chromosomal pairs, because of the heterochromatin amplification observed in these chromosomes, but other specific markers that are shared between these chromosomes need to be analyzed to confirm this hypothesis. The B chromosome could have originated through the amplification and accumulation of repeated DNAs from primordial extra chromosome fragments that were generated from the A complement. This extra element apparently exchanges genetic material with the A complement and could represent a repository of genetic information that could be integrated into A chromosomes, leading to the diversification of genomes.

The variability of organization of multigene families in *D. geminatus* suggests the same mechanisms of evolution of repetitive DNA proposed for other eukaryotes, DNA duplication, non-homologous recombination, translocation and unequal crossover. The process of unequal crossover is likely to have occurred in individuals who do not possess a 5S mark in chromosomal pair 2. In the same manner, the observed heterochromatin variations can be related to the presence of highly repeated DNAs. The repetitive DNAs were long considered to be junk DNA because they had no clearly identified function (Doolittle and Sapienza 1980; Orgel and Crick, 1980). On the other hand, their accumulation in specific genomic areas can cause chromosomal rearrangements through chromosome breakage, deletion, inversion and amplification (Lim and Simmons, 1994; Dimitri *et al.*, 1997) that is possibly involved with the B chromosome origin and can generate genome diversification. In this manner, investigation of the repetitive DNA families that are present in Coleoptera genomes will greatly contribute to our understanding of the basal evolutionary mechanisms involved in the chromosomal diversification of coleopterans.

The results presented in this study will contribute to the elucidation of the genome organization of repetitive elements in Coleoptera and Arthropoda as a whole. Chromosomal mapping of repetitive sequences is a

promising tool in studies of karyotypic repatterning in insects and the origins of supernumerary elements. Moreover, the use of the C_0t -1 DNA fraction in chromosomal hybridization proved to be a valuable approach in the analysis of genome organization and characterization of B chromosomes.

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