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Evolutionary dynamics of heterochromatin in the genome of *Dichotomius* beetles based on chromosomal analysis

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Abstract We comparatively analyzed six *Dichotomius* species (Coleoptera: Scarabainae) through cytogenetic methods and mitochondrial genes sequencing in the aim to identify patterns of chromosomal evolution and heterochromatin differentiation in the group. The chromosomal data were accessed through the classical analysis of heterochromatin and mapping of high and moderately repeated DNAs (C_0t-1 DNA fraction). Mitochondrial data were obtained from nucleotide sequences of the cytochrome oxidase I (COI) and 16S rRNA genes. The heterochromatin distribution was conserved but revealed variability in the base pair richness and repetitive DNA content, and an intense turnover of heterochromatic associated sequences seems to have occurred during *Dichotomius* speciation. Specifically for *D. bos*, an interesting pattern was observed, indicating apparently the presence of heterochromatic sequences composed of low copy-number sequences. Moreover, highly conserved terminal/sub-terminal sequences that could act as a telomeric or telomere-associated DNA were observed. The heterochromatin diversification patterns observed in *Dichotomius* were not accomplished by the diversification of the species studied, which may be a consequence of the intense dynamics that drive the evolution of repeated DNA clusters in the genome. Finally our findings also suggest that the use of C_0t-1 DNA fraction represents a powerful, inexpensive and not time consuming tool to be applied in understanding heterochromatin and repetitive DNA organization.

Keywords C_0t-1 DNA · Evolution · Genome · Heterochromatin · Repetitive DNAs

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Introduction

Repetitive DNAs are ubiquitous components of eukaryotic genomes and are primarily represented by tandem repeats, such as satellite DNAs (satDNA), minisatellite and microsatellite, and some multigenic families as well as by dispersed repeats, such as transposons and retrotransposons (Charlesworth et al. 1994). SatDNAs are normally found in centromeric/telomeric heterochromatic regions, and often show high variability with regard to nucleotide sequence, reiteration frequency and distribution in the genome. Transposable elements (TEs), including DNA transposons, the elements that transpose directly through DNA copies, and retrotransposons, which transpose through an intermediate RNA molecule that is reverse transcribed may be arranged in clusters, thus being easily visualized in the chromosomes by cytogenetic methods. The high dynamic molecular behavior of repeated DNAs is promoted by concerted evolution, which causes a rapid change in repeat sequences between species (Charlesworth et al. 1994; Ugarković and Plohl 2002).

Repetitive sequences are important cytogenetic markers that are used to study species evolution, genome organization, sexual and supernumerary chromosomes, and the

identification of chromosomal rearrangements in diverse organism groups. For the Coleoptera order, chromosomal analyses of repetitive DNAs are scarce, and frequently restricted to descriptions of heterochromatin distribution and AT/GC base pair content (Moura et al. 2003; Schneider et al. 2007; Cabral-de-Mello et al. 2010a). The organization of repeated DNAs based on molecular cytogenetics was mostly conducted on the description of the 45S rDNA location in several taxa (Sánchez-Gea et al. 2000; Moura et al. 2003; Bione et al. 2005a, b; Martínez-Navarro et al. 2004), whereas studies on satDNA organization and distribution have only been conducted primarily in species belonging to Tenebrionidae family (reviewed by Palomeque and Lorite 2008). In Scarabaeidae, the repeated DNAs have also been primarily analyzed only by classical methods and the chromosomal mapping of these elements is concentrated in the description of 45S rRNA gene (Moura et al. 2003; Bione et al. 2005a, b; Colomba et al. 2000, 2006; Cabral-de-Mello et al. 2010a, b). Few data are available for other multigene families, such as the 5S rRNA and histone genes (Cabral-de-Mello et al. 2010b) being other specific repeated sequences, like satDNAs and transposable elements, not cytogenetically mapped until now.

Because Scarabaeinae coleopterans display extensive karyotype variability (Cabral-de-Mello et al. 2008), molecular cytogenetics represents an interesting tool to advance our knowledge regarding their genomes and chromosomal evolution. In addition, molecular cytogenetics will also contribute to the characterization of the repetitive fraction of eukaryotic genomes, which are still poorly understood, even in "completely sequenced genomes". In this way, we analyzed the chromosomal organization of high and moderately repetitive C_0t-1 DNA fraction in six species that belong to *Dichotomius* (Scarabaeinae; Coleoptera), a group of beetles with highly conserved karyotypes, and correlated the chromosomal data to a phylogeny obtained through mtDNA analysis. The location of heterochromatic blocks was highly conserved in the genus, although a differentiation of the heterochromatin associated sequences was observed between *D. geminatus* and the other five studied species. Some heterochromatic regions composed by high and moderately repeated sequences are present in the six investigated species, but in *D. bos* heterochromatin apparently composed of low copy-number of sequences restricted to few chromosomes was also present. Moreover, it was revealed a high conservation of terminal/sub-terminal sequences that could act as a telomeric or telomere-associated DNA. Additionally, our data reinforce the use of the C_0t-1 DNA fraction as a useful tool for studies of repeated DNAs in insects, contributing for understanding heterochromatin differentiation and genome evolution.

Materials and methods

Animals, DNA samples, chromosome preparation and banding

Male samples from six *Dichotomius* species, including *D. bos*, *D. geminatus*, *D. laevicollis*, *D. nisus*, *D. semisquamosus* and *D. sericeus*, were collected from distinct areas in Pernambuco, São Paulo and Paraná States, Brazil, by using pitfall traps. The testes were dissected, fixed in Carnoy (3:1 ethanol:acetic acid) and stored at −20°C. Entire animals of each species were frozen and stored in freezer (−20°C) for DNA extractions. The genomic DNA of individuals from each species was extracted from muscle tissue using the phenol–chloroform procedure (Sambrook and Russel 2001).

Mitotic and meiotic chromosomes were obtained from fixed testicular cells, and the slides were prepared in 45% acetic acid. Coverslips were removed after the preparations were frozen by immersion in liquid nitrogen for a few seconds. C-banding was performed according to the method described by Sumner (1972), and fluorochrome staining with chromomycin A_3/distamycin A/4'-6-diamindino-2-phenylindole (CMA_3/DA/DAPI) was conducted following the method of Schweizer et al. (1983).

Isolation of repetitive DNAs

Enriched samples containing repetitive DNA sequences from the six *Dichotomius* species were constructed based on the renaturation kinetics of C_0t-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences), according the protocol that was described by Zwick et al. (1997) with modifications later published (Ferreira and Martins 2008; Cabral-de-Mello et al. 2010b). DNA samples (200 µl of 100–500 ng/µl of genomic DNA in 0.3 M NaCl) were autoclaved for 30 min at 1.4 atmospheres of pressure at 120°C, and the fragmented DNA was separated by 1% agarose gel electrophoresis. The expected DNA fragments ranged in size from 100 to 1,000 base pairs (bp). The samples of 50 µl of 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. The distinct times for DNA reannealing were tested from 30 s to 5 min, and the samples were subsequently incubated at 37°C for 8 min with 1 U of S1 nuclease to permit the digestion of single-stranded DNA. The samples were immediately frozen in liquid nitrogen, and the DNA was extracted using a traditional phenol–chloroform procedure. The C_0t-1 DNA fractions from each species were used as probes in Fluorescence in situ hybridization (FISH) experiments against their own chromosomes. Moreover, the C_0t-1 DNA fraction obtained from *Dichotomius geminatus* was used as probe

for comparative analyses against the other five *Dichotomius* species.

Fluorescence in situ hybridization

The FISH procedures were performed according to Cabral-de-Mello et al. (2010b). The C_0t-1 DNA fraction probes were labeled by nick translation using biotin-14-dATP (Invitrogen, San Diego, CA, USA) and detected by avidin-FITC (fluorescein isothiocyanate) conjugate (Sigma, St Louis, MO, USA). All of the preparations were counterstained with DAPI and the coverslips were added after the application of Vectashield (Vector, Burlingame, CA, USA) mounting medium. The images were captured using an Olympus BX61 microscope linked to an Olympus DP71 digital camera. The brightness and contrast of the images were optimized using Adobe Photoshop CS2.

Phylogenetic analysis

The sequences for the cytochrome oxidase I (COI) and 16S rRNA genes were amplified by the polymerase chain reaction (PCR). For COI, FishF2 and FishR2 primers designed by Ward et al. (2005) were used, and for the 16S rRNA gene sequence, the primers used (16SscaF- 5′CGC CTG TTT AAC AAA AAC AT and 16SscaR- 5′CTC CGG TTT GAA CTC AGA TCA) were designed based on the 16S rRNA gene sequences of *Dichotomius* species deposited in the NCBI (AY131513-AY131516). The PCR products were purified and sequenced using an ABI Prism 3100 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA) with a Dynamic Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions.

Individual sequences from each species were initially analyzed using the BioEdit 5.0.9 (Hall 1999) software, and a consensus sequence was determined for each DNA segment from each species. The nucleic acid sequences were subjected to BLAST (Altschul et al. 1990) searches at the NCBI website (http://www.ncbi.nlm.nih.gov/) to check for similarities to other previously deposited sequences. The sequences were deposited in the NCBI database under the following accession numbers: HQ824533-HQ824544. All of the sequences were aligned using Muscle software (http://www.ebi.ac.uk/Tools/muscle/index.html) (Edgar 2004). Nucleotide variation and genetic distances were examined using MEGA 4.0 (Tamura et al. 2007). Nucleotide saturation was analyzed by plotting the numbers of observed transitions (Ti) and transversions (Tv) against the genetic distance values that were estimated by the Kimura-2-parameters model (Kimura 1980) using the DAMBE program (Xia and Xie 2001). The genetic distance analyses were based on a hierarchical hypothesis test of alternative models that was implemented using Modeltest 3.06 (Posada and Crandall 1998).

The Bayesian-likelihood method of phylogenetic analysis (Huelsenbeck et al. 2001) was used to evaluate alternative tree topologies through the estimation of posterior probabilities using MrBayes v.3.0 (Ronquist and Huelsenbeck 2003). Four chains were run simultaneously for 3,000,000 generations using the MrBayes analysis. Every 100th generation was sampled, and the asymptote of likelihood score was detected using the SUMP command. All sampled topologies before 1,000,000 generations were discarded from the population of trees that was considered in the subsequent majority-rule consensus tree. The frequency with which a particular clade appeared in the population of retained topologies was interpreted as the posterior probability. The posterior probabilities were interpreted as measures of the likelihood that the clade appeared in the optimal topology, rather than the accuracy of the node with respect to species relationships or clade stability. Consensus trees were produced using the TreeExplorer software that was implemented in MEGA 4 (Tamura et al. 2007). The Scarabaeinae species *Canthidium rufinum*, *Dendropaemon bahianum*, *Phanaeus cambeforti* and *Glyphoderus sterquilinus* were included as outgroups based on the mtDNA sequences of COI (AY131869, AY131947, AY131949, AY131891) and 16S rRNA (AY131507, AY131606, AY131609, AY131534) genes available at NCBI.

Results

Karyotyping and chromosome banding

The six species that were analyzed in this study had a diploid number of 2n = 18 and primarily biarmed chromosomes, with the presence of a remarkably large autosomal bivalent (pair one) (Figs. 1, 4a). All of the species showed similar patterns of heterochromatin distribution with conspicuous heterochromatic blocks in the pericentromeric regions of all of the autosomes (Figs. 1a, d, f, h, j, l, 4a). In *D. geminatus*, additional blocks in the terminal region of the sixth and seventh pairs were observed (Figs. 1f, 4a). The X sex chromosome showed heterochromatic blocks that were exclusively located in the pericentromeric regions of *D. laevicollis* (Fig. 1d), *D. nisus* (Fig. 1h), *D. sericeus* (Fig. 1j) and *D. semisquamosus* (Fig. 1l). However the heterochromatin was distributed in the pericentromeric area and along the short arm of the X chromosomes in *D. bos* (Fig. 1a) and *D. geminatus* (Fig. 1f). With regard to the y chromosome, the precise establishment of the heterochromatic distribution was difficult due to its punctiform size. Nevertheless, small centromeric blocks were observed in the y chromosome from

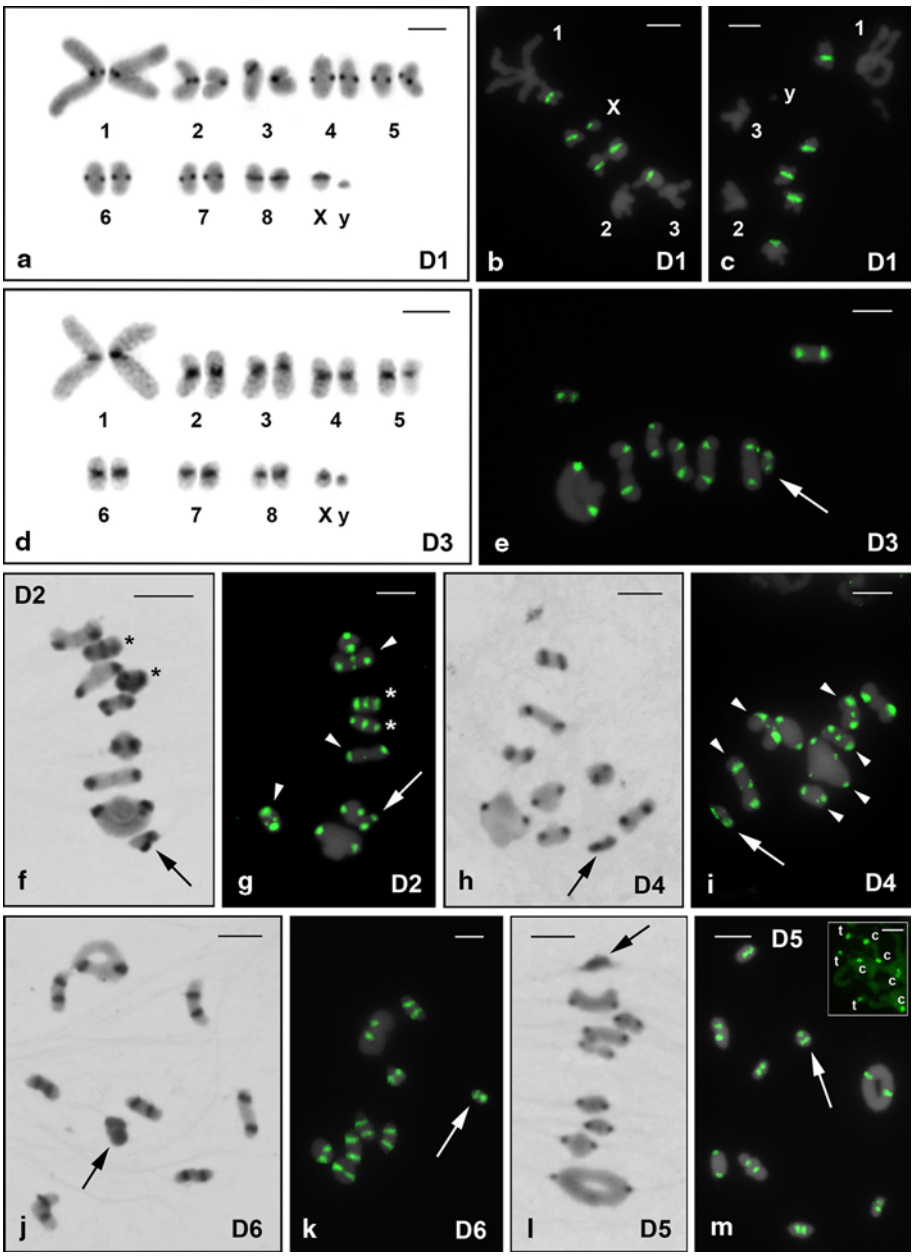

Fig. 1 C-banding and C_0t-1 DNA fraction hybridization in six species of *Dichotomius*. The C_0t-1 DNA fractions were isolated from each species and hybridized against their own chromosomes. C-banded karyotypes from *D. bos* (**a**) and *D. laevicollis* (**d**), and C-banded metaphase I chromosomes from *D. geminatus* (**f**), *D. nisus* (**h**), *D. sericeus* (**j**) and *D. semisquamosus* (**l**) are shown. C_0t-1 DNA fraction hybridization are showed in metaphase II chromosomes from *D. bos* (**b**, **c**), and metaphase I chromosomes from *D. laevicollis* (**e**), *D. geminatus* (**g**), *D. nisus* (**i**), *D. sericeus* (**k**) and *D. semisquamosus* (**m**). The *arrows* indicate the sex bivalents, the *asterisks* denote chromosome pairs containing additional heterochromatic blocks and the *arrowheads* denote the terminal hybridization signals. The *insert* in **m** shows terminal blocks observed in *D. semisquamosus;* (t = terminal region, c = centromere). In **a**–**d**, the X and y chromosomes are indicated. (D1) *Dichotomius bos*, (D2) *D. geminatus*, (D3) *D. laevicollis*, (D4) *D. nisus*, (D5) *D. semisquamosus* and (D6) *D. sericeus*. *Bar* 5 μm

D. bos (Fig. 1a), *D. laevicollis* (Fig. 1d), *D. nisus* (Fig. 1h), *D. semisquamosus* (Fig. 1l) and *D. sericeus* (Fig. 1j). In *D. geminatus*, the y was completely heterochromatic (Fig. 1f). For *D. geminatus*, *D. nisus*, *D. sericeus* and *D. semisquamosus* these results are similar with previous descriptions (Silva et al. 2009; Cabral-de-Mello et al. 2010b), while for the other species it is the first detailed description of karyotypes and C-banding.

The fluorochrome staining combination (CMA_3/DA/DAPI) revealed the presence of GC-rich blocks (CMA_3^+) and the absence of AT rich regions in distinct chromosomes from all studied species. The GC-rich blocks were concentrated in heterochromatic regions of few chromosomes, although euchromatic GC-rich regions were also observed. The pair two had GC-rich pericentromeric heterochromatin in all of the species (Figs. 2, 4a). Additional GC-rich blocks were observed in the terminal euchromatic region of the short arm of pair two in *Dichotomius laevicollis* (Fig. 2b) and *D. sericeus* (Fig. 2e). Moreover, *D. bos* showed CMA_3^+ blocks in the pericentromeric areas of pairs three and six (Fig. 2a), while *D. geminatus* had CMA_3^+ blocks in the heterochromatin of pairs three and four, which extended along the short arm (Fig. 2f). In *D. semisquamosus*, GC richness was observed in pair three (Fig. 2d). With regard to the sex chromosomes, GC-rich heterochromatin was observed in the X chromosomes from *D. bos* (Fig. 2a) and *D. nisus* (Fig. 2c). Additionally, the pericentromeric area of the y chromosome from *D. nisus*

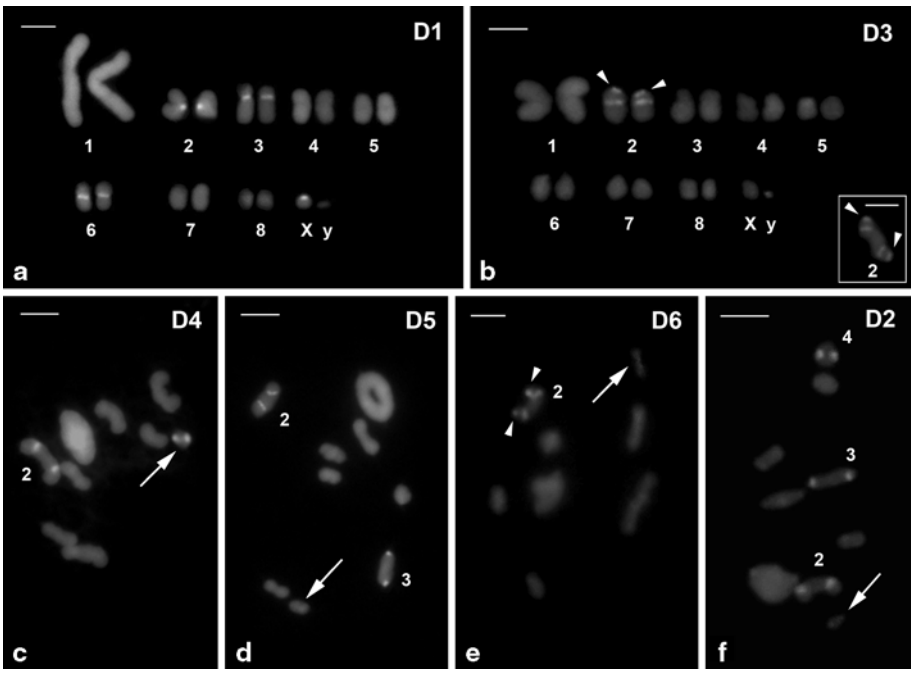

Fig. 2 Fluorochrome staining in the six species of *Dichotomius*. Karyotypes from *D. bos* (**a**) and *D. laevicollis* (**b**), respectively; metaphase I chromosomes from *D. nisus* (**c**), *D. semisquamosus* (**d**), *D. sericeus* (**e**) and *D. geminatus* (**f**) are showed. The *arrows* indicate the sex bivalents, and the *arrowheads* denote the CMA_3^+ euchromatic blocks. The *insert* in **b** indicates the conformation of the pair 2 from *D. laevicollis* in metaphase I. (D1) *Dichotomius bos*, (D2) *D. geminatus*, (D3) *D. laevicollis*, (D4) *D. nisus*, (D5) *D. semisquamosus* and (D6) *D. sericeus*. *Bar* 5 μm

was CMA_3^+ (Fig. 2c). The remaining heterochromatic areas were neutral with regard to the CMA_3 and DAPI fluorochromes. Figure 4a summarizes all patterns of chromosomal distribution of heterochromatin and base-pair richness among the *Dichotomius* species.

C_0t-1 DNA fraction mapping

The C_0t-1 DNA fractions were obtained at different reannealing times (30 s to 5 min) from each *Dichotomius* species (Table 1). The hybridization of C_0t-1 DNA in the six species against their own chromosomes revealed similar patterns of heterochromatin distribution in five species (Fig. 1e, g, i, k, m; Table 1). Moreover, small signals in the terminal regions of chromosomes were observed in some distinct cells of *D. geminatus* (Fig. 1g), *D. nisus* (Fig. 1i) and *D. semisquamosus* (Fig. 1m). In *D. bos*, the pairs one, two and three did not reveal specific marks for C_0t-1 DNA hybridization (Fig. 1b, c), even with the isolated C_0t-1 DNA fraction that underwent 5 min of reannealing. This FISH experiment revealed marks in euchromatic areas, but not in the heterochromatin of pairs one, two and three (results not shown).

The hybridization of the C_0t-1 DNA fraction from *D. geminatus* in the chromosomes of the other five species occurred only in the terminal/sub-terminal regions of all of the autosomes (Fig. 3). Moreover, the X sex chromosome displayed hybridization in all of the species, showing more intense labeling in *D. bos* (Fig. 3c, d), *D. nisus* (Fig. 3i, j) and *D. semisquamosus* (Fig. 3a, b). However, less intense labeling was observed in *D. sericeus* (Fig. 3g, h) and *D. laevicollis* (Fig. 3e, f). The y chromosome was labeled with dots in all of the species (Fig. 3). Low intense hybridization pattern was observed in *D. laevicollis* (Fig. 3e, f) and

Table 1 C_0t-1 DNA fraction reassociation time and chromosomal location for the six *Dichotomius* species investigated in this study

Species	C_0t-1 DNA fraction reassociation times	C_0t-1 DNA fraction chromosomal mapping
Dichotomius bos	2 min 30 s	Pericentromeric heterochromatin, except from pairs 1–3
Dichotomius geminatus	1 min	Pericentromeric heterochromatin, terminal heterochromatic region of the pairs 6, 7 and weak marks in terminal region of other autosomes
Dichotomius laevicollis	30 s	Pericentromeric heterochromatin
Dichotomius nisus	1 min	Pericentromeric heterochromatin and weak marks in terminal region of some chromosomes
Dichotomius semisquamosus	3 min	Pericentromeric heterochromatin and weak marks in terminal region of some chromosomes
Dichotomius sericeus	30 s	Pericentromeric heterochromatin

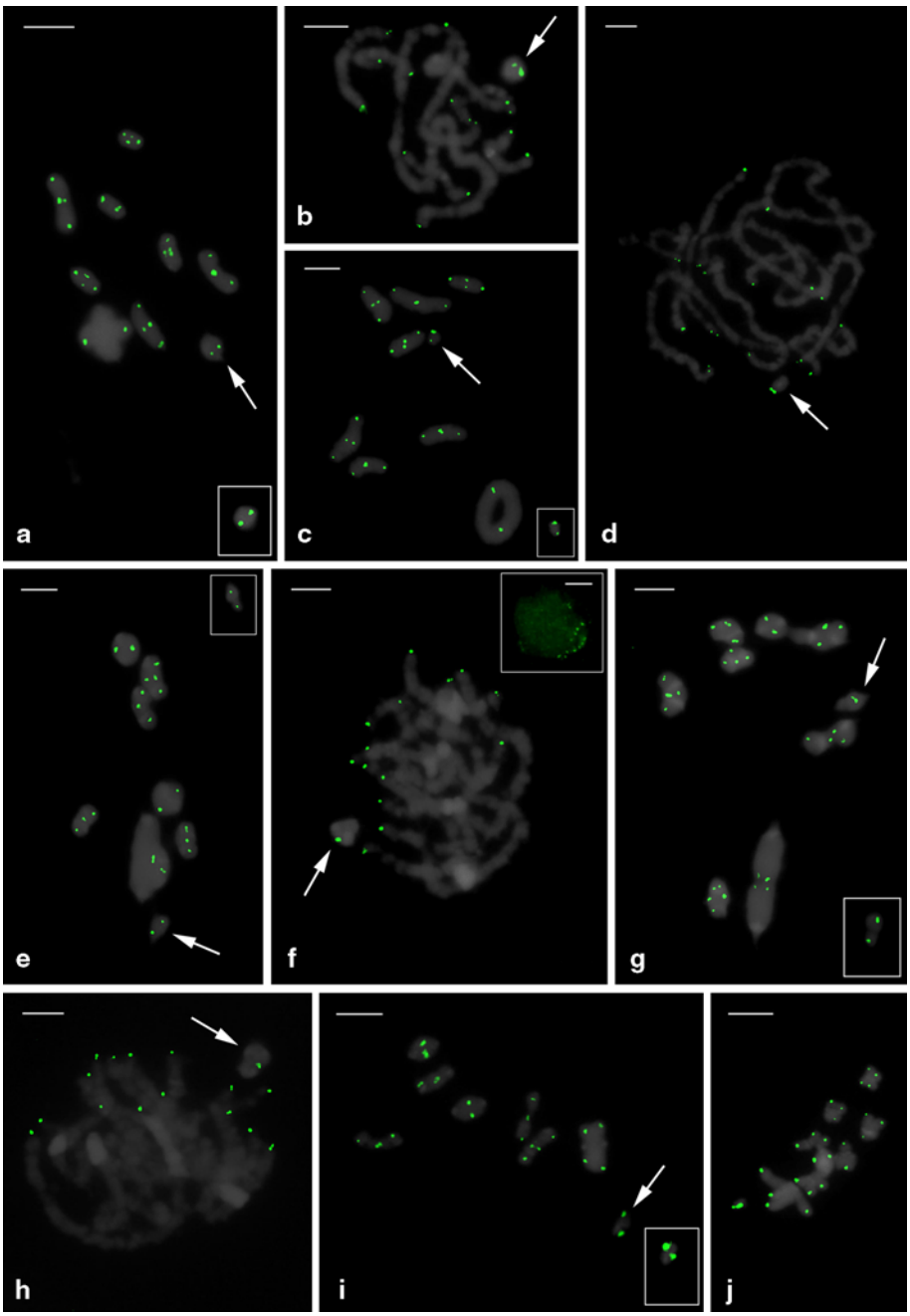

Fig. 3 C_0t-1 DNA fraction obtained from the genome of *Dichotomius geminatus* hybridized against the chromosomes of the other five *Dichotomius* species. Metaphase I chromosomes from *D. semisquamosus* (**a**), *D. bos* (**c**), *D. laevicollis* (**e**), *D. sericeus* (**g**) and *D. nisus* (**i**); the initial pachytenes of *D. semisquamosus* (**b**), *D. bos* (**d**), *D. laevicollis* (**f**) and *D. sericeus* (**h**), and metaphase II of *D. nisus* (**j**) are showed. The *arrows* indicate the sex bivalents. *Inserts* indicate the detail of the sex chromosomes in metaphase I from another cell for each species (**a**, **c**, **e**, **g**, **i**) and the initial meiotic nucleus (**f**) showing the polarization of the hybridization signals. Note that the hybridization signals are restricted to the terminal regions of the chromosomes. *Bar* 5 μm

D. sericeus (Fig. 3g, h), and high intense hybridization was observed in *D. nisus* (Fig. 3i, j). At least fifteen hybridized metaphases or initial meiotic cells from each species were analyzed to define the patterns that are described above.

Phylogeny of *Dichotomius* species based on mtDNA sequence analysis

All phylogenetic analyses resulted in well-resolved trees that exhibited similar topology and maximal or near-maximal indices of support for all of the nodes (Fig. 4b). *Dichotomius laevicollis* and *D. semisquamosus* appeared as a sister group to the other four species. The four remaining species formed two clades of sister taxa. *D. nisus* was grouped together with *D. bos*, and *D. sericeus* was grouped with *D. geminatus*. The topology of the tree was similar independent of the species included as outgroup. In fact the number of species included in the phylogenetic analysis represents only about 7.0% of the representatives of *Dichotomius*, and a wide analysis using more species should be necessary.

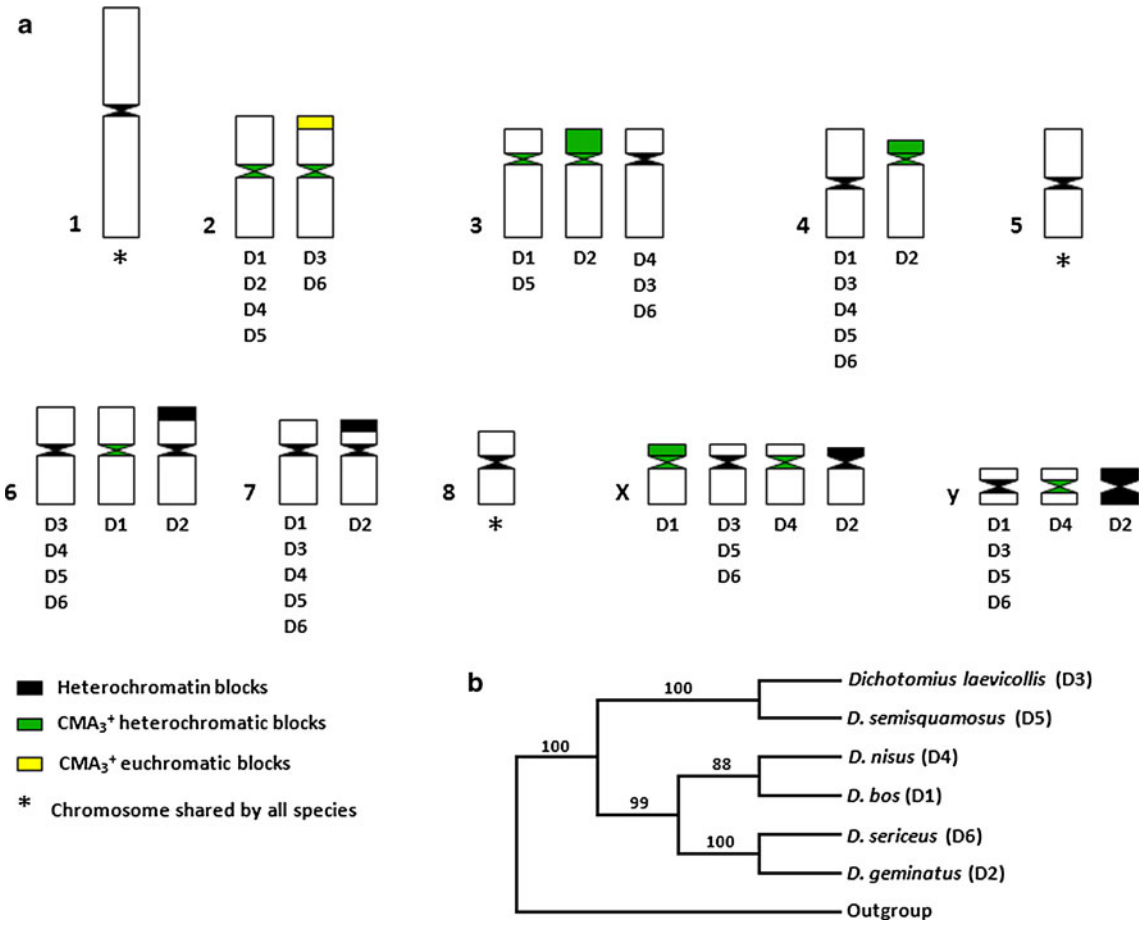

Fig. 4 **a** Idiograms showing the distribution of cytogenetic markers for each chromosome in the six species of *Dichotomius* studied; **b** phylogenetic relationship of the six *Dichotomius* species based on COI and 16S sequences. (D1) *Dichotomius bos*, (D2) *D. geminatus*, (D3) *D. laevicollis*, (D4) *D. nisus*, (D5) *D. semisquamosus* and (D6) *D. sericeus*

Discussion

Similar macro-chromosomal structures were observed in the six *Dichotomius* species studied with $2n = 18,Xy_p$, and biarmed chromosomes. Apparently, this karyotype pattern is highly conserved in the *Dichotomius* genus, including the remarkable presence of a large autosomal pair (pair 1) that most likely arose by a fusion event between autosomes from a $2n = 20$ karyotype. Consistent with this hypothesis, $2n = 20$ is the most common and ancient diploid number for the Scarabaeidae family and Coleoptera order (Smith and Virkki 1978; Yadav and Pillai 1979; Cabral-de-Mello et al. 2008).

The presence of heterochromatin primarily in the pericentromeric regions of all autosomes is a common characteristic in the genus *Dichotomius* and also in coleopterans (Rożek et al. 2004; Silva et al. 2009). Alternatively, the presence of heterochromatic blocks out of pericentromeric region has been reported in *D. geminatus* and other Scarabaeidae species, along with the presence of terminal blocks and diphasic autosomes (Moura et al. 2003; Cabral-de-Mello et al. 2010a, b). The diversified patterns of heterochromatin distribution indicate that the heterochromatin diverged over evolutionary time in the various Scarabaeidae lineages.

With regard to heterochromatin base pair richness, the presence of GC-rich blocks was common in *Dichotomius* and in Scarabaeidae, although neutral and AT rich blocks have also been described (Moura et al. 2003; Colomba et al. 2006). The presence of neutral and GC-rich blocks in *Dichotomius* indicates that there is some level of compartmentalization of heterochromatin in the species. Moreover, the distinct pattern of the distribution of GC-rich blocks among the six species indicates that there is some level of dynamism in the genomic content of heterochromatic areas that have diverged between species. Despite the variation in GC content, some of the CMA_3^+ blocks in specific chromosomes are shared among the studied species, as the GC-rich heterochromatin localized in chromosome pair two. Some other CMA_3^+ blocks are species-specific. For example, the blocks located in the y chromosome are exclusive for *D. nisus,* and the block in

pair six was observed only in *D. bos*. With regard to the sex chromosomes, the observed variability in GC richness indicates that repeated DNAs with distinct composition are present in these chromosomes and did not influence the ancestral structure of chromosome association (Xy_p).

The use of C_0t-1 DNA fractions as probes in the six species of *Dichotomius* revealed the same heterochromatin distribution pattern in five of them, with the exception of *D. bos*, demonstrating that highly and moderately repetitive sequences are present in the heterochromatic areas. Moreover, the presence of weak terminal marks in some of the species, confirms that repetitive DNAs are present in this area, although heterochromatin was not observed in this region by C-banding. The absence of C_0t-1 DNA hybridization in chromosomes 1–3 of *D. bos* indicates that the heterochromatin is composed of low copy-number of sequences or a high diversity of repetitive DNA classes are present in low copy numbers. The presence of low copy numbers of repetitive sequences in the heterochromatin of these three pairs was confirmed using a C_0t-1 DNA fraction that was isolated using 5 min of renaturation. This assay revealed hybridization sites in a euchromatic region, but the three pairs remained without hybridization. These results are inconsistent with the classical idea that most of the segments of constitutive heterochromatin contain high concentrations of highly repeated DNA families (Sumner 2003).

An intense variation in the kinetics of renaturation was observed among the six species when we obtained the C_0t-1 DNA fractions. The rate at which the fragmented DNA sequences reassociate in the C_0t-1 DNA assay is proportional to the copy number in the genome (Peterson et al. 2002); therefore, the variation in the kinetics of renaturation can be attributed to the differences in the amount of repeated DNAs between the genomes of the six species. Based on this parameter, the short C_0t-1 DNA fractions isolation time can be attributed to the presence of more repeated sequences in the genomes of species, such as in the cases of *D. laevicollis* and *D. sericeus*; however, the genome of *D. bos* and *D. semisquamosus* is most likely composed of less quantity of repeated DNA or with more variable composition. These results are clearly correlated with the size of heterochromatic blocks, which are larger in *D. laevicollis* and *D. sericeus* compared to *D. bos* and *D. semisquamosus*.

The use of the C_0t-1 DNA fraction from *D. geminatus* as probes to hybridize in the chromosomes of the other five *Dichotomius* species revealed an interesting pattern of high conservation of terminal/sub-terminal blocks, in contrast to the extensive variation in relation to the pericentromeric heterochromatin. It is a general consensus that rapid modifications in repeated DNAs, even among closely related species, generate species-specific sequences (Miklos 1985; Ugarković and Plohl 2002), as observed among the *Dichotomius* species, at least between *D. geminatus* and the other five species. The copy number variation of repeated DNAs is governed, for example, by unequal crossing-over, replication slippage, rolling-circle replication and other unknown mechanisms (Charlesworth et al. 1994), and the variation in the pool of sequences is attributed to sequences modification and the principles of concerted evolution, in which diverse mechanisms of nonreciprocal transfer induce a high turnover of repeated sequences (Dover 1986). Diversification of repeated DNAs, such satDNA has been described in other insect groups (see Palomeque and Lorite 2008). For example in *D. melanogaster*, the chromosomal banding techniques differentiate heterochromatin into some discrete regions by cytological methods, dependents of the presence and abundance of distinct satDNAs (Brutlag and Peacock 1975; Dimitri 1991; Gatti and Pimpinelli 1992; Lohe et al. 1993). For the first time, the use of the C_0t-1 DNA fraction from beetles permitted a deeper comparison of all of the heterochromatic regions at a molecular level. The analysis of this marker corroborates the results that were obtained using fluorochromes, which already indicated that there was heterochromatin differentiation in the six *Dichotomius* species. Several heterochromatin studies that are only based on C-banding are restricted to the descriptions of location of this genomic component; thus, the C-banding method generated superficial results that can led to erroneous conclusions about the similarities among animal karyotypes and genomes. Moreover, studies of repetitive DNA sequences focus primarily on descriptions of specific sequences in animal or plant chromosomes (for examples, see Yamada et al. 2002; Ansari et al. 2004; Martins et al. 2006; Adega et al. 2008), thereby limiting understanding the whole heterochromatic portion and differentiation of the genomes. Alternatively, the C_0t-1 DNA allows for a wide genomic analysis of repeated DNAs, their relationship to heterochromatin variation and their distribution patterns among the chromosomes without the extensive work and laboratory expenses that are required for cloning and DNA sequencing.

The presence of signals in the terminal/sub-terminal regions of autosomes after using the C_0t-1 DNA fraction obtained from *D. geminatus* in five of the species indicates that the genome portion of chromosome ends among the six species are conserved. However, the possibility of cross-hybridization between centromeric repeated DNAs of *D. geminatus* and terminal/sub-terminal regions of the autosomes from the other five species can not be eliminated. This conservation may be related to structural and/or functional action of specific DNA elements in the terminal/sub-terminal region of the chromosomes. These sequences may play roles as telomeres, telomere-associated DNA, or they may be the telomeric sequences. Some structures that were observed in the initial meiotic cells and in the

interphasic nuclei, such as the bouquet configuration and the polarization of the hybridized signals to one nuclear pole to form a structure that is similar to the *rabl* configuration, led us to propose that these conserved sequences most likely correspond to telomeres or telomere-associated DNA (see Fig. 3).

The variations in pericentromeric repeat sequences that we observed in the five *Dichotomius* species (comparing the C_0t-1 DNA fraction obtained from *D. geminatus*) is a common feature in complex eukaryotes, paradoxically with conserved function of this chromosomal region (Schmidt and Heslop-Harrison 1998; Henikoff et al. 2001; Ugarković and Plohl 2002). On the other hand, the telomeric sequences are highly conserved in some groups, including vertebrates and plants (Meyne et al. 1989; Cox et al. 1993; Fuchs et al. 1995). In insects, the telomeres are more variable, but the telomeric motif (TTAGG)*n* is widespread across several insect orders (Okazaki et al. 1993; Sahara et al. 1999). This high conservation of C_0t-1 DNA hybridization appears to be a common feature in *Dichotomius* species, although we are not certain if the labeled regions correspond to the telomeric sequences or to the telomere-associated DNA sequence. Conserved repeated sequences in the telomeric regions have been reported in insects, including the transposable element TART that is found in distantly related species of *Drosophila* and a complex tandem repeated DNA family that is observed in the telomeres of the *Chironomus* genus (Zhang et al. 1994; Casacuberta and Pardue 2003).

The reduction of the diploid number to 2n = 18 and the presence of a large biarmed bivalent were proposed to have resulted from an autosomal fusion event that may be involved in chromosomal differentiation in the *Dichotomius* species. However, the proposed autosomal fusion event was not corroborated by interstitial hybridization signals in the first bivalent of the five species studied when the *D. geminatus* C_0t-1 DNA fraction that labeled the terminal region of autosomes in the other species was used. These data can be correlated to a rapid modification or loss of terminal sequences that occurred after the chromosomal fusion, leading to the failure of interstitial C_0t-1 DNA site detection in FISH experiments.

With regard to the sex chromosomes in the *Dichotomius* species analyzed in this study, the classical cytogenetic and C_0t-1 DNA hybridization analyses indicate that there are distinct repetitive DNA differentiation patterns for the X and y chromosomes in the genus, despite the conservation of a basic Xy_p system. The X chromosome showed more variation in comparison with the y chromosome with blocks of repeated DNAs concentrated in the pericentromeric areas or extending along the short arm, following the heterochromatin distribution. Using the C_0t-1 DNA fraction from *D. geminatus*, sequence-related variability was also observed. Some of the species had only small size blocks on the X and y chromosomes (*D. sericeus* and *D. laevicollis*), while other species had medium size blocks (*D. bos* and *D. semisquamosus*), and one species had large size blocks (*D. nisus*). Although it was possible to identify sequence variability in the sex chromosomes, it was impossible to determine whether or not these sequences are shared between the autosomal complement and the sex chromosome of *D. geminatus* due to the use of a pool of sequences that was obtained by the C_0t-1 DNA method.

The chromosomal mapping of repeated DNAs using classical and molecular cytogenetic approaches in the six species of *Dichotomius* analyzed indicates that there is conservation of location of heterochromatic blocks as well as modification of sequences, at least between *D. geminatus* and the other five species. Moreover, it was possible to identify some conserved chromosomes within the genus based on the applied chromosomal markers, such as pairs one, five and eight. However, the other chromosomes, including the sex bivalents, have apparently experienced distinct differentiation processes, including heterochromatin differentiation without modification of the macrochromosomal structure. Although we identified some conserved chromosomes in the genus, the general pattern of organization of repeated DNAs does not reflect the relationship between the six species based in COI and 16S rRNA genes. Repeated DNAs are subject to the action of several molecular mechanisms and are thought to be the most rapidly evolving components in genomes (Dover 1986; Charlesworth et al. 1994; Eickbush and Eickbush 2007), displaying intense variability, even in related species such as those in the *Dichotomius* genus.

Finally, the application of C_0t-1 DNA fraction is a useful tool for studies of repeated DNAs in insects, thus contributing to understanding heterochromatin differentiation among related species. Contrary to vertebrates that posses the availability of BAC (Bacterial Artificial Chromosomes) libraries and whole chromosomes as probes, for insects there are few available genes or DNA sequences to be applied as probes for purposes of cytogenetic mapping. In this way, although the C_0t-1 DNA hybridization does not permit the generation of precise information about specific chromosomes or DNA sequences, it allows for a wide comparison of the whole repetitive portion of genomes without expensive applications of DNA cloning and sequencing. This analysis represents an interesting approach for the investigation of karyotype diversification and genome evolution under the focus of cytogenetics.

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