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ORGANIZAÇÃO CROMOSSÔMICA DE ELEMENTOS REPETITIVOS DE DNA EM REPRESENTANTES DA SUBFAMÍLIA SCARABAEINAE (COLEOPTERA: SCARABAEIDAE)

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas (Genética) como parte dos requisitos para obtenção do título de Doutor

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"Dias inteiros de calmaria, noites de ardentia, dedos no leme e olhos no horizonte, descobri a alegria de transformar distâncias em tempo. Um tempo em que aprendi a entender as coisas do mar, a conversar com as grandes ondas e não discutir com o mau tempo. A transformar o medo em respeito, o respeito em confiança. Descobri como é bom chegar quando se tem paciência. E para se chegar, onde quer que seja, aprendi que não é preciso dominar a força, mas a razão. É preciso, antes de mais nada, querer".

Amyr Klink

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SUMÁRIO

RESUMO

O mapeamento cromossômico de seqüências repetitivas de DNA tem se mostrado uma eficiente ferramenta nos estudos comparativos e evolutivos em diversos organismos. Estudos cromossômicos com besouros da subfamília Scarabaeinae têm revelado ampla variabilidade, entretanto a análise da organização cromossômica de DNAs repetitivos neste grupo é escassa e direcionada unicamente ao mapeamento do DNA ribossomal (DNAr) 18S. O presente trabalho teve como objetivo caracterizar cromossomicamente DNAs repetitivos em espécies de Scarabaeinae, utilizando bandeamentos cromossômicos e mapeamento físico cromossômico de seqüências repetitivas, incluindo famílias multigênicas de RNAr 18S, RNAr 5S e histona H3 e a fração de DNA C_0t-1. Ampla variabilidade foi observada relacionada ao número/localização dos sítios de DNAr 18S, aparentemente associada a diversificação da heterocromatina. Por outro lado, os genes de RNAr 5S e histona H3, mostraram-se amplamente conservados e co-localizados em um par cromossômico, com aparente intercalação. Análises em representantes de *Dichotomius* revelaram conservação dos blocos de heterocromatina, entretanto com aparente compartimentalização dos mesmos. O uso da fração DNA C_0t-1 confirmou o enriquecimento em DNAs repetitivos da heterocromatina, que se apresentou diversificada entre as espécies, utilizando como referência *D. geminatus*. Por outro lado, regiões terminais dos cromossomos apresentaram-se amplamente conservadas entre as seis espécies. Além disso, a análise da fração de DNAs repetitivos em *D. geminatus* indicou origem intraespecífica do cromossomo B desta espécie que possivelmente pode estar sofrendo homogeneização com seqüências encontradas no complemento A. Os resultados indicam distintos padrões de diversificação para o DNA repetitivo nos representantes de Scarabaeinae, sugerindo extensiva reorganização microgenômica ao longo da cladogenese do grupo.

Palavras-chave: besouro, citogenética, DNA C_0t-1, evolução cromossômica, famílias multigênicas, hibridização *in situ* fluorescente

ABSTRACT

The chromosomal mapping of repeated DNAs has been used as an efficient tool in comparative and evolutionary studies in some organism. The chromosomal studies in beetles belonging to the subfamily Scarabaeinae have revealed wide variability, although the analysis of chromosomal organization of repeated DNAs in this group is scarce and directed solely for 18S rDNA mapping. The present study aimed in chromosomal characterization of repeated DNAs in Scarabaeinae species using chromosomal banding and physical chromosome mapping of repeated sequences, including the multigene families for 18S and 5S rRNAs and H3 histone genes and the C_0t-1 DNA fraction. Wide variability was observed concerning the number and location of 18S rDNA sites, apparently associated to the heterochromatin diversification. On the other hand, the 5S rRNA and H3 histone genes were widely conserved and co-located in one chromosomal pair, showing apparently interspersion. Analysis in *Dichotomius* representatives revealed conservation for heterochromatic blocks, although an apparent compartmentalization was observed. The use of C_0t-1 DNA fraction confirmed the heterochromatin repeated DNAs enrichment, which is diversified among the species, using as reference *D. geminatus*. On the other hand, the terminal regions of the chromosomes were highly conserved among the six species. Moreover, the analysis of repeated DNA fraction from *D. geminatus* indicated intraspecific origin of a B chromosome in this species that possibly could be suffering homogenization with A complement sequences. The results indicate distinct diversification patterns for repeated DNAs in Scarabaeinae representatives, suggesting extensive microgenomic reorganization along the cladogenesis of the group.

Key-words: beetle, cytogenetics, C_0t-1 DNA, chromosomal evolution, multigene families, fluorescent *in situ* hybridization

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1. INTRODUÇÃO E JUSTIFICATIVA

1.1. Considerações gerais sobre a família Scarabaeidae (Coleoptera)

A ordem Coleoptera compreende a mais diversa dentro da classe Insecta, apresentando cerca de 360.000 espécies com distribuição mundial. Na região Neotropical, este grupo é representado por aproximadamente 72.476 espécies, agrupadas em 6.703 gêneros e 127 famílias (Costa, 2000). Esta riqueza de espécies está associada à extrema diversificação morfológica, ecológica e comportamental, sendo resultado de co-radiação de muitos grupos com angiospermas, mamíferos e mudanças climáticas e geológicas ocorrentes desde o período Cretáceo (Crowson, 1960; Farrell, 1998; Davis *et al.*, 2002; Erwin, 1985). Os besouros apareceram há cerca de 285 milhões de anos atrás e sofreram diversos eventos de radiação adaptativa, gerando os diferentes grupos (Crowson, 1981; Grimaldi e Engel, 2005). Atualmente, Coleoptera é dividida em quatro subordens, Archostemata, Adephaga, Myxophaga e Polyphaga, e a monofilia do grupo é consenso entre diversos autores, apesar das relações entre as quatro subordens permanecerem controvérsias. Dentre as quatro subordens de Coleoptera Polyphaga é a mais diversa agrupando 90% das espécies do grupo (Crowson, 1981; Lawrence e Newton, 1995; Wheeler *et al.*, 2001; Vanin e Ide, 2002).

Para Polyphaga são descritas 16 superfamílias, das quais Scarabaeoidea destaca-se por apresentar ampla radiação adaptativa e alta diversidade de espécies viventes. Este grupo é representado por cerca de 27.800 espécies agrupadas em 13 famílias, Glaressidae, Passalidae, Lucanidae, Diphyllostomidae, Trogidae, Bolboceratidae, Plecomidae, Geotrupidae, Hybosoridae, Ochodaidae, Ceratocanthidae, Glaphyridae e Scarabaeidae (Crowson, 1981; Browne e Scholtz 1999; Vanin e Ide, 2002). Dentre as 13 famílias de Scarabaeiodea Scarabaeidae é a mais diversa e possui cerca de 25.000 espécies e 2.000 gêneros agrupados em 12 subfamílias e diversas tribos distribuídas mundialmente. No neotrópico são registrados 362 gêneros e 4.706 espécies, enquanto no Brasil ocorrem 204 gêneros e 1.777 espécies

(Costa, 2000; Vaz-de-Mello, 2000). São besouros de corpo robusto, ovais ou alongados, usualmente convexos e com antenas lameladas. Variam consideravelmente em hábitos, se alimentando de esterco, matéria vegetal, "carniça", podendo estar associados a formigueiros e cupinzeiros, além de se alimentarem de fungos. A maioria das espécies tem hábitos noturnos e apresentam atração pela luz, entretanto espécies diurnas podem ser encontradas em tecidos vegetais como flores (Lawrence e Newton 1995; Marioni *et al.* 2001; Ratcliffe *et al.* 2002).

Em Scarabaeidae, a subfamília Scarabaeinae apresenta maior quantidade de estudos relacionados a características taxonômicas e ecológicas. É um grupo cosmopolita com ampla diversidade, apresentando 5.000 espécies, 12 tribos e 234 gêneros descritos em todo o mundo (Figura 1). Para região Neotropical tem-se registro de aproximadamente 1.250 espécies e 70 gêneros, enquanto para o Brasil há registro de 618 espécies, das quais 323 são endêmicas (Hanski e Cambefort, 1991; Vaz-de-Mello, 2000). Apesar do grande número de espécies descritas para o grupo, acredita-se que este número seja bastante superior devido à carência de dados para diversas regiões tanto do Brasil quanto do mundo.

Figura 1. Amostra da diversidade de espécies da subfamília Scarabaeinae.

Os escarabeíneos são conhecidos popularmente como “rola bosta” devido ao hábito de rolarem bolas de matéria orgânica em decomposição para realizarem ovoposição e alimentação (Halffter e Mattheus, 1966). Devido a este hábito, os mesmos são importantes no funcionamento de distintos ecossistemas, desde savanas Africanas a matas tropicais, atuando como eficientes recicladores da matéria orgânica, principalmente animal, nestas regiões (Halffter e Mattheus, 1966; Halffter e Favila, 1993). Em decorrência desta importante função ecológica, juntamente com a ampla diversidade do grupo, facilidade de coleta em campo, elevada densidade de indivíduos para as espécies comuns, ocorrência de grupos bem estudados taxonomicamente e por constituírem comunidades bem estruturadas, os escarabeíneos tem sido utilizados em estudos para indicação de qualidade ambiental (Halffter e Edmons, 1982; Hanski e Cambefort, 1991; Halffter e Favila, 1993).

1.2. Citogenética convencional de Scarabaeidae

Embora Coleoptera apresente ampla diversidade de espécies e cromossômica, os estudos citogenéticos são bastante escassos e apenas pouco mais de 1% dos representantes da ordem apresentam cariótipos descritos na literatura. De maneira geral, os estudos são restritos a descrição do número diplóide e dos mecanismo de cromossomos sexuais e, em menor escala, morfologia cromossômica (Smith e Virkki, 1978; Vidal, 1984; Virkki e Santiago-Blay, 1993; Moura *et al*., 2003; Karagyan *et al.*, 2004; Pons, 2004; Schneider *et al*,. 2007; Cabral-de-Mello *et al.*, 2008; de Julio *et al.*, 2010). Além disso, os estudos neste grupo são concentrados em algumas famílias, como por exemplo, Carabidae e Cicindelidae, para subordem Adephaga e Buprestidae, Chrysomelidae, Elateridae, Scarabaeidae e Tenebrionidae para Polyphaga, enquanto para as subordens Myxophaga e Archostemata apenas três espécies foram estudadas (Smith e Virkki, 1978; Mesa e Fontanetti, 1985; Galián e Lawrence, 1993; Galián *et al.*, 2002;

Karagyan *et al.*, 2004; Martinez-Navarro *et al.*, 2004; Pons, 2004; Schneider *et al*,. 2007; Cabral-de-Mello *et al.*, 2008; de Julio *et al.*, 2010).

Para a subordem Polyphaga, onde os estudos citogeneticos têm sido realizados com maior freqüência, o cariótipo considerado modal e primitivo é constituído por número diplóide 2n = 20, Mecanismo sexual Xy_p, cromossomos autossômicos e X com dois braços e y puntiforme (Smith e Virkki, 1978). Apesar desta conservação, tem sido descrito para o grupo ampla variação do cariótipo modal devido a distintos rearranjos cromossômicos, tais como fusão, fissão e inversões. O número diplóide varia de 2n = 4 (*Chalcolepidius zonatus*, Elateridae) a 2n = 64 (*Disonycha bicarinata*, Chrysomelidae) e 11 mecanismos de cromossomos sexuais foram descritos (Xy_p, XY_p, Xny_p, nXy_p, Xy_c, XY_c, X0, Xy, XY, Xy_r, nXnY). Além disso, foram registrados a ocorrência de cromossomos com distintas morfologias (Takenouchi, 1970; Smith e Virkki, 1978; Ferreira *et al.*, 1984; Serrano e Galián, 1998; Dutrillaux e Dutrillaux, 2009; Cabral-de-Mello *et al.*, 2010a).

Para a família Scarabaeidae cerca de 400 espécies foram estudadas citogeneticamente, predominando análises em representantes das subfamílias, Cetoniinae, Dynastinae, Melolonthinae, Rutelinae e Scarabaeinae. Assim como observado para a ordem Coleoptera como um todo, esta família é considerada conservada cariotipicamente e apresenta cariótipo modal e primitivo 2n = 20, Xy_p e cromossomos com dois braços em mais de 50% das espécies estudadas, embora diversas variações foram relatadas (Smith e Virkki, 1978; Yadav e Pillai, 1977, 1979; Moura *et al*., 2003; Bione *et al.*, 2005a; Cabral-de-Mello *et al.*, 2008; Dutrillaux e Dutrillaux, 2009). Com relação ao número diplóide foi observada variação de 2n = 8 em *Eurysternus caribaeus* (Scarabaeinae) a 2n = 30 em *Autoserica assamensis* (Melolonthinae) e dentre os mecanismos sexuais descritos para Coleoptera sete foram observados em Scarabaeidae, sendo quatro quiasmáticos (neo-XY, XY, Xy e Xy_r) e três aquiasmáticos (Xy_p, XY_p e XO) (Dasgupta, 1977; Smith e Virkki, 1978; Cabral-de-Mello *et al.*, 2007). De acordo

com Yadav e Pillai (1979) e Cabral-de-Mello et al. (2008), os rearranjos cromossômicos envolvidos na diferenciação dos cariótipos em Scarabaeidae foram as fusões autossomo-autossomo (A-A) e autossomos e cromossomo X (A-X), perda do cromossomo y ou aumento do mesmo, fissão de autossomos e inversões.

Dentre as cinco subfamílias de Scarabaeidae mais estudadas citogeneticamente, Scarabaeinae apresenta a maior diversidade cariotípica, resultante de distintos rearranjos cromossômicos, tais como fusões, fissões, inversões e aumento ou perda do cromossomo y, assim como descrito para Scarabaeidae como um todo. Para esta subfamília, cerca de 130 das 5.000 espécies conhecidas para o grupo apresentam cariótipos descritos. Em geral os estudos foram realizados com mais freqüência em representantes das tribos Canthonini, Coprini, Ontophagini e Phanaeini. O menor número diplóide registrado para esta subfamília (2n = 8 em *E. caribaeus*) é coincidente com o menor número registrado para Scarabaeidae, enquanto o maior número ocorre em *Tiniocellus spinipes*, 2n = 24 (revisado por Cabral-de-Mello *et al.*, 2008).

Em relação aos cromossomos B em Scarabaeinae, poucos estudos tem relatado a presença destes elementos nos cariótipos dos representantes deste grupo. Além disso, estes estudos têm sido concentrados basicamente apenas na descrição da presença/ausência deste polimorfismo, relatando número de cromossomos B e seus tamanhos em populações naturais (Wilson e Angus, 2005; Angus *et al.*, 2007; Falahee e Angus, 2010). Exemplos de espécies de Scarabaeinae portadoras de cromossomos B ocorrem nos representantes das tribos Onitini e Onthophagini (Wilson e Angus, 2005; Angus *et al.*, 2007; Falahee e Angus, 2010). O maior número de Bs registrado para o grupo, ocorre em *Bubas bubalus* (Onitini), com ocorrência de 2-9 elementos supernumerários de diferentes tamanhos podendo alcançar duas vezes o tamanho do maior par do complemento A (par 1) (Angus *et al.*, 2007). Foram descritos cromossomos B em cinco espécies da tribo Onthophagini: *Euonthophagus amyntas*,

Onthophagus vacca, *O. similis*, *O. gazella* e *O. fracticornis*. Em geral o tamanho dos Bs nestas espécies é puntiforme, similar ao cromossomo y ou pouco maior em relação ao mesmo, entretanto em *O. vacca* o cromossomo B encontrado apresenta tamanho similar ao cromossomo 9. Além da variabilidade em relação ao número e tamanho dos cromossomos B em Scarabaeinae, variações intraindividuais também foram descritas (Wilson e Angus, 2005; Falahee e Angus, 2010).

1.3. DNAs repetitivos

1.3.1. Características gerais dos DNAs repetitivos e organização dos genomas

Os estudos focando tamanho de genomas em eucariotos têm revelado grande variação de quantidade de DNA entre as diferentes espécies dos mais diversos grupos, incluindo até mesmo espécies relacionadas. Esta variação ocorre independentemente da complexidade do organismo e não apresenta relação com a quantidade de genes apresentados pelos organismos, nem nível de ploidia (Gregory 2005). Esta falta de correlação entre número de genes, complexidade do organismo e tamanho de genomas foi definida por Thomas (1971) como o paradoxo do "valor C", onde o "valor C" corresponde à quantidade de DNA de um núcleo haplóide em picogramas (pg).

Para os genomas animais, uma ampla variação de quantidade de DNA tem sido registrada, desde 0,02 pg em *Pratylenchus coffeae* (nemátode) a 132,83 pg em *Protopterus aethiopicus* (peixe) (www.genomesize.com). Esta ampla variação tem sido atribuída a diferentes quantidades de DNAs repetitivos nos diversos genomas, sendo a presença destes elementos uma característica ubíqua em eucariotos. Estas seqüências são caracterizadas por ampla variabilidade, constituindo distintas famílias e podem representar grande parte da quantidade de DNA das células, em alguns casos excedendo mais de 80% da quantidade de DNA da espécie (Charlesworth *et al.*, 1994; Ridley, 1996; Gregory, 2005; Plohl *et al.*, 2008).

Classicamente os DNAs repetitivos foram considerados como *junk DNAs* (DNA lixo) por não apresentarem funções biológicas bem definidas relacionadas à atividade e expressão dos mesmos (Doolittle e Sapienza, 1980; Orgel e Crick, 1980). Entretanto, o envolvimento destes elementos na organização e funcionalidade dos centrômeros, telômeros, perfeita segregação cromossômica, regulação gênica, reparo e replicação do DNA, diferenciação dos cromossomos sexuais foram propostas em diversos estudos (Anleitner e Haymer, 1992; Kraemer e Schmid, 1993; Messier *et al.*, 1996; Martins, 2007). Além disso, análises mais recentes têm demonstrado expressão e envolvimento dos elementos repetitivos, por exemplo, na formação da heterocromatina e em processos de regulação gênica (Shapiro e Sternberg, 2005; Biémont e Vieira, 2006; Feschotte e Pritham, 2007). Por muito tempo os elementos repetitivos foram considerados sem atividade transcricional, fazendo com fossem classicamente divididos em elementos "codificadores" e "não codificadores". Os elementos repetitivos codificadores incluem as famílias multigênicas, enquanto os não codificadores são representados principalmente pelos DNAs satélites e elementos transponíveis (Revisado por Martins *et al.*, in press). Juntamente com as seqüências únicas, moderadamente e pouco repetitivas estes elementos constituem a estrutura básica do genoma nuclear em eucaritos (Figura 2).

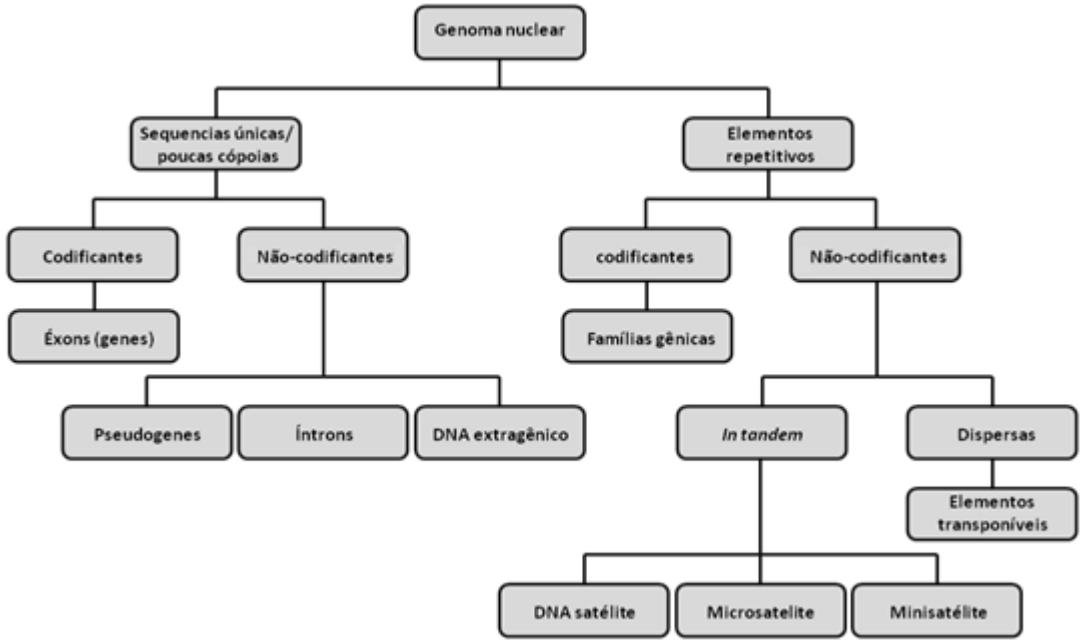

Figura 2. Organização geral das seqüências de DNA do genoma nuclear de eucariotos.

O termo "família multigênica" é utilizado para indicar um grupo de seqüências de DNA (genes) com notável similaridade estrutural e funcional, sendo descendentes de um gene ancestral comum (Nei e Rooney, 2005). Dentre estas seqüências são exemplos bastante conhecidos os genes de RNAs ribossomais (RNAr) e genes codificadores de proteínas histônicas. As seqüências de DNA ribossomal (DNAr) na maioria dos eucariotos são organizadas em dois distintos grupos arranjados *in tandem*. O arranjo maior é formado pelos genes que transcrevem os RNAs ribossomais 18S, 5.8S e 28S (DNAr 45S), sendo estas seqüências separadas por espaçadores intergênicos transcritos internos (ITS-*Internal Transcribed Spacer*); e cada cluster de DNAr 45S separado por espaçadores transcritos externos (ETS-*External Transcribed Spacer*) e por espaçadores integênicos (IGS) (Figura 3a). O outro arranjo é formado pelas seqüências do gene que transcrevem o RNAr 5S. Estes genes são bastante conservados e espaçados por seqüências não transcritas (NTS-*Non Transcribed Spacer*) que são extremamente variáveis em tamanho e composição nucleotídica (Figura 3b) (Long e Dawid, 1980; Williams e Strobeck, 1985; Martins e Wasko, 2004; Eickbush e

Eickbush, 2007). As seqüências codificadores de proteínas histônicas podem ser organizadas em um *cluster* formado por genes que apresentam poucos *introns* (H1, H2A, H2B, H3 e H4) que se encontram espaçados por DNAs não codificantes. Esta estrutura genômica foi descrita, por exemplo, em *Drosophila melanogaster* (Figura 3c). Por outro lado, estes mesmo genes podem estar distribuídos isoladamente como observado em humano, rato e galinha. Além disso, os dois tipos de organização genômica foram observados em *Xenopus laevis* (Lifton *et al.*, 1978; Engel e Dogson, 1981; Ruberti *et al.*, 1982).

Dentre os DNAs não codificadores presentes no genoma dos eucariotos, destacam-se os DNAs satélite (DNAsat), minisatélites, microsatélites e elementos transponíveis, devido à grande abundancia. Os DNAsat são compostos por seqüências altamente repetitivas com ampla variabilidade organizadas *in tandem* com diferentes números de cópias nos genomas das distintas espécies, podendo variar basicamente entre 1.000 e mais de 100.000 cópias. Estas seqüências constituem o principal componente da heterocromatina e estão freqüentemente associadas as regiões centromérica e telomérica dos cromossomos. De uma forma geral estes elementos são bastante diversificados entre diferentes espécies, incluindo espécies relacionadas, resultante dos mecanismos de mutação e evolução em concerto (John e Miklos, 1979; Charlesworth *et al.*, 1994; Ugarković e Plohl, 2002).

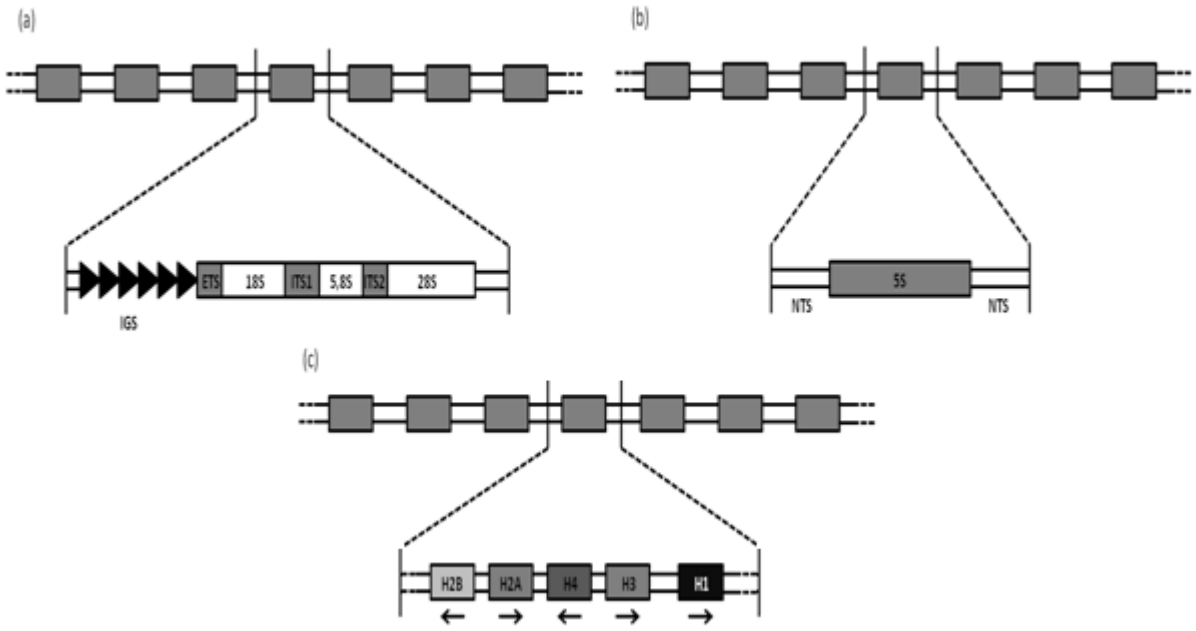

Figura 3. Organização genômica das famílias multigênicas de DNAr e histonas: (a) DNAr 45S, (b) DNAr 5S, (c) genes de histonas em moscas de fruta. ETS = Espaçador transcrito externo; ITS = espaçador transcrito interno; IGS = Espaçador intergênico.

Os minisatélites apresentam variação de tamanho de 10-100 pares de base (pb). Estas seqüências encontram-se dispersas por todo o genoma com variação no número de unidades de repetição (*repeats*) conhecidos como VNTR (Variable Numbers of Tandem Repeats). Os minisatélites são encontrados nos mais distintos grupos de eucariotos, desde leveduras, plantas e animais, sendo uma classe de DNA repetitivo comum nos genomas eucariotos (Jeffreys *et al.*, 1985). As menores seqüências repetidas *in tandem* nos eucariotos são conhecidos como microsatélites, as quais apresentam de 1-6 nucleotídeos por unidade de repetição. Os mesmos podem estar presentes em regiões cromossômicas eucromáticas como podem estar associados com a heterocromatina constitutiva. Dentre os exemplos de microsatélites, podem ser citadas as distintas seqüências de DNA telomérico (Schlötterer, 2000). Estas definições das diferentes classes de DNAs repetitivos *in tandem* são controversas na literatura e diversas exceções para cada classe já foram descritas.

Os elementos transponíveis são diferenciados das outras seqüências repetitivas presentes nos genomas eucariotos simplesmente por sua capacidade de transposição/movimentação para distintas regiões. Grande parte dos diversos elementos repetitivos são originados dos elementos transponíveis e os mesmos representam uma grande porção dos genomas, por exemplo cerca de 40% do genoma humano. Os mesmos são divididos em dois grupos básicos, os transposons e retrotransposons de acordo com o seu mecanismo de transposição. Os transposons se movimentam nos genomas a partir de moléculas de DNA, enquanto os retrotransposons se transpõem utilizando RNAs via transcrição reversa. Diversas famílias destes elementos já foram identificadas nos genomas, podendo ocorrer em espécies não relacionadas ou presentes apenas em grupos específicos (Biémont e Vieira, 2006; Feschotte e Pritham, 2007; Wicker *et al.*, 2007).

Esta ampla variedade em relação à quantidade, seqüências nucleotídicas e localização dos elementos repetitivos de DNA se deve basicamente aos mecanismos evolutivos de conversão gênica, transposição, *crossing-over* desigual, replicação *slippage* e ocorrência de DNAs extracromossômicos circulares (eccDNA). Estes mecanismos podem atuar sinergicamente nas diferentes classes de seqüências ou atuarem isoladamente ao longo do tempo evolutivo (Charlesworth *et al.*, 1994; Ugarković e Plohl, 2002; Cohen e Segal, 2009).

1.3.2. Heterocromatina e mapeamento cromossômico de DNAs repetitivos em Coleoptera

Diferentes técnicas citogenéticas têm sido utilizadas para análise dos DNAs repetitivos em insetos, principalmente relacionadas à distribuição e qualificação da heterocromatina constitutiva. Dentre estas técnicas as mais aplicadas para estudos com representantes da ordem Coleoptera são o bandeamento C e em menor escala o uso de fluorocromos base-específicos (CMA_3 e DAPI), além de poucos estudos com mapeamento de DNAsat. Apesar da grande diversidade em Coleoptera, tanto cromossômica como em número de espécies e da

facilidade e baixo custo da técnica de bandeamento C, os estudos com descrição da localização dos blocos de heterocromatina são escassos. De acordo com Rozèk et al. (2004) o pequeno número de estudos se deve a limitada quantidade de heterocromatina nos cromossomos dos coleópteros, o que dificulta a análise e descrição dos segmentos heterocromáticos, principalmente em fases onde os cromossomos se encontram bastante condensados, tais como metáfases.

Em geral a heterocromatina nos representantes de Coleoptera encontra-se preferencialmente localizada nas regiões pericentroméricas dos cromossomos autossômicos, podendo em algumas espécies serem observados blocos adicionais em regiões intersticiais ou terminais dos cromossomos. No cromossomo X, esta fração genômica encontra-se restrita a região pericentromérica ou distribuída ao longo de toda extensão do cromossomo, enquanto o y em geral é eucromático (Rozèk *et al.*, 2004). Em relação à riqueza de pares de base a heterocromatina neste grupo pode apresentar-se rica em GC, AT, AT/GC ou neutra (Juan *et al.*, 1993; Ugarković *et al.*, 1996; Moura *et al.*, 2003; Bione *et al.*, 2005a). Na família Scarabaeidae, assim como para Coleoptera como um todo, a heterocromatina constitutiva ocorre principalmente nas regiões pericentroméricas dos cromossomos. Entretanto, este grupo destaca-se por apresentar ampla variabilidade, com blocos adicionais de heterocromatina, sendo uma característica bastante marcante a presença de cromossomos difásicos (um braço heterocromático e outro eucromático) em alguns representantes da subfamília Scarabaeinae (Moura *et al.*, 2003; Bione et al., 2005a,b; Wilson e Angus, 2005; Colomba *et al.*, 2006; Cabral-de-Mello *et al.*, 2010a,b; Oliveira *et al.*, 2010). Quanto à riqueza de pares de bases, embora poucas espécies tenham sido analisadas (cerca de 20), a heterocromatina é heterogênea, com espécies apresentando blocos ricos em pares GC, AT, GC/AT e neutros (Moura *et al.*, 2003; Vitturi *et al.*, 2003; Bione *et al.*, 2005a,b; Wilson e Angus, 2005; Colomba *et al.*, 2006; Cabral-de-Mello *et al.*, 2010b; Oliveira *et al.*, 2010).

O uso de seqüências repetitivas de DNA tem se mostrado marcadores cromossômicos informativos em estudos citogenéticos comparativos, análises de estrutura e evolução de genomas, origem e evolução de cromossomos supernumerários e sexuais e para identificação de rearranjos cromossômicos. Estas seqüências são bastante úteis para propósitos de mapeamento citogenético devido a sua organização *in tandem* ou enriquecimento das mesmas em algumas regiões cromossômicas, permitindo a visualização de blocos ao longo dos cromossomos. Em insetos o mapeamento de seqüências de DNAs repetitivos é escasso. Além disso, para maior parte das espécies estudadas as análises têm sido concentradas na descrição do número e localização dos sítios de DNA ribossomal (DNAr), principalmente o DNAr 45S, embora existam descrições de localização de diferentes DNAsat, DNAr 5S, transposons, microsatélites e genes de histonas (Cabrero e Camacho, 2008; Loreto *et al.*, 2008; Cabrero *et al.*, 2009; Nguyen *et al.*, 2010; Teruel *et al.*, 2010).

Para a ordem Coleoptera, assim como para insetos em geral, o mapeamento de seqüências repetitivas tem sido focado na análise do DNAr 45S, embora os estudos sejam ainda escassos (De La Rúa *et al.*, 1996; Gómez-Zurita *et al.*, 2004; Almeida *et al.*, 2010; Cabral-de-Mello *et al.*, 2010b). Neste grupo, além do DNAr 45S, distintos DNAsat foram mapeados em 25 espécies pertencentes as famílias Chrysomelidae, Cicindelidae e Tenebrionidae (revisado por Palomeque e Lorite, 2008). Assim como a heterocromatina, os DNAs satélites em Coleoptera se concentram nas regiões pericentroméricas. Em geral estas seqüências são compartilhadas entre todos os cromossomos autossômicos e para algumas espécies, ocorre também nos cromossomos sexuais. Além disso, as mesmas podem ser compartilhadas entre espécies diferentes pertencentes ao mesmo gênero, tal como observado em espécies do gênero *Tribolium* (Juan *et al.*, 1993; Barceló *et al.*, 1998) ou apresentarem ocorrência espécie-específica como descrito em *Chrysolina americana* (CAMA 189-pb) e *C. carnifex* (CCAH 211-pb) (Lorite *et al.*, 2001; Palomeque *et al.*, 2005).

Em Scarabaeidae o DNAr 45S foi mapeado em apenas 18 espécies, das quais nove pertencem à subfamília Scarabaeinae, revelando ampla variabilidade quanto ao número e localização dos clusters. Neste grupo o DNAr foi localizado restritamente em cromossomos autossômicos, sexuais ou ambos, variando de dois clusters (um bivalente) ocorrente em diversas espécies a 16 sítios em *Coprophanaeus ensifer*, correspondendo ao maior número de sítios para a ordem Coleoptera (Colomba *et al.*, 2000, 2006; Moura *et al*., 2003; Bione *et al.*, 2005a,b; Arcanjo *et al.*, 2009; Silva *et al.*, 2009; Cabral-de-Mello *et al.* 2010b; Oliveira *et al.*, 2010). Enquanto outras famílias multigênicas, como de RNAr 5S e histonas, já foram mapeadas em outros insetos, tais genes até o momento ainda não foram mapeados em Coleoptera.

O uso de elementos repetitivos de DNA tem se mostrado uma ferramenta esclarecedora para diversas questões, desde o entendimento da estrutura de diferentes regiões cromossômicas, tais como centrômeros e telômeros, a análises relacionadas à diversificação cariotípica, incluindo cromossomos autossômicos e origem e evolução de cromossomos sexuais e supernumerários. Além disso, o mapeamento físico cromossômico do genoma tem contribuído no entendimento da estrutura e evolução dos genomas eucariotos, principalmente em relação aos DNAs repetitivos. Em Scarabaeinae, o uso do mapeamento de seqüências repetitivas de DNA apresenta-se como uma ferramenta útil no entendimento da ampla diversidade cariotípica observada no grupo, podendo ser esclarecedora dos processos que governam a evolução de seus cariótipos e genomas como um todo.

2. OBJETIVOS

2.1. Objetivo Geral

- Caracterizar a organização cromossômica de seqüências de DNAs repetitivos em espécies pertencentes à subfamília Scarabaeinae (Coleoptera, Scarabaeidae) através de técnicas citogenéticas clássicas e mapeamento físico cromossômico.

2.2. Objetivos específicos

- Descrever a macro-estrutura cromossômica, incluindo número diplóide, mecanismo de determinação sexual e padrão de localização da heterocromatina constitutiva, em 23 espécies de Scarabaeinae;
- Analisar a heterocromatina constitutiva de seis espécies pertencentes ao gênero *Dichotomius* (*Dichotomius*, *D. bos*, *D. geminatus*, *D. laeveicollis*, *D. nisus*, *D. semisquamosus* e *D. sericeus*) através do bandeamento C, coloração com fluorocromos base específicos (CMA_3 e DAPI) e mapeamento da fração C_0t-1 DNA utilizando hibridizações espécie-especifica e entre espécies;
- Isolar e mapear as sequências nucleotídicas das familias multigênicas de RNAs ribossomais (RNAr 5S e 18S) e codificantes da histona H3 nos cromossomos de distintas espécies de Scarabaeinae analisando a inter-relação das mesmas;
- Analisar a presença de cromossomos B em duas populações da espécie *D. geminatus* e mapear seqüências de DNAs repetitivos (DNAr 18S, 5S, histona H3 e fração C_0t-1 DNA) buscando elucidar a origem deste cromossomo e sua relação com o complemento A.

3. MATERIAIS E MÉTODOS

3.1. Insetos e amostragem

Foram utilizados indivíduos machos adultos de 28 espécies de Scarabaeinae (Coleoptera, Scarabaeidae), coletados em diferentes localidades do estado de Pernambuco, Minas Gerais, Ceará, Paraná, São Paulo e Mato Grosso (Tabela 1) ao longo dos anos de 2008-2010. As coletas foram realizadas com auxilio de armadilhas de solo tipo *pitfall* iscadas com fezes humanas e carne bovina apodrecida. O material coletado encontra-se depositado na coleção do Laboratório de Biodiversidade e Genética de Insetos, Universidade de Pernambuco, Recife/PE, no Laboratório de Genômica Integrativa, Universidade Estadual Paulista, Botucatu/SP e no museu da Universidade Federal de Lavras, Lavras/MG. O material foi coletado com autorização do IBAMA, processo número 16278-1 e identificado pelos especialistas no grupo, Dr. Fernando Vaz-de-Mello (Universidade Federal do Mato Grosso) e Msc. Fernando Augusto Silva (Universidade Federal de Lavras). Além disso, material biológico de algumas espécies analisadas já se encontrava armazenado em freezer -20 ^{o}C no Laboratório de Biodiversidade e Genética de Insetos, Universidade de Pernambuco, Recife/PE.

3.2. Obtenção de cromossomos e análises convencionais e diferenciais

Os cromossomos foram obtidos de acordo com a técnica clássica de esmagamento de folículos testiculares com ácido acético 45% e utilizados para o bandeamento C, coloração com fluorocromos e hibridização *in situ* fluorescente (FISH). Para colorações convencionais foi utilizado orceína lacto-acética 2%. A técnica de banda C seguiu o protocolo descrito por Sumner (1972) e para a coloração com fluorocromos base específicos foi utilizado o protocolo proposto por Schweizer (1983).

Espécies	Origem										
	Terra Roxa, PR	Botucatu, SP	Aldeia, PE	Caruaru, PE	Igarassu, PE	Saloá, PE	Ipojuca, PE	Carrancas, MG	Crato, CE	Barra do Garças, MT	Total
Atheuchus sp		25									25
Canthon staigi				20							20
Coprophanaeus cyanescens				09							09
Coprophanaeus ensifer			03								03
Deltochilum calcaratum				37	32						69
Deltochilum elevatum								06			06
Deltochilum morbillosum					15						15
Deltochilum verruciferum				04							04
Diabroctis mimas				03							03
Dichotomius affinis		15									15
Dichotomius bos		09				17					26
Dichotomius crinicollis								23			23
Dichotomius depresicollis									11		11
Dichotomius geminatus					43		08				51
Dichotomius laevicollis				32							32
Dichotomius mormon		11									11
Dichotomius aff *mundus*		08									08
Dichotomius nisus	05	09		13	04						31
Dichotomius semianeus		14									14
Dichotomius semisquamosus				25	04	07					36
Dichotomius sericeus					26						26
Dichotomius sericeus sp1				35							35
Dichotomius sp										17	17
Digitonthophagus gazella		15									15
Eurysternus caribaeus			12								12
Ontherus appendiculatus	02										02
Ontherus sulcator	10										10
Phanaeus splendidulus				15							15

Tabela 1. Espécies utilizadas neste trabalho com respectivos locais de coleta e número de indivíduos.

3.3. Isolamento de Seqüências Repetitivas e genes mitocondriais por PCR e fração C_0t-1 DNA

A extração de DNA foi realizada a partir de tecido muscular localizado no pronoto e seguiu basicamente o protocolo apresentado por Sambrook and Russel (2001) (protocolo detalhado, anexo 1).

DNAs ribossomais e histona H3

Foi utilizado DNA genômico da espécie *Dichotomius semisquamosus* para amplificação do DNAr 18S e 5S através do uso dos primers: 18S, Sca18SF 5'CCC CGT AAT CGG AAT GAG TA), Sca18SR 5'GAG GTT TCC CGT GTT GAG TC; 5S, Sca5S1F 5'TAC CGG TTC TCG TCC GAT CAC e Sca5S1R 5'TAC AGC GTG CTA TGG CCG TTG. Estes primers foram deduzidos de seqüências codificadoras destes genes de diversas espécies de insetos depositadas no GeneBank para o DNAr 18S e 5S. Para amplificação do gene de histona H3 foram utilizados os seguintes primers degenerados: ScaH3F1 5´GGC NMG NAC NAA RCA RAC; ScaH3R1 5´TGD ATR TCY TTN GGC ATD AT, desenhados através da análise de seqüências desta proteína depositada no GeneBank para diversos animais (protocolo detalhado, anexo 2).

Fração C_0t-1 DNA

DNAs altamente e moderadamente repetitivos (fração C_0t-1 DNA) foram isolados do genoma de seis espécies do gênero *Dichotomius*, *D. bos*, *D. geminatus*, *D. laeveicollis*, *D. nisus*, *D. semisquamosus* e *D. sericeus* através da técnica descrita por Zwick et al. (1997) e adaptada por Ferreira e Martins (2008), o qual está baseado na cinética de reassociação do DNA, com modificações (protocolo detalhado, anexo 3). Esta fração de DNA altamente e

moderadamente repetitivos foi utilizada como sonda em hibridizações espécie-específicas e entre espécies nas seis espécies do gênero *Dichotomius.*

Genes mitocondriais

As seqüências do Citocromo Oxidase I (COI) e do RNAr 16S foram isoladas de seis espécies representativas do gênero *Dichotomius*, *D. bos*, *D. geminatus*, *D. laevicollis*, *D. nisus*, *D. semisquamosus* e *D. sericeus*. Seqüências de COI foram amplificadas utilizando os primers FishF2 e FishR2 (Ward *et al.*, 2005), enquanto para amplificação do rRNA 16S foram utilizados os primers 16SscaF- 5'CGC CTG TTT AAC AAA AAC AT e 16SscaR- 5'CTC CGG TTT GAA CTC AGA TCA desenhados a partir de seqüências depositadas no NCBI (AY131513-AY131516) obtidas de representantes do gênero *Dichotomius*.

3.4. Clonagem, seqüenciamento e Análise das Seqüências

Os fragmentos de DNA isolados através de PCR foram inseridos em vetores plasmidiais e utilizados para transformar bactérias competentes *Escherichia coli* DHα65, utilizado o kit pGEM-T (Promega) de acordo com as recomendações dos fabricantes (protocolo detalhado, anexo 4).

Os plasmídios recombinantes (10-20 clones escolhidos aleatoriamente) foram submetidos à sequenciamento nucleotídico através do seqüenciador automático ABI 3100 (Aplied Biosystems). As seqüências foram processadas retirando-se as regiões dos plasmídios e alinhadas online utilizando-se o programa ClustalW (Thompson *et al.*, 1994), website http://www2.ebi.ac.uk/clustalw para geração de uma seqüência consenso. Além disso, uma busca por similaridades foi realizada através do sistema de pesquisa Blastn (Altschul *et al.,*1990) do National Center for Biotechnology Information (NCBI), website (http://www.ncbi.nlm.nih.gov/blast).

3.5. Análises filogenéticas

As seqüências nucleotídicas para cada espécie foram inicialmente analisadas usando o software BioEdit 5.0.9 (Hall 1999) e uma seqüência consenso foi determinada para cada gene de cada espécie estudada. Para checagem das seqüências dos fragmentos amplificados as mesmas foram submetidas à pesquisa de similaridade com seqüências depositadas no NCBI (http://www.ncbi.nlm.nih.gov/) utilizando-se a ferramenta BLAST (Altschul et al. 1990). As seqüências consenso foram depositadas na base de dados do NCBI com os números de acesso seguintes: HQ824533-HQ824544. Para realização do alinhamento das seqüências obtidas foi utilizado o programa Muscle (http://www.ebi.ac.uk/Tools/muscle/index.html) (Edgar 2004). Variações nucleotídicas e distâncias genéticas foram estimadas utilizando o MEGA 4.0 (Tamura et al. 2007). A possível saturação de substituições de nucleotídeos foi avaliada pelo programa DAMBE plotando-se o número absoluto de transições (Ti) e transversões (Tv) contra os valores de distancia genética estimada pelo modelo Kimura-2-parâmetros. A escolha do melhor modelo de distância genética foi realizada com o programa Modeltest 3.06 (Posada and Crandall 1998).

O método Bayesiano de análise filogenética (Huelsenbeck et al. 2001) foi usado para avaliar topologias alternativas para as árvores filogenéticas através da estimativa das probabilidades posteriores utilizando-se o programa MrBayes v.3.0 (Ronquist and Huelsenbeck 2003) sendo gerada 3.000.000 de gerações. A árvore consenso foi produzida utilizando-se o TreeExplorer como implementado no MEGA 4 (Tamura et al. 2007). Como grupo externo foram utilizadas espécies pertencentes a distintos gêneros da subfamília Scarabaeinae.

3.6. Hibridação *in situ* fluorescente (FISH)

Marcação das sondas

Para FISH as seqüências de DNAr 18S, histona H3 e fração C_0t-1 DNA foram marcadas com biotina através da reação de *nick translation*, utilizando o kit (Bio-Nick, Invitrogen), enquanto a seqüência de DNAr 5S foi marcada com digoxigenina através da reação de *nick translation* utilizando o kit DIG-NICK (Roche) ou através de PCR utilizando como molde DNA plasmidial (protocolo detalhado, anexo 5).

Pré-tratamento das lâminas e hibridização *in situ*

As preparações cromossômicas foram pré-tratadas com RNAse (100 μg/ml) por 1 hora em câmara úmida a 37º C, lavadas em 2xSSC por 5 min (3 lavagens); incubadas em solução de pepsina (10 μg/ml) por 20 min em câmara úmida a 37º C, lavadas em 2xSSC por 5 min (2 lavagens); incubadas em solução de formaldeído (3.7%) por 10 min a temperatura ambiente, lavadas em 2xSSC por 5 min (2 lavagens); em seguida desidratadas em etanol 70% e 100% por 5 min cada.

A hibridização *in situ* seguiu o protocolo descrito por Pinkel et al. (1988), com modificações. Para o mix de hibridização foram utilizados cerca de 40-120ng de sonda, formamida (50%), sulfato de dextrano (10%) e 2xSSC. O mix de sonda foi desnaturado a 95º C durante 10 min, adicionado a lâmina, sendo a mesma incubada a 75º C durante 3-5 min dependendo da espécie. Por fim as preparações foram incubadas em câmara úmida a 37º C por ao menos 18 horas.

Lavagens pós-hibridização, detecção e amplificação do sinal

As lâminas foram lavadas em solução de 2xSSC (2 lavagens), seguida de lavagens em 0,1xSSC (2 lavagens) e 1 lavagem em 2xSSC, todas a temperatura de 42º C e durante 5 min,

para retirada do excesso de sonda. A detecção das sondas marcadas com bitotina foi realizada com uso do fluorocromo FITC associado à avidina (Invitrogen), enquanto as marcadas com digoxigenina foram detectadas com Rodamina associada à anti-digoxigenina (Roche), durante 1 hora a 37º C. O sinal das sondas marcadas com biotina foi amplificado utilizando antiavidina conjugada com biotina (Sigma) durante 10 min a 37º C, e com mais um *round* de FITC conjugado a avidina durante 10 min a 37º C. Após cada passo de detecção três lavagens foram realizadas a 45 ºC durante 5 min utilizando PBD (para cada 100,0 mL: 1,0 g de leite em pó desnatado; 20,0 mL 20xSSC; 0,5 mL de Triton 100x; H_2O q.s.p. 100,0 mL). Os cromossomos foram contra corados e as lâminas foram montadas com meio Vectashield (Vector) conjugado com DAPI (2 μg/mL).

Algumas preparações foram submetidas à FISH mais de uma vez, com objetivo de mapeamento das seqüências de DNAr 18S, 5S e histona H3 na mesma célula. Para o mesmo foi realizado uma double-FISH com as sondas de DNAs ribossomais, em seguida as lâminas foram lavadas em 2xSSC por 30 min e re-hibridadas com as sondas de DNAr 5S e histona H3. Todos os resultados foram certificados através do uso de double-FISH isoladas utilizando DNAr 18S e 5S e DNAr 5S e histona H3.

Registro dos resultados e análise dos dados

Os resultados foram analisados e registrados utilizando o microscópio Olympus BX61 acoplado a câmera DP71. O contraste, brilho e sobreposição das imagens foram realizados utilizando o programa Adobe Photoshop CS2. Além disso, o mesmo foi utilizado para montagem das pranchas, conversão da cor dos cromossomos para cinza e modificação da cor do sinal do DNAr 18S para violeta em algumas células com apresentação de três sinais. Para cada seqüência analisada ao menos três indivíduos foram utilizados e vinte metáfases além de outras fases do ciclo de divisão celular foram estudadas, para confirmação dos resultados.

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5. RESULTADOS E DISCUSSÃO

Os resultados da presente tese estão apresentados em quatro manuscritos a seguir, sendo um publicado na revista Heredity (ISSN 0018-067X) (anexo 6), um aceito na revista Genetica (ISSN 1573-6857) um submetido e em fase de revisão na Cytogenetic and Genome Research (ISSN 1424-8581) e um a ser submetido a Chromosome Research (ISSN 0967-3849). Além disso, também estão apresentados em anexo (anexos 7-9) trabalhos paralelos desenvolvidos com ortópteros envolvendo o mesmo enfoque relacionado à tese, sendo um publicado na revista Cytogenetic and Genome Research (ISSN 1424-8581), um a ser submetido à Cytogenetic and Genome Research (ISSN 1424-8581) e um a Heredity (ISSN 0009-5915).

5.1. Capítulo 1:

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

Cabral-de-Mello DC, Moura RC and Martins C

Manuscrito publicado na revista Heredity (2010), 104:393-400 (anexo 6).

Abstract

Chromosomal banding techniques and repetitive DNA mapping have been useful tools in comparative analysis and elucidation of genome organization of several groups of eukaryotes. In this study, we contribute to the knowledge of Coleoptera genomes by reporting the chromosomal organization of repetitive DNA sequences and the presence and characteristics of a B chromosome in two natural populations of *Dichotomius geminatus* (Coleoptera, Scarabaeidae) by 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 made up almost entirely of heterochromatin. Physical mapping using double FISH was performed for the first time in Coleoptera; using DNA probes for 5S and 18S rRNA and histone H3 genes, we revealed that ribosomal DNAs are located in chromosomes three and four, while 5S rRNA and histone H3 genes are colocalized in chromosomal pair two 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 two, three or four. On the other hand, mapping of the C_0t-1 DNA fraction revealed 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 the genome organization and evolution of repetitive elements in Coleoptera and other insects regarding both A and B chromosomes.

Keywords: cytogenetics, C_0t-1 DNA, FISH, genome evolution, heterochromatin, supernumerary chromosome.

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 (rRNA) (Hadjiolov, 1985; Maxon *et al.*, 1983). 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). Repetitive DNAs in particular 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 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 about 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 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 Scabaraeidae, 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 (Rożek *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 describe the association of 5S rRNA and histone H3 genes, as well as the characteristics of a newly detected B chromosome.

Material and methods

Animals, DNA samples and chromosome preparation

Samples of *Dichotomius geminatus* (Arrow 1913) individuals were collected in Igarassu ($7^{o}50'03"S:34^{o}54'23"W$) (43 males) and in Maracaípe ($8^{o}31'47"S:35^{o}$ 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 Russell (2001).

Meiotic chromosomes were obtained from testicular cells. All individuals from Igarassu provided usable preparations, while only eight animals from Maracaípe were useful to this study. The rest of 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 immersion in liquid nitrogen for a few seconds. C-banding was performed by the method described by Sumner (1972) and the silver nitrate staining according Rufas *et al.* (1987).

Isolation of repetitive DNA

Partial sequences of 18S rRNA, 5S rRNA, and histone H3 genes were obtained by polymerase chain reaction (PCR) of genomic DNA from *Dichotomius semisquamosus*. Primer sets were designed based on 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) and the recombinant constructs were used to transform DH5α *E. coli* competent cells. Positive clones were sequenced with an ABI Prism 3100 automatic DNA sequencer (Applied Biosystems) with a Dynamic Terminator Cycle Sequencing kit (Applied Biosystems), following 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 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 based on the renaturation kinetics of C_0t-1 DNA (DNA enriched for highly and

moderately repetitive DNA sequences) (Zwick *et al.*, 1997, Ferreira and Martins, 2008). DNA samples (200 µL of 100–500 ng/µL genomic DNA in 0.3 M NaCl) were autoclaved for 30 minutes at 1.4 atmospheres of pressure and 120 ^{o}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 µL DNA fragments were denatured at 95 ^{o}C for 10 minutes, placed on ice for 10 seconds, and transferred to a 65 ^{o}C water bath for reannealing. After 1 minute of reannealing, the samples were incubated at 37 ^{o}C for 8 minutes 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 (FISH)

The plasmids containing the 18S rRNA and histone H3 genes, and the C_0t-1 DNA fraction were labeled by nick translation, using biotin-14-dATP (Invitrogen). For simultaneous hybridization (double FISH), the 5S rRNA gene clone was labeled using digoxigenin-11-dUTP (Roche). Meiotic chromosome slides were incubated with RNase (100 µg/mL) for 1.0 hours and with pepsin (10 µg/mL) for 20 minutes in a moist chamber at 37 ^{o}C. The slides were fixed at room temperature using 37 % formaldehyde in Phosphate buffer detergent (PBD) solution and dehydrated in 70 % and 100 % ethanol for 5 minutes. The hybridization mixture (100 ng denatured probe, 50 % formamide, 10 mg/mL dextran sulfate, 2× SSC), in a final volume of 15 µL, was dropped onto slides that were previously denatured using 70 % formamide, 2× SSC for 40-60 seconds at 70 ^{o}C. In the double FISH experiments, 15 µL hybridization mixture of each probe was dropped onto the slides. The slides were covered with coverslips and incubated at 75 ^{o}C for 5 minutes. Hybridization was performed overnight

at 37 ^{o}C in a moist chamber. The probes labeled with biotin were detected by avidin-FITC conjugate (Sigma), and the digoxigenin-labeled probes were detected using anti-digoxigenin-Rhodamine (Roche). All preparations were counterstained with DAPI and mounted with Vectashield (Vector).

Results

Karyotype and chromosomal banding

The standard karyotype observed in *Dichotomius geminatus* was 2n = 18,Xy$_p$, with metacentric (1, 2, 5, 6 and 8), submetacentric (3 and 7) and subacrocentric (4) autosomes, a subacrocentric X and a punctual y chromosome (Fig. 1a). In addition, 9 individuals among the 43 analyzed from Igarassu and 2 of 8 from Maracaípe carried 1B chromosome, corresponding to an average prevalence 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 one 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 (Fig. 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. Additionally, two small chromosomal pairs (six and seven) contained terminal blocks of heterochromatin in one homologue, forming heteromorphic pairs (Fig. 1c,d). The B chromosome was completely heterochromatic (Fig. 1e,f) and the silver nitrate staining revealed only one active NOR (nucleolar organizer region) (Fig. 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 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% to 84% similarity with sequences from species belonging to the Porifera, Cnidaria, Echinodermata and Mollusca groups, as well as sequences from some insects and vertebrates.

FISH with rDNA probes (18S and 5S) revealed the presence of rDNA sites in distinct chromosomes. The 18S rDNA sites were located in the short arm of autosomal pairs three and four, while hybridization of 5S revealed one proximal site in pair two in most of the individuals analyzed (Fig. 2a-c). In three specimens, pair two was heteromorphic for the presence of 5S rDNA sites, possessing only one site in one of the homologous (Fig. 2c). The histone H3 cluster colocalized to the same region as the 5S rDNA site (Fig 2d,e). The C_0t-1 DNA fraction hybridization pattern was coincident with areas of heterochromatin, including the terminal blocks of two small pairs (Fig. 3a).

In the individuals with 1B chromosomes, the hybridized probes of multigenic families (18S and 5S rDNAs and histone H3) showed the same pattern observed in the 0B individuals, and no hybridization was seen in the B chromosome (Fig. 2f-h). In 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 (Fig. 3b,c). All results of hybridized probes are schematized in the figure 3d.

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 performed 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 (i.e. *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 *Isocopris 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 rDNA, 18S rDNA and histone H3 genes against the NCBI database reflect differences in the evolutionary rates of these sequences in the distinct taxa. Although despite these differences, an overall similarity index greater than 80% from the three sequences was observed when compared to 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 Schneider *et al.*, 2007). These results were largely observed using silver nitrate staining, which detects only active NORs. The presence of only one NOR site detected by silver nitrate staining in *D. geminatus* does not correspond to the real genome organization of 45S rDNA clusters (two 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, 2000; Sola, 2000; Martins and Galleti, 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; Vituri *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, revealed by Southern blotting.

With regard to the 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; Barzzoti *et al.*, 2000) and one mussel species (Eirín-López *el al.*, 2004). Studies using Southern blot hybridization and/or fiber-FISH experiments will be necessary to clarify the precise organization of these multigene families, concerning the interspersed or syntenic organization in *D. geminatus* and other invertebrate genomes.

Although the association and/or interspersion of multigene families has been reported in protostome invertebrates (Drouin and Moniz de Sá, 1995; Barzzoti *et al.*, 2000; Vitturi *et al.*, 2002; Eirín-López *el al.*, 2004; Vituri *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 play 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, since 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; Barzzoti *et al.*, 2000) and here 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 approximately 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; Moura *et al.*, 2008; Angus *et al.*, 2007). 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 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, due to 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 two, three or four (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 the 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 two. In the same way, 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 possibly is involved with the B chromosome origin and can generates genome diversification. In this way, 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 work will contribute to 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.

Acknowledgements

The study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco (FACEPE). The authors are grateful to Fernando Augusto Barbosa Silva and Cristiane Maria Queiroz da Costa for the taxonomic identification of the specimens analyzed in this study.

References

As referências citadas neste capítulo encontram-se no anexo 6.

Figure legends

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 inicial 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 (*) indicate the chromosome pairs with additional heterochromatic blocks. Bar = 5 μm.

Figure 2. Fluorescent *in situ* hybridization with 18S rRNA, 5S rRNA and histone H3 gene probes in 0B and 1B individuals of *D. 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; Note the absence of hybridization signals on the B chromosome (f-h) and the heterochromatin highlighted after DAPI staining (a-h). The arrows indicate the sex bivalents (Xy_p), and arrowheads indicate the B chromosome. C = centromere. Bar = 5 μm.

Figure 3. C_0t-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 (*) indicate the chromosome pairs with additional heterochromatic blocks. Bar = 5 μm.

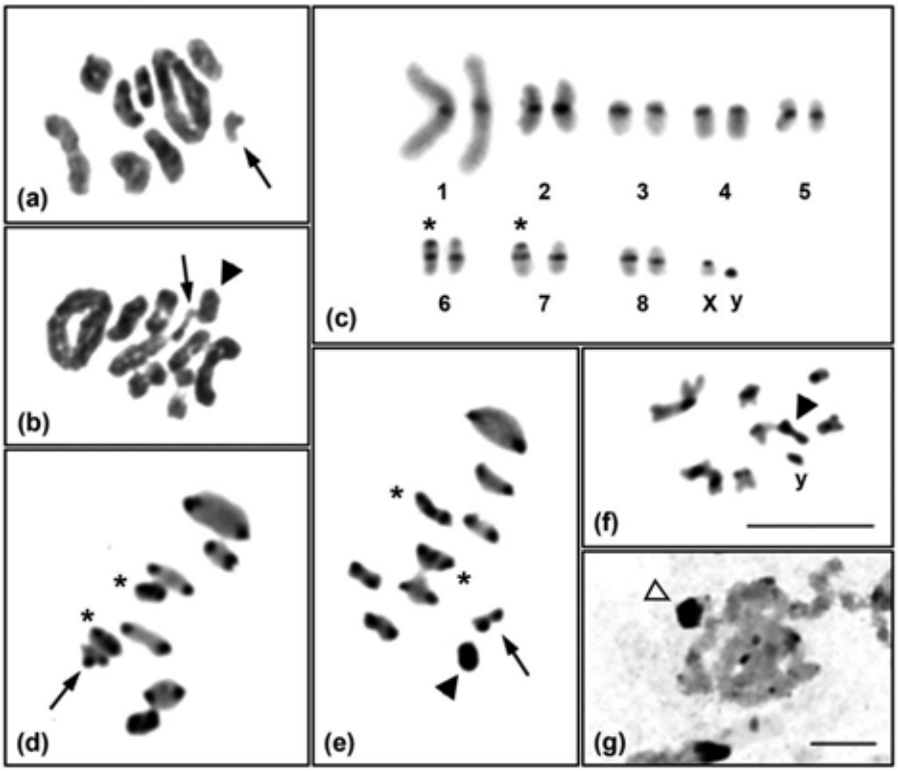


Figure 1

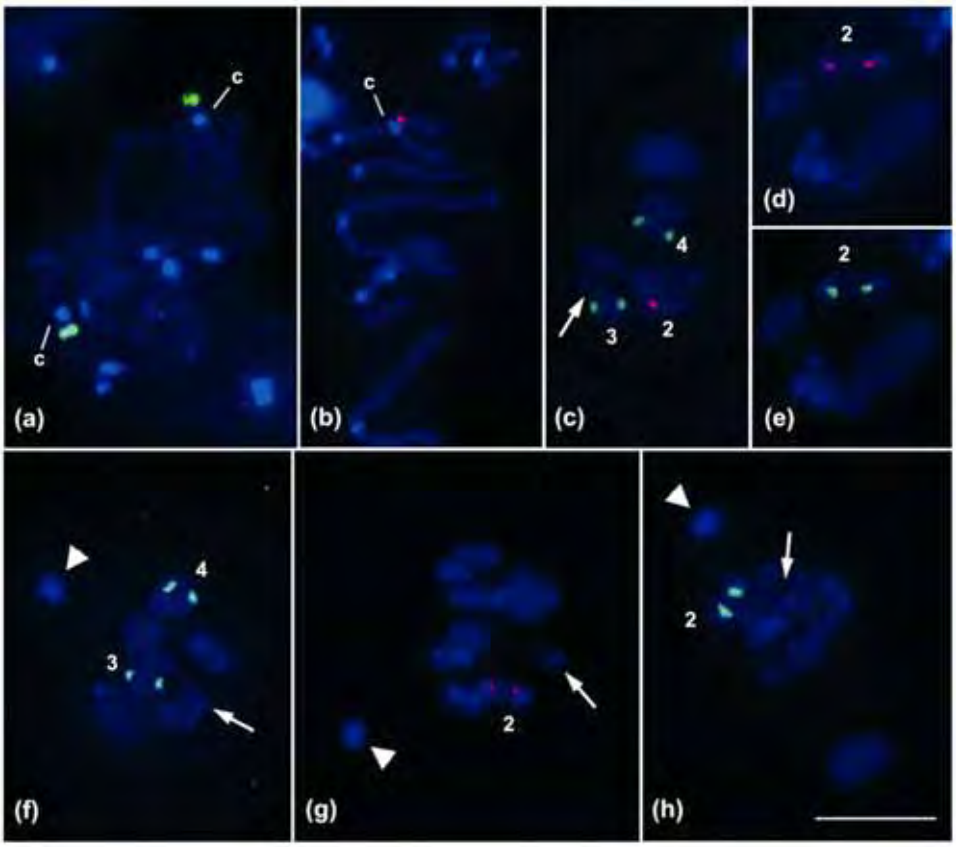
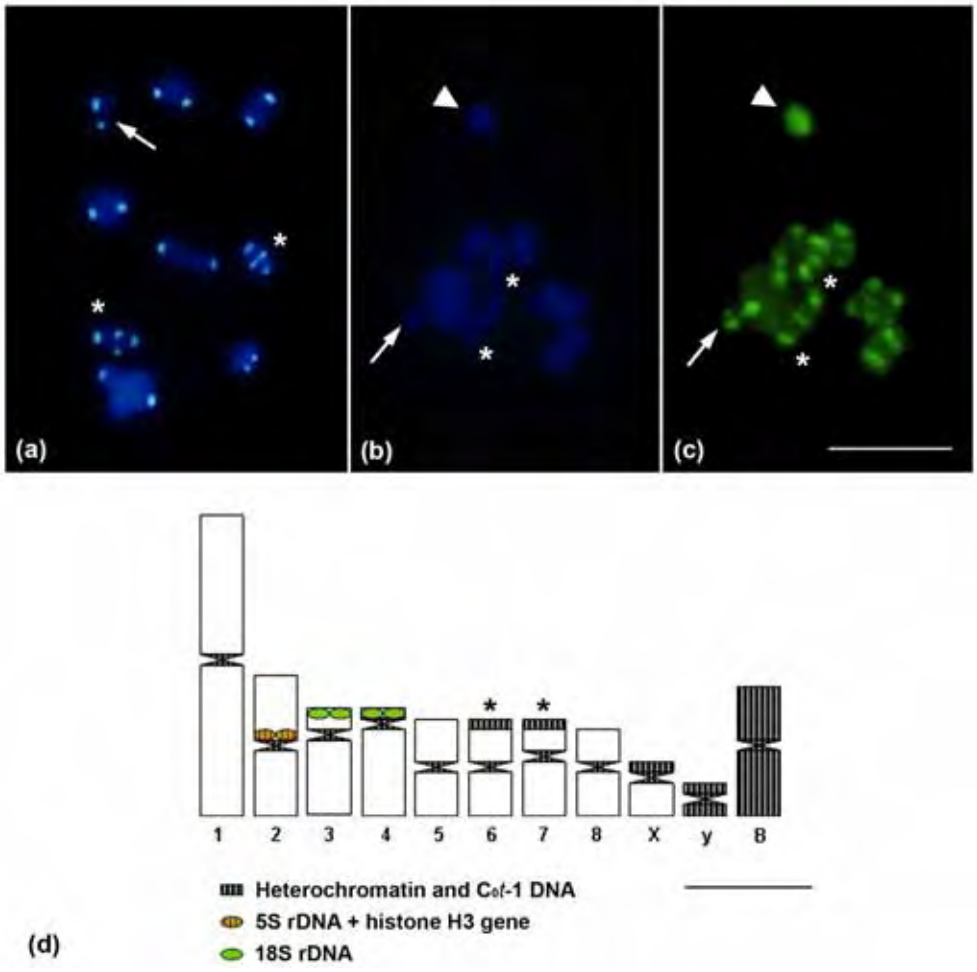


Figure 2


(a)
(b)
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X
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Heterochromatin and Cot-1 DNA
5S rDNA + histone H3 gene
18S rDNA

Figure 3

5.2. Capítulo 2:

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.

Key-words: C_0t-1 DNA, evolution, genome, heterochromatin, repetitive DNAs

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 testis were dissected, fixed in Carnoy (3:1 ethanol:acetic acid) and stored at -20 ^{o}C. Entire animals of each species were frozen and stored in freezer (-20 ^{o}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 ^{o}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 sec and transferred into a 65 °C water bath for reannealing. The distinct times for DNA reannealing were tested from 30 sec 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) (Figure 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 (Figure 1a, d, f, h, j, l; 4a). In *D. geminatus*, additional blocks in the terminal region of the sixth and seventh pairs were observed (Figure 1f; 4a). The X sex chromosome showed heterochromatic blocks that were exclusively located in the pericentromeric regions of *D. laevicollis* (Figure 1d), *D. nisus* (Figure 1h), *D. sericeus* (Figure 1j) and *D. semisquamosus* (Figure 1l). However the heterochromatin was distributed in the pericentromeric area and along the short arm of the X chromosomes in *D. bos* (Figure 1a) and *D. geminatus* (Figure 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 *D. bos* (Figure 1a), *D. laevicollis* (Figure 1d), *D. nisus* (Figure 1h), *D. semisquamosus* (Figure 1l) and *D. sericeus* (Figure 1j). In *D. geminatus*, the y was completely heterochromatic (Figure 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 (Figure 2; 4a). Additional GC-rich blocks were observed in the terminal euchromatic region of the short arm of pair two in *Dichotomius laevicollis* (Figure 2b) and *D. sericeus* (Figure 2e). Moreover, *D. bos* showed CMA_3^+ blocks in the pericentromeric areas of pairs three and six (Figure 2a), while *D. geminatus* had CMA_3^+ blocks in the heterochromatin of pairs three and four, which extended along the short arm (Figure 2f). In *D. semisquamosus*, GC richness was observed in pair three (Figure 2d). With regard to the sex chromosomes, GC-rich heterochromatin was observed in the X chromosomes from *D. bos* (Figure 2a) and *D. nisus* (Figure 2c). Additionally, the pericentromeric area of the y chromosome from *D. nisus* was CMA_3^+ (Figure 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 (thirty sec to five 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 (Figure 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* (Figure 1g), *D. nisus* (Figure 1i) and *D. semisquamosus* (Figure 1m). In *D. bos*, the pairs one, two and three did not reveal specific marks for C_0t-1 DNA hybridization (Figure 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 (Figure 3). Moreover, the X sex chromosome displayed hybridization in all of the species, showing more intense labeling in *D. bos* (Figure 3c, d), *D. nisus* (Figure 3i, j) and *D. semisquamosus* (Figure 3a, b). However, less intense labeling was observed in *D. sericeus* (Figure 3g, h) and *D. laevicollis* (Figure 3e, f). The y chromosome was labeled with dots in all of the species (Figure 3). Low intense hybridization pattern was observed in *D. laevicollis* (Figure 3e, f) and *D. sericeus* (Figure 3g, h), and high intense hybridization was observed in *D. nisus* (Figure 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 and considering also the inclusion of other three *Dichotomius* species (*D. boreus*, *D. pacerpunctatus* and *D.* sp) in the analysis. 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.

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 (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 a low 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 macro-chromosomal 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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Table

Table 1. C_0t-1 DNA fraction reassociation time and chromosomal location for the six *Diichotomius* 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 sec	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 sec	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 sec	Pericentromeric heterochromatin

Figure captions

Figure 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 and 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 = 5µm.

Figure 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 = 5µm.

Figure 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 = 5µm.

Figure 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*.

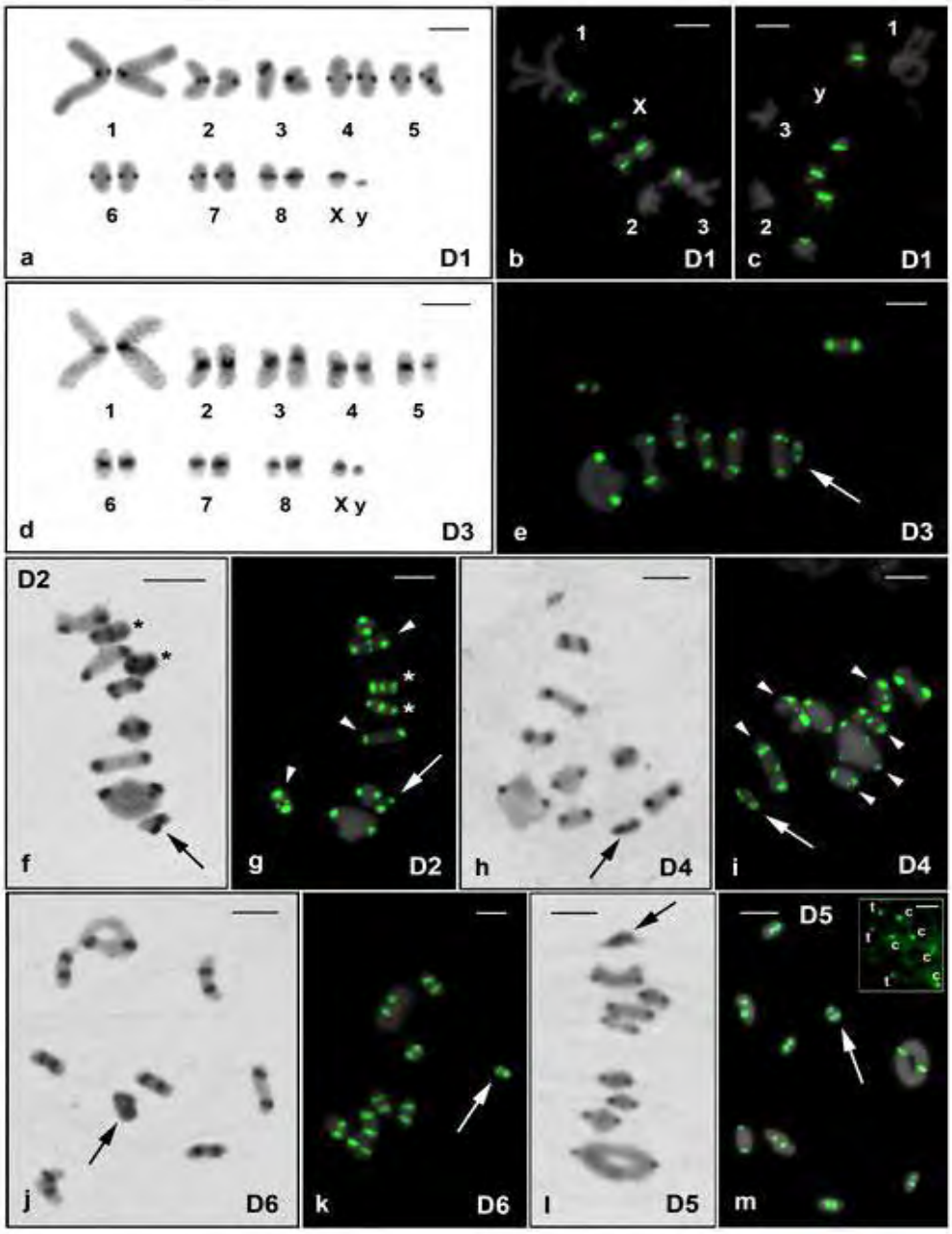

1 2 3 4 5
6 7 8 X y
a D1
1
X
2 3
b D1
1
y
3
2
c D1
1 2 3 4 5
6 7 8 X y
d D3
e D3
D2
f
g D2
h D4
i D4
j D6
k D6
l D5
D5
m

Figure 1

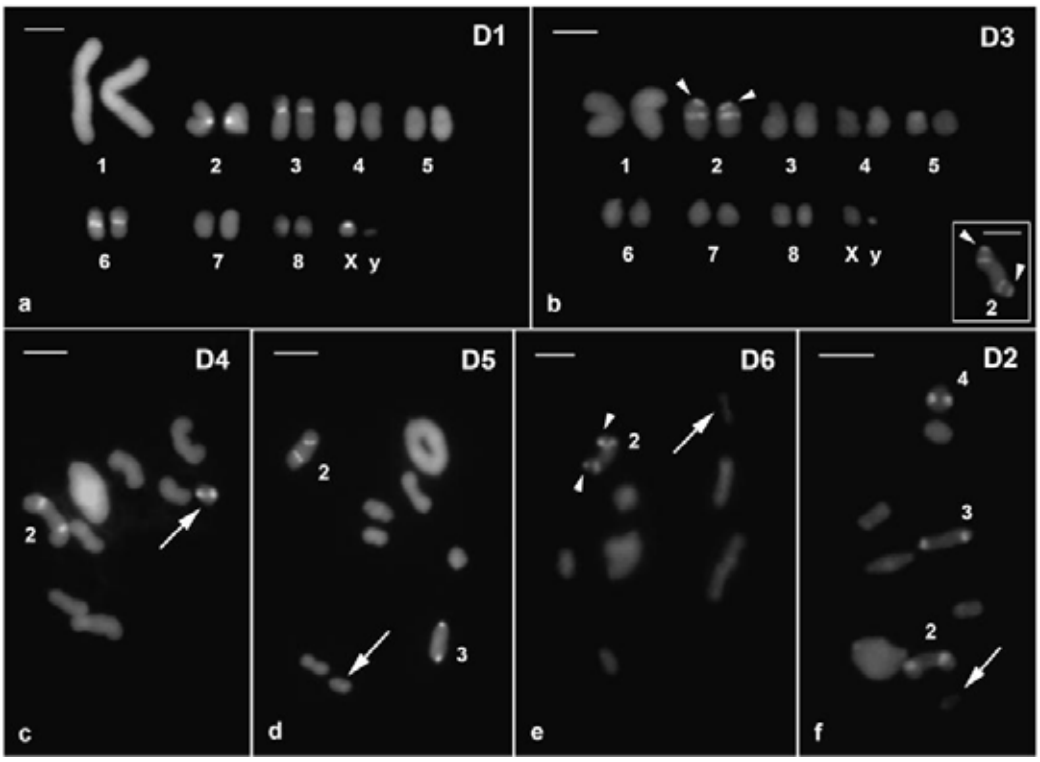


Figure 2

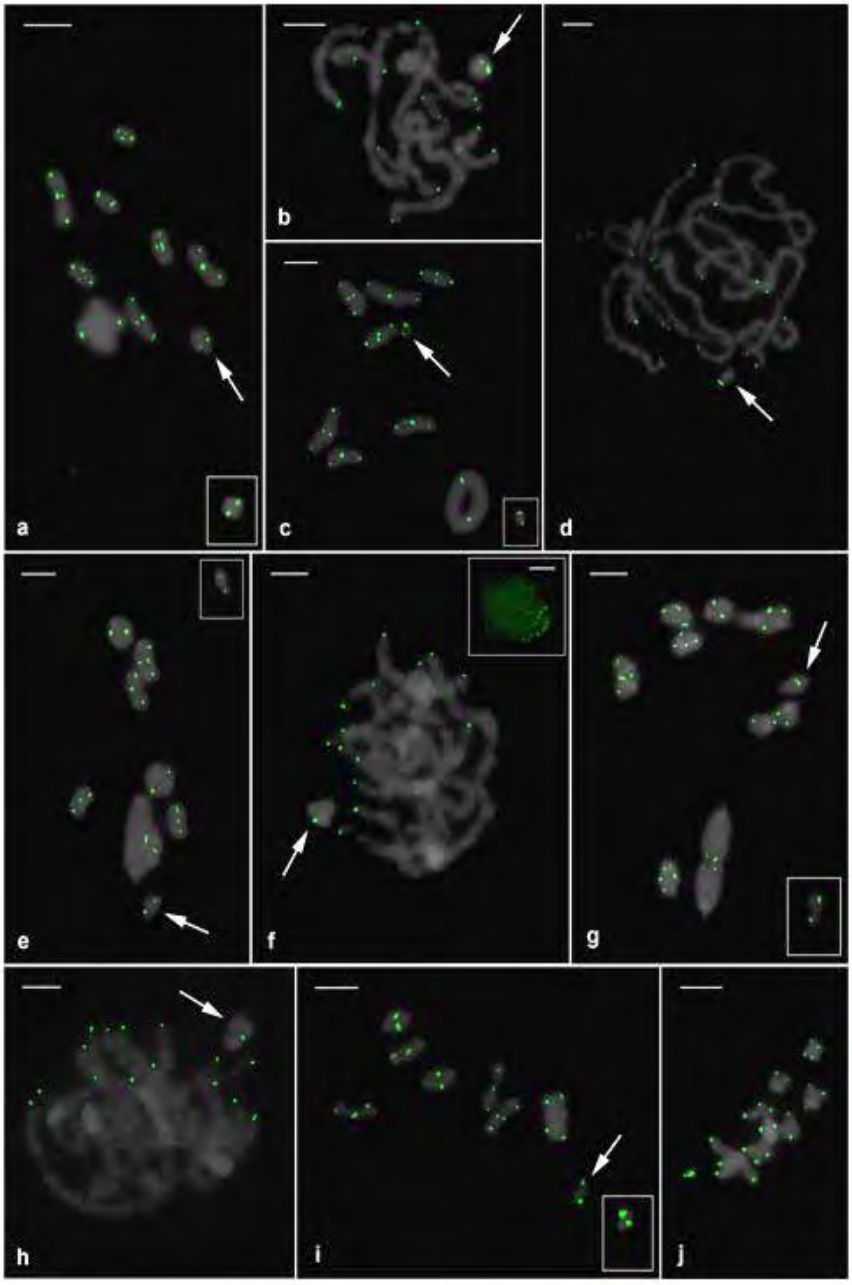
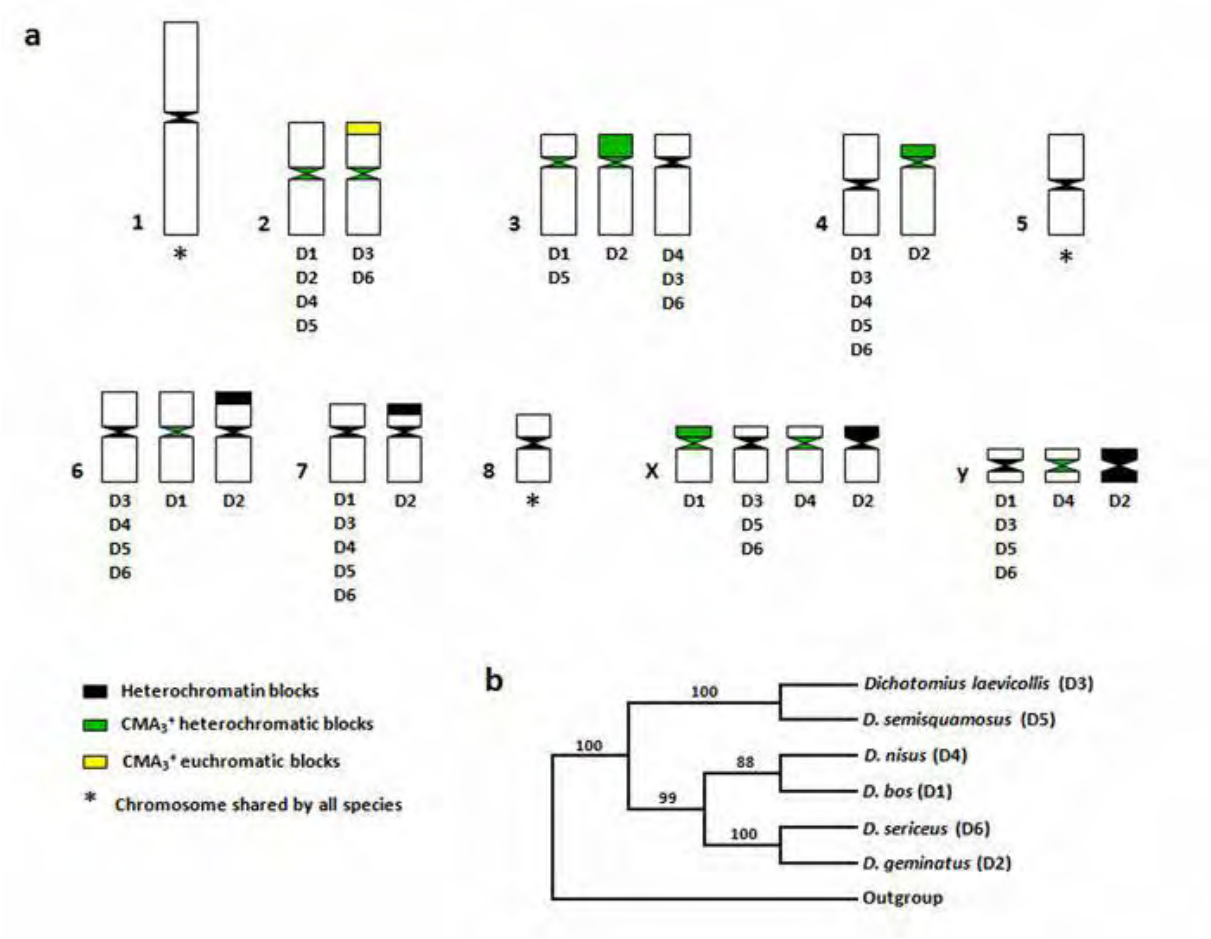


Figure 3


a
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*
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D6
D4
D2
y
D1
D3
D5
D6
D4
D2
Heterochromatin blocks
CMA3+ heterochromatic blocks
CMA3+ euchromatic blocks
* Chromosome shared by all species
b
100
100
88
99
100
Dichotomius laevicollis (D3)
D. semisquamosus (D5)
D. nisus (D4)
D. bos (D1)
D. sericeus (D6)
D. geminatus (D2)
Outgroup

Figure 4

5.3. Capítulo 3:

Cytogenetic mapping of rRNAs and histone H3 genes in 14 species of *Dichotomius* (Coleoptera, Scarabaeidae, Scarabaeinae) beetles

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Abstract

To advance the understanding of chromosomal organization of rRNAs and H3 histone genes in Scarabaeidae beetles and to elucidate the karyotypic differentiation patterns in *Dichotomius* (Coleoptera, Scarabaeidae, Scarabaeinae) genus, classical cytogenetic analysis and chromosomal mapping of the genes for 18S and 5S rRNAs and histone H3 genes were performed in 14 species of the genus. Conserved karyotypes with 2n = 18 and biarmed chromosomes were observed in all species. Moreover the presence of a large metacentric pair (pair one) was characteristic in the studied species, evidencing a remarkable synapomorphy for this genus, probably originated by an ancient autosomal fusion, being conserved the ancestral sex-chromosome bivalent. FISH showed that the 5S rRNA and histone H3 genes are located in the proximal region of pair no. 2, with the two genes co-located. However, the 18S rDNA mapped to one to three chromosomes, being exclusively autosomal, associated with sex elements or both. In most species, the 18S rDNA was observed in pair no. 3, and it was frequently (in about 64.3% of the cases) located in the distal region regardless of the chromosome. The conserved number and position of the 5S rDNA/H3 histone cluster seems to be an ancient pattern shared by all of the studied species. In contrast, the 18S rDNA clusters apparently have suffered distinct patterns of diversification in the karyotypes of the species that could be associated to small inversions, ectopic recombination and transposition. Moreover, we reinforced the association/co-localization between the 5S rRNA and histone H3 genes in this group contributing to the knowledge about the chromosomal organization and diversification patterns of multigene families in beetles and insects.

Keywords: chromosomal evolution, fluorescence *in situ* hybridization, multigene family, Scarabaeidae

Introduction

The ribosomal RNA (rRNA) and histone multigene families are usually arrayed in clusters in eukaryotic genomes located on one or several chromosomes. The major ribosomal cluster (45S rDNA) transcribes 28S, 5.8S and 18S rRNAs being separated from each other by variable intergenic spacers (IGS) and an external transcribed spacer (ETS), whereas the 5S rDNA cluster consists of highly conserved transcribed sequences of 120 bp separated from each other by a variable non-transcribed spacer (NTS) (Long and Dawid, 1980). The histone genes may be clustered in distinct chromosomal regions and among invertebrates these genes are typically clustered as quartets (H2A, H2B, H3 and H4) or quintets (H2A, H2B, H3 and H4 plus H1), although scattered solitary genes were also reported (Lifton et al., 1977; Childs et al., 1981; Engel and Dodgson, 1981; Maxson et al., 1983).

The clustered organization of rDNAs and histone genes makes them useful chromosomal markers that are easily detected, and they have helped to elucidate the karyotypic variation and genomic organization of diverse groups of eukaryotes. Among animals, most studies have focused on the analysis of rDNAs chromosomal location, mostly 45S rDNA (for example Martínez-Navarro et al., 2004; Wang and Guo, 2004; Cabrero and Camacho, 2008; Pisano and Ghigliotti, 2009; Nguyen et al., 2010). The 5S rRNA genes have been primarily mapped in fish (Martins and Galetti, 1999; Martins and Wasko, 2004; Pisano and Ghigliotti, 2009), and the histone genes have been mapped in a few species, for example in mammals, amphibians, fish, molluscs and some insects (Graves et al., 1985; Tripputi et al., 1986; Turner et al., 1988; Hankeln el al., 1993; Pendás et al., 1994; Zhang et al., 2007; Ranz et al., 2003; Cabrero et al., 2009; Cabral-de-Mello et al., 2010a, 2011). These multigene families can be organized in separated clusters or else they can be linked in the same chromosomal region, as have been described, for example in arthropods, annelids, molluscs and fish (Andrews et al., 1987; Drouin et al., 1987, 1992; Barzotti et al., 2000; Vitturi et al.,

2002; Colomba et al., 2002; Eirín-López et al., 2004; Vitturi et al., 2004; Pisano and Ghigliotti, 2009; Cabral-de-Mello et al., 2010a, 2011).

In insects belonging to the order Coleoptera, knowledge about the chromosomal distribution of rDNAs and histone genes is scarce. The 45S rDNAs have primarily been mapped on the chromosomes of representatives of the families Carabidae, Cicindelidae, Scarabaeidae and Chrysomelidae, among others (Sánchez-Gea et al., 2000; Moura et al., 2003; Martínez-Navarro et al., 2004; Bione et al., 2005a; Almeida et al., 2010). In the family Scarabaeidae, the 45S rDNA has been mapped in a few species (about twenty), and the mapping of 5S rRNA and H3 histone genes has been restricted to *Dichotomius geminatus*, which is the only species in the Coleoptera order in which these genes have been mapped (Moura et al., 2003; Vitturi et al., 2003; Bione et al., 2005a, b; Silva et al., 2009; Cabral-de-Mello et al., 2010a, b; Oliveira et al., 2010).

Dichotomius (Coleoptera, Scarabaeidae, Scarabaeinae) is endemic to America, and in Brazil, more than 80 species have been described. Knowledge of the karyotypic organization in this genus is restricted to six species, revealing primarily the presence of 18 chromosomes and a large metacentric pair (pair 1) (Smith and Virkki, 1978; Vidal, 1984; Cabral-de-Mello et al., 2008; Silva et al., 2009). The mapping of repeated multigene families, such as 45S rRNA has been reported for three representatives, and the histone H3 and 5S rRNA genes have been mapped in only one species. The aim of this study was to advance the understanding of the chromosomal organization of rRNAs and H3 histone genes in Scarabaeidae beetles and karyotypic differentiation patterns in the genus *Dichotomius*. To this end, 14 species of *Dichotomius* were karyotyped, and their 5S and 18S RNA and histone H3 genes were mapped. A conserved macro-chromosomal structure and stability for location of 5S rRNA and H3 histone genes were revealed, whereas distinct degrees of variability for 45S rDNA were observed. We also attempt to identify conserved karyological synapomorphy in this genus,

and the data are discussed to elucidate the possible mechanisms involved in the diversification of karyotypes and multigene families.

Material and methods

Adult male samples from fourteen *Dichotomius* species were collected from distinct areas in Ceará (CE), Mato Grosso (MT), Paraná (PR), Pernambuco (PE) and São Paulo (SP) States, Brazil, using pitfall traps (Table 1). The testes were dissected, fixed in Carnoy (3:1 ethanol:acetic acid) and stored at -20 ^{o}C. Mitotic and meiotic chromosomes were obtained from fixed testicular cells, and the slides were prepared in 45% acetic acid by squash technique. Coverslips were removed after the preparations were frozen by immersion in liquid nitrogen for a few seconds.

The FISH procedures were performed according to Cabral-de-Mello et al. (2010a). DNA probes for the 18S and 5S rRNA and histone H3 genes were obtained from fragments cloned from the genome of *Dichotomius geminatus*. The probes for 18S rDNA and histone H3 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). The 5S rDNA was labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany) by polymerase chain reaction (PCR) and detected by anti-digoxigenin-rhodamine (Roche, Mannheim, Germany). Two colour FISH was performed for 18S/5S rDNA and 5S rDNA/histone H3. To map the three multigene families in the same cell, two FISH procedures were performed on the same slides. The probes for the 18S and 5S rRNA genes were hybridized first. After the analysis and image captures, the slides were washed three times for fifteen minutes each in 2× SSC at room temperature. In the second two colour FISH round, the probes for the histone H3 and 5S rRNA genes were hybridized. The multigene family probes were also hybridized singly to eliminate any mistake signal detection interpretation

errors. All of the preparations were counterstained with DAPI, and the coverslips were added after the application of Vectashield (Vector, Burlingame, CA, USA) mounting medium. For some images, the 18S rRNA gene green signals were pseudo-coloured to purple, and the chromosomal images were captured in grayscale. 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.

Results

All species studied showed similar karyotypes with the presence of 2n = 18 chromosomes, Xy_p sex-determining mechanism, biarmed autosomes and X chromosome, whereas the y was punctiform. The presence of a large metacentric bivalent (pair one) almost double the size of the second pair was a remarkable characteristic of these 14 species (Figures 1a-i, 2a-h and 3a). The karyotypes of seven of the 14 species are described here for the first time; moreover, other populations and distinct individuals from populations of previously analyzed species were also karyotyped, such as *D. bos*, *D. geminatus*, *D. laevicollis*, *D. nisus*, *D. semisquamosus* and *D. sericeus*.

The mapping of the three multigene families (18S and 5S rRNA and histone H3) revealed distinct patterns of chromosomal distribution (Figures 1a-i, 2a-h and 3a; Table 1). The 5S rDNA gene was highly conserved in the proximal region of chromosomal pair no. 2 (Figures 1a-f, h, i, 2a-h and 3a). Eight of the fourteen species were randomly chosen for mapping of the histone H3 gene, and similar to 5S rDNA, this sequence was located in the proximal region of chromosomal pair no. 2 and co-localized with the 5S rDNA (Figures 1h, i and 2a-h). Analyses of prophase chromosomes and interphase chromatin (Figure 2c, d) reinforced that the two genes were co-located/associated with each other. However, the 18S

rDNA presented higher variability in the number and location of sites ranging from one to five sites per diploid genome and located in autosomes, sex chromosomes or both, being proximal, interstitial or distal (see Table 1). Although variability was observed for 18S rDNA, in seven species, this sequence was exclusively located in pair three (Figures 1b, c, e, f; 2a, h; Table 1); in three species, it was additionally located in another chromosome: the X chromosome for *D. semisquamosus* (Figure 2f) and *D. crinicollis* (result not shown) and pair no. 4 in *D. geminatus* (Figure 2e). In *D. mormon*, *D.* aff *mundus*, *D. nisus* and *D. semiaeneus*, the 18S rDNA was observed in distinct chromosomes of pair no. 3. It was exclusively autosomal in *D.* aff *mundus* (pair no. 1) (Figure 1a), associated with sex chromosomes in *D. nisus* (chromosomes X and y) (Figure 2g) and *D. semiaeneus* (chromosome X) (Figure 1d) and mapped to both in *D. mormon* (pairs no. 6, 8 and chromosome X) that presented polymorphic condition for the pair no. 8 (Figure 1g).

Concerning autosomes, most of the 18S rDNA sites (18 out 28 sites, corresponding to ~64.3%) were located in distal regions, six (~21.4%) were interstitial and four (~14.3%) were proximal but not far from centromere (Table 1). Interestingly, all distal sites were located in the short chromosomal arm, whereas the interstitial sites were located in the short arm in *Dichotomius affinis* and *D.* sp and in the long arm in *D. depressicollis*. For the sex chromosomes, it was not possible to determinate the precise position of the 18S rDNA due to the condensation and small size of these elements. At least fifteen cells (including distinct mitotic and meiotic cycle phases) per individual and three individuals per species of each population were analyzed by FISH to determine the chromosomal location of the DNA markers assayed. Figure 3b shows chromosome pair 3 in metaphases I and II and spermatogonial metaphases, indicating the precise location of 18S rDNA.

Discussion

Similar macro-chromosomal structure was observed in the fourteen species analyzed here, including distinct populations. The presence of 2n = 18 and the biarmed chromosome pair no. 1 distinct in size was previously reported for distinct populations of *D. bos*, *D. geminatus*, *D. laevicollis*, *D. nisus*, *D. semisquamosus* and *D. sericeus* (Silva et al., 2009; Cabral-de-Mello et al., 2010a, *in press*) and was observed in eight additional species studied here for the first time as well as for two other species (unpublished results). These results indicate karyotype stability in the genus at both intraspecific and interspecific levels, at least with regard to the macro-chromosomal structure. Bearing in mind that 2n = 20 is considered ancient for Coleoptera and Scarabaeidae (Smith and Virkki, 1978; Yadav and Pillai, 1979) the presence of the diploid number 2n = 18 and a large metacentric pair (pair no. 1) in *Dichotomius* could have arisen from autosomal fusion, being conserved the ancestral sex system not involved in the rearrangement. This chromosomal fusion rearrangement likely occurred before the diversification of *Dichotomius* species being the chromosome no. 1 a marker and a remarkable synapomorphy of this genus. However, this chromosomal fusion remains to be elucidated by more refined techniques like hybridization of telomeric sequences, that can reveal interstitial telomeric sites (ITS) in chromosomes involved in chromosomal rearrangements, such as Robertsonian fusions.

Recently, Cabral-de-Mello et al. (*in press*) hybridized a C_0t-1 DNA (a pool of highly and moderately repetitive sequences) fraction obtained from the genome of *D. geminatus* against the chromosomes of five other *Dichotomius* species revealing the occurrence of conspicuous and conserved terminal signals in all autosomal chromosomes (being probably the functional telomeres or telomere-associated DNA), but interstitial sites in pair no. 1 (probably generated by chromosomal fusion) were not observed. However, the authors did not exclude the possibility of origin by fusion of the pair no. 1, and proposed that the absence of

interstitial signals with C_0t-1 DNA probe could be correlated to a rapid modification of terminal sequences after the fusion, leading to the failure of interstitial C_0t-1 DNA site detection in FISH experiments.

The presence of Xy system is common in *Dichotomius*, occurring in all of the species and characterized by the Xy_p configuration, considered ancestral and frequently observed in Scarabaeidae and Coleoptera (Smith and Virkki, 1978; Yadav and Pillai, 1979; Dutrillaux and Dutrillaux 2009). In contrast, in *D. sericeus*, a distinct sex mechanism, Xy_r (rod-shaped), was reported by Silva et al. (2009); however, the *D. sericeus* individuals analyzed here presented Xy_p sex mechanism, indicating the occurrence of polymorphism related to the meiotic behavior of the sex chromosomes, which could represent an initial step of sex chromosomes differentiation. The Xy_r sex mechanism is rare in Coleoptera, and it has only been reported in approximately ten species of Scarabaeidae (Smith and Virkki, 1978; Yadav et al., 1979).

Another remarkable characteristic observed in all species is the presence of 5S rDNA in pair no. 2, revealing an intense conservation of this cluster in the genus. It is impossible to determine if this characteristic is ancient in the family Scarabaeidae and order Coleoptera or if it represents a synapomorphy for *Dichotomius*; thus, the analysis of other species of this group is necessary. In other insects, a distinct scenario has been described for 5S rDNA organization, although until now, the results are incipient. For example, in grasshoppers the presence of single and multiple sites for this gene have been reported (Cabrero et al., 2003; Loreto et al., 2008; Teruel et al., 2010; Cabral-de-Mello et al., 2011). These variable results indicate that distinct evolutionary trends are driving the chromosomal organization of 5S rDNA in the genomes of insects.

In addition to the conservation of 5S rDNA in pair no. 2 in eight of the analyzed species (see Table 1), the histone H3 gene was also conserved and co-located in the same chromosome. An association between these two genes was previously reported in two

crustaceans (Andrews et al., 1987; Barzotti et al., 2000), one mollusc (Eirín-López el al., 2004), four Proscopiidae grasshoppers (Cabral-de-Mello et al., 2011) and in the beetle *D. geminatus* (Cabral-de-Mello et al., 2010a). Besides the association of histone genes and 5S rDNA in Coleoptera, recently Roehrdanz and co-workers (2010) identified interspersion of 45S rDNA and the histone quintet clusters. The number of clusters and specific chromosomal position of the histone H3 gene is apparently highly conserved among animals (see for example Hankeln el al., 1993; Pendás et al., 1994; Cabrero et al., 2009; Cabral-de-Mello et al., 2011), and this was also observed for the *Dichotomius* species analyzed here, indicating that the chromosomal location of the histone gene clusters has been well-conserved over a long period of evolution. In grasshoppers, for example, histone chromosome conservation was recently described for Acrididae, with an absence of changes for the 60 million years since the origin of the group (Cabrero et al., 2009), and for Proscopiidae (Cabral-de-Mello et al., 2011), a more ancient grasshopper family. According to Cabrero et al. (2009), the high conservation at the level of chromosomal location in grasshoppers suggests strong purifying selection operating on this trait, causing most chromosomal mutations to be intolerable. This idea could be also applicable to *Dichotomius* genomes, and bearing in mind this hypothesis, we can propose that the immobility of the 5S rDNA in the chromosomes of *Dichotomius* beetles is a result of its association with histone genes, leading to the same evolutionary patterns at chromosomal level for the two sequences. The association or co-localization at molecular level for these multigene families needs to be investigated further, although the possible association of these sequences could be reinforced here by the observation of interphasic chromatin and initial meiotic cells (see Figures 2c, d), in which the chromosomes are much less condensed than in metaphase.

In contrast to the extensive conservation of the 5S rDNA/histone H3 clusters, more variable patterns were observed for the number and chromosomal positions of the 45S rDNA

clusters. Variations were observed at the intraspecific and interspecific levels for the species studied here. In Coleoptera variability for number and position of rDNA clusters have been also reported (see Schneider et al., 2007), as observed for *Dichotomius*, and the plausible explanation (other than small chromosomal rearrangements) for the variations reported in its number and position is the occurrence of amplification/dispersion of 45S rDNA copies in this genus. On the other hand, using silver nitrate staining the presence of one chromosome responsible for nucleolar organization is the most common pattern in Coleoptera (reviewed by Schneider et al., 2007) and it is possible to speculate that the genomes of the coleopteran species have suffered selection for the activation of rDNA clusters of only one autosomal pair.

Although the presence of 45S rDNA in the pair no. 1 (resulting from fusion) was observed in *D.* aff *mundus*, it seems that the rDNA sites in this chromosome could resultant of a recent transposition occurring after the fusion. This same idea concerning transposition is capable of explaining the presence of 45S rDNA in the sex chromosomes, considering that these species present the ancient sex mechanism (Xy_p) not involved in chromosomal rearrangements. Interestingly, the presence of rDNA sites mapped by FISH in the y chromosome as observed in *D. nisus* has not been extensively reported for Coleoptera; in Scarabaeidae, it was described in a species with a derived sex system (neo-XY) that had undergone chromosomal rearrangements that changed the position of the rDNA from autosomes to the sex bivalent (Arcanjo et al., 2009). In Coleoptera as a whole repositioning of rDNA clusters to sex chromosomes has been well documented, for example, in tiger beetles (Cicindelidae) of the genus *Cicindela*, as result of translocations between autosomes and sex chromosomes and fusion or fissions between X chromosomes (Galián et al., 2007).

The 45S rDNA observed in pair three of distinct species of *Dichotomius* presented variability for its specific position being proximal, interstitial in the short or long arm and distal in the short arm, and the apparent mechanism responsible for its variation may be

associated with small chromosomal rearrangements (such as inversions) that do not modify the chromosomal morphology. In other groups, various mechanisms have been proposed to explain the amplification/spreading and location of 45S rDNA sites at the intra- and interspecific levels: (i) mobility of rDNA associated with transposable elements (Raskina et al., 2008); (ii) equilocal dispersion of heterochromatin and rDNA (Pedersen and Linde-Laursen, 1994); and (iii) chromosomal rearrangements. Similar to proposed here in *Dichotomius*, in other insect such as grasshoppers, butterflies and moths, structural chromosomal rearrangements, ectopic recombination and transposition have been postulated to drive the diversification of 45S rDNA in both number and position (Cabrero and Camacho, 2008; Nguyen et al., 2010). The variation in the 45S rDNA observed in *Dichotomius* could be favored by its usual distal location (64.3% of autosomal sites), that are more dynamic chromosomal regions, which facilitates transposition events and leads to the dispersion of these segments in the genome (Schweizer and Loidl, 1987). Similar ideas were proposed in fish genomes for 45S rDNA in relation to its variation, as well as for the conservation of 5S rDNA, which is interstitially located and seems to be protected from dispersion mechanisms (Martins and Wasko, 2004).

In summary, our results demonstrate that the chromosomal stasis observed for macro-chromosomal structure in the genus *Dichotomius* is not reflected at the microgenomic level, at least concerning 45S rDNA. The other two multigene families mapped (5S rRNA and H3 histone genes) were well conserved in both their number and location, evidencing ancient characteristics of the genus, likewise the diploid number 2n = 18 and a large metacentric pair (pair no. 1). The next steps should be to demonstrate the specific mechanism associated with the dispersion of the 45S rDNA and the conservation of the 5S rDNA/H3 histone sequences, and this genus provides interesting material for this purpose. Moreover, we reinforced the possible co-location/association of 5S rRNA and H3 histone genes in Scarabaeidae by

analyzing less condensed chromosomes and chromatin, and contributed to the knowledge regarding the chromosomal organization and diversification patterns of multigene families in beetles and insects as a whole.

Acknowledgments

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Table

Table 1. Chromosome location of 18S rDNA in 14 species of *Dichotomius*. p= proximal, i= interstitial, d= distal in respect to the centromere. The asterisks indicate the presence of 45S rDNA site in the sex chromosomes in which was impossible to determinate its precise position, and (†) species studied for H3 histone mapping.

Species	2n♂	Chromosomes										Collecting sites in Brazil
		1	2	3	4	5	6	7	8	X	y	
Dichotomius affinis (Felsche, 1910)	18			i								Botucatu, SP (22°53'S, 48°27'W)
D. bos (Blanchard, 1843)	18			p								Botucatu, SP (22°53'S, 48°27'W) Saloá, PE (8°57'S, 36°43'W)
D. crinicollis† (Germar, 1824)	18			d						*		Carrancas, MG (21°28'S, 44°38'W)
D. depresicollis† (Harold, 1867)	18			i								Crato, CE (7°13'S, 39°24'W)
D. geminatus† (Arrow, 1913)	18			d	d							Igarassu, PE (07°48'S, 34°57'W) Maracaípe, PE (8°31'S, 35°01'W)
D. laevicollis† (Felsche, 1901)	18			d								Caruaru, PE (8°42'S, 35° 15' W)
D. mórmon (Ljungh, 1799)	18						d		d	*		Botucatu, SP (22°53'S, 48°27'W)
D. aff *mundus* (Harold, 1869)	18	p										Botucatu, SP (22°53'S, 48°27'W)
D. nisus† (Olivier, 1789)	18									*	*	Botucatu, SP (22°53'S, 48°27'W) Igarassu, PE (07°48'S, 34°57'W) Terra Roxa, PR (24°09'S, 54°06'W)
D. semiaeneus (Germar, 1824)	18									*		Botucatu, SP (22°53'S, 48°27'W)
D. semisquamosus† (Curtis, 1845)	18			d						*		Caruaru, PE (8°42'S, 35° 15' W) Caruaru, PE (08°22'S, 36°05'W) Igarassu, PE (07°48''S, 34°57'W) Saloá, PE (8°57'S, 36°43'W)
D. sericeus† (Harold, 1867)	18			d								Igarassu, PE (07°48'37''S, 34°57'25''W)
D. sericeus sp1 (Harold, 1867)	18			d								Caruaru, PE (08°22'S, 36°05'W)
D. sp Hope, 1838	18			i								Barra do Garças, MT (15°55'S, 52°16'W)

Figure legends

Figure 1. Fluorescent *in situ* hybridization using 18S (green; a-g) and 5S rDNA (red; a-f, h, i) and the histone H3 gene (green; h, i) as probes in nine representative species of *Dichotomius*. Metaphase I of (a) *D.* aff *mundus*, (b) *D. sericeus* sp1, (d) *D. semiaeneus*, (e) *D. affinis*, (f) *D.* sp, (g) *D. mormon* and (h) *D. crinicollis*, and metaphase II of (c, i) *D. depresicollis*. The arrows indicate the sex bivalents, and the inserts in (h, i) show signals from separate probes

for the 5S rRNA (red) and histone H3 (green) genes. Note the large metacentric pair (pair one) indicated by the number. Other chromosomes are also indicated. Scale bar = 5 μm.

Figure 2. Chromosomal mapping of the 18S rRNA (purple), 5S rRNA (red) and histone H3 (green) multigene families in six species of *Dichotomius*. Karyotypes from *D. laevicollis* (a) and *D. sericeus* (b); interphase nucleus from *D. bos* (c); partial pachytene from *D. geminatus* (d); metaphase II from *D. geminatus* (e); metaphase I chromosomes from *D. semisquamosus* (f), *D. nisus* (g) and *D. bos* (h) are shown. The arrows indicate the sex bivalents, and the inserts show the chromosomes labeled with separate probes for the 5S rRNA (red) and histone H3 (green) genes. Note that the 5S rRNA and histone H3 gene sites overlap in all cells, including interphase nuclei (d), less condensed chromosomes in an initial meiotic pachytene (d) and the large metacentric pair (pair one) indicated by the number 1. Other chromosomes are also indicated. Bar = 5 μm.

Figure 3. (a) Ideograms showing the distribution of 18S rDNA (green) and 5S rDNA/H3 histone (red). (D1) *Dichotomius affinis*, (D2) *D. bos*, (D3) *D. crinicollis*, (D4) *D. depressicollis*, (D5) *D. geminatus*, (D6) *D. laevicollis*, (D7) *D. mormon*, (D8) *D.* aff *mundus*, (D9) *D. nisus*, (D10) *D. semiaeneus*, (D11) *D. semisquamosus*, (D12) *D. sericeus*, (D13) *D. sericeus* sp1, (D14) *D.* sp and (*) chromosome shared by all species. The black lines indicate chromosomes that harbor neither 45S/5S rDNA nor the histone cluster in any of the species. (b) Chromosome three selected from metaphase I (1,1',2,2'), metaphase II (3,4) and spermatogonial metaphase (5) showing in detail the position of the 18S rDNA: (1) terminal in the short arm, (2,5) interstitial in the short arm, (3) interstitial in the long arm, and (4) proximal. (1) *D. sericeus* sp1, (2) *D. affinis*, (3) *D. depressicollis*, (4) *D. bos*, (5) *D.* sp. 1' and 2' represent a graphical structure of bivalent three in metaphase I of 1 and 2, respectively. Red arrowheads indicate the centromere and white arrows the positions of chiasmata.

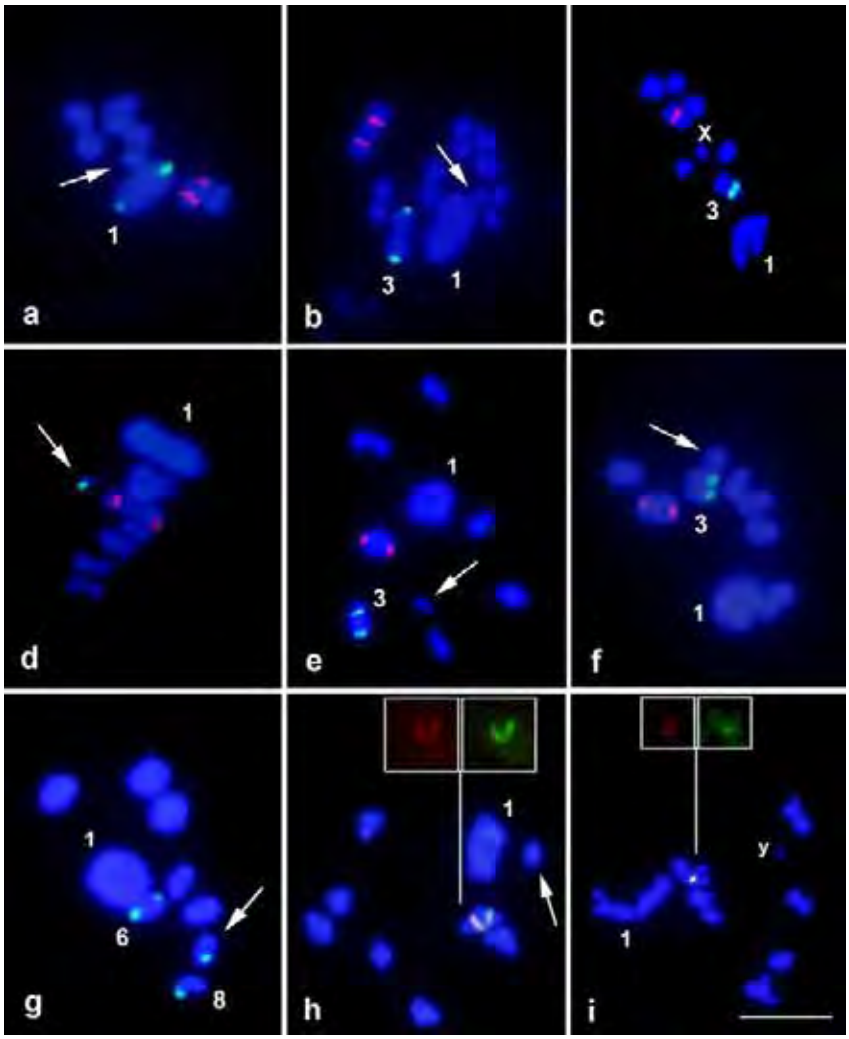


Figure 1

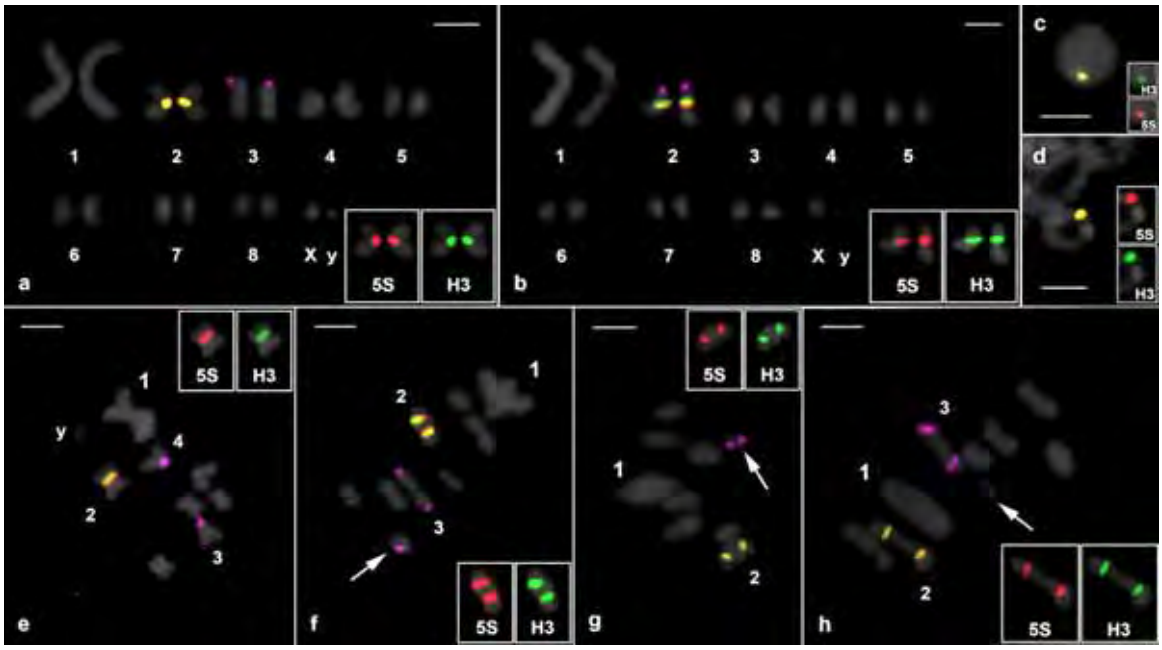
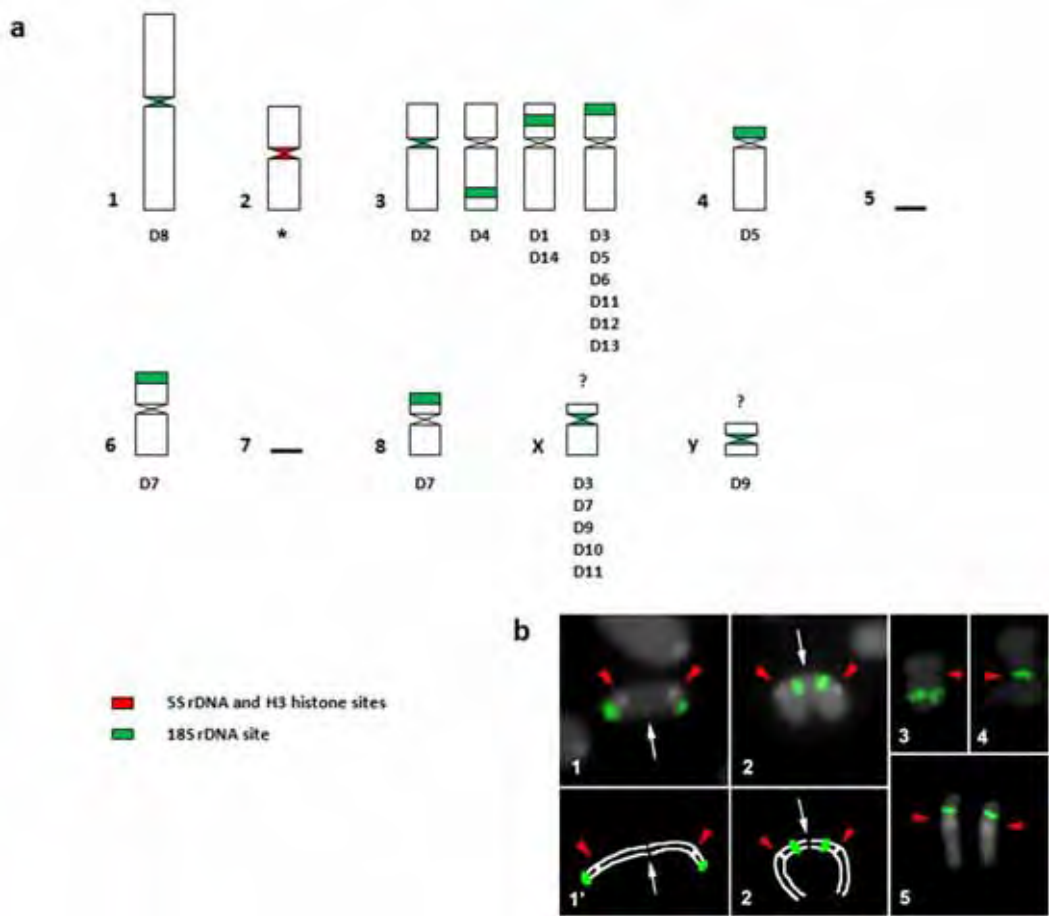


Figure 2


a
1
D8
2
*
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D14
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D6
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D13
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D7
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D3
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D9
D10
D11
Y
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D9
5S rDNA and H3 histone sites
18S rDNA site
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1
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1'
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Figure 3

5.4. Capítulo 4:

Chromosomal organization of 18S and 5S rRNA, and H3 histone genes in Scarabaeinae coleopterans: insights on the evolutionary dynamics of multigene families and heterochromatin

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Abstract

We analyzed the chromosomal location of 5S and 18S rRNA and H3 histone genes in beetles belonging to eight tribes from the subfamily Scarabaeinae (Coleoptera, Scarabaeidae). The number of 18S rDNA sites was variable from two to 16, being located in autosomes, sex chromosomes or both, although the presence of two clusters was the most common pattern. On the other hand, the presence of two clusters (one bivalent) in autosomal chromosomes for 5S rRNA and H3 histone was high conserved. Moreover these genes were apparently interspersed each other, including in initial meiosis and interphasic nucleus. Low correlation (r=0,21) was observed between the diploid number and quantity of rDNA sites. Finally a comparison between the number of rDNA per genome and spread of heterochromatin revealed species with low heterochromatin quantity and few rDNA sites and species with high heterochromatin quantity and spread of rDNA sites. These results point to distinct evolution patterns for major rDNA in Scarabaeinae beetles, primarily non associated with macro-chromosomal rearrangements. Although, the high variability for rDNAs is apparently extensively related with heterochromatin spread, indicating that probably these two genomic elements could be suffering similar evolutionary forces for spreading in the distinct lineages of Scarabaeinae subfamily, evolving by neutral selection. Moreover the high chromosomal variability previously reported for Scarabaeinae is also common in the microevolutionary level, at least for major rDNA genes. On the contrary, the conservation of 5S rRNA and H3 histone gene clusters could represent a basal condition before the diversification of Scarabaeinae and it is still maintained in the group and are under the government of different evolutionary processes compared to the major rDNA.

Keywords: beetles, chromosomal evolution, fluorescence *in situ* hybridization, heterochromatin, multigene families

Introduction

The use of the repetitive sequences has gain a great importance as very useful cytogenetic markers in studies of genome organization, sexual and supernumerary chromosomes, and for identification of chromosomal rearrangements. Among the repeated DNA sequences, ribosomal RNAs (rRNA) and histone genes are grouped in distinct multigene families organized in tandem of hundreds to thousands of copies. The major ribosomal DNA (rDNA) cluster transcribes for 28S, 18S and 5.8S rRNA genes forming the 45S rDNA, while the minor is responsible for the transcription of 5S rRNA gene (Long and David 1980). Concerning the histone gene sequences they may be arranged in repeated clusters tamdemly arrayed composed by intronless genes for H1, H2A, H2B, H3 and H4 histones spaced by noncoding DNA, as observed in for example in *Drosophila*, although some variation have been also reported for this organization (Lifton et al. 1977; Engel and Dodgson 1981; Maxson et al. 1983). The clustered and repeated organization of multigene family members that codes rRNA and histones makes these genes reliable chromosomal markers for studies of karyotypic diversity since they generate easily visible signals in the chromosomes after *in situ* hybridization. For insect chromosomes these sequences have been mapped more frequently in representatives belonging to the orders Coleoptera, Lepdoptera and Orthoptera, although the studies are until now incipient and concentrated in the major rDNA mapping (De la Rúa et al. 1996; Cabrero and Camacho 2008; Cabrero et al 2009; Cabral-de-Mello 2010a, submitted; Nguyen et al 2010).

Leading in consideration the macro karyotypic structure of Scarabaeidae family among the 13 subfamilies of this group, Scarabaeinae has presented the most chromosomal diversity, showing variation of diploid number from 2n = 8 to 2n = 24, distinct sex mechanism systems and chromosomal morphologies (Cabral-de-Mello et al 2008). The heterochromatin in this group apparently has passed through intense variation, in both

chromosomal location and base pair richness, presenting heterochromatic blocks restrict to centromeric region, located in terminal regions and also diphasic chromosomes that presents entire heterochromatic arms (Colomba et al. 1996; Wilson and Angus 2005; Cabral-de-Mello et al. 2010b,c). Moreover, the heterochromatic sequences have shown distinct base pairs richness, with AT or GC rich blocks, besides the presence of neutral blocks (Colomba et al. 2006; Cabral-de-Mello et al. 2010b; Oliveira et al. 2010). Scarabaeidae is poorly studied under the focus of chromosomal mapping of multigene families and the analysis were primarily performed for the description of major rDNA clusters (Moura et al 2003; Bione et al 2005a,b; Colomba et al 2000, 2006; Arcanjo et al 2009; Silva et al 2009; Cabral-de-Mello et al 2010b; Oliveira et al 2010). For the histone genes and 5S rDNA only seven species were analyzed, all of them belonging to the genus *Dichotomius* (Cabral-de-Mello et al 2010c, submitted).

Considering this high chromosomal diversity for Scarabaeinae beetles and in order to understanding the chromosomal and genomic organization, and track changes along the evolution of this group, the distribution of 18S and 5S rDNA, and H3 histone genes array were analyzed in several species of the group. Moreover we compiled the published results on cytogenetic mapping of multigene families location for representatives of this subfamily. Our results showed distinct evolutionary fates for the multigene families studied, being the 5S rRNA and H3 histone genes associated and highly conserved in number. On the other hand, the major rDNA has shown an intense turnover of number and chromosomal location along the Scarabaeinae karyotype diversification, non extensively associated with macro-chromosomal changes and apparently related with heterochromatin dispersion. These data contribute to understanding the organization of multigene families in the genome as well as the chromosomal evolutionary history of beetles and insects as a whole.

Material and methods

Sample of adult males of 27 species of Scarabaeinae beetles belonging to six tribes were collected in distinct cities of the Minas Gerais, Paraná, Pernambuco, Ceará and São Paulo states, Brazil. The testes were fixed in Carnoy (3:1 ethanol:acetic acid) and stored in freezer -20 ^{o}C. Slides for conventional chromosomal analysis, in order to check the male chromosomal structure, were stained with 2% lacto-acetic orcein. The chromosome preparations for C-banding and FISH experiments were made by squashing using a drop of 45% acetic acid and subsequently removing the coverslip with a razorblade after immersion in liquid nitrogen. The C-banding was performed according the protocol described by Sumner (1972).

DNA probes of the 5S and 18S rRNA, and H3 histone genes were obtained from the genome of the beetle *Dichotomius geminatus* (Cabral-de-Mello et al 2010c). The 18S rRNA and H3 histone gene probes were labeled by nick translation using biotin-11-dATP (Invitrogen, San Diego, CA, USA), whereas the 5S rRNA gene was labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany). The FISH procedures were performed according to the method described by Cabral-de-Mello et al (2010c). Preparations were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield (Vector, Burlingame, CA, USA). Images were captured with the Olympus DP71 digital camera coupled to a BX61 Olympus microscope and were optimized for brightness and contrast using Adobe Photoshop CS2.

A statistical analysis was performed using the Pearson rank test to analyze the correlation degree between the number of 45S rDNA sites and diploid number. Moreover a comparative analysis between heterochromatin distribution and number or 45S rDNA sites was also performed.

Results

Karyotypes and heterochromatin distribution

The diploid number and sex mechanism system were for the first time described here for nine species. Moreover, the karyotypic structure of other 21 species was reanalyzed (Table 1). The heterochromatin distribution patterns were determined for *Atheuchus* sp, *Ontherus apendiculatus* and *Digitonthophagus gazella* for the first time and data from previously published works published by our group and other authors were obtained from the literature and also re-analyzed. The heterochromatin distribution patterns were classified in three distinct groups, (i) species presenting heterochromatin restrict to the centromeric/pericentromeric regions (non spread pattern), (ii) species with terminal or subterminal additional heterochromatic blocks (moderately spread pattern), and (iii) the last one with species presenting large quantity of heterochromatin in the karyotypes, represented primarily by diphasic chromosomes and paracentromeric blocks (highly spread pattern) (Table 1).

Mapping of multigene families

Double-FISH performed using the probes for 18S and 5S rDNA revealed mainly conspicuous blocks in distinct chromosomes (Figures 1, 2). Although the most common pattern for 18S rDNA cluster was the presence of two sites (one bivalent), observed in 15 species, the number of clusters for this repeated gene ranged from two to sixteen, with exclusively location in autosomes and/or in sex chromosomes (Figures 1, 2, 5a, Table 1). For 5S gene almost all species presented only two sites (one bivalent), in general located in one autosomal pair, with distinct location of the 18S rDNA sites. Only in three species the sites of 18S and 5S rDNA were coincident. In *Diabroctis mimas* the sites of 18S and 5S rDNA were coincident in one autosomal bivalent and in the X chromosome (Figure 1c, 4). For *Eurysternus caribaeus* and

Digitonthophagus gazella these genes were collocated in the sex bivalent (Figure 2a, b), although in the former species they were restrict to the X chromosome (Figure 2a).

The use of 5S rRNA and H3 histone genes as probes showed these two markers locate in the same chromosomal clusters in all studied species. These two sequences were located only in one autosome bivalent of most species (Figure 3, Table 1). In *Coprophanaeus ensifer* these elements were located in the X and Y chromosomes (Figure 3b) and in *E. caribaeus* exclusively in the X chromosome (Figure 3e). In *D. mimas* these two sequences were located in five autosomal chromosomes plus the X sex element (result not shown). Moreover the 5S and H3 histone sequences were apparently overlapped in the same chromosomal region (Figure 3), independent of the chromosome condensation stage (Figure 4).

Analysis of interphasic nucleus and initial meiotic cells hybridized for 18S and 5S rDNA, and histone H3 sequences revealed similar pattern for co-location of 5S rRNA and H3 histone genes as observed in condensed metaphasic chromosomes (Figure 4). On the other hand the 18S rDNA were located in distinct cell regions of 5S and histone sites, and only in the species with co-location of 5S/18S (*Eurysternus caribaeus* and *Digitonthophagus gazella*) (Figure 2a, b), an overlapped signal was observed (Figure 4e, f).

Another characteristic for the three multigene families studied was the presence of only one site per chromosome, without the presence of, for example, one chromosome with more than one cluster (Figures 1-3). Besides the variability observed for 18S rDNA in the species studied here for the first time, we identified some polymorphism concerning the number of sites in relation to the data previously described, such as in *Deltochilum calcaratum*, *Dichotomius semisquamosus*, *Coprophanaeus ensifer*, *C. cyanescens* and *Diabroctis mimas* (Table 1).

Moreover, it was observed heteromorphism related to the cytogenetic mapping of the three genes in several species regarding to the size and presence/absence of the clusters in the

homologous chromosomes, as observed for example in *E. caribaeus* (Figure 2a, 3e), and for *Ontherus sulcator* (Figure 2d), *Diabroctis mimas* (Figure 1c) and *Phanaeus splendidulus* (Figure 1d), concerning only the 18S rDNA sites. Although we were able to define the number of clusters, the precise positions along the chromosomes were sometimes difficult to define due the small size and condensation level of the chromosomes. It also difficult the determination of the specific chromosome pair that bears the sequences studied in some species. All patterns observed in this work are presented in the table 1, including the data available in the literature.

The data obtained for major rDNA site numbers were compared with diploid numbers (2n) and heterochromatin distribution patterns to evaluate the relationship among these karyotypic characteristics. The relationship between diploid numbers and major rDNA site numbers is showed in the figure 5b and between heterochromatin dispersion and rDNA site numbers in the figure 5c. The variability in the diploid number and number of rDNA sites showed apparently non relationship with low correlation (r= 0.21) (Figure 5b), while the increase of heterochromatin was accomplished by expansion of the number of major rDNA sites (Figure 5c).

Discussion

Here for the first time the chromosomal distribution of rRNAs and histone H3 genes were studied in a reasonable number of species belonging to the subfamily Scarabaeinae. In fact the mapping of 5S rRNA and histone genes were previously performed only in 14 species of beetle belonging exclusively to the genus *Dichotomius*, and for insects as a whole these elements have not been extensively mapped, with few examples most restricted to grasshoppers (Loreto et al. 2005; Cabrero et al. 2009; Cabral-de-Mello et al. 2010a,c; Teruel et al. 2010). Scarabaeinae exhibit a high chromosomal variability, being the most diverse

group of the family Scarabaeidae. According to Cabral-de-Mello et al. (2008) the chromosomal diversity of this subfamily probably results from the occurrence of distinct chromosome rearrangements during their evolution. This variability observed at macro-chromosomal level is also observed concerning the major ribosomal DNA genes, with variation of one to 16 rDNA loci per diploid genome. The variation of number of rDNA sites in Scarabaeinae species indicates that extensive genomic reorganization has occurred during the evolutionary history of the distinct lineages.

The 18S rDNA sites

Two main patterns of distribution of major rRNA genes were detected in Scarabaeinae: (i) only two rDNA sites (one chromosomal bivalent) harboring these genes (15 species) as observed for example in ten *Dichotomius* species, *Canthon staigi*, *Deltochilum elevatum* and *Ontherus apendiculatus*; (ii) more than two rDNA sites (from 3 to 16 clusters), such as *Bubas bison*, *Coprophanaeus ensifer*, *C. cyanescens*, *Diabroctis mimas*, *Ontherus sulcator* and three *Deltochilum* species. This might suggest that the major rRNA genes are apparently under distinct forces that governs the spreading and movement of the copies. Such restriction or intense spreading could be associated to the presence/absence of appropriate molecular mechanism to move the rDNA, such as the presence of transposable elements. The capacity of rDNA clusters movement and number variability was first observed by Schubert (1984) in *Allium*, and since this description some other evidences have been accumulated concerning the ability of rDNA to move in the genome. Recent studies have proposed that transposable elements are a possible source of rDNA (Raskina et al. 2008; Zhang et al. 2008) and other genes (Jiang et al. 2004; Lai et al. 2005) movements to different regions of the genome.

Common patterns of major rDNA distribution could be observed for three tribes (Canthonini, Coprini, Phanaeini - out of eight distinct tribes of Scarabaeinae studied here) that include several species analyzed. On the other hand, for the remaining tribes with few species analyzed, none inference could be proposed. Among Coprini species the clusters of major rDNA have not suffered intense genomic reorganization being primarily associated to only one chromosomal bivalent, as observed in *Dichotomius* species and *Ontherus apendiiculatus*. Phanaeini is characterized by an intense movement of major rDNA sites that have generated different number of clusters spread in several chromosomes. *Coprophanaeus ensifer*, for example, presents the highest number of rDNA clusters (16 sites) for the subfamily and for Coleoptera as a whole. Moreover in this tribe, besides the intense variability of sites in interespecific level some polymorphism were observed between previous studied and the species analyzed here, as observed for *Coprophaneus ensifer*, *C. cyanescens*, and *D. mimas*. For Canthonini species an intermediate condition was observed, with species presenting no spreading of major rDNA clusters, such as *Canthon staigi* and *Deltochilum elevatum*, or with spread rDNA, as three *Deltochilum* representatives. These results indicate that the capacity of movement of major rDNA can be distinct in these three taxonomic groups.

Apparently the ancient condition for this gene in Scarabaeinae is the occurrence of two clusters located in only one autosomal bivalent corroborated by the large number of species with this characteristic (15 out of 26 studied) and the presence of this pattern in sister groups. That feature is also the most common pattern for Coleoptera as a whole, at least for Polyphaga representatives (reviewed by Schneider et al. 2007). Besides the common pattern composed of only one chromosomal pair harboring major rDNA clusters distributed in many species of the group, the intense repositioning in the major rDNA in Scarabaeinae involved increase of rDNA sites and movement to different autosomes and sex chromosomes. The occurrence of major rDNA associated to sex chromosomes in different species apparently have two

evolutionary histories, one involving large chromosomal rearrangements, such as fusions, observed for example in *Deltochilum calcaratum*, *D. merbillosum* and *Eurysternus caribaeus*, and another one with apparently occurrence of transpositions, in *Coprophanaeus* and *Dichotomius* species, *Diabroctis mimas* and *Deltochilum verruciferum*. In *Dichotomius* the occurrence of a chromosomal fusion along the differentiation of the species have been proposed, corroborated by the presence of a large metacentric chromosome and diploid number reduction to 2n = 18. Apparently this macro-chromosomal rearrangement is not related to the presence of major rDNA clusters in the sex bivalents of *D. crinicolis*, *D. nisus* and *D. semisquamosus*, considering the conservation of primitive Xy_p sex chromosomes in these species, being the transposition the most plausible explanation. For Phanaeinae representatives the species with major rDNA in the sex chromosomes conserved the diploid number 2n = 20 considered ancient for Scarabaeidae and Polyphaga suborder, indicating that fusions were not involved in the rDNA repositioning. Similar event could have occurred in *Deltochilum verruciferum*. On the other hand, the fusions might have acted as an important source for the repositioning of rDNA in the sex chromosomes in *Deltochilum calcaratum*, *D. morbilosum* and *E. caribaeus*, indicated by extensive diploid number reduction in these species involving also the formation of fusion derived sex mechanism, as neo-XY.

Although the variation in the major rDNA cluster number could be attributed to chromosomal rearrangements in some species, there is no correlation between the variation of rDNA sites and diploid numbers. There are examples of species with diploid number reduction without modification of rDNA number and species with conservation of the ancestral diploid number and extensive repositioning and expansion of major rDNA clusters. There are evidences of “movement” and “multiplication” of major DNA clusters without the occurrence of fusions and other chromosomal rearrangements (Dubkovsky and Dvorak 1995). In Scarabaeinae these modification can be attributed to ectopic recombination and

transposition and in also to inversions and translocations. In other insects similar mechanisms are responsible for intra- and interespecific variation as observed in Acrididae grasshoppers (Cabrero and Camacho, 2008) and in Lepdoptera (Nguyen et al. 2010). These results indicate distinct evolutionary trends related to the macro-chromosomal structure (diploid number, chromosome morphology and sex chromosomes) and major rDNA organization in the insect genomes.

Our analysis concerning heterochromatin and major rDNA dispersion revealed an interesting relationship pattern for these two genomic components. Species with heterochromatin restrict to centromeric regions were primarily characterized by stability of major rDNA related to number of sites, forming a group of eight species with only two sites (one chromosomal bivalent). Only one species *Ateuchus* sp presented four cluster, and *D. semisquamosus* showed three clusters as a polymorphic condition. On the other hand, extensive variability with variable numbers of rDNA sites was observed in most part of representatives (10 out of 11) in which the heterochromatin is spread, occurring in large quantity in the karyotype, for example whit large paracentromeric heterochromatic blocks and diphasic chromosomes (one chromosomal arm euchromatic and another heterochromatic). In representatives with moderate heterochromatin dispersion (centromeric and terminal blocks), the major rDNA suffered spreading in two species and was restrict to one autosomal bivalent in *Ontherus appendiculatus*. Our results indicated that possibly the same evolutionary forces are acting in these two genomic components of Scarabaeinae genomes, leading to the spreading of the major rDNA together with the heterochromatin. This hypothesized pattern of evolution could be favored by ectopic paring during chromocenter formation in initial meiotic stage. It is a common pattern in this insect group and it seems to play an important role in nucleolar organization and chromosomal segregation (Smith and Virkki 1978; Drets et al. 1983). Association of spreading of heterochromatic sequences and rDNAs was also observed

in other eukaryotic groups, but this association has not been extensively studied. The most intense variation in the number and location of 18S rDNA was observed in the tribe Phanaeini, in which all species studied until now present high heterochromatin quantity. Although the diploid number seems to be conserved with 2n = 20 in some representatives (Cabral-de-Mello et al. 2008), the heterochromatin and 18S rDNA apparently have suffered intense chromosomal reorganization, without extensive karyotypic modifications.

The 5S rRNA and histone H3 genes

Contrary to the variability for major rDNA a high conservation was observed for the 5S rRNA and histone H3 genes, concerning the number of clusters in all analyzed species. For invertebrates the mapping of these sequences are restrict to few species: 5S and H3 were mapped in Mollusca, in insects and in crustaceans; 5S was additionally mapped in few species from Annelida and Echinodermata (Vitturi et al., 2002; Gornug et al. 2005; Zhang et al. 2007; Cabral-de-Mello et al. 2010a,b,c; Cabrero et al. 2009; Teruel et al. 2010). Concerning insects, these studies were mainly concentrated in grasshoppers (Cabrero et al. 2009; Cabral-de-Mello et al. 2010a; Teruel et al. 2010), and for beetles only 14 species were previously studied, belonging to the genus *Dichotomius* (Cabral-de-Mello et al. 2010c, submited). The presence of only two sites (one bivalent) for these two genes in Scarabaeinae indicates that this condition can represent the ancient organization for these sequences, and they have not been suffered extensive changes in number since their origin [estimated in Cretaceous 92-83,5 million-year-old (Krell 2006)], along the species diversification. An intense conservation for number of histone genes was also recently described in grasshoppers (Cabrero et al. 2009; Cabral-de-Mello et al. 2010a) and it is also common in mollusks and was also described in three fish species (Pendás et al., 1994; Zhang et al. 2007). These results could indicate that strong purifying selection can be acting in the invertebrate histone clusters, against the

spreading of these genes in the genome, as proposed for grasshopper by Cabrero et al. (2009). Although some variation related to specific location and also in number of clusters were also reported, as observed in two mollusks, and some dipterans and grasshoppers (Hankeln et al., 1993; Schienman et al., 1998; Ranz et al., 2003; Zhang et al. 2007; Cabral-de-Mello et al. 2010a).

For some studied animal groups the 5S rDNA have an intense dynamic of spreading in the genome, including also the generation of variant copies in some species, as observed in fish (Martins and Galetti 2001). On the contrary the number of clusters for this gene is highly conserved in Scarabaeinae species with primarily two clusters in almost all studies species. This conservation can be resultant of the association of this sequence to histone genes, leading to the same purifying selection against spreading probably suffered by histone sequences in Scarabaeinae and proposed for grasshoppers by Cabrero et al. (2009). An extreme case of spreading of 5S rDNA sequence was observed for Scarabaeinae in *Dicbroctis mimas*. On the other hand reduction of number of sites was revealed in *Eurysternus caribaeus*. In both cases the change in the number of clusters in the species was also followed by the histone H3 gene cluster. In *D. mimas* the 5S rDNA suffered spreading for six chromosomes, being identical pattern observed for H3 histone. For *E. caribaeus* the 5S rDNA located in the sex bivalent (X chromosome), probably translocated from an autosomal pair by chromosomal fusions along karyotypic diversification of this species (that present an extensive diploid number reduction from 2n = 20 to 2n = 8), apparently have been suffered unequal cross-over involved in the sex chromosomes (neo-XY) differentiation (Arcanjo et al. 2009). The same pattern was also observed for histone H3 gene, with this sequence restrict to X chromosome, like the 5S rDNA.

All species studied showed 5S rRNA and H3 histone genes signals overlapped in the same chromosomal region, indicating an interspersed organization for these two multigene

families. This hypothesis can also be corroborated by the cases of combined scattering in *D. mimas* and unequal cross-over in *E. caribaeus* and the presence of overlapped signals in initial meiosis and interphasic nucleus, with less condensed chromosomes. Although some other molecular studies are necessary to clarify this statement, this associated organization was also described in mollusks (Eirín-López el al., 2004), crustacean (Andrews et al 1987; Drouin and Moniz de Sá, 1995; Barzzoti *et al.*, 2000), and in *Dichotomius* coleopterans (Cabral-de-Mello et al. submited) and Proscopiidae grasshoppers (Cabral-de-Mello et al. 2010a).

Although the histone H3 and 5S rRNA genes present co-localized organization, the 18S rDNA has shown a non linked organization with these two multigene families in the genomes of Scarabaeinae species. It was also clear that 5S rRNA/H3 histone genes and the 18S rDNA are organized in distinct nuclear areas, as observed the interphasic nucleus and also in initial meiosis stages. Only in few cases, *Diabroctis mimas* and *Digitonthophagus gazella*, these sequences were co-located, that could be explained by transposition of 18S rDNA, due its intense movement in the genome of some species. This physical separation could be reflected by a functional advantage for these ribosomal sequences. The non association of these two multigene families, that codes for rRNAs, is a common pattern for Eukaryotes and in vertebrate chromosomes, including those in fishes (Sola et al. 2000; Martins and Galetti 2001; Pisano and Ghigliotti 2009). On the other hand, in some invertebrate species the two rRNA genes present co-located organization as related for annelids, mollusks and crustaceans representatives, although the non co-located organization has also been described (Drouin and Moniz de Sá 1995; Vitturi et al. 2002, 2004; Wang and Guo 2004).

These association or co-localization of multigene families in animals is poorly understood and the significance of these associations is still unclear. According to Dover (1986) and Liu and Fredga (1999) this association is important for the maintenance of

conserved and multiple arrays. Kaplan et al. (1993) hypothesized that the association of repetitive multigene families could play a functional role in nucleus organization. The separation of 18S and 5S rDNA arrays could represent a functional advantage, considering that the 18S rRNA is transcribed by the RNA polymerase I and 5S rRNA by the RNA polymerase III. On the other hand, the association of histone H3 and 5S rRNA genes could not be explained by a transcriptional advantage since these two sequences are transcribed by distinct polymerases.

The high variability related to karyotype organization previously observed in Scarabaeinae representatives (Cabral-de-Mello et al. 2008) is also observed in the microevolutionary level, leading in consideration the high variability for the major rDNA clusters. This high variability can be correlated to heterochromatin distribution, indicating that probably these two genomic elements suffer similar evolutionary forces for spreading in the distinct lineages of Scarabaeinae subfamily. Moreover our results indicates that these genomic fractions have suffered neutral selection for dispersion in Scarabaeidae genomes. On the contrary of this differentiation pattern, the conservation of 5S rRNA and H3 histone gene clusters could represent a basal condition before the diversification of Scarabaeinae still maintained in the group and are under the government of different evolutionary processes compared to the major rDNA. Moreover they could indicate that the purifying selection and spreading mechanism might operate differently in the distinct multigene families and for the whole repeated DNAs in the genome. The results also reinforce this intense relationship, concerning possible association, between these two multigene families in Scarabeinae, although this organization need to be more studied at molecular level.

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Tibe *species*	Chromosomal formula (males)	General heterochromatin distribution	45S rDNA		5S rDNA		H3 histone		References
			Aut	Sex	Aut	Sex	Aut	Sex	
Ateuchini									
Atheuchus sp	16 = 7 + Xy	Pericentromeric blocks	4	–	2	–			This work
Canthonini									
Canthon staigi	18 = 8 + Xy_p	Pericentromeric blocks	2	–	2	–			This work
Deltochilum calcaratum	14 = 6 + neoXY	Pericentromeric blocks and diphasic chromosomes	4/ 6*	X/ X,Y*	2	–			Cabral-de-Mello et al (2010); This work
D. elevatum	20 = 9 + Xy_p		2	–	2	–			This work
D. morbillosum	14 = 6 + neoXY	Pericentromeric blocks and diphasic chromosomes	4	X					Cabral-de-Mello et al (2010)
D. verruciferum	20 = 9 +XY_p	Pericentromeric blocks and diphasic chromosomes	4	Y	2	–	2	–	This work
Coprini									
Dichotomius affinis	18 = 8 + Xy_p		2	–	2	–			Cabral-de-Mello et al (submitted); This work
Dichotomius bos	18 = 8 + Xy_p	Pericentromeric blocks	2	–	2	–	2	–	Cabral-de-Mello et al (submitted); This work
D. crinicollis	18 = 8 + Xy_p	Pericentromeric blocks	2	X	2	–	2	–	Cabral-de-Mello et al (submitted); This work
D. depresicollis	18 = 8 + Xy_p	Pericentromeric blocks	2	–	2	–	2	–	Cabral-de-Mello et al (submitted); This work
D. geminatus	18 = 8 +Xy_p	Pericentromeric and terminal blocks	4	–	2	–	2	–	Cabral-deMello et al (2010c, submitted); This work
D. laevicollis	18 = 8 + Xy_p	Pericentromeric blocks	2	–	2	–	2	–	Cabral-de-Mello et al (submitted); This work
D. mormon	18 = 8 + Xy_p		3/4*	X/X*	2	–			Cabral-de-Mello et al (submitted); This work
D. aff *mundus*	18 = 8 + Xy_p		2	–	2	–			Cabral-de-Mello et al (submitted); This work
D. nisus	18 = 8 + Xy_p	Pericentromeric blocks	–	X,Y	2	–	2	–	Cabral-de-Mello et al (submitted); Silva et al (2009); This work
D. semianeus	18 = 8 + Xy_p		–	X	2	–			Cabral-de-Mello et al (submitted); This work
D. semisquamosus	18 = 8 + Xy_p	Pericentromeric blocks	2/ 2*	–/ X*	2	–	2	–	Cabral-de-Mello et al (submitted); Silva et al (2009); This work
D. sericeus	18 = 8 + Xy_p	Pericentromeric blocks	2	–	2	–	2	–	Cabral-de-Mello et al (submitted);

D. sericeus sp1	18 = 8 + Xy_p		2	–	2	–			Silva et al (2009); This work Cabral-de-Mello et al (submitted); This work
D. sp	18 = 8 + Xy_p		2	–	2	–			Cabral-de-Mello et al (submitted); This work
Ontherus appendiculatus	20 = 9 + Xy_p	Pericentromeric and terminal blocks	2	–	2	–			This work
O. sulcator	20 = 9 + Xy_p		7	–					This work
Euristernini									
Eurysternus caribaeus	8 = 3 + neoXY	Pericentromeric blocks	–	X,Y	–	X	–	X	Arcanjo et al (2009) This work
Gymnopleurini									
Gymnopleurus sturmi	20 = 9 +Xy	Pericentromeric and subterminal blocks	4/5*	–					Colomba et al (2000)
Onitini									
Bubas bison	20 = 9 + XY	Pericentromeric and large terminal blocks	8						Colomba et al (2006)
Onthophagini									
Digitonthophagus gazella	20 = 8 + Xy_p/Xy/Xy_r	Pericentromeric blocks	2	–	2	–			This work
Phanaeini									
Coprophanaeus cyanescens	20 = 9 + XY_p	Diphasic chromosomes	5/4*	–/ X*	2	–	2	–	Oliveira et al (2010); This work
C. ensifer	20 = 9 + XY	Diphasic chromosomes	15/ 10*	X/ –*	–	X, Y	–	X, Y	Oliveira et al (2010) This work
Diabroctis mimas	20 = 9 + Xy_p/Xy	Paracentromeric blocks and diphasic chromosomes	4/ 6*	X/ X*	5	X	5	X	Bione et al (2005) This work
Isocopris inhiata	18 = 8 + Xy_p	Peri- and paracentromeric blocks and diphasic chromosomes	2	–					Bione et al (2005)
Phanaeus splendidulus	20 = 9 + xy_p		7	–	2	–	2	–	This work

Table 1. Species and respective diploid numbers, heterochromatin patterns and chromosome location of rDNA clusters and H3 histone gene in 31 Scarabaeinae representatives. The color bars in the diploid number and heterochromatin pattern columns represent the same chromosome characteristics described in figure 5. Asterisks indicate polymorphic condictions.

Figure legends

Figure 1. Fluorescent *in situ* hybridization in metaphases I using 5S rDNA (red) and 18S rDNA (green) in nine representatives species of Scarabaeinae of three distinct tribes (a-d) Phanaeini, (e-h) Canthonini, (i) Ateuchini. (a) *Coprophanaeus ensifer*, (b) *C. cyanescens*, (c) *Diabroctis mimas*, (d) *Phanaeus splendidulus*, (e) *Canthon staigi*, (f) *Deltochilum calcaratum*, (g) *D. verruciferum*, (h) *D. elevatum*, (i) *Atheuchus* sp. The arrows indicate the sex bivalents. Note the co-location of the two sequences in (e) for two chromosomes. Bar = 5µm.

Figure 2. Cytogenetic mapping of 5S (red) and 18S (green) rDNAs in four species of Scarabaeinae from (a) Euristernini, (b) Onthophagini, (c, d) Coprini tribes. (a) *Eurysternus caribaeus*, (b) *Digitonthophagus gazella*, (c) *Ontherus apendiculatus*, (d) *O. sulcator*. The arrows indicate the sex bivalents. Note the co-location of the two sequences in (a) and (b) in two species. Bar = 5µm.

Figure 3. Double-FISH for 5S rRNA and histone H3 genes in five Scarabaeinae representatives. (a) *Coprophanaeus cyanescens*, (b) *C. ensifer*, (c) *Phanaeus splendidulus*, (d) *Deltochilum verruciferum*, (e) *Eurysternus caribaeus*. The arrows indicate the sex bivalents. Note the co-location of the two sequences in all cells, and in (e) the presence of only on site for the two genes in the X chromosome. Bar = 5µm.

Figure 4. Initial meiotic cells (a-e, h, i) and interphasic nucleus (f, g) hybridized with 18S (green) and 5S rDNAs (red) (a-f) and 5S rRNA (red) and H3 histone genes (green) (g-i). (a) *Deltochilum elevatum*, (b) *Deltochilum calcaratum*, (c) *Dichotomius crinicollis*, (d) *Coprophanaeus cyanescens*, (e, f) *Diabroctis mimas*, (g) *Dichotomius bos* (h) *Dichotomius laevicollis*, (i) *Deltochilum verruciferum*. Note the separation of 18S and 5S rDNA signals in (a-e), in (f) two small signals are overlapped, and note in (g-i) the overlapped configuration of

5S rRNA and H3 histone genes. In (a, b, d, e and i) in possible the observation of chromsocenter formation by heterochromatic sequences. The scale bar is not shown.

Figure 5. (a) distribution of rDNA loci number in 31 Scarabaeinae species; Compared distribution of the number of 18S rDNA with diploid number in 31 species of Scarabainae (b) and with heterochromatin distribution in 21 species (c). The species *Deltochilum calcaratum*, *Dichotomius semisquamosus*, *D. mormon*, *Gymnopleurus sturmi*, *Coprophanaeus ensifer* and *Diabroctis mimas* were considered twice due the observation of polymorphisms related to number of sites. Each symbol below the name of species represent the distinct tribes, (Ŧ) Euristernini, (Φ) Ateuchini, (•) Coprini, (Δ) Onthophagini, (+) Gymnopleurini, (*) Canthonini, (o) Onitini. (■) Phaneini.

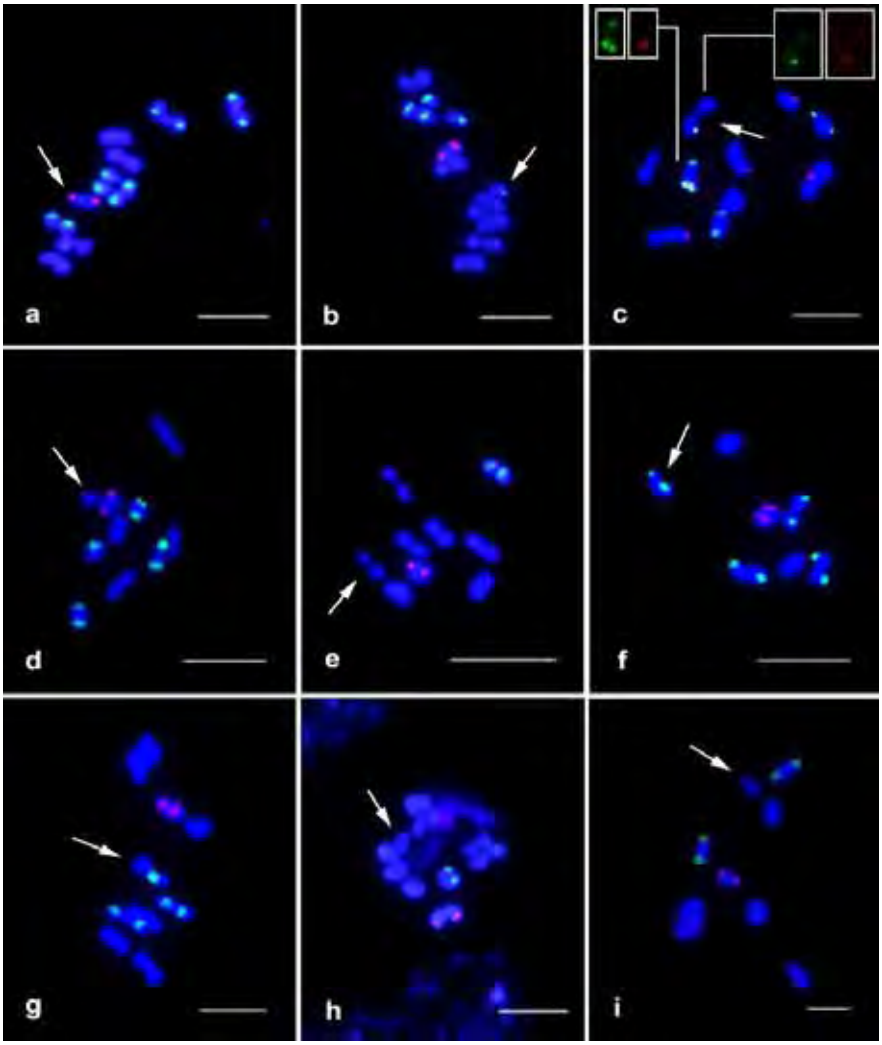

Figure 1

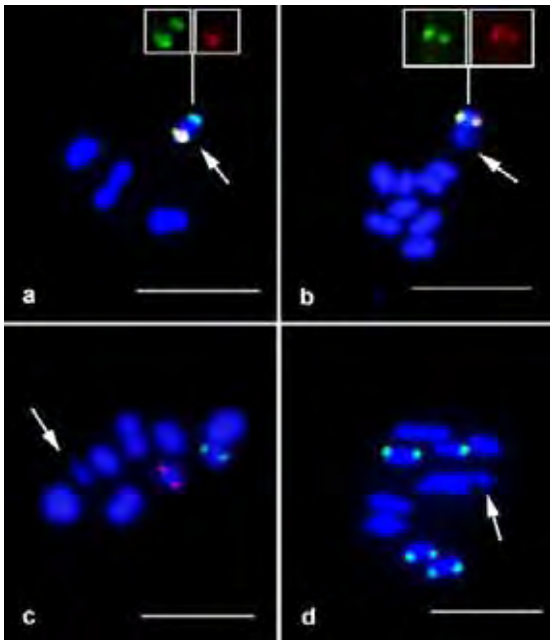

Figure 2

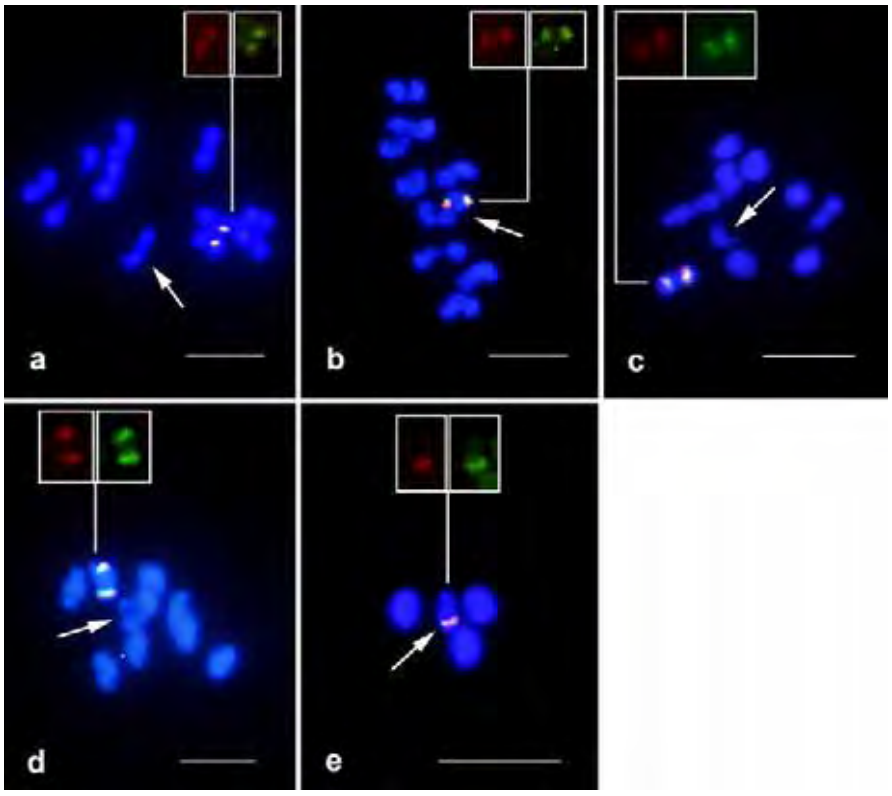

Figure 3

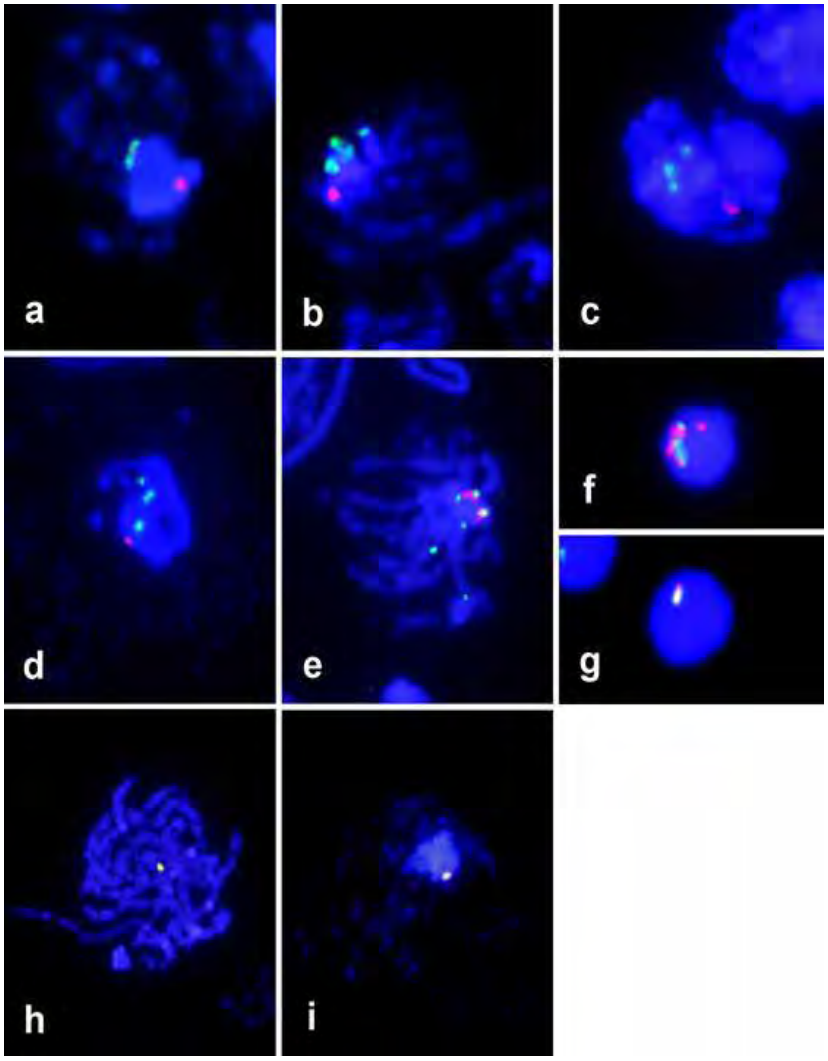

Figure 4

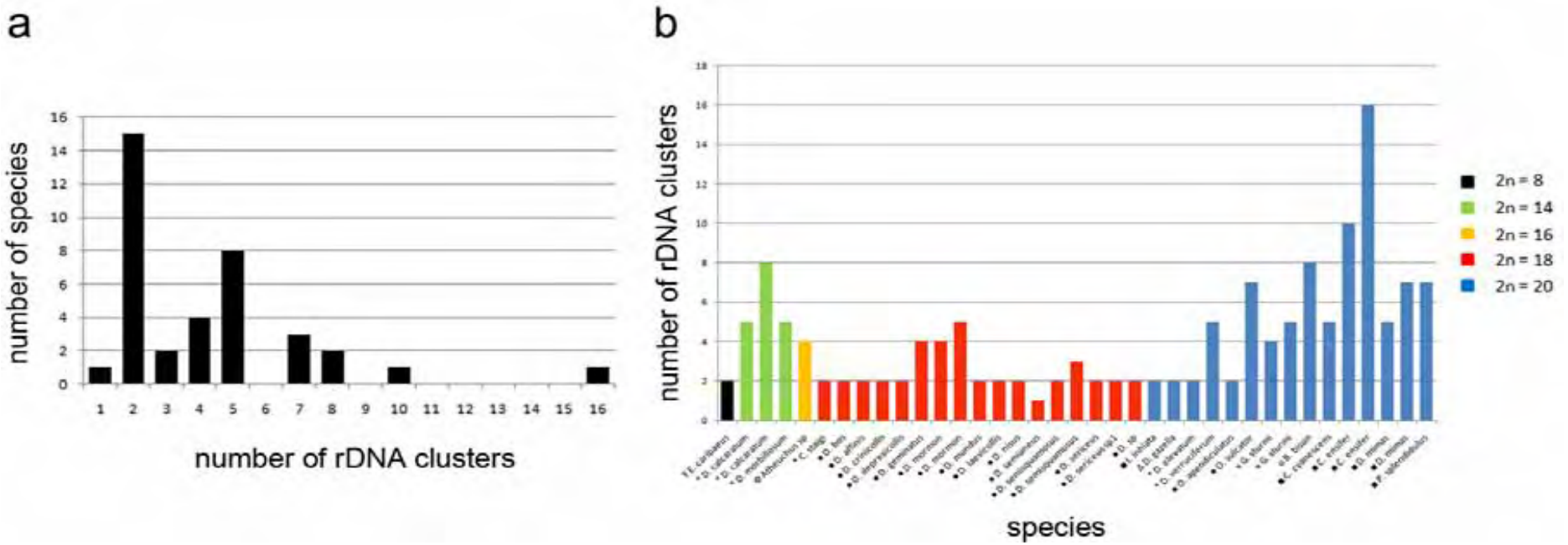
a
number of species
number of rDNA clusters
b
number of rDNA clusters
species
2n = 8
2n = 14
2n = 16
2n = 18
2n = 20

Figure 5

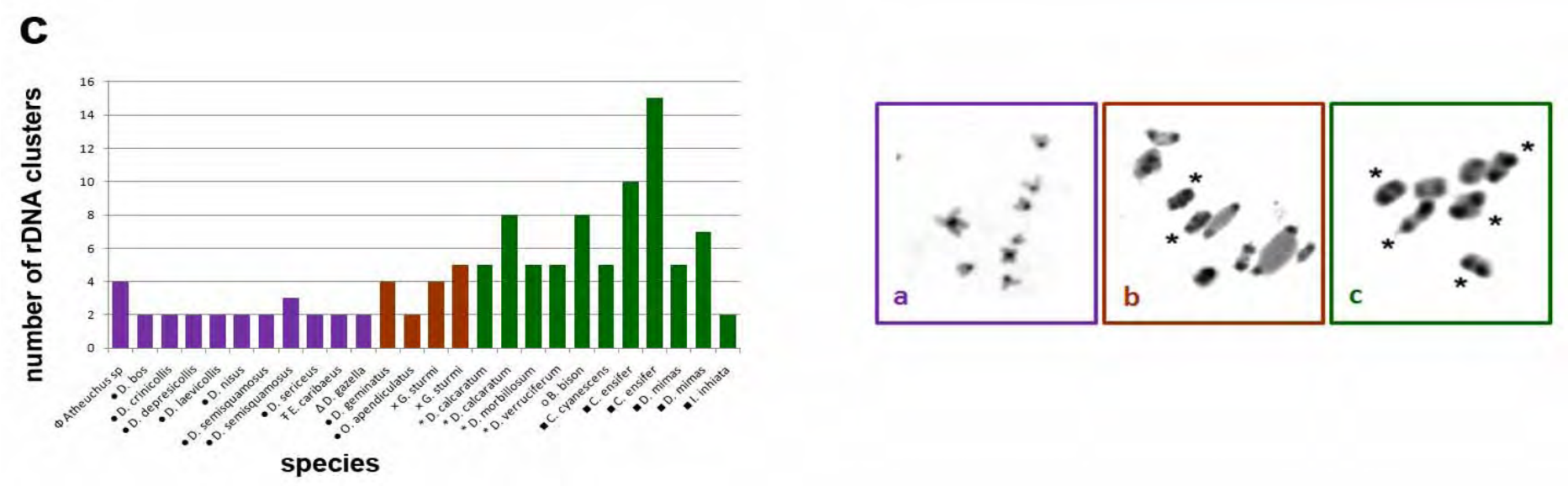
c
number of rDNA clusters
16
14
12
10
8
6
4
2
0
Φ Atheuchus sp
● D. bos
● D. crinicollis
● D. depresicollis
● D. laevicollis
● D. nisus
● D. semisquamosus
● D. semisquamosus
● D. sericeus
Ŧ E. caribaeus
Δ D. gazella
● D. geminatus
● O. apendiculatus
x G. sturmi
x G. sturmi
* D. calcaratum
* D. calcaratum
* D. morbillosum
* D. verruciferum
o B. bison
■ C. cyanescens
■ C. ensifer
■ C. ensifer
■ D. mimas
■ D. mimas
■ I. inhiata
species
a
b
c

Figure 5

6. CONCLUSÕES GERAIS

- As diferentes classes de elementos repetitivos têm sofrido padrões divergentes de diferenciação ao longo da cladogenese da subfamília Scarabaeinae;
- Os genes de RNAr 5S e histona H3, devido sua co-localização possivelmente tem sofrido os mesmos mecanismos de pressão seletiva, impedindo o espalhamento dos mesmos no genoma dos escarabeíneos;
- A heterocromatina constitutiva e o gene de DNAr 45S apresentaram padrões semelhantes de distribuição nos genomas de diversas espécies de Scarabaeinae, indicando que os mecanismos evolutivos atuantes nestas seqüências podem ser similares;
- A heterocromatina dos representantes do gênero *Dichotomius*, embora conservada quanto a sua localização sofreu intensa diversificação entre as espécies, ao menos entre *D. geminatus* e outras cinco espécies do gênero;
- A diversidade cariotípica previamente observada para Scarabaeinae relativa à macro-estrutura cromossômica é também observada em nível microgenômico, levando-se em consideração a variabilidade da heterocromatina constitutiva e do DNAr 18S;
- O uso da fração C_0t-1 DNA é uma ferramenta útil na análise cromossômica em representantes de insetos relacionadas à análise de origem e estrutura de cromossomos Bs, diferenciação da heterocromatina e sistemas sexuais, sendo uma metodologia rápida e barata.

7. ANEXOS

Extração de DNA genômico de tecidos sólidos

(Baseado em Sambrook and Russel 2001)

1. Macerar o tecido muscular obtido do pronoto dentro de um eppendoorf de 1,5mL com auxílio de uma tesoura;
2. Adicional 430 µL de tampão de digestão (ver tabela)

Reagente	**C1**	**V1**	**C2**	**V2**
NaCl	5M	10,0 µL	0,1M	500,0 µL
Tris-HCl pH 8	1M	5,0 µL	0,01M	500,0 µL
EDTA pH 8	0,5M	25,0 µL	0,025M	500,0 µL
SDS	10%	25,0 µL	0,5%	500,0 µL
Proteinase K	10mg/mL	5,0 µL	0,1mg/mL	500,0 µL
H_2O_d q.s.p.	-	430,0 µL	-	500,0 µL

3. Incubar em banho Maria a 50 ^oC por cerca de 1 hora e 30 min (homogeneizar periodicamente);
4. Adicionar 500,0 µL de Fenol:Clorofórmio (1:1) e agitar com movimentos rotatórios durante 15 min até homogeneizar os componentes;
5. Centrifugar a 15.000 RPM durante 15 min;
6. Transferir a camada superior para outro eppendorf de 1,5 mL;
7. Adicionar 0,2x o volume de NaCl 1M e 2x o volume de Etanol 100% gelado, agitar suavemente para precipitar o DNA;
8. Centrifugar a 15.000 RPM durante 15 min;
9. Descartar o sobrenadante e acrescentar 375,0 µL de Etanol 70% (lavagem), sem agitar;
10. Centrifugar a 15.000 RPM durante 15 min;
11. Descartar o sobrenadante e secar o pellet em estufa 37 ^oC;
12. Ressuspender com água mili-Q durante algumas horas;

Reações de PCR DNAr 18S, 5S e histona H3

1. Preparo do mix de reação

Reagente	Quantidade
Tampão Taq 10x	2,5 µL
Taq polymerase (5U/µL)*Invitrogen, 11615-010	0,25 µL
$MgCl_2$ (50mM)	0,75 µL
dNTP (8 µM)	0,8 - 1,0 µL
DNA (150,0 – 200,0 mg/µL)	1,0 µL
Pimer F (10,0 µM)	1,0 µL
Primer R (10,0 µM)	1,0 µL
H_2O mili-Q (q.s.p. 25,0 µL)	q.s.p. 25,0 µL

2. Ciclos para a PCR

Etapa	Temperatura	Tempo	
1	95 °C	5 min	
2	95 °C	40 seg	←
3*	X °C	40 seg	30x
4	72 °C	1 min	
5	72 °C	5 min	
6	4 °C	For ever	

* Ciclo de anelamento do primer: X = 54 (reação DNAr 18S); X = 53 (reação DNAr 5S) X = 48 (reação histona H3).

Isolamento de seqüências repetitivas através da técnica de C_0t-1 DNA

(Baseado em Zwick et al. 1997; Ferreira e Martins 2008)

1. Diluir o DNA genômico a 100,0 – 500,0 ng/µL em NaCl 0,3M;
2. Aliquotar 500 µL do DNA em tubo eppendorf de 1,5 mL;
3. Autoclavar durante 30 min a 1.4 atm/120 ^{o}C;
4. Checar a degradação do DNA em gel de Agarose 1 % (ideal obter fragmentos de 100 a 1.000 pb);
5. Desnaturar a 95 ^{o}C durante 10 min três alíquotas (tubos 0, 1, 5) de 50 µL de DNA autoclavado;
6. Transferir os tubos para gelo durante 10 seg e tratar imediatamente o tubo "0" com S1 nuclease; colocar os tubos "1" e "5" para renaturar o DNA a 65 ^{o}C;
7. Após 1 min tratar com S1 nuclease o tubo "1" e após 5 min tratar o tubo "5"com S1 nuclease;
    a. Para o tratamento com S1 nuclease utilizar 1U da enzima para cada 1 µg de DNA e 5,5 µL do tampão 10x
8. Incubar a 37 ^{o}C durante 8 min;
9. Congelar imediatamente em nitrogênio líquido;
10. Adicionar igual volume de Fenol:Clorofórmio (1:1);
11. Centrifugar durante 5 min a 13.000 RPM; coletar a fase aquosa e transferir para outro tubo eppendorf;
12. Precipitar o DNA com 2,5x do volume de Etanol 100% gelado;
13. Incubar em freezer -80 ^{o}C durante 30 min;
14. Centrifugar durante 15 min a 15.000 RPM a 4 ^{o}C;
15. Secar o pellet em estufa 37 ^{o}C e ressuspender com água mili-Q 30 - 50 µL;
16. Checar em gel de Agarose 1 % o tamanho dos fragmentos.

Clonagem dos fragmentos de PCR

pGEM®-T Easy Vector System (Promega, Cat. No. A1360)

1. Reação de ligação

Reagente	Quantidade
2x rapid ligation buffer, T4 DNA ligase	5,0 µL
pGEM®-T Easy Vector	1,0 µL
T4 DNA ligase	1,0 µL
Produto de PCR (30,0 – 100,0 ng/µL)	1,0 – 3,0 µL
Água mili-Q autoclavada	q.s.p. 10,0 µL

Incubar a 4 ^{o}C durante 18 horas;

2. Transformação bacteriana

2.1. Incubar tubos com bactérias competentes (estocadas a -80 ^{o}C com volume de 50,0 µL) em isopor com gelo até que ocorra descongelamento das mesmas;

2.2. Adicionar no tubo os 10,0 µL da reação de ligação e agitar gentilmente;

2.3. Manter no gelo por 30 min;

2.4. Incubar em banho Maria a 37 ^{o}C durante 1 min;

2.5. Incubar em gelo durante 2 min;

2.6. Adicionar 950,0 µL de maio LB líquido;

2.7. Incubar no Shaker em rotação de 225 RPM a 37 ^{o}C durante 1 hora;

- Durante este tempo preparar as placas com X-gal (20,0 mg/mL): em câmara de fluxo laminar adicionar 20 µL de X-gal a cada placa com meio LB sólido, espalhando-o com uma alça.

2.8. Centrifugar durante 10 – 20 seg a 10.000 RPM;

2.9. Descartar o sobrenadante e plaquear o restante da solução;

2.10. Incubar a 37 ^{o}C durante 18 horas (em câmara úmida com ventilação).

3. Confirmação da presença do inserto

A identificação das colônias recombinantes pode ser realizada através de amplificação via PCR, utilizando primers específicos que fanqueiam o local da inserção do fragmento de DNA de interesse.

Primers M13: M13F 5' AGC GGA TAA CAA TTT CAC ACA GG 3'; m13r 5' CCC AGT CAC GAC GTT GTA AAA 3'

Mix de reação

Reagente	**Quantidade**
Tampão Taq 10x	2,5 µL
Taq polymerase (5U/µL)*Invitrogen, 11615-010	0,25 µL
$MgCl_2$ (50mM)	0,75 µL
dNTP (8 mM)	0,8 - 1,0 µL
DNA (colônia pinçada)	
Pimer F (10,0 µM)	1,25 µL
Primer R (10,0 µM)	1,25 µL
H_2O mili-Q (q.s.p. 25,0 µL)	q.s.p. 25,0 µL

Ciclo da PCR

Etapa	**Temperatura**	**Tempo**	
1	95 °C	5 min	
2	95 °C	40 seg	←
3	50 °C	40 seg	30x
4	72 °C	2 min	
5	72 °C	5 min	
6	4 °C	For ever	

As colônias devem ser pinçadas com palito estéril diretamente da placa de Petri. Pinçar preferencialmente as colônias brancas (recombinantes). Checar em gel de Agarose 1% a amplificação do fragmento. * O vetor possui aproximadamente 250 pb.

Preparo de soluções

Meio LB: *Líquido:* 8,0 g Peptona; 8,0 g NaCl; 4,0 g de extrato de levedura; H2O q.s.p. 800,00 mL; *Sólido:* adicionar 12,0 g de Ágar. Autoclavar e adicionar 200,0 µL de Ampicilina (25,0 µg/mL) para cada 100,0 mL de meio.

Marcação dos fragmentos de DNA para uso como sonda

Marcação através da reação de *Nick translation*

- Kit BiNick™ Labeling System (Invitrogen, Cat. no. 18247-015), Biotina

Mix para 2-4 lâminas

- 200-250 ng de DNA;
- 1,0 µL de dNTP mix;
- 1,0 µL de mix de enzima;
- H_2O q.s.p. 9,0 µL.

1. Misturar os componentes e incubar a 16 ^oC durante 30 min;
2. Interromper a reação adicionando 1,0 µL de *stop buffer*;
3. Adicionar 1/10 do volume (1,0 µL) de Acetato de Sódio 3M e dobrar o volume de Etanol (22,0 µL) 100% gelado;
4. Misturar gentilmente invertendo o tubo e incubar em freezer –80 ^oC durante 30 min;
5. Centrifugar a 15.000 RPM durante 15 min a 4 ^oC;
6. Descartar o sobrenadante e adicionar Etanol 70% gelado;
7. Centrifugar a 15.000 RPM durante 5 min a 4 ^oC;
8. Descartar o sobrenadante e secar o pellet em estufa a 37 ^oC;
9. Resuspender em 6,0 µL de áhua mili-Q autoclavada.

- Kit DIG-Nick translation mix (Roche, Cat. no. 11745816910), Digoxigenina

Mix para 10-20 lâminas

- 1.000-1.200 ng de DNA;
- 4,0 µL de mix de Nick;
- H_2O q.s.p. 20,0 µL.

1. Misturar os componentes e incubar a 15 ^oC durante 90 min;
2. Interromper a reação adicionando 2,0 µL de *stop buffer*;

3. Aquecer a 65 ^{o}C durante 10 min;
4. Adicionar 2,2 µL de Acetato de Sódio 3M e 50,0 µL de Etanol 100% gelado;
5. Misturar gentilmente invertendo o tubo e incubar em freezer –80 ^{o}C durante 30 min;
6. Centrifugar a 15.000 RPM durante 15 min a 4 ^{o}C;
7. Descartar o sobrenadante e adicionar Etanol 70% gelado;
8. Centrifugar a 15.000 RPM durante 5 min a 4 ^{o}C;
9. Descartar o sobrenadante e secar o pellet em estufa a 37 ^{o}C;
10. Resuspender em 30,0 µL de áhua mili-Q autoclavada.

Marcação através da reação de PCR

Utilizar o plasmídio com inserto do gene de RNAr 5S para a reação de PCR. A reação é semelhante a uma reação de identificação de inserto.

Mix de reação

Reagente	**Quantidade**
Tampão Taq 10x	2,5 µL
Taq polymerase (5U/µL)$^{*Invitrogen, 11615-010}$	0,25 µL
$MgCl_2$ (50mM)	0,75 µL
dNTP (8 mM)*	0,8 - 1,0 µL
DNA (1.000 ng/µL)	0,3 µL
Pimer F (10,0 µM)	1,25 µL
Primer R (10,0 µM)	1,25 µL
H_2O mili-Q (q.s.p. 25,0 µL)	q.s.p. 25,0 µL

* Utilizar os dNTPs em separado (2 mM): Adicionar igual volume de dCTP, dGTP, dTTP. Para o dTTP deve ser usado 70% do volume usado para os outros dNTPs e 30% do Digoxigenin-11-dUTP (Roche, cat. no. 11573152910). Checar inicialmente a concentração dos reagentes.

Heredity (2010) 104, 393–400

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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 C_0t-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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Keywords: cytogenetics; C_0t-1 DNA; FISH; genome evolution; heterochromatin; supernumerary chromosome

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 (Rożek *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 DH5α *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 C_0t-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences) (Zwick *et al*., 1997, Ferreira and Martins, 2008). DNA samples (200 μl of 100–500 ng μl^{-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 μl 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 C_0t-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 μg ml^{-1}) for 1.0 h and with pepsin (10 μg 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 × SSC), in a final volume of 15 μl, was dropped onto slides that were previously denatured using 70% formamide, 2 × SSC for 40–60 s at 70 °C. In double FISH experiments, 15 μl 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),

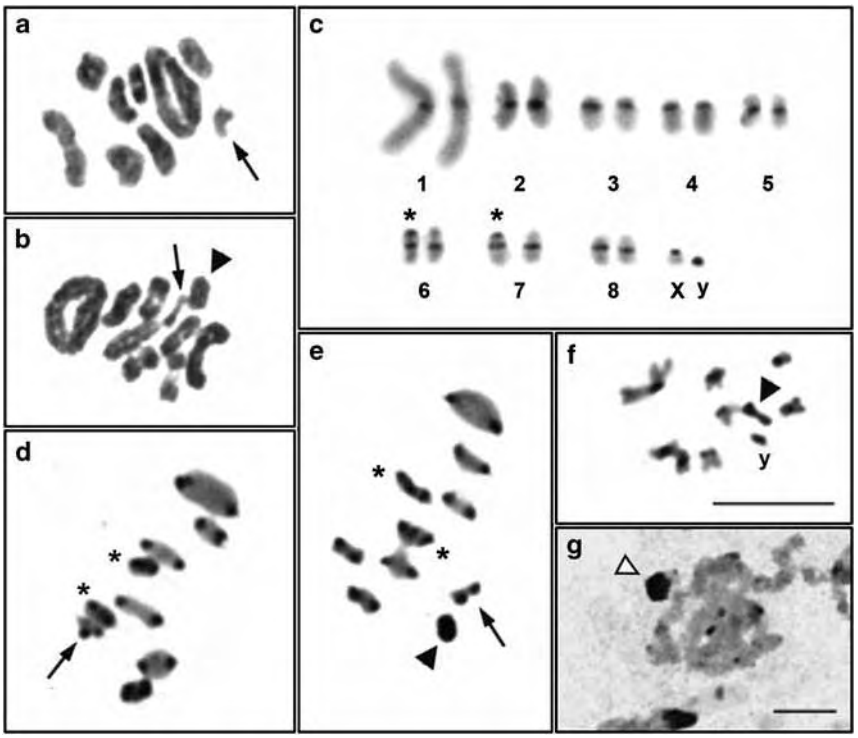

Figure 1 Male meiotic cells and karyotype of *Dichotomius geminatus*. Conventional staining of metaphase I chromosomes of 0B individuals (**a**) and 1B individuals (**b**); C-banded karyotype (**c**), metaphases I of 0B (**d**) and 1B (**e**) individuals and metaphases II of B-carrying individual (**f**). 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 μm.

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 Maracaípe 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.

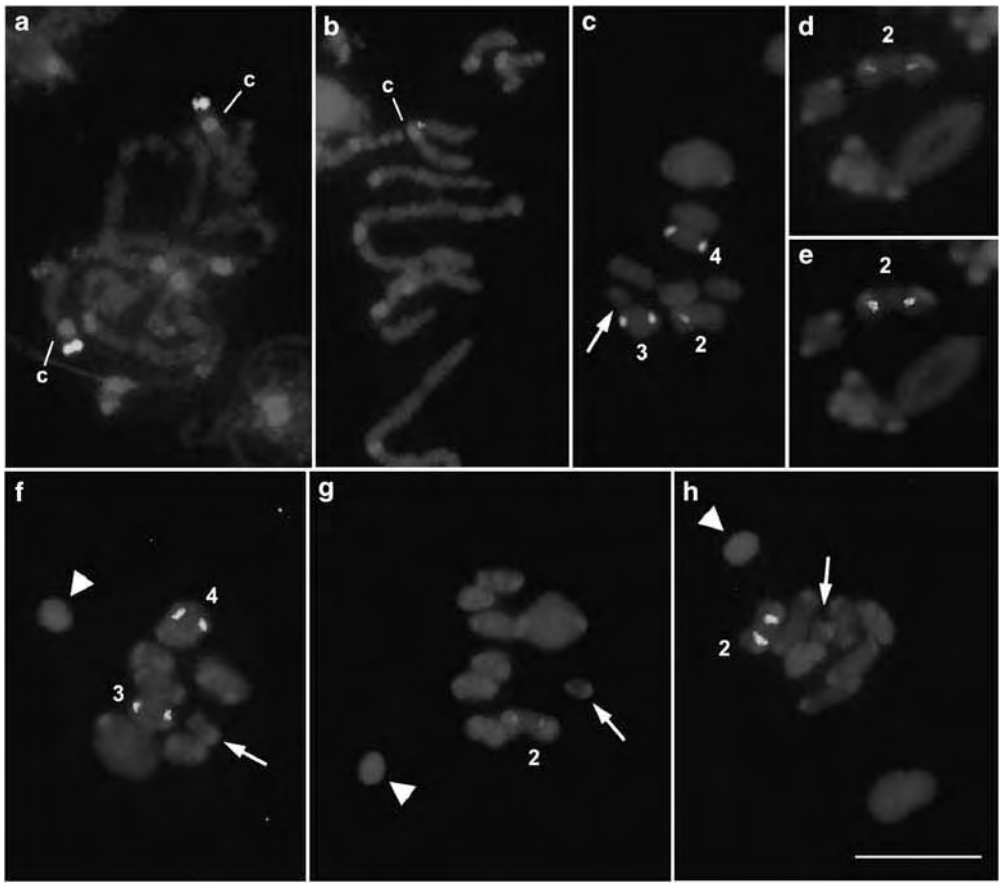

Figure 2 Fluorescent *in situ* hybridization (FISH) with 18S ribosomal RNA (rRNA), 5S rRNA and histone H3 gene probes in 0B and 1B individuals of *Dichotomius geminatus*. Pachytene chromosomes from 0B individuals hybridized using 18S (**a**) and 5S rDNAs (**b**); double FISH with 18S (green) and 5S (red) 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 μm. 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 *Isocopris 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.

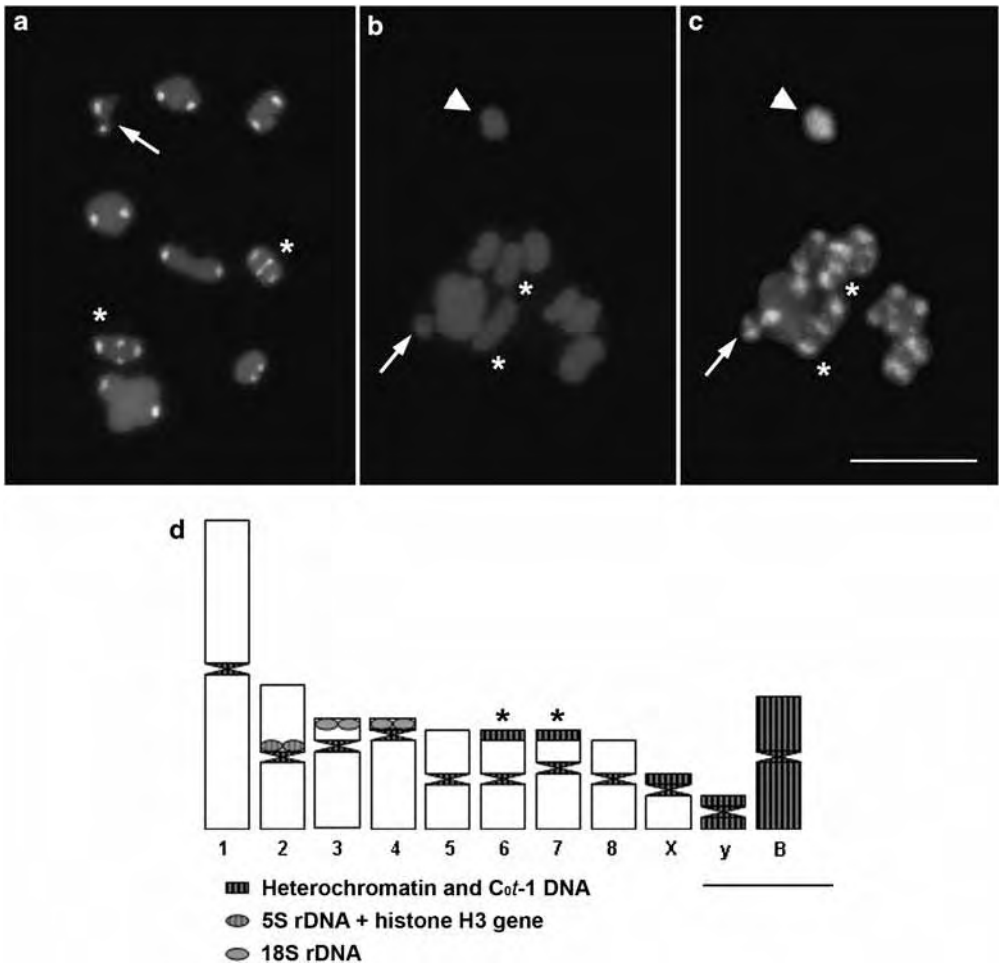

Figure 3 C_0t-1 DNA fraction hybridization in metaphase I chromosomes of 0B individuals (**a**) and 1B individuals (**b**,**c**). Ideogram (**d**) showing the hybridization patterns described in this work. The arrows indicate the sex bivalents (Xy_p), arrowheads indicate the B chromosome, and the * (asterisk) 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 interspersion 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 (Doolitlle 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 generates 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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Cytogenetic Mapping of 5S and 18S rRNAs and H3 Histone Genes in 4 Ancient Proscopiidae Grasshopper Species: Contribution to Understanding the Evolutionary Dynamics of Multigene Families

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Key Words
Chromosomal evolution · FISH · Genome organization · Multigene families · Repetitive DNA

Abstract
This paper reports on the chromosomal location of 18S rRNA, 5S rRNA and H3 histone multigene families in 4 species of a relatively ancient and diversified group of grasshoppers belonging to the family Proscopiidae. The 5S rRNA and H3 histone genes were highly conserved in the number of sites and chromosomal position in the 4th chromosome pair in all species analyzed, whereas the 18S rRNA genes showed slightly more variation because they were present on one or 2 chromosome pairs, depending on the species. The 5S and 18S rRNA gene families occurred in different chromosomes; in contrast, H3 histone and 5S rRNA genes co-localized in the same chromosomal position, with an apparently interspersed organization. Considering that the Proscopiidae family is a relatively ancient group compared with the Acrididae family, the association of the H3 histone and 5S rRNA multigene families can represent a basal condition for grasshoppers, although more research is needed on other representatives of this insect group to confirm this statement. The presence of such an association of 5S rDNA and H3 histone in mussels and arthropods (beetles, grasshoppers and crustaceans) suggests that this linked configuration could represent an ancestral pattern for invertebrates. These results provide new insights into the understanding of the genome organization and the evolution of multigene families in grasshoppers and in insects as a whole.

Repetitive DNA elements constitute a large portion of eukaryotic genomes, including tandem arrays and dispersed repeats. Tandem array repeats comprise mainly satellite DNAs and multigene families [Charlesworth et al., 1994]. The ribosomal RNA (rRNA) and the histone protein gene families include a variable number of copies and locations across the genomes. The use of fluorescence in situ hybridization (FISH) for rRNA and histone genes has provided useful chromosomal markers for comparative analysis, elucidation of genome organization and identification of chromosomal rearrangements in many organisms. Among invertebrates, for instance, mapping

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of rRNA genes has been performed in many groups, such as worms, insects, mollusks, echinoderms and others [Vitturi et al., 2002; Wang and Guo, 2004; Caradonna et al., 2007; Cabrero and Camacho, 2008]. On the other hand, mapping of histone genes has been restricted to 19 chironomid midges [Hankeln et al., 1993], 11 fruit flies [Schienman et al., 1998; Ranz et al., 2003], 5 mollusks [Eirín-López et al., 2004; Zhang et al., 2007], 35 acridid grasshoppers [Cabrero et al., 2009] and one beetle species [Cabral-de-Mello et al., 2010].

The family Proscopiidae comprises about 266 species that have been arranged into 3 subfamilies: Hybusinae, Xeniinae and Proscopiinae, all of which are distributed exclusively throughout Central and South America [Liana, 1980; Otte et al., 2003]. The taxonomic status and the phylogenetic position of this family remain controversial, although it represents a relatively ancient and diversified group if compared with the acridids, forming a quite old Acridomorpha group [Descamps, 1973; Matt et al., 2008]. Proscopiidae are characterized by diploid chromosome numbers ($2n♂ = 15, 17, 19$) lower than those observed in the Acrididae grasshoppers (most species with $2n♂ = 23$), although both families coincide in sharing the XO/XX sex chromosome system and acrocentric chromosomes in most species [Mesa and Ferreira, 1981; Moura et al., 1996; Souza and Moura, 2000]. According to Mesa and Ferreira [1981], fusions, fissions and inversions are responsible for the chromosomal diversity observed in the Proscopiidae family. Other aspects of the chromosomal organization, e.g., the location of repetitive DNA, have scarcely been studied, with only 4 species analyzed for heterochromatin and nucleolus organizer region location, through classical cytogenetic methods [Moura et al., 1996; Souza and Moura, 2000].

With the aim of contributing to the knowledge of grasshopper genomes, we investigated the chromosome location of 3 multigene families (5S rRNA, 18S rRNA and H3 histone genes) in 4 Proscopiidae species, using single and double FISH. Our results revealed the colocalization of 5S rRNA and the H3 histone genes as well as the independent location of the 18S rDNA. These data contribute to a better understanding of the genome organization of multigene families in grasshoppers and in insects as a whole.

Materials and Methods

Adult males of 4 species of Proscopiidae grasshoppers, i.e., *Scleratoscopia protopeirae* (7), *S. spinosa* (8), *Stiphra robusta* (12) and *Tetanorhynchus silvai* (6), were collected from countryside regions in the Pernambuco State, Northeast Brazil. The testes were fixed in Carnoy (3:1 ethanol:acetic acid), and the chromosome preparations were made by squashing in a drop of 45% acetic acid and subsequently removing the coverslip with a razor-blade after immersion in liquid nitrogen.

DNA probes of the 5S rRNA, 18S rRNA and H3 histone genes were obtained from the genome of the beetle *Dichotomius geminatus* [Cabral-de-Mello et al., 2010]. The 18S rRNA and H3 histone gene probes were labeled by nick translation using biotin-11-dATP (Invitrogen, San Diego, CA, USA), whereas the 5S rRNA gene was labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany). The FISH procedures were performed according to the method described by Cabral-de-Mello et al. [2010]. Preparations were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield (Vector, Burlingame, CA, USA). Images were captured with the Olympus DP71 digital camera coupled to a BX61 Olympus microscope and were optimized for brightness and contrast using Adobe Photoshop CS2.

Results

The 4 species studied showed $2n♂ = 19$ chromosomes, with an XO sex-chromosome system. Acrocentric chromosomes were observed in *Stiphra robusta* and *Tetanorhynchus silvai*, whereas the 2 *Scleratoscopia* species showed 2 submetacentric autosomal pairs (the first and 3rd, in order of decreasing size) and a submetacentric X chromosome. The karyotypes of *Scleratoscopia* species consist of 3 pairs of large chromosomes (L1–L3), 3 pairs of medium chromosomes (M4–M6) and 4 pairs of small chromosomes (S6–S10), the X is a medium element (M5). In *S. robusta* and *T. silvai* the chromosomes showed gradual decreasing size, and were not classified in distinct groups. These results for conventional analysis are in agreement with previous studies [Moura et al., 1996; Souza and Moura, 2000].

FISH with the 18S rDNA probe revealed the presence of sites in the 6th and 7th chromosome pairs of *Stiphra robusta* and *Tetanorhynchus silvai*, whereas it was restricted to the 7th pair in the 2 *Scleratoscopia* species (fig. 1). Furthermore, the chromosome location of the 18S rDNA showed some differences among species because it was interstitial in *S. robusta* (see insets in fig. 1c), but it was pericentromeric in the other 3 species (fig. 1). In addition, the 7th chromosome pair in *S. robusta* showed a heteromorphism for the size of the 18S rDNA cluster (see fig. 1c).

All 4 species showed a similar organization pattern for the 5S rRNA and H3 histone genes. Both types of DNA sequences colocalized in the 4th chromosome pair in the 4 species, but they were located in the pericentromeric

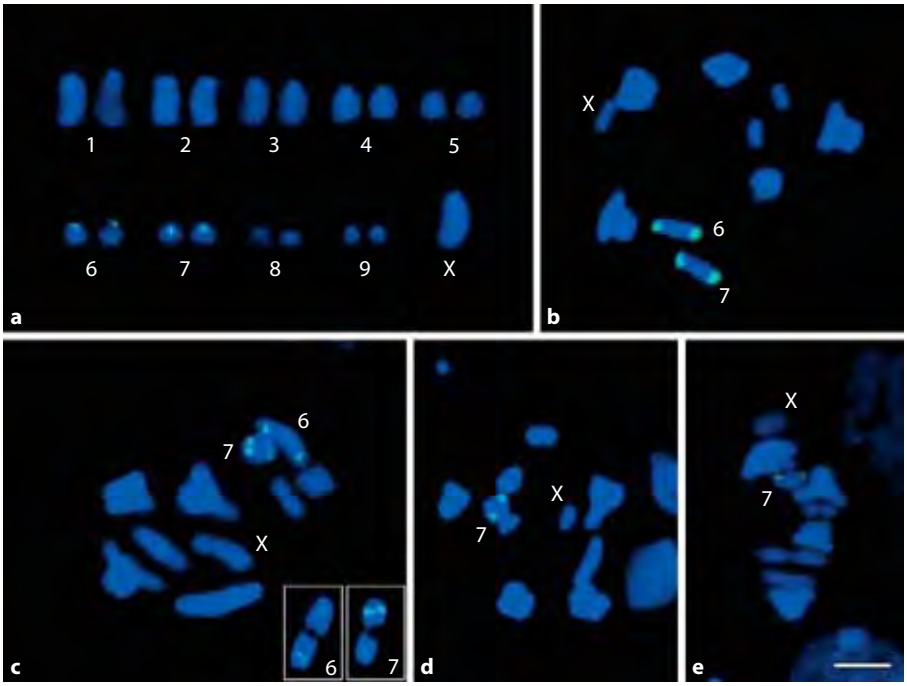

Fig. 1. Fluorescent in situ hybridization of 18S rDNA in the chromosomes of 4 Proscopiidae species. Karyotype (**a**) and metaphase I (**b**) of *Tetanorhynchus silvai*; metaphases I of *Stiphra robusta* (**c**), *Scleratoscopia spinosa* (**d**) and *S. protopeirae* (**e**). The inserts (**c**) show the precise position of the 18S rDNA cluster in chromosomes 6 and 7 in initial anaphase I and the heteromorphism of 18S rDNA clusters between homologues of pair 7. Bar = 5 μm.

region in *Scleratoscopia protopeirae*, *S. spinosa* and *Stiphra robusta* and they were slightly further from the centromere in *T. silvai* (fig. 2).

Discussion

These first data on the chromosome location of the multigene families in Proscopiidae, an ancient family of grasshoppers, indicate a general conservatism within this group because 18S rDNA is restricted in these 4 species from 3 different genera to one or 2 chromosome pairs, and 5S rRNA and H3 histone genes are colocalized at a single chromosome pair. Although more Proscopiidae species need to be analyzed, this family of grasshoppers seems to show less variation than Acrididae grasshoppers, at least for the 2 classes of rDNA analyzed in this investigation.

The location of 18S rDNA sites in the 7th chromosome pair, in order of decreasing size, observed in the 4 species analyzed in this research indicates that this was probably a consensus location in this group and thus suggests that this might be an ancient placement for these genes. The site located in the 6th pair of *Stiphra robusta* and *T. silvai*, however, might represent a derived pattern. In addition, structural rearrangements, e.g., a small paracentric inversion, might have been involved in changing the 18S

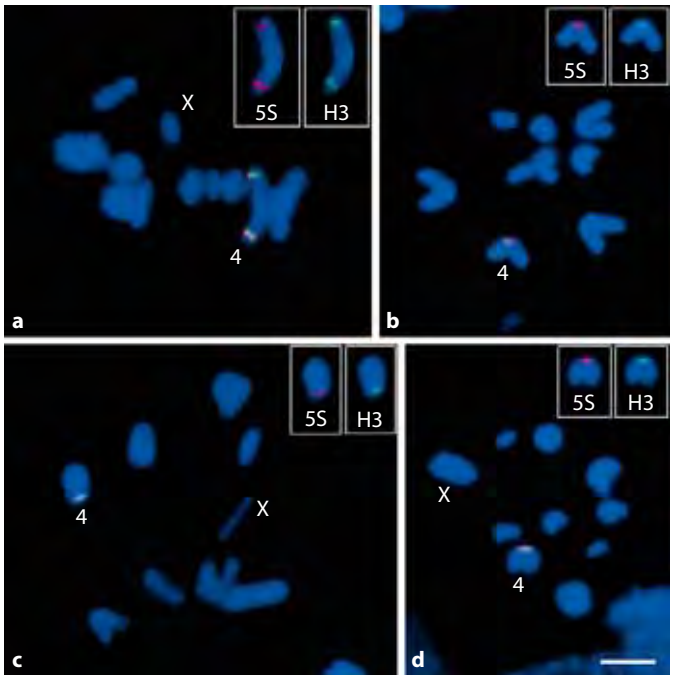

Fig. 2. Double FISH using 5S rRNA (red) and H3 histone (green) genes in metaphase I (**a**, **c**) and metaphase II (**b**, **d**) cells of the species studied in this work. *Tetanorhynchus silvai* (**a**), *Stiphra robusta* (**b**), *Scleratoscopia spinosa* (**c**) and *S. protopeirae* (**d**). Note in the inserts the labeled chromosomes with separated probes. Bar = 5 μm.

rDNA location toward an interstitial position in *S. robusta*, with no modification in the chromosome morphology. Moreover, the size heteromorphism observed for the 18S rDNA in the 7th chromosome pair in this last species suggests that this DNA seems to be currently subjected to mechanisms changing the amount of repeats between homologous chromosomes. Souza and Moura [2000], studying the same *S. robusta* populations, reported the presence of 3 nucleolar organizer regions, detected by silver nitrate staining that suggests the existence of polymorphism for rDNA site number and location.

The 5S rRNA and H3 histone gene clusters were highly conserved in the 4 Proscopiidae species analyzed and were restricted to a single chromosome pair, i.e., the 4th in order of decreasing size. The chromosomal position of the H3 histone genes is also very conserved in representatives in the Acrididae family, with most species showing a single cluster in the 8th chromosome pair in species with $2n♂ = 23$. On the other hand, the H3 histone genes changed position to chromosome 3 as a result of one of the centric fusions that decreased the chromosome number to $2n♂ = 17$ in many genera of the Gomphocerinae subfamily [Cabrero et al., 2009]. This conservatism in the number of clusters for H3 histone could be ancient in grasshoppers and there have been no changes occurring since the divergence of the Proscopiidae and Acrididae families, more than 100 million years ago [see Hewitt, 1979]. The presence of a single cluster for histone genes was also reported in 3 mollusk species [Zhang et al., 2007], 35 grasshopper species [Cabrero et al., 2009] and 3 fish species [Pendás et al., 1994]. On the other hand, more than one histone site was observed in 2 mollusk species [Eirín-López et al., 2004; Zhang et al., 2007] and some dipteran species [Hankeln et al., 1993; Schienman et al., 1998; Ranz et al., 2003].

The presence of a single site for 5S rDNA observed in the 4 Proscopiidae species analyzed here is relatively common in eukaryotes and has been reported in vertebrates and some mollusks [Mandrioli, 2000; Sola et al., 2000; Martins and Galetti, 2001; López-Piñón et al., 2005; Insua et al., 2006; Huang et al., 2007]. In grasshoppers, the 5S rDNA sequences have been previously mapped in only 2 species, *Eyprepocnemis plorans* from 3 populations [Cabrero et al., 2003] and *Rammathocerus brasiliensis* [Loreto et al., 2008]. Both species showed several clusters of 5S rDNA. Therefore, in grasshoppers, the 5S rRNA multigene family appeared to show a more dynamic evolution compared to H3 histone genes. But the Proscopiidae species analyzed here also showed a conservative pattern for the 5S rRNA genes, which might result from their ancient association with the histone genes and their submission to the same constraints that have conserved the chromosomal location of histone genes in grasshoppers in general. Although the 5S rDNA can present high evolutionary dynamics in some genomes, this statement seems to not be applicable in the Proscopiidae family because the 5S rRNA genes are restricted to a single chromosome locus.

The association of 5S rRNA and H3 histone genes has previously been reported in 4 other invertebrate species. In insects, Cabral-de-Mello et al. [2010] described, for the first time, the association of 5S rRNA and H3 genes in the beetle *Dichotomius geminatus*. Likewise, this association has also been reported in 2 crustacean species and one mussel species [Drouin and Moniz de Sá, 1995; Barzotti et al., 2000; Eirín-López et al., 2004]. In the Proscopiidae family, the H3 histone and 5S rRNA genes form a conspicuous cluster, with both multigene families apparently interspersed among each other. However, additional studies by means of fiber-FISH, Southern blot and DNA sequencing are necessary to clarify this statement. Available evidence on the interspersed arrangement of 5S rRNA and histone genes suggests the possibility that it may represent the ancestral condition for arthropods, considering that this arrangement occurs in beetles, grasshoppers and crustaceans. Alternatively, bearing in mind that the 5S rDNA shows the capability to move within the genome, the 5S rDNA repeats could have invaded the histone clusters several times during the evolution of these groups.

The absence of an association among major and 5S rRNA genes is a widespread condition among eukaryotes. The 5S and the major rRNA genes are organized in a linked configuration in fungi but in distinct genomic arrays in higher eukaryotes [Drouin and Moniz de Sá, 1995]. According to Martins and Galetti [2001], the separation of 18S and 5S rRNA genes in distinct genomic arrays could represent a functional advantage, considering that the 18S rRNA is transcribed by the RNA polymerase I and 5S rRNA by the RNA polymerase III. In contrast, the specific association of 5S rRNA and H3 histone genes cannot be explained by an advantage in the co-transcription process since these sequences are also transcribed by different RNA polymerases, RNA polymerase III and II, respectively.

Considering that Proscopiidae is an ancient group of grasshoppers, the condition of association of 5S rDNA and H3 histone genes can represent a basal condition before the diversification of grasshoppers. Further chromosomal studies using these sequences in other representa-

tives of Proscopiidae family and in other grasshopper families, such as Acrididae and Romaleidae, are necessary. Although the molecular organization of the association of 5S and H3 genes is still to be elucidated, our data reinforce the previous findings concerning the possible association of these genes and contribute to understanding the dynamics of multigene families in invertebrate genomes.

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Evolutionary dynamics of rDNAs and H3 histone sequences in the A complement and B chromosome of *Rhammathocerus brasiliensis* (Acrididae, Gomphocerinae): Chromosomal dispersion and co-localization.

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Abstract

The distribution of 18S rRNA, 5S rRNA and H3 histone genes was analyzed by fluorescence *in situ* hybridization in the standard karyotype and B chromosome of three populations of the grasshopper *Rhammatocerus brasiliensis*. The diploid number, chromosomal morphology and number and location of major rDNA were coincident with the previous analysis performed for this species. On the other hand, the 5S rDNA mapped in almost all chromosomes of the standard complement (except in the pair 11) and in the B chromosome, showing a distinct result from other populations previously analyzed. Besides the spreading of 5S rDNA in the genome of *R. brasiliensis* it was also observed multiple sites for H3 histone genes, being located in the same chromosomal regions of 5S rDNAs, including the presence of the H3 histone gene in the B chromosome. These findings indicate a linked organization for these two multigene families in this grasshopper. Due to the intense multiplication of 5S rDNA clusters, its chromosomal distribution was not informative in the clarification of the origin of B elements, at least in the three populations studied, being the present data are in disagreement with autosomal origin hypothesis of the B chromosome in this species, although the sexual origin could not be evidenced. These results reinforce previous findings concerning the association of 5S rRNA and H3 histone genes in some insects, providing new insights to the understanding of genome organization and evolution of multigene families in grasshoppers. Moreover we contribute in the knowledge about B chromosome composition, origin and evolution.

Key-words: B-chromosome, FISH, grasshopper, multigene family

Introduction

In eukaryotes the transcribing sequences for ribosomal RNAs (rRNAs) are organized in two multigenic families tandemly arrayed in genomes. The major one is formed by the 18S, 5.8S and 28S rDNA (45S rDNA) and the minor one is responsible for the transcription of the 5S rRNA (Long and David 1980). Concerning the histone gene sequences they may be arranged in tandemly repeated clusters composed by intronless genes that codes for H1, H2A, H2B, H3 and H4 histone proteins spaced by noncoding DNA, although some variation have been reported for this organization (Maxson et al. 1983; Nei and Rooney 2005).

Supernumerary B chromosomes occur in addition to standard karyotype and have been described in about 15% of eukaryotes. Some studies have been conducted on B elements, concerning their distribution, frequency in populations, structure and origin, effects in standard complement and transmission (Camacho 2000, 2005). The B chromosomes are relatively well studied in grasshopers, with most studies focused, for example in *Eyprepocnemis plorans*, *Locusta migratoria* and *Podisma* species. In this group some variability for B chromosomes have been reported, related to heterochromatin patterns, satellite DNAs organization, and histone multigene families (Cabrero et al. 2003a; Camacho 2005; Bugrov et al. 2007; Loreto et al. 2008a; López-León et al. 2008; Teruel et al. 2009, 2010).

Due to the clustered organization of some repeated DNAs they have been an important source as chromosomal marker for analysis of karyotypic evolution, genomic structure and origin and evolution of B chromosomes in animals. In grasshoppers the mapping of repeated elements is primarily concentrated in analysis of number and location of the major rDNA and histone genes, and in the lesser extend satDNAs and 5S rDNA (Cabrero et al. 2003b; Cabrero and Camacho 2008; Cabrero et al. 2009; Cabral-de-Mello et al. 2010a; Teruel et al. 2010). Concerning multigene families in Acrididae the major rDNA was mapped in 53 species, the

histone genes were located in chromosomes of 39 species and the 5S rDNA distribution was described only in three species (Cabrero et al. 2003a; Cabrero and Camacho 2008; Loreto et al. 2008a; Cabrero et al. 2009; Teruel et al. 2010). The mapping of these sequences together with the satDNA has been an important tool for understanding chromosomal evolution and origin and evolution B chromosomes in some species of this family (Cabrero et al. 2003b; Loreto et al. 2008a; Teruel et al. 2010).

The species *Rhammatocerus brasiliensis* (Acrididae, Gomphocerinae) presents karyotype 2n = 23,XO and acrocentric chromosomes, and bears a distinct B chromosome (Loreto et al. 2008a). Loreto et al. (2008a) have analyzed the occurrence of B chromosomes in nine populations of *R. brasiliensis* and using the mapping of rRNA genes proposed autosomal origin for this supernumerary element. Here we performed an analysis of three populations of *R. brasiliensis* bearing B chromosomes under the focus of chromosomal mapping of rRNAs and histone H3 multigene families. Our results indicated an intense spreading of 5S rDNAs in this grasshopper species accompanied by dispersion of H3 histone genes for the same chromosomal regions, including B chromosomes. These results indicated an interspersion and linked arrangement of these two multigene families, besides the association of the two rDNAs families in three chromosomes. Moreover our results are in disagreement with the autosomal origin hypothesis of the B chromosome proposed by Loreto et al. (2008a) using 5S rDNA, at least for the populations analyzed here, although the sexual origin could not be evidenced.

Material and Methods

DNA and chromosomal spreads were obtained from ten individuals collected in Serra Talhada (07º 59’ 31’’ S 38º 17’ 54’’ W), six from Surubim (07º 49’ 59’’ S 35º 45’ 17’’ W) and four from Vitória de Santo Antão (08º 07’ 05’’ S 35º 17’ 29’’ W), Pernambuco State, Brazil. 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. Slides used for Fluorescence *in situ* hybridizations (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.

Partial sequences of 5S rRNA and histone H3 genes were isolated by PCR of genomic DNA from *R. brasiliensis* using the primers designated by Loreto et al. (2008) and Cabral-de-Mello et al. (2010b), 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). The 18S rDNA sequence was obtained from a cloned fragment previously isolated from the genome of *Dichotomius geminatus* (Cabral-de-Mello et al. 2010b).

The plasmid containing the 18S rRNA and the histone H3 genes were labeled by nick translation using biotin-14-dATP (Invitrogen, San Diego, CA, USA). For simultaneous hybridization (double-FISH) the 5S rDNA was labeled using digoxigenin-11-dUTP (Roche, Mannheim, Germany). The FISH procedures were performed according to Cabral-de-Mello et al. (2010b). The preparations were counterstained using the 4',6-diamidine-2'-*phenylindole dihydrochloride* (DAPI) and mounted in Vectashield (Vector). Images were captured with the Olympus DP71 digital camera coupled to a BX61 Olympus microscope and were optimized for brightness and contrast using Adobe Photoshop CS2.

Results

All populations of *R. brasiliensis* showed diploid number 2n =23, XO sex system and acrocentric chromosomes, organized in three large pairs (L1-L3), five medium (M4-M8) and

three small (S9-S11). The X chromosome was identified as medium element and the B element when present showed similar size to the X, as described by Loreto et al. (2008a,b).

The *in situ* hybridization using the probes for 18S rDNA revealed the presence of this sequence in three autosomal chromosomes (M4, M6 and S9) located in the pericentromeric region in all populations studied (Figure 1a). The 5S rDNA was located in the pericentromeric region of all chromosomes of complement, including the X, except in the smallest autosome (S11) (Figure 1b). The double-FISH indicated co-localization of 5S and 18S rRNA genes in the pairs M4, M6 and S9 (Figure 1a,b).

The FISH for histone H3 gene in one individual from Surubim showed the presence of only one site for this gene in the pair M7 (Figure 1c), while for the other individuals analyzed for this population and for the other two populations studied this gene occupied all chromosomes, except the S11. In the population from Vitória de Santo Antão and Surubim the double-FISH using 5S rRNA and H3 histone genes as probes confirmed the patterns obtained by single hybridization revealing loci dispersion for these sequences, being co-located in almost all chromosomes, except in the pair S11 (Figure 1d-f).

The FISH for 5S rRNA and H3 histone genes in individuals with 1B revealed the presence of these genes in the B chromosome (Figure 1g,h). In this same population the double-FISH (5S rDNA and H3 histone gene) was performed, revealing co-localization for these genes in the centromeric region of the B element (Figure 1h-inserts).

Discussion

Standard chromosomal complement

The number of sites for 18S rDNA observed in the three populations of *Rammathocerus brasiliensis* is coincident with the results published by Loreto et al. (2008a,b) for this species. The chromosomal mapping of 45S rDNA in grasshoppers has revealed an

intense variability related to number and chromosomal location of this gene cluster. According to Cabrero and Camacho (2008) the variability of rDNA in grasshoppers is caused probably by three mechanisms, structural chromosome rearrangements, ectopic recombination and transpositions. For *R. brasiliensis* the most plausible explanation is the transposition or occurrence of ectopic-recombination of major rDNA sites, since this species maintains the ancestral diploid number for Acrididae. On the other hand, for other Gomphocerinae the action of large chromosomal rearrangements were important in changing of rDNA location, as described for species with 2n = 17,X0 (Cabrero and Camacho 2008).

The mapping of 5S rDNA in grasshoppers is scarce and only seven species were analyzed under this focus, including four Proscopiidae and three Acrididae, being the number and chromosomal location conserved in the former and variable in the later (Cabrero et al. 2003a; Loreto et al. 2008a; Cabral-de-Mello et al. 2010a; Teruel et al. 2010). In *R. brasiliensis* besides the spreading of major rDNA sites it was also observed a more intense dispersion for the 5S rDNA, for almost all chromosomes of the complement. In this work the number of sites observed in this species for all populations was distinct of the previously described by Loreto et al. (2008a). Our result points to a divergent condition of 5S rDNA organization and dispersion among the distinct populations of this species. The differential spreading for this sequence could be explained by differences in appropriate molecular mechanisms for 5S rDNA dispersion in the distinct populations, as proposed for major rDNA in grasshoppers as a whole (Cabrero and Camacho 2008).

The FISH using H3 histone sequence as probe also revealed dispersion for this gene sequence, being the pattern coincident to the same location of 5S rDNA, confirmed by double-FISH. In 35 species of Acrididae and four species belonging to Proscopiidae grasshoppers the number and location of histone genes were extremely conserved, with the presence of only one site (Cabrero et al. 2009; Cabral-de-Mello et al. 2010b). Cabrero et al.

(2009) suggested that strong purifying selection could be operating in the non-spreading of histones in Acrididae grasshopper genomes, and Cabral-de-Mello et al. (2010b) by the analysis of four ancient grasshoppers (Proscopiidae) proposed that the genomic association of histones and 5S rRNA genes can be ancient for grasshoppers as a whole. Bearing in mind these hypotheses, it can be proposed a posterior dispersion of histone H3 gene in *R. brasiliensis*, being the ancestral characteristic the occurrence of only one site that occasionally was observed in the population sampled in Surubim. The spreading of H3 histone can be directly associated to the dispersion of 5S rDNA, indicating a linked organization of these two multigene families in the genome of *R. brasiliensis*, which causes the same evolutionary patterns for the two sequences. The linked organization of 5S rRNA and histone genes was observed for other invertebrate species, by molecular approach in one mussel (Eirín-López et al. 2004) and two crustaceans (Andrews et al 1987; Barzotti et al. 2000;). Moreover by chromosomal analysis Cabral-de-Mello et al. (2010a,b) have proposed the association of these sequences in the genomes of insect representatives, such as in one beetle (*Dichotomius geminatus*) and in four ancient grasshoppers (Proscopiidae). Although molecular analysis needs to be performed in *R. brasiliensis* and in other insects to confirm the genomic nature of the association of 5S rRNA and histone H3 genes.

Besides the co-localization of 5S rDNA and H3 histone sequences it was also observed a similar pattern for 18S and 5S rDNA in the chromosomal pairs M4, M6 and S9 of all populations. Loreto et al. (2008a) described these two ribosomal genes located in distinct chromosomes of *R. brasiliensis*, being identical the position of 18S rDNA observed here. Considering that the location of 18S rDNA is stable among the populations of *R. brasiliensis*, the co-location of ribosomal elements in the three studied populations might be explained by invasion of 18S rDNA or a near region by the 5S rRNA genes through transposition events. The movement of 5S rDNA throught trasnpositon has been well documented for several

mammals (Drouin 2000) Association of major and 5S rRNA genes was also described in other invertebrates, such as annelids, mollusks and crustaceans and also in fungi. On the other hand, in most eukaryotic organisms these sequences show distinct and independent genomic arrays (Drouin and Moniz de Sá, 1995; Colomba et al. 2002; Vitturi et al. 2002, 2004; Wang and Guo, 2004).

The B chromosome

The B chromosome of *R. brasiliensis* is widely distributed among all population analyzed until the present time. Moreover a possible autosomal origin for this element using *in situ* hybridization for 5S and 45S rDNAs was proposed (Loreto et al. 2008). This conclusion was based in the presence of 5S rDNA sites restrict to autosomes and B element, and absent in the X chromosome. On the contrary, our results can not evidence an autosomal origin of the B chromosome in *R. brasiliensis* due the presence of 5S rDNA sites in almost all autosomes and also in the X chromosome, being this chromosomal marker non informative for B origin in this species. An alternative explanation can be the multiple origin of the B element in the distinct populations of *R. brasisiensis* studied with distinct conditions of 5S rDNA location. Multiregional origin for B chromosomes in grasshoppers was also previously proposed by Cabrero et al. (2003b) in *E. plorans*, using the mapping of rDNAs and a satellite DNA with 180bp repeat sequences. Another limitation of the use of 5S rDNA sequence as an informative marker for definition of B origin and evolution is related to the intense evolutionary dynamics of this sequence in grasshopper genomes (as described above). In fact the presence of 5S rDNA in the B chromosome of *R. brasiliensis* could be a characteristic generated after the origin of this chromosome by transposition, and not a characteristic inherited from the chromosome responsible for its origin.

Besides the presence of 5S rDNA in B chromosomes of grasshoppers, satellite DNAs and 45S rDNA were also identified in this genomic element, as for example, in *Eyprepocnemis plorans* and in some other animal groups, such as fish and mammals (Stitou et al. 2000; Cabrero et al. 2003b; Silva and Yonenaga-Yassuda 2004; Poletto et al. 2010). On the other hand, the presence of histone genes in B chromosomes was described only in one species until now, *Locusta migratoria*, being a good marker for discrimination of ancestry of this chromosome in this species (Teruel at al. 2010). The presence of H3 histone gene in the B chromosome of *R. brasiliensis* is the second case for grasshopper and for animal as a whole, indicating that in fact these chromosomes can harbor more gene sequences than was observed until now. Moreover due the clustered organization of histone genes they can be a useful marker in the investigating of B origin. On the other hand, in the case of *R. brasiliensis*, this sequence, as well as the 5S rDNA, was not informative due their high dispersion. The presence of H3 histone gene in the B chromosome can be also directly associated with the dispersion of 5S rDNA, reinforcing the association at genomic level of these two multigene families.

The results presented here reinforce the hypothesis of association of the 5S rDNA and H3 histone genes in some insect groups, and other invertebrates. This association led to the same patterns of chromosomal dispersion of these two multigene families in the genome, including both A complement and B chromosomes. These results also contribute to a better knowledge about B chromosomes content in animal kingdom. Moreover, we bring attention in the definition of chromosomal markers to be applied for analysis of B origin, depending on the mechanisms of evolution for the chosen sequence in the genomes to be investigated. Specifically for the case of *R. brasiliensis* the ribosomal DNAs and histone genes apparently are not sufficient for definition of B origin, due their intense evolutionary dynamics leading to spread of clusters in the genome and remarkable differences among population of the species.

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Figure caption

Fig. 1 Fluorescence *in situ* hybridization of 18S rRNA, 5S rRNA and H3 histone gene sequences in *Rammathocerus brasiliensis* with 0B and 1B chromosome. (a-f) Metaphases I and (g,h) terminal diplotenes; (a) 18s rDNA; (b,e,g) 5S rDNA; (c,f,h) H3 histone; (d) DAPI only. In (c) is showed the H3 histone location in one individual from Surubim and in the inserts in (h) is shown the hybridization patterns of 5S and H3 in the X and B chromosomes by double-FISH. The X and B chromosomes, and chromosomes bearing specific hybridization signals are indicated. Bar = 5µm.

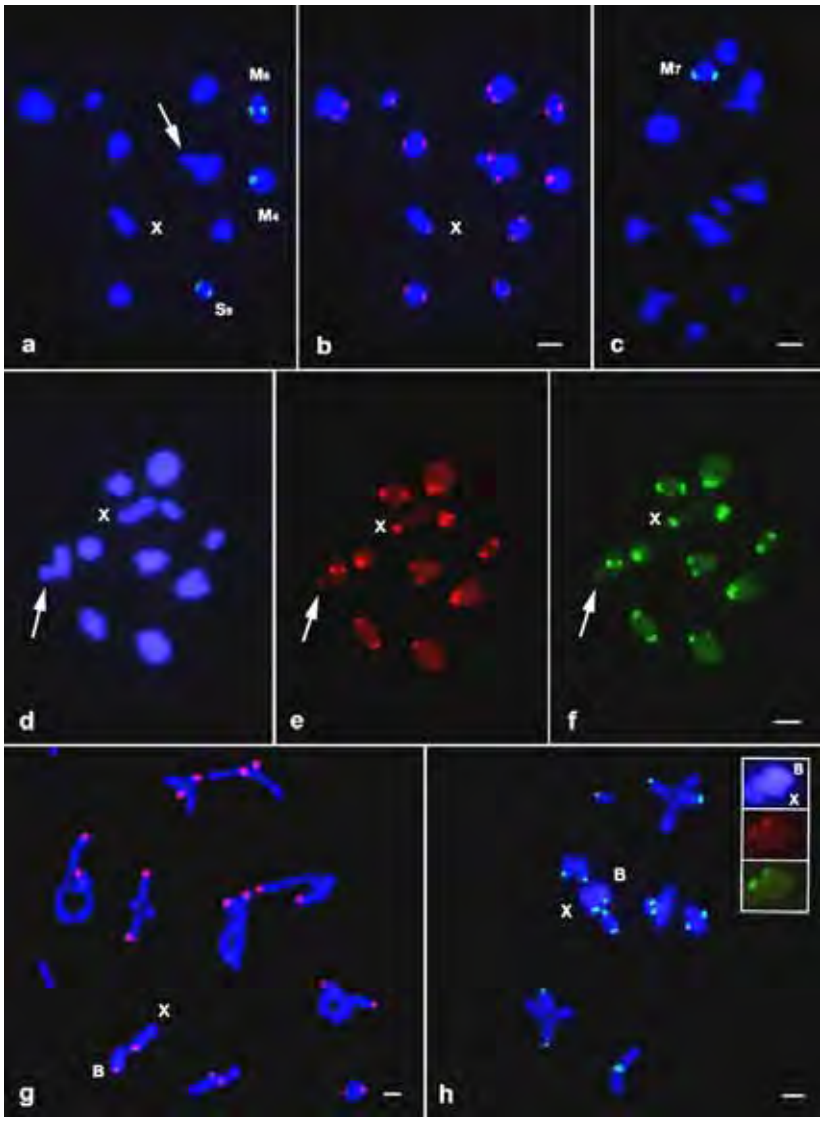

Figure 1

Evolutionary dynamics of 5S rDNA location in acridid grasshoppers and its relationship with histone gene and 45S rDNA location

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Abstract

We analyze the chromosomal location of 5S rRNA gene clusters in 29 species of grasshoppers belonging to the family Acrididae. There was extensive variation among species for the number and location of 5S rDNA sites. Out of 148 sites detected, 75% were proximally located, 21.6% were interstitial, and only 3.4% were distal. The number of 5S rDNA sites per species varied from a single chromosome pair (in six species) to all chromosome pairs (in five species), with a range of intermediate situations. Thirteen chromosomes from eight species carried two 5S rDNA clusters. At intraspecific level, differences among populations were detected in *Eyprepocnemis plorans*, and some heteromorphisms have also been observed in some species. Double FISH for 5S rDNA and H3 histone gene DNA, performed on 17 of these 29 species, revealed that both markers are sometimes placed in a same chromosome but at different location, whereas they appeared to co-localize in five species (*Calliptamus barbarus*, *Heteracris adpersa*, *Aiolopus strepens*, *Oedipoda charpentieri* and *O. coerulescens*). Double fiber-FISH in *A. strepens* and *O. coerulescens* showed that the two DNAs are closely interspersed with variable relative amounts of both classes of DNA. Finally, no correlation was observed between the number of 5S and 45S rDNA clusters in 23 species where this information was available. These results are discussed in the light of possible mechanisms of spread that led to the extensive variation in the number of clusters observed for both rDNA types in acridid grasshoppers.

Keywords: fluorescence *in situ* hybridization, grasshopper, histone genes, multigene families, rDNA.

Introduction

Multigene families are groups of genes descended from a common ancestral gene and showing similarity in structure and function (Nei and Rooney 2005). In eukaryote genomes, the multigene families for ribosomal RNA (rRNA) genes are tandemly arrayed in clusters located in one or several chromosomes. The major ribosomal DNA (45S rDNA) cluster codes for 28S, 5.8S and 18S rRNA, whereas the minor rDNA codes for 5S rRNA (Long and Dawid 1980). The histone multigene family is organized in one or more clusters including all intronless histone gene sequences (H1, H2A, H2B, H3 and H4), spaced by noncoding DNA sequences, although some variation for this organization has been reported, including H1 location outside the main histone cluster (Maxson et al. 1983; Nei and Rooney 2005).

The clustered organization of rDNAs and histone genes makes them good chromosomal markers for physical mapping, for their high value for understanding karyotypic evolution, to elucidate genome organization and identification of specific chromosomal rearrangements. In animals, chromosome location of 45S rDNA has been analysed in many species of both invertebrate and vertebrate organisms. Among the former, extensive variation has been found among adephagan beetles (Sánchez-Gea et al. 2000; Martínez-Navarro et al. 2004), mollusks (Wang and Guo 2004), acridid grasshoppers (Cabrero and Camacho 2008), and Lepidoptera (Nguyen et al. 2010). Among vertebrates, extensive variation has been reported mostly in fish (Pisano and Ghigliotti 2009).

In contrast, the 5S rRNA and histone genes have been mapped in only a few number of species within a scarce number of groups. The 5S rDNA has been extensively mapped in fish, but it has scarcely been mapped in other animals (see, for instance, Vitturi et al. 2002; Martins and Wasko 2004; Wang and Guo 2004; Gornug et al. 2005; Cabral-de-Mello et al. 2011). This DNA sequence is generally located in one or more chromosomal sites, mostly interstitial in amphibians (Vitelli et al. 1982; Schmid et al. 1987; Lucchini et al. 1993),

mammals (Mellink et al. 1996; Frederiksen et al. 1997), fish (Martins and Wasko 2004) and mollusks (Wang and Guo 2004). The histone genes have hitherto been mapped in a few species of mammals (Graves et al. 1985; Tripputi et al. 1986), amphibians (Turner et al. 1988), midges (Hankeln et al. 1993), fish (Pendás et al. 1994), fruit flies (Ranz et al. 2003), mollusks (Eirín-López et al. 2004; Zhang et al. 2007), grasshoppers (Cabrero et al. 2009; Cabral-de-Mello et al. 2011) and beetles (Cabral-de-Mello et al. 2010). In comparison with the rDNA multigene families, the histone genes are much more conserved among relative species concerning the number of clusters and chromosome position (Hankeln et al. 1993; Ranz et al. 2003; Cabrero et al. 2009; Cabral-de-Mello et al. 2011).

These three multigene families can be organized in the genome as separate clusters or else they can be linked in a same chromosome and can even be apparently syntenic. Association between the two rDNA families has been reported, for instance, in nematodes (Vahidi et al. 1988), crustaceans (Drouin et al. 1987, 1992), spiders (Drouin et al. 1992), fungi (Drouin and Moniz de Sá 1995), annelids (Vitturi et al. 2002), mollusks (Colomba et al. 2002; Vitturi et al. 2004) and fish (Pisano and Ghigliotti 2009). In addition, histone genes have been found to be associated with 5S rDNAs in two crustaceans (Andrews et al. 1987; Barzzoti et al. 2000), one mussel (Eirín-López et al. 2004), one beetle (Cabral-de-Mello et al. 2010) and four Proscopiidae grasshoppers (Cabral-de-Mello et al. 2011).

In insects, most studies of multigene families have focused on the major rDNA location. In grasshoppers, however, other multigene families have also been mapped by FISH, so that major rDNA location is known in 53 species (Cabrero and Camacho 2008; Cabral-de-Mello et al. 2011), the H3 histone gene has been located in 39 species (Cabrero et al. 2009; Cabral-de-Mello et al. 2011), but 5S rDNA location is known in only 7 species (Cabrero et al. 2003; Loreto et al. 2008; Teruel et al. 2010; Cabral-de-Mello et al. 2011). Whereas the 45S rDNA shows high interspecies variation in the number of clusters per genome (Cabrero and

Camacho 2008), the histone genes are very conserved in respect to the number of sites (a single cluster in all species) and chromosome location (interstitial in the eighth autosome, in order of decreasing size, in species with 2n♂= 23, and interstitial in the short arm of the third metacentric autosome, in species with 2n♂= 17) (Cabrero et al. 2009). The scarce information for 5S rDNA location pointed to its close association with H3 histone genes, at least in the four Proscopiidae grasshopper species analysed by Cabral-de-Mello et al. (2011).

Here we analyze chromosome location of 5S rDNA in 29 species of grasshoppers belonging to the family Acrididae. In addition, in those species where 5S rDNA was located in the same chromosome as the H3 histone gene (as reported in Cabrero et al. 2009), we performed double-FISH experiments on mitotic or meiotic chromosomes to analyse the relative location of both multigene families. Moreover, in two species where both markers appeared to co-localize, we carried out the fiber-FISH technique to analyse the degree of interspersion of both DNA sequences. Finally, we analysed the relationship between the number of clusters of 45S and 5S rDNA families, by comparing our present results with those previously published by Cabrero and Camacho (2008) on 45S rDNA location.

Materials and methods

Adult males of 29 species of Acrididae grasshoppers were collected in Spain, Greece and Morocco (see localities in Cabrero and Camacho 2008; Cabrero et al. 2009). Testes were fixed in 1:3 acetic acid - ethanol and chromosome preparations were made by squashing two testis tubules in a drop of 50% acetic acid. The coverslip was subsequently removed with a razorblade after immersion in liquid nitrogen. In several species (*Eyprepocnemis plorans*, *Chorthippus jacobsi* and *Stauroderus scalaris*) embryos were also obtained and fixed for cytological analysis, as described in Camacho et al. (1991).

Fluorescesce *in situ* hybridization (FISH), using the 5S rDNA sequence as probe, was performed in the 29 species. To test the possible association of 5S rDNA with H3 histone genes, double-FISH was performed in 17 species where both appeared to be located in the same chromosome. In *E. plorans*, seven Spanish populations were analyzed for 5S rDNA distribution, and four of them were also analysed for H3 location.

DNA probes for 5S rRNA and H3 histone genes were obtained from cloned fragments obtained from the genomes of *E. plorans* and *Locusta migratoria*, respectively. The 5S rDNA was labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany) by polymerase chain reaction (PCR), while the plasmid containing H3 fragment was submitted to a nick-translation reaction using biotin-11-dATP (Invitrogen, San Diego, CA, USA). The H3 histone probe was detected by avidin-FITC (fluorescein isothiocyanate) conjugate (Sigma, St Louis, MO, USA), and the 5S probe was detected using anti-digoxigenin-Rhodamine (Roche, Mannheim, Germany). The *in situ* hybridization followed the protocol described in Cabral-de-Mello et al. (2010). All preparations were counterstained with DAPI and mounted in Vectashield (Vector, Burlingame, CA, USA). Photographs were made using an Olympus microscope equipped with the DP70 cooled digital camera. Images were merged and optimized for brightness and contrast with the Gimp freeware.

At least five cells per individual, and three individuals per species were analysed by FISH in order to determine chromosome location of the DNA markers assayed. Correlation analysis between the number of 5S clusters (present results) and 45S rDNA sites (in Cabrero and Camacho 2008) was performed by the Gnumeric spreadsheet.

Results

We detected a total of 148 sites for 5S rDNA in the 29 species analyzed, 75% of which were located close to centromere (proximal), 21.6% were interstitial, and only 3.4% were distally

located (Table 1). The number of 5S rDNA sites per species varied from a single chromosome pair (e.g. in *Pezotettix giornae*, *Heteracris adspersa*, *Chorthippus apicalis*, *Stauroderus scalaris*, *Oedipoda coerulescens* and *Tropidopola graeca*) to all chromosome pairs (in *Chorthippus binotatus*, *Ch. jacobsi*, *Ch. nevadensis*, *Omocestus bolivari* and *Sphingonotus azurescens*). In several species, one (*Eyprepocnemis plorans*, *Chorthippus binotatus*, *Ch. vagans*, *Omocestus panteli*, *Acrotylus patruelis* and *Locusta migratoria*) or two (*Ch. jacobsi* and *Ch. nevadensis*) chromosome pairs carried two 5S rDNA clusters (Figure 1). Therefore, there was high variation for the number of 5S rDNA sites per species, averaging 5.1 sites, 3.83 of which were proximal, 1.1 interstitial and 0.17 distal (Table 1). But we also found intraspecific variation. The analysis of seven Spanish populations of *Eyprepocnemis plorans* showed the existence of variation among populations (Table 2). In all seven populations, chromosome 5 carried two interstitial 5S rDNA sites, and chromosomes 6, 8-11 carried a proximal site. In addition, chromosome 4 carried an interstitial site in all populations except Fuengirola, where it was proximal, and chromosome 7 carried a proximal site of 5S rDNA in Torrox and Fuengirola only.

In order to investigate the possible association or co-localization of the 5S rRNA and H3 histone genes, we performed double-FISH with both DNA probes in 17 out of the 18 species where some of the 5S rDNA clusters were located on the same chromosome as the H3 histone DNA sequence reported in Cabrero et al. (2009) (this was deduced by comparing Table 1 in the present paper with Table 1 in Cabrero et al. 2009). As shown by these authors, most Acrididae species with n♂=11+X0 chromosomes carry a single locus for H3 genes located in the eighth autosome in order of decreasing size, whereas most species with n♂=8+X0 carry it in the short arm of the third autosome. As Table 1 shows, 10 species with n♂=11+X0 and 8 with n♂=8+X0 carried a 5S rDNA cluster in the same chromosome harboring the H3 histone cluster. The double FISH results showed that H3 genes and 5S

rDNA were sometimes located in the same chromosome but at clearly different sites (Figure 2a-g). For instance, in some species with n♂=8+X0, the third autosome showed H3 located in the short arm but the 5S rDNA was sited in the centromeric region or in the long arm (Figure 3a-c). Likewise, some species with n♂=11+X0 showed the 5S rRNA and H3 genes in the eighth autosome, but at different sites, e.g. interstitial H3 and proximal 5S rDNA (Figure 2d-g). On the contrary, in five species (*Calliptamus barbarus*, *Heteracris adpersa*, *Aiolopus strepens*, *Oedipoda charpentieri* and *O. coerulescens*) the H3 and 5S rRNA genes showed apparently the same location and possible interspersion (Figure 2h-j). To visualize more closely the structure of the chromosome regions where the sites for both DNAs appeared to co-localize, we performed the fiber-FISH technique for both DNA probes simultaneously in *A. strepens* and *O. coerulescens*. Figure 2k shows that the two DNAs are closely interspersed in *O. coerulescens*, although the relative amount of both classes of DNA seems to be variable from one site to another. Similar results were obtained for *A. strepens* (results not shown).

In several species, we observed conspicuous heteromorphism for the 5S rRNA gene in both cluster size and presence/absence (Figure 3). For instance, one of the homologous chromosomes in the fourth autosome of *Oedipoda charpentieri* failed to show a FISH signal for 5S rDNA in one of the males analysed (Figure 3a). In *Calliptamus barbarus*, we observed size variation for the 5S rDNA cluster located in the smallest autosome (Figure 3b). Presence/absence heteromorphisms were also observed in *Acrotylus patruelis* (autosome 11), *Chorthippus jacobsi* (autosome 6), *C. nevadensis* (autosome 6), and *Euchorthippus albolineatus* (autosome 8) (Figure 3c-f).

Since Cabrero and Camacho (2008) had shown a comparably high variation for chromosome location of 45S rDNA in acridid grasshoppers, we investigated for a possible association between the two classes of rRNA genes. Figure 4 shows the number of sites for 5S and 45S rDNA in the 23 species where this information was available (*E. plorans* was not

included because of interpopulation variation, as shown in Table 2). Given the functional relationship between these two classes of rRNA, by which an equimolecular amount of them is needed in all species, it could be conceivable that the number of clusters for both rDNA families would show positive correlation (assuming about similar number of genes per cluster). However, no significant correlation was found (r= 0.071, P= 0.747). Figure 4 also illustrates that the observed variation in the number of clusters for both rDNA families follows no taxonomical pattern since, within the two most represented subfamilies, i.e. Gomphocerinae and Oedipodinae, there are species with few or many rDNA clusters for any family.

Discussion

Chromosome location of 5S rDNA in acridid grasshoppers

Our present results have shown extensive variation for the number of 5S rDNA sites in Acrididae grasshoppers, with some species carrying it in a single chromosome pair (e.g. *P. giornae*, *H. adspersa*, *Ch. apicalis*, *S. scalaris*, *O. coerulescens* and *T. graeca*) and others in all chromosomes (e.g. *Ch. binotatus*, *Ch. jacobsi*, *Ch. nevadensis*, *O. bolivari* and *S. azurescens*), with intermediate situations (see Table 1). This high variation is also found within the two most represented subfamilies in our sample, i.e. Gomphocerinae and Oedipodinae. In both cases, there were species with a single 5S rDNA cluster (*Ch. apicalis* and *S. scalaris* in Gomphocerinae and *O. coerulescens* in Oedipodinae) and species with 5S rDNA in all chromosomes (*Ch. binotatus*, *Ch. jacobsi*, *Ch. nevadensis* and *O. bolivari* in Gomphocerinae and *S. azurescens* in Oedipodinae). High resemblance is apparent among closely related species, suggesting possible common descent of the observed 5S rDNA chromosome distribution (e.g. *Ch. binotatus*, *Ch. jacobsi*, *Ch. nevadensis*). But other species within the same genus (e.g. *Ch. apicalis* and *Ch. vagans*) may show very few sites (1 and 3,

respectively). Molecular phylogenetic information suggests that *Ch. jacobsi* is more closely related to *Ch. binotatus* than to *Ch. vagans* (Contreras and Chapco 2006). This would support the possibility of common descent for the 5S distribution in *Ch. binotatus*, *Ch. jacobsi* and *Ch. nevadensis*. The high similarity among species in other genera (e.g. *Sphingonotus*) could also be due to common descent. In any case, the extensive variation among acridid species suggests that 5S rDNA have spread between non-homologous chromosomes in some genomes. The existence of a single cluster is presumably the ancestral situation, from which arose the saturated genomes (with 5S rDNA in all chromosomes) passing through intermediate situations as those observed in many current species (see Table 1). Although the specific spread mechanisms remain to be shown, it seems that, in acridid grasshoppers, these mechanisms have acted profusely, in some species, expanding 5S rDNA to many or all chromosomes (with even two clusters in some chromosomes) whereas they have failed to operate in species where 5S rDNA is restricted to a single chromosome pair.

It is remarkable that 75% of 5S rDNA clusters in acridid grasshoppers were proximal to the centromeric region. This suggests that this location could be ancestral in the Acrididae. This is also supported by the proximal location of rDNA found in the three species of Proscopiidae grasshoppers hitherto analysed (Cabral-de-Mello et al. 2011), a family considered relatively ancient compared to the Acrididae (Descamps 1973; Matt et al. 2008). The analysed Proscopiidae, however, did not show variation for the number of 5S rDNA, all four species showing a single cluster in the fourth autosome, in order of decreasing size. In other organisms, e.g. fish, 5S rDNA clusters are predominantly interstitial, which has been interpreted as a protection against transposition and exchange events (Martins and Wasko 2004). If 5S rDNA evolution would have had a common startpoint in the Proscopiidae and Acrididae grasshopper families, we would expect that those acridid species conserving the putative ancestral position for this rDNA would show a single cluster in a proximal location.

However, Table 1 shows that, in the Acrididae species with only one 5S rDNA site, it is proximal in *T. graeca* only, whereas it is interstitial (but close to the paracentromeric heterochromatin) in *H. adspersa*, *Ch. apicalis*, *S. scalaris* and *O. coerulescens*, and distal in *P. giornae*.

Relationship between 5S rDNA and histone genes location

In five out of the 17 acridid species where 5S rRNA and H3 histone genes were located on a same chromosome and were analyzed by double-FISH, we observed that both markers were placed in the same chromosomal location, with apparently interspersed organization. Recently Cabral-de-Mello et al. (2011) observed the same fact in four Proscopiidae grasshopper species and, given the basal phylogenetic position of this family, they proposed that 5S rDNA and H3 gene DNA colocalization could be an ancestral feature, given that it has been observed in other invertebrate groups, such as crustacean (Andrews et al. 1987; Cruces et al. 1989; Barzotti et al. 2000), mussels (Eirín-López et al. 2004) and beetles (Cabral-de-Mello et al. 2010).

However, our present results have shown that this condition is scarcely observed in Acrididae grasshoppers, which is a logical consequence of the extreme degree of conservatism shown by histone gene location (Cabrero et al. 2009) and the extensive variation shown by 5S rDNA location (this paper). If both DNAs were placed together ancestrally, we would expect a tendency to 5S rDNA and H3 histone gene colocalization in species still bearing a single 5S rDNA cluster. However, a comparison between Table 1 in this paper and Table 1 in Cabrero et al. (2009) indicates that this condition is met in only two species (*Heteracris adspersa* and *Oedipoda coerulescens*), whereas they do not colocalize in the other three (*Pezotettix giornae*, *Chorthippus apicalis* and *Stauroderus scalaris*). In the two former species, both DNA families are apparently interspersed, as shown by FISH techniques,

whereas in the three latter species they are located at different chromosomes. In *Aiolopus strepens* and *Oedipoda coerulescens* the co-location of 5S rRNA and H3 histone genes was corroborated by fiber-FISH experiments, although *A. strepens* carried 5S rRNA genes with no histone genes in two other chromosomes. It is thus clear that, in acridid grasshoppers, 5S rDNA has moved among heterologous chromosomes, whereas histone genes have mostly conserved a single location. The analysis of 5S rDNA and histone gene location in other grasshopper families, in combination with molecular phylogenetic analysis, could help to trace back the origin of the present patterns of co-localization of these two repetitive DNA families.

Relationship between 5S rDNA and 45S rDNA location

Our present analysis of 5S rDNA chromosome distribution (Table 1) included 24 species in common with that by Cabrero and Camacho (2008) on 45S rDNA location. A comparative analysis revealed that ten of these species (*E. plorans*, *Ch. apicalis*, *Ch. binotatus*, *Ch. jacobsi*, *Ch. vagans*, *D. maroccanus*, *O. bolivari*, *A. strepens*, *O. coerulescens* and *S. coerulans*) carried some 5S rDNA clusters in a similar location (i.e. proximal or interstitial; never distal) as 45S rDNA. About one third of the 127 clusters of 5S rDNA observed in these 24 species were located in a chromosome also carrying 45S rDNA. But only 25 out of the 127 5S rDNA clusters (19.7%) were placed at the same proximal (15%) or interstitial (4.7%) location as the 45S rDNA. The remaining 80.3% were thus located at separate sites from the 45S rDNA. In vertebrates, both rDNA types are also most frequently located on different chromosomes (Lucchini et al. 1993; Martínez et al. 1996; Shrikulnhat et al. 2009; Henderson et al. 1972; Steffensen and Duffey 1974).

As shown in Figure 6, grasshopper genomes show extensive variation in the number of rDNA clusters, but this variation does not run in parallel for the 5S and 45S rRNA gene

families. Some species carry a small number of clusters for both rDNAs, whereas other species carry one family in only a few chromosomes but the other family in most chromosomes. There is even one species (*O. bolivari*) carrying both rDNA types in all chromosomes. We do not know how many of the 5S rDNA clusters are functionally active in every species, but the analysis of NOR activity by Cabrero and Camacho (2008) showed that some of the 45S rDNA clusters are inactive, whereas some cryptic loci were active at chromosome locations where FISH showed no evidence of 45S rDNA presence. In *S. scalaris*, a species with 45S rDNA in all chromosomes, only those copies located on the third autosome are active (López-León et al. 1999). This suggests that, in species where one or both rDNA gene families have extensively spread over the genome, it might occur that many gene copies of one or both families are inactive.

Independent changes of 5S and 45S rDNA families, suggesting separate evolutionary pathways, have previously been reported in plants, (Hanson et al. 1996; Adams et al. 2000; Pedrosa-Harand et al. 2006). The independent variation in cluster number might rest on differences in the mechanisms by which both kinds of DNA repeats move across the genome, so that, in some species, it is paved the way for 5S rDNA movement whereas, in others, it is easier the pathway for 45S rDNA movement.

Mobile nature of rDNA

The mobility of nucleolus organizer regions (NORs) was first vindicated in *Allium* by Schubert (1984) and Schubert and Wobus (1985). Later on, other authors have also claimed for 45S rDNA transposition in other organisms, as a major explanation for the observed variation in rDNA loci number and location (e.g. Castro et al. 2001; Shishido et al. 2000; Raskina et al. 2004; Cai et al. 2006; Datson and Murray 2006; Cabrero and Camacho 2008). In most cases, variation in rDNA location is not associated with apparent chromosome

structural rearrangements, on which basis, Dubcovsky and Dvorak (1995) suggested that it may occur through the dispersion and magnification of minor rDNA loci. In grasshoppers, it is a frequent phenomenon to find nucleolar activity at chromosome regions where FISH do not reveals presence of 45S rDNA genes (Cabrero and Camacho 2008). These cryptic loci are equivalent to the minor loci hypothesized by Dubcovsky and Dvorak (1995), and may represent the germ for future changes in rDNA location, once they undergo significant amplification.

The colonization of new chromosome domains by a single (or few) 45S rDNA copies might be facilitated by association to transposable elements (TEs). It has been shown that rDNA regions are common targets for TEs (Belyayev et al. 2001, 2005; Eickbush and Eickbush 2003; Penton and Crease 2004; Averbeck and Eickbush 2005). In addition, Raskina et al. (2004) showed that *En/Spm* transposons were involved in rDNA spread in *Aegilops speltoides*, and Raskina et al. (2008) claimed for TE-mediated intragenomic transfer of rDNA fragments. Some other authors have reported the association of TEs with rDNAs (see Mandrioli 2000; Gillespie et al. 2006; Rezende-Teixeira et al. 2009). Remarkably, Kapitonov and Jurka (2003) reported a novel class of SINE elements derived from 5S rRNA in the zebrafish genome, and Raskina et al. (2004) found the presence of combined DNA fragments consisting of the *En/Spm* transposon and 5S rDNA. In addition, the retrotransposon *Cassandra*, present in all vascular plants, carries conserved 5S rDNA sequences, and its activity would explain part of the high variation observed in the distribution of 5S rDNA loci (Kalendar et al. 2008). Recently, Cioffi et al. (2010) have found colocalization of 5S rDNA and the *Rex3* non-LTR retrotransposon in the fish *Erythrinus erythrinus*. These authors found a remarkable difference between two karyomorphs (A and D) in chromosome distribution of 5S rDNA/*Rex3* sites, with only two sites (chromosome pair no. 8) in karyomorph A, but 22 sites in females and 21 in males, including chromosome 8 and the X_1X_2Y sex chromosomes.

They hypothesized that *Rex3* might have inserted into 5S rDNA sequences and that the 5S rDNA/*Rex3* complex had moved and dispersed throughout the genome.

Another conceivable way for 5S movement is through extrachomosomal circular DNA (eccDNA), which has been detected in many organisms. EccDNA usually consists of tandemly repeated DNA and has been found carrying 5S rDNA in *Drosophila* (Pont et al. 1987; Degroote et al. 1989; Cohen et al. 2003), *Xenopus* embryos (Cohen et al. 1999), the plants *Arabidopsis thaliana* and *Brachycome dichromosomatica* (Cohen et al. 2008) and human beings (Cohen et al. 2010).

The extensive variation for the number and location of 5S and 45S rDNA clusters found in acridid grasshoppers was observed at both intra- and interspecific levels, and grasshoppers thus constitute an excellent material to investigate the possible association of rDNA with several types of mobile elements. The huge variation in the number of clusters among species allows predicting that the putative associations between mobile elements and rDNA should most likely be found in species with rDNA clusters in most heterologous chromosomes, but less likely in those showing a single cluster.

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Table 1. Chromosomal location of 5S rRNA genes in 29 Acrididae grasshoppers. †In *Eyprepocnemis plorans* there was variation among populations for autosomes 4 and 7, as shown in Table 2. p= proximal, i= interstitial, d= distal in respect to the centromere. Grey areas correspond to absent chromosome numbers in 2n♂= 17 species. Asterisks indicate the species analysed by double-FISH for 5S rDNA and H3 histone gene DNA probes.

		Chromosome no. (in order of decreasing size)												no. 5S rDNA clusters			
Subfamily	Species	1	2	3	4	5	6	7	8	9	10	11	X	p	i	d	Total
Calliptaminae	*Calliptamus barbarus**		p	p		p		p	i	p	p	p	p	8	1	0	9
	Calliptamus wattenwylianus					p	i							1	1	0	2
Cantatopinae	*Pezotettix giornae*					d								0	0	1	1
Cyrtacanthacridinae	*Anacridium aegyptum**			p			p		p	p	p	p	p	7	0	0	7
Eyprepocnemidinae	*Heteracris adpersa**								i					0	1	0	1
	Eyprepocnemis plorans				i,p†	2i	p	p†	p	p	p	p		7	3	0	10
Gomphocerinae	*Dociostaurus maroccanus*			p		p	p	p		p				5	0	0	5
	Chorthippus apicalis						i							0	1	0	1
	Chorthippus binotatus	p	p	p, i	p	p	p	p	p				p	9	1	0	10
	*Chorthippus jacobsi**	p	p	p, i	p	p	p, d	p	p				p, i	9	2	1	12
	*Chorthippus nevadensis**	p	p	p, i	p	p	p, d	p	p				p, i	9	2	1	12
	*Chorthippus vagans**	p		p, i										2	1	0	3
	Euchorthippus albolineatus	i							i					0	2	0	2
	*Omocestus bolivari**	p	p	p	p	p	p	p	p				p	9	0	0	9
	*Omocestus burri**	p	p	p										3	0	0	3
	*Omocestus panteli**	p, d	p	p										3	0	1	4
	Stauroderus scalaris		i											0	1	0	1
	*Stenobothrus festivus**	i	i	i			i	d						0	4	1	5
Oedipodinae	*Acrotylus patruelis*			2i								p		1	2	0	3
	*Aiolopus strepens**			i					i		i			0	3	0	3
	*Aiolopus thalassinus**			i				p	p					2	1	0	3
	Locusta migratória			2i						p		p		2	2	0	4
	*Oedipoda charpentieri**			i	i				i					0	3	0	3

	*Oedipoda coerulescens**								i					0	1	0	1
	Oedipoda fuscocincta	p											p	2	0	0	2
	*Paracinema tricolor**	p	p	p	p				p	p	p	p		8	0	0	8
	*Sphingonotus azurescens**	p	p	p	p	p	p	p	p	p	p	p	p	12	0	0	12
	*Sphingonotus coerulans**	p	p	p	p	p	p	p	p	p	p		p	11	0	0	11
Tropidopolinae	*Tropidopola graeca*									p				1	0	0	1
Total														111	32	5	148
Percent														75	21.6	3.4	
Average														3.83	1.1	0.17	5.10

Table 2. Chromosome location of 5S rDNA in 7 Spanish populations of *Eyprepocnemis plorans*. The Spanish province is indicated in brackets. The asterisks indicate the populations also analyzed for histone H3 gene location. p= proximal, i= interstitial, d= distal in respect with the centromere.

	Chromosome no.											
Population	1	2	3	4	5	6	7	8	9	10	11	X
El Gallego (Albacete)*				i	2i	p		p	p	p	p	
Río Mundo (Albacete)*				i	2i	p		p	p	p	p	
Jete (Granada)				i	2i	p		p	p	p	p	
Melegí (Granada)				i	2i	p		p	p	p	p	
Salobreña (Granada)*				i	2i	p		p	p	p	p	
Torox (Málaga)*				i	2i	p	p	p	p	p	p	
Fuengirola (Málaga)				p	2i	p	p	p	p	p	p	

Figure captions

Fig. 1 Fluorescent *in situ* hybridization using 5S rDNA as probe in nine species of Acrididae grasshoppers. (a) *Stauroderus scalaris*, (b) *Chorthippus jacobsi*, (c) *Omocestus panteli*, (d) *Anacridium aegyptum*, (e) *Pezotettix giornae*, (f) *Tropidopola graeca*, (g) *Sphingonotus azurescens*, (h) *Sphingonotus coerulans* (i) *Acrotylus patruelis*. Scale bar = 10 μm.

Fig. 2 Selected chromosomes of ten Acrididae species showing double FISH for 5S rDNA (red) and H3 histone gene (green) DNA probes (a-j), and fiber-FISH for 5S rDNA (red) and histone H3 genes (green) (k). (a) *Chorthippus jacobsi*, (b) *Stenobothrus festivus*, (c) *Chorthippus vagans*, (d) *Anacridium aegyptum*, (e) *Sphingonotus azurescens*, (f) *Aiolopus thalassinus*, (g) *Sphingonotus coerulans*, (h) *Oedipoda charpentieri*, (i) *Aiolopus strepens*, (j, k) *Oedipoda coerulescens*. Arrowheads in (a-j) indicate centromeres and in (k) indicates the difference in relative bright of both signals in some locations. Note in (k) the colocalization of both DNAs in most places Scale bar = 10 μm (a-j) and 1 μm (k).

Fig. 3 Polymorphism for 5S rDNA in six species belonging to the family Acrididae. (a) *Oedipoda charpentieri*, (b) *Calliptamus barbarus*, (c) *Acrotylus patruelis* (d) *Chorthippus jacobsi*, (e) *C. nevadensis*, (f) *Euchorthippus albolineatus*. Scale bar = 10 μm.

Fig. 4 Distribution of the number of clusters for 5S and 45S rDNA in 23 grasshopper species. The species *Eyprepocnemis plorans* was not included due to the observed interpopulation variation. The symbols indicate subfamilies Cantatopinae (▌), Oedipodinae (●), Eyprepocneminae (▲) and Gomphocerinae (■).

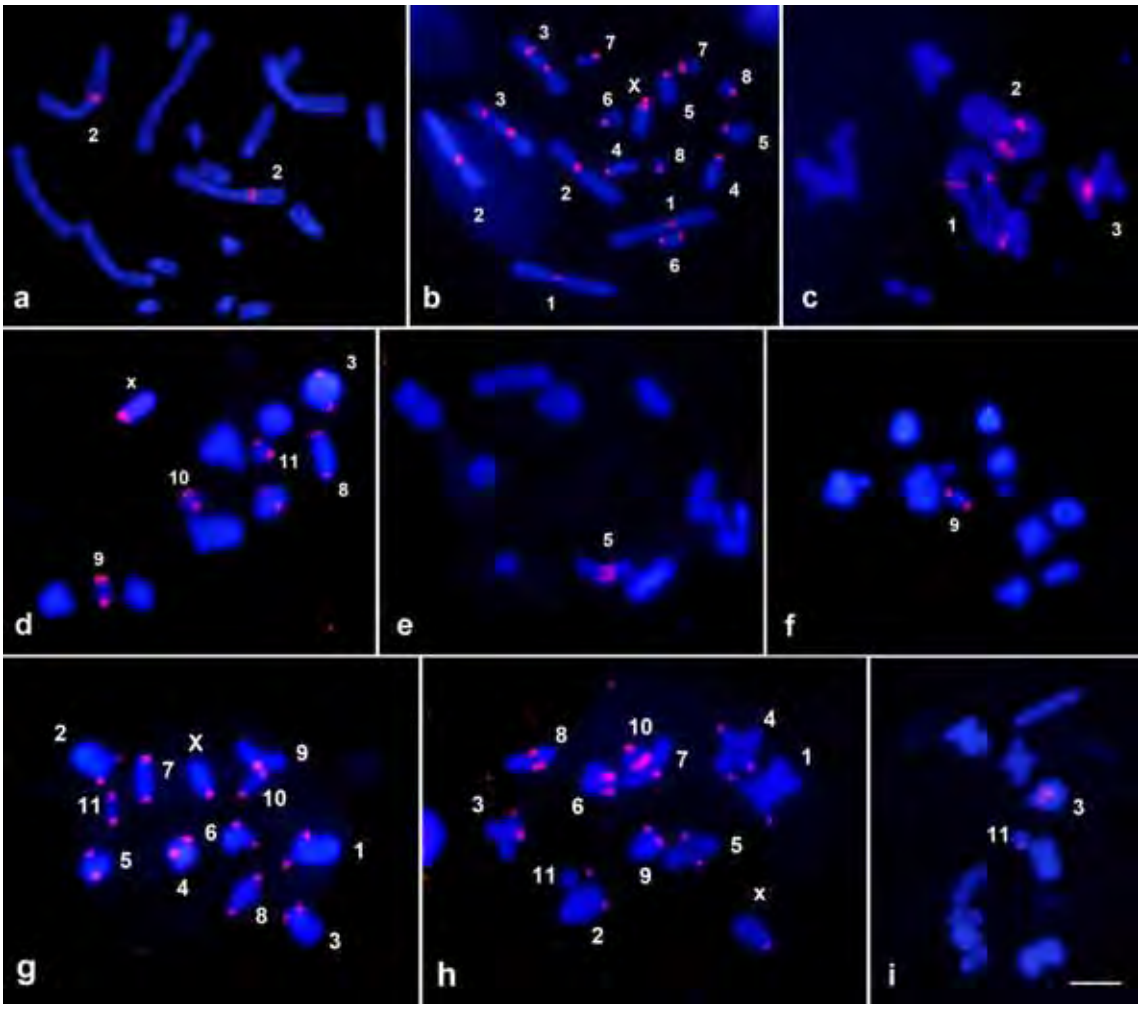
a
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Figure1

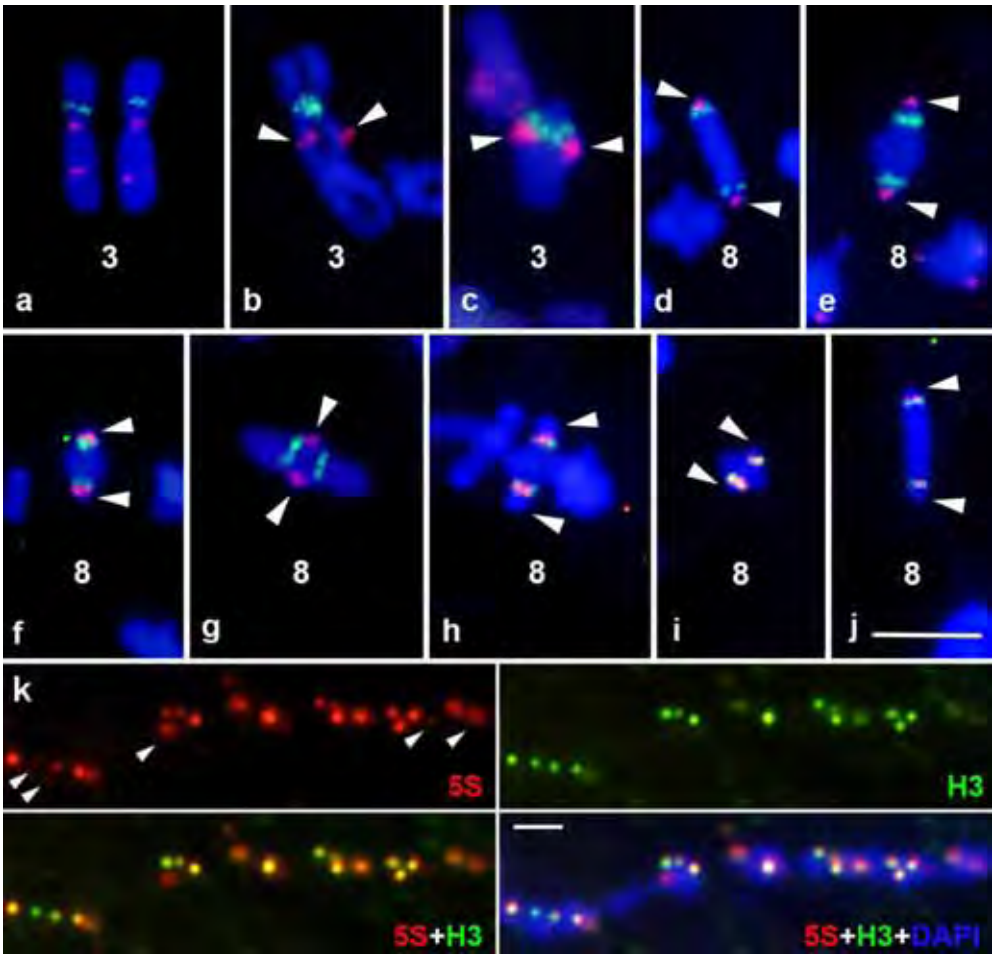


Figure 2

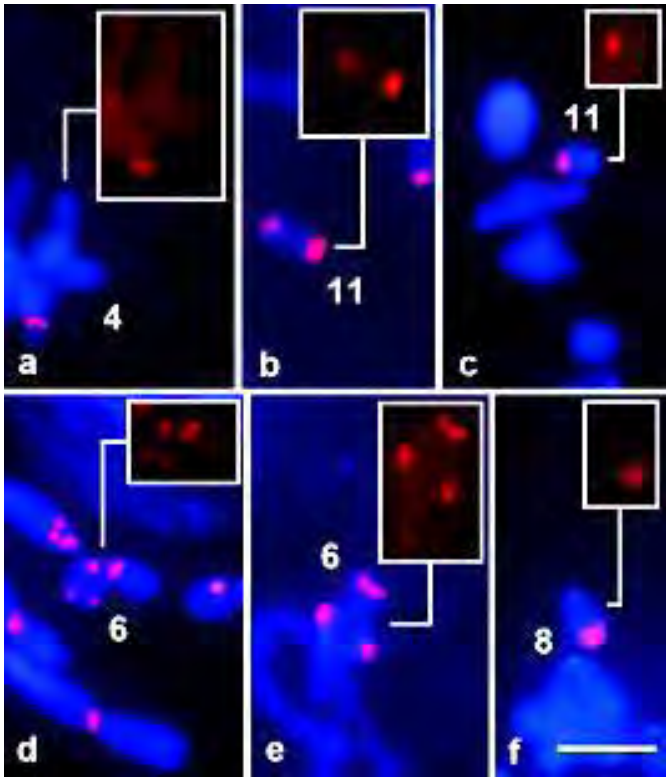
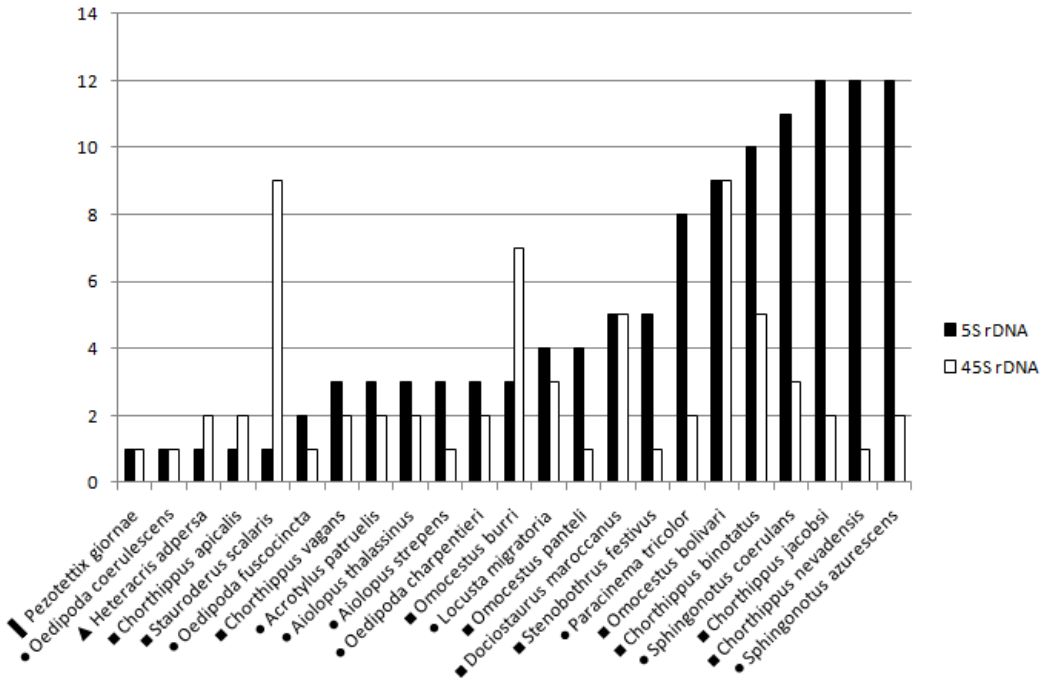


Figure 3


14
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4
2
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5S rDNA
45S rDNA
Pezotettix giornae
Oedipoda coerulescens
Heteracris adpersa
Chorthippus apicalis
Stauroderus scalaris
Oedipoda fuscocincta
Chorthippus vagans
Acrotylus patruelis
Aiolopus thalassinus
Aiolopus strepens
Oedipoda charpentieri
Omocestus burri
Locusta migratoria
Omocestus panteli
Dociostaurus maroccanus
Stenobothrus festivus
Paracinema tricolor
Omocestus bolivari
Chorthippus binotatus
Sphingonotus coerulans
Chorthippus jacobsi
Chorthippus nevadensis
Sphingonotus azurescens

Figure 4

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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 (separated by '-') C_0t-1 DNA - Evolution - Genome - Heterochromatin - Repetitive DNAs

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7. 10. Anexo 10

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

Diogo Cavalcanti Cabral-de-Mello · Rita de Cássia de Moura · Adriana de Souza Melo · Cesar Martins

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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

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

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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 testis 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

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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

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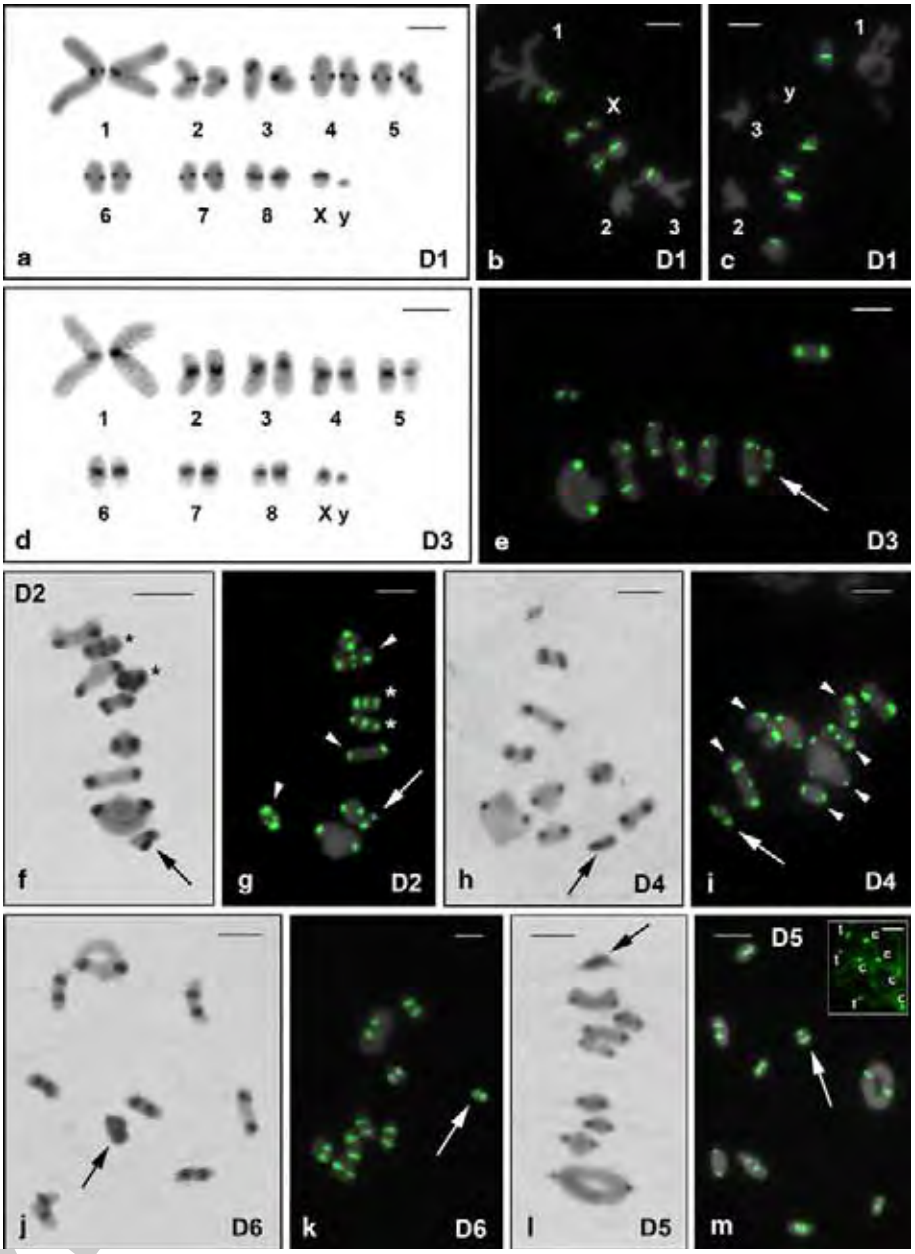

Fig. 1 C-banding and C_0t-1 DNA fraction hybridization in six species of *Dichotomius*. The C_0t-1 DNA fractions were isolated from each species and hybridized against their own chromosomes. C-banded karyotypes from *D. bos* (**a**) and *D. laevicollis* (**d**), and C-banded metaphase I chromosomes from *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*

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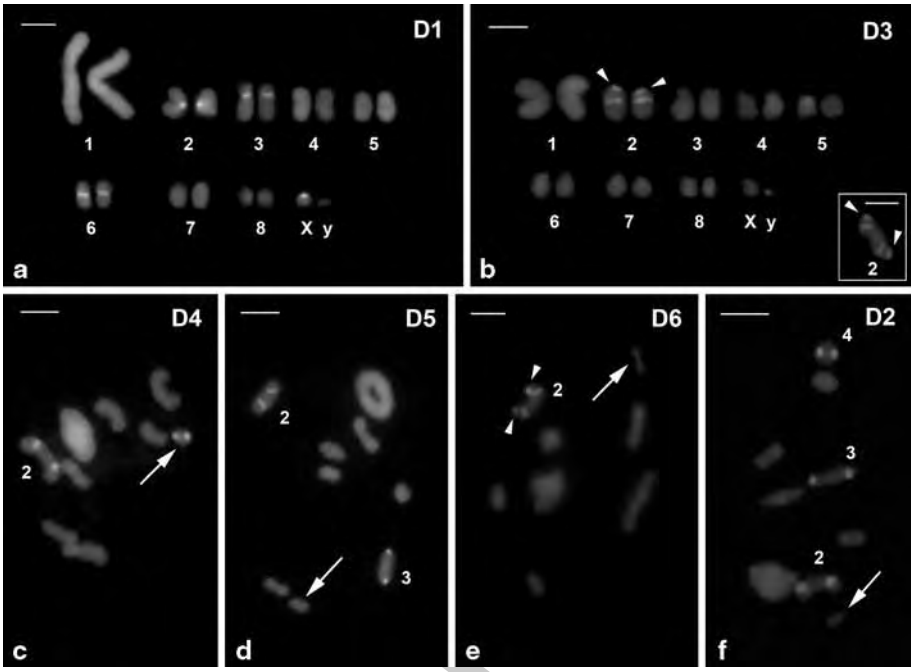

Fig. 2 Fluorochrome staining in the six species of *Dichotomius*. Karyotypes from *D. bos* (**a**) and *D. laevicollis* (**b**), respectively; metaphase I chromosomes from *D. nisus* (**c**), *D. semisquamosus* (**d**), *D. sericeus* (**e**) and *D. geminatus* (**f**) are showed. The *arrows* indicate 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*

Table 1 C_0t-1 DNA fraction reassociation time and chromosomal location for the six *Diichotomius* 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

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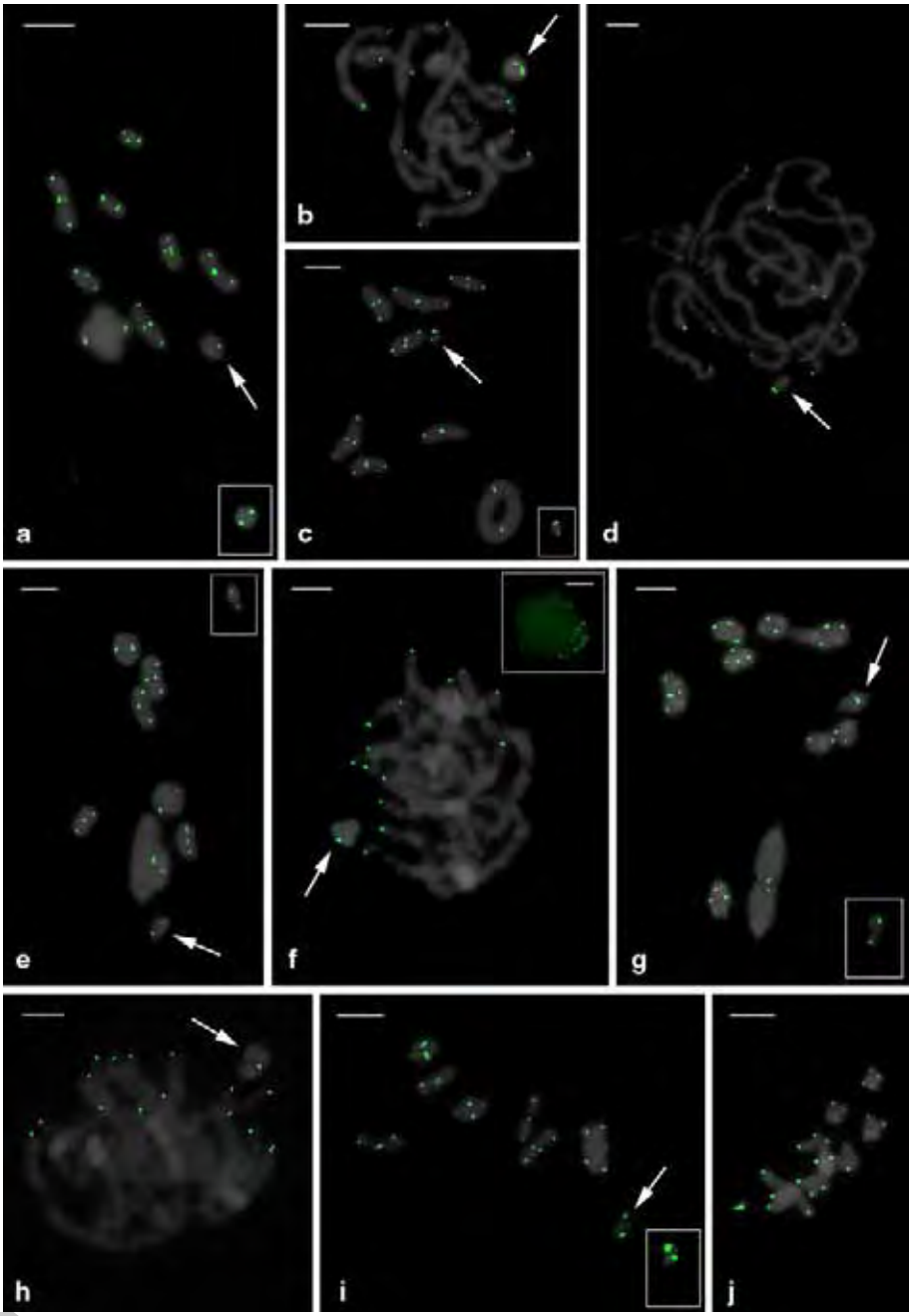

Fig. 3 C_0t-1 DNA fraction obtained from the genome of *Dichotomius geminatus* hybridized against the chromosomes of the other five *Dichotomius* species. Metaphase I chromosomes from *D. semisquamosus* (**a**), *D. bos* (**c**), *D. laevicollis* (**e**), *D. sericeus* (**g**) and *D. nisus* (**i**); 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

(Fig. 3e, f) and *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.

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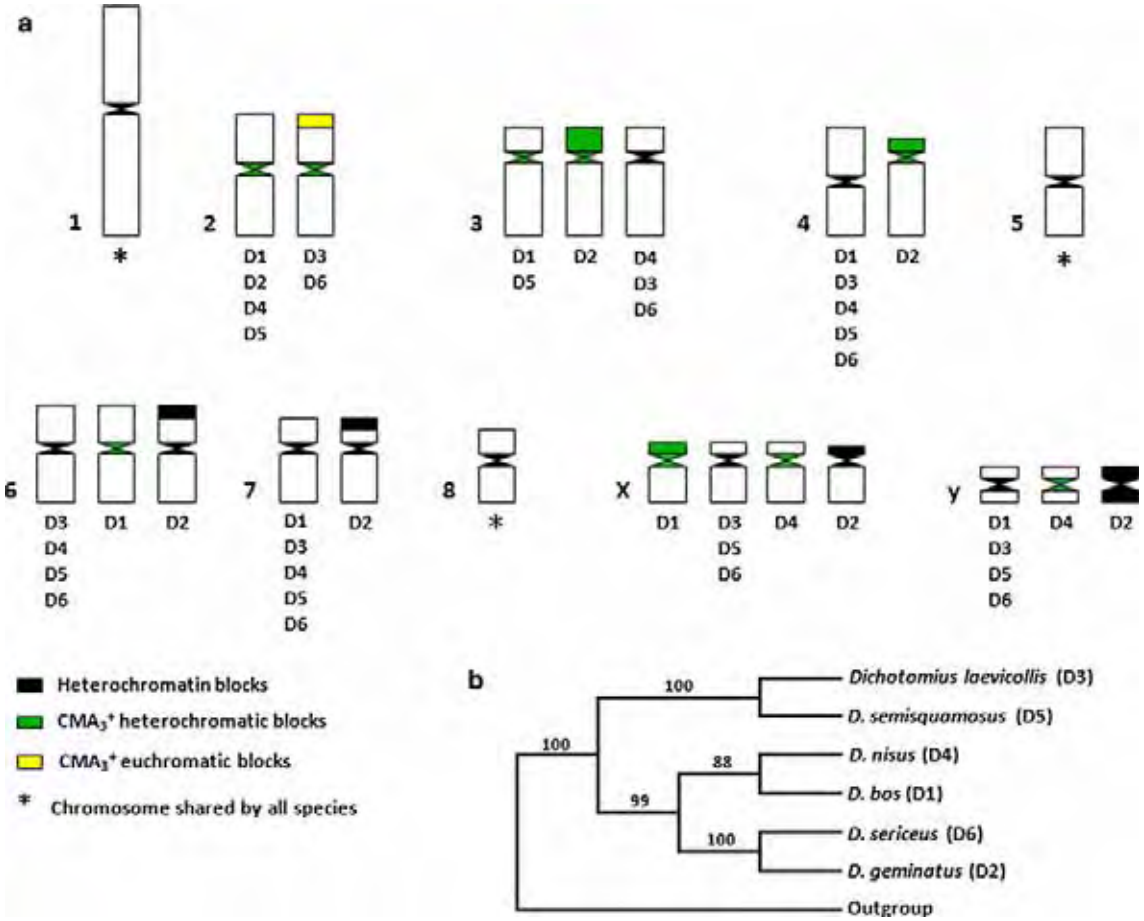

Fig. 4 **a** Idiograms showing the distribution of cytogenetic markers for each chromosome in the six species of *Dichotomius* studied; **b** phylogenetic relationship of the six *Dichotomius* species based on COI and 16S sequences. (D1) *Dichotomius bos*, (D2) *D. geminatus*, (D3) *D. laevicollis*,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

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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 a low 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

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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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