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Nahanna Zimmermann Menezes de Carvalho

Padrões evolutivos de famílias multigênicas de RNAs (RNAsn e RNAr) no genoma do gafanhoto *Abracris flavolineata*, com ênfase no cromossomo B

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NAHANNA ZIMMERMANN MENEZES DE CARVALHO

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Orientador: PROF. DR. DIOGO CAVALCANTI CABRAL DE MELLO

Co-orientador: OCTAVIO MANUEL PALACIOS GIMENEZ

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RESUMO

Os cromossomos B são elementos supernumerários dispensáveis definidos como não homólogos aos cromossomos do complemento A e não se recombinam com os mesmos, portanto seguem seu próprio caminho evolutivo. Sua presença é relatada em aproximadamente 15% dos eucariotos. A maioria desses cromossomos são heterocromáticos, o que está relacionado ao acúmulo de elementos repetitivos. Dentre as classes de elementos repetitivos encontrados nos cromossomos B estão principalmente genes ribossomais, DNAs satélites e elementos transponíveis. Em gafanhotos através da FISH (hibridização *in situ* fluorescente) foi relatado a presença de distintos DNAs repetitivos nos cromossomos B. Especificamente em indivíduos *Abracris flavolineata* coletados em Rio Claro/SP, um cromossomo B eucromático submetacêntrico foi descrito. Neste cromossomo foi observada a presença de genes de RNAsn U2 em ambos braços cromossômicos, além de distintos microssatélites e dois elementos de transposição do tipo *Mariner*. O presente estudo teve como objetivo observar a taxa evolutiva do cromossomo B comparando a mesma com os cromossomos do complemento A, utilizando como base sequências de famílias multigênicas. Para isso, foi realizada a microdissecção do cromossomo B e seu produto amplificado foi usado em experimentos de pintura cromossômica e também como modelo de amplificação para cinco famílias multigênicas (18S e 5S DNAr, U1 e U2 DNAsn e histona H3). Além disso, também amplificamos as mesmas famílias multigênicas para o genoma 0B, para fazer análises comparativas e inferir a evolução específica da sequência nucleotídica do complemento A e cromossomo B. Os dados gerados permitiram melhor conhecimento dos mecanismos atuantes na diversificação das sequencias de DNA presentes no B e compartilhadas como o genoma A.

Palavras-chave: DNAs repetitivos, microdissecção, pintura cromossômica, evolução molecular

SUMÁRIO

1. Introdução

1.1 DNAs repetitivos com ênfase em famílias multigênicas de RNAr e RNAsn

Os DNAs repetitivos compõem uma fração grande dos genomas eucariotos e estão organizados em tandem (famílias multigênicas, DNAs satélite, microsatélites, minissatélites) o dispersos (transposons e retrotransposons) ao longo do genoma. O termo "família multigênica" é utilizado para indicar um grupo de sequências de DNA (genes) com notável similaridade estrutural e funcional, sendo descendentes de um gene ancestral comum (NEI e ROONEY 2005). Basicamente, as famílias multigênicas estão envolvidas na codificação de importantes proteínas e RNAs que atuam na organização e metabolismo celular. Além disso, estes elementos estão envolvidos na organização e funcionalidade dos centrômeros, telômeros, segregação cromossômica, regulação gênica, reparo e replicação do DNA, além da diferenciação dos cromossomos sexuais (ANLEITNER e HAYMER 1992, KRAEMER e SCHMID 1993, MESSIER et al 1996, MARTINS 2007).

Dentre algumas famílias multigênicas, os genes de RNAs ribossomais (RNAr) estão representados pelo DNAr maior 45S e o DNAr menor 5S. O cluster do DNAr 45S corresponde a região organizadora de nucléolos (RON) e consiste de unidade repetitiva de três genes ou regiões codificantes (18S, 5.8S e 28S) separadas por espaçadores transcritos internos entre estes genes (ITS1 e ITS2) e um espaçador intergênico (IGS). A unidade repetitiva do DNAr 5S consiste de um gene de aproximadamente 120 pares de base (pb) separadas por um espaçador não transcrito (NTS), este último altamente variável em tamanho e sequência nucleotídica (LONG e DAWID 1980, NEI e ROONEY 2005, EICKBUSH e EICKBUSH 2007). Outras famílias multigênicas, por exemplo os genes de RNAs nucleares pequenos (RNAsn), estão envolvidas no processo de *splicing* alternativo (RNAsn). Estes genes incluem U1, U2, U4, U5 e U6 (BRINGMANN e LÜHRMANN 1986; NILSEN 2003; VALADKHAN 2005; WEST 2012). Do ponto de vista cromossômico, tanto o DNAr maior 45S como o DNAr menor 5S estão organizados em um ou mais locus cromossômicos (CABRERO e CAMACHO 2008, CABRERO et al. 2009, CABRAL-DE-MELLO et al. 2011), enquanto que os genes RNAsn se apresentam mais conservados em número e localização cromossômica comparados com os clusters de DNAr (NEI e ROONEY 2005, CABRAL-DE-MELLO et al. 2012, ANJOS et. al. 2015).

1.2 Cromossomo B

Os cromossomos B, também conhecidos como elementos supernumerários, foram definidos por Jones and Rees (1982) como cromossomos não homólogos aos cromossomos do complemento normal (complemento A) que não apresentam capacidade de recombinação com os mesmos e são completamente dispensáveis. A presença de cromossomos Bs, desde sua descoberta, foi relatada para cerca de 15% das espécies de eucariotos, sendo esta frequência também relatada em Orthoptera (CAMACHO 2005, PALESTIS et al. 2010). Esses elementos podem ser originados intraespecíficamente no genoma a partir do complemento A ou através de hibridação interespecífica (CAMACHO et al. 2000).

O tamanho e composição molecular dos cromossomos B é bastante variável entre os diferentes grupos, podendo os mesmos apresentar tamanhos similares aos elementos A, serem maiores, ou com tamanho diminuto, o que dificulta sua identificação e análise. O tamanho dos cromossomos Bs interferem diretamente em sua estabilidade ao longo das divisões celulares, sendo os grandes ou com tamanhos medianos mais estáveis comparados aos Bs diminutos (CAMACHO 2005, HOUBEN et al. 2013). Quanto à composição, a maioria dos cromossomos B são heterocromáticos ou compostos basicamente de elementos repetitivos. Dentre os elementos de DNA mais encontrados nos cromossomos B destacam-se genes ribossomais, DNAs satélites e elementos transponíveis. O acúmulo de elementos repetitivos nos cromossomos B está diretamente associado a sua natureza heterocromática e ausência de recombinação com os elementos A, e assim, seguem seu próprio caminho evolutivo, divergindo entre espécies distintas e até entre indivíduos da mesma espécie (BEUKEBOOM 1994; CAMACHO et. al. 2000; HOUBEN et al. 2013, 2014).

Em Orthoptera, cromossomos B foram relatados em pelo menos 191 espécies, sendo observado em cerca de 15% em Acridoidea (PALESTIS et al. 2010). Em gafanhotos é frequente a ocorrência de DNAs repetitivos nos distintos cromossomos B, detectados através da FISH (hibridização *in situ* fluorescente), como por exemplo observado o gene de histona H3 em *Locusta migratoria* e *Rhammatocerus brasiliensis*; genes de RNAr 45S em *Dichroplus pratensis* e *Eyprepocnemis plorans*; genes de RNAr 5S em *R. brasiliensis* e *E. plorans*; DNAs satélites e distintos elementos de transposição em *E. plorans* (TERUEL et al. 2010; OIIVEIRA et al 2011.; BIDAU et al. 2004; TERUEL 2013; CABRERO et al. 2003). No gafanhoto *Abracris flavolineata* foi relatada a presença de genes de RNAsn U2 nos dois braços cromossômicos em indivíduos pertencentes à população de Rio Claro/SP (BUENO et al. 2013), além de dois elementos tipo *Mariner* (PALACIOS-GIMENEZ et al. 2014). A

presença destes elementos repetitivos nos cromossomos B de *A. flavolineata* podem ser marcadores úteis no entendimento dos padrões de evolução destes cromossomos em relação ao complemento A a partir da comparação das sequências nucleotídicas obtidas de cromossomo B microdissectado e do genoma A sem cromossomos B.

2. Objetivos

Neste estudo buscamos um conhecimento mais profundo no que diz respeito à relação do cromossomo B e os cromossomos do complemento A de *Abracris flavolineata*, através do estudo do padrão de diversificação de famílias gênicas de RNAr (18S e 5S), RNAsn (U1 e U2) e gene histona H3 no genoma de *Abracris flavolineata*, com ênfase em cromossomos B.

- Realizar a microdissecção cromossômica do cromossomo B de *A. falvolineata* para obtenção do DNA específico deste cromossomo visando obter por PCR distintas famílias multigênicas de RNAr e RNAsn.
- Comparar sequências nucleotídicas obtidas por PCR do genoma 0B e das presentes no cromossomo B microdissectado para entendimento dos padrões de evolução molecular destes elementos supranumerários em *A. flavolineata.*

3. Materiais e Métodos

Os indivíduos utilizados para as análises estavam armazenados em freezer -20°C do Laboratório de Citogenética da UNESP/Rio Claro. Indivíduos com cromossomos B foram utilizados para obtenção de lâminas coradas com Giemsa 5% para realização da microdissecção cromossômica, para a qual foram selecionadas células em diplóteno e metáfase I, pois nessas fases o cromossomo B é facilmente reconhecido devido ao seu tamanho e heteropicnose. Após a microdissecção os cromossomos B foram amplificados utilizando os kits WGA4 (Sigma-Aldrich, St Louis, MO, USA) seguido do WGA3 (Sigma-Aldrich) de acordo com as recomendações do fabricante. Este produto foi utilizado com dois propósitos: (i) experimentos de FISH (pintura cromossômica) e (ii) como modelo para amplificação por PCR convencional de cinco familias multigênicas.

A sonda para pintura cromossômica foi marcada com Digoxigenin-11-dUTP (Roche, Mannheim, Germany) através de nick-translation. O experimento foi realizado seguindo as adaptações propostas por Cabral-de-Mello et al. (2010). A sonda foi detectada utilizando anti-digoxigenin rhodamine (Roche) e as preparações foram contrastadas usando 4', 6-diamidine-2'-phenylindole (DAPI) e montadas em VECTASHIELD (Vector, Burlingame, CA, USA). As imagens foram obtidas pela câmera digital DP70 acoplada ao microscópio Olympus BX61 equipado com uma lâmpada de fluorescência e filtros apropriados.

O DNA genômico de indivíduos 0B foi obtido seguindo o método de Sambrook e Russel (2001). Para a realização das PCR foram utilizados os primers dos genes de RNAr 18S e 5S e dos genes de RNAsn U1 e U2 (CABRAL-DE-MELLO et al. 2010; BUENO et al. 2013). A PCR foi realizada utilizando como molde o DNA genômico obtido de indivíduos 0B e do B microdissectado. Os produtos de PCR positivos foram clonados utilizando-se o vetor pGEM®-T vector (Promega, Madison, WI, USA) e a linhagem DH5 α da bactéria *Escherichia coli*. Clones positivos foram recuperados e enviados para sequenciamento nucleotídico na empresa Macrogen. As sequências foram inicialmente analisadas no programa Geneious (DRUMMOND et al. 2009) para checagem de sua qualidade e montagem das sequências consenso. Em seguida para a análise de polimorfismos de DNA e reconhecimento de haplótipos foi utilizado o programa DNAsp v.5.10.01 (LIBRADO, P. and ROZAS, J. 2009) e por fim a árvore neighbor-joining e as análises filogenético-moleculares foram realizadas no software MEGA 5.2 (TAMURA et al. 2011) e no Network (http://www.fluxus-engineering.com/sharepub.htm#a10)

4. Resultados e discussão

Esta etapa do trabalho está apresentada no referente manuscrito publicado na revista Molecular Genetics and Genomics (anexo 1).

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Anexo 1.

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ORIGINAL PAPER

High similarity of U2 snDNA sequence between A and B chromosomes in the grasshopper *Abracris flavolineata*

Nahanna Zimmermann Menezes-de-Carvalho[1] · Octavio Manuel Palacios-Gimenez[1] · Diogo Milani[1] · Diogo Cavalcanti Cabral-de-Mello[1]

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Abstract B chromosomes are frequently enriched for a wide variety of repetitive DNAs. Among grasshoppers in the species *Abracris flavolineata* (Ommatolampidinae) the B chromosomes are submetacentric, C-negative and harbor repetitive DNAs such as, U2 snDNA, C_0t-1 DNA, two *Mariner*-like elements and some microsatellites. Here, we provide evidence showing the intragenome similarity between the B chromosome and the A complement in *A. flavolineata*, combining analysis of microdissection and chromosome painting and B chromosome-specific amplification through polymerase chain reaction (PCR) of U2 snDNA. Chromosome painting revealed signals spread through the C-negative regions, including the A and B chromosomes. Moreover, significant clustered signals forming bands were observed in some A chromosomes, and for the B chromosome, significant signals were located on both arms, which could be caused by accumulation of repetitive DNA sequences. The C-positive regions did not reveal any signals. Sequence comparison of U2 snDNA between that obtained from a genome without the B chromosome and that from μB-DNA revealed high similarity with the occurrence of four shared haplotypes, one of them (i.e., Hap1) being highly prevalent and putatively ancestral. The highest divergence from Hap1 was observed for Hap3, which was caused by only six mutational steps. These data support an intraspecific origin of the B chromosome in *A. flavolineata* that is highly similar with the A complement, and the low U2 snDNA sequence diversity observed in the B chromosome could be related to its recent origin, besides intrachromosomal concerted evolution for U2 snDNA repeats in the B chromosome.

Keywords FISH · Splicing · Microdissection · Multigene families · Repetitive DNAs

Introduction

Supernumerary, accessory or B chromosomes are dispensable for normal growth components of the genome that have been widely reported in eukaryotes, approximately 15 % (Jones and Rees 1982; Camacho 2005; Houben et al. 2013, 2014). These elements have arisen intraspecifically from the genome of carrier species or through interspecific hybridization (Camacho et al. 2000). B chromosomes do not recombine with the A complement (normal set of chromosomes) and also show non-Mendelian inheritance, which allows them to accumulate in the germ line due to higher transmission rates exceeding those of autosomes (Camacho et al. 2000; Houben et al. 2013, 2014). As a possible consequence, B chromosomes could follow their proper species-specific evolutionary trajectory (Houben et al. 2013).

Generally, B chromosomes are heterochromatic and composed specially of repetitive DNAs, i.e., multigene families, microsatellites, satellite DNAs and transposable elements (Camacho et al. 2000; Camacho 2005; Jones et al. 2008; Houben et al. 2013, 2014), although some single copy protein coding genes have been recently reported (Graphodatsky et al. 2005; Yoshida et al. 2011; Martis et al. 2012; Trifonov

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✉ Diogo Cavalcanti Cabral-de-Mello
mellodc@rc.unesp.br

[1] Departamento de Biologia, Instituto de Biociências/IB, UNESP - Univ Estadual Paulista, Rio Claro, São Paulo CEP 13506-900, Brazil

et al. 2013; Valente et al. 2014). Among these repetitive DNAs, some multigene families have been found in B chromosomes, including in some grasshopper species, as for example, major rDNA in *Eyprepocnemis plorans* (López-León et al. 1994) and *Dichroplus pratensis* (Bidau et al. 2004), 5S rDNA in *Rhammatocerus brasiliensis* (Oliveira et al. 2011), histone genes in *Locusta migratoria* (Teruel et al. 2010) and *Rhammatocerus brasiliensis* (Oliveira et al. 2011), and U2 snDNA in *Abracris flavolineata* (Bueno et al. 2013), and have been informative about their evolution at chromosomal and molecular levels. The isolation of B chromosomes through microdissection followed by painting have also been used to understand B chromosome diversification (see for example Teruel et al. 2009a, b; Voltolin et al. 2010; Vicari et al. 2011; Martins et al. 2013; Pansonato-Alves et al. 2014) but in a few cases, this microdissected B chromatin has been used as a template to recover repetitive sequences to address specific molecular evolution in comparison to the A genome (Teruel et al. 2010; Silva et al. 2014).

In Orthoptera, B chromosomes have been reported in at least 191 species, having been observed in approximately 15 % of Acridoidea representatives (Palestis et al. 2010), including the South American grasshopper *A. flavolineta* (Bueno et al. 2013). The chromosome complement of *A. flavolineata* is characterized by a diploid number $2n = 23$,X0♂/24,XX♀ plus one or two B chromosomes in individuals collected in Rio Claro, São Paulo, Brazil. This B chromosome is submetacentric, lacks C-positive bands, and the mapping of some repetitive DNAs revealed the presence of U2 snDNA in both arms (Bueno et al. 2013), some spread and clustered microsatellite motifs along its entire length (Milani and Cabral-de-Mello 2014), two *Mariner*-like elements, *Afmar1* scattered along its entire length and *Afmar2* scattered in the proximal/interstitial regions of the long arm (Palacios-Gimenez et al. 2014), and a C_0t-DNA fraction (enriched for highly and moderately repetitive DNAs) restricted to the centromeric region (Bueno et al. 2013).

In this study, our aim was to obtain a deeper understanding concerning the origin, relationship with the A complement and specific sequence evolution in the B chromosome of *A. flavolineata*. For this aim, we used the microdissected B chromosome for chromosome painting and as a template for specific amplification through Polymerase Chain Reaction (PCR) of multigene families comparing the data of successfully amplified repeats (i.e., U2 snDNA) with sequences recovered from genome without B chromosomes.

Materials and methods

Male individuals with and without the B chromosome of *A. flavolineata* previously collected in the Parque Estadual Edmundo Navarro de Andrade in Rio Claro, São Paulo, Brazil, with the authorization of ICMBio SISBIO (process number 16009-1) and stored at −20 °C were used in this study. For chromosome microdissection we selected diplotene and metaphase I cells, in which the B chromosome is easily recognized due to its heteropycnosis and size, being smaller than the X chromosome. Ten B chromosomes were microdissected using an Eppendorf 5171 micromanipulator coupled with a Nikon Axiphot inverted microscope and subjected to amplification using GenomePlex Single Cell Whole Genome Amplification Kit WGA4 (Sigma–Aldrich, St Louis, MO, USA), followed by reamplification using the GenomePlex WGA3 kit (Sigma–Aldrich) according to manufacturer's recommendations.

The probe for chromosome painting was labeled with Digoxigenin-11-dUTP (Roche, Mannheim, Germany) through nick-translation. The chromosome painting experiment was performed following the adaptations proposed for fluorescent in situ hybridization (FISH) by Cabral-de-Mello et al. (2010). The probe was detected using anti-digoxigenin rhodamine (Roche) and the preparations were counterstained using 4′, 6-diamidine-2′-phenylindole (DAPI) and mounted in VECTASHIELD (Vector, Burlingame, CA, USA). Images were recorded using a DP70 cooled digital camera coupled to the Olympus microscope BX61 equipped with a fluorescent lamp and appropriate filters.

The PCR for the five multigene families, i.e., 18S and 5S rRNAs, U1 and U2 snRNAs and H3 histone genes was conducted using as templates the genomic DNA obtained from individuals without the B chromosome (0B-gDNA) following the protocol proposed by Sambrook and Russell (2001) and the DNA obtained from the microdissected B chromosome (μB-DNA). The primers used were previously published by Cabral-de-Mello et al. (2010) for 18S rDNA and 5S rDNA, Cabral-de-Mello et al. (2012) for U1 snDNA, Bueno et al. (2013) for U2 snDNA and Colgan et al. (1998) for H3 histone gene. For each distinct sequence the PCR reactions were performed at the same time using 0B-gDNA and μB-DNA as templates under distinct temperatures for primer annealing. The PCR conditions included an initial denaturation at 94 °C for 5 min and 30 cycles at 94 °C (30 s), 53–61 °C (30 s), and 72 °C (80 s), plus a final extension at 72 °C for 5 min. The PCR products were resolved using a 1 % agarose gel and the positive bands were isolated and purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., The Epigenetics Company, USA) according to the manufacturer's recommendations.

The purified PCR products were cloned into a pGEM-T vector (Promega, Madison, WI, USA) and transfected into DH5α *E. coli.* Positive clones were screened by PCR using the M13 primers (F 5′CCCAGTCACGACGTTGTAAAACG and R

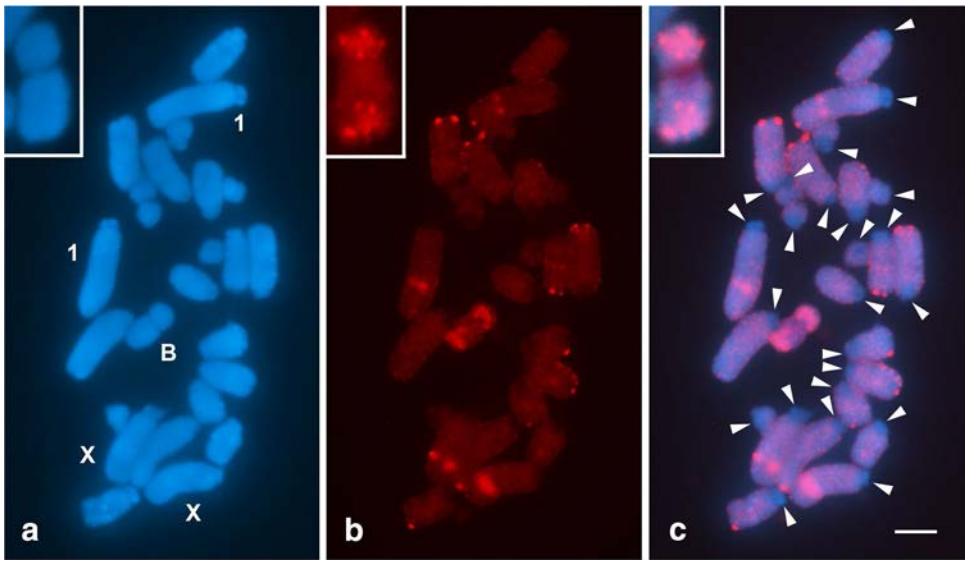

Fig. 1 Chromosome painting using the microdissected B chromosome (μB-DNA) as a probe of *Abracris flavolineata* in female mitotic metaphase. Note the spread signals on the C-negative regions and clustered signals forming bands on the B chromosome and some A chromosomes, including both pair 1 and X. **a** DAPI, **b** probe signal and **c** DAPI + signal. The *arrowheads* point to the regions without signals, corresponding to the C-positive regions. *Bar* 5 μm

Table 1 Polymorphisms to U2 snDNA obtained from 0B-gDNA and the μB-DNA from *Abracris flavolineata*

	0B-gDNA	μB-DNA	All
Number of sequences	22	27	49
Number of haplotypes	6	5	7
Number of sites	179	179	179
Number of variable sites	10	5	11
Number of mutations	10	5	11
Nucleotide diversity (π)	0.0112	0.00430	0.00749
Standard deviation (π)	0.00332	0.00129	0.00186
Haplotypes diversity (Hd)	0.645	0.447	0.537

5′AGCGGATAACAATTTCACACAGG), recovered and sequenced in both directions using the service of Macrogen Inc. (Korea). For a more accurate analysis, we discarded the singleton sequences (sequences that were unique among the sequenced clones). The resultant sequenced products of U2 snDNA were 177–179 bp, which included 22 sequences from 0B-gDNA and 27 from μB-DNA. The sequences were analyzed with the Geneious software (Drummond et al. 2009) to check their quality and to exclude the vector sequences and assemble consensus sequences. Then, the DnaSP v.5.10.01 software (Librado and Rozas 2009) was used for DNA polymorphism analysis and haplotype recognition. To obtain the graphical haplotype network with the unique haplotypes set, we built a minimum spanning network using the Network 4.6.1.2 software (http://www.fluxus-engineering.com) previously built in DnaSP v.5.10.01. The U2 snDNA sequence of *Drosophila ananassae* (Diptera) was used to anchor the network. The sequences corresponding to distinct haplotypes were deposited in NCBI database under the following accession numbers, KP975079-KP975085. See the alignment of distinct haplotypes in supplementary material 1.

Results

Chromosome painting using the B chromosome revealed spread signals through the C-negative regions, which corresponded to long arms of autosomes and X chromosomes and the entire extension of the B chromosome. Moreover, clustered signals were also observed in some chromosomes from A complement, i.e., interstitial in pair 1 and X chromosomes and terminal in three other autosomal pairs. For the B chromosome, besides the spread signals, significant clustered signals forming bands were located on the short arm and the second distal half of the long arm. The C-positive regions, which include the vicinity of the centromeric region and the short chromosomal arms of the A complement being absent in the B chromosome (Bueno et al. 2013; Milani and Cabral-de-Mello 2014), did not reveal signals (Fig. 1).

For PCR of the five multigene families using the μB-DNA as template, only the U2 snDNA was successfully obtained (Supplementary material 2). Previous FISH experiments revealed the presence of this sequence in the B chromosome of *A. flavolineata* (Bueno et al. 2013). For comparative analysis, the U2 snDNA was also amplified from 0B-gDNA. The sequence analysis revealed a number of variable sites and mutations two times higher in sequences obtained from 0B-gDNA than the μB-DNA. Higher variability associated to sequences from 0B-gDNA was also noticed for nucleotide and haplotype diversity (Table 1). Six and five haplotypes were observed for 0B-gDNA and μB-DNA, respectively, with four of them

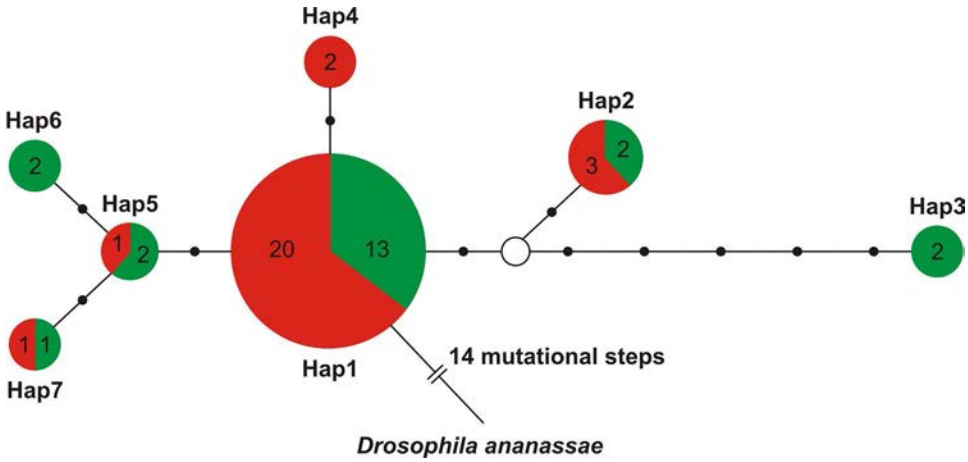

Fig. 2 Haplotype network showing the relationship between the seven haplotypes based on DNA sequences for U2 snDNA in the genome of *A. flavolineata*, 0B-gDNA (*green*) and the μB-DNA (*red*). *Black dots* correspond to mutational steps (substitutions) and circle diameters for each haplotype are proportional to their abundance. *White circle* symbolize an hypothetical haplotype (color figure online)

being shared between A and B genomes. Two haplotypes were exclusive for A genome (Hap3 and Hap6) and one for B chromosome (Hap4). Hap1, which is shared between A and B chromosomes, was the most frequent, corresponding to 33 of the 49 sequences and with a central position in the network, being the most similar to the haplotype from the outgroup, i.e., *D. ananassae*. In general the connected haplotypes differed between them only by one mutational step (only substitutions), but between Hap1 and Hap3, six mutations were noticed (Fig. 2).

Discussion

Microdissection followed by painting has been used for the study of B chromosomes, revealing clues about the origin (intra- or interspecific) and evolutionary diversification of these chromosomes, for example in fishes (Vicari et al. 2011; Pansonato-Alves et al. 2014), insects (Teruel et al. 2009a; Martins et al. 2013) and mammals (Rubtsov et al. 2004). As observed here for *A. flavolineata* and other grasshoppers, such as *L. migratoria* (Teruel et al. 2009a) and *E. plorans* (Teruel et al. 2009b), shared signals between A and B chromosomes using the B painting probe supports an intraspecific origin for these B chromosomes. However, in other animals, such as the bee *Partamona helleri* (Martins et al. 2013) and *Prochilodus* fish species (Voltolin et al. 2010, 2013) the B probes painted exclusively the B chromatin, suggesting interspecific origin or substantial genetic differentiation due to independent evolution, a common placement for B chromosomes after their origin.

The origin of the B chromosome in *A. flavolineata* is related to the largest autosomal pair due to the sharing of U2 snDNA clusters (Bueno et al. 2013) and the presence of this sequence could be the cause of significant clustered signals forming bands on the B chromosome and on the first autosomal pair. However, we cannot exclude the possibility of the presence of other non-identified repetitive sequences. These clustered signals on the *A. flavolineata* B chromosome also reinforce the notion of the isochromosome formation followed by the enlargement of one arm (current long arm) in the evolutionary history of this element, as previously suggested by the mapping of U2 snDNA and *Afmar2 Mariner*-like element (Bueno et al. 2013; Palacios-Gimenez et al. 2014). However, we could not precisely define the type of sequence present in this region due to the anonymity of the probe obtained from the μB-DNA.

Our painting results contrast with the data obtained using the C_0t-1 DNA fraction as a probe, in which few shared signals between the B and A complement were reported (Bueno et al. 2013). This difference could be due to the fraction of DNAs isolated by the two approaches, i.e., C_0t-1 DNA isolation and B microdissection, that could represent highly repetitive sequences in the former, as satDNAs, while in the latter interspersed euchromatic repeats, such as mobile elements (e.g., *Mariner*-like elements) that are abundant in the C-negative regions of *A. flavolineata* chromosomes (Palacios-Gimenez et al. 2014). The shared repeats harboring the C-positive regions of A chromosomes and the centromere of the B element, such as C_0t-1 DNA (Bueno et al. 2013) and satDNA (Milani et al. unpublished data), were not represented or only occurred in a small quantity in the μB-DNA, revealing no evident signals through chromosome painting. Based on the occurrence of sequences shared with C-positive regions of the A complement restricted to the B centromeric region, the enrichment of C-negative sequences in the B chromosome and their evident sharing between the B chromosome and the A complement, we suggest that the B chromosome is a recent element in the genome of *A. flavolineata*. Information related to both the repetitive DNAs previously mapped and the chromosome painting in *A. flavolineata* suggests that its B chromosome accumulated some repetitive DNAs, which seem to be a common fate in the B

chromosome differentiation and might facilitate the heterochromatinization process for these elements (Camacho 2005; Houben et al. 2013, 2014). Although for *A. flavolineata* this is a distinctive case because the repeats are more related to C-negative than to C-positive chromosomal regions.

The sequence analysis of U2 snDNA revealed a high similarity between the U2 copies present in A and B chromosomes of *A. flavolineata*, with four out of the seven observed haplotypes being present in both A and B chromosomes. The low variability and sharing of sequences between the two samples (0B-gDNA and µB-DNA) also support the idea that the B chromosome could be relatively young with low diversification from A chromosomes, at least for U2 snDNA sequences, although we could not estimate its age due to the absence of data concerning the evolutionary rate of U2 snRNA genes in related species. The low variation also suggests a recent amplification of U2 snDNA repeats in the B chromosome, thus also pointing to a relatively young B chromosome. In high contrast to observed for *A. flavolineata,* analysis of histone genes and 18S rRNA gene in the grasshopper *L. migratoria* (Teruel et al. 2010) and in the fish *Astyanax paranae* (Silva et al. 2014), respectively, revealed higher variability for sequences from B chromosomes, which is conceivable considering that the purifying selection is more relaxed in this chromosome. The lower variability in the B chromosome of *A. flavolineata* could be also attributed to low mutation rate and intrachromosomal homogenization of U2 snDNA sequences due to concerted evolution in the repeats of the B chromosome.

Among the five distinct U2 snDNA haplotypes observed in the B chromosome, four are shared with those obtained from the A complement (0B-gDNA), including the most common, Hap1, which might be the ancestral state due its central position in the network and higher proximity with the sequence of *D. ananassae* used as an outgroup. Considering this result, Hap1 could represent the haplotype with potential activity and the repeats present in the B chromosome have the potential of activity. Putative activity for sequences located in the B chromosome, based in the similarity of sequences from the A complement, was previously reported for H3 and H4 histone genes in the grasshopper *L. migratoria* (Teruel et al. 2010) and the characid fish *A. paranae* (Silva et al. 2014). It will be interesting to investigate if the presence of the B chromosome influences the transcript quantity for U2 snDNA, considering that the putative functional haplotype is highly abundant in this element, and for other U snRNA genes, which could be advantageous, considering the central role of U snRNAs in the process of alternative splicing.

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