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Estrutura e evolução cariotípica em espécies da infraordem Cicadomorpha (Hemiptera: Auchenorrhyncha) baseadas na análise de DNAs repetitivos

ALLISON KLEITON DOS ANJOS

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Celular e Molecular)

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TÍTULO DA TESE: Estrutura e evolução cariotípica em espécies da infraordem Cicadomorpha (Hemiptera: Auchenorrhyncha) baseadas na análise de DNAs repetitivos

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“É preciso tentar não sucumbir sob o peso de nossas angústias e continuar a lutar.”

J.K. Rowling

“Passamos toda a vida nos preocupando com o futuro. Fazendo planos para o futuro. Tentando prever o futuro. Como se desvendá-lo fosse aliviar o impacto. Mas o futuro está sempre mudando. O futuro é o lar dos nossos medos mais profundos e das nossas maiores esperanças. Mas uma coisa é certa: quando ele finalmente se revela, o futuro nunca é como imaginamos.”

Meredith Grey

Resumo

Os hemipteros da infraordem Cicadomorpha são representados por aproximadamente 30.000 espécies de insetos sugadores distribuídos mundialmente. Apesar de se destacarem por causarem muitos prejuízos na agricultura e pecuária pouco se sabe sobre a variabilidade genética e cromossômica desses animais que apresentam cromossomos holocentricos. Os DNAs repetitivos são uma ferramenta útil em estudos de diversificação cariotípica, organização e evolução dos genomas. Deste modo, este trabalho teve como objetivo contribuir com o conhecimento sobre a dinâmica dos cariótipos de representantes de Cicadomorpha e a organização de DNAs repetitivos, especificamente: I. analisar o cariótipo de espécies pertencentes a quatro famílias de Cicadomorpha, bem como caracterizar a heterocromatina constitutiva quanto a riqueza de pares de base; II. inferir a respeito da dinâmica evolutiva de famílias gênicas por meio do mapeamento cromossômico; III. analisar a organização cromossômica e testar a conservação interespecífica da sequência telomérica TTAGG_n do pool de DNAs repetitivos obtidos (fração C_0t) de espécies do gênero *Mahanarva* e das sequências teloméricas; IV. Analisar o satelitoma de *Mahanarva quadripunctata* e comparar os diferentes DNAs satélites de seu genoma com o de outras espécies do gênero visando entender a organização e evolução dos satDNAs neste grupo. Os dados obtidos revelam ampla variabilidade cromossômica entre as distintas famílias, causada principalmente por fusões cromossômicas, entretanto dentro de uma mesma família os cariótipos tendem a apresentar menos variações ao nível macrocromossômico. Embora os Cicadomorpha apresentem variabilidade em números diplóides a organização dos DNAs repetitivos é bastante conservada, mesmo em famílias distantes filogeneticamente, sugerindo estabilidade. Alguns dados foram analisados baseados na filogenia da infraordem, sendo sugerido os possíveis padrões ancestrais. Além disso, as possíveis causas da variabilidade em relação aos padrões modais são sugeridos. Os dados apresentados são um avanço no conhecimento da organização de DNAs repetitivos em Cicadomorpha, sendo para algumas sequências a primeira vez que se realiza algum estudo.

Palavras-chave: cigarrinhas, Cicadomorpha, DNAs repetitivos, evolução cromossômica, cromossomos holocentricos.

Abstract

The hemipterans of the Cicadomorpha infraorder are represented by approximately 30.000 species of sucking insects distributed worldwide. Although they stand out by cause many damages in agriculture and livestock, they are poorly studied regarding the genetic and chromosomal variability of these animals that present holocentric chromosomes. Repetitive DNAs are a useful tools in studies of karyotypic diversification, organization and evolution of genomes. The aim of this work was to contribute with knowledge about the dynamics of the karyotypes of Cicadomorpha and the organization of repetitive DNAs, specifically: I. Analyze the karyotype of species belonging to four families of Cicadomorpha, as well as to characterize the constitutive heterochromatin regarding base pairs richess; II. Infer about the evolutionary dynamics of multigene families through the chromosomal mapping; III. Analyze the chromosomal organization and test the interspecific conservation of the telemetric repeat $TTAGG_n$ from the pool of repetitive DNA fraction (C_0t fraction) of *Mahanarva* species and telomeric sequences; IV. Analyze the satellitome of *Mahanarva quadripunctata* and compare the different satellite DNAs of its genome with that from other species of the genus in order to understand the organization and evolution of satDNAs in this group. The data obtained reveal a wide chromosomal variability among the different families, caused mainly by chromosomal fusions, however within a same family the karyotypes tend to present less variations at the macro-chromosomal level. Although Cicadomorpha exhibit variability in diploid numbers, the organization of repetitive DNAs is highly conserved, even in phylogenetically distant families, suggesting stability. Some data were analyzed based on the phylogeny of the infraorder, suggesting possible ancestral patterns. In addition, the possible causes of variability relative to modal patterns are suggested. The data presented is an advance in the knowledge of the organization of repetitive DNAs in Cicadomorpha, being for some sequences the first time that some study is done.

Key words: spittlebugs, Cicadomorpha, repeated DNAs, chromosome evolution, holocentric chromosomes.

SUMÁRIO

1 Introdução

1.1 Considerações gerais sobre a infraordem Cicadomorpha (Hemiptera)

A infraordem Cicadomorpha, incluída na subordem Auchenorrhyncha, se destaca por ser uma das mais diversas linhagens de hemípteros fitófagos e chama atenção pela grande variabilidade morfológica (Figura 1). Cicadomorpha apresenta aproximadamente 30.000 espécies amplamente distribuídas mundialmente (Dietrich 2002; Cryan 2005; Cryan e Urban 2012). Essas espécies estão agrupadas em quatro superfamílias: Cicadoidea, Cercopoidea, Membracoidea, and Myerslopioidea, e que juntamente com a infraordem Fulguromorpha constituem a subordem Auchenorryncha (Cryan 2005; Cryan e Urban 2012). Cryan (2005), propôs a seguinte relação filogenética: (Myerslopioidea (Membracoidea (Cicadoidea + Cercopoidea))), onde Myerslopioidea seria grupo irmão de Membracoidea, ocupando uma posição mais basal na árvore filogenética de Cicadomorpha, enquanto Cicadoidea e Cercopoidea estariam agrupadas juntas. Dentre as características que distinguem o grupo dos Cicadomorpha, a principal delas é a presença de estruturas bucais especializadas para alimentação por sucção da seiva das plantas hospedeiras, o que torna algumas espécies e gêneros pertencentes a subordem Auchenorrhyncha economicamente importantes, sendo consideradas pragas e causando prejuízos na produção agrícola e agropecuária (Dietrich 2002; Dietrich 2005; Peck e Thompson 2008; Valério 2009).

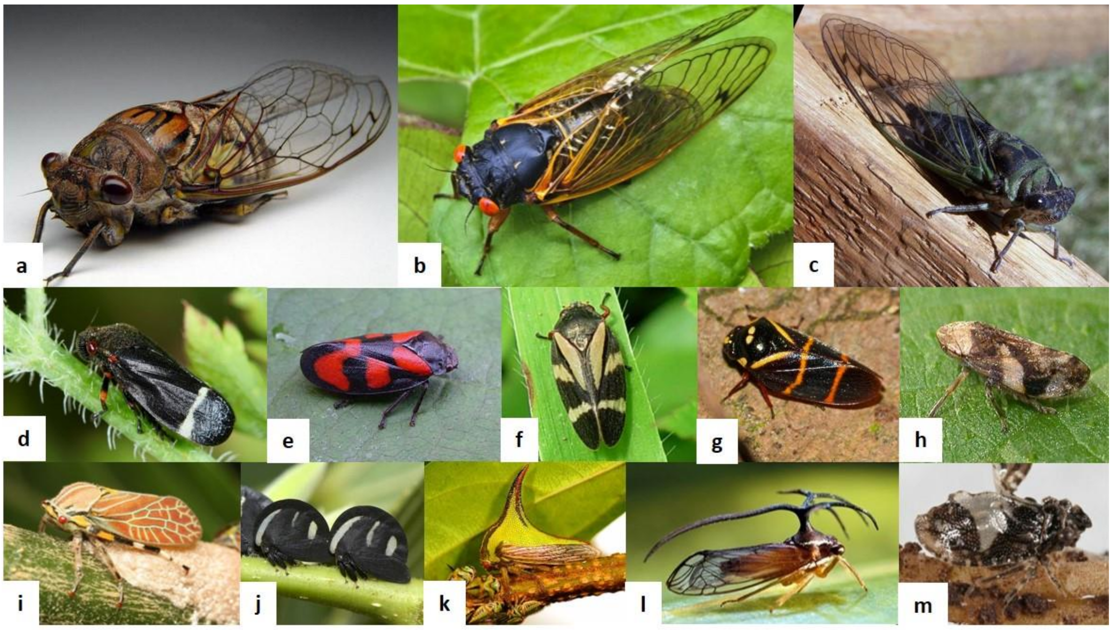

Figura 1: Diversidade de espécies incluídas na infraordem Cicadomorpha. (a-c) Cicadoidea; (d-h) Cercopoidea; (i-l) Membracoidea; (m) Myerslopioidea.

Dentre as superfamílias de Cicadomorpha, os membros de Cicadoidea se destacam por apresentar um complexo sistema de produção de sons, desenvolvimento larval longo (em algumas espécies o estágio larval chega a durar 17 anos), onde depois desse período, as larvas cavam túneis, sobem nas árvores e sofrem uma metamorfose, a ecdise (Figura 2a), se tornando adultas e prontas para o acasalamento (Cryan 2005). De forma semelhante, as ninfas de algumas espécies da superfamília Cercopoidea se alimentam do xilema das plantas hospedeiras, porém liberam uma massa de espuma que lhes confere proteção contra perda de umidade e predação (Figura 2b,c), enquanto os adultos apresentam compostos fitotóxicos na saliva que são injetados nas folhas à medida que eles se alimentam, interrompendo a atividade fotossintética e causando lesões necróticas que se espalham longitudinalmente até o ápice das folhas (Dietrich 2002; Peck e Thompson 2008; Valério 2009; Lohmann et al. 2010). Adicionalmente, a superfamília Myerslopioidea inclui apenas 19 espécies de pequenos insetos sem asas encontradas em Madagascar, Nova Zelândia, Austrália e Chile (Szwedo 2004).

Enquanto Membracoidea é a mais diversa das quatro superfamílias com cerca de 25.000 espécies descritas, que frequentemente são encontradas formando agregados de ninfas ou de ninfas e adultos (Figura 2d), como um mecanismo de proteção contra predadores, além de apresentar espécies conhecidas por se alimentarem de fluídos de floema ao invés de xilema (Cryan 2005).

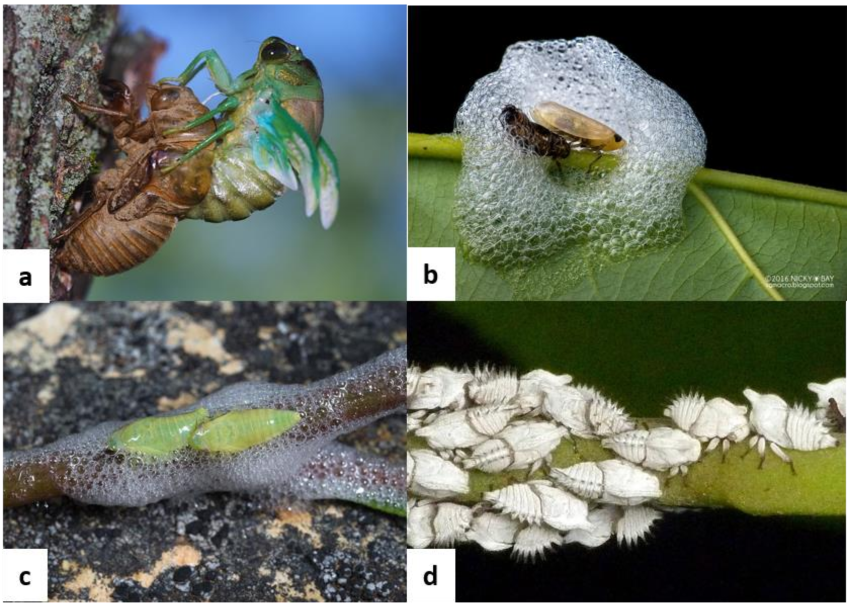

Figura 2: Hábitos das espécies da infraordem Cicadomorpha. (a) Cigarra realizando ecdise; (b,c) ninfas de Cercopidae se alimentando do xilema da planta hospedeira enquanto liberam uma massa de espuma; (d) agregados de ninfas de Membracidae.

1.2 Estudos cromossômicos em Auchenorrhyncha

Considerando a grande diversidade observada para a subordem Auchenorrhyncha com mais de 42.000 espécies descritas (Dietrich et al. 2001), pouco se sabe a respeito da citogenética do grupo, uma vez que apenas cerca de 2% desse total foi estudado cromossomicamente até o momento (Kuznetsova e Aguin-Pombo 2015), essencialmente com técnicas de coloração convencional. Os primeiros dados citogenéticos em Auchenorrhyncha foram obtidos por Boring (1907), que comparou o cariótipo de 22 espécies pertencentes as famílias Membracidae, Jassidae, Cercopidae e

Fulgoridae. A partir de então outros estudos foram publicados, utilizando em sua grande maioria técnicas de coloração convencional apenas com o foco de descrever o número cromossômico e o sistema de determinação sexual das espécies, evidenciando a presença de cromossomos holocentricos e uma extensa variabilidade nos números diploides (Halkka 1959; Halkka 1964; Kirillova 1986; Kirillova 1987; Kuznetsova et al. 1998; Kuznetsova e Aguin-Pombo 2015).

Assim como em Cicadomorpha, a presença de cariótipos compostos por cromossomos holocêntricos é uma característica comum para todos os Hemiptera. Este tipo cromossômico surgiu ao longo da diversificação de distintos grupos de plantas e animais de maneira independente (Maddox et al. 2004; Haizel et al. 2005; Papeschi e Bressa 2006; Nguyen et al. 2010; Melters et al. 2012; Mattos et al. 2013; Kuznetsova e Aguin-Pombo 2015). Esses cromossomos não apresentam constrição primária e dessa forma a fibra de fuso pode ligar-se em qualquer região do cromossomo durante a divisão celular. Acredita-se que eventos de diversificação cariotípica sejam possibilitados por esses cromossomos, uma vez que, fissões cromossômicas criariam fragmentos que mantem atividade centromérica, segregando normalmente durante a divisão celular, da mesma forma que fusões entre cromossomos não originam cromossomos dicêntricos (Dernburg 2001; Mandrioli e Manicardi 2012; Melters et al. 2012).

Apesar dos poucos estudos com a finalidade de realizar uma caracterização citogenética no grupo dos Auchenorrhyncha, uma extensa variabilidade em relação ao número diploide tem sido observada. Halkka (1959) descreveu que o número diploide das espécies analisadas variava de 2n=10 à 2n=39. Semelhante ao observado por Halkka (1964), onde as quatro espécies de Cercopidae analisadas (*Aphrophora forneri*, *A. alni*, *Neophilaenus lineatus* e *N. exclamationis*), apresentaram cariótipos variando de 2n=16 à

2n=20, enquanto as 37 espécies de Fulgoroidea analisadas por Kuznetsova et al. (1998) apresentaram números cromossômicos variando de 2n=19 à 2n=31. Adicionalmente, no estudo conduzido por Castanhole et al. (2010) foi possível observar que *Deois flavopicta, Mahanarva fimbriolata* e *Notozulia entreriana* apresentavam cariótipos com 2n=19, 2n=19 e 2n=15, respectivamente.

De acordo com Kuznetsova e Aguin-Pombo (2015), o número diploide em Auchenorrhyncha varia entre 8 e 38 cromossomos (nas fêmeas), enquanto para a infraordem Cicadomorpha a maior parte das espécies analisadas até o momento apresenta números diploides variando entre 16 e 22 cromossomos, com poucas exceções para mais e para menos, como por exemplo *Orosius* sp. (2n=8) e *Peuceptyelus coriaceus* (2n=32). Essa variação cariotípica pode ter sido produto de rearranjos cromossômicos, como fusões e/ou fissões que ocorreram no curso da diversificação cromossômica desses organismos. Em algumas espécies, como *Opio multistrigia* (2n=13) e *Cixius cunicularius* (2n=29), é possível observar a presença de cariótipos bimodais, reforçando a teoria da ocorrência de fusões cromossômicas (Whitten 1965; Kuznetsova et al. 1998b). Além disso, essa variabilidade parece estar relacionada com táxons ao nível de tribos e famílias, enquanto cariótipos aparentemente com o mesmo número cromossômico são observados ao nível de espécies e gêneros, assim como observado nas espécies dos gêneros *Cosmoscarta* (2n=28), *Mahanarva* (2n=19), e nas espécies da tribo Issini (2n=27) (Dey 1991; Marin-Morales et al. 2002; Maryańska-Nadachowska et al. 2006).

Apesar da variação observada quanto aos números diploides, a maior parte das espécies de Auchenorrhyncha apresentam sistema sexual bastante conservado do tipo XX♀/X0♂, sendo esta característica considerada como ancestral para o grupo como um todo (Kuznetsova and Aguin-Pombo 2015). Por outro lado, variações deste padrão já foram descritas, como nas espécies *Nilaparvata lugens* (2n=30,XY), *Philaenus arslani* (2n=20,XY) e *Philaenus italosignus* (2n=23,neo-X1X2Y) (Noda and Tatewaki 1990; Maryańska-Nadachowska et al. 2008; Maryańska-Nadachowska et al. 2012). Além disso, outros casos de polimorfismos também já foram descritos como a presença de cromossomos B nas espécies *Alebra albostriella* e *A. wahlbergi* (Kuznetsova et al. 2013), reforçando a variabilidade cromossômica no grupo.

1.3 Classificação, estrutura e organização genômica dos DNAs repetitivos nos genomas eucarióticos

O genoma dos eucariotos apresenta uma fração substancial de elementos repetitivos constituindo distintas famílias e podendo representar grande parte do conteúdo de DNA (Charlesworth et al. 1994; Nowak 1994). Sabe-se que estas sequências repetitivas estão envolvidas em importantes processos celulares, como codificação de importantes proteínas e RNAs, organização e funcionalidade dos centrômeros, telômeros, perfeita segregação cromossômica, regulação gênica, reparo e replicação do DNA e diferenciação de cromossomos sexuais (Anleitner e Haymer 1992; Kraemer e Schmidt 1993).

Estes elementos se encontram organizados basicamente em dois distintos grupos: (i) sequências codificadoras, que incluem diversas famílias multigênicas; (ii) sequências classicamente conhecidas como não codificadoras (embora atualmente sabe-se que estes DNAs podem transcrever importantes sequências regulatórias), representadas pelos DNAs satélites, microssatélites, minissatélites e elementos de transposição (transposons e retrotransposons) (Charlesworth et al. 1994; Martins et al. 2011). Além disso, quanto à organização genômica estes elementos podem estar dispersos como os elementos de transposição ou arranjados em tandem com as repetições dispostas em cadeias contínuas como os DNAs satélites e algumas famílias multigênicas (Kidwell 2002; Eirín-López et al. 2012; López-Flores e Garrido-Ramos 2012).

As famílias multigênicas são compostas por um grupo de sequências de DNA similares em estrutura e funcionalidade e que apresentam um mesmo gene ancestral comum (Nei e Rooney 2005). Dentre estas sequências são exemplos bastante conhecidos os genes de RNAs ribossomais (RNAr), genes codificadores de proteínas histônicas e genes de pequenos RNAs nuleares (RNAsn) envolvidos na produção de pequenos RNAs que atuam na maquinaria de *splicing*. As sequências de DNA ribossomal (DNAr) 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 sequências 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 intergênicos (IGS). O outro arranjo é formado pelas sequências do gene que transcrevem o RNAr 5S, que por sua vez são bastante conservados e espaçados por sequências não transcritas (NTS-*Non Transcribed Spacer*) que são extremamente variáveis em tamanho e composição nucleotídica (Long e Dawid 1980; Eickbush e Eickbush 2007).

As sequências codificadoras de proteínas histonas (H1, H2A, H2B, H3 e H4) são conhecidos por conter genes moderadamente repetidos, podendo estar organizados em um *cluster* único formado por genes que apresentam poucos *introns* e que se encontram espaçados por DNAs não codificantes (Kedes 1979; Nagoda et al. 2005). Essa estrutura genômica foi descrita, por exemplo, em *Drosophila melanogaster* (Lifton et al. 1978).

Por outro lado, estes mesmos genes podem estar distribuídos isoladamente, como observado em humanos (Albig et al. 1991), rato (Sittman et al. 1981) e *Gallus domesticus* (Engel e Dodgson 1981). Além disso, organismos apresentando os dois tipos de organização genômica também foram descritos, como em *Xenopus laevis* (Ruberti et al. 1982).

Outro exemplo de família multigênica são as sequências responsáveis pela produção de pequenos RNAs nucleares (RNAsn), envolvidos no processo de *splicing* e que são codificados por uma família multigênica que inclui U1, U2, U4, U5 e U6 (Bringmann e Lührmann 1986; Nilsen 2003; Valadkhan 2005; West 2012). Devido a sua grande importância para o metabolismo celular, os genes RNAsn U constituem uma distinta classe de genes conservados evolutivamente e do ponto de vista molecular podem apresentar múltiplas cópias dispersas no genoma, como observado em humanos (Manser e Gesteland 1982) e ratos (Marzluff et al. 1983), ou podem estar organizados em tandem, assim como no sapo *Xenopus laevis* (Mattaj e Zeller 1983), no ouriço *Strongylocentrotus purpuratus* (Yu et al. 1991) e em alguns peixes teleósteos (Marz et al. 2008).

No que diz respeito às sequências de DNAs repetitivos classicamente conhecidas como não codificadoras e repetidas em tandem, se destacam os DNAs satélites (DNAsat), os minissatélites e os microssatélites. Os DNAsat são sequências altamente repetitivas que variam em sequência de nucleotídeos, número de cópias e organização cromossômica, constituindo uma parte considerável dos genomas eucariotos (Palomeque e Lorite 2008; López-Flores e Garrido-Ramos 2012). Além disso, essas sequências são consideradas como o principal componente da heterocromatina e são comumente encontradas em regiões pericentroméricas e teloméricas (Plohl et al. 2008; Garrido-Ramos 2017). Por não condificarem proteínas, foram por muito tempo considerados DNA lixo, porém atualmente sabe-se que essas sequências estão relacionadas com uma série de funções celulares, como organização e pareamento cromossômico, além de estarem envolvidas com processos de especiação (Plohl et al. 2008; Ferree e Prasad 2012).

De forma semelhante, os minissatélites, que consistem de sequências moderadamente repetidas, apresentam uma tendência a se localizar mais próximos das regiões terminais dos cromossomos e são importantes no pareamento cromossômico e recombinação genética (Amarger et al. 1998; Sybenga 1999; Martins et al. 2011), enquanto os microssatélites representam as sequências repetitivas compostas de curtas unidades de repetição e que estão envolvidas na organização da cromatina, replicação do DNA, recombinação e regulação da atividade gênica (Li et al. 2002; Martins et al. 2011). Estas sequências apresentam um grande polimorfismo e são encontradas amplamente distribuídas pelo genoma.

Do ponto de vista cromossômico o uso de elementos repetitivos de DNA descritos acima tem se mostrado uma ferramenta esclarecedora para diversas questões, desde o entendimento da composição de diferentes regiões cromossômicas, tais como centrômeros e telômeros, a análises relacionadas à diversificação cariotípica, incluindo cromossomos autossômicos, origem e evolução de cromossomos sexuais e supernumerários, além de entendimento de rearranjos cromossômicos.

1.4 Estudos cromossômicos de DNAs repetitivos em Auchenorrhyncha

Considerando a riqueza de sequências repetitivas, como os DNAsat e elementos de transposição, frequentemente presentes nas regiões de heterocromatina constitutiva (HC), estudos envolvendo análises da heterocromatina através do bandeamento C em espécies de Auchenorrhyncha são bastante escassos. Nos estudos realizados até o momento foi possível observar que a maioria das espécies analisadas apresentam blocos heterocromáticos dispersos em vários cromossomos, distribuídos tanto em regiões terminais, quanto regiões intersticiais (Noda e Tatewaki 1990; Perepelov et al. 2002; Kuznetsova et al. 2003; Maryańska-Nadachowska et al. 2008; Maryańska-Nadachowska et al. 2012; Kuznetsova e Aguin-Pombo 2015). As exceções foram *Alebra viridis* que apresentou apenas blocos HC terminais (Kuznetsova et al. 2015b) e *Philaenus tesselatus* com blocos heterocromáticos apenas nos bivalentes maiores e no cromossomo X (Maryańska-Nadachowska et al. 2012).

De forma semelhante, o uso dos fluorocromos base específicos CMA_3 e DAPI evidenciou uma grande variabilidade com relação à riqueza de pares de base AT e GC na heterocromatina, com uma predominância de regiões ricas em GC (Kuznetsova et al. 2003; Maryańska-Nadachowska et al. 2008; Kuznetsova et al. 2015a; Kuznetsova e Aguin-Pombo 2015). Os resultados variaram desde a baixa especificidade para pares de base AT ou GC, como observado em *Philaenus tesselatus* e *Philaenus italosignus* (Maryańska-Nadachowska et al. 2012), até espécies com blocos CMA_3^+ (GC) e $DAPI^+$ (AT) distribuídos por vários cromossomos, como observado em *Hysteropterum albaceticum* (Kuznetsova et al. 2009).

Estudos envolvendo o mapeamento cromossômico de sequências repetitivas através da hibridização *in situ* fluorescente (FISH) em espécies de Auchenorrhyncha são ainda mais escassos e restritos a descrição da localização das sequências DNA ribossomal 18S e a presença da sequência telomérica $(TTAGG)_n$ (Maryańska-Nadachowska et al. 2013; Golub et al. 2014; Maryańska-Nadachowska et al. 2016). A sequência $(TTAGG)_n$ é considerada como a sequência telomérica ancestral para os insetos (Sahara et al. 1999; Frydrychová et al. 2004) e tem sido conservada nas regiões terminais dos cromossomos da maioria das linhagens do grupo dos artrópodes, incluindo as espécies de Auchenorrhyncha, com 18 espécies analisadas até o momento (Maryańska-Nadachowska et al. 2013; Golub et al. 2014; Kuznetsova et al. 2015b; Maryańska-Nadachowska et al. 2016). Por outro lado, em relação à distribuição das sequências de DNAr 18S, a maioria das espécies apresentou clusters localizados em autossomos, como em *M. chilensis*, cinco espécies do gênero *Alebra* e 11 espécies da família Issidae (Golub et al. 2014; Kuznetsova et al. 2015b; Maryańska-Nadachowska et al. 2016). No entanto, clusters localizados em cromossomos sexuais também foram observados, como em três espécies do gênero *Philaenus* (Maryańska-Nadachowska et al. 2013).

2 Objetivos

2.1 Objetivo geral

O conhecimento relativo à variabilidade genética em insetos infraordem Cicadomorpha, incluindo a variabilidade cromossômica, é bastante escasso. Marcadores cromossômicos obtidos a partir de DNAs repetitivos e que podem auxiliar no entendimento da história evolutiva dos cromossomos foram estudados apenas em quatro estudos (Maryańska-Nadachowska et al. 2013; Golub et al. 2014; Kuznetsova et al. 2015b; Maryańska-Nadachowska et al. 2016). O uso de marcadores obtidos por FISH juntamente com análises cromossômicas clássicas irão auxiliar no entendimento da estrutura cariotípica, padrões de evolução cromossômica e organização genômica nos representantes de Cicadomorpha. Estes dados em conjunto permitirão a construção de um cenário evolutivo do ponto de vista inter- e intraespecífico no grupo.

Deste modo, o objetivo geral desta tese é auxiliar no entendimento dos processos envolvidos na diversificação cariotípica de espécies de insetos pertencentes a quatro famílias da infraordem Cicadomorpha (Aethalionidae, Cercopidae, Cicadellidae e Membracidae), baseado na análise de marcadores cromossômicos obtidos utilizando ferramentas de citogenética clássica e distintas classes de DNAs repetitivos.

2.2 Objetivos específicos e metas

- Compreender a variabilidade cromossômica a partir da caracterização dos cariótipos (número diploide e sistema cromossômico sexual) de distintas espécies da infraordem Cicadomorpha, incluindo representantes de quatro famílias;

- Entender a organização geral dos DNAs repetitivos em distintos representantes de Cicadomorpha utilizando a qualificação da heterocromatina por bandeamentos com fluorocromos base-específicos e a fração C_0t, utilizando estes marcadores em análises comparativas;
- Analisar os padrões de organização e variabilidade de sequências teloméricas e famílias multigênicas do ponto de vista cromossômico, utilizando estes marcadores no entendimento da evolução cromossômica em Cicadomorpha;
- A partir de dados de sequenciamento Illumina de baixa cobertura, estudar o satelitoma da espécie *Mahanarva quadripunctata*, buscando entender os padrões evolutivos destas sequências nos cromossomos de espécies do gênero *Mahanarva*.

3 Material e Métodos

3.1 Animais, extração de DNA e preparações cromossômicas

Indivíduos de 19 espécies pertencentes às famílias Aethalionidae (1 espécie), Cercopidae (10 espécies), Cicadellidae (2 species) e Membracidae (6 espécies), da infraordem Cicadomorpha (Tabela 1), foram coletadas em áreas naturais da cidade de Rio Claro, São Paulo, Brasil (Latitude 22º 25’ S Longitude 47º 33’W). Os testículos dos machos foram fixados em solução Carnoy (3:1 etanol absoluto:ácido acético) e armazenados em freezer -20 ºC. Animais inteiros foram armazenados em etanol 100% para identificação. DNA genômico foi extraído de *Aetalion reticulatum*, *Mahanarva quadripunctata*, *Membracis foliatafasciata* e *Oncometopia facialis* seguindo o método fenol-clorofórmio-álcool isoamílico, proposto por Sambrook e Russell (2001). As preparações cromossômicas foram obtidas por maceração dos folículos testiculares em uma gota de ácido acético 45% e as lâminas foram secas em uma plataforma aquecida com temperatura entre 40-45 ºC. Os cromossomos foram corados com Giemsa 5%, enquanto a coloração com fluorocromos CMA_3/DA/DAPI foi realizada segundo Schweizer et al. (1983).

Tabela 1. Número cromossômico de 19 espécies pertencentes a 4 famílias da infraordem Cicadomorpha (Auchenorrhyncha, Hemiptera)

Família **Espécies**	**Cariótipo (2n)**
Cercopoidea	
Cercopidae	
Deois flavopicta (Stål, 1854)	2n=19,X0
Deois mourei Cavichioli & Sakakibara, 1993	2n=19,X0
Deois schach (Fabricius, 1787)	2n=19,X0
Mahanarva fimbriolata (Stål, 1854)	2n=19,X0
Mahanarva liturata (Le Peletier & Serville, 1825)	2n=19,X0
Mahanarva quadripunctata (Walker, 1858)	2n=19,X0
Mahanarva spectabilis (Distant, 1909)	2n=19,X0
Mahanarva tristis (Fabricius, 1803)	2n=19,X0
Mahanarva vittata (Walker, 1851)	2n=19,X0

Notozulia entreriana (Berg, 1879)	2n=15,X0
Membracoidea	
Aetalionidae	
Aetalion reticulatum (Linneaeus, 1758)	2n=21, X0
Cicadellidae	
Ferrariana trivittata (Signoret, 1854)	2n=19,X0
Oncometopia facialis (Signoret, 1854)	2n=17,X0
Membracidae	
Amblyophallus exaltatus (Fabricius, 1803)	2n=20,neo-XY
Bolbonota melaena (Germar, 1835)	2n=21,X0
Enchenopa sp.	2n=19,X0
Horiola picta (Coquebert, 1801)	2n=21,X0
Membracis foliatafasciata (DeGeer, 1773)	2n=13,X0
Neotynelia pubescens (Fabricius, 1803)	2n=21,X0

3.2 Isolamento de sequências repetitivas, obtenção de sondas e sequenciamento Sanger

Amostras enriquecidas contendo DNA repetitivo (sequencias altamente e moderadamente repetidas) foram obtidas baseadas no coeficiente de renaturação C_0t-1 de acordo com o protocolo descrito por Zwick et al. (1997) seis espécies de *Mahanarva* (manuscrito 1). Amostras de DNA (200 µl com 100–500 ng/ml de DNA genômico em NaCl 0,3 M) foram digeridos com Deoxyribonuclease I 0,01U/ml (Sigma) por 45 segundos e os fragmentos foram separados através de eletroforese em gel de agarose 1%, onde os fragmentos variavam entre 100 até 1000 pares de base. As amostra com os fragmentos de DNA foram desnaturadas à 95 °C por 10 minutos, transferidas para gelo por 10 segundos e em seguida transferidas para termociclador à 65 ºC por 25 minutos para reanelamento. Subsequentemente as amostras foram incubadas à 37 ºC por 8 minutos com S1 nuclease 1U, permitindo a digestão de fragmentos de DNA fita simples, seguido de purificação utilizando o método fenol-clorofórmio-álcool isoamílico.

As sequências parciais de DNAr 18S, histona H3 e U1 snDNA foram obtidas através de Reação em Cadeia da Polimerase (PCR) do genoma de *Aetalion reticulatum*, *Mahanarva quadripunctata*, *Membracis foliatafasciata* e *Oncometopia facialis*, utilizando primers universais descritos por Cabral-de-Mello et al. (2010), Cabrero et al. (2009), e Cabral-de-Mello et al. (2012), respectivamente. A sonda de sequência telomérica foi obtida através da PCR utilizando primers complementares $(TTAGG)_5$ e $(CCTAA)_5$ seguindo o protocolo proposto por Ijdo et al. (1991). As sondas de DNAr 18S e fração C_0t foram marcadas usando biotin-14-dATP através da reação de nick translation (Invitrogen, San Diego, CA, USA), enquanto as sondas de histona H3, U1 snDNA e sequência telomérica foram marcadas através da PCR com digoxigenin-11-dUTP (Roche, Mannheim, Germany).

Para o sequenciamento, foram utilizadas as amostras que possuíam concentração por volta de 50ng de DNA/µL. Estas amostras passaram pelo tratamento com a enzima EXOSAP (GE Healthcare) onde foi adicionado a um tubo eppendorf 10µL de produto de PCR, 2µL da enzima EXOSAP e 2µL de água Milli Q autoclavada. Os tubos foram levados ao termociclador em um ciclo de 1 hora à 37ºC e 15 minutos à 80ºC. As amostras foram mantidas no freezer (-20ºC). O produto de PCR purificado foi enviado para sequenciamento Sanger na empresa MacroGen (Seoul, Coréia).

3.3 Hibridização *in situ* fluorescente (FISH) e dot blot

A técnica de FISH foi realizada de acordo com o método proposto por Pinkel et al. (1986), com modificações (Cabral-de-Mello et al. 2010). Sondas marcadas com digoxigenin-11-dUTP foram detectadas usando anti-digoxigenin rhodamine (Roche), enquanto sondas marcadas com biotin-14-dATP foram detectadas usando Streptavidin Alexa Fluor 488 (Invitrogen). Todas as preparações foram coradas com 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) e posteriormente montadas usando Vectashield (Vector, Burlingame, CA, USA). Os cromossomos e os sinais da FISH foram observadas usando microscópio Olympus BX61 equipado com uma lâmpada fluorescente e filtros apropriados acoplados a uma câmera digital DP70. As imagens tiveram ajustes de brilho/contraste no Adobe Photoshop CS2 software.

Para a técnica de dot blot foram aplicados aproximadamente 100 ng de DNA genômico de cada uma das espécies analisadas em uma membrana Hybond N+ nylon (GE Healthcare). A membrana foi desnaturada com NaOH 0,5 M, lavada em 5xSSC por 1 minuto e finalmente submetida a secagem à 80 °C por 90 minutos. A hibridização seguiu overnihght à 37 ºC usando 200 ng de sonda marcada e desnaturada, diluída na solução tampão de hibridização ECL gold (GE Healthcare), com adição de albumina do soro bovino (0,05% w/v) e NaCl (2,5 M). Após a hibridização foram realizadas as seguintes lavagens: 5xSSC por 5 min à 42 °C; tampão primário contendo ureia 6M, SDS 0,4% (w/v), e 0.1xSSC 3vezes por 10 minutos cada à 42 °C; e 20× SSC por 5 min à temperatura ambiente. Finalmente, foi utilizado o kit de detecção de biotina (K0661, Thermo Scientific) seguindo as recomendações do fabricante.

3.4 Sequenciamento Illumina e análises de DNAs satélites

O genoma da espécie *Mahanarva quadripunctata* foi sequenciado através do Next-generation sequencing (NGS) pela plataforma Solexa da Illumina que permite a produção de milhares de leituras de uma só vez. Os reads obtidos do sequenciamento genômico foram agrupados usando o “fastq-join” da ferramenta FASTX-TOOLKIT (Gordon e Hannon 2010), onde os dados foram filtrados e agrupados seguindo parâmetros pré-estabelecidos no próprio programa. Os clusters foram obtidos a partir do pipeline proposto no RepeatExplorer (Novák et al. 2013) utilizando os parâmetros padrões. A procura de sequências de DNA satélite foi primeiramente realizada através da análise do formato e densidade dos gráficos de acordo com Novák et al. (2010). Em seguida foi utilizado o programa Tandem Repeat Finder (Benson 1999) para identificar sequências com máximos escores de alinhamento entre diferentes monômeros, usando os parâmetros de alinhamento 2, 3, 5 para correspondência, falta de correspondência e indels, respectivamente, e um mínimo de 50 pontos de alinhamento. Além disso, para identificar monómeros da mesma família e para confirmar a organização em conjunto, utilizou-se a ferramenta de alinhamento gráfico dotplot implementada em Dotlet (Junier e Pagni 2000). O monômero com o comprimento máximo foi usado como a cópia representativa para uma determinada família de DNAsat e como a sequência base para consulta de similaridade nos bancos de dados do NCBI (http://www.ncbi.nlm.gov/Blast/) e Repbase (http://www.girinst.org/repbase/) para verificar a semelhança com sequências já publicadas. Os alinhamentos de sequências de cópias de DNAsat foram realizados com MUSCLE (Edgar 2004) implementado no programa MEGA5 (Tamura et al. 2011).

As sequências de consenso de cada família de DNAsat foram usadas para desenhar primers usando o software Primer3 (Rozen e Skaletsky 1999) ou manualmente. Para verificar a presença de famílias de DNAsat no genoma de *M. quadripunctata*, foram realizadas reações de PCR usando 10x PCR Rxn Buffer, 0,2 mM $MgCl_2$, 0,16 mM dNTPs, 2 mM de cada iniciador, 1U de Taq Platinum DNA Polimerase (Invitrogen, San Diego, CA, EUA) e 50-100 ng/µl de DNA molde. As condições de PCR incluíram uma desnaturação inicial a 94°C durante 5 min e 30 ciclos a 94°C (30 s), 55°C (30 s) e 72°C (80 s), mais uma extensão final a 72°C por 5 min. Os produtos foram visualizados num gel de agarose a 1% e as bandas monoméricas foram isoladas e purificadas utilizando o Kit de Recuperação de DNA de Gel Zymoclean ™

(Zymo Research Corp., The Epigenetics Company, EUA) de acordo com as recomendações do fabricante e depois utilizadas como fonte para reamplificação. As sondas dos clusters MquSat29, MquSat113 e MquSat138 foram marcadas usando biotin-14-dATP através da reação de nick translation (Invitrogen, San Diego, CA, USA), enquanto MquSat115, MquSat117 e MquSat153 foram marcadas através da PCR com digoxigenin-11-dUTP (Roche, Mannheim, Germany).

4 Resultados e Discussão

Os resultados da presente tese estão apresentados nos quatro manuscritos descritos a seguir:

4.1 “**Karyotypes and Repetitive DNA Evolution in Six Species of the Genus *Mahanarva* (Auchenorrhyncha: Cercopidae)**”, publicado na revista Cytogenetic and Genome Research (ISSN: 1424-8581);

4.2 “**U1 snDNA chromosomal mapping in ten spittlebug species (Cercopidade, Auchenorrhyncha, Hemiptera)**” publicado na revista Genome (ISSN: 1480-3321);

4.3 “**Physical mapping of two multigene families and base-pair richness in Cicadomorphan insects reveal conservative chromosomal organization**”;

4.4 “**Unveiling some satellite DNAs in the holocentric species Mahanarva**”.

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Karyotypes and Repetitive DNA Evolution in Six Species of the Genus *Mahanarva* (Auchenorrhyncha: Cercopidae)

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Key Words
Agricultural pests · Heterochromatin · Holocentric chromosomes · Repetitive DNAs · Telomeres

Abstract
Insects of the Cercopidae family are widely distributed and comprise 59 genera and 431 species in the New World. They are xylemophagous, causing losses in agricultural and pasture grasses, and are considered as emerging pests. Chromosomally, these insects have been studied by standard techniques, revealing variable diploid numbers and primarily X0 sex chromosome systems (males). We performed chromosome studies in 6 *Mahanarva* (Cercopidae) species using standard and differential chromosome staining as well as mapping of repetitive DNAs. Moreover, the relationship between the repetitive DNAs was analyzed at the interspecific level. A diploid chromosome number of 2n = 19,X0 was documented, with chromosomes gradually decreasing in size. Neutral or GC-rich regions were detected which varied depending on the species. Fluorescence in situ hybridization with a $(TTAGG)_n$ telomeric motif probe revealed terminal signals, matching those of the Cot DNAs obtained from each species, that were also restricted to the terminal regions of all chromosomes. Dot blot analysis with the Cot fraction from *M. quadripunctata* showed that at least part of the repetitive genome is shared among the 6 species. Our data highlight the conservation of chromosomal features and organization of repetitive DNAs in the genus *Mahanarva*, suggesting a low differentiation for chromosomes and repetitive DNAs in most of the 6 species studied.

Cercopidae belong to the hemipteran infraorder Cicadomorpha, suborder Auchenorrhyncha. These insects are distributed worldwide and represent the largest cercopoid family, with 431 species grouped in 59 genera in the New World [Carvalho and Webb, 2005; Cryan and Urban, 2012; Paladini and Cryan, 2012]. They are xylemophagous insects, and due to this habit, they cause losses in agricultural and pasture grasses, as pests of forage grasses, sugarcane, and occasionally other cultivated grass, such as rice [Bernardo et al., 2003; Peck and Thompson, 2008]. At least 11 genera of Cercopidae are considered emerging pests, including *Mahanarva*, with 39 species widely distributed in South and Central America [Paladini and Carvalho, 2007].

Chromosomal studies in Auchenorrhyncha have been performed in ~820 species, mostly using standard chro-

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mosome staining with description of diploid numbers, that range from 2n = 8 to 2n = 38 in females, and sex chromosomes, revealing predominance of XX♀/X0♂ [Boring, 1913; Halkka, 1964; Dey, 1991; Kuznetsova and Aguin-Pombo, 2015]. For Cercopidae, ~31 species were karyotyped showing variability for diploid number and sex chromosomes, e.g., 2n = 28, XY♂ in *Cosmoscarta* species *(C. dimidiata*, *C. septempunctata*, *C. decisa*, *C. egens*, and *C. fulviceps)*; 2n = 19, X0♂ in *Mahanarva fimbriolata*, *M. posticata,* and *Deois flavopicta*; and 2n = 15, X0♂ in *Notozulia entreriana* [Dey, 1991; Marin-Morales et al., 2002; Castanhole et al., 2010].

Repetitive DNAs are abundant in eukaryotic genomes, including in-tandem and dispersed elements, and could play an important role in genome and chromosome diversification [Cavalier-Smith, 1982; Charlesworth et al., 1994; Elder and Turner, 1995; Kidwell, 2002; Feschotte and Pritham, 2007]. These repetitive sequences represent excellent chromosomal markers and have been successfully used for the understanding of chromosomal evolution in distinct insect orders [e.g., Palomeque and Lorite, 2008; Nguyen et al., 2010; Anjos et al., 2015]. In species with holocentric chromosomes, repetitive DNA markers are even more important to understand evolution, due to the lack of other chromosomal features, such as the primary constriction, allowing the identification of specific chromosomes or chromosomal regions [Pita et al., 2014; Kuznetsova and Aguin-Pombo, 2015].

Among insects with holocentric chromosomes, the organization of heterochromatin has mostly been studied by conventional techniques, and studies using isolated repetitive fractions or specific repetitive DNAs have been undertaken in a few species [Kuznetsova et al., 2009; Bardella et al., 2014; Pita et al., 2014; Kuznetsova and Aguin-Pombo, 2015]. The telomeric repeat $(TTAGG)_n$ is considered ancestral for arthropods [Vítková et al., 2005], although in insects this motif was repeatedly lost [Frydrychová et al., 2004], including representatives of distinct orders, like Coleoptera, Hemiptera, Diptera [Frydrychová et al., 2004], and Hymenoptera [Gokhman et al., 2014].

Here, we performed chromosome studies in 6 species of *Mahanarva* applying standard staining and banding techniques, and FISH with the ancestral arthropod telomeric motif $(TTAGG)_n$. In addition, a repetitive DNA-enriched fraction (Cot-DNA) was obtained for each species, and the location of the pool of repetitive DNAs was established by FISH to the species of origin. The conservation of the repetitive DNA pools between the 6 species was tested by cross-hybridization using dot blot assays. The similarities and differences in the chromosomal markers were used to infer the evolutionary processes that led to chromosomal and repetitive DNA diversification in the species studied.

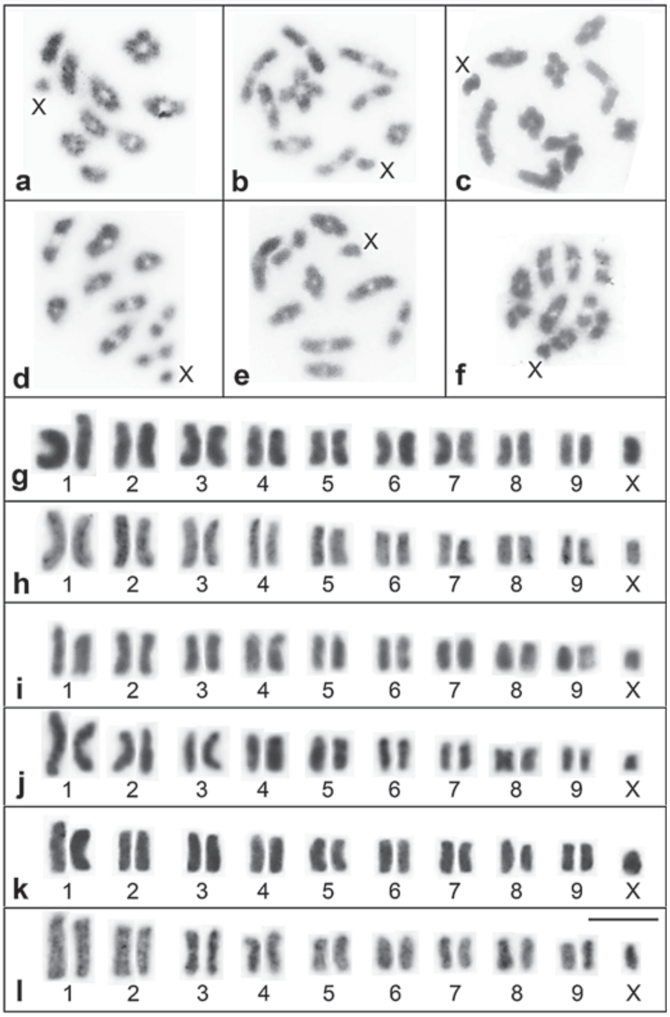

Fig. 1. Meiotic (**a–f**) and mitotic (**g–l**) chromosomes of 6 *Mahanarva* species stained with 5% Giemsa. **a**, **g** *M. fimbriolata*; **b**, **h** *M. liturata*; **c**, **i** *M. quadripunctata*; **d**, **j** *M. spectabilis*; **e**, **k** *M. tristis*; **f**, **l** *M. vittata*. **a–c**, **e** Diakinesis; **d**, **f** metaphase I; **g–l** arranged karyotypes. In **a–f**, the X chromosome is indicated. Bar = 5 μm.

Materials and Methods

Adult males of 6 *Mahanarva* species *(M. fimbriolata*, *M. liturata*, *M. quadripunctata*, *M. spectabilis*, *M. tristis*, and *M. vittata)* were sampled in natural areas of Rio Claro, São Paulo State, Brazil. Animals were anesthetized, the testes were dissected, left in distilled water for 5 min, and then fixed in modified Carnoy's solution (absolute ethanol:acetic acid, 3:1). Whole animals were stored in

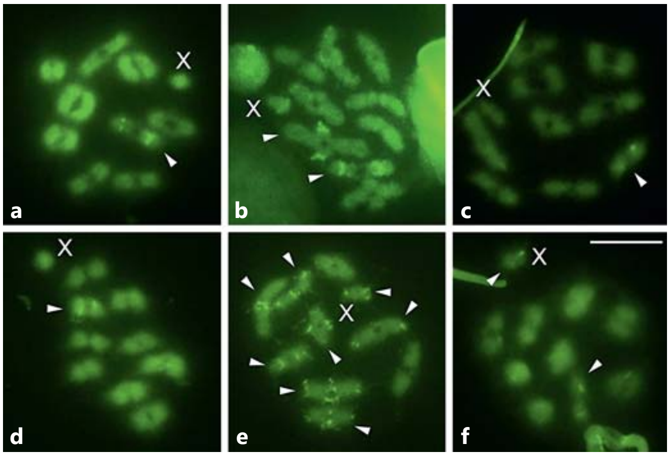

Fig. 2. Fluorochrome staining with CMA_3 in meiotic cells of 6 *Mahanarva* species. **a** *M. fimbriolata*; **b** *M. liturata*; **c** *M. quadripunctata*; **d** *M. spectabilis*; **e** *M. tristis*; **f** *M. vittata*. **b**, **e** Diakinesis; **a**, **c**, **d**, **f** metaphase I. The X chromosome is indicated, and chromosomes bearing positive signals are shown with arrowheads. Bar = 5 μm.

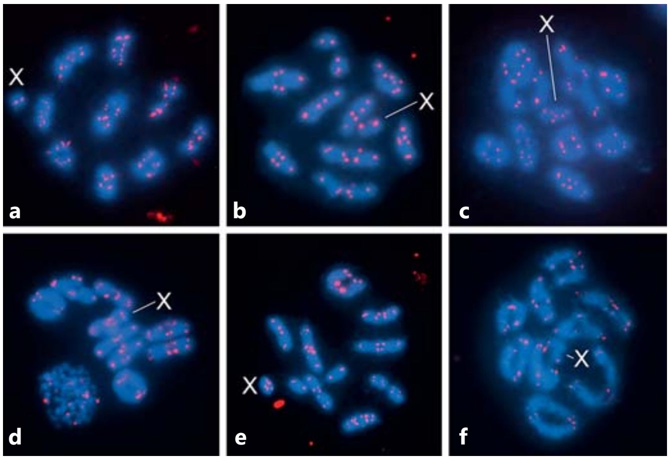

Fig. 3. FISH using the telomeric motif $(TTAGG)_n$ in meiotic chromosomes of 6 *Mahanarva* species. **a** *M. fimbriolata*; **b** *M. liturata*; **c** *M. quadripunctata*; **d** *M. spectabilis*; **e** *M. tristis*; **f** *M. vittata*. **a**, **b**, **d**, **e** Metaphase I; **c** diakinesis; **f** diplotene. The X chromosome is indicated in each cell. Bar = 5 μm.

100% ethanol for DNA extraction, following the protocol of Sambrook and Russell [2001], and for species identification. The testis follicles were macerated in a drop of 50% acetic acid, and the slides were dried using a hot plate at 40–45°C for chromosome preparation. The chromosomes were stained with 5% Giemsa, and fluorochrome staining with chromomycin A_3/distamycin A/DAPI (CMA_3/DA/DAPI) was performed as proposed by Schweizer et al. [1983].

The telomeric probe was obtained through PCR using the complementary primers $(TTAGG)_5$ and $(CCTAA)_5$ following the protocol of Ijdo et al. [1991]. Cot-DNA samples (DNA enriched for highly and moderately repetitive DNA sequences) were obtained according to the protocol described by Zwick et al. [1997] using the reannealing time of 25 min.

FISH was performed according to the protocol of Pinkel et al. [1986], with modifications described by Cabral-de-Mello et al. [2010]. The telomeric probe was labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany) through PCR, and Cot-DNA fractions were labeled with biotin-14-dATP through nick translation (Invitrogen, San Diego, Calif., USA). Probes labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin rhodamine (Roche), and probes labeled with biotin-14-dATP were detected using Streptavidin Alexa Fluor 488 (Invitrogen). All preparations were counterstained with DAPI and mounted in Vectashield (Vector, Burlingame, Calif., USA). Chromosomes and signals were observed using an Olympus microscope BX61 equipped with a fluorescence lamp and appropriate filters. Photographs were recorded with a DP70 cooled digital camera. The images were merged and optimized for brightness and contrast with Adobe Photoshop CS2.

For dot blot analysis, the biotin-14-dATP-labeled Cot-DNA fraction of *M. quadripunctata* was used as probe. Approximately 100 ng of genomic DNA from each of the 6 *Mahanarva* species was applied to a Hybond N+ nylon membrane (GE Healthcare) and denatured with 0.5 M NaOH for 2 min. The membranes were then washed in 5× SSC for 1 min. Next, the membrane was dried for 90 min at 80°C. The hybridization was carried out overnight (16 h) at 37°C using 200 ng of denatured labeled probe diluted in ECL gold hybridization buffer hybridization solution (GE Healthcare), with the addition of bovine serum albumin (0.05% w/v) and NaCl (2.5 M). After hybridization, the membrane washes were performed as follows: 5× SSC for 5 min at 42°C; 3 times in primary buffer containing 6 M urea, 0.4% SDS (w/v), and 0.1× SSC for 10 min at 42°C each; and 20× SSC for 5 min at room temperature. Finally, the biotin chromogenic detection kit (K0661, Thermo Scientific) was used, following the manufacturer's recommendations for detection.

Results

All 6 *Mahanarva* species showed 2n = 19, X0♂ (fig. 1), with holocentric chromosomes decreasing gradually in size (fig. 1g–l). This pattern made the accurate identification of each pair difficult for meiotic cells, except for the X chromosome, which can be observed as a univalent during meiosis (fig. 1a–f). Fluorochrome staining was homogeneous for DAPI in all species (results not shown), but distinct patterns were detected for CMA_3 (fig. 2): interstitial blocks in 1 autosomal bivalent of *M. fimbriolata*, *M. quadripunctata,* and *M. spectabilis* (fig. 2a, c, d); 2 bivalents with GC-rich blocks, 1 located interstitially and the other terminally, in *M. liturata* (fig. 2b); an interstitial block in 1 autosomal bivalent and in 1 terminus of the X

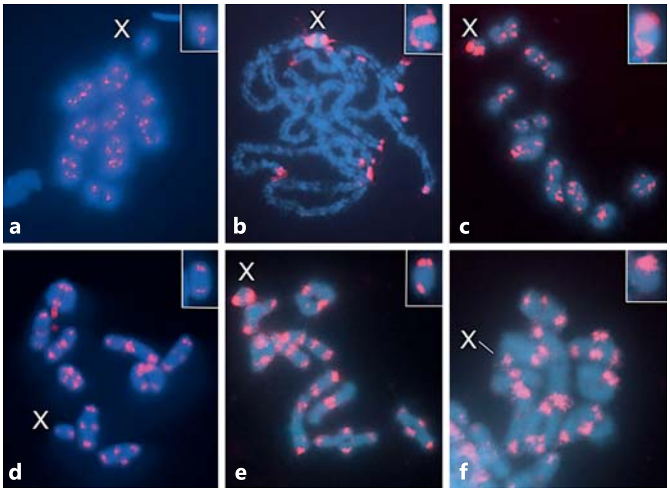

Fig. 4. FISH mapping of the Cot-DNA fraction obtained from each species (self-FISH) in meiotic cells. **a** *M. fimbriolata*; **b** *M. liturata*; **c** *M. quadripunctata*; **d** *M. spectabilis*; **e** *M. tristis*; **f** *M. vittata*. **a**, **c–f** Metaphase I; **b** pachytene. The X chromosome is indicated in each cell and is highlighted in the insets. Bar = 5 μm.

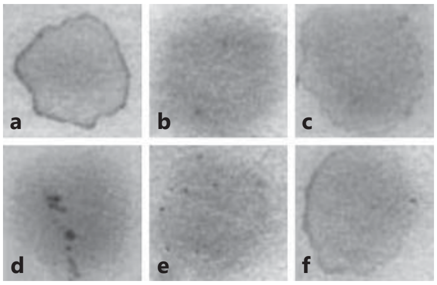

Fig. 5. Labeled Cot-DNA obtained from *M. quadripunctata* hybridized on a membrane with *Mahanarva* species unlabeled genomic DNAs. **a** *M. quadripunctata*; **b** *M. liturata*; **c** *M. spectabilis*; **d** *M. vittata*; **e** *M. tristis;* **f** *M. fimbriolata*.

chromosome in *M. vittata* (fig. 2f); and multiple sites, including 7 autosomal pairs and the X chromosome in *M. tristis*. For *M. tristis,* 2 autosomes presented signals in 1 terminus, 4 showed signals in both termini, 1 showed an interstitial signal, and the X harbored signals in both termini (fig. 2e).

The telomeric motif was restricted to the terminal regions of all chromosomes (fig. 3). These terminal regions were also strongly labeled with the Cot-DNA fraction obtained from each species, revealing stronger signals than those of the telomeric probes (fig. 4). A remarkable difference between the species for the location of Cot-DNA was the presence of only 1 terminal block in the X chromosome of *M. vittata* (fig. 4f), while the other species exhibited blocks in both X chromosome termini (fig. 4a–e). Finally, the dot blot using the Cot-DNA fraction obtained from *M. quadripunctata* as a probe revealed positive hybridization against the genomic DNA of the other *Mahanarva* species (fig. 5).

Discussion

Chromosomal studies in Cercopidae are scarce, and for *Mahanarva,* the karyotypes of *M. fimbriolata* and *M. posticata* were previously described [Marin-Morales et al., 2002]. Invariably, the 7 *Mahanarva* species studied until now share 2n = 19, X0♂, and this could represent the plesiomorphic character for the genus, which is also observed in other Cercopidae species, such as *D. flavopicta* [Castanhole et al., 2010], although other additional species should be karyotyped. Considering the ancestral diploid number for Cercopoidea, i.e., 2n = 26–28 [Kuznetsova and Aguin-Pombo, 2015], *Mahanarva* has a derived constitution that originated most parsimoniously through chromosomal fusions, leading to a reduction in 2n. Although extensive variability in diploid numbers has been reported in Auchenorrhyncha in high rank taxa such as tribes, families, and subfamilies [Kuznetsova and Aguin-Pombo, 2015], species within the same genus could present more stable karyotypes as noticed here for *Mahanarva*, and in *Cosmoscarta* [Dey, 1991] and *Alebra* [Kuznetsova et al., 2013].

The telomeric probe (TTAGG)$_n$ did not identify internal telomeric repeats that could be the result of chromosomal fusion suggested here for *Mahanarva*. The (TTAGG)$_n$ internal sequences could have been lost during chromosomal evolution, or may not have been identified due to low repeat numbers and classical FISH technique resolution limitations. The (TTAGG)$_n$ motif is canonical and considered ancestral for insects and arthropods as a whole [Sahara et al., 1999; Frydrychová et al., 2004], although it has been variably lost during the evolution of some orders, such as Diptera, Coleoptera, Hymenoptera, and Hemiptera [Sahara et al., 1999; Frydrychová et al., 2004; Gokhman et al., 2014]. Among hemipterans, the apomorphic heteropterans Cimicomorpha (families Miridae, Cimicidae, and Tingidae) and Pentatomomorpha (families Pyrrhocoridae and Pentatomidae) lost the (TTAGG)$_n$ ancestral motif [Frydrychová et al., 2004; Grozeva et al., 2011; Golub et al., 2015]. However, (TTAGG)$_n$ was not lost in other groups, including 4 aphid species [Monti et al., 2011], in the coccid *Planococcus li-*

lacinus [Mohan et al., 2011], in the suborder Coleorrhyncha [Kuznetsova et al., 2015], in the heteropteran infraorder Nepomorpha [Kuznetsova et al., 2012], and in Auchenorrhyncha representatives [Frydrychová et al., 2004; Maryańska-Nadachowska et al., 2012; Golub et al., 2014; Kuznetsova et al., 2015b]. For Auchenorrhyncha, $(TTAGG)_n$ is present in the families Aphrophoridae [Maryańska-Nadachowska et al., 2012], Cicadellidae [Kuznetsova et al., 2015b], Myerslopiidae [Golub et al., 2014], and Delphacidae [Frydrychová et al., 2004]. The data presented here for Cercopidae expand the knowledge of telomere types in this insect group, supporting the hypothesis that the ancestral arthropod telomere motif is conserved in Auchenorrhyncha.

The limited data for C-heterochromatin distribution in Auchenorrhyncha have shown accumulation of heterochromatin in one or both terminal/subterminal regions of the chromosomes [Kuznetsova et al., 2003, 2009, 2015b]. Here, the use of the anonymous Cot-DNA fraction that is enriched with highly repetitive DNA which is also abundant in heterochromatin, like satDNAs, revealed the accumulation of this genomic fraction mainly in terminal chromosome regions, a common location in *Mahanarva* as in other species of Auchenorrhyncha [Kuznetsova et al., 2009, 2015b] and as frequently reported in species with holocentric chromosomes [Spence et al., 1998; Bardella et al., 2014]. The observed difference in the size of Cot-DNA signals could suggest differential accumulation of repetitive DNAs between the species. Specifically, differences in the size of the X chromosome blocks were noticed, also indicating variability in the accumulation of repetitive DNAs. This pattern is more evident for *M. vittata*, in which the Cot-DNA hybridized in only 1 X terminus. The X chromosome of *M. vittata* could represent a variation related to the subgenus *Ipiranga* in which *M. vittata* is included, in comparison to the subgenus *Mahanarva* that includes the other species. These data also suggest that differential processes of expansion or contraction of repetitive DNAs act in the X chromosome of *Mahanarva* that led to its diversification among the 6 species.

Interestingly, the use of the Cot-DNA fraction from *M. quadripunctata* as a probe in dot blotting against genomic DNA from the 5 other *Mahanarva* species revealed conservation of at least part of the highly and moderately repetitive DNA among the species. It is known that highly repetitive DNAs, especially the satDNAs, are subject to the action of several molecular mechanisms causing variation and allowing rapid evolution, generating species-specific sequences [Dover, 1986; Charlesworth et al., 1994; López-Flores and Garrido-Ramos, 2012]. These molecular mechanisms could have less impact in the *Mahanarva* genomes, as evidenced by the conservation detected here, although we do not know the type of sequences conserved because the Cot-DNA is anonymous. Therefore, analysis of specific families of satDNAs should be undertaken. Among insects, conservation of a portion of the repetitive DNA pools was observed; for example, in terminal chromosomal regions of *Dichotomius* beetles [Cabral-de-Mello et al., 2011] through Cot-DNA interspecies FISH, and in Triatominae species using inter-specific GISH analysis [Pita et al., 2014].

Regarding repetitive DNA base pair composition, *Mahanarva* species appear to have a low specificity for AT or GC richness, except for *M. tristis*, with a predominance of GC blocks. Although few studies have addressed the heterochromatin base pair constitution in Auchenorrhyncha, the data show wide variation, evidencing heterogeneity for the constitutive heterochromatin composition of some species and homogeneity for others [Kuznetsova et al., 2003, 2009, 2015b]. The distinct patterns of GC-rich and neutral block distribution observed here reveal that the repetitive DNAs have diverged differently at the intragenomic level and between species, mainly between the species with 1 GC-rich block, i.e., *M. fimbriolata*, *M. quadripunctata*, and *M. spectabilis*, and the other species with 2 or multiple GC-rich blocks. However, some repetitive DNAs remained conserved, as demonstrated by Cot-DNA hybridization. Distinct families of repetitive DNAs differing in base pair composition could be compartmentalized in specific chromosomes among the 6 species, and are analyzed for 1 species of the genus using data from a sequenced genome [Anjos et al., in preparation]. A remarkable invariable feature among the 6 species is the presence of an interstitial GC-rich block in 1 medium-sized autosome pair, suggesting that this pair could be conserved, although other markers should be employed to confirm this statement. Finally, the data obtained from CMA_3 reinforce the differentiation of the X chromosome (that harbors a GC-rich block) of *M. vittata* in relation to the other species. This differentiation was also indicated by the results of Cot-DNA FISH, and could be a characteristic of the subgenus *Ipiranga*.

According to Melters et al. [2012], there are no consequences associated with chromosomal rearrangements arising from fission or fusion in holocentric chromosomes, because they can segregate perfectly after these rearrangements, encouraging rapid chromosomal evolution. However, in some other clades, the rate of karyotypic evolution is not increased [Gokhman and Kuznetsova, 2006; Panzera et al., 2012], as also observed here for *Mahanarva*. In contrast, there are some examples in which chromosomal diversity is noticed, such as *Myzus persicae* [Mandrioli et al., 2014] and Buthidae scorpions [Mattos et al., 2013]. Our data show that besides macrochromosomal stability, conservation for the pool of repetitive DNAs can be documented in *Mahanarva* species.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

Anjos A, Ruiz-Ruano FJ, Camacho JPM, Loreto V, Cabrero J, et al: U1 snDNA clusters in grasshoppers: chromosomal dynamics and genomic organization. Heredity 114:207–219 (2015).

Bardella VB, Rosa JA, Vanzela AL: Origin and distribution of AT-rich repetitive DNA families in *Triatoma infestans* (Heteroptera). Infect Genet Evol 23:106–114 (2014).

Bernardo ERA, Rocha VDF, Puga O: Espécies de cigarrinhas-das-pastagens (Hemiptera: Cercopidae) no meio-norte do Mato Grosso. Cienc Rural 33:369–371 (2003).

Boring AM: The chromosomes of the Cercopidae. Biol Bull 24:133–146 (1913).

Cabral-de-Mello DC, Moura RC, Martins C: 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. Heredity 104: 393–400 (2010).

Cabral-de-Mello DC, de Moura RC, de Souza Melo A, Martins C: Evolutionary dynamics of heterochromatin in the genome of *Dichotomius* beetles based on chromosomal analysis. Genetica 139:315–325 (2011).

Carvalho GS, Webb MD: Cercopid Spittle Bugs of the New World (Hemiptera, Auchenorrhyncha, Cercopidae). (Pensoft, Sofia 2005).

Castanhole MMU, Pereira LLV, de Souza HV, Valério JR, Barbosa LR, Itoyama MM: Meiotic chromosomes and nucleolar behavior in testicular cells of the grassland spittlebugs *Deois flavopicta*, *Mahanarva fimbriolata* and *Notozulia entreriana* (Hemiptera, Auchenorrhyncha). Genet Mol Biol 33:244–252 (2010).

Cavalier-Smith T: Skeletal DNA and the evolution of genome size. Annu Rev Biophys Bioeng 11:273–302 (1982).

Charlesworth B, Sniegowski P, Stephan W: The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371:215–220 (1994).

Cryan JR, Urban JM: Higher-level phylogeny of the insect order Hemiptera: is Auchenorrhyncha really paraphyletic? Syst Entomol 37: 7–21 (2012).

Dey SK: Chromosomes of five species of spittle bugs (Homoptera, Cercopidae). Cytologia 56: 523–526 (1991).

Dover GA: Molecular drive in multigene families: how biological novelties arise, spread and are assimilated. Trends Genet 2:159–165 (1986).

Elder JF, Turner BJ: Concerted evolution of repetitive DNA sequences in eukaryotes. Q Rev Biol 70:297–320 (1995).

Feschotte C, Pritham EJ: DNA transposons and the evolution of eukaryotic genomes. Annu Rev Genet 41:331–368 (2007).

Frydrychová R, Grossmann P, Trubac P, Vítková M, Marec F: Phylogenetic distribution of TTAGG telomeric repeats in insects. Genome 47:163–178 (2004).

Gokhman VE, Kuznetsova VG: Comparative insect karyology: current state and applications. Entomol Rev 86:352–368 (2006).

Gokhman VE, Anokhin BA, Kuznetsova VG: Distribution of 18S rDNA sites and absence of the canonical TTAGG insect telomeric repeat in parasitoid Hymenoptera. Genetica 142: 317–322 (2014).

Golub NV, Kuznetsova VG, Rakitov RA: First karyotype data on the family Myerslopiidae (Hemiptera, Auchenorrhyncha, Cicadomorpha). Comp Cytogenet 8:293–300 (2014).

Golub NV, Golub VB, Kuznetsova VG: Variability of 18S rDNA loci in four lace bug species (Hemiptera, Tingidae) with the same chromosome number. Comp Cytogenet 9:513–522 (2015).

Grozeva S, Kuznetsova VG, Anokhin BA: Karyotypes, male meiosis and comparative FISH mapping of 18S ribosomal DNA and telomeric $(TTAGG)_n$ repeat in eight species of true bugs (Hemiptera, Heteroptera). Comp Cytogenet 5:355–374 (2011).

Halkka O: Recombination in six homopterous families. Evolution 18:81–88 (1964).

Ijdo JW, Wells RA, Baldini A, Reeders ST: Improved telomere detection using a telomere repeat probe $(TTAGGG)_n$ generated by PCR. Nucleic Acids Res 19:4780 (1991).

Kidwell MG: Transposable elements and the evolution of genome size in eukaryotes. Genetica 115:49–63 (2002).

Kuznetsova V, Aguin-Pombo D: Comparative cytogenetics of Auchenorrhyncha (Hemiptera, Homoptera): a review. ZooKeys 538:63–93 (2015).

Kuznetsova VG, Maryanska-Nadachowska A, Nokkala S: A new approach to the Auchenorrhyncha (Hemiptera, Insecta) cytogenetics: chromosomes of the meadow spittlebug *Philaenus spumarius* (L.) examined using various chromosome banding techniques. Folia Biol 51:33–40 (2003).

Kuznetsova VG, Maryanska-Nadachowska A, Nokkala S: Karyotype characterization of planthopper species *Hysteropterum albaceticum* Dlabola, 1983 and *Agalmatium bilobum* (Fieber, 1877) (Homoptera: Auchenorrhyncha : Issidae) using AgNOR-, C- and DAPI/CMA_3-banding techniques. Comp Cytogenet 3:111–123 (2009).

Kuznetsova VG, Grozeva SM, Anokhin BA: The first finding of $(TTAGG)_n$ telomeric repeat in chromosomes of true bugs (Heteroptera, Belostomatidae). Comp Cytogenet 6:341–346 (2012).

Kuznetsova V, Golub N, Aguin-Pombo D: Karyotypes, B-chromosomes and meiotic abnormalities in 13 populations of *Alebra albostriella* and *A. wahlbergi* (Hemiptera, Auchenorrhyncha, Cicadellidae) from Greece. Comp Cytogenet 7:305–325 (2013).

Kuznetsova VG, Grozeva SM, Hartung V, Anokhin BA: First evidence for (TTAGG)$_n$ telomeric sequence and sex chromosome post-reduction in Coleorrhyncha (Insecta, Hemiptera). Comp Cytogenet 9:523–532 (2015a).

Kuznetsova VG, Maryanska-Nadachowska A, Anokhin B, Aguin-Pombo D: Evidence for TTAGG telomere repeats and rRNA gene clusters in leafhoppers of the genus *Alebra* (Hemiptera: Auchenorrhyncha: Cicadellidae). Eur J Entomol 112:207–214 (2015b).

López-Flores I, Garrido-Ramos MA: The repetitive DNA content of eukaryotic genomes. Genome Dyn 7:1–28 (2012).

Mandrioli M, Zanasi F, Manicardi GC: Karyotype rearrangements and telomere analysis in *Myzus persicae* (Hemiptera, Aphididae) strains collected on *Lavandula* sp. plants. Comp Cytogenet 8:259–274 (2014).

Marin-Morales MA, Zefa E, Bertagna M, Camargo Mathias MI, Arrigoni E: Chromosome analysis of two species of sugar cane pests of the genus *Mahanarva* (Homoptera, Cercopidae). Caryologia 55:357–360 (2002).

Maryańska-Nadachowska A, Kuznetsova VG, Lachowska D, Drosopoulos S: Mediterranean species of the spittlebug genus *Philaenus*: modes of chromosome evolution. J Insect Sci 12:54 (2012).

Mattos VF, Cella DM, Carvalho LS, Candido DM, Schneider MC: High chromosome variability and the presence of multivalent associations in buthid scorpions. Chromosome Res 21: 121–136 (2013).

Melters DP, Paliulis LV, Korf IF, Chan SWL: Holocentric chromosomes: convergent evolution, meiotic adaptations, and genomic analysis. Chromosome Res 20:579–593 (2012).

Mohan KN, Rani BS, Kulashrestha PS, Kadandale JS: Characterization of TTAGG telomeric repeats, their interstitial occurrence and constitutively active telomerase in the mealybug *Planococcus lilacinus* (Homoptera; Coccoidea). Chromosoma 120:165–175 (2011).

Monti V, Giusti M, Bizzaro D, Manicardi GC, Mandrioli M: Presence of a functional (TTAGG)$_n$ telomere-telomerase system in aphids. Chromosome Res 19:625–633 (2011).

Nguyen P, Sahara K, Yoshido A, Marec F: Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). Genetica 138:343–354 (2010).

Paladini A, Carvalho GS: Descrição de três novas espécies de *Mahanarva* (Hemiptera, Cercopidae, Ischnorhininae). Iheringia. Série Zool 97: 57–66 (2007).

Paladini A, Cryan JR: Nine new species of Neotropical spittlebugs (Hemiptera: Cercopidae: Ischnorhininae). Zootaxa 3519:53–68 (2012).

Palomeque T, Lorite P: Satellite DNA in insects: a review. Heredity 100:564–573 (2008).

Panzera Y, Pita S, Ferreiro MJ, Ferrandis I, Lages C, et al: High dynamics of rDNA cluster location in kissing bug holocentric chromosomes (Triatominae, Heteroptera). Cytogenet Genome Res 138:56–67 (2012).

Peck DC, Thompson V: Spittlebugs (Hemiptera: Cercopoidea), in Capinera JL (ed): Encyclopedia of Entomology, pp 3512–3516 (Springer, Dordrecht 2008).

Pinkel D, Straume T, Gray JW: Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83:2934–2938 (1986).

Pita S, Panzera F, Sánchez A, Panzera Y, Palomeque T, Lorite P: Distribution and evolution of repeated sequences in genomes of Triatominae (Hemiptera-Reduviidae) inferred from genomic in situ hybridization. PLoS One 9:e114298 (2014).

Sahara K, Marec F, Traut W: TTAGG telomeric repeats in chromosomes of some insects and other arthropods. Chromosome Res 7:449–460 (1999).

Sambrook JW, Russell D: Molecular Cloning: A Laboratory Manual, ed 3 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor 2001).

Schweizer D, Mendelak M, White MJD, Contreras N: Cytogenetics of the parthenogenetic grasshopper *Warramaba virgo* and its bisexual relatives. X. Patterns of fluorescent banding. Chromosoma 88:227–236 (1983).

Spence JM, Blackman RL, Testa JM, Ready PD: A 169-base pair tandem repeat DNA marker for subtelomeric heterochromatin and chromosomal rearrangements in aphids of the *Myzus persicae* group. Chromosome Res 6:167–175 (1998).

Vítková M, Král JJJ, Traut W, Zrzavý J, Marec F: The evolutionary origin of insect telomeric repeats, (TTAGG)$_n$. Chromosome Res 13: 145–156 (2005).

Zwick MS, Hanson RE, Islam-Faridi MN, Stelly DM, Wing RA, et al: A rapid procedure for the isolation of C_0t-1 DNA from plants. Genome 40:138–142 (1997).

U1 snDNA chromosomal mapping in ten spittlebug species (Cercopidade, Auchenorrhyncha, Hemiptera)

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Abstract

Spittlebugs, which belong to the Cercopidae family (Auchenorrhyncha, Hemiptera), form a large group of xylem-feeding insects that are best known for causing damage to plantations and pasture grasses. The holocentric chromosomes of these insects remain poorly studied regarding to the organization of different classes of repetitive DNA. To improve chromosomal maps based on repetitive DNAs and to better understand the chromosomal organization and evolutionary dynamics of multigene families in spittlebugs, we physically mapped U1 snRNA gene with fluorescence *in situ* hybridization (FISH) in ten Cercopidae species belonging to three different genera. All the U1 snDNA clusters were autosomal and located in interstitial position. In seven species, they were restricted to one autosome per haploid genome, while three *Mahanarva* species showed two clusters in two different autosomes. Although it was not possible to precisely define the ancestral location of this gene, it was possible to observe the presence of at least one cluster located in a small bivalent in all karyotypes. The karyotype stability observed in Cercopidae is also observed in respect to the distribution of U1 snDNA. Our data are discussed in light of possible mechanisms for
U1 snDNA conservation and compared with the available data from other species.

Keywords: Fluorescent *in situ* hybridization, holocentric chromosome, multigene family, repetitive DNA, U1 snDNA

Introduction

Cercopidae (Auchenorrhyncha), also known as spittlebugs, are Hemipteran insects that are well-known for causing losses in agricultural and pasture grasses by feeding on plant fluids (Bernardo et al. 2003, Peck and Thompson 2008). Auchenorrhyncha has holocentric chromosomes (without located centromeric activity) that have been primarily studied using standard chromosome staining to describe the diploid numbers and sex chromosomes, which have been revealed to be 2n ranging from 8 to 38 (females), and be predominantly XX♀/X0♂ (Boring 1907, Halkka 1959, Dey 1991, Marin-Morales et al. 2002, Castanhole et al. 2010, Kuznetsova and AguinPombo 2015, Anjos et al. 2016). Studies concerning repetitive DNA chromosomal organization, which could serve as useful markers for understanding karyotypic diversification, have only been performed in few Auchenorrhyncha species (MaryańskaNadachowska et al. 2013; Anjos et al. 2016). In Cercopidae, these studies are restricted to mapping of telomeric modal insect motifs (TTAGG)n and C_0t-DNA fractions in six species, all of which belong to the *Mahanarva* genus (Anjos *et al*. 2016).

Small nuclear RNAs (snRNAs) are a class of small RNA molecules that play an important role in processing messenger RNA through the splicing in eukaryotic cells (West 2012). The major spliceosome complex is formed by a protein set associated with U snRNAs, which are encoded by a multigene family that includes the U1, U2, U4, U5 and U6 snRNA genes. U1 snDNA is commonly found arranged in tandem (Zeller et al. 1984, Yu et al. 1991, Bretagne et al. 1991), and it has been used as a chromosomal marker to understand genome organization and chromosomal diversification (Anjos et al. 2015; Silva et al. 2015; García-Souto et al. 2015). Furthermore, chromosome mapping of U1 snDNA has been primarily performed in organisms with monocentric chromosomes, usually revealing conservation with regards to the location among related species and the presence of clusters in one or a few chromosomes (Lund et al. 1983; Lund and Nesbitt 1988; Pelliccia et al. 2001; Cabral-de-Mello et al. 2012; Anjos et al. 2015; Silva et al. 2015; García-Souto et al. 2015).

To improve the chromosomal maps based on repetitive DNAs in Cercopidae representatives and to better understand the chromosomal evolution, genome organization and evolutionary dynamics of repetitive sequences in their chromosomes, we mapped the U1 snDNA sequence in chromosomes from ten species with fluorescent *in situ* hybridization (FISH). The data were analyzed comparatively between species and indicated a highly conserved for position of U1 snDNA clusters, with a slight variation in the number of clusters.

Materials and methods

Adult males from ten Cercopidae species belonging to three genera were sampled from natural areas in Rio Claro, São Paulo State, Brazil, (*Deois flavopicta*, *Deois mourei*, *D. schach*, *Mahanarva fimbriolata*, *M. liturara*, *M. quadripunctata*, *M. spectabilis*, *M. tristis*, *M. vittata* and *Notozulia entreriana*). After anesthetizing, the animals were dissected and their testes removed, fixed in 3:1 absolute ethanol:acetic acid and stored at 20 °C until use. Entire animals were stored in 100% ethanol for species identification and DNA extraction, which followed the protocol of Sambrook and Russell (2001). The follicular testes were macerated in a drop of 50% acetic acid, and the slides were dried using a hot plate at 40–45 °C.

The U1 snDNA sequence was obtained through PCR using primers described by Cabral-de-Mello et al. (2012) from the genome of *M. quadripunctata*. To check the isolation of U1 sDNA PCR fragment was sequenced and submitted to a BLAST search. FISH procedures were performed according to Pinkel et al. (1986), with some modifications (Cabral-de-Mello et al. 2010), using a digoxigenin-11-dUTP (Roche, Mannheim, Germany) labeled PCR probe, which was detected through antidigoxigenin-rhodamine (Roche). The chromosomes were counterstained with 4,6diamidino-2-phenylindole (DAPI) and mounted using Vectashield (Vector, Burlingame, CA, USA). The chromosomes and signals were observed using an Olympus microscope BX61 equipped with a fluorescence lamp and appropriate filters. The photographs were recorded using a DP70 cooled digital camera in greyscale, and the images were merged and optimized for brightness and contrast using Adobe Photoshop CS2. For a better visualization for signals the chromosomes were pseudocolored in red while the signals were pseudocolored in green.

Results and Discussion

The analyzed species belonging to the *Mahanarva* and *Deois* genera showed karyotype similarities with presence of 2n=19 and X0 (Figures 1a-i), while *Notozulia entreriana* displayed 2n=15 and X0 (Figure 1j), which was previously reported by other authors (Marin-Morales et al. 2002, Castanhole et al. 2010, Anjos et al. 2016). The karyotypes of *Deois mourei* (Figure 1b) and *D. schach* (Figure 1c) are described here for the first time; they display the same characteristics that are frequently observed in Cercopidae species, including 2n=19 and X0 (Marin-Morales et al. 2002, Castanhole et al. 2010, Anjos et al. 2016). The chromosome numbers observed here are in contrast with the modal and ancestral number proposed for Cercopoidea, which is 2n=26-28 (Emeljanov and Kirillova 1992, Kuznetsova and Aguin-Pombo 2015), suggesting the presence of divergent karyotypes that could have originated though chromosomal fusions, which has been proposed by Anjos et al. (2016) for six *Mahanarva* species. In

N. entreriana, the presence of at least two chromosomal fusions are supported by the diploid number reduction to 2n=15 compared with the 2n=19 observed in other species. Moreover, the presence of two large chromosomes that are not observed in species with the 2n=19 reinforce the chromosomal fusions, which generated a bimodal karyotype.

U1 snDNA mapping revealed invariable interstitial clusters located in autosomes (Figure 1). Regarding the number of clusters per haploid genome, two main patterns were observed: (i) there was one cluster per haploid genome in seven species (Figures 1a-d, g, i, and j) or (ii) two clusters in *M. liturata* (Figure 1e), *M. quadripunctata* (Figure 1f) and *M. tristis* (Figure 1h). Studies involving the chromosomal mapping of U snRNA genes in organisms with holocentric chromosomes are scarce and before this study have only been performed once in Heteroptera. Contrasting with our results for chromosomal position, the physical mapping of U snDNA (U2) clusters in ten true bugs performed by Bardella et al. (2016) revealed one terminal cluster in one autosomal bivalent per genome, except in *Leptoglossus neovexillatus* and *Hypselonotus fulvus*, which showed two clusters located in distinct autosomal pairs. The U snDNA clusters distribution patterns observed here, restricted to one or a few chromosomes, has also been reported for species with monocentric chromosomes, such as orthopterans (Anjos et al. 2015; Palacios-Gimenez et al. 2015), fishes (Cabral-de-Mello et al. 2012, Silva et al. 2015), mice (Lund and Nesbitt 1988) and crustaceans (Barzotti et al. 2003). However, scattered U snDNA clusters have also been described (Úbeda-Manzanaro et al. 2010; Palacios-Gimenez et al. 2013; Anjos et al. 2015).

The presence of one cluster per haploid genome could be the modal pattern, which also seems to be the ancestral placement, at least for Tomaspidini tribe, as it is observed in three distinct genera belonging to different lineages (Paladini et al. 2015). The two clusters per haploid genome, which were observed in three *Mahanarva* species, is probably a derived condition caused by amplification followed by transposition or ectopic recombination, similar to what has been proposed for other repetitive sequences in insect chromosomes (Cabrero and Camacho 2008; Cabral-de-Mello et al. 2011; Anjos et al. 2015). According to Anjos et al. (2015), the association of U1 clusters with transposable elements in grasshopper genomes could have caused the dispersion, as observed in *Eyprepocnemis plorans*. The most frequent occurrence of one U1 snDNA cluster or a slight variation with a few chromosomes per genome bearing these genes is similar to the pattern reported for histone genes in other insects, such as grasshoppers (Cabrero et al. 2009), tortricid moths (Síchová et al. 2013) and true bugs (Bardella et al. 2016), in which histone sites are frequently interstitial or terminal in one autosomal bivalent. It has been proposed that purification at the chromosomal level preserves the histone cluster locations in grasshopper genomes (Cabrero et al. 2009) and it could also be occurring in the spittlebugs, which favor an interstitial location for U snRNAs genes and few clusters.

Due to the slight differences in the autosomes regarding size, it was not possible to precisely determine which chromosomes contained the U1 clusters, although they were located in one small bivalent in all species, which corresponded to pair 8 or 9 for species with 2n=19 chromosomes (Figure 1a-i), and pair 5 or 6 in *N. entreriana* (2n=15) (Figure 1j). The additional cluster in the three *Mahanarva* species was located on a large bivalent, which corresponds to pair 2 or 3 (Figures 1e, f, and h). This observation suggests conserved features for this repetitive DNA in the karyotypes of the studied species, similar to what was previously observed in *Mahanarva* species concerning a C_0t-DNA distribution (Anjos et al. 2016). The U1 snDNA cluster on a small autosome is likely the ancestral location of this gene, though there is occasional multiplication and dispersion for the large pair in some *Mahanarva* species. According to the phylogeny proposed for neotropical cercopids (Paladini et al. 2015), two of three *Mahanarva* with multiple clusters are sister species, suggesting an ancestral cluster dispersion for U1 snDNA, though this hypothesis needs to be tested with a larger taxon sampling.

Finally, the karyotypic stability observed in the cercopid species is also observed in respect to the distribution of the chromosomal markers, including U1 snDNA and C_0t-DNA (Anjos et al. 2016), which were maintained at a specific number and chromosomal position throughout the evolution of some species. Other species with divergent diploid numbers among Cercopidae should be studied to infer in more details the possible conservation U1 snDNA clusters. Our data contribute to knowledge on the chromosomal evolutionary history in Auchenorrhyncha. The use of chromosomal marker presented here that is limited because identify only few chromosomes, together with other chromosomal markers that can be obtained from repetitive DNAs will permit a broader picture regarding chromosomal evolutionary history in Cercopidae.

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References

Anjos, A., Rocha, G.C., Paladini, A., Mariguela, T.C., and Cabral-de-Mello, D.C. 2016. Karyotypes and repetitive DNA evolution in six species of the genus *Mahanarva*

(Auchenorrhyncha: Cercopidae). Cytogenet. Genome Res. **149**(4): 321–327. doi:10.1159/000450730.

Anjos, A., Ruiz-Ruano, F.J., Camacho, J.P.M., Loreto, V., Cabrero, J., de Souza, M.J., and Cabral-de-Mello, D.C. 2015. U1 snDNA clusters in grasshoppers: chromosomal dynamics and genomic organization. Heredity (Edinb). **114**(2): 207–219. doi:10.1038/hdy.2014.87.

Bardella, V.B., Fernandes, J.A.M., and Cabral-de-Mello, D.C. 2016. Chromosomal evolutionary dynamics of four multigene families in Coreidae and Pentatomidae (Heteroptera) true bugs. Mol. Genet. Genomics. **291**(5):1919-1925. doi:10.1007/s00438-016-1229-5.

Barzotti, R., Pellicia, F., and Rocchi, A. 2003. Identification and characterization of U1 small nuclear RNA genes from two crustacean isopod species. Chromosom. Res. **11**: 365–373.

Bernardo, E.R. de A., Rocha, V.D.F., and Puga, O. 2003. Espécies de cigarrinhas-daspastagens (Hemiptera : Cercopidae) no meio-norte do Mato Grosso. Cienc. Rural **33**(2): 369–371.

Boring, A.M. 1907. A study of the spermatogenesis of 22 species of the Membracidae, Jassidae, Cercopidae and Fulgoridae with special reference to the behavior of the odd chromosome. J. Exp. Zool. **4**(1): 469–514.

Bretagne, S., Robert, B., Vidaud, D., Goossens, M., and Houin, R. 1991. Structure of the *Echinococcus multilocularis* U1 snRNA gene repeat. Mol. Biochem. Parasitol. **46**(2): 285–292. Available from http://www.ncbi.nlm.nih.gov/pubmed/1840625.

Cabral-de-Mello, D.C., Cabrero, J., López-León, M.D., and Camacho, J.P.M. 2011. Evolutionary dynamics of 5S rDNA location in acridid grasshoppers and its relationship with H3 histone gene and 45S rDNA location. Genetica **139**: 921–931. doi:10.1007/s10709-011-9596-7.

Cabral-de-Mello, D.C., Moura, R.C., and Martins, C. 2010. 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. Heredity (Edinb). **104**(4): 393–400. doi:10.1038/hdy.2009.126.

Cabral-de-Mello, D.C., Valente, G.T., Nakajima, R.T., and Martins, C. 2012. Genomic organization and comparative chromosome mapping of the U1 snRNA gene in cichlid fish, with an emphasis in *Oreochromis niloticus*. Chromosom. Res. **20**(2): 279–292. doi:10.1007/s10577-011-9271-y.

Cabrero, J., and Camacho, J.P.M. 2008. Location and expression of ribosomal RNA genes in grasshoppers: Abundance of silent and cryptic loci. Chromosom. Res. **16**(4): 595–607. doi:10.1007/s10577-008-1214-x.

Cabrero, J., López-León, M.D., Teruel, M., and Camacho, J.P.M. 2009. Chromosome mapping of H3 and H4 histone gene clusters in 35 species of acridid grasshoppers. Chromosom. Res. **17**(3): 397–404. doi:10.1007/s10577-009-9030-5.

Castanhole, M.M.U., Pereira, L.L.V., Souza, H.V. de, Valério, J.R., Barbosa, L.R., and Itoyama, M.M. 2010. Meiotic chromosomes and nucleolar behavior in testicular cells of the grassland spittlebugs *Deois flavopicta*, *Mahanarva fimbriolata* and *Notozulia entreriana* (Hemiptera, Auchenorrhyncha). Genet. Mol. Biol. **33**(2): 244–252. doi:10.1590/S1415-47572010005000025.

Dey, S.K. 1991. Chromosomes of five species of spittle bugs (Homoptera, Cercopidae). Cytologia. **56**(4): 523–526. doi:ISSN:0011-4545.

Emeljanov, A.F., and Kirillova, V.I. 1992. Trends and types of karyotype evolution in Cicadina (Homoptera). II. Peculiarities and evolutionary changes of the karyotypes in the superfamilies Cercopoidea, Cicadoidea, Fulgoroidea and in the Cicadina as a whole. Entomol. Rev. **71**: 59–81.

García-Souto, D., Troncoso, T., Pérez, M., and Pasantes, J.J. 2015. Molecular Cytogenetic analysis of the european hake *Merluccius merluccius* (Merlucciidae, Gadiformes): U1 and U2 snRNA gene clusters map to the same location. PLoS One **10**(12): e0146150. doi:10.1371/journal.pone.0146150.

Halkka, O. 1959. Chromosome studies on the Hemiptera Homoptera Auchenorrhyncha. Ann. Acad. Sci. Fenn. **43**: 1–72.

Kuznetsova, V., and Aguin-Pombo, D. 2015. Comparative cytogenetics of Auchenorrhyncha (Hemiptera, Homoptera): a review. Zookeys **538**: 63–93. doi:10.3897/zookeys.538.6724.

Lund, E., Bostock, C., Robertson, M., Christie, S., Mitchen, J.L., and Dahlberg, J.E. 1983. U1 small nuclear RNA genes are located on human chromosome 1 and are expressed in mouse-human hybrid cells. Mol. Cell. Biol. **3**(12): 2211–2220. Available from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=370092&tool=pmcentrez&rendertype=abstract.

Lund, E., and Nesbitt, M.N. 1988. The embryonic and adult mouse U1 snRNA genes map to different chromosomal loci. Somat. Cell Mol. Genet. **14**(2): 143–148. Available from http://www.ncbi.nlm.nih.gov/pubmed/2894719.

Marin-Morales, M.A., Zefa, E., Bertagna, M., Camargo Mathias, M.I., and Arrigoni, E. 2002. Chromosome analysis of two species of sugar cane pests of the genus

Mahanarva (Homoptera, Cercopidae). Caryologia **55**(4): 357–360.

Maryańska-Nadachowska, A., Kuznetsova, V. G., Karamysheva, T. V. 2013. Chromosomal location of rDNA clusters and TTAGG telomeric repeats in eight species of the spittlebug genus *Philaenus* (Hemiptera: Auchenorrhyncha: Aphrophoridae). European Journal of Entomology **110**(3): 411-418.

Palacios-Gimenez, O.M., Carvalho, C.R., Ferrari Soares, F.A., and Cabral-De-Mello Cabral-de-mello, D.C. 2015. Contrasting the chromosomal organization of repetitive DNAs in two Gryllidae crickets with highly divergent karyotypes. PLoS One **10**(12): 1–18. doi:10.1371/journal.pone.0143540.

Palacios-Gimenez, O.M., Castillo, E.R., Martí, D. a, and Cabral-de-Mello, D.C. 2013. Tracking the evolution of sex chromosome systems in Melanoplinae grasshoppers through chromosomal mapping of repetitive DNA sequences. BMC Evol. Biol. **13**: 167. doi:10.1186/1471-2148-13-167.

Paladini, A., Takiya, D.M., Cavichioli, R.R., and Carvalho, G.S. 2015. Phylogeny and biogeography of Neotropical spittlebugs (Hemiptera: Cercopidae: Ischnorhininae): revised tribal classification based on morphological data. Syst. Entomol. **40**(1): 82–108. doi:10.1111/syen.12091.

Peck, D.C., and Thompson, V. 2008. Spittlebugs (Hemiptera: Cercopoidea). *In* Encyclopedia of Entomology. Springer Netherlands, Dordrecht. pp. 3512–3516. doi:10.1007/978-1-4020-6359-6_4341.

Pelliccia, F., Barzotti, R., Bucciarelli, E., and Rocchi, A. 2001. 5S ribosomal and U1 small nuclear RNA genes: A new linkage type in the genome of a crustacean that has three different tandemly repeated units containing 5S ribosomal DNA sequences. Genome **44**(3): 331–335. doi:10.1139/gen-44-3-331.

Pinkel, D., Straume, T., and Gray, J.W. 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc. Natl. Acad. Sci. U. S. A. **83**(9): 2934–8. doi:10.1073/pnas.83.9.2934.

Sambrook, J., and Russell, D.W. 2001. Molecular cloning: A laboratory manual. Cold Spring Harb. Lab. Press. Cold Spring Harb. NY: 999. Available from http://books.google.com/books?id=YTxKwWUiBeUC&printsec=frontcover%5Cn papers2://publication/uuid/BBBF5563-6091-40C6-8B14-06ACC3392EBB.

Síchová, J., Nguyen, P., Dalíková, M., and Marec, F. 2013. Chromosomal evolution in tortricid moths: conserved karyotypes with diverged features. PLoS One **8**(5): e64520. doi:10.1371/journal.pone.0064520.

Silva, D.M.Z.A., Utsunomia, R., Pansonato-Alves, J.C., Oliveira, C., and Foresti, F. 2015. Chromosomal mapping of repetitive DNA sequences in five species of *Astyanax* (Characiformes, Characidae) reveals independent location of U1 and U2 snRNA sites and sssociation of U1 snRNA and 5S rDNA. Cytogenet. Genome Res. **146**(2): 144–152. doi:10.1159/000438813.

Úbeda-Manzanaro, M., Merlo, M.A., Palazón, J.L., Cross, I., Sarasquete, C., and Rebordinos, L. 2010. Chromosomal mapping of the major and minor ribosomal genes, (GATA)n and U2 snRNA gene by double-colour FISH in species of the Batrachoididae family. Genetica **138**: 787–794. doi:10.1007/s10709-010-9460-1.

West, S. 2012. The increasing functional repertoire of U1 snRNA. Biochem. Soc. Trans. **40**(4): 846–849. doi:10.1042/BST20120058.

Yu, J.C., Wendelburg, B., Sakallah, S., and Marzluff, W.F. 1991. The U1 snRNA gene repeat from the sea urchin (*Strongylocentrotus purpuratus*): the 70 kilobase tandem repeat ends directly 3’ to a U1 gene. Nucleic Acids Res. **19**(5): 1093–1098.

Available from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=333786&tool=pmcentrez&rendertype=abstract.

Zeller, R., Carri, M., Mattaj, I.W., and Robertis, E.M. De. 1984. *Xenopus laevis* U1 snRNA genes: characterisation of transcriptionally active genes reveals major and minor repeated gene families. EMBO J. **3**(5): 1075–1081.

Figures

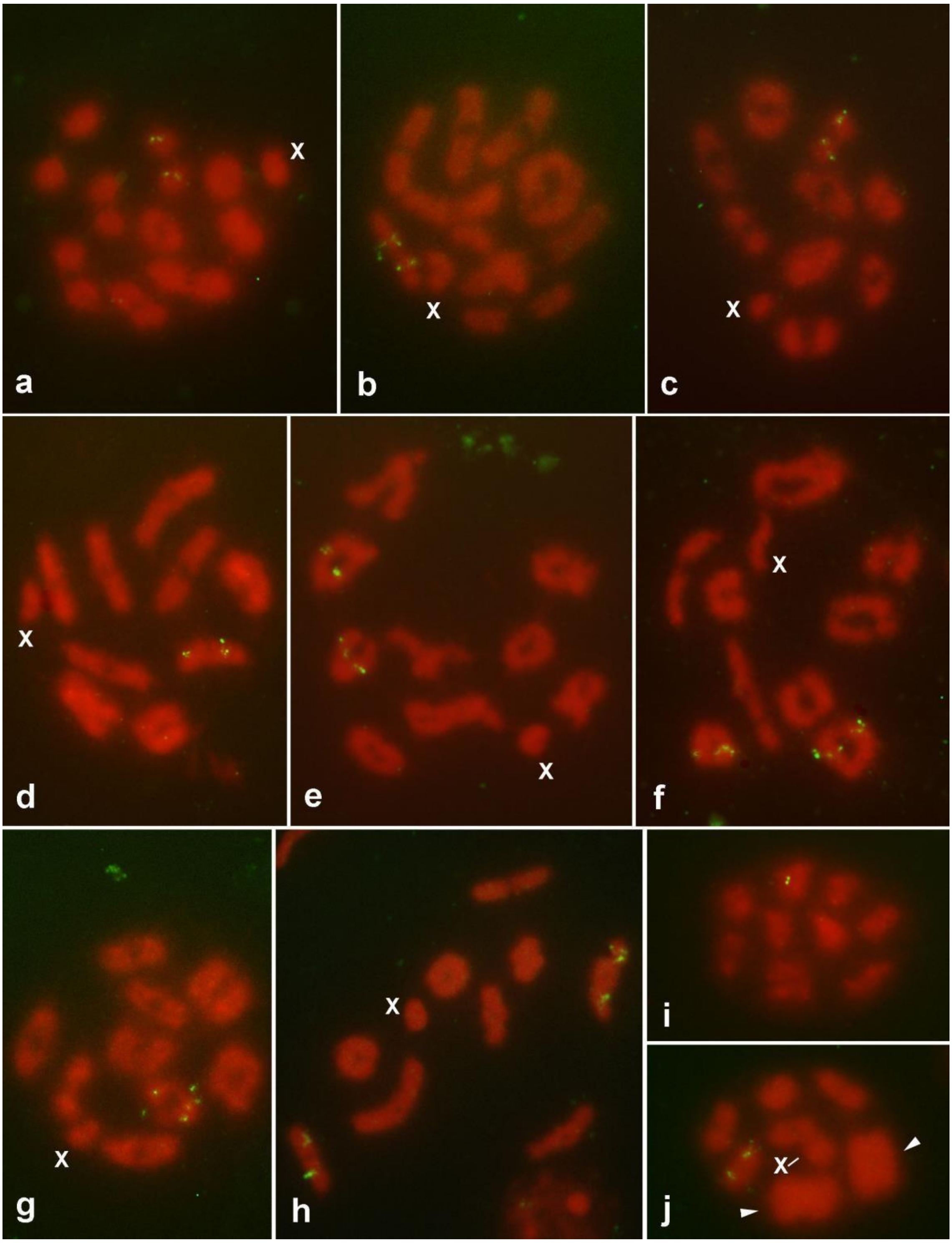

Figure 1. Fluorescent *in situ* hybridization of U1 snDNA as probe in ten species of Cercopidae. (a) *Deois flavopicta*, (b) *D. mourei*, (c) *D. schach*, (d) *Mahanarva fimbriolata*, (e) *M. liturata*, (f) *M. quadripunctata*, (g) *M. spectabillis*, (h) *M. tristis*, (i)

M. vittata (j) *Notozulia entreriana*. (a-h,j) metaphase I, (i) metaphase II. In all metaphases I the X chromosome is indicated; the arrowheads in (j) indicate the two largest chromosomes.

Physical mapping of two multigene families and base-pair richness in Cicadomorphan insects reveal conservative chromosomal organization

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Abstract

The infraorder Cicadomorpha (Hemiptera) is amongst the most diverse lineages of phytophagous insects. They have holocentric chromosomes that are highly variable in number in different families, although X0 is the prevalent sex determination mechanism. Here, we advance in the understanding of chromosomal mapping of repetitive DNAs in four families of Cicadomorpha encompassing the spittlebugs, leafhoppers and treehoppers (Aetalionidae, Cercopidae, Cicadellidae and Membracidae). Our data shows diploid number variability, which could have resulted from fusions between autosomes and between autosomes and ancient X, although some species shared the same karyotype. Instead of diploid number variability the occurrence of CMA_3^+ blocks were more conserved, primarily occurring in low numbers and placed in autosomes. 18S rDNA and H3 were present in one autosomal bivalent in most species. Based on the Cicadomorpha phylogeny and bearing in mind that these arrangements occur in some families, and also in sister groups for 18S rDNA, they could represent the ancestral for Cicadomorpha. The observed variation may be related to chromosomal rearrangement and transpositions in different few species. Our data corroborates the reported karyotypic variability, while conservatism is noticed for repetitive DNAs studied here in most species, revealing stability. On the other hand, the similar size of chromosomes prevents to ascertain if the elements harboring the distinct chromosomal markers are in fact homeologous. The data expands the poor knowledge concerning repetitive DNAs organization in Cicadomorpha.

Keywords: chromosomal evolution, Cicadomorpha, FISH, holocentric chromosome, multigene families

Introduction

The hemipteran infraorder Cicadomorpha is one of the most diverse lineages of phytophagous insects, and comprises approximately 30,000 described species distributed worldwide (Dietrich 2002; Cryan 2005). The species are grouped into the following four superfamilies: Cicadoidea (cicadas), Cercopoidea (froghoppers and spittlebugs), Membracoidea (leafhoppers and treehoppers), and Myerslopiioidea (ground-dwelling leafhoppers), which together with the Fulgoromorpha infraorder constitute the suborder Auchenorrhyncha (Cryan 2005; Cryan and Urban 2012). These insects are characterized by their specialized piercing-sucking mouthparts that enable them to feed on plant sap, a feeding habit that can inflict direct damage to crops and pasture or facilitate transmission of plant pathogens (Dietrich 2002; Dietrich 2005; Peck and Thompson 2008).

Cicadomorphans are cytogenetically characterized by holocentric chromosomes that lack the primary constriction (centromere). Despite their outstanding diversity and the economical significance of many species, the karyotypes of auchenorrhynchan insects are still poorly characterized with regard to chromosome banding and mapping of repetitive DNAs (Kuznetsova and Aguin-Pombo 2015). In Auchenorrhyncha, diploid numbers range from 2n=8 to 2n=38 in females, and the XX♀/X0♂ is the most predominant sex chromosome system (Boring 1907; Halkka 1964; Kuznetsova and Aguin-Pombo 2015). Earlier contributions have shown extensive variability in heterochromatin in auchenorrhynchans, including species from the same genus, such as differences in the amount of C-heterochromatin and its chromosomal location (Noda and Tatewaki 1990; Perepelov *et al*. 2002; Kuznetsova *et al*. 2003; Kuznetsova *et al*. 2009; Kuznetsova and Aguin-Pombo 2015), including related ones (Maryańska-Nadachowska *et al*. 2012). Species level differences in base pair richness with more occurrence of G+C-rich regions in comparison to A+T-rich chromosomal blocks were also noticed (Maryańska-Nadachowska *et al*. 2008; Kuznetsova *et al*. 2009; Kuznetsova *et al*. 2015b; Kuznetsova and Aguin-Pombo 2015; Anjos *et al*. 2016). Mapping of repetitive DNA sequences by fluorescent *in situ* hybridization (FISH) has been performed in only four studies in Auchenorrhyncha. The chromosomal location of the ribosomal 18S DNA and the presence of the telomeric repeats $(TTAGG)_n$ at chromosome ends has been described, although mapping of other repetitive DNAs is still not well surveyed (Maryańska-Nadachowska *et al*. 2013; Golub *et al*. 2014; Kuznetsova *et al*. 2015a; Anjos *et al*. 2016; Maryańska-Nadachowska *et al*. 2016).

Repetitive DNAs are good markers for tracking evolutionary chromosomal history and genome organization. In species with holocentric chromosomes, obtaining markers based on this types of sequence could improve the understanding of karyotype evolution (Pita *et al*. 2013; Nguyen and Carabajal Paladino 2016; Bardella *et al*. 2016). With the aim of understanding the dynamics of karyotypes and repetitive DNAs in Auchenorrhyncha we analyzed the chromosomes from 19 species belonging to four families (Aetalionidae, Cercopidae, Cicadellidae and Membracidae) through classical and fluorochrome staining and FISH for 18S rDNA and histone H3 probes. We comparatively discussed the data, trying to understand the putative ancestral patterns and causes for observed variation.

Material and Methods

Our taxonomic sampling encompasses 19 cicadomorphan representatives circunscribed to the following families (with number of species in parantheses): Aetalionidae (1), Cercopidae (10), Cicadellidae (2) and Membracidae (6). Male and female representatives of each species were collected in the municipality of Rio Claro, São Paulo State, southeastern Brazil (Table 1). Testis from anesthetized males were immersed in distilled water for five minutes, and subsequently fixed in Carnoy's solution (3:1, absolute ethanol: acetic acid). The whole body of studied exemplars (minus the abdomen) was stored in 100% ethanol for DNA extraction and species identification. The testis follicles were macerated in a drop of 50% acetic acid, and the slides were dried using a hot plate at 40-45°C. Chromosomes were stained with 5% Giemsa and fluorochrome staining (CMA_3/DA/DAPI) according to the procedure outlined in Schweizer *et al*. (1983).

Genomic DNA was extracted for one representative in each family (*Aetalion reticulatum*, *Mahanarva quadripunctata*, *Membracis foliatafasciata* and *Oncometopia facialis*), using the phenol-chloroform procedure as proposed by Sambrook and Russell (2001). The 18S rDNA and histone H3 were amplified through polymerase chain reaction (PCR) using primers available in the literature (Cabral-de-Mello *et al*. 2010; Cabrero *et al*. 2009). The 18S rDNA probe was labeled with biotin-14-dATP through nick translation (Invitrogen, San Diego, CA, USA), while the histone H3 probe was labeled through PCR with digoxigenin-11-dUTP (Roche, Mannheim, Germany). Two-color FISH assays were performed using each probe according to Pinkel *et al*. (1986), with modifications from Cabral-de-Mello *et al*. (2010). Probes labeled with biotin-14-dATP and digoxigenin-11-dUTP were detected using streptavidin-Alexa Fluor 488 (Invitrogen) and anti-digoxigenin-Rhodamine (Roche), respectively. All preparations were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield (Vector, Burlingame, CA, USA). Chromosomes and banding and FISH signals were observed using an Olympus BX61 epifluorescence microscope equipped with appropriate filters. Photographs were acquired with a DP70 cooled digital camera to produce images, which were stacked and edited for brightness and contrast in Adobe Photoshop CS2.

Results

Karyotypes

Sampled species showed karyotypes with diploid numbers ranging from 2n=13 (*Membracis foliatafasciata*) to 2n=21 (*Neotynelia pubescens*, *Bolbonota melaena* and *Horiola picta*), with slight variation in chromosome size. The X0 sex system was predominant (Table 1, Figure 1), except for *Amplyphallus exaltatus*, which showed a neo-XY sex system (Figure 1e). Membracidae and Cercopidae, the most densely sampled families, were the most variable with regard to number of chromosomes. In Cercopidae, 2n=19 was prevalent, whereas 2n=21 was predominant in Membracidae (Table 1). Amongst sampled species, 10 had their karyotypes described for the first time (Figure 1), as indicated in Table 1.

Fluorochrome staining

Fluorochrome staining assays revealed homogeneous DAPI staining in all sampled species (results not shown) and species-specific banding patterns for the CMA_3^+ staining (Table 1; Figure 2). Most species showed a single CMA_3^+ block per autosomal pair, which was interstitially or terminally located in some cercopids (Anjos *et al*. 2016; Table 1, Figures 2a-b). CMA_3^+ blocks, when present, were terminally located on autosomes in treehoppers and leafhoppers (Figure 2c-i). A few exceptions to this case were observed in *Amblyophallus exaltatus*, with one terminal CMA_3^+ block located on the neo-X (Figure 1i); and *Membracis foliatafasciata* (Table 1, Figure 2i), *Enchenopa* sp. (Table 1), and *Bolbonota melaena* (Table 1, Figure 2g), in which no CMA_3^+ blocks were visible. Three Cercopidae species previous studied revealed the same pattern described by Anjos *et al*. (2016) whit multiple CMA3+ sites occupying distinct chromosomal locations (Table 1).

FISH mapping of 18S rDNA and H3

Fluorescence *in situ* hybridization (FISH) assays revealed clusters of 18S rDNA and H3 on a single autosomal bivalent in most species (Table 1, Figure 3), except for *Mahanarva vittata* and *Amblyophallus exaltatus*, in which 18S rDNA clusters were located on sex chromosomes (Figures 3c-h). *Membracis foliatafasciata* showed multiple 18S rDNA clusters in almost all chromosomes (Figure 3l). Regarding the H3 histone gene, A*etalion reticulatum* was the only species with more than one cluster, displaying four clusters located on two autosomal bivalents (Figure 3e). No specific pattern was observed with regard to the position of these clusters, which were observed in both interstitial and terminal locations (Table 1 and Figure 3). Two-color FISH assays performed for 18S rDNA and H3 in 18 species have identified these clusters in distinct chromosomes (Figure 3).

Discussion

The putative ancestral diploid numbers to Cercopoidea is 2n=26-28 and to Membracoidea is 2n=22 (Kuznetsova and Aguin-Pombo 2015). Representatives of Cercopoidea studied here showed lower chromosome numbers compared with the ancestral placement; a similar situation was observed in a few representatives of Membracoidea. The lower chromosome number observed in several species could have resulted from chromosomal fusions that occurred over the course of repeated chromosome diversification events. The reduced diploid numbers, bimodal karyotypes and large chromosomes in distantly related cicadomorphan species, such as *Membracis* *foliatafasciata* (2n=13) and *Notozulia entreriana* (2n=15), reinforces the hypothesis of independent chromosomal fusions. For instance, the origin of neo-XY sex systems (observed in *Amblyophallus exaltatus*) could also be explained based on a fusion of the original X with an autosome. Despite a distinct variation in diploid numbers across Membracoidea and Cecopoidea species (ranging from 2n=13 to 2n=22 in males), the X0 system was found in most species, which is in fact a widespread sex determination mechanism in Auchenorrhyncha insects (Blackman 1995, Kuznetsova and Aguin-Pombo 2015). The observed variation in Membracoidea and Cercopoidea karyotypes could have been favored by the presence of holocentric chromosomes, which are in theory known to encouraging rapid karyotype evolution without compromising chromosome inheritance, because chromosome fragments retain centrometic activity (Melters *et al*. 2012).

Instead of diploid number variability noticed the bivalent carrying a CMA_3^+ block tend to be more evolutionarily conserved, besides the low occurrence of A+T or G+C rich blocks. Low specificity in base pair richness appears to be common in Auchenorrhyncha, with most species presenting no base pair richness or a few A+T or G+C-rich regions (Maryańska-Nadachowska *et al*. 2012, Anjos et al. 2016). For example, a few autosomal CMA_3^+ blocks were previously recorded in two species of spittlebugs, *Philaenus spumarius* and *Ph. tesselatus* (Maryańska-Nadachowska *et al*. 2012), like in some *Mahanarva* (Anjos et al. 2016) and most species studied here. Although, as well as in *Mahanarva tristis*, some species also showed multiple blocks enriched for A+T or G+C base pairs. *Agalmatium bilobum* and *Hysteropterum albaceticum* presented A+T blocks spread to the chromosome (Kuznetsova et al. 2009). The occurrence of G+C rich blocks placed on autosomes is the modal condition, differing in specific position between Membracoidea and some species of Cercopidae.

The divergent placements could be explained by (i) chromosomal rearrangements, like the putative fusion responsible for neo-XY origin in *Amblyophallus exaltatus* and previously reported in *Ph. Italosignum* (Maryańska-Nadachowska *et al*. 2012); and by (ii) spreading of G+C repetitive DNAs in species with multiple CMA_3^+ blocks, such as in some species of *Mahanarva*. Variation for base pair richness was also previously observed in other hemipteran insects. CMA_3^+ restricted to the X chromosome and scattered $DAPI^+$ blocks are common in aphids (Bizzaro *et al*. 1999; Criniti *et al*. 2005; Marco *et al*. 2009; Mandrioli *et al*. 2011), while several patterns have been described in heteropterans, ranging from absence of blocks to blocks distributed throughout some chromosomes (Bardella *et al*. 2014a; Bardella *et al*. 2014b).

One autosomal 18S rDNA loci per haploid genome are conserved, suggesting a putative ancestral placement in Cicadomorpha. Besides some species studied here that belongs to four distinct families, i.e. Membracidae, Cercopidae, Athelionidae and Cicadellidae, similar rDNA position were described in other species belonging to Cicacellidae, *Alebra* species (Kuznetsova *et al*. 2015b) and in Aphrophoridae representatives (Maryańska-Nadachowska et al. 2013). The putative ancestral location for 18S rDNA in Cicadomorpha is supported (i) by its common occurrence in non related families, including families with a basal placement in Cicadomorpha phylogenetic hypothesis (Cryan and Urban 2012), i.e. Myerslopiidae (Golub et al. 2014), and (ii) its similar distribution in sister groups, like Issidae (Fulgoroidea) representatives (Maryańska-Nadachowska *et al*. 2016).

On the contrary to one autosomal loci for 18S rDNA, multiple loci dispersed on different chromosomes, as observed in *Membracis foliatafasciata*, are rare in Cicadomorpha. Although, it has been reported in other insects (Cabrero and Camacho 2008; Cabral-de-Mello et al. 2012), including holocentric species, like assassin bugs

(Reduviidae; Panzera *et al*. 2012) and moth and butterflies (Nguyen *et al*. 2010). This divergent pattern could be resultant from amplification followed by spreading as has been proposed for some repetitive sequences in insect chromosomes (Cabrero and Camacho 2008; Cabral-de-Mello *et al*. 2011; Anjos *et al*. 2015). Besides these mechanism putatively transposition and chromosomal rearrangements are involved with allocation of 18S rDNA to sex chromosomes in *Mahanarva tristis* and *Amblyophallus exaltatus*, respectively. Sex chromosomes bearing 18S rDNA genes as consequence of chromosomal fusions were also reported for several *Philaenus* species (Maryańska-Nadachowska *et al*. 2013). Movement of 18S rDNA in representatives of the families studied here is also supported also by occurrence of interstitial and terminal loci.

This is the first time that the histone gene is mapped in Cicadomorpha representatives. Like 18S rDNA, H3 histone gene loci was conserved in number and autosomal location, although varying in interstitial or terminal position. The recurrent placement in autosomes in distinct families suggests that this is a putatively ancestral condition, although we take care with this hypothesis because sister groups should be studied. Similar conservatism was noticed in other few insects with holocentric chromosomes, like in Coreidae and Pentatomidae bugs (Bardella et al. 2016), aphids (Mandrioli and Manicardi 2013) and Tortricid moths (Šíchová et al. 2013). Insects with monocentric chromosomes also showed conservation for histone loci (Cabrero *et al*. 2009; Teruel *et al*. 2010; Cabral-De-Mello *et al*. 2011; Cabral-de-Mello *et al*. 2011b), and according to Cabrero *et al*. (2009), these may be undergoing a strong purifying selection for locations at the chromosomal level in Acrididae grasshoppers. The variability for H3 loci specific chromosomal position, i.e., interstitial or terminal, including in the related *Mahanarva* species, could be consequence of chromosomal inversions or transpositions.

In conclusion our study reinforces the chromosomal variability in Cicadomorpha insects with regard to their diploid numbers (Kuznetsova and Aguin-Pombo 2015). On the other hand, conservatism is noticed comparing most species for the chromosomal markers based in repetitive DNAs studied here, revealing stability. Conservatism was also reported for the pool of repetitive DNAs (C0t-DNA) and U1 snDNA loci in Cercopidae representatives (Anjos et al. 2015, 2017). Although some repetitive sequences are conserved in number and loci position in karyotypes of Cicadomorpha it still challenging the assessing of specific chromosome homologies due to the similar size of chromosomes, that allow most precise establishment of chromosomal evolutionary history. The data expands the poorly knowledge concerning repetitive DNAs organization in Cicadomorpha and the increase in number of species studied will allow confirming patterns observed here.

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References

Anjos A, Rocha GC, Paladini A, et al (2016) Karyotypes and Repetitive DNA Evolution in Six Species of the Genus Mahanarva (Auchenorrhyncha: Cercopidae). Cytogenet Genome Res 149:321–327. doi: 10.1159/000450730

Anjos A, Ruiz-Ruano FJ, Camacho JPM, et al (2015) U1 snDNA clusters in grasshoppers: chromosomal dynamics and genomic organization. Heredity (Edinb) 114:207–219. doi: 10.1038/hdy.2014.87

Bardella VB, da Rosa JA, Vanzela ALL (2014a) Origin and distribution of AT-rich repetitive DNA families in Triatoma infestans (Heteroptera). Infect Genet Evol 23:106–114. doi: 10.1016/j.meegid.2014.01.035

Bardella VB, Fernandes JAM, Cabral-de-Mello DC (2016) Chromosomal evolutionary dynamics of four multigene families in Coreidae and Pentatomidae (Heteroptera) true bugs. Mol Genet Genomics. doi: 10.1007/s00438-016-1229-5

Bardella VB, Grazia J, Fernandes JAM, Vanzela ALL (2014b) High diversity in CMA3/DAPI-banding patterns in Heteropterans. Cytogenet Genome Res 142:46–53. doi: 10.1159/000355214

Bizzaro D, Barbolini E, Mandrioli M (1999) Cytogenetic characterization of the holocentric chromosomes in the aphids Myzus varians and Myzus cerasi. Caryologia 52:81–85.

Bizzaro D, Mandrioli M, Zanotti M, et al (2000) Chromosome analysis and molecular characterization of highly repeated DNAs in the aphid Acyrthosiphon pisum (Aphididae, Hemiptera). Genetica 108:197–202. doi: 10.1023/A:1004128314728

Blackman RL (1995) Sex deterination in insects. In: Leather SR, Hardie J (eds) Insect Reproduction. CrC press, Taylor & Francis, Florida, pp 54–97

Boring AM (1907) A study of the spermatogenesis of 22 species of the Membracidae, Jassidae, Cercopidae and Fulgoridae with special reference to the behavior of the odd chromosome. J Exp Zool 4:469–514.

Cabral-de-Mello DC, Cabrero J, López-León MD, Camacho JPM (2011a) Evolutionary dynamics of 5S rDNA location in acridid grasshoppers and its relationship with H3 histone gene and 45S rDNA location. Genetica 139:921–931. doi: 10.1007/s10709-011-9596-7

Cabral-de-Mello DC, de Moura RDC, de Souza Melo A, Martins C (2011b) Evolutionary dynamics of heterochromatin in the genome of Dichotomius beetles based on chromosomal analysis. Genetica 139:315–25. doi: 10.1007/s10709-011-9551-7

Cabral-De-Mello DC, Martins C, Souza MJ, Moura RC (2011) 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. Cytogenet Genome Res 132:89–93. doi: 10.1159/000317476

Cabral-de-Mello DC, Moura RC, Martins C (2010) 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. Heredity (Edinb) 104:393–400. doi: 10.1038/hdy.2009.126

Cabral-de-Mello DC, Moura RC, Martins C (2011c) Cytogenetic mapping of rRNAs and histone H3 genes in 14 species of Dichotomius (Coleoptera, Scarabaeidae, Scarabaeinae) beetles. Cytogenet Genome Res 134:127–35. doi: 10.1159/000326803

Cabrero J, Camacho JPM (2008) Location and expression of ribosomal RNA genes in grasshoppers: Abundance of silent and cryptic loci. Chromosom Res 16:595–607. doi: 10.1007/s10577-008-1214-x

Cabrero J, López-León MD, Teruel M, Camacho JPM (2009) Chromosome mapping of H3 and H4 histone gene clusters in 35 species of acridid grasshoppers. Chromosom Res 17:397–404. doi: 10.1007/s10577-009-9030-5

Criniti A, Simonazzi G, Cassanelli S, et al (2005) X-linked heterochromatin distribution in the holocentric chromosomes of the green apple aphid Aphis pomi. Genetica 124:93–8. doi: 10.1007/s10709-004-8154-y

Cryan JR (2005) Molecular phylogeny of Cicadomorpha (Insecta: Hemiptera: Cicadoidea, Cercopoidea and Membracoidea): adding evidence to the controversy. Syst Entomol 30:563–574. doi: 10.1111/j.1365-3113.2004.00285.x

Cryan JR, Urban JM (2012) Higher-level phylogeny of the insect order Hemiptera: Is Auchenorrhyncha really paraphyletic? Syst Entomol 37:7–21. doi: 10.1111/j.1365-3113.2011.00611.x

da Silva CRM, Quintas CC, Vanzela ALL (2010) Distribution of 45S and 5S rDNA sites in 23 species of Eleocharis (Cyperaceae). Genetica 138:951–957. doi: 10.1007/s10709-010-9477-5

Dietrich CH (2005) Keys To the Families of Cicadomorpha and Subfamilies and Tribes of Cicadellidae (Hemiptera: Auchenorrhyncha). Florida Entomol 88:502–517. doi: 10.1653/0015-4040(2005)88[502:KTTFOC]2.0.CO;2

Dietrich CH (2002) Evolution of Cicadomorpha (Insecta, Hemiptera). Denisia 176:155–170.

Golub N, Kuznetsova V, Rakitov R (2014) First karyotype data on the family Myerslopiidae (Hemiptera, Auchenorrhyncha, Cicadomorpha). Comp Cytogenet

8:293–300. doi: 10.3897/CompCytogen.v8i4.8813

Grozeva S, Anokhin BA, Kuznetsova VG (2014) Bedbugs (Hemiptera). In: Protocols for cytogenetic mapping of arthropod genomes. CrC press, Taylor & Francis, pp 285–326

Halkka O (1964) Recombination in Six Homopterous Families. Evolution (N Y) 18:81. doi: 10.2307/2406421

Heckmann S, Schroeder-Reiter E, Kumke K, et al (2011) Holocentric chromosomes of luzula elegans are characterized by a longitudinal centromere groove, chromosome bending, and a terminal nucleolus organizer region. Cytogenet Genome Res 134:220–228. doi: 10.1159/000327713

Kuznetsova V, Aguin-Pombo D (2015) Comparative cytogenetics of Auchenorrhyncha (Hemiptera, Homoptera): a review. Zookeys 538:63–93. doi: 10.3897/zookeys.538.6724

Kuznetsova VG, Grozeva SM, Hartung V, Anokhin BA (2015a) First evidence for (TTAGG)n telomeric sequence and sex chromosome post-reduction in Coleorrhyncha (Insecta, Hemiptera). Comp Cytogenet 9:523–532. doi: 10.3897/CompCytogen.v9i4.5609

Kuznetsova VG, Maryańska-Nadachowska A, Anokhin BA, Aguin-Pombo D (2015b) Evidence for TTAGG telomere repeats and rRNA gene clusters in leafhoppers of the genus Alebra (Hemiptera: Auchenorrhyncha: Cicadellidae). Eur J Entomol 112:207–214. doi: 10.14411/eje.2015.045

Kuznetsova VG, Maryanska-Nadachowska A, Nokkala S (2003) A new approach to the auchenorrhyncha (Hemiptera, Insecta) cytogenetics: chromosomes of the meadow spittlebug Philaenus spumarius (L.) examined using various chromosome banding techniques. Folia Biol 51:33–40.

Kuznetsova VG, Maryanska-Ndachowska A, Nokkala S (2009) Karyotype characterization of planthopper species Hysteropterum albaceticum Dlabola , 1983 and Agalmatium bilobum (Fieber , 1877) (Homoptera: Auchenorrhyncha : Issidae) using AgNOR. C and DAPI/CMA3 banding techniques. Comp Cytogenet 3:111–123. doi: 10.3897/compcytogen.v3i2.18

Mandrioli M, Azzoni P, Lombardo G, Manicardi GC (2011) Composition and epigenetic markers of heterochromatin in the aphid Aphis nerii (Hemiptera: Aphididae). Cytogenet Genome Res 133:67–77. doi: 10.1159/000323510

Mandrioli M, Manicardi GC (2013) Chromosomal mapping reveals a dynamic organization of the histone genes in aphids (Hemiptera: Aphididae). Entomologia 1:e2. doi: 10.4081/entomologia.2013.e2

Marco R, Cassanelli S, Mazzoni E, et al (2009) Heterochromatin and rDNA localization on the holocentric chromosomes of black bean aphid, Aphis fabae Scop . (Hemiptera: Aphididae). Caryologia 62:341–346. doi: 10.1080/00087114.2004.10589700

Maryańska-Nadachowska A, Anokhin BA, Gnezdilov VM, Kuznetsova VG (2016) Karyotype stability in the family Issidae (Hemiptera, Auchenorrhyncha) revealed by chromosome techniques and FISH with telomeric (TTAGG)n and 18S rDNA probes. Comp Cytogenet 10:347–369. doi: 10.3897/CompCytogen.v10i3.9672

Maryańska-Nadachowska A, Kuznetsova VG, Abdul-Nour H (2008) A chromosomal study on a Lebanese spittlebug Philaenus arslani (Hemiptera: Auchenorrhyncha: Aphrophoridae). Eur J Entomol 105:205–210. doi: 10.14411/eje.2008.029

Maryańska-Nadachowska A, Kuznetsova VG, Karamysheva T V. (2013)

Chromosomal location of rDNA clusters and TTAGG telomeric repeats in eight species of the spittlebug genus Philaenus (Hemiptera: Auchenorrhyncha: Aphrophoridae). Eur J Entomol 110:411–418. doi: 10.14411/eje.2013.055

Maryańska-Nadachowska A, Kuznetsova VG, Lachowska D, Drosopoulos S (2012) Mediterranean species of the spittlebug genus Philaenus: modes of chromosome evolution. J Insect Sci 12:54. doi: 10.1673/031.012.5401

Melters DP, Paliulis L V, Korf IF, Chan SWL (2012) Holocentric chromosomes: convergent evolution, meiotic adaptations, and genomic analysis. Chromosom Res 20:579–93. doi: 10.1007/s10577-012-9292-1

Nguyen P, Carabajal Paladino L (2016) On the Neo-Sex Chromosomes of Lepidoptera. In: Pontarotti P (ed) Evolutionary Biology: Convergent Evolution, Evolution of Complex Traits, Concepts and Methods. Springer International Publishing, Cham, pp 171–185

Nguyen P, Sahara K, Yoshido A, Marec F (2010) Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). Genetica 138:343–354. doi: 10.1007/s10709-009-9424-5

Noda H, Tatewaki R (1990) Re-examination of Chromosomes of Three Species of Rice Planthoppers(Homoptera : Delphacidae). Appl Entomol Zool 25:538–540. doi: 10.1303/aez.25.538

Panzera Y, Pita S, Ferreiro MJ, et al (2012) High dynamics of rDNA cluster location in kissing bug holocentric chromosomes (Triatominae, Heteroptera). Cytogenet Genome Res 138:56–67. doi: 10.1159/000341888

Peck DC, Thompson V (2008) Spittlebugs (Hemiptera: Cercopoidea). In: Encyclopedia of Entomology. Springer Netherlands, Dordrecht, pp 3512–3516

Perepelov E, Bugrov AG, Maryańska-Nadachowska A (2002) Constitutive heterochromatin in karyotypes of two cicadidae species from Japan (Cicadoidea, Hemiptera). Folia Biol (Praha) 50:217–219.

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci U S A 83:2934–8. doi: 10.1073/pnas.83.9.2934

Pita S, Panzera F, Ferrandis I, et al (2013) Chromosomal divergence and evolutionary inferences in Rhodniini based on the chromosomal location of ribosomal genes. Mem Inst Oswaldo Cruz 108:376–382. doi: 10.1590/0074-0276108032013017

Roa F, Guerra M (2012) Distribution of 45S rDNA sites in chromosomes of plants: structural and evolutionary implications. BMC Evol Biol 12:225. doi: 10.1186/1471-2148-12-225

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harb Lab Press Cold Spring Harb NY 999.

Schweizer D, Mendelak M, White MJD, Contreras N (1983) Cytogenetics of the parthenogenetic grasshopper Warramaba virgo and its bisexual relatives. Chromosoma 88:227–236. doi: 10.1007/BF00285625

Síchová J, Nguyen P, Dalíková M, Marec F (2013) Chromosomal evolution in tortricid moths: conserved karyotypes with diverged features. PLoS One 8:e64520. doi: 10.1371/journal.pone.0064520

Teruel M, Cabrero J, Perfectti F, Camacho JPM (2010) B chromosome ancestry revealed by histone genes in the migratory locust. Chromosoma 119:217–225. doi: 10.1007/s00412-009-0251-3

Vanzela ALL, Cuadrado A, Jouve N, et al (1998) Multiple locations of the rDNA sites in holocentric chromosomes of Rhynchospora (Cyperaceae). Chromosom Res 6:345–349. doi: 10.1023/A:1009279912631

Table 1. Chromosome number, occurrence and distribution of CMA$_3$/DAPI bands and location of multigene families in diploid genomes of different species belonging to the infraorder Cicadomorpha (Auchenorrhyncha).

Family **Species**	**Karyotype (2n)**	**CMA$_3^+$ banding**	**FISH**		**Reference**
			45S	**H3 Histone**	
Cercopoidea					
Cercopidae					
Deois flavopicta (Stål, 1854)	2n=19,X0	2tA	2iA	2iA	This paper
*Deois mourei** Cavichioli & Sakakibara, 1993	2n=19,X0	2tA	2iA	2iA	This paper
*Deois schach** (Fabricius, 1787)	2n=19,X0	2tA	2iA	2iA	This paper
Mahanarva fimbriolata (Stål, 1854)	2n=19,X0	2iA	2iA	2iA	This paper, Anjos et al. 2016
Mahanarva liturata (Le Peletier & Serville, 1825)	2n=19,X0	2tA,2iA	2iA	2iA	This paper, Anjos et al. 2016
Mahanarva quadripunctata (Walker, 1858)	2n=19,X0	2iA	2iA	2iA	This paper, Anjos et al. 2016
Mahanarva spectabilis (Distant, 1909)	2n=19,X0	2iA	2iA	2iA	This paper, Anjos et al. 2016
Mahanarva tristis (Fabricius, 1803)	2n=19,X0	7tA,1tX	2iA	2iA	This paper, Anjos et al. 2016
Mahanarva vittata (Walker, 1851)	2n=19,X0	2iA,1tX	1tX	2tA	This paper, Anjos et al. 2016
Notozulia entreriana (Berg, 1879)	2n=15,X0	2iA	2tA	2tA	This paper
Membracoidea					
Aetalionidae					
Aetalion reticulatum (Linneaeus, 1758)	2n=21, X0	2tA	2tA	4tA	This paper
Cicadellidae					
*Ferrariana trivittata** (Signoret, 1854)	2n=19,X0	2tA	2tA	2iA	This paper
*Oncometopia facialis** (Signoret, 1854)	2n=17,X0	2tA	2tA	2iA	This paper
Membracidae					
*Amblyophallus exaltatus** (Fabricius, 1803)	2n=20,neo-XY	1tX	2tXY	2tA	This paper
*Bolbonota melaena** (Germar, 1835)	2n=21,X0	absent	2iA	2iA	This paper
Enchenopa sp.*	2n=19,X0	absent	2tA	2tA	This paper
*Horiola picta** (Coquebert, 1801)	2n=21,X0	2tA	2tA	2tA	This paper
*Membracis foliatafasciata** (DeGeer, 1773)	2n=13,X0	absent	4iA,8tA,1tX	2tA	This paper
*Neotynelia pubescens** (Fabricius, 1803)	2n=21,X0	2tA	2tA	2tA	This paper

A autosome, *t* terminal, *i* interstitial. * Species with karyotypes published for the first time.

Figures

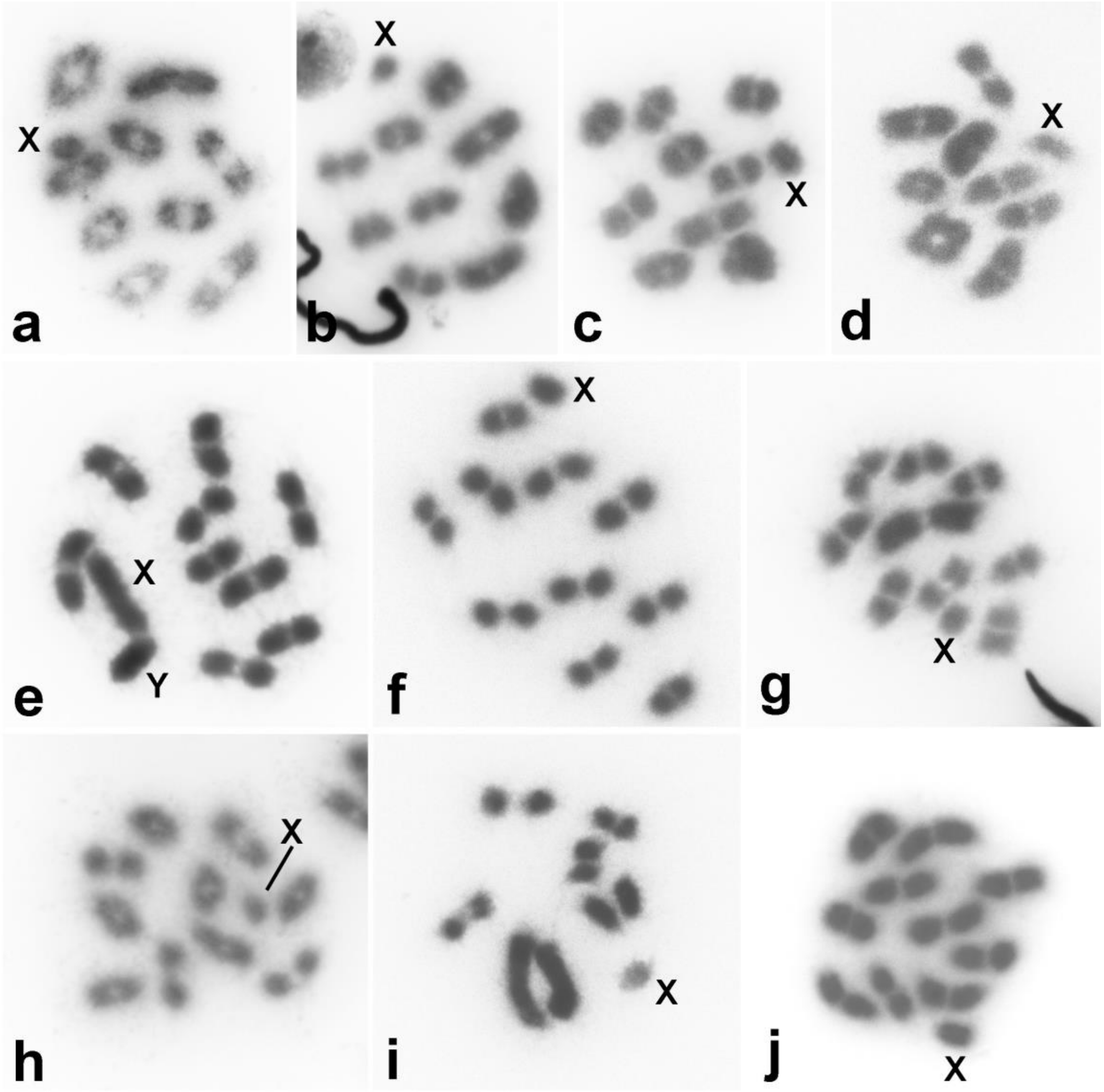

Figure 1. Conventional staining with Giemsa 5% in metaphase I of species in which the karyotype is described for the first time in this work. (a) *Deois incompleta*, (b) *D. sach*, (c) *Ferrariana trivittata*, (d) *Oncometopia facialis*, (e) *Amblyophallus exaltatus*, (f) *Bolbonota melaena*, (g) *Enchenopa* sp., (h) *Horiola picta*, (i) *Membracis foliatafasciata*, (j) *Neotynelia pubescens*. The sex chromosomes are indicated.

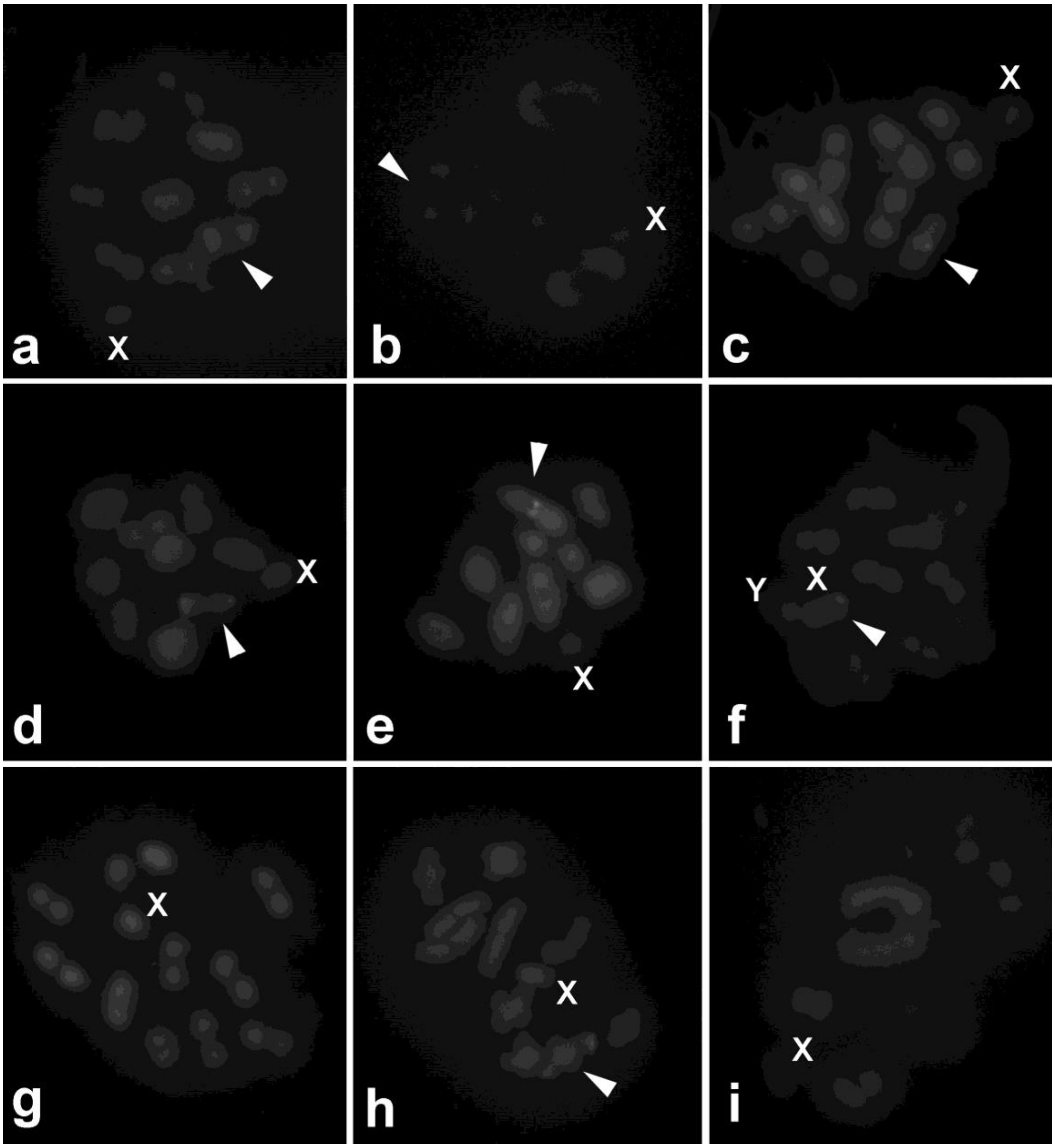

Figure 2. Examples of CMA_3 banding patterns in Cicadomorpha representatives. (a) *Deois flavopicta*, (b) *Notozulia entretiana*, (c) *Aetalion reticulatum*, (d) *Ferrariana trivittata*, (e) *Oncometopia facialis*, (f) *Amblyophallus exaltatus*, (g) *Bolbonota melaena*, (h) *Horiola picta*, (i) *Membracis foliatafasciata*. (a-i) metáfases I. The arrowhead indicate the chromosomes harboring CMA_3^+ blocks. The sex chromosomes are indicated.

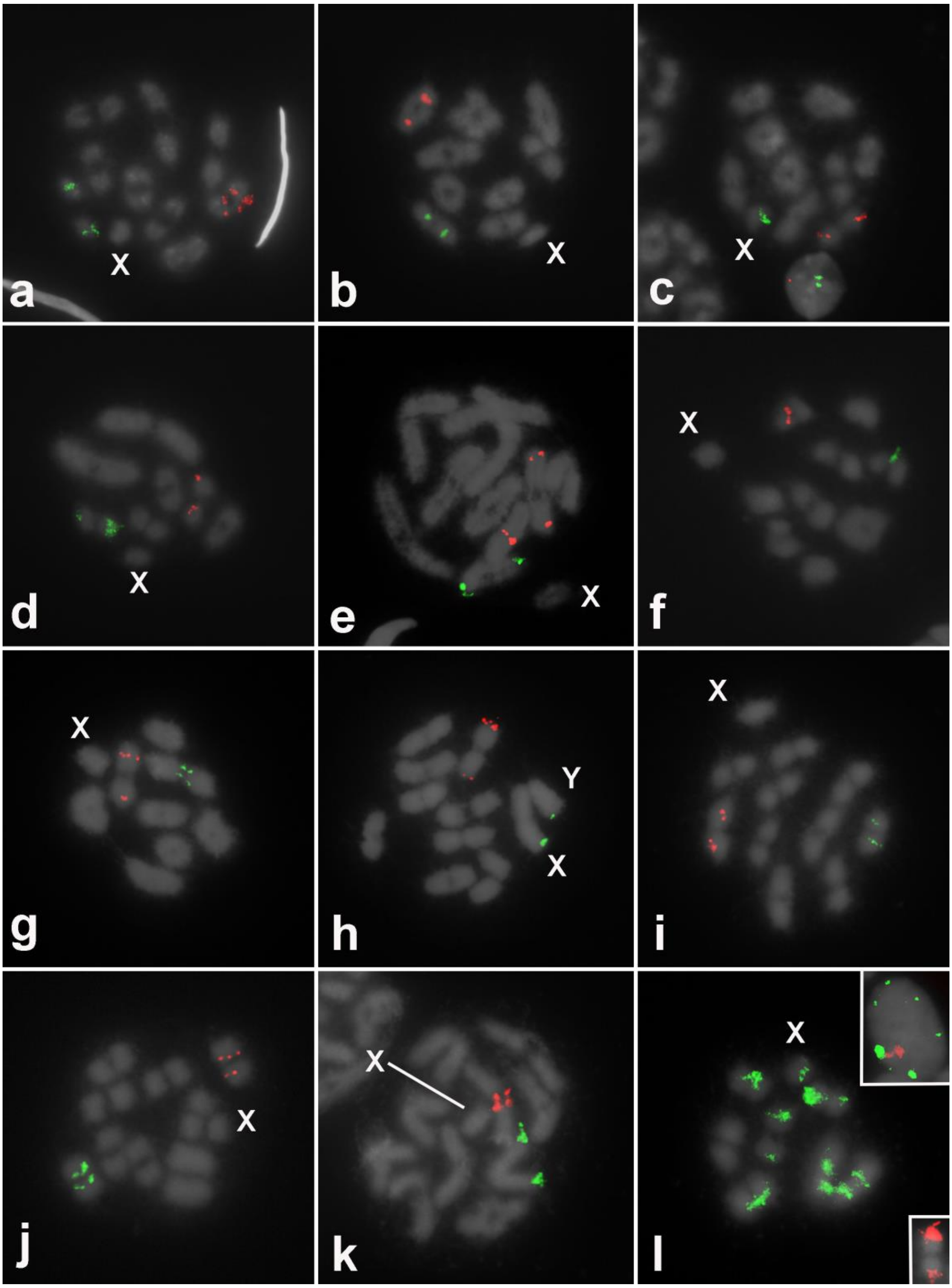

Figure 3. Fluorescent *in situ* hybridization using as probes 18S rDNA (green) and H3 histone genes (red) in twelve Cicadomorpha representatives. (a) *Deois flavopicta*, (b) *Mahanarva fimbriolata*, (c) *M. vittata*, (d) *Notozulia entretiana*, (e) *Aetalion*

reticulatum, (f) *Ferrariana trivittata*, (g) *Oncometopia facialis*, (h) *Amblyophallus exaltatus*, (i) *Bolbonota melaena*, (j) *Enchenopa* sp., (k) *Horiola picta*, (l) *Membracis foliatafasciata*. (a-d,fj,l) Metaphases I, (e,k) late Diplotene. The sex chromosomes are indicated.

Unveiling some satellite DNAs in the holocentric hemipteran species of *Mahanarva*

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Short title: SatDNAs in *Mahanarva*

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Abstract

Satellite DNAs repeats (satDNAs), the large component of heterochromatin, are non-protein-coding tandemly repeated sequences. They constitute a considerable portion of eukaryote genomes and could be involved in chromosomal organization and evolution. The repetitive DNA content in Auchenorrhyncha species are poorly known and no studies addressing satDNAs were performed. Here, we examined the most abundant satellite DNAs from *Mahanarva quadripunctata* through high-throughput analysis of Illumina reads. We found 30 satDNA families *M. quadripunctata*, representing less than 5% of the species genome, however, two of the 30 sequences were highly abundant, representing together 3.72% of the genome, accounting to more than 80% of satDNA in the species. The FISH results revealed variable chromosomal organization for the satDNAs families in *M. quadripuctata*, with clustered and non-clustered families. The most abundant satDNAs are terminal indicating that the *M. quadripunctata* heterochromatin is enriched of satellite DNAs. The interspecific investigation evidenced distinct degree of satDNA conservation among *Mahanarva* species in accordance with the “library” hypothesis, with species diversification accompanied by satDNA library diversification. The putative causes of variable abundance and chromosomal organization, and the conservation of distinct satDNAs in *Mahanarva* are discussed.

Keywords: Auchenorrhyncha, Cicadomorpha, FISH, holocentric chromosome, satelliome.

Introduction

Eukaryotic genomes are largely composed of several classes of highly repetitive DNA sequences. Among them there are the satellite DNAs (satDNA), which are in tandem organized (Smith 1976, Charlesworth et al. 1994, Palomeque and Lorite 2008). These sequences are characterized by their abundance and great diversity related to monomer length and nucleotide sequence composition (Richard et al. 2008, López-Flores and Garrido-Ramos 2012, Garrido-Ramos 2017). It is known that satDNAs follow the concerted evolution model leading to the homogenization of repeats, often associated with events such as unequal crossing over, slippage replication and even rolling circle replication followed by reinsertion of the repeat sequence into the genome (Smith 1976, Walsh 1987, Mestrović et al. 1998). The satDNAs families most abundant in a genome are diverse between different species, even among phylogenetically related ones. According to the library hypothesis, related species share conserved satDNA sequences (library) that could be amplified causing the emergence of a most abundant satDNA in a particular species (Salser et al. 1976, Mestrović et al. 1998).

SatDNAs are considered the major component of heterochromatin (Charlesworth et al. 1994, Richard et al. 2008, Palomeque and Lorite 2008), and are commonly found located in centromeric, telomeric and interstitial regions of monocentric chromosomes (Plohl et al. 2008, López-Flores and Garrido-Ramos 2012). Despite this, satDNAs located in euchromatic regions have been also described, where they could be involved in regulatory processes (Kuhn et al. 2012, Brajković et al. 2012). Among species with holocentric chromosomes the satDNAs were characterized for example in nematodes and insects species (Naclerio et al. 1992, Castagnone-Sereno et al. 1998) and *Rhynchospora* and *Luzula* plants (Heckmann et al. 2013, Ribeiro et al. 2017). Among insects the studies evidenced the preferential location of these sequences for heterochromatic regions and in sex chromosomes of Lepidoptera and Hemiptera representatives (Lu et al. 1994, Bizzaro et al. 1996, Mandrioli et al. 2003, Palomeque and Lorite 2008, Bardella et al. 2014, Věchtová et al. 2016, Dalíková et al. 2017).

The chromosomal organization of heterochromatin in insects belonging to the Auchenorrhyncha suborder is poorly known and only general characterization was done through C-banding, fluorochrome staining and C_0t-DNA (repetitive DNA pool) mapping (Kuznetsova and Aguin-Pombo 2015, Anjos et al. 2016). The genus *Mahanarva* (Cicadomorpha, Auchenorrhyncha, Cercopidae) is among those cytogenetically characterized so far and is comprised by 42 species distributed into two subgenus, i.e., *Mahanarva s. str.* and *M.* (*Ipiranga*) (Fennah 1968, Paladini and Carvalho 2007). Recently we started chromosomal studies in *Mahanarva* aiming to understand evolution and organization of repetitive DNAs (Anjos et al. 2016). Like other Cercopidae, *Mahanarva* species have holocentric chromosomes and the seven species studied until now share the same karyotype, 2n=19,X0 with chromosomes presenting similar size (Marin-Morales et al. 2002, Anjos et al. 2016). The repetitive DNA pools are conserved in the six species studied, presenting similar chromosomal distribution. Although there is variability regarding the number and distribution of G+C bands (Anjos et al. 2016).

The Next Generation Sequencing (NGS) followed by RepeatExplorer (Novák et al. 2013) analysis have been used as a powerful tool to characterize a huge quantity of satDNAs repeats and to obtain probes for comparative chromosomal studies. Among insects this strategy has been insightful about chromosomal organization and genome evolution of satDNAs, mainly in monocentric species (see for exemple Ruiz-Ruano et al. 2016b, 2016a, Palacios-Gimenez et al. 2017), and in the distinct lineages of holocentric Triatominae *Triatoma infestans* (Pita et al. 2017b). Here we aimed to characterize the organization and evolution of satDNAs in Cicadomorpha species, using as model the genus *Mahanarva*. For this proposes, we take the advantage of low coverage genome sequencing to characterize the satDNAs that populate the genome of *M. quadripunctata*. Moreover we comparatively studied the presence of the most abundant *M. quadripunctata* satDNAs in the genome of other *Mahanarva* species. Our results provided the first insights on the organization and evolution of satDNAs in *Mahanarva* and in Cicadomorpha. The intraspecific investigation revealed variable chromosomal organization for the satDNAs families and, at interspecific level, distinct degree of conservation and variable chromosomal distribution for satDNAs families.

Material and methods

Samples, chromosome preparations and genomic DNA extraction

Males of six *Mahanarva* (*M. fimbriolata*, *M. liturata*, *M. quadripunctata*, *M. vittata*, *M. tristis* and *M. spectabilis*) were sampled in natural areas of Rio Claro, São Paulo State, Brazil. Testis were removed and fixed in Carnoy's solution (3:1 100% Ethanol:Absolute Acetic Acid). The testis follicles were macerated in a drop of 50% acetic acid, and the slides were dried using a hot plate at 40-45 ºC for chromosome preparation obtaining. Whole animals were stored in 100% ethanol for species identification and for DNA extraction, which followed the phenol/chloroform-based procedure described by Sambrook and Russell (2001).

Illumina sequencing and graph-based clustering of sequencing reads

Paired-ends libraries (2x300) from a male individual genomic DNA of *M. quadripunctata* were prepared as recommended by Illumina (Illumina Inc., San Diego, CA, USA) using Nextera DNA Library Prepartion Kit v3. The sequencing was performed using Miseq Sequencing System. The FASTQC (Simon Andrews 2010) software were used to process the pair-ends reads to check the quality of the reads, while the quality trimming were performed using the FASTX-Toolkit suit (Gordon and Hannon 2010). The paired-end reads were joined using the “fastq-join” software of the FASTX-Toolkit suit (Gordon and Hannon 2010) with default options, and to search for satDNAs the joined reads were used for clustering and assembly of theses sequences using the RepeatExplorer pipeline (Novák et al. 2010, 2013).

satDNAs searching and sequence analysis

We searched for clusters that show circular graph, which is a typical characteristic of satDNAs families (Novák et al. 2010). Selected contigs were given to the Tandem Repeats Finder (TRF) algorithm (Benson 1999) to identify the DNA sequence that maximized the alignment scores between the different monomers that could be defined in tandem, using the alignment parameters 2, 3, 5 for match, mismatch and indels, respectively, and a minimum alignment score of 50 required for reporting. To identify monomers of the same family and to confirm the in tandem organization we used the dotplot graphic alignment tool implemented in Dotlet (Junier and Pagni 2000). The monomer with maximum length was used as the representative copy for a given satDNA family, and as the query sequence in further BLAST and Repbase searches using NCBI blast (http://www.ncbi.nlm.gov/Blast/) and RepeatMasking tools (http://www.girinst.org/repbase/) to check similarity with published sequences. Sequence alignments of satDNAs copies were performed with MUSCLE (Edgar 2004) implemented in MEGA5 (Tamura et al. 2011). The sat DNAs were named considering their abundance following the indications of Ruiz-Ruano et al. (2016b).

The consensus sequences of each satDNA family were used to design primers using the Primer3 software (Rozen and Skaletsky 1999) or manually (Supplementary Material 1). In order to isolate the satDNAs families in *M. quadripunctata* genome we performed polymerase chain reactions (PCR) using 10× PCR Rxn Buffer, 0.2 mM $MgCl_2$, 0.16 mM dNTPs, 2 mM of each primer, 1 U of *Taq* Platinum DNA Polymerase (Invitrogen, San Diego, CA, USA) and 50-100 ng/µl of template DNA. The PCR conditions included an initial denaturation at 94°C for 5 min and 30 cycles at 94°C (30 s), 55°C (30 s), and 72°C (80 s), plus a final extension at 72°C for 5 min. The products were visualized on a 1% agarose gel, and the monomeric 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 and then used as source for re-amplification. Monomer consensuses were deposited in GenBank under the accession numbers XXX-XXX.

Probes and Fluorescence *in situ* Hybridization and dot blot

The PCR products for satDNA families with more than 50 bp were labelled by nick translation using biotin-14-dATP (Invitrogen) or digoxigenin-11-dUTP (Roche, Mannheim, Germany). SatDNAs with less than 50 bp were labelled directly at the 5’ end with biotin-14 dATP (Sigma-Aldrich, St Louis, MO, USA) during their synthesis. Fluorescent *in situ* hybridization (FISH) was carried out according to Pinkel et al. (1986), with modifications (Cabral-de-Mello et al. 2010) using meiotic chromosome preparations. The probes that were labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin rhodamine (Roche), while the probes that were labelled with biotin-14-dATP were detected using Streptavidin Alexa Fluor 488-conjugated (Invitrogen).

The preparations were counterstained using 4’,6-diamidine-2’-phenylindole (DAPI) and mounted in VECTASHIELD (Vector, Burlingame, CA, USA). The chromosomes and hybridization signals were observed using an Olympus microscope BX61 that was equipped with a fluorescent lamp and appropriate filters. Fluorescent images were recorded using a DP71 cooled digital camera in grayscale. The images were pseudocolored, merged and optimized for brightness and contrast using Adobe Photoshop CS2.

For dot blot analysis, the biotin labelled probes from the two most abundant satDNA of *M. quadripunctata* (see results) were used and it was followed the procedure described by Anjos et al. (2016). It was used approximately 100 ng of genomic DNA from the six *Mahanarva* species.

Results

SatDNAs identification and sequence characterization

The total of 2,272,325 paired-ends reads generated by Illumina Next Generation Sequencing were added to the RepeatExplorer pipeline (Novák et al. 2010, 2013), that grouped 83.5% of reads (1,670,450) in 496,725 clusters variable in monomer size composition and abundance. The set of most abundant clusters, i.e. 330, were examined and 30 of them were putatively satDNAs, representing 4.53% of the *M. quadripunctata* genome (Table 1, Supplementary Material 2). The occurrence of satDNAs were confirmed using TRF algorithm, besides dotplots. None of the sequences revealed similarity with other sequences deposited in GenBank or Repbase.

Considering the genomic abundance among the 30 satDNAs distinct monomers we go ahead with followed analysis for 13 of them. Among them it is remarkable the discrepancy of abundance for MquSat01-18 and MquSat01-22 in comparison to other satDNAs sequences that together represented 3.72% of the 4.53% of the 30 satDNAs prospected, accounting to more than 80% of satDNAs of the *M. quadripunctata* genome. The next most abundant satDNA, i.e. MquSat03-19, composed only 0.157% of the genome and the less abundant repeat MquSat30-90 represented only 0.011% (Table 1, Supplementary Material 2). Due to difficulties to obtain some of the sequences through PCR we were able to map chromosomally only eight of the 13 most abundant satDNAs. The MquSat01-18 and MquSat02-22 were also not amplified through PCR and considering their abundance we synthetized a directly labeled sequence for FISH use. Characteristically the sequences successfully amplified through PCR presented the latter pattern in agarose gel when submitted to electrophoresis, common for satDNAs.

Chromosomal localization of satDNAs in *M. quadripunctata* and the sharing of MquSat01-18 and MquSat02-22 in *Mahanarva* species

The eight satDNAs successfully isolated from *M. quadripunctata* genome showed different chromosomal organization patterns revealed by FISH, as follows: (i) two satDNAs with a clustered pattern with signals placed in multiple loci; MquSat01-18 occurring in terminal and interstitial regions in the whole chromosome complement (Figure 1a,b), while MquSat02-22 showed terminal and interstitial blocks in seven autosomes and in the X chromosome (Figure 1c). (ii) Two satDNAs (MquSat07-161 and MquSat12-94) presented interstitial clusters located in a single autosomal bivalent (Figure 1d,e). (iii) Four satDNAs (MquSat05-84, MquSat08-72, MquSat09-107 and MquSat13-115) that although amplified through PCR did not show signals, i.e. a non-clustered patterns of hybridization through FISH (result not shown).

Based in the results obtained through FISH in *M. quadripunctata*, we tested the occurrence of MquSat01-18, MquSat02-22, MquSat07-161 and MquSat12-94 repeats in other five *Mahanarva* species to test the evolutionary conservation of these sequences. Dot-blot and FISH using MquSat01-18 and MquSat02-22 as probes revealed signals in other *Mahanarva* species (Figure 2, 3), while for MquSat07-161 and MquSat12-94 no signal was observed for any of the analyzed species (result not shown). Through Dot-blot the MquSat01-18 probe showed positive signals only for *M. quadripunctata*, *M. liturata* and *M. tristis* (Figure 2a), while MqSat2 exhibited positive signals for *M. quadripunctata*, *M. fimbriolata*, *M. liturata*, *M. spectabilis* and *M. tristis* (Figure 3a). The sharing of MquSat01-18 and MquSat02-22 between *Mahanarva* species was confirmed through FISH, and their chromosomal position assigned. Like in *M. quadripuctata* the signals for MquSat01-18 and MquSat02-22 formed a band-like pattern, with both interstitial and terminal clusters without preferential location (Figure 2b,c, 3b-e). *M. liturata* showed blocks of MquSat01-18 located in seven chromosomes, including a single terminal cluster in the X chromosome (Figure 2b). While *M. tristis* showed MquSat01-18 blocks in the whole chromosome complement, with two terminal blocks in the X chromosome (Figure 2c). The MquSat01-22 sequence was located in four chromosomes of *M. fimbriolata* (Figure 3e) and *M. spectabilis* (Figure 3c), in five chromosomes of *M. liturata* (Fig. 3b), and in the whole chromosome complement of *M. tristis* (Fig. 3d). In all these species the X chromosome showed terminal signals in both ends.

Discussion

SatDNAs in *Mahanarva quadripunctata*

The amount and chromosomal organization of satDNAs in genomes of insects with holocentric chromosomes is poorly known (Věchtová et al. 2016, Dalíková et al. 2017, Pita et al. 2017a). Here, through the high-throughput of satellitome analysis we unveil the occurrence of at least 30 satDNAs families that accounted for less than 5% of the genome of *Mahanarva quadripuctata*. The low account of satDNAs in *M. quadripuctata* suggests a quantity of TEs composing the repeatome of the species. The amount of satDNAs observed here is similar to other Hemiptera, *Nilaparvata lugens*, Delphacidae (about 6.4% of the genome) (Xue et al. 2014), but it is highly discrepant to observed in *Triatoma infestans* lineages, Reduviidae (about 30% of the genome) (Pita et al. 2017b). Remarkably only two sequences accounted for about 80% of the satDNAs in the species. This discrepancy regarding to the abundance of distinct satDNAs families is a recurrent pattern already been described for other holocentric species. Among plants it was observed in *Luzula elegans* (Heckmann et al. 2013) and some *Rhynchospora* species (Ribeiro et al. 2017) and among insects the unique species with holocentric chromosomes studied by means of Next Generation Sequencing (NGS) data, *Triatoma infestans* (Pita et al. 2017b) revealed a similar pattern, with a few satDNA families highly abundant.

Regarding to the chromosomal organization and distribution pattern of the different satDNAs that populate *M. quadripunctata* genome, two sequences MquSat01-18 and MquSat02-22 were the only ones organized into loci dispersed in several chromosomes. It could be the result of the amplification and spreading processes, caused by events known to disseminate tandem-repeated sequences in the genome, such as unequal crossing over, gene conversion, rolling-circle replication and re-insertion, and transposon-mediated exchange (Smith 1976, Charlesworth et al. 1994, Cohen et al. 2005, Ruiz-Ruano et al. 2016b). The distribution of these two most abundant satDNAs primarily in terminal regions of chromosomes follows the described pattern for heterochromatin distribution in several Auchenorrhyncha species (Noda and Tatewaki 1990, Perepelov et al. 2002, Kuznetsova et al. 2003, Maryańska-Nadachowska et al.

2008, Kuznetsova and Aguin-Pombo 2015) and correlates with the general distribution of repetitive DNAs in *M. quadripunctata* revealed by C_0t-DNA (Anjos et al. 2011). This indicate that the repetitive DNA pool enriched regions of *M. quadripunctata* chromosomes are composed by satellite DNAs. Additionally, the data reveals that interstitial regions of *M. quadripuctata* chromosomes are enriched with satDNAs. For other holocentric insect species, although several studies suggest the preferential location of repetitive sequences for heterochromatic regions (Lu et al. 1994, Bizzaro et al. 1996, Mandrioli et al. 2003, Palomeque and Lorite 2008, Bardella et al. 2014), apparently satDNAs are highly dynamics occurring also in non-heterochromatic areas, including spread in multiple parts of the chromosomes, like observed here (Věchtová et al. 2016, Pita et al. 2017b). Euchromatic satDNAs is not uncommon among insects (Kuhn et al. 2012; Pavlek et al. 2015; Ruiz-Ruano et al. 2016a; Palacios-Gimenez et al. 2017). During meiosis the end-to-end association of homologous chromosomes in holocentric species may involve the terminal heterochromatin (Guerra et al. 2010, Heckmann and Houben 2012). In *M. quadripuctata* the MquSat01-18 and MquSat02-22 could perform this association and these sequences could also be relevant for chromosomal segregation.

The other two satDNAs with clustered organization MquSat07-161 and MquSat12-94 in *M. quadripuctata* are restrict to one chromosome. The difference in number of clusters and genome multiplication for the four clustered satDNAs (sequences MquSat01-18, MquSat02-22, MquSat07-161 and MquSat12-94) in *M. quadripuctata* chromosomes could be related with their specific chromosomal placement, where sequences in terminal position (MquSat01-18 and MquSat01-22) would be more able to be spread than the placed interstitially (MquSat07-161 and MquSat12-94). The distal or sub-distal regions of holocentric chromosomes tend to be enriched of satDNAs (Mandrioli et al. 2011, Heckmann et al. 2013, Bardella et al. 2014). SatDNAs terminally located could follows the same patterns described by Sousa et al. (2011) for ribosomal DNA that are known to present high frequency of inter-locus rearrangements when located terminally. In this way when these sequences are situated in the terminal regions of the chromosomes the non-allelic rearrangements would have little or no effect on the genetic balance of organisms. Besides, considering that interstitial regions in Auchenorrhyncha are usually areas of euchromatin (Noda and Tatewaki 1990, Perepelov et al. 2002, Kuznetsova et al. 2003, Maryańska-Nadachowska et al. 2008, Kuznetsova and Aguin-Pombo 2015), it is expected less satDNAs. These sequences remain more stable in terminal regions where there it is a higher concentration of heterochromatin.

The absence of FISH signals for four of the isolated satDNAs could be consequence of their non-clustered organization. According to Ruiz-Ruano et al. (2016b), the link between monomer length and repeat number per locus could influence the sensitivity of FISH to detect specific sequences at cytological level. In *M. quadripunctata* the absence of FISH signals related to non-clustered organization is supported by the observation of FISH signals even for repeats with similar or less genomic abundance. As observed in *Locusta migratoria*, a species with monocentric chromosomes, in *M. quadripuctata* the non-clustered organization for stDNAs occurs independent of their monomer size. Moreover our data suggests that in species with holocentric chromosomes the satDNAs sequences evolution, involve birth, multiplication and clusterization, like the model proposed for a species with monocentric chromosomes (Ruiz-Ruano et al. 2016b).

Sharing of satDNAs between some *Mahanarva*

Among the satDNAs found on *M. quadripuctata* genome in which their presences were tested in other species only MquSat01-18 and MquSat02-22 were shared. This sharing and differential multiplication or loss in distinct species is in accordance with “library” hypothesis (Mestrović et al. 1998), where related species could present a set of shared repetitive sequences that amplified differently in each genome. Our data Although MquSat01-18 and MquSat02-22 are shared in some species belonging to the subgenus *Mahanarva* (*Mahanarva*), they are not observed in *M. vittata*, which belongs to the subgenus *Mahanarva* (*Ipiranga*). MquSat02-22, was present in the genomic DNA of four other species (*M. fimbriolata*, *M. liturata, M. spectabilis* and *M. tristis*), while MquSat01-18 was present in the genomic DNA of two species (*M. liturata* and *M. tristis*). Although they presented similar results regarding the clusters location, the number of clusters was quite variable, with each species presenting a different amount. Considering the putative functional constraints for terminal heterochromatin in holocentric chromosome pairing during meiosis (Guerra et al. 2010, Heckmann and Houben 2013) different satDNAs could perform this function in *Mahanarva* genus, including diverse families in the same genome. Although in some species conserved shared sequences could be assigned to this role, like for MquSat01-18 and MquSat02-22 in *M. quadripuctata* and *M. tristis*.

Our results make evident the evolutionary distance between the two subgenus of *Mahanarva* (*Mahanarva* and *Ipiranga*), since apparently none of any analyzed satellites are shared with *M.* (*Ipiranga*) *vittata*. In previous studies the X chromosome of *M. vittata* already presented a certain degree of differentiation in relation to the other species of the genus, presenting a cluster of rDNA 18S (Anjos et al. in preparation), a block of CMA_3^+ and difference for location of C_0t-DNA fraction (Anjos et al. 2016).

Considering the rapid evolution by which the satDNAs can undergo, several studies point that this divergence in the satellite DNA content could serve as a reproductive barrier (Sawamura et al. 1993, Jagannathan et al. 2017), even between related species, probably caused by process like disruption of chromosome pairing, alteration of the chromatin structure of the satellites themselves or their surrounding sequences, or even involvement of satellites in meiotic or post meiotic chromosome drive systems (Ferree and Prasad 2012).

Taking together our data indicate high dynamism in copy number variability and chromosomal organization of satDNAs in *M. quadripuctata*. Considering the genus *Mahanarva* the species diversification have been accompanied by changes in the composition and content of repetitive DNAs, like for some satellite DNAs. However, we must take into account that despite only two satDNA sequences analyzed here were specific to the subgenus *Mahanarva* (*mahanarva*), the C_0t fraction of *M. quadripunctata* was shared by all species, including *M. vittata* (Anjos et al. 2016), indicating that the repetitive DNA content of the species still maintains a degree of similarity and other repetitive sequences may rather be shared between species.

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References

Anjos, A., Rocha, G.C., Paladini, A., Mariguela, T.C., and Cabral-de-Mello, D.C. 2016. Karyotypes and Repetitive DNA Evolution in Six Species of the Genus Mahanarva (Auchenorrhyncha: Cercopidae). Cytogenet. Genome Res. **149**(4): 321–327. doi:10.1159/000450730.

Bardella, V.B., da Rosa, J.A., and Vanzela, A.L.L. 2014. Origin and distribution of AT-rich repetitive DNA families in Triatoma infestans (Heteroptera). Infect. Genet. Evol. **23**: 106–114. doi:10.1016/j.meegid.2014.01.035.

Benson, G. 1999. Tandem Repeats Finder: a program to analyse DNA sequences. Nucleic Acids Res. **27**(2): 573–578.

Bizzaro, D., Manicardi, G.C., and Bianchi, U. 1996. Chromosomal localization of a highly repeated EcoRI DNA fragment in Megoura viciae (Homoptera, Aphididae) by nick translation and fluorescence in situ hybridization. Chromosom. Res. **4**(5): 392–6. Available from http://www.ncbi.nlm.nih.gov/pubmed/8871828.

Brajković, J., Feliciello, I., Bruvo-Mađarić, B., and Ugarković, D. 2012. Satellite DNA-like elements associated with genes within euchromatin of the beetle Tribolium castaneum. G3 (Bethesda). **2**(8): 931–41. doi:10.1534/g3.112.003467.

Cabral-de-Mello, D.C., Moura, R.C., and Martins, C. 2010. 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. Heredity (Edinb). **104**(4): 393–400. The Genetics Society. doi:10.1038/hdy.2009.126.

Castagnone-Sereno, P., Leroy, H., Semblat, J.P., Leroy, F., Abad, P., and Zijlstra, C. 1998. Unusual and strongly structured sequence variation in a complex satellite

DNA family from the nematode Meloidogyne chitwoodi. J. Mol. Evol. **46**(2): 225–233. doi:10.1007/PL00006297.

Charlesworth, B., Sniegowski, P., and Stephan, W. 1994. The evolutionary dynamics of repetitive DNA in eukaryotes. Nature **371**: 215–220. doi:10.1038/371215a0.

Cohen, S., Agmon, N., Yacobi, K., Mislovati, M., and Segal, D. 2005. Evidence for rolling circle replication of tandem genes in Drosophila. Nucleic Acids Res. **33**(14): 4519–4526. doi:10.1093/nar/gki764.

Dalíková, M., Zrzavá, M., Kubíčková, S., and Marec, F. 2017. W-enriched satellite sequence in the Indian meal moth, Plodia interpunctella (Lepidoptera, Pyralidae). Chromosom. Res. **25**(3–4): 241–252. doi:10.1007/s10577-017-9558-8.

Edgar, R.C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32**(5): 1792–1797. doi:10.1385/1-59259-192-2:365.

Fennah, R.G. 1968. Revisionary notes on the new world genera of Cercopid froghoppers (Homoptera: Cercopoidea). Bull. Entomol. Res. **58**(1): 165–190. Cambridge University Press. doi:10.1017/S0007485300055954.

Ferree, P.M., and Prasad, S. 2012. How Can Satellite DNA Divergence Cause Reproductive Isolation? Let Us Count the Chromosomal Ways. Genet. Res. Int. **2012**(Figure 1): 1–11. doi:10.1155/2012/430136.

Garrido-Ramos, M.A. 2017. Satellite DNA: An Evolving Topic. Genes (Basel). **8**(9): 1–44. doi:10.3390/genes8090230.

Gordon, A., and Hannon, G.F. 2010. FASTX-Toolkit. FASTQ/A short-reads pre-processing tools.

Guerra, M., Cabral, G., Cuacos, M., González-García, M., González-Sánchez, M., Vega, J., and Puertas, M.J. 2010. Neocentrics and holokinetics (holocentrics): chromosomes out of the centromeric rules. Cytogenet. Genome Res. **129**(1–3): 82–96. doi:10.1159/000314289.

Heckmann, S., and Houben, A. 2013. Holokinetic Centromeres. *In* Plant Centromere Biology. Wiley-Blackwell, Oxford, UK. pp. 83–94. doi:10.1002/9781118525715.ch7.

Heckmann, S., MacAs, J., Kumke, K., Fuchs, J., Schubert, V., Ma, L., Novák, P., Neumann, P., Taudien, S., Platzer, M., and Houben, A. 2013. The holocentric species Luzula elegans shows interplay between centromere and large-scale genome organization. Plant J. **73**(4): 555–565. doi:10.1111/tpj.12054.

Jagannathan, M., Warsinger-Pepe, N., Watase, G.J., and Yamashita, Y.M. 2017. Comparative Analysis of Satellite DNA in the Drosophila melanogaster Species Complex. G3 Genes|Genomes|Genetics **7**(2): 693–704. doi:10.1534/g3.116.035352.

Junier, T., and Pagni, M. 2000. Dotlet: diagonal plots in a web browser. Bioinformatics **16**(2): 178–179. doi:10.1093/bioinformatics/16.2.178.

Kuhn, G.C.S., Küttler, H., Moreira-Filho, O., and Heslop-Harrison, J.S. 2012. The 1.688 repetitive DNA of drosophila: Concerted evolution at different genomic scales and association with genes. Mol. Biol. Evol. **29**(1): 7–11. doi:10.1093/molbev/msr173.

Kuznetsova, V., and Aguin-Pombo, D. 2015. Comparative cytogenetics of Auchenorrhyncha (Hemiptera, Homoptera): a review. Zookeys **538**: 63–93. doi:10.3897/zookeys.538.6724.

Kuznetsova, V.G., Maryanska-Nadachowska, A., and Nokkala, S. 2003. A new approach to the auchenorrhyncha (Hemiptera, Insecta) cytogenetics: chromosomes of the meadow spittlebug Philaenus spumarius (L.) examined using various chromosome banding techniques. Folia Biol **51**(1–2): 33–40. Available from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14686645.

López-Flores, I., and Garrido-Ramos, M. a. 2012. The repetitive DNA content of eukaryotic genomes. Genome Dyn. **7**: 1–28. doi:10.1159/000337118.

Lu, Y.-J., Kochert, G.D., Isenhour, D.J., and Adang, M.J. 1994. Molecular characterization of a strain-specific repeated DNA sequence in the fall armyworm Spodoptera frugiperda (Lepidoptera: Noctuidae). Insect Mol. Biol. **3**(2): 123–130. doi:10.1111/j.1365-2583.1994.tb00159.x.

Mandrioli, M., Azzoni, P., Lombardo, G., and Manicardi, G.C. 2011. Composition and epigenetic markers of heterochromatin in the aphid Aphis nerii (Hemiptera: Aphididae). Cytogenet. Genome Res. **133**(1): 67–77. doi:10.1159/000323510.

Mandrioli, M., Manicardi, G.C., and Marec, F. 2003. Cytogenetic and molecular characterization of the MBSAT1 satellite DNA in holokinetic chromosomes of the cabbage moth, Mamestra brassicae (Lepidoptera). Chromosom. Res. **11**(1): 51–56. doi:10.1023/A:1022058032217.

Marin-Morales, M.A., Zefa, E., Bertagna, M., Camargo Mathias, M.I., and Arrigoni, E. 2002. Chromosome analysis of two species of sugar cane pests of the genus Mahanarva (Homoptera , Cercopidae). Caryologia **55**(4): 357–360.

Maryańska-Nadachowska, A., Kuznetsova, V.G., and Abdul-Nour, H. 2008. A chromosomal study on a Lebanese spittlebug Philaenus arslani (Hemiptera: Auchenorrhyncha: Aphrophoridae). Eur. J. Entomol. **105**(2): 205–210.

doi:10.14411/eje.2008.029.

Mestrović, N., Plohl, M., Mravinac, B., and Ugarković, Đ. 1998. Evolution of satellite DNAs from the genus Palorus--experimental evidence for the “library” hypothesis. Mol. Biol. Evol. **15**(8): 1062–1068. doi:10.1093/oxfordjournals.molbev.a026005.

Naclerio, G., Cangiano, G., Coulson, A., Levitt, A., Ruvolo, V., and La Volpe, A. 1992. Molecular and genomic organization of clusters of repetitive DNA sequences in Caenorhabditis elegans. J. Mol. Biol. **226**(1): 159–168. doi:10.1016/0022-2836(92)90131-3.

Noda, H., and Tatewaki, R. 1990. Re-examination of Chromosomes of Three Species of Rice Planthoppers (Homoptera : Delphacidae). Appl. Entomol. Zool. **25**(4): 538–540. doi:10.1303/aez.25.538.

Novák, P., Neumann, P., and Macas, J. 2010. Graph-based clustering and characterization of repetitive sequences in next-generation sequencing data. BMC Bioinformatics **11**(1): 378. doi:10.1186/1471-2105-11-378.

Novák, P., Neumann, P., Pech, J., Steinhaisl, J., and MacAs, J. 2013. RepeatExplorer: A Galaxy-based web server for genome-wide characterization of eukaryotic repetitive elements from next-generation sequence reads. Bioinformatics **29**(6): 792–793. doi:10.1093/bioinformatics/btt054.

Palacios-Gimenez, O.M., Dias, G.B., de Lima, L.G., Kuhn, G.C. e S., Ramos, É., Martins, C., and Cabral-de-Mello, D.C. 2017. High-throughput analysis of the satellitome revealed enormous diversity of satellite DNAs in the neo-Y chromosome of the cricket Eneoptera surinamensis. Sci. Rep. **7**(1): 6422. doi:10.1038/s41598-017-06822-8.

Paladini, A., and Carvalho, G.S. 2007. Descrição de três novas espécies de Mahanarva

(Hemiptera, Cercopidae, Ischnorhininae). Iheringia. Série Zool. **97**(1): 57–66. doi:10.1590/S0073-47212007000100009.

Palomeque, T., and Lorite, P. 2008. Satellite DNA in insects: a review. Heredity (Edinb). **100**(6): 564–73. doi:10.1038/hdy.2008.24.

Perepelov, E., Bugrov, A.G., and Maryańska-Nadachowska, A. 2002. Constitutive heterochromatin in karyotypes of two cicadidae species from Japan (Cicadoidea, Hemiptera). Folia Biol. (Praha). **50**(3–4): 217–219.

Pinkel, D., Straume, T., and Gray, J.W. 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc. Natl. Acad. Sci. U. S. A. **83**(9): 2934–8. doi:10.1073/pnas.83.9.2934.

Pita, S., Lorite, P., Vela, J., Mora, P., Palomeque, T., Thi, K.P., and Panzera, F. 2017a. Holocentric chromosome evolution in kissing bugs (Hemiptera: Reduviidae: Triatominae): diversification of repeated sequences. Parasit. Vectors **10**(1): 410. Parasites & Vectors. doi:10.1186/s13071-017-2349-4.

Pita, S., Panzera, F., Mora, P., Vela, J., Cuadrado, Á., Sánchez, A., Palomeque, T., and Lorite, P. 2017b. Comparative repeatome analysis on Triatoma infestans Andean and Non-Andean lineages , main vector of Chagas disease. PLoS One (12(7): e0181635): 1–13.

Plohl, M., Luchetti, A., Meštrović, N., and Mantovani, B. 2008. Satellite DNAs between selfishness and functionality: Structure, genomics and evolution of tandem repeats in centromeric (hetero)chromatin. Gene **409**(1–2): 72–82. doi:10.1016/j.gene.2007.11.013.

Ribeiro, T., Marques, A., Novák, P., Schubert, V., Vanzela, A.L.L., Macas, J., Houben, A., and Pedrosa-Harand, A. 2017. Centromeric and non-centromeric satellite DNA organisation differs in holocentric Rhynchospora species. Chromosoma **126**(2): 325–335. Chromosoma. doi:10.1007/s00412-016-0616-3.

Richard, G.-F., Kerrest, A., and Dujon, B. 2008. Comparative genomics and molecular dynamics of DNA repeats in eukaryotes. Microbiol. Mol. Biol. Rev. **72**(4): 686–727. doi:10.1128/MMBR.00011-08.

Rozen, S., and Skaletsky, H. 1999. Primer3 on the WWW for General Users and for Biologist Programmers. *In* Bioinformatics Methods and Protocols. Humana Press, New Jersey. pp. 365–386. doi:10.1385/1-59259-192-2:365.

Ruiz-Ruano, F.J., Cabrero, J., López-León, M.D., and Camacho, J.P.M. 2016a. Satellite DNA content illuminates the ancestry of a supernumerary (B) chromosome. Chromosoma. doi:10.1007/s00412-016-0611-8.

Ruiz-Ruano, F.J., López-León, M.D., Cabrero, J., and Camacho, J.P.M. 2016b. High-throughput analysis of the satellitome illuminates satellite DNA evolution. Sci. Rep. **6**(January): 28333. Nature Publishing Group. doi:10.1038/srep28333.

Salser, W., Bowen, S., Browne, D., el-Adli, F., Fedoroff, N., Fry, K., Heindell, H., Paddock, G., Poon, R., Wallace, B., and Whitcome, P. 1976. Investigation of the organization of mammalian chromosomes at the DNA sequence level. Fed Proc **35**(1): 23–35. Available from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1107072.

Sambrook, J., and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harb. Lab. Press. Cold Spring Harb. NY: 999. Available from http://books.google.com/books?id=YTxKwWUiBeUC&printsec=frontcover%5Cn papers2://publication/uuid/BBBF5563-6091-40C6-8B14-06ACC3392EBB.

Sawamura, K., Yamamoto, M.T., and Watanabe, T.K. 1993. Hybrid lethal systems in the Drosophila melanogaster species complex. II. The Zygotic hybrid rescue (Zhr) gene of D. melanogaster. Genetics **133**(2): 307–313.

Simon Andrews. 2010. FastQC: a quality control tool for high throughput sequence data. Available from http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [accessed 12 July 2017].

Smith, G.P. 1976. Evolution of repeated DNA sequences by unequal crossover. doi:10.1126/science.1251186.

Sousa, A., Barros e Silva, A.E., Cuadrado, A., Loarce, Y., Alves, M. V., and Guerra, M. 2011. Distribution of 5S and 45S rDNA sites in plants with holokinetic chromosomes and the “chromosome field” hypothesis. Micron **42**(6): 625–631. doi:10.1016/j.micron.2011.03.002.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28**(10): 2731–2739. doi:10.1093/molbev/msr121.

Věchtová, P., Dalíková, M., Sýkorová, M., Žurovcová, M., Füssy, Z., and Zrzavá, M. 2016. CpSAT-1, a transcribed satellite sequence from the codling moth, Cydia pomonella. Genetica **144**(4): 385–95. doi:10.1007/s10709-016-9907-0.

Walsh, J.B. 1987. Persistence of tandem arrays: implications for satellite and simple-sequence DNAs. Genetics **115**(3): 553–567.

Xue, J., Zhou, X., Zhang, C.-X., Yu, L.-L., Fan, H., Wang, Z., Xu, H., Xi, Y., Zhu, Z.-R., Zhou, W.-W., Pan, P.-L., Li, B.-L., Colbourne, J.K., Noda, H., Suetsugu, Y., Kobayashi, T., Zheng, Y., Liu, S., Zhang, R., Liu, Y., Luo, Y.-D., Fang, D.-M., Chen, Y., Zhan, D.-L., Lv, X.-D., Cai, Y., Wang, Z.-B., Huang, H.-J., Cheng, R.-L., Zhang, X.-C., Lou, Y.-H., Yu, B., Zhuo, J.-C., Ye, Y.-X., Zhang, W.-Q., Shen, Z.-C., Yang, H.-M., Wang, J., Wang, J., Bao, Y.-Y., and Cheng, J.-A. 2014. Genomes of the rice pest brown planthopper and its endosymbionts reveal complex complementary contributions for host adaptation. Genome Biol. **15**(12): 521. doi:10.1186/s13059-014-0521-0.

Tables

Table 1. Main features of the satDNAs families isolated from *Mahanarva quadripunctata* genome and their chromosomal distribution.

satDNA family	Monomer lenght (bp)	A+T (%)	Abundance (%)	Chromosomal organization
MquSat01-18	18	66.7	2.160	9A (t, i); 2X (t)
MquSat02-22	22	36.4	1.460	7A (t, i); 2X (t)
MquSat03-19	19	42.1	0.157	Not mapped
MquSat04-37	37	67.6	0.104	Not mapped
MquSat05-84	84	64.3	0.100	nc
MquSat06-60	60	60.7	0.051	Not mapped
MquSat07-161	161	59.0	0.034	1A (i)
MquSat08-72	72	63.9	0.034	nc
MquSat09-107	107	52.8	0.033	nc
MquSat10-37	37	73.0	0.031	Not mapped
MquSat11-77	77	55.8	0.030	Not mapped
MquSat12-94	94	55.3	0.027	1A (i)
MquSat13-115	115	54.8	0.024	nc

bp=base pairs, A=autosomes, X=X chromosome; t= terminal, i=interstitial, nc=non-clustered.

Figures

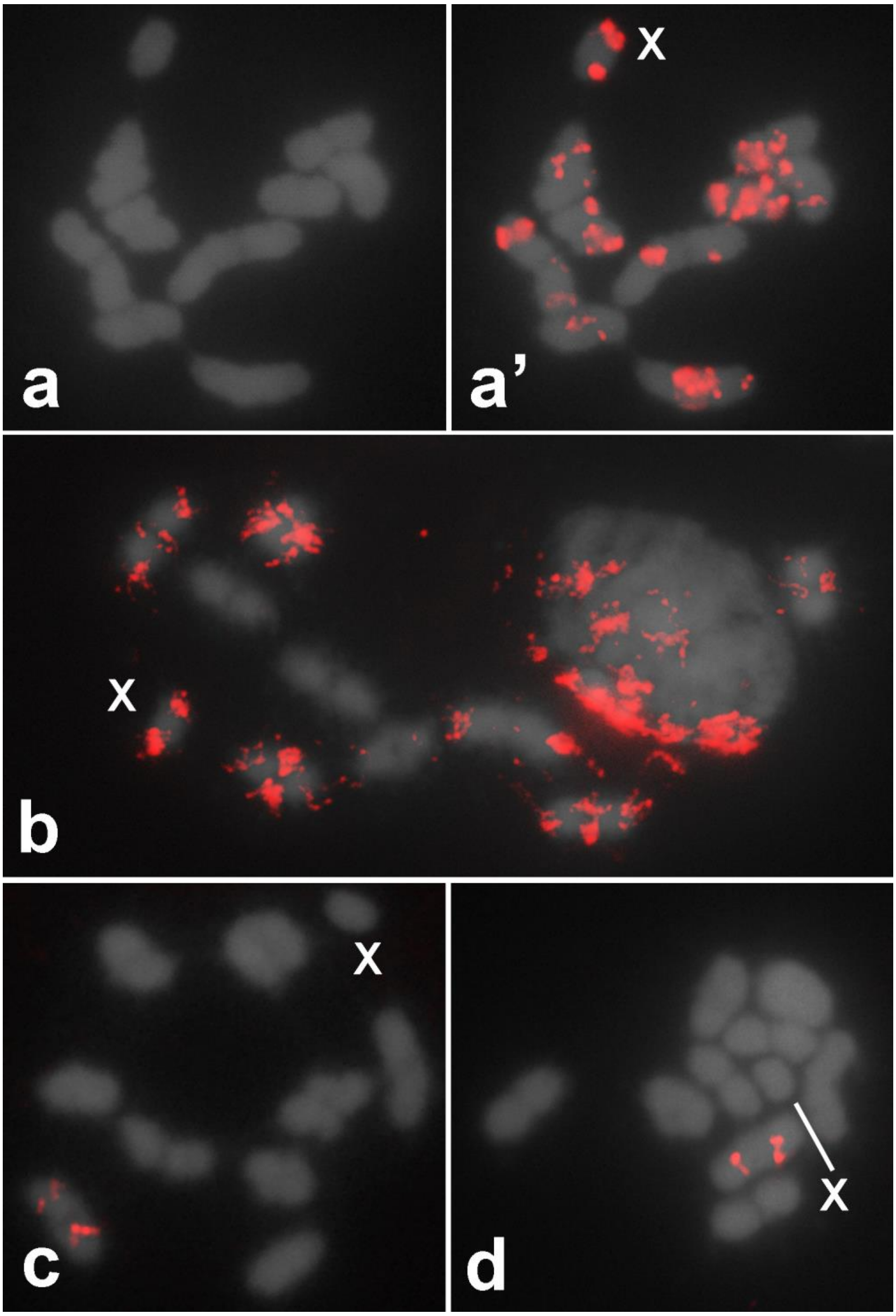

Figure 1. Physical mapping through FISH of four clustered satDNAs found in the *Mahanarva quadripunctata* genome. (a,a') MquSat01-18, (a) DAPI, (a,a') merge; (b) MquSat02-22; (c) MquSat07-161; and (d) MquSat12-94. The univalent X chromosome is indicated.

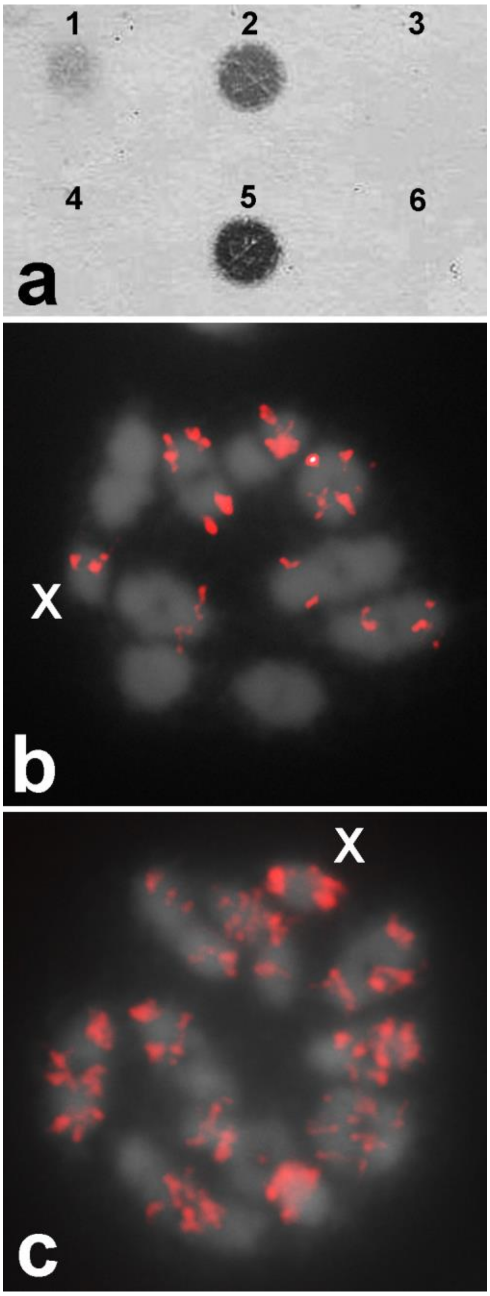

Figure 2. Dot-blot and FISH analysis for MquSat01-18 in *Mahanarva* species. (a) Dot-blot of MquSat01-18 hybridized against the unlabeled genomic DNA from *Mahanarva* revealing positive silgnals only for three species; 1- *M. quadripunctata*, 2- *M. liturata*, 3- *M. spectabilis*, 4- *M. vittata*, 5- *M. tristis*, 6- *M. fimbriolata*. (b,c) MquSat01-18 chromosomal location assigned through FISH in *M. liturata* and *M. tristis*, respectively. The univalent X chromosome is indicated.

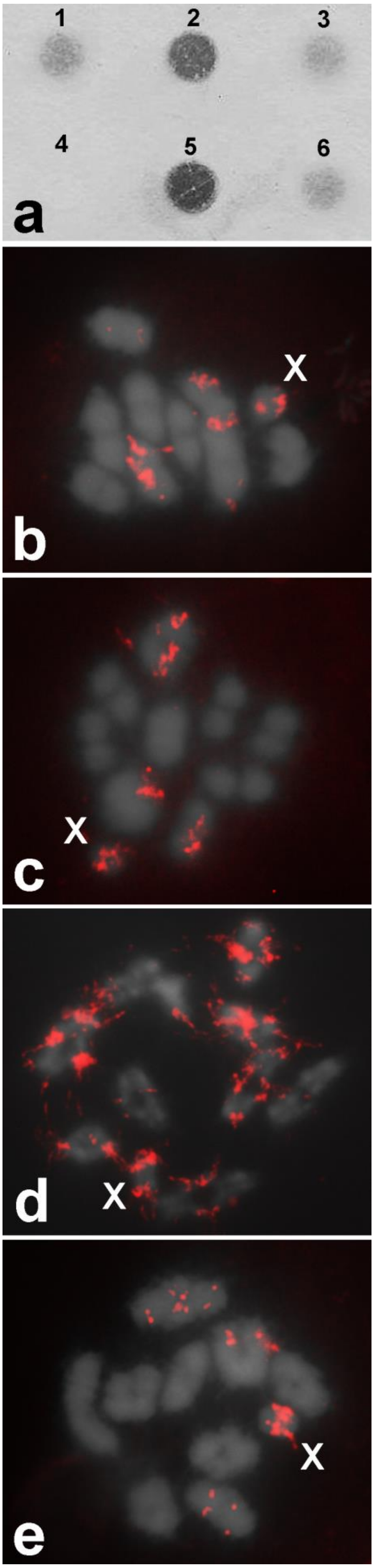
1
2
3
4
5
6
a
X
b
X
c
X
d
X
e

Figure 3. Dot-blot and FISH data for MquSat02-22 in *Mahanarva* species. (a) Dot-blot from MquSat02-22 hybridized against the unlabeled genomic DNA from *Mahanarva* showing positive signs for five species; 1- *M. quadripunctata*, 2- *M. liturata,* 3- *M. spectabilis*, 4- *M. vittata*, 5- *M. tristis*, 6- *M. fimbriolata*. FISH data revealing loci distribution for MquSat02-22 in *M. liturata* (b), *M. spectabilis* (c), *M. tristis* (d) and *M. fimbriolata*. The univalent X chromosome is indicated.

Supplementary materials

Supplementary material 1. Primers designed to amplify each satDNA family from the genome of *Mahanarva quadripunctata*

Repeat family	**Foward**	**Reverse**
MquSat03-19	CAACAGAATGCAGGGCCA	GGCCCTGCATTCTGTTGG
MquSat04-37	AATAGATAGAGTAGGCCT	TTTACATGTAAAAGTGCA
MquSat05-84	TGAGCTGCTGAATAATTACA	ATCATTTAGTCTTCAGAGTC
MquSat06-60	CACGACACAGACGGTCTTGG	TTAACTACAGTCTACGTAGC
MquSat07-161	TCCCTTCCCCAAAACAGTAA	TGAAAATACAACAACAAAACACTGA
MquSat08-72	GGGCCTATGTCAATGTAGGA	CCGAATACGAAAAAATCCAT
MquSat09-107	GCGTATCTCTACTCCGCGTTC	ATGTTTTGCGTTTGGTTAGTAGC
MquSat10-37	TTCAATAAAAGTAACTTA	TTTACATGTAAAAGTGCA
MquSat11-77	TAACGGTAATACAAAGGC	CTTATAGAATCGTTTCCG
MquSat12-94	CCTACCTGGGGTGAGTAA	CTTCGCTATGACTCTTGTG
MquSat13-115	AAGGTGGTGGAACCATTCAG	TGCCTACGTACCCAATCTGTT

Supplementary material 2. Length (bp), A+T content (%) and abundance (% of the genome) of the 17 satDNA from the genome of *M. quadripunctata*. These satDNA families were not studied under FISH.

satDNA family	**Monomer lenght (bp)**	**A+T (%)**	**Abundance (%)**
MquSat14-40	40	40.0	0.023
MquSat15-168	168	61.3	0.022
MquSat16-53	53	60.4	0.021
MquSat17-58	58	74.1	0.020
MquSat18-159	159	50.3	0.020
MquSat19-63	63	60.3	0.020
MquSat20-65	65	63.1	0.019
MquSat21-91	91	53.8	0.018
MquSat22-17	17	70.6	0.018
MquSat23-25	25	32.0	0.017
MquSat24-149	149	42.3	0.016
MquSat25-71	71	53.5	0.014
MquSat26-83	83	51.8	0.014
MquSat27-66	66	60.6	0.013
MquSat28-102	102	52.9	0.012
MquSat29-115	115	65.2	0.011
MquSat30-90	90	56.7	0.011

5 Considerações Finais

1. A variação de número diploide é bastante ampla entre os representantes das distintas famílias de Cicadomorpha, embora o sistema sexual X0 é bastante conservado. Esta variação se deve principalmente a fusões e fissões cromossômicas. Entretanto dentro das distintas famílias os cariótipos são mais conservados.

2. A pouca especificidade quanto a riqueza de pares de base (AT ou GC) específicos na heterocromatina constitutiva com prevalência de poucas regiões ricas em GC é um padrão bastante comum em Cicadomorpha, indicando pouca variabilidade da heterocromatina constitutiva. Embora estudos com sequências específicas são necessários.

3. Os resultados obtidos através da fração C_0t evidenciaram a riqueza de DNAs repetitivos nas regiões terminais no gênero *Mahanarva*. Além disso, o conteúdo de repetitivos das espécies do gênero parece ser conservado, uma vez que ao menos parte do conteúdo de DNAs repetitivos de *M. quadripunctata* é compartilhado com outras espécies do gênero.

4. No que diz respeito a distribuição das sequências de famílias multigênicas, também foi observada uma grande conservação no número e localização dos clusters de DNAr 18S, histona H3 e RNAsn U1, sugerindo estabilidade. Além disso, a presença de clusters em um único bivalente autossômico é mais um indicativo de que esses marcadores se mantiveram conservados mesmo com os processos que levaram a variação nos números cromossômicos observados em Cicadomorpha.

5. A análise do satelitoma de *M. quadripunctata* permitiu observar uma grande variabilidade na organização cromossômica dos diferentes DNAs satélites da espécie com sequências muito mais abundantes do que outras, organizadas ou não em *clusters*. Além disso, a comparação com outras espécies do gênero evidenciou o

compartilhamento de algumas dessas sequências, enquanto outras não são compartilhadas, sugerindo dinâmica interespecífica para esta fração do genoma.

6 Referências bibliográficas

Albig W, Kardalinou E, Drabent B, et al (1991) Isolation and characterization of two human H1 histone genes within clusters of core histone genes. Genomics 10:940–8.

Amarger V, Gauguier D, Yerle M, et al (1998) Analysis of distribution in the human, pig, and rat genomes points toward a general subtelomeric origin of minisatellite structures. Genomics 52:62–71.

Anleitner JE, Haymer DS (1992) Y enriched and Y specific DNA sequences from the genome of the Mediterranean fruit fly, *Ceratitis capitata*. Chromosoma 101:271–8.

Benson G (1999) Tandem Repeats Finder: a program to analyse DNA sequences. Nucleic Acids Res 27:573–578.

Boring AM (1907) A study of the spermatogenesis of 22 species of the Membracidae, Jassidae, Cercopidae and Fulgoridae with special reference to the behavior of the odd chromosome. J Exp Zool 4:469–514.

Bringmann P, Lührmann R (1986) Purification of the individual snRNPs U1, U2, U5 and U4/U6 from HeLa cells and characterization of their protein constituents. EMBO J 5:3509–16.

Cabral-de-Mello DC, Moura RC, Martins C (2010) 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. Heredity (Edinb) 104:393–400.

Cabral-de-Mello DC, Valente GT, Nakajima RT, Martins C (2012) Genomic organization and comparative chromosome mapping of the U1 snRNA gene in cichlid fish, with an emphasis in *Oreochromis niloticus*. Chromosom Res 20:279–

92.

Cabrero J, López-León MD, Teruel M, Camacho JPM (2009) Chromosome mapping of H3 and H4 histone gene clusters in 35 species of acridid grasshoppers. Chromosom Res 17:397–404.

Castanhole MMU, Pereira LLV, Souza HV de, et al (2010) Meiotic chromosomes and nucleolar behavior in testicular cells of the grassland spittlebugs *Deois flavopicta*, *Mahanarva fimbriolata* and *Notozulia entreriana* (Hemiptera, Auchenorrhyncha). Genet Mol Biol 33:244–252.

Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371:215–220.

Cryan JR (2005) Molecular phylogeny of Cicadomorpha (Insecta: Hemiptera: Cicadoidea, Cercopoidea and Membracoidea): adding evidence to the controversy. Syst Entomol 30:563–574.

Cryan JR, Urban JM (2012) Higher-level phylogeny of the insect order Hemiptera: Is Auchenorrhyncha really paraphyletic? Syst Entomol 37:7–21.

Dernburg AF (2001) Here, there, and everywhere: kinetochore function on holocentric chromosomes. J Cell Biol 153:F33-8.

Dey SK (1991) Chromosomes of Five Species of Spittle Bugs (Homoptera, Cercopidae). Cytologia (Tokyo) 56:523–526.

Dietrich CH (2005) Keys To the Families of Cicadomorpha and Subfamilies and Tribes of Cicadellidae (Hemiptera: Auchenorrhyncha). Florida Entomol 88:502–517.

Dietrich CH (2002) Evolution of Cicadomorpha (Insecta, Hemiptera). Denisia 176:155–170.

Dietrich CH, Mckamey SH, Deitz LL (2001) Morphology-based phylogeny of the treehopper family Membracidae (Hemiptera: Cicadomorpha: Membracoidea). Syst

Entomol 26:213–239.

Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797.

Eickbush TH, Eickbush DG (2007) Finely orchestrated movements: evolution of the ribosomal RNA genes. Genetics 175:477–85.

Eirín-López JM, Rebordinos L, Rooney a. P, Rozas J (2012) The birth-and-death evolution of multigene families revisited. Genome Dyn 7:170–196.

Engel JD, Dodgson JB (1981) Histone genes are clustered but not tandemly repeated in the chicken genome. Proc Natl Acad Sci U S A 78:2856–60.

Ferree PM, Prasad S (2012) How Can Satellite DNA Divergence Cause Reproductive Isolation? Let Us Count the Chromosomal Ways. Genet Res Int 2012:1–11.

Frydrychová R, Grossmann P, Trubac P, et al (2004) Phylogenetic distribution of TTAGG telomeric repeats in insects. Genome 47:163–78.

Garrido-Ramos MA (2017) Satellite DNA: An Evolving Topic. Genes (Basel) 8:1–44.

Golub N, Kuznetsova V, Rakitov R (2014) First karyotype data on the family Myerslopiidae (Hemiptera, Auchenorrhyncha, Cicadomorpha). Comp Cytogenet 8:293–300.

Gordon A, Hannon GF (2010) FASTX-Toolkit. FASTQ/A short-reads pre-processing tools.

Haizel T, Lim YK, Leitch AR, Moore G (2005) Molecular analysis of holocentric centromeres of *Luzula species*. Cytogenet Genome Res 109:134–143.

Halkka O (1959) Chromosome studies on the Hemiptera Homoptera Auchenorrhyncha. Ann Acad Sci Fenn 43:1–72.

Halkka O (1964) Recombination in Six Homopterous Families. Evolution (N Y) 18:81.

Ijdo JW, Wells R a, Baldini A, Reeders ST (1991) Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. Nucleic Acids Res 19:4780.

Junier T, Pagni M (2000) Dotlet: diagonal plots in a web browser. Bioinformatics 16:178–179.

Kedes LH (1979) Histone genes and histone messengers. Annu Rev Biochem 48:837–70.

Kidwell MG (2002) Transposable elements and the evolution of genome size in eukaryotes. Genetica 115:49–63.

Kirillova VI (1986) Chromosome numbers of world Homoptera Auchenorrhyncha. 1. Fulgoroidea, Cercopodea, and Cicadoidea. Entomol Rev 65:34–47.

Kirillova VI (1987) Chromosome numbers of world Homoptera Auchenorrhyncha. II. Cicadelloidea. Entomol Rev 67:80–107.

Kraemer C, Schmidt ER (1993) The sex determining region of *Chironomus thummi* is associated with highly repetitive DNA and transposable elements. Chromosoma 102:553–62.

Kuznetsova V, Aguin-Pombo D (2015) Comparative cytogenetics of Auchenorrhyncha (Hemiptera, Homoptera): a review. Zookeys 538:63–93. doi: 10.3897/zookeys.538.6724

Kuznetsova V, Golub N, Aguin-Pombo D (2013) Karyotypes, B-chromosomes and meiotic abnormalities in 13 populations of *Alebra albostriella* and *A. wahlbergi* (Hemiptera, Auchenorrhyncha, Cicadellidae) from Greece. Comp Cytogenet 7:305–325.

Kuznetsova VG, Grozeva SM, Hartung V, Anokhin BA (2015a) First evidence for (TTAGG)n telomeric sequence and sex chromosome post-reduction in Coleorrhyncha (Insecta, Hemiptera). Comp Cytogenet 9:523–532.

Kuznetsova VG, Maryańska-Nadachowska A, Anokhin BA, Aguin-Pombo D (2015b) Evidence for TTAGG telomere repeats and rRNA gene clusters in leafhoppers of the genus *Alebra* (Hemiptera: Auchenorrhyncha: Cicadellidae). Eur J Entomol 112:207–214.

Kuznetsova VG, Maryanska-Nadachowska A, Nokkala S (2003) A new approach to the auchenorrhyncha (Hemiptera, Insecta) cytogenetics: chromosomes of the meadow spittlebug *Philaenus spumarius* (L.) examined using various chromosome banding techniques. Folia Biol 51:33–40.

Kuznetsova VG, Maryańska-Nadachowska A, Yang CT, O'Brien LB (1998) Karyotypes, sex- chromosome systems and testis structure in Fulgoroidea (Auchenorrhyncha, Homoptera, Insecta). Folia Biol 46:23–40.

Kuznetsova VG, Maryanska-Ndachowska A, Nokkala S (2009) Karyotype characterization of planthopper species *Hysteropterum albaceticum* Dlabola , 1983 and *Agalmatium bilobum* (Fieber , 1877) (Homoptera: Auchenorrhyncha : Issidae) using AgNOR. C and DAPI/CMA3 banding techniques. Comp Cytogenet 3:111–123.

Li Y-C, Korol AB, Fahima T, et al (2002) Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Mol Ecol 11:2453–65.

Lifton RP, Goldberg ML, Karp RW, Hogness DS (1978) The Organization of the Histone Genes in *Drosophila melanogaster*: Functional and Evolutionary Implications. Cold Spring Harb Symp Quant Biol 42:1047–1051.

Lohmann TR, Pietrowski V, Bressan DF (2010) Population dynamics of spittlebugs in the Western Region of Paraná State. Semin Ciências Agrárias 31:1291–1298.

Long EO, Dawid IB (1980) Repeated genes in eukaryotes. Annu Rev Biochem 49:727–764.

López-Flores I, Garrido-Ramos M a. (2012) The repetitive DNA content of eukaryotic genomes. Genome Dyn 7:1–28.

Maddox PS, Oegema K, Desai A, Cheeseman IM (2004) “Holo”er than thou: Chromosome segregation and kinetochore function in *C. elegans*. Chromosom Res 12:641–653.

Mandrioli M, Manicardi G (2012) Unlocking Holocentric Chromosomes: New Perspectives from Comparative and Functional Genomics? Curr Genomics 13:343–349.

Manser T, Gesteland RF (1982) Human U1 loci: genes for human U1 RNA have dramatically similar genomic environments. Cell 29:257–64.

Marin-Morales MA, Zefa E, Bertagna M, et al (2002) Chromosome analysis of two species of sugar cane pests of the genus *Mahanarva* (Homoptera , Cercopidae). Caryologia 55:357–360.

Márquez-Corro JI, Escudero M, Luceño M (2017) Do holocentric chromosomes represent an evolutionary advantage? A study of paired analyses of diversification rates of lineages with holocentric chromosomes and their monocentric closest relatives. Chromosom Res 1–14.

Martins C, Cabral-de-Mello DC, Valente GT, et al (2011) Animal Genomes Under the Focus of Cytogenetics. Nova Science

Maryańska-Nadachowska A, Anokhin BA, Gnezdilov VM, Kuznetsova VG (2016) Karyotype stability in the family *Issidae* (Hemiptera, Auchenorrhyncha) revealed by chromosome techniques and FISH with telomeric (TTAGG)n and 18S rDNA probes. Comp Cytogenet 10:347–369.

Maryańska-Nadachowska A, Kuznetsova VG, Abdul-Nour H (2008) A chromosomal study on a Lebanese spittlebug *Philaenus arslani* (Hemiptera: Auchenorrhyncha:

Aphrophoridae). Eur J Entomol 105:205–210.

Maryańska-Nadachowska A, Kuznetsova VG, Gnezdilov VM, Drosopoulos S (2006) Variability in the karyotypes, testes and ovaries of planthoppers of the families Issidae, Caliscelidae, and Acanaloniidae (Hemiptera: Fulgoroidea). Eur J Entomol 103:505–513.

Maryańska-Nadachowska A, Kuznetsova VG, Karamysheva T V. (2013) Chromosomal location of rDNA clusters and TTAGG telomeric repeats in eight species of the spittlebug genus *Philaenus* (Hemiptera: Auchenorrhyncha: Aphrophoridae). Eur J Entomol 110:411–418.

Maryańska-Nadachowska A, Kuznetsova VG, Lachowska D, Drosopoulos S (2012) Mediterranean species of the spittlebug genus *Philaenus*: modes of chromosome evolution. J Insect Sci 12:54.

Marz M, Kirsten T, Stadler PF (2008) Evolution of Spliceosomal snRNA Genes in Metazoan Animals. J Mol Evol 67:594–607.

Marzluff WF, Brown DT, Lobo S, Wang S-S (1983) Isolation and characterization of two linked mouse Ulb small nuclear RNA genes. Nucleic Acids Res 11:6255–6270.

Mattaj IW, Zeller R (1983) Xenopus laevis U2 snRNA genes: tandemly repeated transcription units sharing 5’ and 3’ flanking homology with other RNA polymerase II transcribed genes. EMBO J 2:1883–1891.

Mattos VF, Cella DM, Carvalho LS, et al (2013) High chromosome variability and the presence of multivalent associations in buthid scorpions. Chromosom Res 21:121–36.

Melters DP, Paliulis L V, Korf IF, Chan SWL (2012) Holocentric chromosomes: convergent evolution, meiotic adaptations, and genomic analysis. Chromosom Res

20:579–93.

Nagoda N, Fukuda A, Nakashima Y, Matsuo Y (2005) Molecular characterization and evolution of the repeating units of histone genes in *Drosophila americana*: Coexistence of quartet and quintet units in a genome. Insect Mol Biol 14:713–717.

Nei M, Rooney AP (2005) Concerted and birth-and-death evolution of multigene families. Annu Rev Genet 39:121–152.

Nguyen P, Sahara K, Yoshido A, Marec F (2010) Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). Genetica 138:343–354.

Nilsen TW (2003) The spliceosome: the most complex macromolecular machine in the cell? Bioessays 25:1147–9.

Noda H, Tatewaki R (1990) Re-examination of Chromosomes of Three Species of Rice Planthoppers (Homoptera : Delphacidae). Appl Entomol Zool 25:538–540.

Novák P, Neumann P, Macas J (2010) Graph-based clustering and characterization of repetitive sequences in next-generation sequencing data. BMC Bioinformatics 11:378.

Novák P, Neumann P, Pech J, et al (2013) RepeatExplorer: A Galaxy-based web server for genome-wide characterization of eukaryotic repetitive elements from next-generation sequence reads. Bioinformatics 29:792–793.

Nowak R (1994) Mining treasures from “junk DNA”. Science (80-) 263:608–610.

Paladini A, Takiya DM, Cavichioli RR, Carvalho GS (2015) Phylogeny and biogeography of Neotropical spittlebugs (Hemiptera: Cercopidae: Ischnorhininae): revised tribal classification based on morphological data. Syst Entomol 40:82–108.

Palomeque T, Lorite P (2008) Satellite DNA in insects: a review. Heredity (Edinb) 100:564–73.

Papeschi A, Bressa M (2006) Evolutionary cytogenetics in Heteroptera. J Biol Res 5:3–21.

Peck DC, Thompson V (2008) Spittlebugs (Hemiptera: Cercopoidea). In: Encyclopedia of Entomology. Springer Netherlands, Dordrecht, pp 3512–3516

Perepelov E, Bugrov AG, Maryańska-Nadachowska A (2002) Constitutive heterochromatin in karyotypes of two cicadidae species from Japan (Cicadoidea, Hemiptera). Folia Biol (Praha) 50:217–219.

Plohl M, Luchetti A, Meštrović N, Mantovani B (2008) Satellite DNAs between selfishness and functionality: Structure, genomics and evolution of tandem repeats in centromeric (hetero)chromatin. Gene 409:72–82.

Rozen S, Skaletsky H (1999) Primer3 on the WWW for General Users and for Biologist Programmers. In: Bioinformatics Methods and Protocols. Humana Press, New Jersey, pp 365–386

Ruberti I, Fragapane P, Pierandrei-Amaldi P, et al (1982) Characterization of histone genes isolated from Xenopus laevis and Xenopus tropicalis genomic libraries. Nucleic Acids Res 10:7543–7559.

Sahara K, Marec F, Traut W (1999) TTAGG telomeric repeats in chromosomes of some insects and other arthropods. Chromosome Res 7:449–60.

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harb Lab Press Cold Spring Harb NY 999.

Schweizer D, Mendelak M, White MJD, Contreras N (1983) Cytogenetics of the parthenogenetic grasshopper *Warramaba virgo* and its bisexual relatives. Chromosoma 88:227–236.

Sittman DB, Chiu IM, Pan CJ, et al (1981) Isolation of two clusters of mouse histone genes. Proc Natl Acad Sci U S A 78:4078–82.

Sybenga J (1999) Chromosoma Focus What makes homologous chromosomes find each other in meiosis ? A review and an hypothesis. Chromosoma 108:209–219.

Szwedo J (2004) An annotated checklist of Myerslopiidae with notes on the distribution and origin of the group (Hemiptera: Cicadomorpha). Zootaxa 425:1–15.

Tamura K, Peterson D, Peterson N, et al (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739.

Valadkhan S (2005) snRNAs as the catalysts of pre-mRNA splicing. Curr Opin Chem Biol 9:603–608.

Valério JR (2009) Cigarrinhas-das-pastagens. Embrapa Gado Corte 51.

West S (2012) The increasing functional repertoire of U1 snRNA. Biochem Soc Trans 40:846–9.

Whitten MJ (1965) Chromosome numbers in some Australian leafhoppers (Homoptera, Auchenorrhyncha) Author(s): Proc Linn Soc New Wales 90:407–409.

Yu JC, Wendelburg B, Sakallah S, Marzluff WF (1991) The U1 snRNA gene repeat from the sea urchin (Strongylocentrotus purpuratus): the 70 kilobase tandem repeat ends directly 3’ to a U1 gene. Nucleic Acids Res 19:1093–8.

Zwick MS, Hanson RE, Islam-Faridi MN, et al (1997) A rapid procedure for the isolation of C_0t-1 DNA from plants. Genome 40:138–142.