UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

Vanessa Bellini Bardella

Comportamento e organização dos cromossomos holocêntricos de heterópteros da região Sul do Brasil baseados em sequências repetitivas DNA, meiose e análise ultraestrutural

Tese apresentada ao Programa de Pós-Graduação em Genética, para a obtenção do Título de Doutora em Genética

São José do Rio Preto-SP
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Resumo

As espécies da subordem Heteroptera possuem cromossomos holocinéticos, variação do número cromossômico, meiose invertida e aquiasmática nos cromossomos sexuais e acúmulo de heterocromatina predominantemente nas extremidades cromossômicas. Das 30 espécies analisadas das infraordens Pentatomomorpha e Cimicomorpha, foram observadas variações nos números cromossômicos e cariótipos assimétricos, sobretudo em Reduviidae. O bandamento C-CMA_3/DAPI também mostrou que há variabilidade na distribuição de bandas heterocromáticas entre autossomos e alossomos, contudo, a localização da heterocromatina predominou nos terminais cromossômicos. Os sítios de DNAr 18S foram localizados principalmente nas regiões terminais, com tendência à distribuição entre os autossomos nos Pentatomomorpha, enquanto que nas espécies de Cimicomorpha houve uma maior variação entre autossomos e alossomos. Para um estudo mais aprofundado sobre a origem e a organização de famílias de DNA repetitivo, foi escolhida *Triatoma infestans* como modelo. Este estudo mostrou as duas sequências de DNA satélite ricas em AT que predominam nas regiões terminais dos cromossomos, são compostas por motivos curtos com 79 bp e 33 bp de comprimento, que foram originadas possivelmente de elementos transponíveis gigantes conhecidos como Polintons. As comparações citogenéticas de todas as amostras estudadas neste trabalho mostraram algumas tendências, tais como a predominância do sistema sexual XY/XX, a localização preferencial da heterocromatina e dos sítios de DNAr 18S nas regiões terminais dos cromossomos e a ocorrência de cromossomos holocinéticos. Contudo, as variações na organização e assimetria dos cariótipos observadas de cada grupo mostram uma relativa dinâmica nos genomas desses heterópteros.

Palavras-chave: citogenética; cromossomos holocinéticos; cromossomos sexuais; DNAr 18S; DNA satélite; heterocromatina.

Abstract

The species of the suborder Heteroptera have holokinetic chromosomes, variation in chromosome number, inverted/achiasmatic meiosis in the sex chromosomes and accumulation of heterochromatin predominantly in chromosome ends. In the 30 species analyzed of the infraorders Pentatomomorpha and Cimicomorpha, variations in chromosome numbers and asymmetrical karyotypes were observed, especially in Reduviidae. The C-CMA_3/DAPI banding also showed that there is variability in the distribution of heterochromatic bands between autosomes and alossomos, however, the predominant location of heterochromatin were in the chromosome terminals. The 18S rDNA sites were located mainly in the terminal regions, with a tendency to distributed among the autosomes in Pentatomomorpha, whereas in species Cimicomorpha there was greater variation between autosomes and alossomos. To a more detailed study of the origin and organization of families of repetitive DNA, *Triatoma infestans* was chosen as a model. This study showed the two sequences of AT-rich satellite DNA that predominate on the ends of chromosomes, are composed of short motifs with 79 bp and 33 bp, which were possibly originated from transposable elements known as giant Polintons. Cytogenetic comparisons of all samples studied in this work showed some trends, such as the predominance of the sexual system XY/XX, the preferred location of heterochromatin and 18S rDNA sites on the ends of chromosomes and the occurrence of holokinetic chromosomes. However, changes in the organization and asymmetry of karyotypes observed in each group show a relative dynamic in the genomes of these heteropteran.

Key-words: cytogenetic; DNA satellite; holokinetic chromosomes; heterochromatin; sex chromosomes; 18S rDNA.

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1. Introdução

1.1. Subordem Heteroptera

Heteroptera apresenta mais de 40.000 espécies descritas e, entre os hemimetábolos, é um dos grupos com maior sucesso na radiação de insetos (WEIRAUCH; SCHUH, 2011). Os heterópteros são conhecidos popularmente como percevejos e apresentam distribuição geográfica nas regiões tropicais e subtropicais do planeta (SCHUH; SLATER, 1995). Essa subordem é considerada um grupo monofilético de Hemiptera. Carver et al. (1991) e Wheeler et al. (1993) sugeriram como sinapomorfias de Heteroptera *i*) o labium inserido anteriormente na cabeça, ao contrário de posteriormente como em Sternorrhyncha, Auchenorrhyncha e Coleorrhyncha (Figura 1A), *ii*) presença de glândulas metatoráxicas nos adultos (Figura 1B) e *iii*) ninfas com glândula abdominal dorsal.

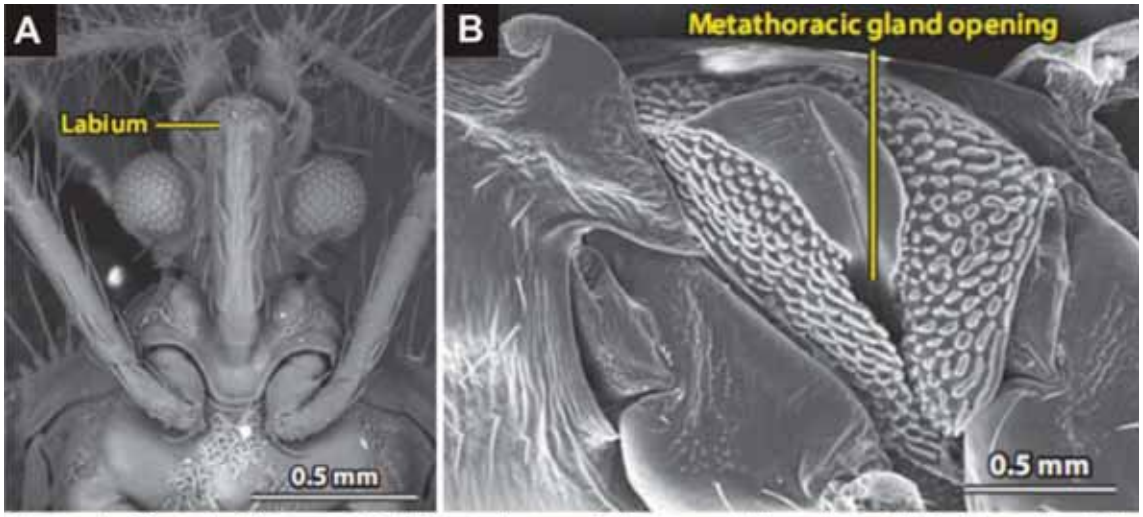

Figura 1. Características morfológicas sinapomórficas em Heteroptera. (A) Labium inserido anteriormente na cabeça. (B) Presença de glândulas metatoráxicas na cabeça. (Fonte: WEIRAUCH, SCHUH, 2011).

Existem diferentes propostas acerca do relacionamento filogenético e da quantidade de infraordens em Heteroptera. O estudo Wheeler et al. (1993) é o mais aceito por utilizar um grande número de espécies (29) e dados moleculares e morfológicos. Heteroptera está organizada em sete infraordens (Figura 2). A infraordem Cimicomorpha possui mais de

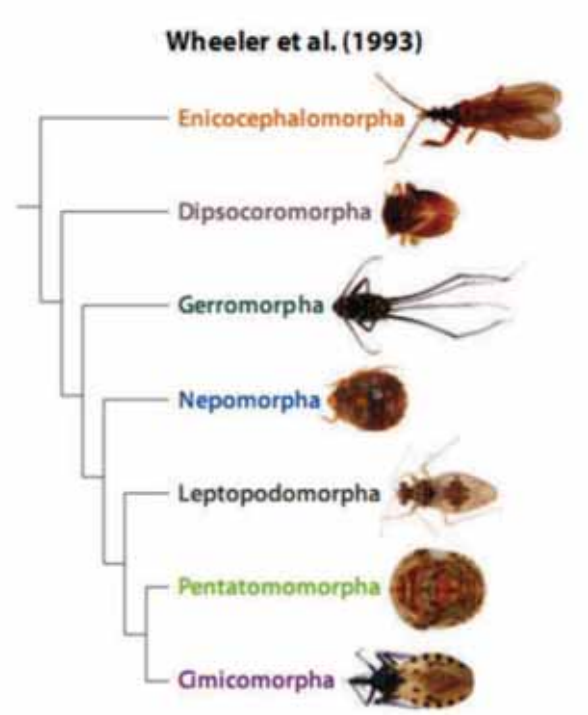

Figura 2. Filogenia das infraordens de Heteroptera com base em caracteres morfológicos e moleculares. (Fonte: WEIRAUCH, SCHUH, 2011).

20.000 espécies, as quais apresentam diferentes hábitos alimentares, tais como, a predação, a hematofagia, o ectoparasitismo e a fitofagia (SCHUH; SLATER, 1995; WEIRAUCH; SCHUH, 2011). A família Reduviidae, com cerca de 7.000 espécies, é destacada entre as demais de Cimicomorpha por apresentar alta variabilidade na morfologia corporal (WEIRAUCH; MUNRO, 2009) (Figura 3). Além disso, apresenta subfamílias diferenciadas, como Harpatorinae, na qual grande parte dos insetos possui modificações morfológicas como "patas pegajosas" que auxiliam na captura das presas, e Triatominae, que abrange 140 insetos hematófagos e vetores da doença de Chagas (SCHOFIELD; GALVÃO, 2009; ZHANG; WEIRAUCH, 2013). Uma diversidade similar nos hábitos alimentares é observada na infraordem Pentatomomorpha (Figura 4), a qual compreende 14.000 espécies divididas em 5 superfamílias (Aradoidea, Coreoidea, Lygaeoidea, Pentatomoidea e Pyrrhocoroidea). Contudo, o relacionamento filogenético entre as infraordens vem sendo considerado controverso, devido a divergências entre os dados morfológicos e moleculares (SCHAEFER, 1993).

Figura 3. Exemplos de espécies da infraordem Cimicomorpha. (Fonte:WEIRAUCH; SCHUH, 2011).

Além da importância biológica, relacionada com a participação dos heterópteros na diversidade de espécie e no equilíbrio das populações, os heterópteros possuem destaque agronômico por serem considerados pragas de lavouras, tais como soja e

milho (KONO, 1989; YAMANAKA et al., 1990). Indivíduos pertencentes aos gêneros *Nezara* e *Piezodorus* (Pentatomidae) e a espécie *Riptortus pedestris* da família Alydidae possuem hábito fitófago e são outros exemplos de heterópteros que causam danos econômicos em lavouras (KONO 1989, YAMANAKA et al., 1990).

Figura 4. Exemplares da infraordem Pentatomomorpha. (Fonte:WEIRAUCH; SCHUH, 2011).

1.2 Citogenética convencional de Heteroptera

1.2.1. Cromossomos holocêntricos

Os cromossomos holocêntricos são caracterizados por não apresentarem constrição primária e a organização do cinetócoro ocorrer ao longo dos cromossomos (GUERRA et al., 2010). Esta disposição cinetocórica parece aceitar mais facilmente eventos de fissão e fusão cromossômica, e permite que eventuais fragmentos cromossômicos permaneçam viáveis em divisões celulares subsequentes. Isto aumenta a chance de existência de variação cromossômica numérica inter e intrapopulacional (HUGHES-SCHRADER; SCHRADER, 1961; MELTERS et al., 2012).

Os cromossomos holocêntricos foram observados em plantas, insetos, aracnídeos e nematóides (MELTERS et al., 2012). Dado os diferentes organismos que possuem os cromossomos holocêntricos e inferindo como mais parcimonioso a presença de cromossomos monocêntricos no último ancestral comum de todos os eucariotos, Melters et al. (2012) sugeriram que os cromossomos holocêntricos surgiram independentemente 13 vezes durante a

história evolutiva dos eucariotos. No reino Animal os cromossomos holocêntricos sugiram nove vezes, sendo uma no filo Nematoda e oito no filo Arthropoda (MELTERS et al., 2012). Na classe Insecta os cromossomos holocêntricos ocorrem em diferentes ordens como Epheneroptera (efêmeras), Odonata (libélulas), Zoraptera, Dermaptera (tesourinhas), Trichoptera (moscas-de-água), Lepidoptera (borboletas e mariposas) e na superordem Paraneoptera (piolhos casca, piolhos verdadeiros e percevejos), com os estudos sobre segregação cromossômica concentrados nos hemípteros (WOLF, 1996; MELTERS et al., 2012).

Nos hemípteros e em outros organismos com cromossomos holocêntricos como *Rhynchospora tenuis*, Cyperaceae (GUERRA et al., 2006), certas características citogenéticas são observadas como a migração paralela das cromátides em anáfase mitótica, não existindo a configuração de *Rabl* durante a atividade cinética, como observado para os cromossomos monocêntricos (HUGHES-SCHRADER; SCHRADER, 1961; MELTERS et al., 2012). Entretanto, na anáfase I da meiose, os cromossomos apresentam migração telocinética, com funcionamento aleatório da região terminal na qual ocorrerá a atividade cinética (PÉREZ et al., 2000; MELTERS et al., 2012).

1.2.2. Número cromossômico

O número cromossômico em Heteroptera varia de $2n = 4$ (*Lethocerus* sp., Belostomatidae) a $2n = 80$ (quatro espécies de *Lopidea* Uhler, Miridae), com o predomínio de cinco números cromossômicos modais: *i*) $2n = 14$ (460 espécies), *ii*) $2n = 16$ (186 espécies), *iii*) $2n = 34$ (92 espécies), *iv*) $2n = 12$ (89 espécies) e *v*) $2n = 13$ (80 espécies) (UESHIMA, 1979; PAPESCHI; BRESSA, 2006). Embora existam análises citogenéticas em muitas espécies, os estudos são concentrados nas infraordens Cimicomorpha e Pentatomomorpha,

com abordagem principalmente relacionada aos números cromossômicos (PAPESCHI; BRESSA, 2006).

Para a infraordem Cimicomorpha, os dados citogenéticos foram obtidos em 465 espécies e evidenciaram a variação numérica de $2n = 6$ em *Hesperoctenes fumarius*, Polyctenidae (UESHIMA, 1979) até $2n = 80$ em quatro espécies do gênero *Lopidea*, Miridae (AKINGBOHUNGBE, 1974). Esta infraordem exibe ainda famílias com grande variabilidade numérica: Miridae, com $2n = 14$ a 80 e Cimicidae, com $2n = 10$ a 50 (KUZNETSOVA et al., 2011). A subfamília Triatominae, que apresenta 58% das espécies analisadas quanto ao número cromossômico, contrasta com os dados citogenéticos da infraordem, sobretudo pela uniformidade no número cromossômico dos triatomíneos, que varia apenas de $2n = 21$ até $2n = 25$, nos machos (PANZERA et al., 2010).

Na infraordem Pentatomomorpha, as análises citogenéticas são centralizadas nas famílias Coreidae, Lygaeidae e Pentatomidae (PAPESCHI; BRESSA, 2006). A maior variabilidade no número cromossômico é encontrada em Coreidae e Lygaeidae, com cariótipos contendo desde $2n = 13$ até $2n = 28$ e $2n = 10$ a $2n = 30$, respectivamente (BRESSA et al., 2008; UESHIMA, 1979). Embora ocorra variação no número cromossômico em Pentatomidae ($2n = 6$ a $2n = 27$), 85% das espécies analisadas apresentam $2n = 14$ (REBAGLIATI et al., 2005).

Apesar de existirem muitos dados sobre o número cromossômico em Heteroptera, ainda é difícil traçar o sentido da evolução cromossômica do grupo. Muitos autores sugerem como cariótipos basais aqueles que apresentam os números modais, conceito aplicado em muitos casos para famílias, tribos ou gêneros (PAPESCHI; BRESSA, 2006). Entretanto, os rearranjos cromossômicos envolvidos na organização e diferenciação dos cariótipos são pouco conhecidos. Um exemplo desses raros casos é a translocação recíproca observada em *Mepraia gajardoi*, Reduviidae, a qual resultou em irregularidades cromossômicas como

presença de um trivalente autossômico, univalentes e fragmentos cromossômicos, que interferiram na segregação tanto dos autossomos quanto dos cromossomos sexuais (PÉREZ et al., 2004).

1.2.3. Cromossomos sexuais

Nos heterópteros, os estudos citogenéticos dos cromossomos sexuais são concentrados na descrição do sistema cromossômico do sexo das espécies, bem como na apresentação de características citogenéticas básicas como tamanho cromossômico, presença de bandas heterocromáticas e comportamento meiótico. Há diferentes sistemas cromossômicos do sexo nos heterópteros. Os sistemas cromossômicos simples (XY/XX e X0/XX) ocorrem em 86,1% das espécies, já os sistemas cromossômicos múltiplos (X_nY/X_nX_n, X_n0/X_nX_n, XY_n/XX) são encontrados em 13,5% espécies. Apenas 0,4% das espécies analisadas apresentaram o sistema de neo-X/neo-Y (PAPESCHI; BRESSA, 2006).

Nas espécies com o sistemas cromossômicos simples os estudos são concentrados no comportamento meiótico dos cromossomo sexuais. Nos heterópteros os cromossomos sexuais apresentam o comportamento invertido e aquiasmático durante a meiose I (SOLARI, 1979). Entretanto, em algumas espécies das famílias Notonectidae (*Anisops fieberi*, *A. nivea* e *A. sardea*), Reduviidae (*Ectrychotes dispar*), Coreidae (gênero *Archimerus* e *Pachylis*) e Tingidae (27 espécies) a meiose invertida não foi constatada (UESHIMA, 1979; GROZEVA; NOKKALA, 2001; PAPESCHI et al., 2003). O comportamento aquiasmático dos cromossomos sexuais foi observado em diferentes heterópteros como nos pentatomídeos *Antiteuchus mixtus* (LANZONE; SOUZA, 2006) e *Nezara viridula* (PAPESCHI et al., 2003) e em *Triatoma infestans*, Reduviidae (PÉREZ et al., 2000). Nesses insetos, bem como em outros heterópteros, foram relatados em meiose II o comportamento de *"touch-and-go-*

pairing" dos cromossomos sexuais, o qual auxiliaria na segregação correta destes cromossomos (UESHIMA, 1979; PAPESCHI; BRESSA, 2006).

Os sistemas cromossômicos múltiplos ocorrem nas infraordens Pentatomomorpha e Nepomorpha, mas são reportados sobretudo na infraordem Cimicomorpha (FRANCO et al., 2006; POGGIO et al., 2007; KUZNETSOVA et al., 2011; CHIRINO et al., 2013). O caso mais extremo de fragmentação do cromossomo sexual pré-existente foi observado em *Cimex lectularius*, Cimicidae, o qual apresentou no cariótipo, 20 cromossomos X (SADÍLEK et al., 2013).

Os casos de neo-X/neo-Y observados na literatura são provenientes do sistema de determinação sexual simples XY/XX, exceto em *Dysdercus albofasciatus*, Pyrrhocoridae (PAPESCHI; BRESSA, 2006). Nesta espécie ocorreu a formação dos cromossomos neo-X/neo-Y a partir do sistema cromossômico do sexo X0/XX. Além disso, há relatos que a inversão cromossômica foi envolvida na formação do cromossomo neo-X, evento o qual foi confirmado pela observação de sinais de DNAr 18S em diferentes posições do cromossomo neo-X de *Dysdercus albofasciatus* (BRESSA et al., 1999; BRESSA et al., 2009).

1.2.4. m-chromossomos

Os m-cromossomos são caracterizados pelo tamanho reduzido e pelo comportamento meiótico diferenciado, com ausência de pareamento, sem presença de quiasmas e associação apenas no final da diacinese (WILSON, 1905; PAPESCHI; BRESSA, 2006). Esses cromossomos foram descritos em diferentes famílias de Heteroptera como Micronectidae (Nepomorpha), Coreidae (Pentatomomorpha), Alydidae (Pentatomomorpha), Rhopalidae (Pentatomomorpha) e Miridae (Cimicomorpha) (ITUARTE; PAPESCHI, 2004; BRESSA et al., 2005; SOUZA et al., 2009; KUZNETSOVA et al., 2011). Em *Pachylis pharaonis*, $2n = 12 + 2m + X0$, Coreidae a hipofosforilação da H3S10 no m-cromossomo em meiose II foi

associada a heteropcinose negativa do m-cromossomo durante a meiose (SOTERO-CAIO et al., 2011). O comportamento como bivalente foi apenas reportado por Suja et al. (2000) em *Coreus marginatus*, Coreidae, com a observação da organização do complexo sinaptonêmico. Embora algumas características citogenéticas dos m-cromossomos sejam conhecidas, os aspectos como sua origem e evolução nas diferentes famílias ainda permanecem sem soluções.

1.3. Organização dos genomas de eucariotos

Os genomas dos eucariotos são compostos por sequências únicas de DNA e, em sua maior parte, por famílias de DNA de natureza repetitiva. Essas últimas podem ser divididas em porções moderadamente repetitivas e altamente repetitivas. O DNA repetitivo pode ser organizado também em grupos, de acordo com a organização *in tandem* ou dispersa, ou ainda pela funcionalidade em expressão (LÓPEZ-FLORES; GARRIDO-RAMOS, 2012). As famílias de DNA moderadamente repetitivas, as quais são formadas de sequências de DNA imediatamente adjacentes um ao outro com orientação *head-to-tail*, têm caráter funcional e seus principais representantes são as sequências que codificam o RNA ribossômico e as proteínas histônicas (LÓPEZ-FLORES; GARRIDO-RAMOS, 2012) As sequências altamente repetitivas de DNA, também têm distribuição *in tandem*, mas podem aparecer dispersas nos genomas, podendo ou não apresentar expressão genética. Dentre essas, podemos mencionar os micro, mini e DNAs satélites não codificantes, e os elementos de transposição autônomos e não autônomos, que podem ou não ser codificantes (JURKA et al., 2007; LÓPEZ-FLORES; GARRIDO-RAMOS, 2012).

1.3.1. DNAr 18S

Os genes nucleares que codificam as moléculas de RNA ribossomal (RNAr) são organizados *in tandem* e distribuídos em duas famílias multigênicas no genoma: uma representada pelo DNAr 45S, o qual consiste dos genes que transcrevem o RNAr 18S, 5,8S e 28S, sendo estas sequências separadas por espaçadores transcritos internos (ITS: *Internal Transcribed Spacer* ITS1 e ITS2), e cada cluster de DNAr 45S separado por espaçadores transcritos externos (ETS: *External Transcribed Spacer*) e por espaçadores intergênicos (IGS) (LONG; DAWID, 1980; ALVES-COSTA et al., 2006; LÓPEZ-FLORES; GARRIDO-RAMOS, 2012). A segunda família multigênica corresponde às sequências do gene que transcreve o RNAr 5S, sendo essas sequências separadas entre si pelos espaçadores não transcritos (*NTS- Non-Transcribed Spacer*) (LONG; DAWID, 1980; COSTA et al., 2006).

Nos heterópteros os dados sobre a distribuição do DNAr 5S ainda não são conhecidos, e os dados sobre a distribuição dos sítios de DNAr 18S ocorrem sobretudo na subfamília Triatominae. Esses insetos hematófagos exibem variações tanto no número quanto na distribuição dos sítios de DNAr 18S (BARDELLA et al., 2010; PANZERA et al., 2012). Panzera et al. (2012) analisou cerca de 38 espécies de triatomíneos e observou que o número de sinais varia de 1 até 3. A distribuição dos sinais de DNAr 18S ocorre tanto nos autossomos, como em *Triatoma pseudomaculata* (PANZERA et al., 2012), quanto nos cromossomos sexuais, como observado em *Triatoma sordida* e *Triatoma garciabesi* (PANZERA et al., 2012).

Uma característica distinta de *Triatoma infestans* é a variação intraespecífica na localização dos sítios de DNAr 18S. Nesta espécie as populações Andinas exibiram os sinais de hibridização em um bivalente, enquanto que as populações não-Andinas apresentaram os sitíos de DNAr 18S no cromossomo X (PANZERA et al., 2012). Em Pentatomomorpha os poucos estudos sobre os sítios de DNAr 18S apontam a presença de dois sinais localizados nos autossomos (*Camptischium clavipes* e *Holhymenia rubiginosa*) ou nos cromossomos

sexuais (*Graphosoma lineatum*) (CATTANI et al., 2004, BRESSA et al., 2008; GROZEVA et al., 2011). Uma característica observada em diferentes grupos de heterópteros é a diferença na intensidade do sinal dentro dnos cariótipos. Isso foi relatado para *Triatoma matogrossensis*, *Triatoma rubrovaria* (Reduviidae, Cimicomorpha) e para *Holhymenia rubiginosa* (Coreidae, Pentatomomorpha) (BRESSA et al., 2008; BARDELLA et al., 2010). Esses casos provavelmente refletem a variação no número de cópias do DNAr, os quais podem ser originados por eventos de crossing-over desigual ou rearranjos cromossômicos (BARDELLA et al., 2010).

1.3.2. DNA satélite e heterocromatina

Embora os estudos envolvendo DNA satélites na subordem Heteroptera (Hemiptera) seja ausente, existem abordagens sobre o tema na subordem Sternorrhyncha (Hemiptera), sobretudo em Aphidoidea (pulgões). Nos afídeos, a distribuição heterocromática, detectada pela técnica de bandamento-C, ocorre nas regiões terminais dos autossomos e/ou nos cromossomos sexuais. Em raros casos foram observadas em regiões cromossômicas intersticiais (MANICARDI et al., 1991; BIZZARO et al., 1999; CRINITI et al., 2005).

A partir da década de 90, foram intensificados os estudos visando reconhecer a natureza das famílias de DNA repetitivo que compõem os DNAs satélites de insetos nos Aphididae. A diversidade nas bandas heterocromáticas no cromossomo X de *Megoura viciae* foi evidenciada por Bizzaro et al. (1996) por meio da clivagem do DNA genômico de *Megoura viciae* com *Eco*RI. Neste experimento foi obtido um *repeat* com 600 pb de comprimento localizado na região intercalar do cromossomo X, o qual também apresentou uma banda rica em AT e uma banda associada à Região Organizadora Nucleolar (RON). Em *M. persicae*, diferentemente, foi reportada uma sequência com 169 bp de comprimento que foi comum a região subtelomérica de todos os cromossomos (SPENCE et al.,1998). Outra

sequência com 177 bp de comprimento, obtida após a clivagem com *Dra*I, foi específica para a região intercalar do cromossomo X em *Rhopalosiphum padi*, Aphididae, a qual possibilitará a diferenciação de populações nesta espécie (MONTI et al., 2010).

O padrão heterocromático mais observado nos heterópteros é a localização de bandas nos terminais cromossômicos (PAPESCHI; BRESSA, 2006). Esse perfil ocorre em diferentes famílias, como em Pentatomidae (Pentatomomorpha), Coreidae (Pentatomomorpha), Reduviidae (Cimicomorpha) e Belostomatidae (Nepomorpha) (REBAGLIATI et al., 2003; BRESSA et al., 2005; PANZERA et al., 2010; CHIRINO et al., 2013). A localização de pequenos *dots* heterocromáticos intersticiais em autossomos ou nos cromossomos sexuais também foi observada. Os *dots* heterocromáticos são muitas vezes associados às Regiões Organizadoras Nucleolares (RONs) (CAMACHO et al., 1985; GONZÁLEZ-GARCÍA et al., 1996). Já as bandas intersticiais localizadas nos autossomos são raras nos heterópteros, com relatos apenas nos coreídeos *Spartocera batatas* e *Holhymenia rubiginosa* (FRANCO et al., 2006, BRESSA et al., 2008).

Embora exista a descrição da localização heterocromática em diferentes grupos, pouco é conhecido sobre a natureza das bandas descritas. Nos casos de acúmulo de heterocromática nas regiões terminais dos cromossomos, as bandas são caracterizadas como $DAPI^+/CMA_3^+$, como em *Edessa meditabunda*, Pentatomidae, Pentatomomorpha (REBAGLIATI et al., 2003), ou apenas ricas em AT, como em *Phthia picta*, Coreidae, Pentatomomorpha (BRESSA et al., 2005). A composição das bandas terminais de *T. infestans* é um caso peculiar por exibir regiões CMA_3^+adjacentes as bandas terminais $DAPI^+$ (BARDELLA et al., 2010). Já os *dots* heterocromáticos associados as RONs são caracterizados como CMA_3^+, com exceção de *Carlisis wahlbergi* (Coreidae, Pentatomomorpha) e *T. infestans melanosoma* nas quais os *dots* foram $DAPI^+$ (FOSSEY; LIEBENBERG, 1995; BARDELLA et al., 2010).

1.3.3. Elementos transponíveis

Os elementos transponíveis compreendem a diversos arranjos de sequências de DNA com capacidade de movimento, direto ou indireto, no genoma (JURKA et al., 2007; FEDOROFF, 2012). Eles podem ser organizados em autônomos e não-autônomos. Os autônomos sintetizam todas as enzimas características de suas famílias e são auto-suficientes quanto a transposição, enquanto que um elemento não autônomo utiliza as proteínas sintetizadas pelos elementos autônomos relativos ou similares (JURKA el al., 2007). Os elementos transponíveis são ainda divididos quanto ao modo de transposição em retrotransposons ou transposons de DNA. Os retrotransposons utilizam um intermediário de RNA para a transposição. O RNA intermediário é transcrito de um elemento transponível (DNA), e então pela transcriptase reversa novamente é codificado em DNA. Este é inserido no DNA genômico por meio das nucleases e integrases, sintetizadas pelos elementos autônomos (JURKA et al., 2007). Os retroelementos podem ser divididos em: i) LTR retrotransposons (*LTR*: *long terminal repeat*), ii) não-LTR retrotransposons, iii) *Penelope* e iv) *DIRS* (JURKA et al., 2007; KAPITONOV; JURKA, 2008).

Os transposons de DNA, diferentemente dos retrotransposons, não utilizam RNA intermediários para mobilização de uma região cromossômica para outra de seu material genético. Além disso, a maioria dos DNA transposons é flanqueada por *target site duplications* (TSDs), os quais são resultantes do processo de inserção do transposons de DNA no genoma hospedeiro (JURKA et al., 2007). Esses elementos transponíveis são divididos em: *i*) transposons *cut-and-paste*, *ii*) *Helitrons* e *iii*) Polintons, os quais, respectivamente, apresentam os mecanismos de transposição *cut-and-paste* (CRAIG, 1995), *rolling-cincle replicative* (KAPITONOV; JURKA, 2001) e *self-synthesizing* (KAPITONOV; JURKA, 2006).

Os Polintons, também conhecidos como *Mavericks* (PRITHAM et al., 2007), possuem extensão de 15-20 kb, 6 bp formando a TSDs, 100 a 1000 bp de *terminal inverted repeats* (TIRs) em ambos os terminais, com pequenas sequências repetidas *in tandem* (KAPITONOV; JURKA, 2006). As repetições estão relacionadas com o mecanismo de *sliding-back* da polimerase B (POLB), a qual necessita da leitura de pelo menos duas timinas para iniciar o processo de replicação do Polinton (MÉNDEZ et al., 1992; KAPITONOV; JURKA, 2006; PRITHAM et al., 2007)

2. Justificativa

Estima-se que haja aproximadamente um milhão as espécies de insetos (GALLO et al., 2002). Dentre esses, estão os heterópteros, que fornecem um material biológico rico para o estudo dos cromossomos holocêntricos. Há espécies de heterópteros fitófagos, como *Dichelops melacanthus, Nezara viridula* e *Corecoris fuscus*e, que são alvos de estudos diversos, sobretudo por serem consideradas pragas em lavouras de trigo, soja e arroz, respectivamente, bem como os hematófagos (SCHOFIELD, 2000; GALVÃO, 2009), os quais são vetores da doença de Chagas (COURA; DIAS, 2009). Embora este grupo de insetos seja muito numeroso, os estudos citogenéticos relacionados até o momento são em sua maior parte limitados à contagem cromossômica, sendo poucos os estudos relacionados à organização e o mapeamento físico de DNA repetitivos. Tendo em vista isso mais estudos são necessário para a compreensão da organização genômica nestes insetos.

3. Objetivos

Analisar comparativamente a estrutura cariotípica, bem como a ocorrência e a distribuição de sequências repetitivas nos cromossomos holocinéticos em heterópteros, e utilizá-las para estudar o comportamento dos cromossomos holocinéticos, sobretudo quanto aos aspectos envolvendo a evolução cariotípica destes insetos.

3.1. Objetivos específicos

(a) Descrever o número cromossômico, sistema cromossômico do sexo e a localização de regiões heterocromáticas em 25 espécies pertencentes à infraordem Pentatomomorpha;

(b) Localizar os sítios de DNAr 18S em 25 espécies pertencentes à infraordem Pentatomomorpha;

(c) Descrever o número cromossômico, sistema cromossômico do sexo, a localização das regiões heterocromáticas e os sítios de DNAr 18S em 5 espécies pertencentes à família Reduviidae (Cimicomorpha);

(d) Isolar, caracterizar e mapear fisicamente famílias de DNAs satélites em *Triatoma infestans*, e propor a origem de famílias de DNA repetitivo.

4.1 Capítulo 1

High diversity in CMA_3/DAPI-banding patterns in heteropterans

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High Diversity in CMA_3/DAPI-Banding Patterns in Heteropterans

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Key Words

CMA_3/DAPI banding · Chromosome number · Heteroptera · Holokinetic chromosomes · Repetitive DNA

Abstract

Heteroptera is the most numerous and diverse suborder of Hemiptera, with about 38,000 species. This diversity also involves cytogenetic features, including chromosome number and a sex determining system. Information about heterochromatin occurrence and distribution is scarce in heteropterans, but still, there is some evidence of variability. We determined the chromosome number and CMA_3/DAPI-banding pattern of 179 individuals of 25 heteropteran species from Brazil. Eight species of Pentatomidae exhibited a constant chromosome number (2n = 12 + XY), but in Coreidae (12 species), Largidae (1 species), Rhopalidae (1 species), and Pyrrhocoridae (3 species), the numbers ranged from 2n = 10 + 2m + X0 to 2n = 24 + 2m + X0. Although there were no large differences in the chromosome size between species, the CMA_3/DAPI-banding patterns differed markedly. Among the genera, species of *Edessa, Spartocera, Hypselonotus, Phtia, Holhymenia* and *Euryophthalmus* showed a large accumulation of heterochromatin, while the other species exhibited few or no heterochromatic bands. In general, when heterochromatin was more accumulated, this occurred preferentially at terminal positions, except in *Holhymenia histrio*, which exhibited intercalary bands. This study made it possible to identify some chromosome rearrangements and to enhance our knowledge of the evolutionary mechanisms that determine karyotype differentiation in Heteroptera.

Heteroptera is considered the largest group of Hemiptera, with about 38,000 species organized in 80 families [Weirauch and Schuh, 2011]. These insects have a wide geographical distribution [Resh and Cardé, 2009] and diversity in karyotype structure, with numbers varying from 2n = 4 (*Lethocerus*, Belostomatidae) to 2n = 80 (4 species of *Lopidea* Uhler, Miridae) [Ueshima, 1979]. This diversity can be attributed to the occurrence of holokinetic chromosomes, which tolerate changes arising from chromosome fusions and fissions [Hughes-Schrader and Schrader, 1961].

Pentatomomorpha, particularly the families Coreidae, Lygaeidae and Pentatomidae, shows chromosome number variation from 2n = 13–28 in Coreidae [Ueshima, 1979; Bressa et al., 2008], from 2n = 10–30 in Lygaeidae and from 2n = 6–27 in Pentatomidae [Ueshima, 1979; Papeschi and Bressa, 2006]. The numbers can also vary according to changes in the sex and m chromosomes

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[Ueshima, 1979; Papeschi and Bressa, 2006]. The m chromosomes are especially interesting because they show differential meiotic behavior (achiasmatic/inverted meiosis) and are frequently small and weakly staining when compared with other chromosomes [Wilson, 1905; Papeschi and Bressa, 2006].

Although few heteropterans have been studied by chromosome banding, a large variation in heterochromatin distribution has been detected. In the Pentatomidae, the majority of species show only one CMA_3^+ heterochromatic dot [González-García et al., 1996; Papeschi et al., 2003]. On the other hand, some species of Belostomatidae and Coreidae have large heterochromatic bands located especially in terminal chromosome regions [Bressa et al., 2005]. Two intriguing cases are *Spartocera batatas* (Coreidae), which displays terminal and intercalary bands [Franco et al., 2006], and *Triatoma infestans melanosoma*, which shows terminal AT-rich bands adjacent to more internal GC-rich ones in the 3 large autosomal pairs [Bardella et al., 2010].

Given the wide variation in banding patterns in heteropterans, the aim of this study was to compare the karyotypes of 25 species according to the occurrence and distribution of CMA_3/DAPI bands as well as to propose mechanisms of amplification/reduction of heterochromatin in representatives of 5 families of Pentatomomorpha.

Material and Methods

Biological Material

Twenty-five species of Heteroptera belonging to the families Pentatomidae (8 species), Coreidae (12), Pyrrhocoridae (3), Rhopalidae (1), and Largidae (1) were collected in South and Southeast Brazil. Information about the number of individuals, localities and collection numbers is shown in online supplementary table 1 and figure 1 (see www.karger.com/doi/10.1159/000355214 for all online suppl. material).

Chromosome Preparation

Only male gonads were dissected, and the seminiferous tubules were fixed in methanol-acetic acid (3:1, v:v) and stored at –20°C. Seminiferous tubules were first incubated in 45% acetic acid for 10 min, and slides were then prepared by dissection and squash in a drop of 45% acetic acid. The coverslips were removed after freezing the slides in liquid nitrogen, and they were then air-dried.

Chromosome Banding

For chromosome banding [Sumner, 1982, with small modifications], slides were aged for 3 days after removal of coverslips. Afterwards, the slides were incubated in 0.2 N HCl for 10 min at room temperature, 5% barium hydroxide at 60°C for 2 min, and 2× SSC, pH 7.0, at 60°C for 60 min. Slides were treated with fluorochromes: 0.5 mg/ml CMA_3 for 1.5 h and 2 μg/ml DAPI for 30 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer, pH 7.0 (1:1, v:v), plus 2.5 mM $MgCl_2$. Images were captured in grayscale. After washing the slides in SSC buffer, pH 7.0, and post-fixation in methanol-acetic acid (3:1, v:v) solution, slides were stained with 2% Giemsa and permanently mounted using Entellan (Merck).

Images

All images were acquired using a Leica DM 4500 B microscope, equipped with a DFC 300FX camera. For DAPI/CMA_3 banding, the images were pseudocolored (blue for DAPI and greenish-yellow for CMA_3) and overlapped using the Leica IM50 4.0 software. The images were optimized for best contrast and brightness with iGrafx Image software.

Results

Pentatomidae

All 8 species of 5 genera of Pentatomidae showed 2n = 12A + XY, with autosomes decreasing gradually in size (fig. 1A, D, G, J, M; table 1). CMA_3/DAPI banding revealed a large amount of heterochromatin in species of *Edessa* (fig. 1B, C, E, F). These species always exhibited terminal $DAPI^+$ and CMA_3^+ bands, which varied substantially in amount. *Edessa meditabunda* showed CMA_3^+ bands at both chromosome ends in almost all autosomal pairs (fig. 1B, C). In contrast, *E. rufomarginata* (fig. 1E, F) and *E. impura* showed only 1 terminal CMA_3^+ band on 1 autosomal pair, but we could not distinguish it due to similarity in size between chromosome pairs.

The other species of Pentatomidae exhibited only small CMA_3^+ dots on the autosome pairs (fig. 1H, I, K, L, N, O). Except for *Euschistus heros* (fig. 1N, O), in which the dot was intercalary, the other 4 species showed 1 CMA_3^+ dot terminally located at one end. A representative of *E. heros* collected in Maringá, PR, Brazil, also showed a polymorphism involving the CMA_3^+ dot (fig. 1O).

The sex chromosomes differed in size in all species except *Arvelius albopunctatus* (fig. 1J). In *E. heros*, only one sex chromosome appeared totally $DAPI^+$ (fig. 1N), whereas in *A. albopunctatus,* one sex chromosome was totally $DAPI^+$ and the other CMA_3^+ (fig. 1K, L). The opposite was observed in *E. meditabunda* and *E. rufomarginata*, where $DAPI^+$ bands were detected at both ends of sex chromosomes (fig. 1B, C, E, F). Images for other species can be found in online supplementary figures 2 and 5.

Coreidae

The chromosome number ranged from 2n = 19–27, with a gradual decrease in chromosome size (figs. 2A, G, 3A, D, G; table 1), except for *H. interruptus* (fig. 2D),

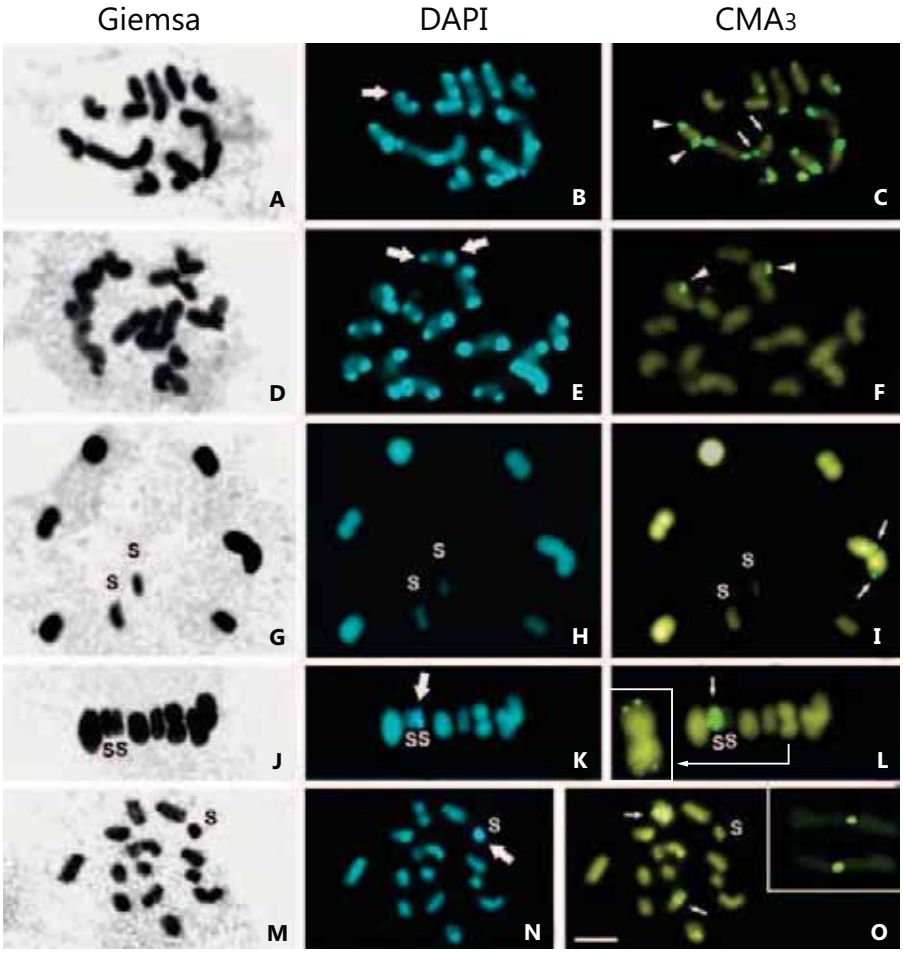

Fig. 1. Karyotype comparison between species of Pentatomidae using Giemsa conventional staining and DAPI/CMA_3 banding. **A–C** Mitotic metaphase in *E. meditabunda* showing the $DAPI^+$, CMA_3^+ and $DAPI^+/CMA_3^+$ bands. **D–F** Mitotic metaphase in *E. rufomarginata*. Note the occurrence of $DAPI^+$ and $DAPI^+/CMA_3^+$ bands. **G–I** Metaphase I in *Antiteuchus tripterus*. Observe the presence of terminal CMA_3^+ dots in one autosomal pair. **J–L** Metaphase I in *A. albopunctatus* showing terminal CMA_3^+ dots (**inset**) and one sex chromosome totally $DAPI^+$ and the other completely CMA_3^+. **M–O** Mitotic metaphase in *E. heros*. Note the intercalary CMA_3^+ dots in one autosomal pair, which was confirmed in diplotene (**inset**), and one sex chromosome totally $DAPI^+$. $DAPI^+$ = Large arrows (pseudocolored in blue); CMA_3^+ = small arrows (pseudocolored in greenish-yellow); $DAPI^+/CMA_3^+$ = arrowheads; S = sex chromosomes. Scale bar = 5 μm.

which showed 3 autosomal pairs larger than other autosomes, and for *A. hahni* and *Cebrenis* sp., which showed one pair larger than the other bivalents (online suppl. fig. 3G, J). In general, chromosome banding exhibited large contrast in the amount and location of heterochromatin in these 12 species of Coreidae (table 1). *Phtia picta* (fig. 2B, C) and *H. fulvus* showed terminal CMA_3^+/$DAPI^+$ bands on both chromosome ends in all autosomes. In *H. interruptus* (fig. 2E, F) and in *S.* cf. *fusca*, most autosomes exhibited terminal bands. *H. histrio* was highlighted by the presence of $DAPI^-/CMA_3^+$ bands in the intercalary position of 11 autosomes and in the terminal positions of 2 chromosomes, but it showed intraspecific variation in band number and size (fig. 2H, I). Chromosome banding in diplotene of *H. histrio* showed 2 heteromorphic bivalents with regard to the presence of a CMA_3^+ band (fig. 2K, L). For *L. zonatus* (fig. 3B, C) and *A. hahni,* only CMA_3^+ dots were detected. For other species, *A. haematicus* (fig. 3H, I), *Cebrenis* sp., *A. obscurator*, and *C. armatus*, bands were not visualized.

The sex determination system was X0, except for *S.* cf. *fusca* exhibiting X_1X_20. In all species, the X chromosome varied in size from intermediate to small, except for *H. histrio*, where it was larger than the autosomes (fig. 2G, J). This sex chromosome also showed one intercalary $DAPI^-/CMA_3^+$ band, whose pattern was different from that of other sex chromosomes of Coreidae (fig. 2H, I). Terminal $DAPI^+/CMA_3^+$ bands were found on the sex chromosomes of *P. picta*, *H. fulvus* and *S.* cf. *fusca* (fig. 2B, C; online suppl. fig. 3B, C, E, F), while in *H. interruptus,* the sex chromosome was totally $DAPI^+$/CMA_3^+ (fig. 3E, F).

Species of Coreidae showed m chromosomes of different sizes (figs. 2, 3; table 1), except for *A. hahni* in which an m chromosome was not detected (online suppl. fig. 3G, I). *P. picta* (fig. 2B, C), *S.* cf. *fusca* and *H. fulvus* displayed

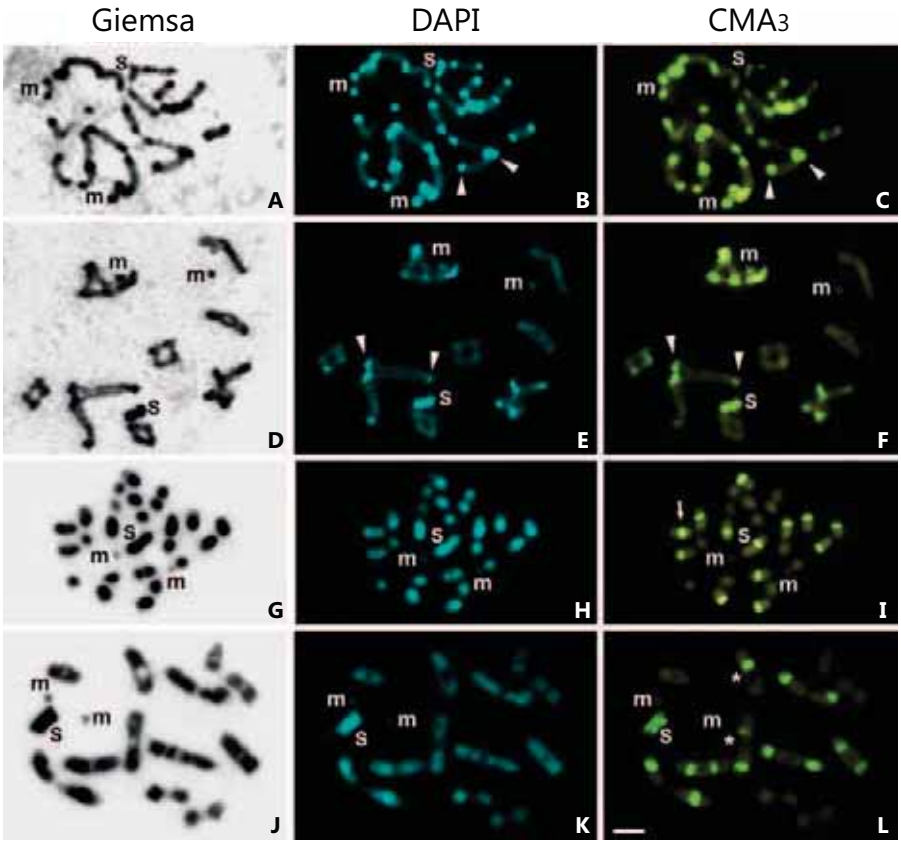

Fig. 2. Karyotype comparison between species of Coreidae using Giemsa conventional staining and DAPI/CMA_3 banding. **A–C** Mitotic metaphase in *P. picta* showing $DAPI^+/CMA_3^+$ bands on all chromosomes. **D–F** Diplotene in *H. interruptus* indicating the presence of terminal $DAPI^+/CMA_3^+$ bands on 4 autosomal pairs and the sex chromosome totally $DAPI^+/CMA_3^+$. **G–I** Mitotic metaphase in *H. histrio*. Note the occurrence of intercalary CMA_3^+ bands on 11 chromosomes. **J–L** Diplotene in *H. histrio*: with 2 heteromorphic bivalents for the intercalary CMA_3^+ bands (asterisk). S = Sex chromosome; m = m chromosome. Scale bar = 5 μm. For further details see figure 1.

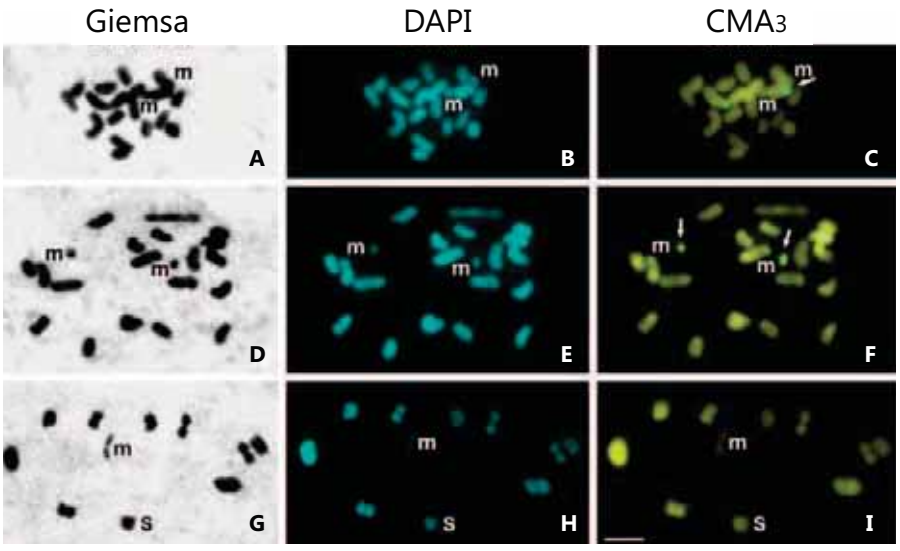

Fig. 3. Karyotype comparison between species of Coreidae using Giemsa conventional staining and DAPI/CMA_3 banding. **A–C** Mitotic metaphase in *L. zonatus* showing terminal CMA_3^+ band on one autosomal pair. **D–F** Mitotic metaphase in *L. gonagra*. Note the presence of CMA_3^+ bands on the m chromosomes. **G–I** Metaphase I in *A. haematicus* without detectable bands. Scale bar = 5 μm. For further details see figure 1.

Table 1. Summary of chromosome number, occurrence and distribution of CMA_3/DAPI bands in the 25 heteropterans analyzed

Family/species	2n	Autosomes		Sex chromosomes		m chromosomes		Figures
		DAPI	CMA_3	DAPI	CMA_3	DAPI	CMA_3	
Pentatomidae								
Antiteuchus tripterus (Fabricius, 1787)	14	0	+	0	0	W	W	1
Euschistus cornutus (Dallas, 1851)	14	0	+	0	0	W	W	SF 2
Euschistus heros (Fabricius, 1798)	14	0	+	+	0	W	W	1, SF 5
Arvelius albopunctatus (De Geer, 1773)	14	0	+	+	+	W	W	1
Mormidea v-luteum (Lichtenstein, 1796)	14	0	+	0	0	W	W	SF 2
Edessa rufomarginata (De Geer, 1773)	14	+++	+	+	0	W	W	1, SF 5
Edessa impura (Bergroth, 1891) [Det. Fernandes-UFPA]	14	+++	+	+	0	W	W	SF 2, 5
Edessa meditabunda (Fabricius, 1784)	14	+++	+++	+	+	W	W	1, SF 5
Coreidae								
Spartocera cf. *fusca* (Thunberg, 1783) (= *Corecoris fuscus*)	24	+++	+++	+	+	+	+	SF 3, 5
Chariesterus armatus (Thunberg, 1825)	25	0	0	0	0	0	0	2, SF 5
Leptoglossus gonagra (Fabricius, 1775)	21	0	0	0	0	0	+	3, SF 5
Leptoglossus zonatus (Dallas, 1852)	21	0	+	0	0	0	0	3, SF 5
Phtia picta (Drury, 1770)	21	+++	+++	+	+	+	+	2, SF 5
Athaumastus haematicus (Stål, 1860)	21	0	0	0	0	0	0	SF 3
Holhymenia histrio (Fabricius, 1803)	27	0	+++	0	+	0	0	2
Hypselonotus interruptus (Hahn, 1831)	19	++	++	+	+	0	0	SF 3
Hypselonotus fulvus (De Geer, 1775)	19	+++	+++	+	+	+	+	2
Acanonicus hahni (Stål, 1860)	19	0	+	0	0	W	W	SF 3
Cebrenis sp.	23	0	0	0	0	0	0	SF 3
Althos obscurator (Fabricius, 1803)	25	0	0	0	0	0	0	SF 3
Rophalidae								
Harmosthes prolixus (Stål, 1860)	13	0	+	0	0	+	+	SF 4, 5
Largidae								
Euryophthalmus rufipennis (Laporte, 1832)	13	+++	+++	+	+	W	W	SF 4, 5
Pyrrhocoridae								
Dysdercus ruficollis (Linnaeus, 1758)	13	0	+	0	0	W	W	SF 4
Dysdercus imitator (Blöte, 1931)	13	0	0	0	0	W	W	SF 4
Dysdercus fulvoniger (De Geer, 1773)	13	0	+	0	0	W	W	SF 4, 5

SF = Supplementary figures; W = without m chromosome; 0 = without bands; + = 1 bivalent pair with a band; ++ = 2–6 bivalents with bands; +++ = all bivalents with 7–14 chromosomes with bands.

terminal $DAPI^+/CMA_3^+$ bands on the m chromosomes. Interestingly, the only visible band in the metaphase spread of *L. gonagra* was a terminal dot on the m chromosome (fig. 3E, F). Images for other species can be found in online supplementary figures 3 and 5.

Rhopalidae, Largidae and Pyrrhocoridae

The karyotype information of these 3 families was grouped due to the small number of analyzed species. *E. rufipennis* (Largidae) showed 2n = 12A + X0 (online suppl. figs. 4A, 5K) with one pair larger than the other bivalents and with terminal $CMA_3^+/DAPI^+$ bands on all chromosome pairs (online suppl. fig. 4B, C). *Harmostes prolixus* (Rhopalidae) showed 2n = 10A + 2m + X0 and only one terminal CMA_3^+ heterochromatic dot on one autosomal pair (online suppl. figs. 4D, F, 5J). The 3 species of *Dysdercus* (Pyrrhocoridae) showed 2n = 12A + X0 (online suppl. figs. 4J, O, 5L) with terminal CMA_3^+ heterochromatic dots on one autosomal pair of *D. imitator* and *D. fulvoniger* (online suppl. fig. 4H, I, K, L). However, *D. ruficollis* exhibited chromosomes without heterochromatic bands (online suppl. fig. 4N, O). Images of these families can be found in online supplementary figures 4 and 5.

Discussion

Conventional Karyotypes

A few number of heteropterans have been studied by more sophisticated cytogenetic tools [Papeschi and Bressa, 2006], but they have been sufficient to show a great diversity of karyotypes between infraorders. However, they were insufficient to establish parameters such as modal number and main evolutionary lines of karyotype differentiation for all suborders. The infraorders with a higher number of studied species were Cimicomorpha, Pentatomomorpha and Nepomorpha, and in some cases, the modal numbers were accepted only for families and/or tribes [Papeschi and Bressa, 2006]. Pentatomidae is the most studied family of Pentatomomorpha, and for all 294 studied species (85%), the number 2n = 12 + XY has been conserved [Rebagliati et al., 2005]. For the 4 species of the families Largidae *(E. rufipennis)* and Pyrrhocoridae *(D. imitator, D. fulvoniger* and *D. ruficollis)* studied here, the chromosome number 2n = 12 + XO was conserved and/or agrees with the range proposed by Ueshima [1979].

The family Coreidae, unlike other families studied here, displays a larger numerical variation, from 2n = 10 + 2m + X0 in *Ceraleptus obtusus* [Xavier, 1945] to 2n = 24 + 2m + X_1X_20 in *Anisocelis foliacea* [Piza, 1945]. The more common number is 2n = 18 + 2m + X0 [Ueshima, 1979; Papeschi and Bressa, 2006]. Besides this number, our data show a variation from 2n = 16 + 2m + X0 to 2n = 24 + 2m + X0. Many of these numerical variations found in heteropterans have been attributed to dysploidy events, such as fragmentation and fusion of holocentric chromosomes [Ueshima, 1979]. Thomas [1987] has suggested that nondisjunction of holocentric chromosomes is the main reason for chromosome number variation in heteropterans. However, Guerra [2008] suggested that organisms with holocentric chromosomes seem to be more prone to dysploidy than aneuploidy. We did not observe any evidence of nondisjunction in these heteropterans, and only diploids without evidence of univalents and/or homomorphic trivalents were observed at meiosis. Thus, our results do not indicate the occurrence of aneuploidy in these species.

Many of the numerical chromosome variations in heteropterans have been attributed to fragmentation of sex chromosomes, such as in *S. batatas* [Franco et al., 2006], and/or by the occurrence of m chromosomes, such as in *Pachylis argentinus* [Papeschi et al., 2003]. Although we observed fragmentation of sex chromosomes only in *S. fusca* (discussed below), m chromosomes were observed in 11 species of coreids, excluding *A. hahni*. These 'special' chromosomes were first mentioned by Wilson [1905], and later referred to by Sotero-Caio et al. [2011]. These chromosomes appeared as univalents in coreids, without pairing or chiasmata. Although the m chromosomes found here exhibited no signs of pairing, the literature shows at least one record of m bivalents in *Coreus marginatus* [Nokkala, 1986; Suja et al., 2000].

Heterochromatin Distribution

CMA_3/DAPI bands showed a large variation in the distribution (number, location and size) of heterochromatin in these 25 species of Heteroptera. We found 5 different profiles of band distribution: (i) 5 species without detectable bands; (ii) 10 species with only 1 dot in autosomes; (iii) 1 species with 1 dot on the m chromosomes; (iv) 8 species with a high accumulation of bands, and (v) 1 species with intercalary bands. Our results corroborate the findings of Bressa et al. [2005], who also reported a variability in heterochromatin occurrence and distribution in 5 species of Coreidae, including *A. haematicus* (without bands) and *L. impictus* and *P. picta* (with terminal bands). A variation in the heterochromatic distribution was also observed in other organisms with holocentric chromosomes, as observed in representatives of Psocoptera and Odonata [Golub et al., 2004; De Gennaro et al., 2008].

E. heros (Pentatomidae) was interesting because this species showed a polymorphism in a CMA_3^+ band, which could be associated with differences in amplification/deletion of repeats between homologues. We also observed that in *E. cornutus* the position of a CMA_3^+ dot was terminal, while in *E. heros* this dot was intercalary. This is some evidence of the importance of chromosome rearrangements in Pentatomidae, even when compared with large rearrangements described for the Reduviidae *T. infestans* [Poggio et al., 2013].

Species that showed large accumulation of bands belong to Pentatomidae, Coreidae and Largidae. Among Pentatomidae, only species of *Edessa* accumulated bands. *E. meditabunda* showed CMA_3/DAPI terminal bands, while *E. rufomarginata* and *E. impura* showed predominantly DAPI terminal bands. Rebagliati et al. [2003] also reported the occurrence of terminal CMA_3/DAPI bands in all chromosomes of *E. meditabunda* and *E. rufomarginata*, but their results were different from ours, especially in relation to CMA_3^+ bands in *E. rufomarginata*. *Euryophthalmus rufipennis* (Largidae) and *P. picta*, *S.* cf. *fusca*, *H. interruptus* and *H. fulvus* (Coreidae) showed the same band distribution as in *E. meditabunda*. These band profiles are another indication of the immense variability involving heterochromatin, and they show that the Pen-

tatomidae, Coreidae and Largidae share similar dynamics of amplification/reduction of this repetitive DNA family.

The accumulation of intercalary bands, which are uncommon among Coreidae, was observed here only in *H. histrio* (CMA_3 bands). Intercalary bands were described also for *S. batatas* [Franco et al., 2006] and *Holhymenia rubiginosa* [Bressa et al., 2008], but only this last species exhibited a large number of bands equilocally arranged. Additionally, *H. histrio* showed heteromorphic bands on 2 bivalents, in 5 samples of a Londrina-PR population. Bressa et al. [2008] also reported different polymorphisms involving the size, number and position of heterochromatic bands on different chromosome pairs of *H. rubiginosa*. This suggests that these rearrangements could be common in the genus *Holhymenia*.

In general terms, when heterochromatin appears abundantly accumulated in the chromosomes of heteropterans, it appears equilocally distributed, either in terminal regions in Pentatomidae, Coreidae or Largidae, or in intercalary positions, such as in *H. histrio* (Coreidae). We cannot explain this equilocality on the basis of the Rabl configuration due to the lack of primary constriction of holocentrics, but the proposal of Guerra [2000] for the equidistribution of heterochromatin based on the receptivity of some regions (structural or functional) to the accumulation and/or amplification of repetitive DNA sequences seems to be reasonable. At least for the terminal bands, the equilocal dispersion could be explained by the proximity of telomeres to the nuclear envelope in interphase to organize the 'bouquet polarization'. According to Rodríguez-Iñigo et al. [1996], it could provide a cytological basis for the satellite DNA homogenization on both homologous and non-homologous chromosomes. However, there is other evidence that heterochromatin in holocentrics does not disperse following the equilocal model as in Aphidae (Hemiptera) [Criniti et al., 2005].

Characterization of Sex Chromosomes

Three sex determining systems were observed for the 25 species of heteropterans studied here: XY/XX, XX/X0 and X_nX_n/X_n0. These results are consistent with the literature, which describes the XX/XY, XX/X0, X_nX_n/X_nY_n and neo-XneoY systems [Ueshima, 1979; Franco et al., 2006; Bressa et al., 2009]. Pentatomidae always exhibited the XY system, with a clear difference in the size of these chromosomes, except for *A. albopunctatus*, which showed sex chromosomes of similar size. We drew our attention to this species because one sex chromosome was $DAPI^+$ while the other was CMA_3^+. Among the other pentatomids, only *E. heros* showed one sex chromosome totally $DAPI^+$. Although Camacho et al. [1985], González-García et al. [1996] and Lanzone and Souza [2006] observed that in some Pentatomidae the X chromosome is always larger than Y, mainly on the basis of studies of male and female karyotypes, we found that the difference in size was not large enough to assume which one was X or Y, especially in *A. albopunctatus*. Moreover, there was a large difference in the occurrence and distribution of heterochromatin in these sex chromosomes with at least 3 distinct situations: (i) without any band, (ii) with only $DAPI^+$ bands, and (iii) with only CMA_3^+ bands. This heterochromatic diversity could lead to mistakes in sex chromosome determination (X or Y).

The coreids, rophalids, pyrrhocorids and largids showed the X0 determining system, except for *S. fusca* (Coreidae). In general, sex chromosomes were smaller than autosomes. Besides the X chromosome of *H. histrio*, the sex chromosomes of other species seem to follow the trend of intermediate to small size in relation to autosomes [Papeschi et al., 2003; Lanzone and Souza, 2006; Bressa et al., 2008].

Our findings also indicate that there is extensive variation in the sex chromosomes with regard to size and organization, which can include chromosomes of similar size or not and the presence or absence of CMA_3 and DAPI bands. Thus, these results do not allow the discrimination between X or Y sex chromosomes, except in cases where males and females are studied separately and with evident chromosome markers for each of these.

Conclusions

Our findings in 25 species of 5 families of heteropterans demonstrate that the chromosome number is variable, from 2n = 13–27, but that it can be conserved in some groups, such as the Pentatomidae with 2n = 14. Despite this conserved status, members of this family show a large variation in heterochromatin (occurrence, accumulation and distribution). When we compared Pentatomidae with Coreidae, the latter family exhibited more varied karyotypes with regard to both chromosome number and size and/or heterochromatin distribution. In conclusion, we present a good sampling of karyotype diversity and new insights for future studies on the processes of karyotype differentiation and evolution in heteropterans.

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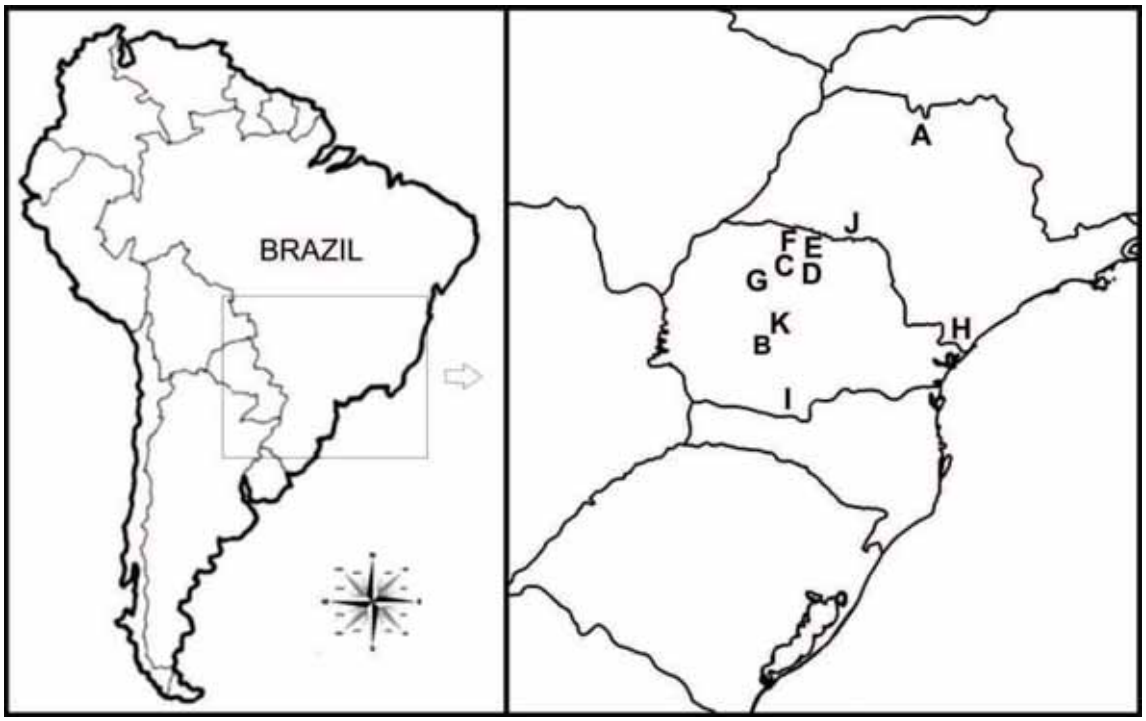
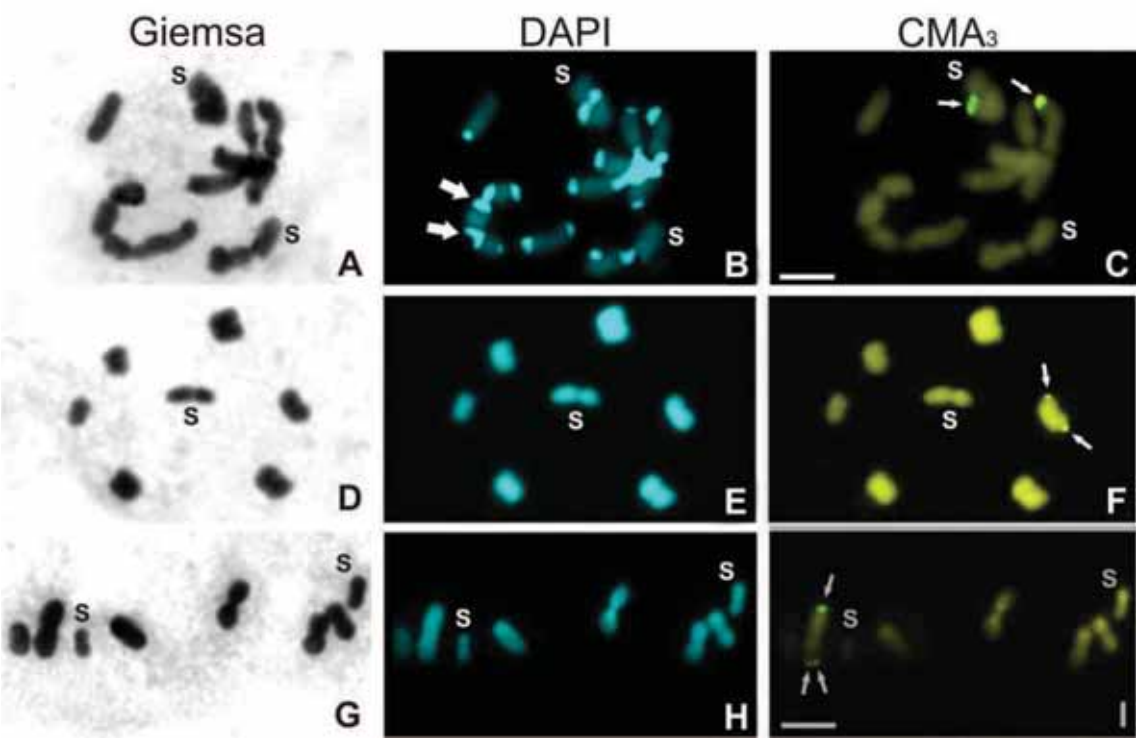
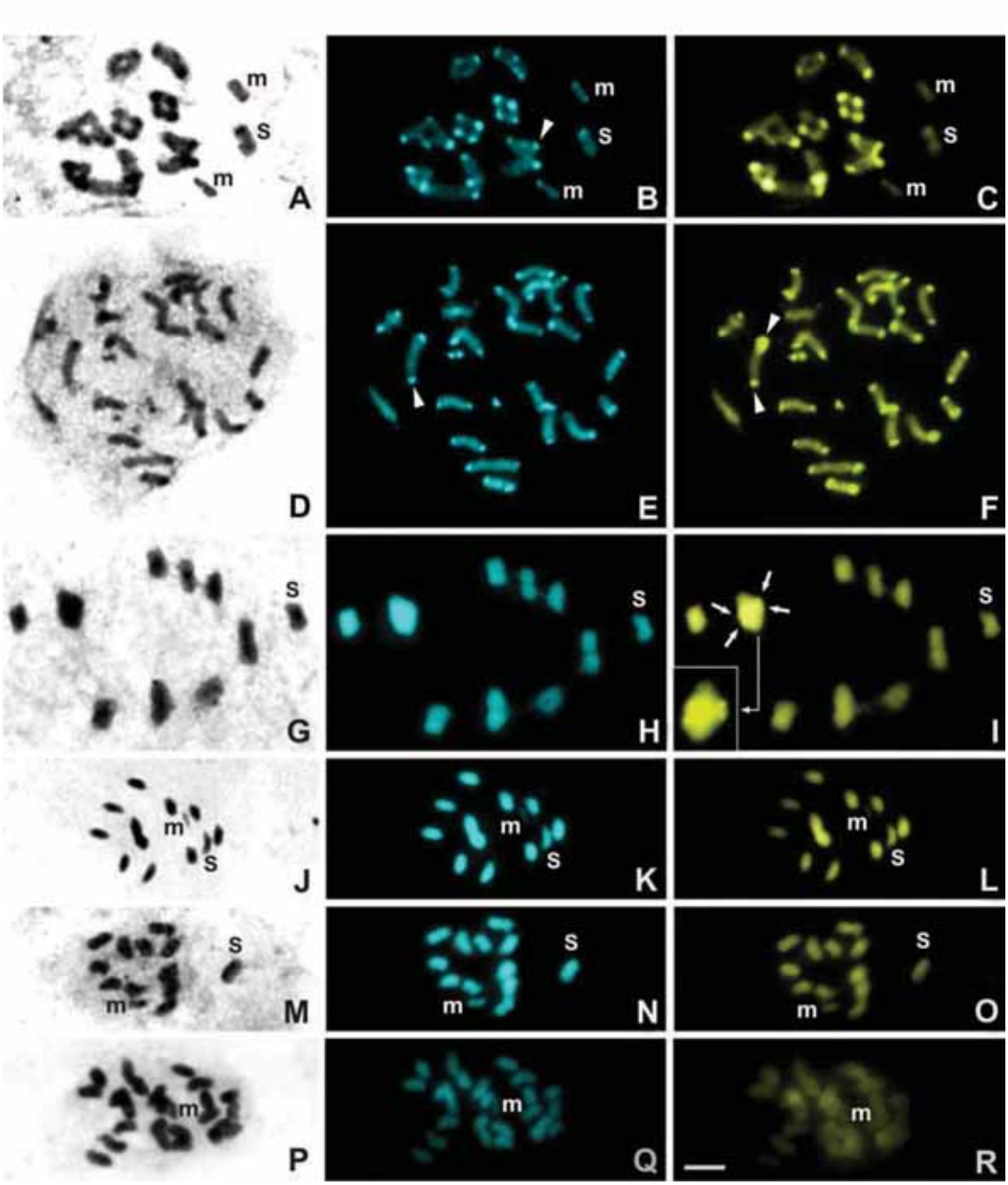


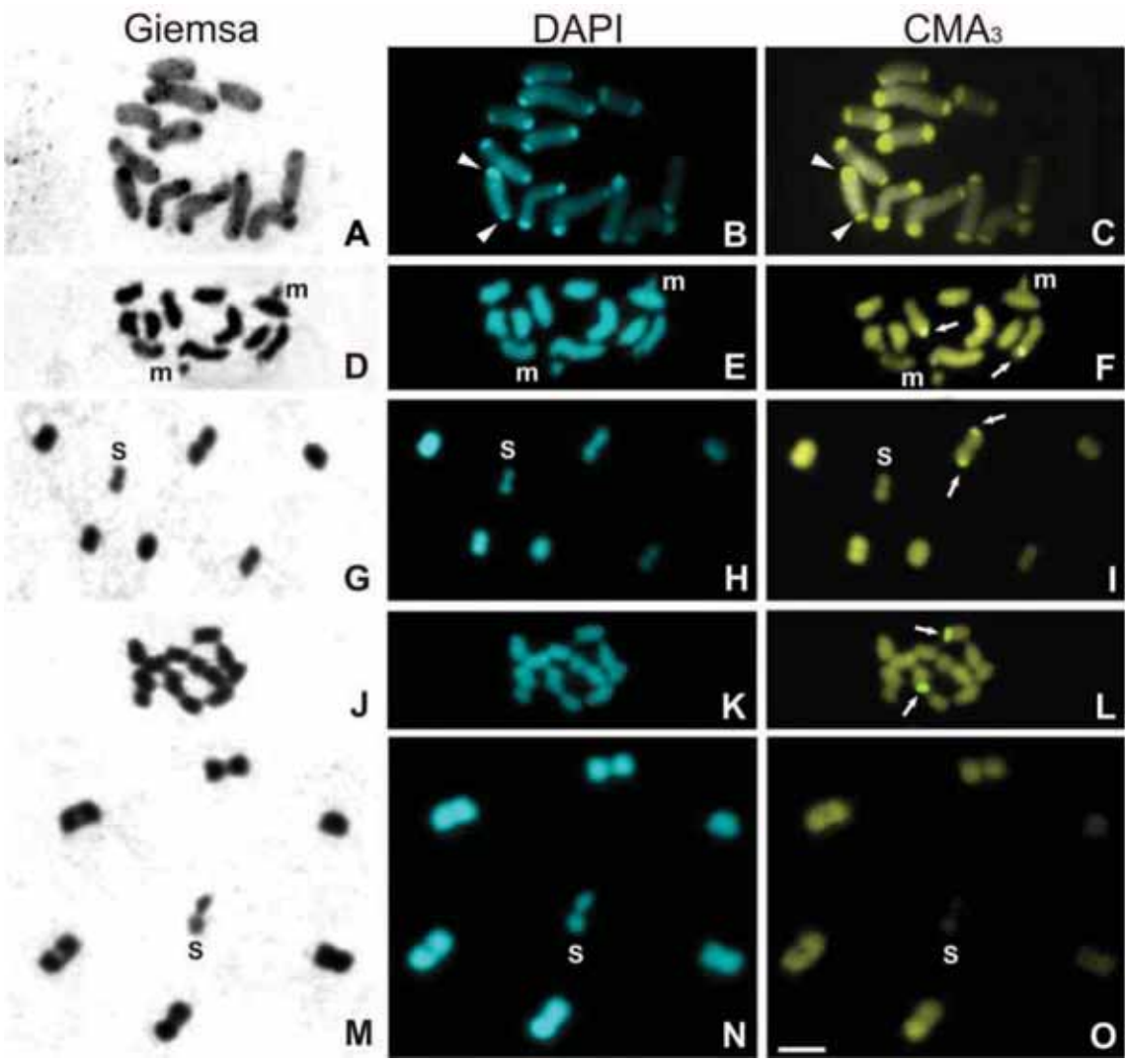
Supplementary Figure 1



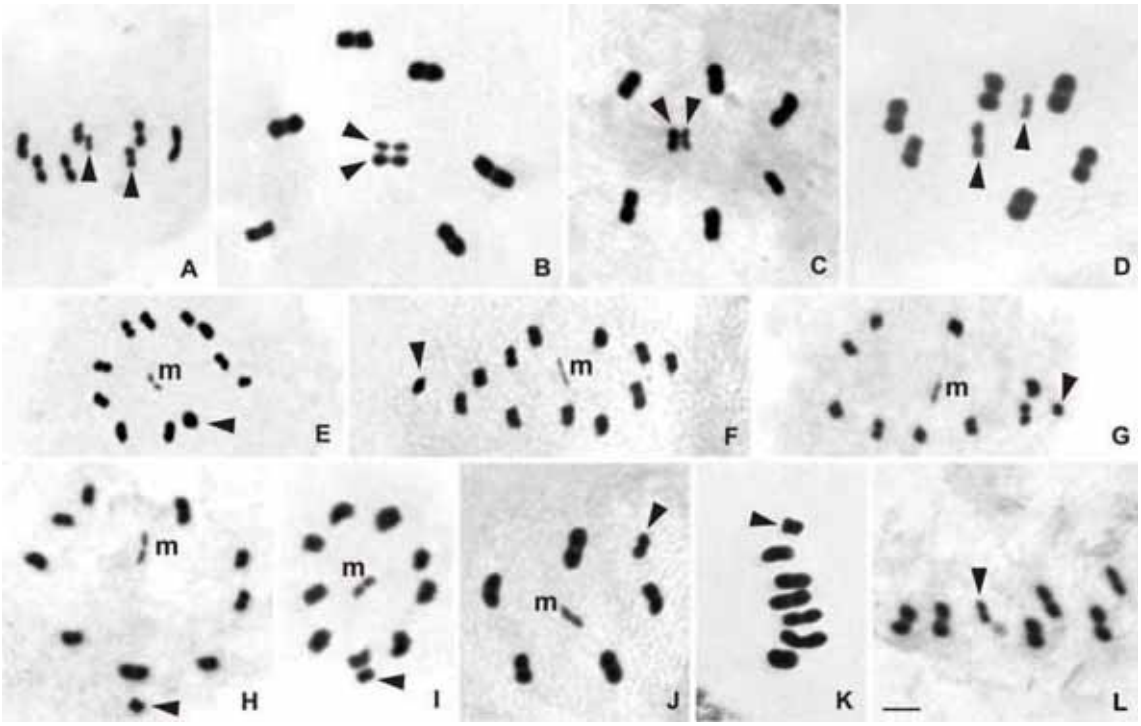
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5

Supplementary Figure 1. Map of Brazil. The letters indicate the cities where heteropterans were collected.

Supplementary Figure 2. Karyotype comparison between species of Pentatomidae using Giemsa conventional staining and C-DAPI/CMA banding. A-C) Mitotic metaphase in *Edessa impura.* Note the occurrence of a terminal $DAPI^+$ band (large arrows) on most autosomal pairs and a terminal $DAPI^+/CMA^+$ bands on one autosomal pair (small arrows). D-F) Metaphase II in *Euschistus cornutus*. Note the occurrence of a terminal CMA^+ dots (small arrows) on one autosomal pair. G-I) Metaphase I in *Mormidea v-luteum*. Note the occurrence of a terminal CMA^+ dots (small arrows) on one autosomal pair. S indicates the sex chromosome. Bar = 5µm. (DAPI is pseudocolored in blue and CMA is pseudocolored in greenish-yellow).

Supplementary Figure 3. Karyotype comparison between species of Coreidae using Giemsa conventional staining and C-DAPI/CMA banding. A-C) Diplotene in *Hypselonotus fulvus*. Note the occurrence of a terminal $DAPI^+/CMA^+$ band on all chromosomes (arrowheads). D-F) Mitotic metaphase in *Spartocera* cf. *fusca*. Note the occurrence of a terminal $DAPI^+/CMA^+$ band on most chromosomes (arrowheads). G-I) Metaphase I in *Acanonicus hahni*. Note the occurrence of a terminal CMA^+ dots on one autosomal pair (small arrows). J-L) Metaphase II in *Cebrenis* sp. M-O) Metaphase II in *Althos obscurator.* P-R) Metaphase I in *Chariesterus armatus*. The three last species presented no bands. S indicates the sex chromosome and m points to the m chromosome. Bar = 5µm. (DAPI is pseudocolored in blue and CMA is pseudocolored in greenish-yellow).

Supplementary Figure 4. Karyotype comparison between species of Largidae, Rophalidae and Pyrrhocoridae using Giemsa conventional staining and C-DAPI/CMA banding. A-C) Mitotic metaphase in *Euryophthalmus rufipennis* (Largidae). Note the occurrence of a terminal $DAPI^+/CMA^+$ band on all chromosomes (arrowheads). D-F) Mitotic metaphase in *Harmostes prolixus* (Rophalidae). Note the presence of a terminal CMA^+ dot on one autosomal pair (small arrows). G-I) Metaphase I in *Dysdercus imitator* (Pyrrhocoridae). Note the presence of a terminal CMA^+ dot on one autosomal pair (small arrows). J-L) Mitotic metaphase in *Dysdercus fulvoniger*. Note the presence of a terminal CMA^+ dot on one autosomal pair (small arrows). J-L) Metaphase I in *Dysdercus ruficollis*. S indicates the sex chromosome and m points to the m chromosome. Bar = 5µm. (DAPI is pseudocolored in blue and CMA is pseudocolored in greenish-yellow).

Supplementary Figure 5. Conventional analyses in metaphase I of meiosis in different species of Heteroptera. (A) *Euschistus heros*. (B) *Edessa impura*. (C) *Edessa rufomarginata*. (D) *Edessa meditabunda*. A-D) Note the central location of sex chromosomes. (E) *Spartocera fusca*. (F) *Chariesterus armatus.* (G) *Leptoglossus gonagra*. (H) *Leptoglossus zonatus*. (I) *Phtia picta.* (J) *Harmostes prolixus.* (K) *Euryophthalmus rufipennis.* (L) *Dysdercus fulvoniger.* E-J) Note the central location of the m-chromosomes and the sex chromosomes arranged away from the circle formed by autosomes. Arrowheads indicate the sex chromosomes and m points to the m chromosomes. Bar = 5µm.

Supplementary Table 1. List of species studied, containing number of males and location with coordinates.

Supplementary Table 1.

Families/Species	Males collected	Localities	Coordinates	Identification
Pentatomidae				
Antiteuchus tripterus (Fabricius, 1787)	9	Olímpia-SP (1)	20º 44.139'S 48º 54.907'W	1
Euschistus cornutus Dallas, 1851	1	Pitanga-PR (2)	24º 42.776'S 51° 46.061'W	1
	1	Arapongas-PR (3)	23º 25.157'S 51º 25.546'W	
Euschistus heros (Fabricius, 1798)	7	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	1
	6	Rancho Alegre-PR (5)	23° 00.077'S 50° 56.565'W	
	4	Primeiro de Maio-PR (6)	22° 51.782'S 50° 59.865'W	
	2	Maringá-PR (7)	23º 30.051'S 51º 45.788'W	
Arvelius albopunctatus (De Geer, 1773)	3	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	1
Mormidea v-luteum (Lichtenstein, 1796)	3	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	1
Edessa rufomarginata (De Geer, 1773)	1	Cananéia-SP (8)	25° 53.955'S 51° 37. 251'W	1
	2	Arroio Bonito-PR (9)	25° 53.955'S 51° 37. 251'W	
Edessa impura Bergroth, 1891 [Det. J.A.M.Fernandes-UFPA]	1	Arroio Bonito-PR (9)	25° 53.955'S 51° 37. 251'W	1
Edessa meditabunda (Fabricius, 1784)	9	Assis-SP (10)	22° 28.645'S 50° 20.983'W	1
	2	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	
Coreidae				
Spartocera cf. *fusca* (Thunberg, 1783) (= *Corecoris fuscus*)	9	Cananéia-SP (8)	25º 1.4965'S 47º 55.989'W	*3*
	8	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	
Chariesterus armatus (Thunberg, 1825)	1	Rancho Alegre-PR (5)	23° 00.077'S 50° 56.565'W	2
	1	Primeiro de Maio-PR (6)	22° 51.782'S 50° 59.865'W	
	9	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	
	1	Maringá-PR (7)	23º 30.051'S 51º 45.788'W	
Leptoglossus gonagra (Fabricius, 1775)	3	Rancho Alegre-PR (5)	23° 00.077'S 50° 56.565'W	2
	1	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	
Leptoglossus zonatus (Dallas, 1852)	10	Rancho Alegre-PR (5)	23° 00.077'S 50° 56.565'W	2

	3	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	
	1	Primeiro de Maio-PR (6)	22° 51.782'S 50° 59.865'W	
	1	Arapongas-PR (2)		
Phtia picta (Drury, 1770)		Londrina-PR (4)	23º 18.394'S 51º 12.139'W	2
Athaumastus haematicus (Stål, 1860)	1	Rancho Alegre-PR (5)	23° 00.077'S 50° 56.565'W	2
	2	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	
Holymenia histrio (Fabricius, 1803)	6	Londrina-PR (4)	23º 19.452' S 51º 11.891'W	2
Hypselonotus interruptus Hahn, 1831	2	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	2
	3	Rancho Alegre-PR (5)	23° 00.077'S 50° 56.565'W	
Hypselonotus fulvus (De Geer, 1775)	2	Pitanga-PR (2)	24º 42.776'S 51° 46.061'W	2
Acanonicus hahni (Stål, 1860)	10	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	2
Cebrenis sp.	1	Assis-SP (10)		2
Althos obscurator (Fabricius, 1803)	1	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	2
Rophalidae				
Harmosthes prolixus Stål, 1860	12	Londrina-PR (4)	23º 19.452' S 51º 11.891'W	2
Largidae				
Euryophthalmus rufipennis Laporte, 1832	9	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	2
Pyrrhocoridae				
Dysdercus ruficollis (Linnaeus, 1758)	8	Manuel Ribas-PR (11)	24° 30.712'S 51° 41.343'W	2
	5	Primeiro de Maio-PR (6)	22° 51.782'S 50° 59.865'W	
	1	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	
Dysdercus imitator Blöte, 1931	4	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	2
Dysdercus fulvoniger (De Geer, 1773)	15	Londrina-PR (4)	23º 18.394'S 51º 12.139'W	2

The letters from A to K refer to the localities on the map of Brazil in Supplementary Figure 2. The numbers 1 and 2 refer to refer to the experts who conducted the identification, namely, 1= Jocélia Grazia, 2 = José Antônio Marin Fernandes and 3 = Hélcio Gil-Santana.

4.2 Capítulo 2

The conservation of number and location of 18S sites indicates the relative stability of rDNA in species of Pentatomomorpha (Heteroptera)

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The conservation of number and location of 18S sites indicates the relative stability of rDNA in species of Pentatomomorpha (Heteroptera)

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Running title: Physical location of 18S rDNA in species of Pentatomomorpha

Abstract:

FISH with rDNA probes has been used for comparative cytogenetics studies in different groups of organisms. Although heteropterans are a large suborder of Hemiptera, studies using rDNA are limited to the infraorder Cimicomorpha, in which the rDNA sites are present in the autosomes or in the sex chromosomes. We isolated and sequenced a conserved 18S rDNA region of *Antiteuchus tripterus* (Pentatomidae) and used it as a probe against chromosomes of 25 species belonging to five different families of Pentatomomorpha. The clone p*At*05 with a length of 736 bp exhibited a conserved stretch of 590 bp. FISH using p*At*05 as probe demonstrated hybridization signals always in sub-terminal positions, except for *Euschistus heros*. Apparently, there is a tendency for 18S rDNA sites to locate in autosomes, except in *Leptoglossus gonagra* and *Euryophthalmus rufipennis*, which showed signals in the m- and sex chromosomes, respectively. Although FISH has produced evidence that rearrangements are involved in rDNA repositioning, whether in different autosomes or between sex and m-chromosomes, we have no conclusive evidence of what were the pathways of these rearrangements based on the evolutionary history of the species studied here. Nevertheless, the diversity in the number of species analyzed here showed a tendency of 18S rDNA to remain among the autosomes.

Key-words: chromosome heteromorphism, holocentric chromosomes, in situ hybridization, 45S rDNA, m-chromosomes.

Introduction

The genomes of eukaryotes can accumulate repetitive DNA from different families, which may vary from hundreds to thousands of copies depending on the

species and/or taxonomic group (Charlesworth et al. 1994). Moderately repeated sequences, which include the multigene families of ribosomal genes (45S and 5S rDNA), have been used as cytogenetic markers for studies of karyotype differentiation in both plants (Roa and Guerra 2012) and animals (Cabral de Mello et al. 2011). The 45S rDNA generally appears as repeated and clustered sequences at particular chromosomal positions, especially at the chromosome ends (Martínez-Navarro et al. 2004). Variations in the rDNA sites can occur in size and homologue bearer. An example was reported by Bardella et al. (2010) for four species of *Triatoma* (Reduviidae). These authors showed that in *Triatoma brasiliensis* and *T. rubrovaria* the hybridization signal with rDNA probe was located on one autosome pair, while in *T. matogrossensis* and *T. infestans melanosoma,* signals were detected in the sex chromosomes. Triatominae may be highlighted because of the greater number of studies using FISH with rDNA probes (Panzera et al. 2012); the species of other infraorders studied using molecular cytogenetics are still very scarce. Consequently, there is a gap of information that leads to several difficulties in understanding the evolutionary mechanisms involved in the karyotype diversification of heteropterans.

For other insect groups, such as Diptera, Orthoptera, Lepidoptera and Coleoptera, there are a large number of cytogenetic studies that examine karyotype diversification using information from FISH with rDNA probes. Proença et al. (2002, 2005) showed that in the order Coleoptera, for instance, the number of rDNA sites decreases with a reduction in chromosome number, especially in the Cicindelidae. A different case was reported in Lepidoptera, where the variation in the number of rDNA sites was also detected in different families, but without relation to chromosome number. According to Nguyen et al. (2010), the main mechanisms involved with rDNA variation in this group seems to be associated with ectopic recombination, besides other

chromosome rearrangements, especially in species of the superfamilies Pyraloidea and Papilionoidea.

To understand the dynamics of distribution of 18S rDNA on karyotypes of heteropterans, i.e., the number, size and position of cistrons, a clone containing an insert with a conserved partial sequence of 18S rDNA of *Antiteuchus tripterus* (Pentatomidae, Pentatomomorpha) was isolated, sequenced and used against the chromosomes of 25 species of Pentatomomorpha (Heteroptera). The findings are discussed in a comparative manner, seeking to highlight the evolutionary processes associated with this repetitive family within and between taxonomic groups, especially the placement of cistrons within and between karyotypes.

Materials and Methods

Biological material and chromosome preparation

Heteropterans belonging to the families Pentatomidae, Coreidae, Pyrrhocoridae, Rhopalidae and Largidae were collected in the South and Southeast regions of Brazil. Information about the collection localities is given in Table 1.

Gonads were dissected out and the seminiferous tubules were fixed in methanol-acetic acid (3:1, v:v) and stored at −20ºC. Samples were first incubated in 45% acetic acid for 10 min, and slides were then prepared using dissection and squash in a drop of 45% acetic acid. The coverslips were removed after freezing in liquid nitrogen, and the slides air-dried.

DNA extraction and 18S rDNA characterization

The genome of *Antiteuchus tripterus* (Pentatomidae, Pentatomomorpha) was chosen for isolation and characterization of 18S rDNA, mainly due to large abundance of individuals sampled in relation to the others studied here. DNA extraction was done using a standard phenol/chloroform protocol. 18S rDNA was amplified using the primers 18S ai 5'-CCTGAGAAACGGCTACCACATC-3' and 18S bi 5'-GAGTCTCGTTCGTTATCGGA-3', reported by Whiting et al. (2002). PCR was done in a final volume of 25 µL containing 20 ng template, 0.4 µM each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1× PCR buffer and 2 U Taq polymerase (Invitrogen). The PCR program consisted of one step at 94ºC for 2 min followed by 40 cycles at 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min, and one final step at 72ºC for 5 min. The PCR product was cloned using the pMOS Blunt-Ended Cloning Kit (Roche), with *E. coli* TOP 10 competent cells. Clones previously selected by FISH were sequenced using a 3500xL automatic sequencer (Applied Biosystems). Determination of sequence quality and contig construction were performed with Phred-PhrapConsed software. The consensus sequence was compared with sequences of the GenBank database using BLAST tools (http://www.ncbi.nlm.nih.gov/blast).

Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) was done as described in Bardella et al. (2010) and performed in at least two individuals. The p*At*05 clone, containing a partial sequence of the 18S rDNA of *Antiteuchus tripterus* was labeled with digoxigenin-11dUTP by nick translation (Dignick translation Roche). Preparations were treated with 30 µL of hybridization mixture containing 4 µL of labeled probe (100 ng), 15 µL of 100% formamide, 6 µL of 50% polyethylene glycol, 3 µL of 20× SSC, 1 µL of 10% SDS and 1 µL of water. Chromosome denaturation/renaturation was done at 90ºC for 10

119 min using a thermal cycler, and hybridization was performed for 12 h at 37ºC in a
120 humidified chamber. Post-hybridization washes were carried out in SSC buffer, with a
121 60% stringency due to heterologous hybridization. For detection, anti-digoxigenin-
122 rhodamine in 5% BSA/4× SSC/0.2 Tween 20 (1:100, v:v) was used. The post-detection
123 washes were performed in 4× SSC/0.2 Tween 20 at room temperature. Slides were
124 mounted with 26 µL of DABCO solution (1,4-diaza-bicyclo (2.2.2)-octane (2.3%), 20
125 mM Tris-HCl, pH 8.0 (2%) and glycerol (90%) in distilled water), 2 µL of 2 µg/mL
126 DAPI and 1 µL of 50 mM $MgCl_2$.
127
128 *Image acquisition and processing*
129 All chromosome images were acquired separately in grayscale mode using a
130 Leica DM 4500 B microscope equipped with a DFC 300FX camera and overlapped
131 with red colors for DAPI and greenish-yellow for rhodamine, using Leica IM50 4.0.
132
133 **Results and Discussion**
134 We sought to determine the distribution profile of the 45S rDNA multigene
135 family in species of Pentatomomorpha. This repetitive family is composed of 18S, 5.8S
136 and 28S rRNAs, which are separated from each other by internal transcribed spacers
137 (Stage and Eickbush 2007). We used here a fragment of 18S obtained by PCR from the
138 genome of *Antiteuchus tripterus*, Pentatomidae, which was cloned and sequenced. The
139 consensus sequence with a length of 736 bp inserted in the p*At*05 clone showed a stretch
140 of 590 bp highly similar (99-96%) to the conserved 18S rDNA region of several
141 heteropterans (Figure 1), such as *Coleotichus costatus* (gb: EF641219.1), *Oechalia*
142 *schellenbergii* (gb: EF641205.1), *Riptortus pedestris* (gb: AB725684.1), besides some
143 others deposited in GenBank (NCBI).

When the p*At*05 clone was labeled and hybridized in situ against chromosomes of 25 species belonging to the families Pentatomidae, Coreidae, Rhopalidae, Largidae and Pyrrhocoridae, all species exhibited hybridization signals in only one chromosome pair, except for *Edessa meditabunda* (Pentatomidae) and *Euryophthalmus rufipennis* (Largidae) (Figures 2 and 3). In the first case, *E. meditabunda* showed three signals of 18S rDNA, one being bivalent and other a heteromorphic pair (Figure 2F). In the second case, *E. rufipennis* showed only one hybridization site, located on the X chromosome (Figure 3A). The occurrence of one bivalent with 18S signals was recurrent in the few species of Pentatomomorpha analyzed (Papeschi et al. 2003; Cattani et al. 2004; Bressa et al. 2008). On the other hand, in species of the infraorder Cimicomorpha, which are close to Pentatomomorpha, the variation in the number of rDNA sites is frequent, with species showing one hybridization signal: *Triatoma infestans melanosoma* and *Deraeocoris rutilus* (Bardella et al. 2010; Grozeva et al. 2011, respectively), species with two signals: *Triatoma rubrovaria* and *Cimex lectularius* (Bardella et al. 2010; Grozeva et al. 2011), and species with three signals: *Triatoma delpontei* (Panzera et al. 2012). Martínez-Navarro et al. (2004) proposed that in Coleoptera the conservation of rDNA site number is associated with chromosome number maintenance. In heteropterans, however, it seems to be different. We detected here that the rDNA sites number is relatively conserved, even when close species exhibit large variation in chromosome number.

Another feature that looked highly conserved was the sub-terminal positioning of the hybridization signals, except for *Euschistus heros* (Pentatomidae), which exhibited an intercalary signal (Figure 2B). It was very interesting because there are only two other species of Pentatomidae exhibiting intercalary positioning of 18S rDNA sites: *Nezara viridula* (Papeschi et al. 2003) and *Pyrrhocoris apterus* (Grozeva et al.

2011). The conservation of 18S rDNA sites on the chromosome ends was also reported for Coleoptera (Galián and Hudson 1999; Martínez-Navarro et al. 2004). Our results indicate that in *Euschistus heros* the intercalary positioning may be an apomorphy in relation to the other heteropterans, which occurred by inversion since there is no evidence of change in chromosome number. Changes in the rDNA location associated with inversions have also been reported in the superfamily Noctuoidea of Lepidoptera; however, these intercalary rDNA sites have been considered plesiomorphic in relation to terminal locations in this group (Monti et al. 1998; Nguyen et al. 2010).

Heteromorphisms in the rDNA sites were detected in 14 species (Table 1). Of these, we can highlight *Leptoglossus zonatus* (Figure 3E) because one of the signals appeared to be twice the size of the other. Heteromorphisms involving the 18S rDNA have been reported for *Holhymenia rubiginosa* (Bressa et al. 2008) and *Triatoma rubrovaria* (Bardella et al. 2010), suggesting an association with mechanisms of amplification and/or unequal crossing-over. Other insect groups also exhibit heteromorphisms, such as *Polyommatus bellargus*, Lepidoptera (Nguyen et al. 2010).

Bardella et al. (2013) studied these same 25 species using conventional Giemsa analysis and C-CMA/DAPI banding. These authors reported that, although there is a high variation in chromosome banding, there is little variation in size between autosomes, which hinders the accurate recognition of pairs for the purpose of ordering by size. Faced with this obstacle, we opted for a broader comparison, only considering: i) autosomes and ii) sex and iii) m-chromosomes. A comparison of our results with those of ten other species previously published (see Table 1), indicated that the presence of 18S rDNA is predominant in the autosomes, with 30 species of the 35 already reported (Figures 2 and 3 and Table 1). Hybridization signals in the sex chromosomes have been observed in *Eurydema oleracea, Graphosoma lineatum* (Grozeva et al. 2011)

and *Dysdercus albofasciatus* (Bressa et al. 2009) and *Euryophthalmus rufipennis*, analyzed here (Figure 3A and Table 1). The m-chromosomes appeared to bear the 18S rDNA signal only in *Leptoglossus gonagra* (Coreidae) (Figure 3F and Table 1).

According to Panzera et al. (2012), despite the maintenance of chromosome number in the genus *Triatoma* (Cimicomorpha), the mobility of rDNA sites located on autosomes and the sex chromosomes is substantial. The most extreme case reported by these authors was for *Triatoma infestans*, in which different populations showed the location in both autosomes and sex chromosomes. Mobility of rDNA was also observed in different orders of insects, such as Orthoptera, Coleoptera and Lepidoptera (Nguyen et al. 2010; Cabral-de-Mello et al. 2011). According to Nguyen et al. (2010), ectopic recombination and transposition could be responsible for rDNA mobility in insects, increasing consequently the number of rDNA sites. Multiplication and dispersion of 18S rDNA sites are uncommon among species of Pentatomomorpha, and in the few cases where there was suspicion of rDNA mobility, i.e. the occurrence of 18S rDNA sites in m- and sex-chromosomes; our results did not revealed the real mechanism that originated these events. Our results using 25 species of different groups of Pentatomomorpha significantly extend the information on the distribution of 18S rDNA in heteropterans, especially showing the trends for this family to occupy the sub-terminal region of the autosomes. These data are different from those obtained for the infraorder Cimicomorpha, and it points to the different evolutionary ways of 18S rDNA distribution within Heteroptera. Future studies should be directed to understanding the variability of rDNA and to screening for the occurrence of other associated repetitive DNA families that could be responsible for the mobility of rDNA.

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295
296

297 **Legends**

298 **Figure 1.** Sequence of p*At*05 clone

299

300 **Figure 2.** Localization of 18S rDNA in chromosomes of *Antiteuchus tripterus* (A),
301 *Euschistus heros* (B), *Euschistus cornutus* (C), *Edessa impura* (D), *Edessa*
302 *rufomarginata* (E), *Edessa meditabunda* (F), *Arvelius albopunctatus* (G), *Dysdercus*
303 *ruficollis* (H), *Dysdercus fulvoniger* (I) and *Dysdercus imitator* (J). Note hybridization
304 signals in only one autosomic pair (arrows). Observe also the intercalary position of the
305 18S rDNA site in *Euschistus heros* (box in B). Arrowheads indicate the sex
306 chromosomes. Bar = 5µm.

307

308 **Figure 3.** Localization of 18S rDNA in chromosomes of *Euryophthalmus rufipennis*
309 (A), *Acanonicus hahni* (B), *Harmosthes prolixus* (C), *Chariesterus armatus* (D),
310 *Leptoglossus zonatus* (E), *Leptoglossus gonagra* (F), *Phtia picta* (G), *Athaumastus*
311 *haematicus* (H), *Holhymenia histrio* (I), *Spartocera* cf. *fusca* (J), *Althos obscurator* (K),
312 *Zicca annulata* (L), *Cebrenis* sp. (M), *Hypselonotus fulvus* (N) and *Hypselonotus*
313 *interruptus* (O). Observe that the hybridization signals of 18S rDNA probe is located in
314 the autosomes, except for *E. rufipennis* (A), which is located in the X chromosome, and
315 for *Leptoglossus gonagra* (F), whose signal is detected in the m-chromosomes (arrow).
316 Arrowheads indicate the sex chromosomes. Bar = 5µm.

Table 1. Species of Pentatomomorpha with information about karyotype number and positioning of 18S rDNA sites.

Families/Species	(2*n*)	L/P	SN	CL	H	References / Localities
Pentatomidae						
Antiteuchus tripterus	12+XY	A/LP	2	T	+	Olímpia-SP
Euschistus cornutus	12+XY	A/IP	2	T	+	Pitanga-PR/Arapongas-PR
Euschistus heros	12+XY	A/IP	2	I	+	Londrina-PR
Arvelius albopunctatus	12+XY	A/IP	2	T	+	Londrina-PR
Edessa rufomarginata	12+XY	A/LP	2	T	+	Cananéia-SP
Edessa impura	12+XY	A/LP	2	T	-	Arroio Bonito-PR
Edessa meditabunda	12+XY	A/LP	2-3*	T	+	Assis-SP
Eurydema oleracea	12+XY	S/X-Y	2	-		Grozeva et al. 2011
Graphosoma lineatum	12+XY	S/X	1	T		Grozeva et al. 2011 González-García et al. 1996
Nezara viridula	12+XY	A	2	I		Papeschi et al. 2003
Coreidae						
Spartocera cf. *fusca*	20+2m+X0	A/LP	2	T	+	Cattani and Papeschi 2004/Cananéia-SP
Chariesterus armatus	22+2m+X0	A/IP	2	T	-	Londrina-PR
Leptoglossus gonagra	18+2m+X0	m	2	T	-	Rancho Alegre-PR
Leptoglossus zonatus	18+2m+X0	A/IP	2	T	+	Rancho Alegre-PR
Phtia picta	18+2m+X0	A/IP	2	T	-	Londrina-PR
Athaumastus haematicus	18+2m+X0	A/LP	2	T	+	Rancho Alegre-PR
Holhymenia histrio	24+2m+X0	A/LP	2	T	+	Londrina-PR
Hypselonotus interruptus	16+2m+X0	A/IP	2	T	-	Londrina-PR
Hypselonotus fulvus	16+2m+X0	A/IP	2	T	-	Pitanga-PR
Acanonicus hahni	18+X0	A/LP	2	T	+	Londrina-PR
Cebrenis sp.	20+2m+X0	A/LP	2	T	-	Assis-SP
Zicca annulata	20+2m+X0	A/LP	2	T	-	Londrina-PR
Althos obscurator	22+2m+X0	A/IP	2	T	+	Londrina-PR
Pachylis argentinus	12+2m+X0	A	2	-		Papeschi et al. 2003
Holhymenia rubiginosa	24+2m+X0	A/LP	1-2*	T		Bressa et al. 2008
Camptischium clavipes	18+2m+X0	A	2	T		Cattani et al. 2004
Rhopalidae						
Harmosthes prolixus	10+2m+X0	A/LP	2	T	+	Londrina-PR
Largidae						
Euryophthalmus rufipennis	12+X0	S/X	1	T	-	Londrina-PR
Pyrrhocoridae						
Dysdercus ruficollis	12+X0	A	2	T	-	Bressa et al. 2009/ Manuel Ribas-PR
Dysdercus imitator	12+X0	A	2	T	+	Londrina-PR
Dysdercus fulvoniger	12+X0	A	2	T	-	Londrina-PR
Pyrrhocoris apterus	22+X0	A/LP	2	I		Grozeva et al. 2011
Dysdercus albofasciatus	10+neo-X neo-Y	S/neo-X	2	-		Bressa et al. 2009
D. chaquensis	12+X0	A	2	T		Bressa et al, 2009
Lygaeidae						
Oxycarenus lavaterae	14+2m+XY	A/LP	2	-		Grozeva et al. 2011

2*n* = diploid number; L/P= Location/Pair; SN = sites number; CL = chromosome location; H = heteromorphisms.
* Corresponds to species with heteromorphism in sites number of rDNA 18S. For the chromosome types: A = autosomes, S = sex chromosomes and m = m-chromosome. For the chromosome sizes: LP = largest pair. IP = intermediate pair. For the chromosome positions: T = terminal. I = intercalary

Figure 1. Conserved sequence of p*At*05 clone

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1........|........|........|........|........|........|........|........80
ACAAATTAGAGTGCTTCAAGCAGGCTCTTAAAGCAGCCTGAATAGTGGTGCATGGAATAATGGAACAGGACCTTGGTTCT
ATTTTGTTGGTTTTCGGAATCCAAGGTAATGATCAATAAGGACAGGCGGGGGCATTCGTATTGCGACGTTAGAGGTGAAA
TTCTTGGATCGTCGCAAGACGCACTAGAGCGAAAGCATTTGCCAAGTATGTCTTAATTGATCAAGAACGAAAGTTAGAGG
TTCGAAGGCGATCAGATACCGCCCTAGTTCTAACCATAAACGATGCCAGCCAGCGATCCGCCGATGTTCCTCCGATGACT
CGGCGGGGAGCTTTCCCCGGGAAACCAAAGCTTTTGGGTTCCGGGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGGAAT
TGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAACCTCACCAGGCCCGGACATTG
GAAGGATTGACAGATCGATAGCTCTTTCTCGATTCAGTGGGTAGTGGTGCATGGCCGTTCTTAGTTGGTGGACTGATTTG
TCTGGTTAATTCCGATAACGAACGAGACTC
.........|.........|.........590
```

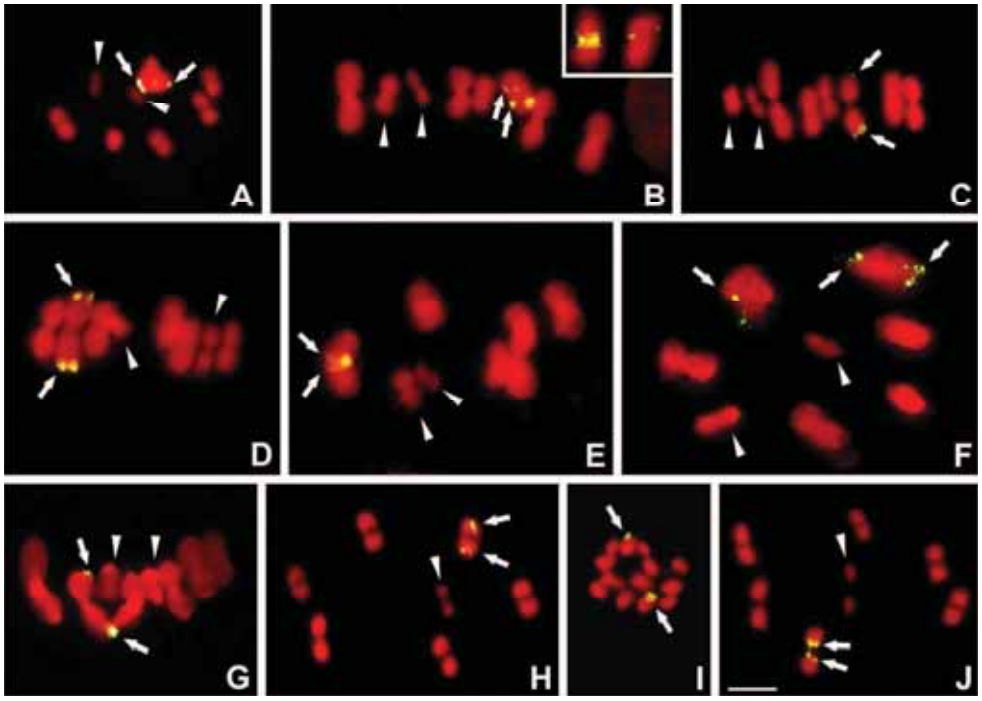

Figure 2. Localization of 18S rDNA in chromosomes of Antiteuchus tripterus (A), Euschistus heros (B), Euschistus cornutus (C), Edessa impura (D), Edessa rufomarginata (E), Edessa meditabunda (F), Arvelius albopunctatus (G), Dysdercus ruficollis (H), Dysdercus fulvoniger (I) and Dysdercus imitator (J). Note hybridization signals in only one autosomic pair (arrows). Observe also the intercalary position of the 18S rDNA site in Euschistus heros (box in B). Arrowheads indicate the sex chromosomes. Bar = 5µm.
176x125mm (300 x 300 DPI)

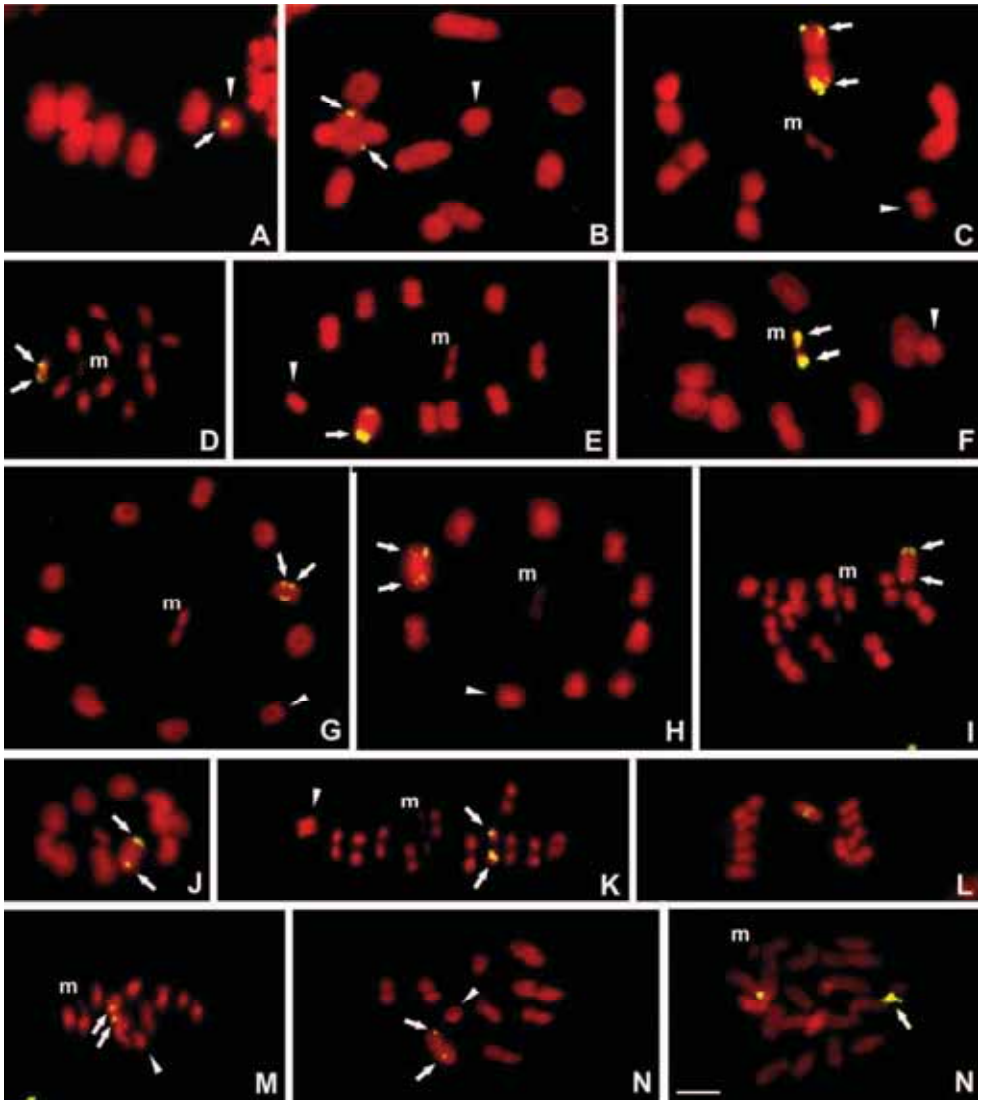

Figure 3. Localization of 18S rDNA in chromosomes of Euryophthalmus rufipennis (A), Acanonicus hahni (B), Harmosthes prolixus (C), Chariesterus armatus (D), Leptoglossus zonatus (E), Leptoglossus gonagra (F), Phtia picta (G), Athaumastus haematicus (H), Holymenia histrio (I), Spartocera cf. fusca (J), Althos obscurator (K), Zicca annulata (L), Cebrenis sp. (M), Hypselonotus fulvus (N) and Hypselonotus interruptus (O). Observe that the hybridization signals of 18S rDNA probe is located in the autosomes, except for E. rufipennis (A), which is located in the X chromosome, and for Leptoglossus gonagra (F), whose signal is detected in the m-chromosomes (arrow). Arrowheads indicate the sex chromosomes. Bar = 5µm.
198x223mm (300 x 300 DPI)

4.3 Capítulo 3

Origin and distribution of AT-rich repetitive DNA families in *Triatoma infestans* (Heteroptera)

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Origin and distribution of AT-rich repetitive DNA families in *Triatoma infestans* (Heteroptera)

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Abstract

Triatoma infestans, one of the most important vectors of *Trypanosoma cruzi*, is very interesting model, because it shows large interpopulation variation in the amount and distribution of heterochromatin. This polymorphism involved the three large pairs up to almost all autosomal pairs, including the sex chromosomes. To understand the dynamics of heterochromatin variation in *T. infestans*, we isolated the AT-rich satDNA portion of this insect using reassociation kinetics (C_0t), followed by cloning, sequencing and FISH. After chromosome localization, immunolabeling with anti-5-methylcytosine, anti-H4K5ac and anti-H3K9me2 antibodies was performed to determine the functional characteristics of heterochromatin. The results allowed us to reorganize the karyotype of *T. infestans* in

accordance with the distribution of the families of repetitive DNA using seven different markers. We found that two arrays with lengths of 79 bp and 33 bp length have a strong relationship with transposable element sequences, suggesting that these two families of satDNA probably originated from Polintons. The results also allowed us to identify at least four chromosome rearrangements involved in the amplification/dispersion of AT-rich satDNA of *T. infestans*. These data should be very useful in new studies including those examining the cytogenomic and population aspects of this very important species of insect.

Key-words: heterochromatin, holocentric chromosomes, immunolabeling, molecular cytogenetic, satDNA, Reduviidae

Abbreviations: TRF: Tandem Repeats Finder; TIRs: terminal inverted repeats; SSC: saline-sodium citrate; SDS: sodium dodecyl sulfate; FITC: fluorescein isothiocyanate; DABCO: 1,4-diazabicyclo[2.2.2]octane; PBS: phosphate-buffered saline; BSA: bovine serum albumin; TRITC: tetramethylrhodamine-5-(and-6-)isothiocyanate; FISH: fluorescence in situ hybridization; HCl: hydrochloric acid; CMA_3: chromomycin A_3; DAPI: 4'-6-diamidino-2-phenylindole; Tris: tris(hydroxymethyl)aminomethane hydrochloride; $MgCl_2$: magnesium chloride; NCBI: National Center of Biotechnology Information.

1. Introduction

Heterochromatin is known as a nuclear fraction with high methylation/condensation state, low functional activity, late replication and lower susceptibility to recombination events. These characteristics are common in regions rich is repetitive DNA families, such as satDNA, and they can vary between species in size, composition and amount of motifs

(Schmidt and Heslop-Harrison, 1998; Subirana and Messeguer, 2013). This diversity of heterochromatic regions can be exemplified by proximal chromosome regions of some coleopterans, whose composition of repetitive DNA families depends on the species or group of species. *Palorus subdepressus*, for instance, shows an array with a length of 72 bp, tandemly repeated in the pericentromeric region, whose sequence is also found in other coleopterans, such as *Chysolina americana* (Plohl et al., 1998; Lorite et al., 2001). In Aphididae, a family of Hemiptera with holokinetic chromosomes, three families of repetitive DNA were found: i) an array of 3.000 bp length associated with a terminal GC-rich region in the X chromosome, ii) a 200 bp length sequence associated with an AT-rich region of X chromosome and iii) a satDNA obtained after *Hae*III cleavage associated with AT-rich regions of autosomes (Mandrioli et al., 1999).

The traditional methods of chromosome banding (C-Giemsa and CMA/DAPI) have been useful for revealing the location, size and AT- or GC-rich composition of heterochromatic regions in the chromosomes and nucleus, and also for producing markers to compare karyotypes of different species and/or groups of insects (Schneider et al., 2006; Bressa et al., 2008; Cabral-de-Mello et al., 2010; Panzera et al., 2010; Bardella et al., 2014). Especially for Triatominae, chromosome banding allows us to identify intra- and interspecific variations in the amount and distribution of heterochromatin (Panzera et al., 1992; Panzera et al., 1995). An example is the intraspecific polymorphism of chromosome band patterns between natural populations of *Triatoma infestans* (Panzera et al., 1992; Panzera et al., 1995). Panzera et al. (2004) suggested that Andean population of *T. infestans* exhibit heterochromatin in 14 to 20 chromosomes, and about 1.825 ± 0.49 pg per haploid nucleus. However, non-Andean populations seems to be from four to seven chromosomes carrying bands and a C-value with about 1.401 ± 0.111 pg. These data suggested that

polymorphisms were associated with migration and adaptation of genomes during the occupation of this species in new environments in South America. Karyotype changes were also observed in the distribution of 18S rDNA in distinct population of *T. infestans*, whose hybridization signals can occur in autosomes or alosomes (Panzera et al., 2012).

Triatoma infestans has holokinetic chromosomes and $2n = 20 + XY$, like the majority of species of Triatominae (Ueshima, 1979; Panzera et al., 1996). Karyotypes of *T. infestans* are asymmetrically organized into three large pairs and the remainder smaller pairs, with the Y chromosome always larger than X in the non-Andean group (Pérez et al., 2000), because the X not have C-heterochromatin. But in Andean group, both sex chromosomes are the similar size and both of them with C-heterochromatin (Panzera et al., 2004). This species is also particularly intriguing because it belongs to a hematophagous insect group that is important to public health in Latin America, since they are the main vectors of the parasite *Trypanosoma cruzi*, responsible for Chagas disease. According to data from the World Health Organization (http://www.who.int/topics/chagas_disease/en/), about 10 million people were infected with *T. cruzi* in 2012.

To understand the origin and variation of heterochromatic regions in *T. infestans*, we used C_{ot} DNA reassociation to isolate and to produce a plasmid microlibrary with short DNA fragments, followed by cloning and SANGER sequencing. This strategy was useful in selecting different repetitive DNA fragments for physical mapping. The main goal of this study was to determine the mechanisms that account for the origin, distribution and diversity of AT-rich heterochromatin that occupy predominantly the terminal chromosome regions.

2. Material and Methods

2.1. Biological material

Ten males and eight females of *T. infestans* from Andean regions of Peru, South America, were obtained from the insectarium of the Faculty of Pharmaceutical Sciences, Department of Biological Sciences, UNESP, Araraquara, Brazil. The insects were obtained from a colony that was founded from seven nymphs in August 1983. Samples were used for the removal seminiferous tubules and DNA extraction. Gonads were dissected out and the tubules were directly fixed in a freshly prepared solution of methanol:acetic acid (3:1, v:v) and then stored at -20ºC. Genomic DNA was extracted from the leg muscles of males and females using the DNeasy Blood & Tissue Kit (Qiagen).

2.2. Obtaining the C_0t fraction and microlibrary construction

Repetitive DNA was isolated using renaturation kinetics (Zwick et al., 1997), with modifications, using a pool of males and females of *T. infestans*. About 10 µg DNA dissolved in 0.3 M NaCl were autoclaved at 1.4 atm (120 ºC) for 15 min, and the size of fragments was checked by electrophoresis in 1% agarose. The sample was denatured at 95 ºC for 10 min, placed on ice for 10 s and transferred to a water bath at 65 ºC for 10 min. The sample was then incubated at 37 ºC for 8 min with S1 nuclease and the reaction stopped by the addition of liquid nitrogen. DNA was purified using phenol-chloroform (1:1, v:v), and subsequently cloned using the Blunt-Ended PCR Cloning Kit (GE Healthcare Life Sciences), with *E. coli* TOP10 competent cells. Six hundred fifty-three clones were used in a pre-selection by PCR with the primers M13 F (5'-

120 GTAAAACGACGGCCAG-3') and M13 R (5'-CAGGAAACAGCTATGAC-3') to obtain
121 fragments larger than 200 bp.
122 Inserts were sequenced on a 3500xL automatic sequencer (Applied Biosystem),
123 according to the manufacturer's procedures. The quality of sequences was tested with
124 Phred-PhrapConsed software. After the identification and removal of parts of the vectors
125 using Vector Screen (NCBI), the consensus sequences were contrasted against the NCBI
126 (http://www.ncbi.nlm.nih.gov/blast), Flybase (http://flybase.org) and RepeatMasker
127 (http://www.girinst.org/censor/index.php) gene banks. To get repetitive stretches, the
128 sequences were tested with RepFind (http://cagt.bu.edu/page/REPFIND_submit) and
129 Tandem Repeats Finder (TRF) (http://tandem.bu.edu/trf/trf.html) software.
130
131 2.3. Cytogenetics
132 Seminiferous tubules were incubated in 60% acetic acid for 10 min, and then
133 minced and squashed. Coverslips were removed by freezing in liquid nitrogen. For
134 chromosome banding (Sumner, 1982, with small modifications) all the seminiferous
135 tubules of six males were used. Slides were aged at room temperature and also incubated in
136 0.2 M HCl for 10 min at room temperature, 5% barium hydroxide for 2 min at 60 ºC and 2×
137 SSC, pH 7.0, for 60 min at 60 °C. The samples were dehydrated in an alcohol series, air
138 dried and stained with fluorochromes: 0.5 mg/mL chromomycin A_3 (CMA_3) for 1.5 h and 2
139 µg/mL 4'-6-diamidino-2-phenylindole (DAPI) for 30 min. Slides were mounted with a
140 medium composed of glycerol/McIlvaine buffer (pH 7.0) 1:1, plus 2.5 mM $MgCl_2$. After
141 image acquisition, slides were treated with ethanol:acetic acid (3:1, v:v) for 15 min and
142 stained with 2% Giemsa.

For FISH, the slides were prepared as previously described, and probes were labeled with biotin or digoxigenin using nick translation (Invitrogen). 18S rDNA, which was isolated from *Antiteuchus tripterus* [see 16] was also labeled by nick translation, and used as control for FISH. Preparations were treated with a mixture composed of 100% formamide (15 μL), 50% polyethylene glycol (6 μL), 20× SSC (3 μL), 100 ng sonicated calf thymus DNA (1 μL), 10% SDS (1 μL), and 100 ng probe (4 μL each). This mixture was denatured at 70 °C for 10 min, immediately chilled on ice for 5 min, and added to the slide. Chromosome denaturation/hybridization was done at 90 °C for 10 min, 48 °C for 10 min, and 38 °C for 5 min, using a thermal cycler (MJ Research, Inc., USA). Samples were incubated in a humidified chamber at 37 °C overnight. Post-hybridization washes were carried out in SSC, with 80% stringency. Probes were detected with avidin-FITC or anti-digoxigenin-rhodamine. The post-detection washes were performed in 4× SSC/0.2% Tween 20, all at room temperature. Slides were mounted with 25 μL of a solution composed of glycerol (90%), DABCO (2.3%), 20 mM Tris-HCl, pH 8.0 (2%), 2.5 mM $MgCl_2$ (4%), and distilled water (1.7%), plus 1 μL of 2 μg/mL DAPI.

2.4.Immunolabeling

For detection of hypermethylated chromatin, seminiferous tubules were fixed in methanol-acetic acid (3:1, v:v), washed in 1× PBS buffer, dissected in a drop of 60% acetic acid and squashed. After removing the coverslips by freezing, slides were placed in 1× PBS for 5 min. The material was blocked in a solution of 3% BSA in 1× PBS plus 0.2% Tween 20 (w/v) at room temperature for 10 min. Afterwards, samples were incubated with a 1:100 (v:v) solution of mouse anti-5-methylcytosine primary antibody (Eurogentec) diluted in 1× PBS with 3% BSA plus 0.2% Tween 20 (w/v), in a moist chamber at 4°C overnight.

TRITC-conjugated goat anti-mouse secondary antibody (DAKO R0270) was used for detection.

For the detection of modified histones, H3K9me2 and H4K5ac (Feitoza et al., 2011, with modifications], the seminiferous tubules were fixed in freshly prepared 4% (w/v) paraformaldehyde for 60 min at room temperature and squashed in a drop of cold 1× PBS. After removing the coverslips by freezing, samples were blocked in a solution of 3% BSA in 1× SSC plus 0.2% Tween 20 (*w/v*) at room temperature for 10 min. Afterwards, samples were incubated with anti-H4K5ac and anti-H3K9me2 antibodies (Upstate Biotechnology, USA), both diluted 1:100 in 1× PBS with 3% BSA, in a moist chamber for 24 h at 4 ºC. Samples were washed in 1× PBS and developed with FITC-conjugated goat anti-rabbit IgG secondary antibody (Sigma), diluted 1:100 in 1× PBS with 3% BSA, for 3 h at 37 ºC. Slides were mounted as described for FISH.

2.5. Images and idiogram

Light microscopy images were acquired in grayscale using a Leica DM 4500 B microscope, equipped with a DFC 300FX camera. For FISH and immunolabeling, the images were acquired in grayscale and pseudocolored (red or blue for DAPI, greenish-yellow for FITC or TRITC and red for rhodamine) and overlapped using the Leica IM50 4.0 software. All images were optimized for best contrast and brightness using Adobe Photoshop CS software. The idiogram was organized comparing at least five doubly hybridized metaphases, using each probe, and that were subsequently treated for CMA_3/DAPI-banding.

188

189 **3. Results**

190 3.1. Conventional analysis and chromosome banding

191 Males of *T. infestans* from Peru showed karyotypes with $2n = 20 + XY$. The
192 chromosomes were arranged into eight large (pairs 1 to 4), six medium (pairs 5 to 7) and
193 six small (pairs 8 to 10) chromosomes plus two sex chromosomes (Figure 1A and J). C-
194 CMA/DAPI staining showed bands located preferentially in the terminal/subterminal
195 regions of large and medium pairs and sex chromosomes. Pairs 1-3 displayed bands at both
196 ends, with CMA bands always adjacent and more internally located in relation to DAPI
197 bands, except for pair 4, which showed only one CMA band on one end of one homologous
198 chromosome (Figure 1B-D and J). Pairs 5 and 6 exhibited only a terminal DAPI band on
199 one end and pair 7 showed a heteromorphism between homologous chromosomes, with one
200 DAPI band terminal and the other intercalary (Figure 1B-D and J). Heteromorphism were
201 also observed in pair 4 (Figures 1B-C and J). Pairs 8-10 exhibited no bands. The sex
202 chromosomes showed differences in size. The X chromosome was the larger and displayed
203 two DAPI bands on the ends, while the smaller Y showed a large DAPI band, which
204 occupied more than half the length of the chromosome (Figure 1B-D and J). To recognize
205 the sex chromosomes, C-CMA/DAPI staining was performed in ganglions of eggs, where
206 the X chromosomes showed DAPI bands at both ends (data not shown).

207

208 3.2. C_{0t} microlibrary

209 It was produced a microlibrary containing 653 clones, and these, 97 clones with
210 inserts over than 300 bp length were chosen for sequencing. Fifty-nine of them (60.82%)
211 displayed a tandem repetitive sequence of 79 bp length. The p*Ti*-103 clone stood out

because it possessed 9.6 copies of repeats with 79 bp containing 30% A, 19% C, 21% G and 29% T (Figure 2A). This AT-rich repeated family did not show identity with other sequences deposited in databases until now. The TRF software produced a consensus sequence for the clone p*Ti*-103, which differed from other clones only by 6% mismatches and 1% indels (Figure 2A).

The p*Ti*-95 clone with a length of 536 bp (Figure 3A) showed two repeats of 79 bp with high identity as those described above, directly adjacent to a stretch of 370 bp with 67% identity (*E-value* of 5e-14) for the Polinton-1_Deu of *Drosophyla eugracilis* (Figure 3A). The 370-bp stretch was used to search protein domains to determine its location in the complete sequence of Polinton-1_Deu, which we used as a reference. The search in the Blast*p* showed of 92% identity (*E-value* of 4e-54) to an ATPase domain of the Maverick Polinton of the wasp *Cortesia congregata*, gi|531033916 (Figure 3B). Another clone, p*Ti*-34 clone with a length 336 bp (Figure 3C), also showed a stretch of 85 bp with 73% identity (*E-value* of 0.099) to Polinton-1 HM of the cnidarian *Hydra magnipapillata* (Figure 3C). Although these segments are small in relation to the large size of Polintons (≥ 20 kb), the recurrent appearance of stretches of these Polintons adjacent to 79 bp repetitive family did not seem to be a coincidence.

Seven clones from the microlibrary (7.22%) exhibited a tandem repetitive sequence of 33 bp length. The p*Ti*-78 was one of these clones, with 42% A, 14% C, 17% G and 26% T (Figure 2B). The analysis using the TRF software produced a consensus sequence for the p*Ti*-78 clone, which differed from other motifs by 6% mismatches and 1% indels (Figure 2B). Interestingly, two clones containing the 33 bp repetitive family (p*Ti*-399 and p*Ti*-646) exhibited adjacent fragments or entire sequences identical to those of the 79 bp repetitive

family (described above). It was more evident in the p*Ti*-646 clone, which was 282 bp length with 26% A, 21% C, 13% G and 40% T, and had a consensus sequence with 7% mismatches and 0 indels (Figure 2C). It showed that these repetitive families are co-located and interspersed in chromosomes. The sequences with 79 bp and 33 bp exhibited no identity with each other. The p*Ti*-382 clone with a length of 225 bp exhibited an 8 bp tandem repetitive motif, with 27 occurrences and 38% A, 28% C, 1% G and 33% T. The TRF software produced an AT-rich consensus sequence with 18% mismatches and 0 indel (Figure 2D).

3.3. FISH and immunolabeling

Probes from five clones (p*At*05, p*Ti*-103, p*Ti*-95, p*Ti*-78 and p*Ti*-382) were used to generate markers in the mitotic chromosomes of *T. infestans*. The p*At*05 clone containing the 18S rDNA showed a hybridization signal at one end of pair 4 (Figure 1E and J). The p*Ti*-78 probe containing motifs of 33 bp hybridized in both terminal regions of pairs 1 and 2, at one end of pairs 4 and 5 and on the X chromosome (Figure 1F, H and J). The p*Ti*-103 probe with motifs of 79 bp hybridized in both terminal regions of pairs 1, 2 and 3, and the X chromosome (Figure 1G, H and J). Hybridization signals were also detected in one terminal region of pairs 4 and 5 (Figure 1G, H and J). The p*Ti*-95 probe produced signals in the same that p*Ti*-103 probe (data not shown). The p*Ti*-382 probe hybridized in one of the terminal regions of pairs 2 and 3 (Figure 1I and J).

Because the p*Ti*-646 clone has shown the motifs with 79 and 33 bp positioned immediately adjacent in the insert, we performed FISH with the p*Ti*-103 and p*Ti*-78 probes in the diffuse stage at prophase I of meiosis to detect the accurate co-location of these satDNA families. The slightly condensed state of chromatin in early meiotic prophase,

allowed to detect completely independent signals with the p*Ti*-78 probe (Figure 4A-B (box) and D), as well as the accurate co-location in at least at four positions in prophase cells (Figure 4B-C, E-F). This co-location was also observed in the nuclei of spermatocytes, when all heterochromatic regions appeared to be associated, creating one single chromocenter (Figure 5A-B, box).

Spermatocytes exhibited one or two chromocenters concentrating all heterochromatic regions in a "bouquet-like" structure (Figure 5A-B). This highly methylated chromocenter was certified after immunolabeling using anti-5-methylcytosine (Figure 5C-D), and also in subterminal and terminal chromosome regions of autosomes and sex chromosomes (Figure 5I-K). This idea was also supported by the spermatocyte immunolabeling results, using the H3K9me2 and H4K5ac antibodies against modified histones typical of heterochromatin and euchromatin, respectively. In this case, the chromocenters evidenced by DAPI (Figure 5E, G) appeared immunolabeled with anti-H3K9me2 but not with anti-H4K5me (Figure 5F-H).

4. Discussion

Heteroptera exhibits chromosome numbers from 2 to 80, but the subfamily Triatominae is differentiated within this suborder of insects because a chromosome number of $2n = 20 + XY$ or XX is maintained with small variations related to sex system (Ueshima, 1979). Intra- and interspecific variations in chromosome sizes are associated with the accumulation of heterochromatin, which can be in one or both chromosome ends (Panzera et al., 1992; Panzera et al., 1995). Our results using samples of *T. infestans* from the Andes exhibited a large number of terminal bands (14-20), including those of the sex chromosomes as proposed in the literature (Panzera et al., 2004). Our analysis allowed a

283 better definition of the karyotype of *T. infestans*. We found it arranged into four large, three
284 medium and three small pairs, and two sex chromosomes, the X being slightly larger than
285 Y. Although Panzera et al. (2004) have reported the occurrence of heterochromatic bands in
286 both sex chromosomes, there was no a perfect definition to differentiate each sex
287 chromosomes based in the bands profile. Our results, using chromosome banding in
288 embryonic ganglia of samples from Peru, allowed to visualize that X chromosome was
289 always bigger than Y, due to differential accumulation of heterochromatin.

290 The genus *Triatoma* is recognized by maintenance of chromosome number as well
291 as by mobility of rDNA sites, in both sex and autosomes (Panzera et al., 2012). The
292 location of 18S rDNA sites was also reported for 25 species of Pentatomomorpha, but with
293 predominance of signals in the terminal region in autosomes (Bardella et al., 2013). The
294 Andean samples of *T. infestans* used here exhibited the 18S rDNA sites in the terminal
295 region of fourth autosomal pair, differently for non Andean samples studied by Bardella et
296 al. (2010), that showed hybridization signals with 18S rDNA probe in the X chromosome,
297 as previously suggested by (Panzera et al., 2012). Although the variations in the 18S rDNA
298 are well documented in the literature, in comparative terms this of moderately repetitive
299 DNA family appears to contribute less to the karyotype variability of *T. infestans* than the
300 heterochromatin.

301 Seven chromosome profiles were observed according to distribution and co-location
302 of repetitive DNA families, which were: i) without bands (pairs 8, 9 and 10), ii) only with
303 DAPI bands (pairs 6, 7 and Y), iii) with CMA/DAPI bands co-locating with p*Ti*-78/p*Ti*-103
304 signals (pair 1), iv) with CMA/DAPI bands co-locating with p*Ti*-78/p*Ti*-103/18S rDNA
305 signals (pair 4), v) with CMA/DAPI bands co-locating with p*Ti*-78/p*Ti*-103/p*Ti*-382 signals
306 (pair 2), vi) with CMA/DAPI bands co-locating with p*Ti*-103/p*Ti*-382 signals (pair 3) and

307 vii) with DAPI bands co-locating with p*Ti*-78/p*Ti*-103 signals (pair 5 and X chromosome).
308 This complexity in the occurrence and distribution of satDNA families in the
309 heterochromatic regions of *T. infestans* shows that each repetitive DNA family can present
310 distinct and independent levels of amplification/reduction and dispersion along the
311 karyotype. These results reinforce the idea that heterochromatin is more dynamic than the
312 euchromatic fraction in relation to the process of karyotypes differentiation among
313 populations of *T. infestans* in South America (Panzera et al., 1995). An example of
314 differential distribution of satDNA families along karyotypes was described in *Ixodes*
315 *scapularis* (Acari - Ixodida), where AT-rich satDNA was preferentially located at terminal
316 positions (ISR-1 and 2) on the chromosomes, with one pair bearing an interstitial site with
317 ISR-3 (Meyer et al., 2010).

318 Examples such as these of *T. infestans* and *I. scapularis,* which show the sharing of
319 specific chromosomal regions with different types of satDNA, or of *Megoura viciae*
320 (Aphidae) where the heterochromatin is restricted to the X chromosome (Bizzaro et al.,
321 1996), or still of *Chrysolina americana* (leaf beetle) which display the same satDNA in all
322 chromosomes of complement (Lorite et al., 2001), could demonstrate the immense
323 variability of behavior and distribution of satDNA in insects. Especially for co-location of
324 different repetitive DNA families, *T. infestans* can be a good example because different
325 kinds of AT-rich satDNA appeared to co-locate with 18S rDNA, microsatellites and
326 transposable elements, as showed here.

327 The sequence of the p*Ti*-95 clone showed two tandem DNA families, namely the
328 satDNA of 79 bp immediately adjacent to the other sequence 370 bp in length, which
329 exhibited identity to a stretch of Polinton-1_Deu of *Drosophila* and to a stretch of the
330 ATPase gene from the Maverick-like polinton of *Cortesia congregata*. The literature

331 contains some examples of co-location of satDNA with transposable elements. In
332 *Drosophila melanogaster*, for instance, some classes of TEs such as *copia*, *gypsy*, *element*
333 *G* and *Bari-1*, are located in the heterochromatin of autosomes and sex chromosomes
334 (Pimpinelli et al., 1995). This has also been seen in vertebrates (cichlids), where *Rex*
335 retrotransposons are found co-locating with pericentromeric heterochromatin (Valente et
336 al., 2011).

337 The p*Ti*-78 clone was particularly interesting because the 33-bp motif exhibited
338 identity with the TIRs of Polintons. These regions show a universal fragment 5'-AG
339 (Kapitonov and Jurka, 2007; Pritham et al., 2007), which was also identified in the 33-bp
340 motifs of seven clones, represented here by the p*Ti*-78 and p*Ti*-646 clones. Besides, the
341 motif of clone p*Ti*-78 showed a TTTCAT fragment that was duplicated, inverted and
342 internally positioned in the 33 bp motif. This drew our attention because the presence of
343 two T residues in the TTTCAT motif is one of the determinant factors for elongation of
344 Polintons by polymerase B (Méndez et al., 1992). In the p*Ti*-646 clone, for instance, we
345 found two internal motifs (TACTTC and TTTCCAT) separated by 12 nucleotides. The
346 substitution of T by C in the first fragment and the addition of a C in the second fragment
347 could be enough to initiate a sliding-back replication, which may explain the tandem
348 amplification of the 33-bp DNA family in *T. infestans*. This case could be similar to that
349 described by Méndez et al. (1992) for bacteriophage ϕ29. The origin of satDNAs from
350 TIRs or other fragments of transposable elements has been previously reported in the
351 literature. In the coleopteran *Misolampus goudoti*, for example, two satDNA families
352 (*Eco*RI with 196 bp and *Psd*I with 1.2 kb), showed similarity with TIRs of class II
353 transposable elements (Pons, 2004).

Another fact that drew out attention is the short length of the motifs of the clones p*Ti*-103 (79 bp), p*Ti*-78 and p*Ti*-646 (33 bp) and p*Ti*-382 (8 bp). The satDNA sequences of more than 100 bp in length seem to be more common in plants and animals (Heslop-Harrison and Schwarzacher, 2011; Palomeque and Lorite, 2008). As examples we can mention the 180 bp repeat of *Eyprepocnemis plorans*, Orthoptera (López-León et al., 1995) and 189 bp repeat of *Myzus persicae*, Hemiptera (Mandrioli et al., 1999), and some are even much larger, such as the 1.2 kb repeat described for *Misolampus goudoti*, Coleoptera (Pons, 2004). But short motifs, such as the AT-rich satDNA reported here for *T infestans*, were also reported for *Messor structor*, Hymenoptera, whose motif showed a length of 79 bp (Lorite et al., 1999), and *Anopheles gambiae*, Diptera, with a length of 53 bp (Krzywinski et al., 2005). At least for *T. infestans*, these short motifs may have originated and amplified from Polintons, arranging the end the AT-rich heterochromatin. This idea is reinforced by AT-rich chromosomal regions that show the main cytological features defining heterochromatin, such as hypermethylation and lack of euchromatin, which were recognized here by immunolabeling with anti-5-Mc, anti-H3K9me2 and anti-H4K5ac, respectively. Evidences produced after immunolabeling with anti-H3K9me2 antibody showed that the heterochromatic regions in insects, such as *Mamestra brassicae* (Lepidoptera) and *Acyrthosiphon pisum* (Aphidae), are also rich in lysine-methylated histone H3 (Borsatti and Mandrioli, 2005; Mandrioli and Borsatti, 2007).

The variability in chromosome band patterns between distinct populations of *T. infestans* is well documented in the literature (Panzera et al., 1995; Panzera et al., 2004). However, the origin of this variability is poorly understood. Besides learning a little more about the origin of the AT-rich portion in *T. infestans*, our study produced new chromosome markers that can be used to check the dynamics of rearrangements that play a

role in karyotype differentiation in this species, as well as in other species of the genus *Triatoma*. Besides evidence of the participation of a Polinton-like element in the origin of AT-rich repeat arrays in *T. infestans*, we can recognize different mechanisms of rearrangement occurring at the ends of chromosomes, such as sliding-back (33-bp motif), slippage replication (8-bp motif), amplification associated with equilocal dispersion (at the chromosome ends) and inversion (pair 7). The greatest impediment was related to obtaining the complete sequence of the Polinton, because these giant TEs have been reconstructed only in silico. Future advances in this area depend on large-scale genomic sequencing to identify, characterize and certify the class or kind of Polinton-like element that now seems to be elected as the source of this large variation in AT-rich heterochromatin in *T. infestans*.

5. Acknowledgements

The authors thank CNPq, Fundação Araucária and CAPES for financial support, as well as Prof. Dr. Marcelo Guerra (UFPE, Brazil) for providing the antibodies and Dr. A. Leyva for English review of the manuscript.

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504
505 **Legends**
506 **Figure 1.** Chromosome mapping in spermatogonial metaphases of *Triatoma. infestans*. (A)
507 Chromosome stained with 2% Giemsa. (B) C-DAPI banding. Note one chromosome of pair
508 7 with a interstitial band (arrowhead). The X chromosome presented $DAPI^+$ bands on each
509 chromosomal end, while the Y chromosome is almost entirely $DAPI^+$. (C) C-CMA
510 banding. Note CMA^+ bands at subterminal regions in the pairs 1-3, as well as the absence
511 of bands in the sex chromosomes. Observe also a heteromorphism in pair four
512 (arrowheads). (D) C-CMA/DAPI band overlapping. Note the adjacent positioning of bands
513 with those CMA more internally located in relation to DAPI bands. (E) FISH with 18S
514 rDNA (p*At*05). Ribosomal signals were detected at subterminal region of pair 4. (F) FISH
515 with p*Ti*-78 probe showing a terminal distribution in pairs 1, 2, 4, 5 and X chromosome.
516 (G) FISH with p*Ti*-103 probe showing a terminal distribution in pairs 1, 2, 3, 4, 5 and X
517 chromosome. (H) Overlap of the images F and G showing the adjacent and independent

positioning of 33 bp and 79 bp motifs at chromosome ends, respectively. (I) FISH with p*Ti*-382 at one end of pairs 2 and 3. (J) Idiogram showing all chromosome signals in *T. infestans*. Bar = 5µm

Figure 2. Information on the sequences obtained from the clones of C_0t microlibrary of *T. infestans*. (A) Sequence of p*Ti*-103 clone with 760 bp length showing a tandemly repeated of 79 bp. Note the predominance of A and T bases. (B) Sequence of p*Ti*-78 clone with 348 bp length showing a tandemly repeated of 33 bp. Note also the predominance of A and T bases. (C) Sequence of p*Ti*-646 clone with of length 282 bp. Note that tandemly repeated sequence with 33 bp motifs is immediately adjacent to 79 bp motif (box). Highlighting in yellow in the clones p*Ti*-78 and p*Ti*-646 identifies the conserved region 5'-AG, and highlighting in gray corresponds to sequences related to Polinton elongation. (D) Sequence of p*Ti*-382 clone with length of 225 bp, which contains a AT-rich CTATCTAA (box) motif 27 times repeatedly.

Figure 3. Information on the sequences obtained from the clones of C_0t microlibrary of *T. infestans*. (A) Sequence of p*Ti*-95 clone with 536 bp length. The gray and yellow colors are indicating two adjacent stretches, 79 bp and 370 bp, respectively. The last sequence (370 bp) exibits 67% of identity with Polinton-1_Deu of *D. eugracilis* (Kojima and Jurka, 2012), separated by 26 bp. (B) Scheme showing the positioning of ATPase gene in a partial organization of Polinton of *Cortesia congregate*, whose protein domain have identity with the fragment of 370 bp of p*Ti*-95 clone. Different amino acids between sequences are represented with gray box. (C) Sequence of p*Ti*-34 clone with length of 336 bp. The yellow

box highlights a 85 bp length sequence that shows 73% similarity with a Polinton-1_HM of *Hydra magnipapillata* (Bao and Jurka, 2008).

Figure 4. Location of probes p*Ti*-78 and p*Ti*-103 in the diffuse stage chromatin of prophase I of *Triatoma infestans*. (A) DAPI staining. (B) Double FISH using the p*Ti*-103 and p*Ti*-78 probes. Arrowhead indicates a terminal chromosome region that presents a yellow signal referent to the 79 bp motif (p*Ti*-103 probe). Note in the top box another terminal chromosome region containing only red signals relative to 33 bp of p*Ti*-78 clone. (C-F) Boxes with high magnification that are showing co-locations between sequences of 33 bp and 79 bp after FISH. Arrows in B indicate part of two bivalents labeled by probes. Bar = 5µm.

Figure 5. Composition and heterochromatic behavior in spermatocytes of *Triatoma infestans*. (A-B) FISH using the p*Ti*-103 and p*Ti*-78 probes in spermatocytes of *T. infestans*. (A) DAPI staining and (B) chromocenter hybridized with both probes. Note regions with co-location of signals (top of chromocenter) and only with 79 bp motifs (yellowish green) on down of chromocenter (see the box). (C, E and G) Spermatocytes counterstained with DAPI. (D) Immunolabeling with anti-5mC. Note the hypermethylated chromocenters. (F) Immunolabeling with anti-H3K9. Note the strong signals in chromocenters. (H) Immunolabeling with anti-H4K5ac. Note absence of signal in chromocenters. (I) Mitotic metaphase with C-banding stained with 2% Giemsa. (J) C-Mitotic metaphase counterstained with DAPI. (K) Immunolabeling with anti-5mC. Note the signals of hypermethylation at the end of chromosomes and in almost all the sex chromosomes (arrows in I, J and K). Bar = 5µm.

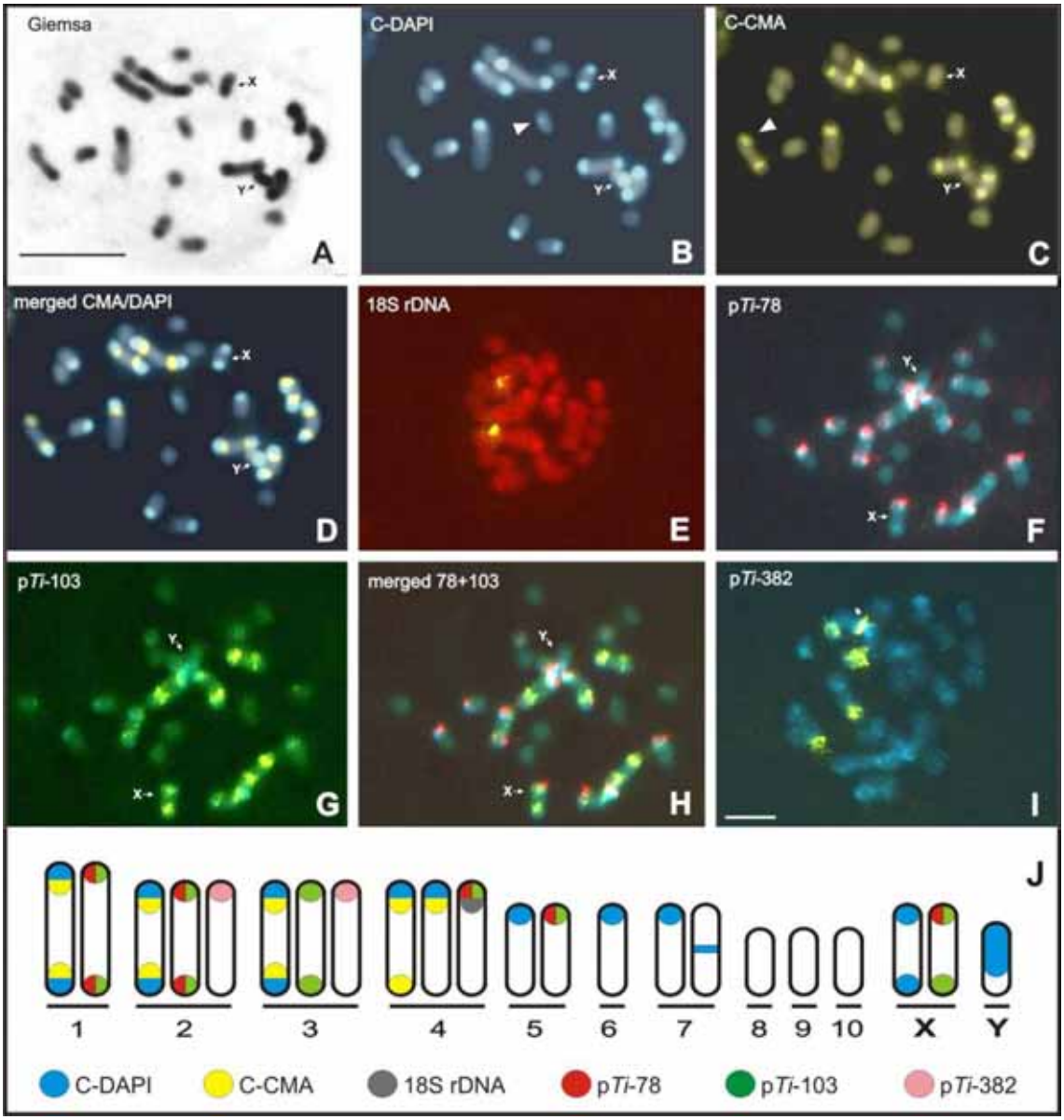
Giemsa
A
C-DAPI
B
C-CMA
C
merged CMA/DAPI
D
18S rDNA
E
pTi-78
F
pTi-103
G
merged 78+103
H
pTi-382
I
J
1
2
3
4
5
6
7
8
9
10
X
Y
C-DAPI
C-CMA
18S rDNA
pTi-78
pTi-103
pTi-382

Figure 1

A) pTi-103 (760 pb length)

```
Consensus pattern with 79 bp length:
AGTTTTCGAGCAACACTCACTTCAGTTGGTTGTAAGGTTCGAGAAAATCCCTAAAATCGGCGTTTCAAACGTAAGAGTG

1........|.........|.........|.........|.........|.........|.........|.........79
AGTTTTCGAGCAACACTCACTTCAGTTGGTTGTAAGGTTYWAGAAAATCCCTAAAATCGGCGTTTCAAACGTAAGAGTG
AGTTTTCGAGCAACACTCACTTCAGTTGGTTGTAAGGTTCAAGAAAATCCCTAAAATCGGCGTTTCAAACGTAAGAGTG
AGTTTTYGAGCAACACTCACTTCAGTTAGTTGTAAGGTTTAAGAAAATCCCTAAAATCGGCGTTTCAAACGTAAGAGTG
AGTTTTCGAGCAACACTMACTTCACTTGGTTGTAAGGTTCGAGAAAATCTTTCAAATCGGCGTTTCAACCGTACGAGTG
AGTTTCCGAGCAACACTMACTTCAGTTGGTTGTAAGGTTCGAGAAAATCCCTAAAATCGGTGTTTCAACCGTAAGAGTG
AGTTTTCGAGCAACACTCACTTCAGTTGGTTGTAAGGTTCGAGAAAATCCCTAGAATCGGTGTTTCAACCGTAAGAGTG
AGTTTTCGAGCAACACTCACTTCACTTGGTTGTAAGGTTCGAGAAGATCCCTAAAATCGGCGTTTCAAACGTAAGAGTG
AGTTTTCGAGCAACACTCACTTCACTTAGTTGTAAGGTTCGAGAAAATCCCTAAAATCGGCGTTTCAAACTTAAGAGTG
AGTTTCCGAGCAACACTCACTTCAGTTGGTTGTAAGGTTCACGAAAATCCCTAAAATCGGCGTTTCAAACCTAAGAGTG
AGTTTTTGAGCAACACTCACTTCAGTTAGTTGTAAGGTTTAAGAAAATC
.........|.........|.........|.........|........760

Matches: 637 (93%), Mismatches: 42 (6%), Indels: 4 (1%)
ACGTcount: A:30%, C:19%, G:21%, T:29%
```

B) pTi-78 (348 pb length)

```
Consensus pattern (33 bp): AGTAATTACGAAGTAATAGACTTATGGAAACCC

1........|.........|.........|..33
............GTAATAGACTTATGTAAATCC
AGTAATTACAAAGTAATAGACTTATGGAAACCC
AGTAATTACGAAGTAATAGTCTTATGAAAAACC
AGTAATTACGAAGTAATAGACTTATGGAAATCC
CGTAATTACGAAGTAATAGACTTATGGAAAACC
AGTAATTACGAAGTAATAGACTTATAGAAAACC
AGTAATTACGAAGTAATAGACTTATGGATACCC
AGTAATTGCGAAGTAATAGACTTATGGAAACCC
AGTAATTACGAAGTAATAGACTTATGGAAACCC
AGTAATTACGCAGTAATAGACTTATGGAAACCC
AGTAATTACGAAGTAATAGACTTATGTAAA
.........|.........|.........348

Matches: 290 (93%), Mismatches: 19 (6%), Indels: 4 (1%)
ACGTcount: A:42%, C:14%, G:17%, T:26%
```

C) pTi-646 (282 pb length)

```
Consensus pattern (33 bp): AGTCTATTACTTCGCAATTACTGGGTTTCCATA
1........|.........|.........|..33
...CTATTACTTCGCAATTACTGGGTTTCCATA
AGTCTATTACTTCGTAATTACTGGGTTTCCATA
AGTCTATTACTTCGCAATTACTGGGTATCCATA
AGTCAATTACTTCGTAATTACTGGGTTTCCATA
AATCTATTACTTCGTAATTACTGGGTTTCCATA
ACTCTATTACTTCGCAAAAGAGTGAGTTTCCGAGCAACACTCACTTCAGTTGGTTGTAAGGTTCACGAAAATCCCTAAAA
TCGGCGTTTCAAACCTAAGNGANNAGATACGCTCATNGCC
.........|.........|.........|.........282

Matches: 136 (93%), Mismatches: 10 (7%)
 ACGTcount: A:26%, C:21%, G:13%, T:40%
```

D) pTi-382 (225 pb length)

```
Consensus pattern with 8 bp length: CTATCTAA

1........|.........|.........|.........|.........|.........|.........|.........|.........90
CTATCTACCTATCTAACAATCCAACTATCTAACTATCTAACTATCTAACCATCTAACTATCTATCTATCTAAGTATCTTACTATCCAAAT
ATCCAACTATCTAACTATCTAACTATCTAACTATCGAACTACCTAACTATCTAACTATATAAATATCTAACTATCCAACTAACTAACTAT
CTAACTATCCAACTATCTAACAACCTATCTATCTAACGATCATCG
.........|.........|.........|.........|....225

Matches: 172 (82%), Mismatches: 37 (18%)
ACGTcount: A:38%, C:28%, G:1%, T:33%
```

Figure 2

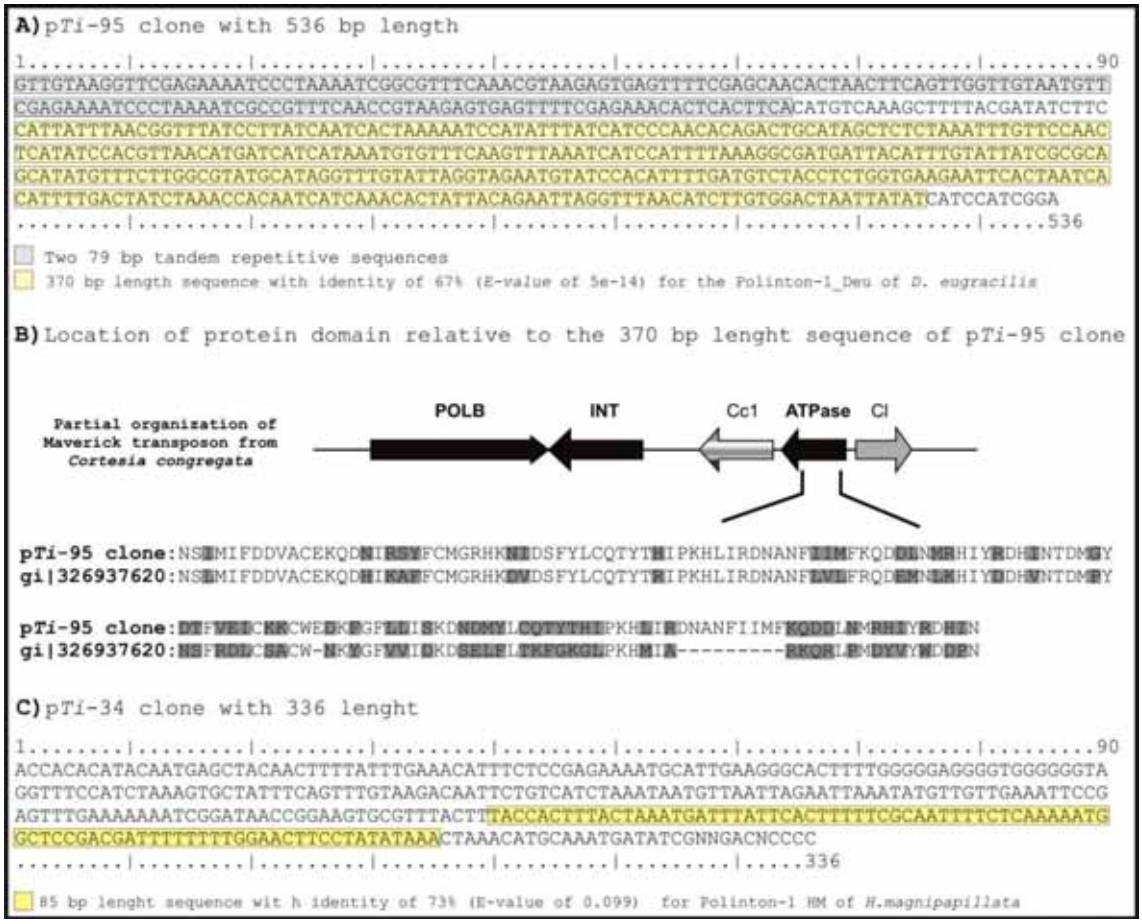
A) pTi-95 clone with 536 bp length
Two 79 bp tandem repetitive sequences
370 bp length sequence with identity of 67% (E-value of 5e-14) for the Polinton-1_Dmu of D. eugracilis
B) Location of protein domain relative to the 370 bp lenght sequence of pTi-95 clone
Partial organization of Maverick transposon from Cortesia congregata
POLB
INT
Cc1
ATPase
CI
pTi-95 clone:
gi|326937620:
C) pTi-34 clone with 336 lenght
85 bp lenght sequence wit h identity of 73% (E-value of 0.099) for Polinton-1 HM of H.magnipapillata

Figure 3

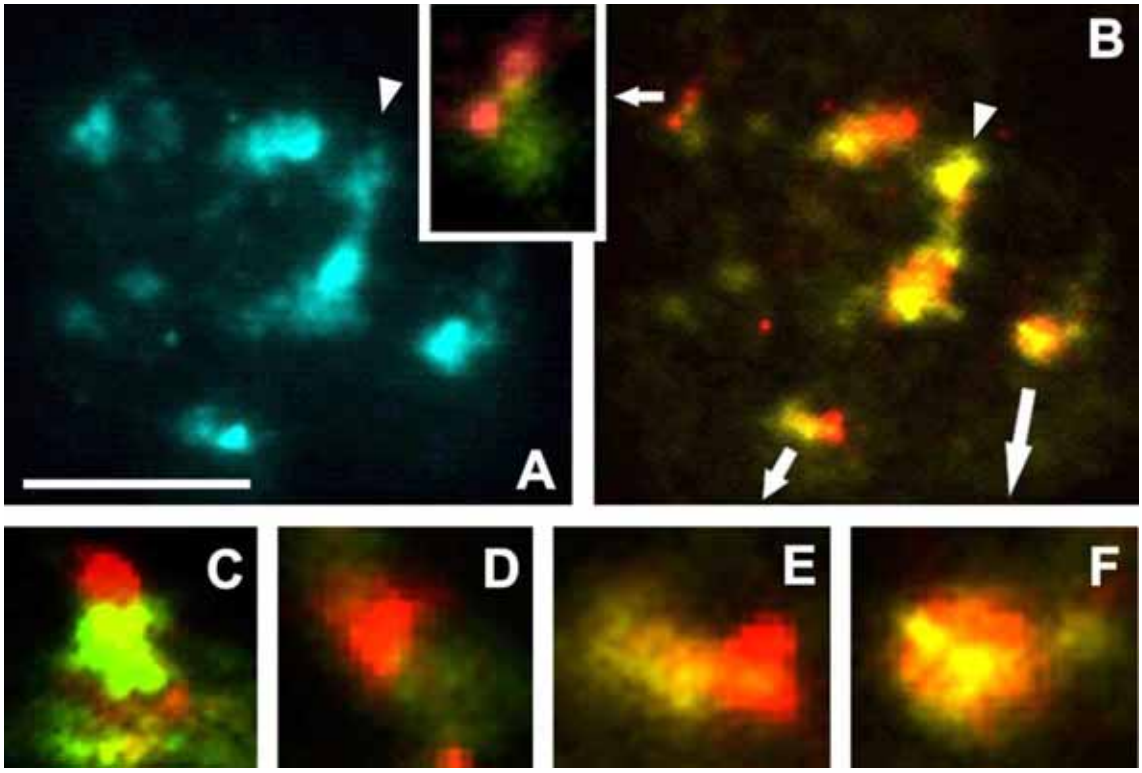

Figure 4

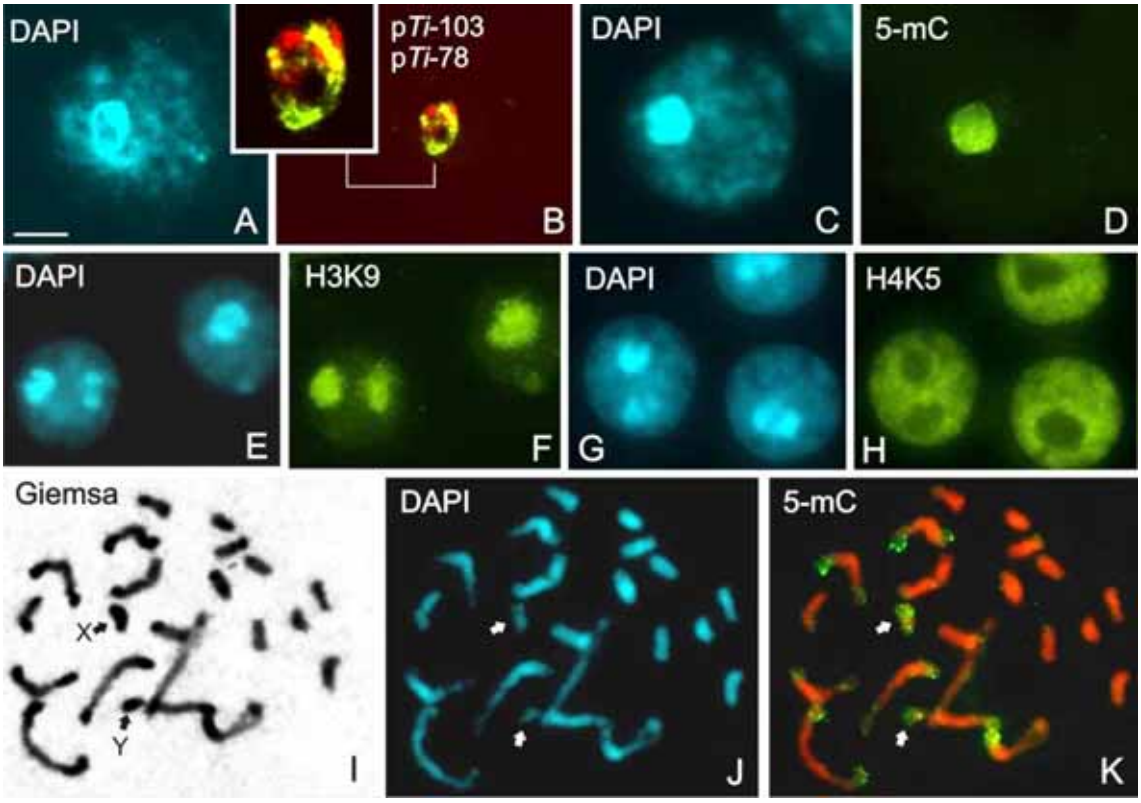
DAPI
A
pTi-103
pTi-78
B
DAPI
C
5-mC
D
DAPI
E
H3K9
F
DAPI
G
H4K5
H
Giemsa
X
Y
I
DAPI
J
5-mC
K

Figure 5

4.4 Capítulo 4

Karyotype relationships among predators of Reduviidae (Cimicomorpha, Heteroptera)

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Fator de impacto: 2.413

Karyotype relationships among insect predators of the family Reduviidae (Heteropterans)

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Short running title: Karyotype evolution in heteropteran predadors

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Abstract

Species of infraorder Cimicomorpha of Heteroptera exhibit holokinetic chromosomes, with inverted meiosis for alosomes and high diversity in chromosome numbers. The family Reduviidae that belongs to this infraorder is also recognized by high variability of occurrence and distribution of heterochromatic bands and 18S rDNA sites. We studied here five species of Reduviidae with predator habit, which are especially interesting because individuals are found solitary and dispersed in nature. Karyotypes exhibited a large variation in chromosome number, karyotype asymmetry and distribution of repetitive DNA families. Although some of these species it was possible to detect the role of chromosome rearrangements in karyotype differentiation, the constancy of location of heterochromatin in the terminal chromosome regions in some

species, seems to be reinforcing the model of dispersion of repetitive DNA families based in the “bouquet configuration”.

Key-words: Chromosome number, CMA_3/DAPI banding, Heteroptera, molecular cytogenetics, 18S rDNA

Introduction

Species of Heteroptera share several cytogenetical features, such as the occurrence of holokinetic chromosomes, inverted meiosis for sex chromosomes, and large variation in chromosome numbers (Ueshima, 1979; Pérez *et al*., 2000; Papeschi and Bressa, 2006; Bardella *et al*., 2013). Chromosome numbers vary from $2n = 4$ in Nepomorpha, Belostomatidae to $2n = 80$ in Cimicomorpha, Miridae (Ueshima, 1979), and this last infraorder presents the greatest karyotype diversity among Heteroptera (Kuznetsova *et al*., 2011). These insects exhibit also a large diversity in the heterochromatic patterns (Bressa *et al*., 2005; Panzera *et al*., 2010; Bardella *et al*., 2014a; Chirino *et al*., 2013), although heterochromatin tend to occur predominantly at terminal chromosome regions (Papeschi and Bressa, 2006). Among Reduviidae (Cimicomorpha), the species of Triatominae exhibit large differences in the amount and number of heterochromatic bands (Panzera *et al*., 2004; Panzera *et al*., 2010). In this taxonomical group, *Triatoma infestans* is the most well studied species in relation to interpopulational heterochromatic variation (Panzera *et al*., 2004; Bardella *et al*., 2010).

The repetitive DNA family that has been located in chromosomes of Pentatomomorpha and Cimicomorpha is the 18S rDNA (Bardella *et al*., 2010; Poggio *et al*., 2011; Panzera *et al*., 2012). In Triatominae the number of hybridization sites varied from 1 to 3, which were located in both auto and alosomes, like observed in other

species of Cimicomorpha (Grozeva *et al*., 2011; Poggio *et al*., 2011; Panzera *et al*., 2012). The location of 18S rDNA in Pentatomomorpha drew attention because of the 36 species studied until now, only four of them hybridization sites appeared in sex chromosomes (González-García *et al*., 1996; Bressa *et al*., 2008; Grozeva *et al*., 2011; Bardella *et al*., 2013).

Cimicomorpha includes species with different habits, such as predators and hematophagous (Reduviidae), phytophagous (Miroidae) and ectoparasites (Cimicidae and Polyctenidae) (see Schuh and Slater, 1995). Reduviidae predators are interesting because they act in the biological control of other insects, either in natural or agricultural environment (Schaefer and Panizzi 2000); however, the study of these insects is difficult because they are always found scattered in nature, without the formation of colonies. The small number of individuals obtained in collects limits the comparative analyzes of relatedness, karyotype evolution, as well as population approaches. We done a considerable investment to obtain the largest possible number of predators of Cimicomorpha to increase knowledge of the karyotypical structure of these insects. Our goal was generate a great volume of data to compare with results produced until now for other groups of heteropterans, but especially to verify the tendency of distribution of heterochromatin and 18S rDNA sites based in the model of bouquet for dispersion of repetitive DNA families.

Materials and Methods

Five species of Heteroptera belonging to the family Reduviidae (subfamily Harpactorinae) were collected in the South and Southeast regions of Brazil. Information about the collection localities are given in the Table 1. Gonads were dissected out and

the seminiferous tubules were fixed in a solution of methanol-acetic acid (3:1, v:v) and stored at −20 ºC. For slides preparation, tubules were incubated in 45% acetic acid for 10 min at room temperature, and immediately squashed in a drop of 45% acetic acid. Coverslips were removed after freezing in liquid nitrogen, and the slides air-dried. For conventional staining the slides were treated with HCl 1M for 6 min and stained with 2% Giemsa. The samples were air-dried and mounted with Entellan. Chromosome measurements were made in five metaphases for each species, where were determined the haploid relative length of each chromosome pair and the symmetry index, which was calculated from the ratio between the sizes of the largest and the smallest chromosomes (Stebbins, 1971).

For chromosome banding (Sumner, 1982, with small modifications), slides were aged for three days after removal of coverslips. Afterwards, the slides were incubated in 0.2 N HCl for 10 min at room temperature, 5% barium hydroxide at 60ºC for 2 min, and 2× SSC, pH 7.0, at 60ºC for 60 min. Samples were treated with fluorochromes: 0.5 mg/mL chromomycin A_3 (CMA_3) for 1.5 h and 2 µg/mL 4'-6-diamidino-2-phenylindole (DAPI) for 30 min. Preparations were mounted with a medium composed of glycerol/McIlvaine buffer, pH 7.0 (1:1, v:v), plus 2.5 mM $MgCl_2$.

Fluorescent in situ hybridization (FISH) was done as described in Bardella *et al*. (2010) and performed in samples of at least two individuals. The p*At*05 clone, containing a partial sequence of the 18S rDNA of *Antiteuchus tripterus* (Pentatomidae, Pentatomomorpha) was labeled with digoxigenin-11dUTP by nick translation (Dignick translation Roche). Preparations were treated with 30 µL of hybridization mixture containing 4 µL of labeled probe (100 ng), 15 µL of 100% formamide, 6 µL of 50% polyethylene glycol, 3 µL of 20× SSC, 1 µL of 10% SDS and 1 µL of water. Chromosome denaturation/renaturation was done at 90ºC for 10 min using a thermal

cycler, and hybridization was performed for 12 h at 37 ºC in a humidified chamber. Post-hybridization washes were carried out in SSC buffer, with a 60% stringency due to heterologous hybridization. For detection, anti-digoxigenin-rhodamine in 5% BSA/4× SSC/0.2 Tween 20 (1:100, v:v) was used. The post-detection washes were performed in 4× SSC/0.2 Tween 20 at room temperature. Slides were mounted with 26 µL of DABCO solution (1,4-diaza-bicyclo (2.2.2)-octane (2.3%), 20 mM Tris-HCl, pH 8.0 (2%) and glycerol (90%) in distilled water), 2 µL of 2 µg/mL DAPI and 1 µL of 50 mM $MgCl_2$.

All chromosome images were acquired separately in grayscale mode using a Leica DM 4500 B microscope equipped with a DFC 300FX camera. Pseudo coloration of blue/red colors for DAPI, greenish for CMA and greenish-yellow for rhodamine were done using Leica IM50 4.0, as well as the overlapping of images.

Results

The chromosome number of five species of Reduviidae varied from $2n = 12 + XY$ (Figure 1D), with chromosomes measuring from 4,95 µm to 2 µm in *M. confusa*, until $2n = 24 + X_1X_2X_3Y$ (Figure 1B), with chromosomes measuring from 2,89 µm to 1,44 µm in *C. nigroannulatus* (Table 2). Karyotypes appeared asymmetrical (Figure 2), especially in *M. confusa*, which exhibited a almost bimodal karyotype with three major and three smaller autosomal pairs (Figure 1D). In the remainder species, such as *A. lanipes* (Figure 1A), the asymmetry happens because the largest bivalent is much larger than the smallest chromosome of the complement (Figure 1 and 2 and Table 2). If we compare the karyotype asymmetry using only autosomes, ie. without sex chromosomes, we can note that differences in the asymmetry index among species is very small.

The predominant sex determination system was the simple XY, except to *C. nigroannulatus* that presented $X_1X_2X_3Y$ (Figure 1B). The difficult of keeping these species in captivity made it impossible to obtain eggs, and this prevented the differentiation of the sex chromosomes X and Y. Then, these chromosomes are named here generically as only "sex chromosomes". The comparison of measurements of sex chromosomes showed that $X_1X_2X_3$ of *C. nigroannulatus* are five times smaller than sex chromosomes of *M. confusa, R. flavicans and Z. laticornis*, until ten times smaller than sex chromosomes of *A. lanipes* (Table 2, Figure 1).

The chromosome banding exhibited a large variability in the occurrence C-$DAPI^+/CMA_3^+$ bands among five species. *Apiomerus lanipes* presented C-$DAPI^+/CMA_3^+$ terminal bands only in the largest autosome and in the sex chromosomes (Figure 3A-B). However, the C-$DAPI^+$ band occupied both sex chromosomes, while the C-CMA_3^+ bands appeared as a subterminal dot in one sex chromosome, but not in the other, which was totally C-CMA_3^+ (Figure 3A-B). In *M. confusa*, the two largest autosomes and the sex chromosomes exhibited C-$DAPI^+/CMA_3^+$ bands in both terminal regions (Figure 3 D-E). The third largest bivalent showed a C-$DAPI^+/CMA_3^+$ band in only one terminal region, while the three smaller pairs were totally C-$DAPI^+/CMA_3^+$ (Figure 3D-E). Unlike *A. lanipes* and *M. confusa*, who showed a larger concentration of bands, *C. nigroannulatus* and *Z. laticornis* exhibited C-$DAPI^+/CMA_3^+$ band in only one of the sex chromosomes (Figures 3G-H and J-K, respectively). *Repipta flavicans* exhibited no heterochromatic bands (Figure 3M-N).

FISH showed variation in the number, location and signal intensity of 18S rDNA, but the signals appeared always in terminal position of the five species analyzed. In *A. lanipes*, one of the sex chromosomes showed hybridization signal in only one chromosome end, while the other sex chromosome exhibited sites of 18S rDNA in both

terminal chromosome regions (Figure 3C). In *C. nigroannulatus* one of the signals of 18S rDNA was located in the largest sex chromosome, while the other hybridization signal was observed in one of fragmented X chromosomes (Figure 3I). Only in *R. flavicans* one hybridization signal in one of the sex chromosomes was observed (Figure 3O). In *M. confusa* (Figure 3F) and *Z. laticornis* (Figure 3L) the signals of 18S rDNA appeared in large autosome bivalents. The size of 18S rDNA signals varied also among species. The brighter signals was detected in both sex chromsomes of *A. lanipes* (Figure 2C), and the signal was less intense in one of sex chromosome of *R. flavicans* (Figure 2O).

Discussion

Species of Reduviidae present a variation in chromosome numbers ranging from $2n = 10 + XY$ in the genus *Polididus* (Manna and Deb-Mallich, 1981) until $2n = 32 + XY$ in the genus *Bagauda* (Ueshima, 1979), and the species analyzed here are within this range. In the context of Cimicomorpha, species of Reduviidae present low chromosome numbers variation, when these species are compared with members of Miridae (from $2n = 14$ to 80) and Cimicidae with $2n = 14$ to 50 (Kuznetsova *et al*., 2011). The large variation in the chromosome numbers within Heteroptera has been associated with chromosome rearrangements, such as fusion and fission of holokinetic chromosomes (Ueshima, 1979).

Although dysploidy is recognized as an important evolutionary mechanism for karyotype differentiation in organisms with holokinetic chromosomes, for both plants (Guerra, 2008) and animals (Svartman *et al*., 2004), the lack of global phylogenetic analyzes, as well as the lack of chromosome markers for most heteropterans, it becomes very speculative indicate which karyotypes could be plesiomorphic or apomorphic, and

also evolutionary direction for certain rearrangements. This seems to be more complicated when we considered the group of Heteroptera predators. However, there are occasional cases where this variation can be well perceived, such as in *C. nigroannulatus*, where the numerical diversity is clearly linked to the fragmentation of sex chromosomes, which produced the system $X_1X_2X_3Y$ (Ueshima, 1979; Poggio *et al.* 2007).

The five karyotypes of Reduviidae studied here can be considered relatively asymmetrical, due to relative decrease in size of autosomes, or by differences in the size of the sex chromosomes. The karyotype asymmetry observed in *C. cosmoclopius*, *R. flavicans* and *Z. laticornis* is very similar to those found in members of other families of Heteroptera, such as *Holhymenia rubiginosa*, Coreidae (Bressa *et al*., 2008) and *Edessa rufomarginata*, Pentatomidae (Rebagliati *et al*., 2003). The karyotype asymmetry observed in *A. lanipes* drew attention due to occurrence of a big bivalent in comparison to other chromosomes of the complement, which could be associated to reduction in the chromosome number ($2n = 22 + XY$), when it is compared with the modal number of subfamily Harpactorinae ($2n = 24 + XY$). One similar case was of *Dichelops furcatus* with $2n = 10 + XY$, Pentatomidae, where one much larger bivalent derived by chromosome fusion was reported (Rebagliati *et al*., 2001). The most striking case of asymmetry found here was the bimodal karyotype of *M. confusa*. Grozeva *et al.* (2006) reported also other case of almost bimodal karyotypes for *Macrolophus costalis*, Miridae. When we compared these cases with *Triatoma infestans*, we can perceive that asymmetry may be associated with other mechanisms of differentiation of karyotypes, such as the differential accumulation of repetitive DNA families at the chromosome ends (Panzera *et al*., 1995; Panzera *et al*., 2004; Bardella *et al*., 2014b). However, this

seems not be the case of *A. lanipes* and *M. confusa*, because if we disregard the heterochromatin, the karyotypes will remain asymmetrics.

The high variation in the occurrence and distribution of heterochromatin in auto and alosomes among species of Heteroptera is very well documented in the literature, even to these bands that occur predominantly at terminal chromosome regions (Bressa *et al*., 2005; Panzera *et al*., 2010; Bardella *et al*., 2014a). Among the predator species studied here, *M. confusa* drew attention by accumulation of heterochromatin in the terminal regions of all chromosomes, while in other species, such as *A. lanipes*, the heterochromatin was restricted in terminal regions of one bivalent and in all extension of both sex chromosomes. The best studied species of Reduviidae in relation to the distribution of heterochromatin was *Triatoma infestans*. This species exhibits bands always in terminal chromosome regions, but there is a large variation in the chromosome pairs that are carrying bands, which is associated with the geographic distribution of each population in the South America (Panzera et al., 2004). High interspecific variation in distribution of heterochromatin was also reported to other species of infraorder Cimicomorpha (Grozeva and Nokkala, 2001, Panzera *et al*., 2010), and Pentatomomorpha (Bardella *et al*., 2014a). Despite this high variability found in the occurrence of heterochromatin, the constancy in the positioning of bands in terminal chromosome regions, suggests that mechanisms of dispersion of heterochromatin could be associated with positioning of satDNA in interphase. The model of "bouquet polarization", which postulates that the chromosomes can be closely associated by the ends to the nuclear envelope, could support the idea of sharing of repetitive DNA families at terminal chromosomal regions. This model was proposed by Rodriguez Iñigo *et al*. (1996) when cells in the transition interphase-prophase I of *Dociostaurus genei*, Orthoptera were studied. Among Heteroptera, the "bouquet configuration" was

mentioned for *Pyrrhocoris apterus,* Pyrrhocoridae (Suja *et al*., 2000), *Graphosoma italicum*, Pentatomidae (Viera *et al.*, 2009) and *Triatoma infestans* (Bardella *et al.*, 2014b). Except for *Holhymenia histrio* that presents interstitial bands in all chromosomes (see Bardella *et al.*, 2014a), the terminal pattern of heterochromatin distribution, such as found here in *M. confusa*, was also found in all species of Heteroptera that accumulate or not bands. The total absence of bands, such as found here in *R. flavicans* and in two species of Coreidae: *Athaumastus haematicus* (Bressa *et al*., 2005) and *Chariesterus armatus* (Bardella *et al*., 2014a), as well as *Microtomus lunifer*, Reduviidae (Poggio *et al*., 2011) and *Mormidea v-luteum*, Pentatomidae (Bardella *et al*., 2014a), where there are a small number of heterochromatic bands observed, suggest that accumulation or not of heterochromatin might be intrinsic in each genome, regardless of the phylogenetic relationships of the species studied to date.

The five species of predators studied here presented a variation in number (1-3) and distribution (auto- and alosomes) of 18S rDNA sites. This finding is within the range previously reported to Reduviidae (Bardella et al., 2010; Panzera *et al*., 2012). There was a proposal that species of Reduviidae presented 18S rDNA sites always associated sex chromosomes, when these ones happen in a simple sex system, and that 18S rDNA sites appears always in autosomes when there are multiple sex system (Poggio *et al.*, 2011). However, our data in *C. nigroannulatus*, that present fragmentation of X chromosome, suggest an additional situation for distribution of 18S rDNA sites, since this site appears in one of the X chromosome fragmented and in the Y chromosome. There is at least one example in the *Dysdercus albofasciatus*, Pyrrhocoridae, where the 18S rDNA site anteriorly present in one autosome was rearranged to one X chromosome after a fusion event, generating a sex system composed by neo-X and neo-Y (Bressa *et al*., 2009). Even though we have not observed

chromosomal rearrangements directly associated with the mobility of 18S rDNA sites, for that we can not rule out this possibility given the great variations in the distribution of 18S rDNA sites in 49 species of Cimicomorpha studied until now. On the other hand, in the infraorder Pentatomomorpha, there is a trend for distribution of 18S rDNA sites among autosomes, with always two hybridization sites (Bardella *et al*., 2013).

The five species of predator studied here belong to family Reduviidae, tribe Harpactorinae, however, these species are not closely related within of this tribe. Despite of phylogenetical distance among them, it was possible to recognize some cytogenetical features that are common to Reduviidae, and even with other groups of Heteroptera, such as: i) large variation in chromosome number, ii) karyotype asymmetry, iii) XY/XX sex system, iv) terminal location of heterochromatin and 18S rDNA sites and v) high heterochromatic diversity among sex chromosomes, including chromosomes without bands, with C-CMA$^+$/DAPI$^+$, only C-CMA$^+$, only C-DAPI$^+$ and with dots C-CMA$^+$. The group of predators need of more attention and investments in field collection because, as these insects are solitary life, the knowledge of intra-and inter-diversity seems to be always fragmented. In our view, studies involving species of Reduviidae predators to produce an evolutionay model, such as already described for *T. infestans*, will require a long time.

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Legends

Table 1. Number of males analyzed and localities collection with chromosome number of each species.

Table 2. The average of the absolute sizes of chromosomes with all measurements displayed in µm.

Figure 1. Conventional staining with Giemsa 2% in five species of Reduviidae. (A) *Apiomerus lanipes* with $2n = 22 + XY$. (B) *Cosmoclopius nigroannulatus* with $2n = 24 + X_1X_2X_3Y$. (C) *Zelus laticornis* with $2n = 24 + XY$. (D) *Montina confusa* with $2n = 12 + XY$. (E) *Repipta flavicans* with $2n = 18 + XY$. The arrowheads indicate the sex chromosomes. Bar = 5µm.

Figure 2. Comparison of symmetry index with sex chromosomes and without sex chromossomes among the five species of Reduviidae. Note the symmetry index with sex chromosomes larger than the symmetry index without sex chromosomes, except for *A. lanipes*.

Figure 3. C-CMA/DAPI banding and FISH with 18S rDNA probe in meiotic cells of five Cimicomorpha species. (A-C) *Apiomerus lanipes*. Note the terminal $DAPI^+/CMA^+$ bands in the largest bivalent (arrows), one of the sex chromosomes totally $DAPI^+/CMA^+$ (arrowhead) and other sex chromosome fully $DAPI^+$ (arrowhead) with a terminal CMA^+ dot. Note the signals of 18S rDNA in both sex chromosomes (arrow). (D-F) *Montina confusa*. Note the $DAPI^+/CMA^+$ terminal bands in most chromosomes and 18S rDNA sites in one of the largest bivalent (arrow). (G-I) *Cosmoclopius nigroannulatus*. Note the

Y chromosome entirely $DAPI^+/CMA^+$ (asterisk) and hybridization signals of 18S rDNA probe in the sex chromosomes (arrows). (J-L) *Zelus laticornis*. Observe one of the sex chromosomes totally $DAPI^+/CMA^+$ (asterisk) and sites of 18S rDNA in one bivalent (arrow). (M-O) *Repipta flavicans.* Note the hybridization signals in one of the sex chromosomes (arrows). Arrowheads indicated the sex chromosomes. Bar = 5µm.

Table 1. Number of males analyzed and collection localities in Brazil (MS = state of Mato Grosso do Sul; PR = state of Paraná; SP = state of São Paulo).

Species/Tribe	Males	Localities	Coordinates	Chromosome number
Apiomerini				
Apiomerus lanipes (Fabricius, 1803)	6	Nova Alvorada do Sul-MS	21° 23.058'S 54° 23.012'W	2*n*=22+XY
Harpactorini				
Cosmoclopius nigroannulatus (Stål, 1986)	4	Londrina-PR	23° 18.394'S 51° 12.139'W	2*n*=24+$X_1X_2X_3Y$
Zelus laticornis (Herrich-Schäffer, 1853)	5	Assis-SP	22° 28.645'S 50° 20.983'W	2*n*=24+XY
Montina confusa (Stål, 1859)	3	Assis-SP	22° 28.645'S 50° 20.983'W	2*n*=12+XY
Repipta flavicans (Amyot & Serville, 1843)	5	Borrazópolis-PR	23° 56.225'S 51° 35.280'W	2*n*=18+XY

Table 2. The average of the absolute sizes of chromosomes with all measurements displayed in µm.

Species/ pairs	*A. lanipes*	*C. nigroannulatus*	*Z. laticornis*	*M. confusa*	*R. flavicans*
1	4,64 ± 0,33	2,89 ± 0,27	3,79 ± 0,62	4,95 ± 0,64	3,83 ± 0,45
2	3,76 ± 0,29	2,74 ± 0,33	3,13 ± 0,40	4,83 ± 0,53	3,09 ± 0,28
3	3,50 ± 0,27	2,58 ± 0,11	2,99 ± 0,48	4,10 ± 0,35	2,96 ± 0,17
4	3,50 ± 0,27	2,50 ± 0,00	2,88 ± 0,52	2,63 ± 0,47	2,79 ± 0,17
5	3,40 ± 0,22	2,26 ± 0,13	2,70 ± 0,35	2,63 ± 0,47	2,65 ± 0,10
6	3,30 ± 0,00	2,20 ± 0,00	2,54 ± 0,09	2,56 ± 0,35	2,60 ± 0,12
7	3,30 ± 0,00	2,14 ± 0,13	2,54 ± 0,09	2,00 ± 0,31*	2,48 ± 0,21
8	3,25 ± 0,11	2,14 ± 0,13	2,42 ± 0,22	-	2,40 ± 0,24
9	3,08 ± 0,25	1,96 ± 0,25	2,36 ± 0,23	-	2,33 ± 0,35
10	2,96 ± 0,26	1,96 ± 0,25	2,26 ± 0,25	-	1,79 ± 0,33*
11	2,64 ± 0,43	1,78 ±0,16	2,20 ± 0,21	-	-
12	3,33 ± 0,17*	1,60 ± 0,00	2,08 ± 0,34	-	-
13	-	1,44 ± 0,18*	1,70 ± 0,58*	-	-

The asterisks indicate the average absolute size of the sex chromosomes; -: points the absence of the pair in the species.

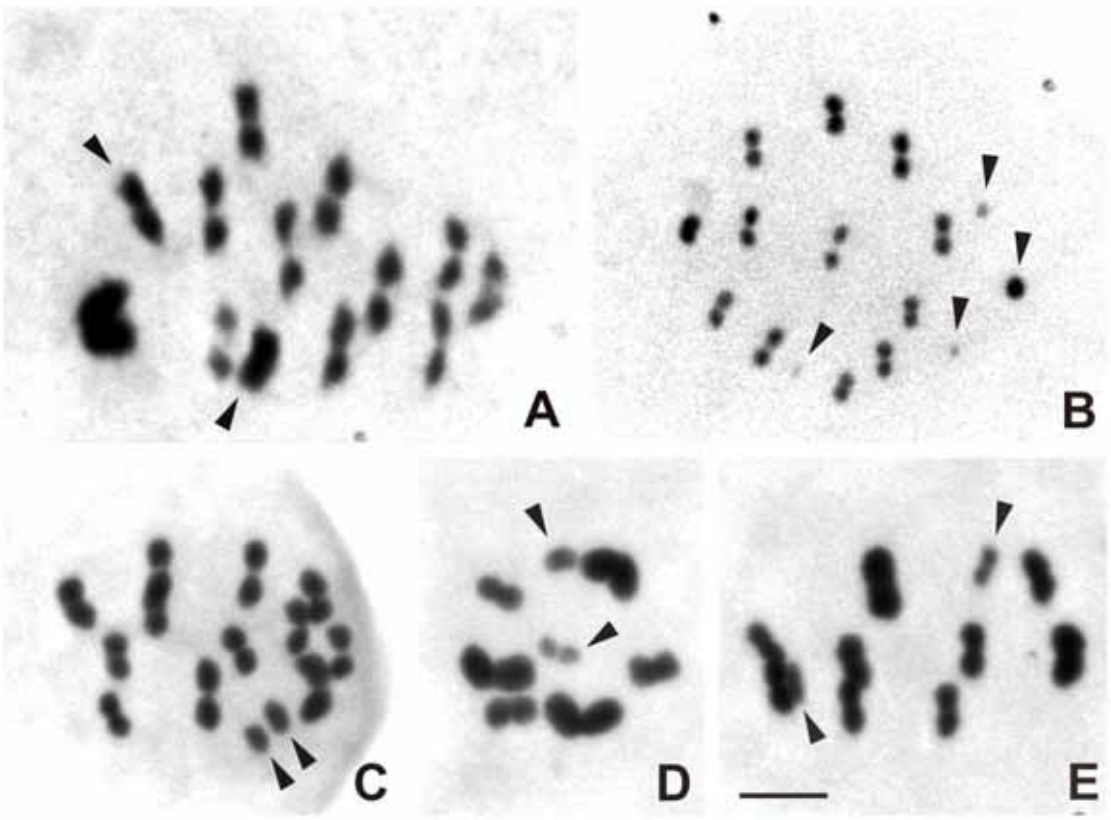

Figure1

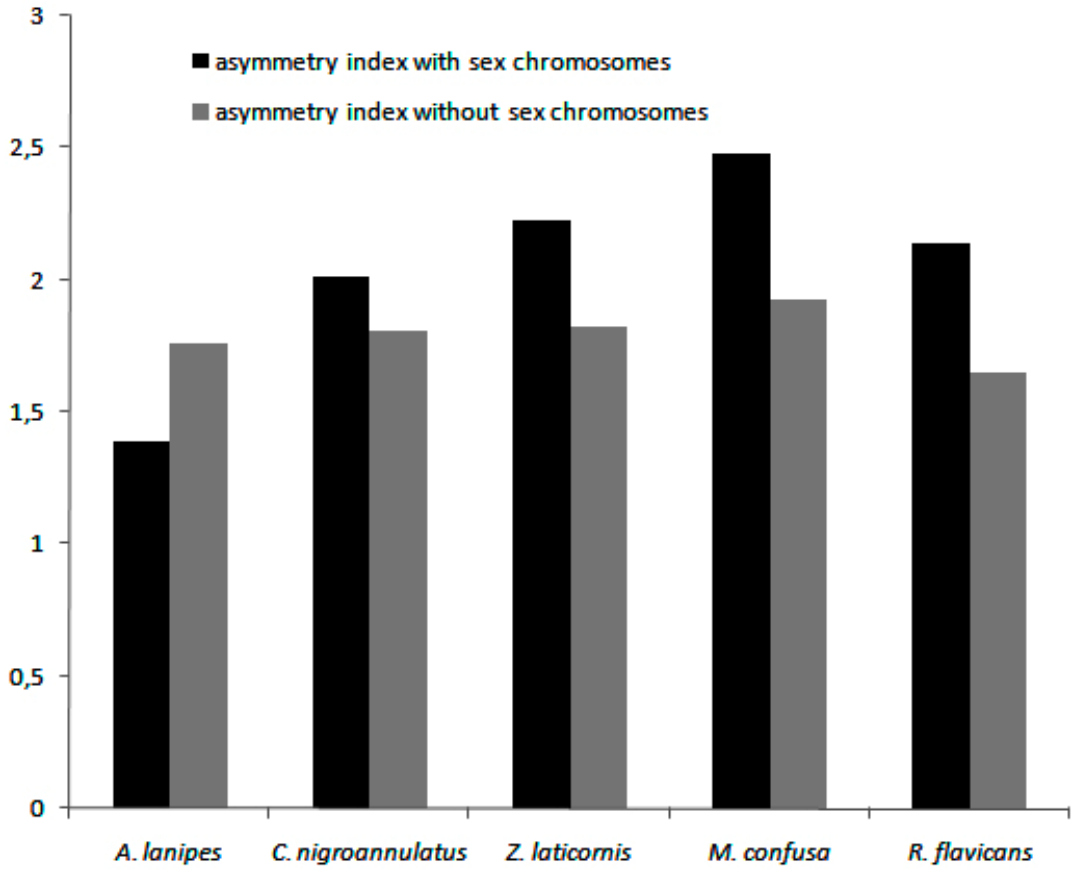
3
2,5
2
1,5
1
0,5
0
asymmetry index with sex chromosomes
asymmetry index without sex chromosomes
A. lanipes
C. nigroannulatus
Z. laticornis
M. confusa
R. flavicans

Figure 2

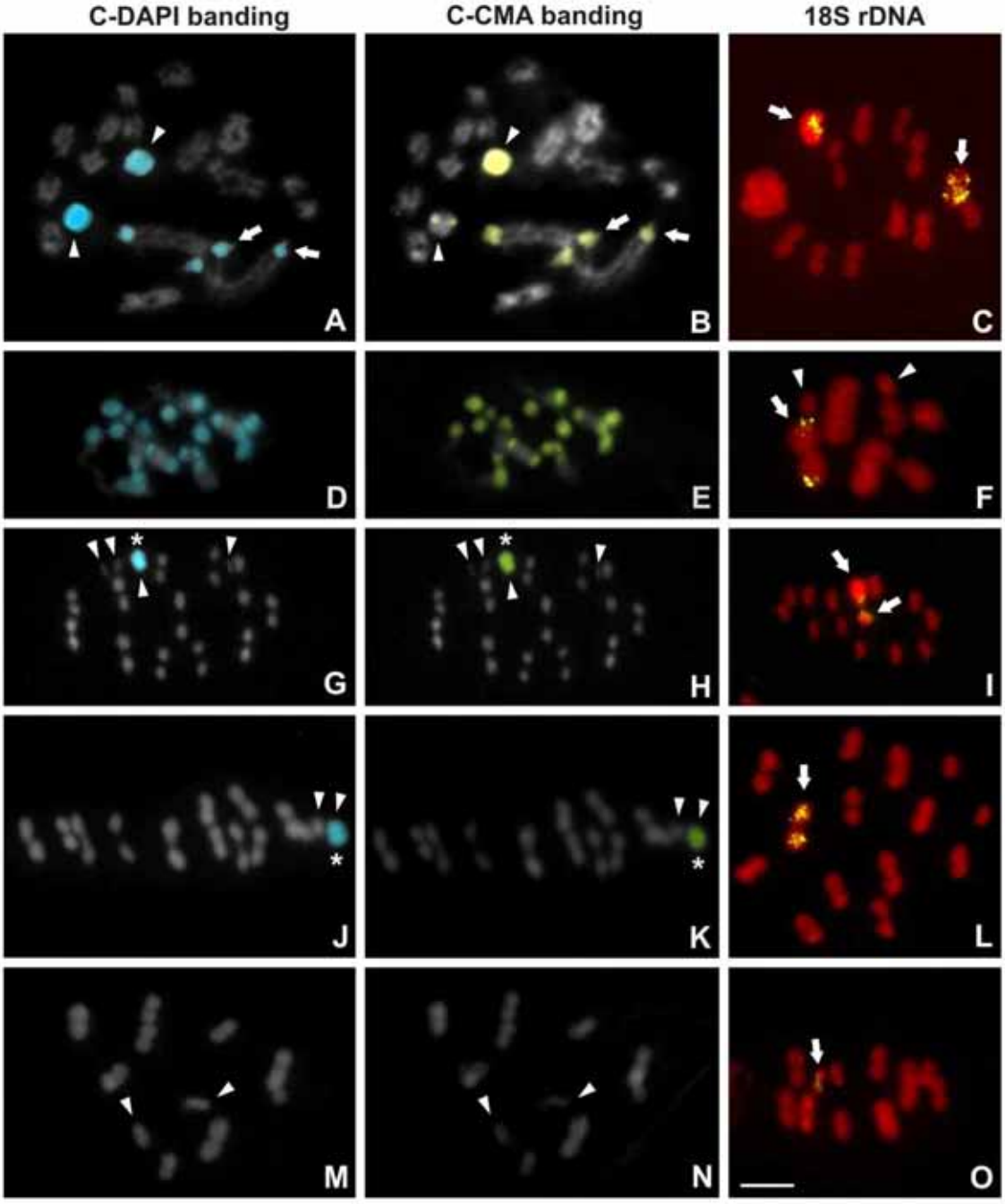

Figure 3

5. Discussão Geral (concisa)

Nos heterópteros pertencentes à infraordem Pentatomomorpha a variação no número cromossômico é recorrente em diferentes famílias como Coreidae e Lygaeidae (PAPESCHI; BRESSA, 2006). Em nossas análises abrangendo 30 espécies de Pentatomomorpha e Cimicomorpha a variação no número cromossômico foi confirmada e associada a eventos de disploidia.

Diferentes estudos exibem como uma das características citogenéticas dos heterópteros a localização terminal da heterocromatina (BRESSA et al., 2005; PANZERA et al., 2010; CHIRINO et al., 2013). Nós observamos uma grande diversidade na distribuição heterocromática desses insetos com a distinção de seis perfis: *i*) 6 espécies (*Athaumastus haematicus*, *Cebrenis* sp., *Althos obscurator*, *Chariesterus armatus*, *Dysdercus ruficollis* e *Repipta flavicans*) sem bandas detectáveis, *ii*) 10 espécies (*Antiteuchus tripterus*, *Arvelius albopunctatus*, *Euschistus cornutus*, *Euschistus heros*, *Leptoglossus zonatus*, *Acanonicus hahni*, *Harmostes prolixus*, *Harmostes prolixus*, *Dysdercus fulvoniger* e *Mormidea v-luteum*) com apenas um *dot* nos autossomos , *iii*) 1 espécie (*Leptoglossus gonagra*) com *dot* nos m cromossomos, *iv*) 2 espécies (*Cosmoclopius nigroannulatus* e *Zelus laticornis*) com heterocromatina apenas nos cromossomos sexuais, *v*) 10 espécies (*Edessa rufomarginata*, *Edessa impura*, *Edessa meditabunda*, *Spartocera fusca*, *Phtia picta*, *Hypselonotus interruptus*, *Hypselonotus fulvus*, *Euryophthalmus rufipennis*, *Montina confusa* e *Apiomerus lanipes*) com acumulação de bandas terminais, e *vi*) 1 espécie (*Holhymenia histrio*) com banda intercalar. O modelo de dispersão heterocromática sugerido para as espécies com acúmulo de heterocromatina terminal foi a dispersão equilocal, sustentado pela associação das pontas dos cromossomos em uma estrutura em "bouquet" proposta por Rodriguez-Iñigo et al. (1996) em *Dociostaurus genei*, Orthoptera. Com isso, o posicionamento intersticial de bandas como em *Holhymenia histrio*, Coreidae seria uma característica derivada.

Outra característica dos heterópteros, relatada principalmente na subfamília Triatominae, Reduviidae (Cimicomorpha), é a distribuição dos sinais de DNAr 18S nos autossomos ou nos cromossomos sexuais, com a variação no número de sítios entre espécies e populações (PANZERA et al., 2012). A mesma diversidade observada por Panzera et al. (2012) nos triatomíneos foi confirmada em cinco espécies de Reduviidae. Diferentemente, nossas análises em 25 espécies de Pentatomomorpha evidenciaram uma predominância no número de sinais de hibridização (2) e a tendência de distribuição dos sítios de DNAr 18S entre os autossomos.

Um dos principais modelos citogenéticos dentro dos heterópteros é *Triatoma infestans*, Reduviidae. Nesta foram observadas variações heterocromáticas intraespecíficas em insetos de regiões geográficas distintas (Panzera et al., 2004). Entretanto, pouco era conhecido sobre a natureza das famílias de DNAs repetitivos neste organismo. Com a obtenção das sondas p*Ti*-78 e p*Ti*-103 compostas, respectivamente, pelas sequências curtas de 33 bp e de 79 bp de comprimento, conseguimos identificar a composição e origem do DNA satélite rico em AT. Essas sequências foram possivelmente originadas a partir de elementos de transposição gigantes, conhecidos como Polintons. Além dessas sequências, foi encontrada um família de microsatélite (p*Ti*-382) com 8 bp de comprimento e também associada a uma das regiões terminais dos pares 2 e 3 de *T. infestans*.

6. Conclusões gerais

Os esforços nas coletas dos heterópteros ocorridas durante esses quatro anos resultaram na captura de 30 espécies diferentes. Essas nos forneceram um material biológico rico para o estudo citogenético. Entretanto, como espécies pertencem a grupos taxonômicos distintos e pouco relacionados, as nossas expectativas em elaborar um modelo contemplando a evolução cromossômica do grupo ficou deficiente. Embora essa dificuldade tenha ocorrido, nós evidenciamos algumas características citogenéticas presentes nos diferentes grupos tais como: *i*) predomínio do sistema cromossômico sexual XY/XX; *ii*) variação no número cromossômico; *iii*) tendência para a localização de bandas heterocromáticas e DNAr 18S nos terminais cromossômicos e a *iv*) a grande variação heterocromática nos cromossomos sexuais;

1. A assimetria dos cariótipos, bem como a grande variabilidade na distribuição heterocromática, a qual foi evidenciada pela ocorrência de bandas: *i*) C-CMA_3^+/$DAPI^+$, *ii*) C-CMA_3^+, *iii*) C-$DAPI^+$, *iv*) *dots* C-CMA_3, ou até mesmo v) a presença de heterocromatina por toda a extensão dos cromossomos sexuais, indicaram uma relativa dinâmica genômica desses insetos associada aos DNAs repetitivos detectáveis por bandeamento cromossômico.

2. Os sítios de DNAr 18S foram distribuídos preferencialmente entre os autossomos em Pentatomomorpha evidenciando a estabilidade desta regiões nesta infraordem. Por outro lado, em Cimicomorpha os sítios de DNAr 18S ocorreram nos autossomos ou nos cromossomos sexuais, seguindo assim, a variabilidade observada para a família Reduviidae.

3. O estudo aprofundado sobre a composição do DNA satélite de *Triatoma infestans* indicou a composição de duas sequências curtas no DNA satélite rico em AT, além de um microsatélite. A origem dessas sequências foi associada às regiões terminais do

elemento transponível conhecido como Polinton. As sondas obtidas aumentaram o número de marcas cromossômicas no cariótipo de *T. infestans* possibilitando a observação da organização complexa desse genoma na espécie.

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