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(Biologia Celular e Molecular)

OCTAVIO MANUEL PALACIOS-GIMENEZ

CONTRIBUIÇÕES A ELUCIDAÇÃO DA ESTRUTURA, ORIGEM E EVOLUÇÃO DE NEO-SISTEMAS CROMOSSÔMICOS DE DETERMINAÇÃO SEXUAL EM GAFANHOTOS UTILIZANDO COMO MODELO ESPÉCIES DOS GÊNEROS *Chlorus*, *Dichromatos* E *Eurotettix* (MELANOPLINAE; ACRIDIDAE)

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas. Área de Concentração: Biologia Celular e Molecular.

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TÍTULO: CONTRIBUIÇÕES A ELUCIDAÇÃO DA ESTRUTURA, ORIGEM E EVOLUÇÃO DE NEO-SISTEMAS CROMOSSÔMICOS DE DETERMINAÇÃO SEXUAL EM GAFANHOTOS UTILIZANDO COMO MODELO ESPÉCIES DOS GÊNEROS Chlorus, Dichromatos e Eurotettix (MELANOPLINAE; ACRIDIDAE)

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A co-existência dos sucessos mais maravilhosos com a mais profunda ignorância é uma das características da biologia atual.

Albert Szent-Györgyi (1940).

RESUMO

Os cromossomos sexuais se originam independentemente de um par de homólogos autossômicos e em várias linhagens apresentam características comuns, representando um exemplo fascinante de convergência evolutiva. Algumas destas características incluem a acumulação de vários tipos de DNAs repetitivos, restrição de recombinação e perda ou ganho de genes que derivam na diferenciação morfológica e genética entre os cromossomos sexuais X e Y ou Z e W. Em Orthoptera, o sistema cromossômico sexual comumente encontrado na maioria das espécies estudadas é do tipo X0♂/XX♀; entretanto, na subfamília Melanoplinae sistemas cromossômicos sexuais derivados dos tipos neo-XY♂/XX♀ e neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀ são frequentemente observados, tendo evoluído repetidamente por fusões Robertsonianas (Fusão Rb) entre autossomos e cromossomos sexuais ancestrais. Sendo assim, o objetivo do presente trabalho foi analizar os sitemas cromossômicos sexuais em Melanoplinae utilizando como modelo as espécies dos gêneros filogeneticamente relacionados *Chlorus*, *Eurotettix* e *Dichromatos*, e adicionalmente *Ronderosia bergi.* Assim, nós integramos citogenética clásica, FISH para distintos DNAs repetitivos, microdissecção de cromossomos sexuais e imunolocalização para diferentes modificações de histonas. Além disso, em *R. bergi* os cromossomos sexuais microdissectados foram usados como molde para a amplificação específica de famílias multigênicas com o objetivo de entender a variação destas sequências nos cromossomos sexuais. Com relação as espécies filogeneticamente relacionadas, nossos dados revelam não propagação de blocos heterocromáticos e da fração de DNA C_0t-1, mas foi observada uma remarcável propagação de famílias multigênicas entre os neo-sistemas sexuias de *Eurotettix* e *Dichromatos*, pricipalmente para o DNAr 5S. Estes resultados também sugeriram uma origem comun e subsequente acumulação diferencial de DNAs repetitivos nos cromossomos sexuais de *Dichromatos*, e uma origem independente dos sistemas sexuais neo-XY e neo-X_1X_2Y intragenericamente. Na espécie *R. bergi*, nossos dados suportam um neo-Y altamente diferenciado e degenerado (incluindo genes repetitivos), refletindo o papel potencial das fusões-Rb e inversões como primeiro passo para originar novos cromsossomos sexuais e regiões livres de recombinação em gafanhotos. As análises para três famílias multigênicas revelam homogeneização entre autossomos e o neo-Y para o DNAsn U2, divergência para DNAsn U1 entre o neo-Y e autossomos, e adicionalmente homogeneização/diversificação entre neo-X, neo-Y e autossomos para sequências de DNAr 5S. Embora o neo-XY de *R. bergi* seja bem diferenciado, o ex-autossomo incorporado ao novo sistema sexual mantém as modificações pós-traducionais de histonas tipo autossomos, sugerindo que i) o conteúdo molecular não influencia os padrões transducionais de histonas, ii) que estas regiões poderiam conter alguns genes importantes que seguem as mesmas modificações que os autossomos atuais, iii) e que o estabelecimento de modificações funcionais nos cromossomos sexuais são mais lentas que as modificações moleculares. Estes resultados fornecem novas informações com relação a variabilidade cromossômica para distintos DNAs repetitivos e a composição molecular específica para neo-sistemas cromossômicos de determinação sexual em gafanhotos, sendo os primeiros dados relacionados a composição específica destes cromossomos.

Palavras-chave: Fusão-Rb, inversão, neo-cromossomos sexuais, FISH, DNAs repetitivos, modificações de histonas.

ABSTRACT

Sex chromosomes have been evolved independently from a pair of homologous autosomes and several lineages share common characteristics, representing fascinating examples of evolutionary convergence. Some of those features include accumulation of various kinds of repetitive DNAs, recombination constraint, loss or gain of genes that derived in the genetic and morphological differentiation among the sex chromosomes X and Y or Z and W. In Orthoptera, sex chromosome systems commonly found in the most studies species is the type X0♂/XX♀; however, in the subfamily Melanoplinae, derived variants neo-XY or neo-X_1X_2Y evolved several times by repeated autosome-sex chromosome Robtersonian fusions (Rb-fusions). Thus, the aim of this study was to analyze sex chromosome systems in Melanoplinae using as model species of the phylogenetically related genera *Chlorus*, *Eurotettix* and *Dichromatos*, in addition *Ronderosia bergi*. For this proposes it was integrated classical cytogenetic analysis, cytogenetic mapping for distinct repetitive DNAs and microdisssected sex chromosomes and immunolocalization for distinct histone modifications. Moreover in *R. bergi* the microdissected chromosomes were used as source for specific amplification of multigene families in order to address the specific variation of these sequences in the sex chromosomes. Concerning to the phylogenetically related genera, our data indicate a non-spreading of heterochromatic blocks and pool of repetitive DNAs (C_0t-1 DNA) in the sex chromosomes, but the spreading of multigene families among the neo-sex chromosomes of *Eurotettix* and *Dichromatos* was remarkable, particularly for 5S rDNA. These results also suggest a common origin and subsequent differential accumulation of repetitive DNAs in the sex chromosomes of *Dichromatos* and an independent origin of the sex chromosomes of the neo-XY and neo-X_1X_2Y systems intragenerically. In the species *R. bergi*, our data supports the argument that the *R. bergi* neo-Y is an element highly differentiated and degenerates (including repetitive genes), reflecting the potential role of the Rb-fusions and inversions as first step to create new sex chromosomes and free regions recombination in sex chromosomes of grasshoppers. The sequence analysis for three multigene families revealed homogenization among autosomes and neo-Y for U2 snDNA, divergence for U1 snDNA between the neo-Y and autosomes and mixed (diversification/homogenization) among neo-X, neo-Y and autosomes for 5S rDNA. However, and although the *R. bergi* neo-XY sex-chromosomes are well differentiated the ex-autosomes incorporated in the new systems retain autosomal post-translational histone modifications, suggesting that i) the molecular content do not influence the post-translational modification patterns for histones; ii) that these regions could retain some important autosomal genes that follow similar modifications to actual autosomes; iii) and that the establishment of functional modifications in neo-sex chromosomes are slower than the molecular diversification. All of these results provide new information regarding chromosomal variability for repetitive DNAs and the specific molecular composition of neo-sex chromosomes in grasshoppers, being these the first data concerning the specific composition of these chromosomes.

Keywords: Rb-fusions, inversion, neo-sex chromosomes, FISH, repetitive DNAs, histone modification.

SUMÁRIO

1. INTRODUÇÃO

1.1 A ordem Orthoptera

A ordem Orthoptera originou-se há cerca de 300 milhões de anos, com uma importante radiação durante o Permiano, Triássico e Jurássico, e com a radiação da subordem Caelifera posterior a Ensifera. A característica mais importante durante o Terciário e Quaternário foi a expansão da superfamília Acridoidea que aconteceu há 30 milhões de anos (HEWITT, 1979).

Atualmente, os ortópteros estão representados por mais de 25.000 espécies distribuídas mundialmente, reconhecidas principalmente por caracteres morfológicos, capacidade de salto e por seus aparelhos estridulatórios (CIGLIANO e LANGE, 1998; POCCO et al., 2010). Este táxon faz parte de uma das faunas mais importantes de insetos terrestres, sendo em sua maioria espécies fitófagas, ocupando uma grande variedade de habitats e confinadas especialmente na região tropical, com poucas espécies que ocupam latitudes frias (HEWITT, 1979; CIGLIANO e LANGE, 1998). A ordem inclui três subordens: Grylloblattinea, Ensifera e Caelifera. A subordem Grylloblattinea tem apenas seis espécies descritas, enquanto que Ensifera inclui os ortópteros de antenas longas nas superfamílias Grylloidea, Gryllacridoidea, Gryllotalpoidea e Tettigonioidea, contendo um total de 9.500 espécies descritas. Por outro lado, Caelifera inclui os gafanhotos de antenas curtas na superfamília Acridoidea com 10.000 espécies de gafanhotos e bichos pau, Tetrigoidea com aproximadamente 1.000 espécies conhecidas e Trydactiloidea com cerca de 146 espécies (HEWITT, 1979; CIGLIANO e LANGE, 1998).

1.2 A família Acrididae e os Melanolplinae neotropicais: Uma visão geral

A superfamília Acridoidea contém as famílias Acrididae, Ommexechidae, Romaleidae, Tristiridae, Pyrgomorphidae, Pyrgacrididae, Pamphagidae, Lathiceridae, Dericorythidae, Lithidiidae e Lentulidae, a grande maioria destes com distribução Neotropical (HEWITT, 1979; CIGLIANO e LANGE, 1998). A família de maior biodiversidade é Acrididae, e dentro deste taxón se encontra a subfamília Melanoplinae, que constitui o maior grupo de acrídidos do Novo Mundo com 43 gêneros e 232 espécies descritas até o momento, se distribuindo amplamente desde o Alasca até o ponto mais austral do Chile e Argentina (CIGLIANO, 2007; CIGLIANO e LANGE, 2007). Na região Neotropical os melanoplíneos se caracterizam por serem maioritariamente dominantes em número de espécies e indivíduos nos estudos de

ocorrência de ortópteros, incluindo, além disso, diversas pragas prejudiciais a agricultura da América (CIGLIANO, 2007), tais como *Melanoplus bivitattus*, *M. sanguinipes* e algumas espécies do gênero *Dichroplus*.

Os melanoplíneos Sul-americanos pertenecentes a tribo Dicrhoplini: *Chlorus*, *Eurotettix*, *Dichromatos*, *Scotussa, Leiotettix*, *Atrachelacris* e *Ronderosia* foram incluídos no grupo informal de gêneros ‘‘Paranaense-Pampeano’’ ultimamente chamado *Scotussae,* baseados principalmente em caracteres da genitália masculina (DINGHI et al., 2009). As espécies de *Chlorus*, *Eurotettix, Dichromatos* e *Ronderosia*, algumas das quais são discutidas neste trabalho, estão distribuídas nas províncias fitogeográficas Paranaense, Cerrado e Catinga (CIGLIANO, 1997; CIGLIANO e LANGE, 2007), ocorrendo frequentemente diferentes espécies em simpatria.

Eurotettix com doze espécies (*E. femoratus, E. minor, E. monnei*, *E. carbonnelli, E. raphaelandrearum, E. robustus, E. procerus, E. brevicerci, E. bugrensis, E. concavus, E. latus* e *E. similraphael*) se encontra principalmente nas províncias fitogeográficas Paranaense e Cerrado, no centro-sul do Brasil e Paraguai (CIGLIANO, 2007); *Dichromatos* que compõe quatro espécies (*D. curupa, D. lilloanus, D. montanus* e *D. schrottkyi*) está melhor representado no estado do Paraná, ao sul do Brasil, leste do Paraguai e noroeste da Argentina; enquanto que *Chlorus* (*Ch. spatulus; Ch. chiquitensis; Ch. bolivianus; Ch. vittatus, Ch. attenuatus; Ch. brunneus* e *Ch. borellii*) e *Ronderosia* (*Rondrosia bergi*, *Ronderosia cintipes*, *Ronderosia dubius*, *Ronderosia gracilis*, *Ronderisa malloi*, *Ronderosia paraguayensis*, *Ronderosia forcipatus*, *Ronderosia piceomaculatus* e *Ronderosia robustus*) são os de mais ampla distribução (CCIGLIANO, 1997; CIGLIANO e LANGE, 2007).

As análises filogenéticas recentes, baseadas em dados morfológicos, propõem que os gêneros *Eurotettix* e *Dichromatos* juntamente com *Chlorus* são grupos monofiléticos (CIGLIANO e LANGE, 2007; CIGLIANO, 2007). Por outro lado, as hipóteses filogenéticas são contraditórias e as relações permanecem incertas. Levando-se em consideração os estudos de morfologia da genitália masculina o gênero *Chlorus* se posiciona como grupo irmão de *Eurotettix*, enquanto se considerado os caracteres exo-morfológicos o mesmo seria filogeneticamente mais próximo de *Dichromatos* (CIGLIANO e LANGE, 2007).

1.3 Origem e evolução de cromossomos sexuais em eucariotos: Uma visão geral

Os cromossomos sexuais X e Y (ou Z e W) morfologicamente e geneticamente distintos se originaram diversas vezes de forma independente a partir de um par de homólogos autossômicos (OHNO, 1967; BULL, 1983). Embora os cromossomos sexuais tenham evoluído independendemente tanto em plantas como animais eles apresentam algumas características comuns. Em princípio, a aquisição de um gene determinante do sexo inicia a evolução de cromossomos sexuais, e em muitas linhagens, cromossomos sexuais originalmente homomórficos se diferenciam em cromossomos sexuais heteromórficos (BULL, 1983; CHARLESWORTH, 1996).

Um pré-requisito para que os cromossomos sexuais se diferenciem e evoluam independentemente é a diminuição ou abolição da recombinação entre os proto cromossomos sexuais homomórficos. Neste contexto, alelos antagônicos sexuais benéficos para um sexo, mas prejudiciais para o outro, poderiam se acumular nos proto cromossomos sexuais e seriam seleccionados para não recombinar (CHARLESWORTH, 1996; KAISER e BACHTROG, 2010). Sendo assim, a ausência de reombinação em parte ou toda a extensão dos cromossomos sexuais limitados a um sexo (Y ou W) resultaria na degeneração da região não recombinante; ou seja, a porção não recombinate dos cromossomos Y ou W perdem a maior parte dos seus genes ancestralmente presentes no proto-Y ou proto-W (CHARLESWORTH et al., 1994; RICE, 1996; STEINEMANN e STEINEMANN, 2005; KAISER e BACHTROG, 2010). O cromosomo Y muitas vezes contém uma abundância incomun de DNAs repetitivos (CHARLESWORTH et al., 1994), e em vários casos, a presença de um cromossomo Y degenerado está associado com mecanismos de compensação de doses no cromossomo X (RICE, 1996; MARIN et al., 2000), de modo tal que a atividade da maioria dos genes ligados a o X é a mesma em machos e fêmeas (CHARLESWORTH, 1996; CHARLESWORTH e CHARLESWORTH, 2000; STEINEMANN e STEINEMANN, 2005).

Em alguns grupos como por exemplo humanos (SKALESTKY et al., 2003) e *Drosophila* (CARVALHO, 2002) os cromossomos sexuais exibem poucos remanescentes de sua história evolutiva, enquanto outros táxons como peixes (EZAZ et al., 2006) e cobras (EZAZ et al., 2006; VICOSO, et al., 2013a) apresentam vários estados de transição de cromossomos sexuais morfologicamente idênticos até completamente diferenciados. Atualmente, ainda não está claro por que algumas espécies suprimem a recombinação ao longo dos cromossomos sexuais e adquirem

cromossomos sexuais heteromórficos XY ou ZW, enquanto que outros conservam cromossomos sexuais homomórficos. Uma interpretação possível poderia ser devido a falta de mutações antagônicas sexuais em alguns grupos, ou também pela resolução de conflitos impostos por mutações antagônicas sexuais para evolução sexo específico ou expressão enviesada dos genes (VICOSO et al., 2013b).

Adicionalmente além da supressão de recombinação os cromossomos sexuais teriam a tendência de acumular rapidamente sequências repetitivas, tais como elementos de transposição, DNA satélites, microssatélites e famílias multigênicas (STEINEMANN et al., 1992; STEINEMANN at al., 1993; MATSUNAGA, 2009). Tal situação foi observada nas regiões não codificantes do neo-cromossomo Y de *Drosophila miranda*, caracterizada por uma alta densidade de transposons neste neo-cromossomo sexual, se comparado com as regiões homólogas do cromossomo X (BACHTROG, 2005, 2006). Este acúmulo de sequências repetitivas poderia explicar também porque o cromossomo Y de *D. miranda* tem um tamanho maior que o cromossomo X, embora possua menor quantidade de genes. Por sua vez o acúmulo dos elementos repetitivos poderia contribuir com a degeneração do cromossomo Y, afetando seu modo de expressão gênica (STEINEMANN et al., 1992; STEINEMANN at al., 1993; BACHTROG, 2005; BERGERO e CHARLESWORTH, 2008). Esta "degeneração genética" seria a causa da divergência morfológica e consequentemente genética dos neo-cromossomos sexuais (CHARLESWORTH et al., 2005; BACHTROG, 2006; KAISER e BACHTROG, 2010). Assim, o neo-Y talvez é selecionado para não recombinar em resposta ao acúmulo de sequências que são benéficas para o macho, mas não para as fêmeas (RICE, 1996; CHARLESWORTH et al., 2005).

1.4 Evolução de neo-sistemas de determinação sexual

As evidências citológicas e moleculares em vários grupos de plantas e animais postulam que os principais estagios durante a evolução dos cromossomos sexuais são o estabelecimento de genes determinates do sexo, supressão da recombinação, perda de genes, aumento massivo de elementos repetitivos e heterocromatina em regiões não recombinantes, além de múltiplos rearranjos estruturais no cromossomo Y (RICE, 1996; CHARLESWORTH et al., 1994, CHARLESWORTH et al., 2005; SKALESTKY et al., 2003; STEINEMANN e STEINEMANN, 2005; HOBZA et al., 2007 KEJNOVSKY et al., 2009; MATSUNAGA, 2009). Com base nestas evidências,

vários pesquisadores postulam que os cromossomos sexuais neo-XY originados por fusões Rb entre o cromossomo X e um autososomo são excelentes modelos experimentais para estudar os processos envolvidos na degeneração do cromossomo Y (CHARLESWORTH et al., 2005; VELTSOS et al., 2008; CASTILLO et al., 2010b).

Há provas suficientes onde as fusões, inversões e inserção de elementos repetitivos (principalmente elementos transponíveis e DNAs satélites) podem afetar a recombinação entre cromossomos sexuais, e portanto produzir heteromorfismos cromossômicos (STEINEMANN et al., 1993; CHARLESWORTH et al., 1994; 2005; NICOLAS et al., 2003; BERGERO e CHARLESWORTH, 2008; MATSUNAGA, 2009). As fusões Rb envolvendo autossomos e cromossomos sexuais, como por exemplo em *Podisma pedestris,* podem gerar uma forte ligação entre genes antagonistas sexuais e cromossomos sexuais, e portanto ser favorecidos pela seleção natural (VELTSOS et al., 2008; VELTSOS et al., 2009).

Além disso, a ligação de genes produto da fusão podem exercer efeitos durante o *crossing over*, com a exclusão da recombinação nas regiões próximas aos pontos de ruptura dos heterozigotos estruturais (CHARLESWORTH et al., 2005). Por outro lado, depois da restrição da recombinação os neo-cromossomos sexuais podem rapidamente acumular sequências repetitivas, como por exemplo em *D. miranda* (STEINEMANN e STEINEMANN, 1992; STEINEMANN et al., 1993; CARVALHO, 2002; BACHTROG, 2005), *Rumex acetosa* (NAVAJAZ-PÉREZ et al., 2005; NAVAJAZ-PÉREZ et al., 2006; NAVAJAZ-PÉREZ et al., 2009) e *Silene latifolia* (NICOLAS et al., 2003; MATSUNAGA, 2009; KEJNOVSKY et al., 2013).

1.5. Evolução de cromossomos sexuais em Orthoptera

Em vários táxons como por exemplo Orthoptera, Blattodea, Mantodea, Phasmatodea e Lepidoptera o sistema cromossômico de determinação do sexo X0♂/XX♀ e Z0♀/ZZ♂ evoluiu a partir do sistema sexual ancestral do tipo XY♂/XX♀ ou ZW♀/ZZ♂, sugerindo que a reversão para o estado X0 ou Z0 ocorreu em um antepassado destas linhagens (WHITE, 1973; HEWITT, 1979; CASTILLO et al., 20010a; CHARLESWORTH e MANK, 2010; KAISER e BACHTROG, 2010; YOSHIDO et al., 2013).

O sistema cromossômico de determinação sexual (X0♂/XX♀) de Orthoptera é bastante conservado evolutivamente comparado com as variedades de mecanismos de

determinação sexual encontrados em Diptera, Coleoptera e Lepidoptera (HEWITT, 1979; YOSHIDO et al., 2013; NGUYEN et al., 2013). O mecanismo cromossômico de determinação sexual X0♂/XX♀ envolve um equilíbrio entre o X e autossomos (WHITE, 1973; HEWITT, 1979; CHARLESWORTH, 1996), como foi descoberto por Bridges em seus estudos com *Drosophila melanogaster* (CHARLESWORTH, 1996). Isto sugere que os genes presentes no cromossomo X controlam o desenvolvimento feminino e os genes presentes em autossomos controlam o desenvolvimento masculino (CHARLESWORTH, 1996; AYLING e GRIFFIN, 2002).

Em machos de grilos, tetigonidos e gafanhotos, o cromossomo X representa um elemento único não pareado e na meiose exibe heteropicnose ao longo de toda sua extensão (WHITE, 1973; HEWITT, 1979). A heteropicnose do cromossomo X em Orthoptera indica uma condição genética inerte, o que foi também demostrado pelos baixos níveis de acetilação de histonas, uma modificação pós-translacional associada com silenciamento genético (CABRERO et al., 2007). Assim então, poderia-se esperar que o X carregue um número pequeno de genes ou regiões determinantes do sexo intercaladas ao longo de toda sua extensão. Entretanto, atualmente permanece um mistério se o cromossomo X de Orthoptera apresenta genes determinantes do sexo ou alguns genes relacionados com a fertilidade dos indivíduos.

Além do sistema sexual X0♂/XX♀, a literatura científica relata várias espécies com sistemas sexuais derivados do tipo neo-XY♂/XX♀ e neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀ (WHITE, 1973; JOHN e FREEMAN, 1976; HEWITT, 1979; MESA et al., 1982; MESA et al., 2001, MESA et al., 2002; BIDAU E MARTÍ, 2001; VELTSOS et al., 2008, VELTSOS, 2009; CASTILLO et al., 2010a; CASTILLO et al., 2010b, FERREIRA e MESA, 2010), originados principalmente por fusões cêntricas o Robertsonianas (Rb) entre o cromossomo X ancestral e autossomos.

Com o objetivo de diferenciar os braços dos neo-cromossomos sexuais XY, White (1973) definiu uma terminologia a qual é ainda utilizada nos trabalhos recentes; o neo-X metacêntrico é formado pelos braços XL e XR, onde o XL corresponde ao cromossomo X do antigo sistema sexual X0 e o XR é resultado do autossomo envolvido na fusão. Assim o cromossomo homólogo ao braço XR passa a denominar-se neo-Y, de morfologia acro-telocêntrica e restrito a linhagem masculina (WHITE, 1973; HEWITT, 1979). Em Orthoptera, exemplos de sistemas cromossômicos sexuais neo-XY podem ser encontrados em espécies Sul-Americanas,

como por exemplo *Dichroplus silveiraguidoi* (CARDOSO et al., 1974; SÁEZ e PÉREZ-MOSQUERA, 1977), *D. maculipennis*, *D. vittigerum*, *Zygoclistron nasicum*, *Z. falconicum*, *Z. trachysticum* (MESA et al., 2001) e no gênero *Ronderosia* (CASTILLO et al., 2010a). Dentro das espécies mencionadas acima, *D. silveiraguidoi* é um exemplo de extrema divergência do número diploide neste grupo, com ocorrência de 2n=8,XY com NF= 13♂/14♀ (CARDOS et al., 1974; SÁEZ e PÉREZ-MOSQUERA, 1977).

Segundo Hewitt (1979) e Rice (1996) é possível que alelos antagônicos sexuais se acumulem em machos e poderiam surgir novos mecanismos de compensação de dose como resposta ao acúmulo de alelos femeninos no cromossomo X. Uma vez que o novo mecanismo for estabelecido em todos os indivíduos da espécie uma série de transformações ocorrem em autossomos que foram incorporados ao novo sistema cromossômico de determinação sexual, como por exemplo heterocromatinização do neo-Y, ocorrência de segmentos diferenciais e alterações na distribuição de quiasmas entre os ex-homólogos autossômicos, ou mesmo um segundo evento de fusão Rb Y-A, originando um sistema complexo do tipo neo-X_1X_2Y (MESA et al., 2001; FERREIRA e CELLA, 2006; FERREIRA e MESA, 2010).

Para o caso de sistemas cromossômicos sexuais múltiplos (neo-X_1X_2Y), os braços do metacêntrico neo-X_1 são chamados como descrito anteriormente para o sistema cromossômico de determinação sexual neo-XY; enquanto que o neo-Y metacêntrio está formado pelos braços YL e YR, os quais compartilham homologia com os braços XR e o neo-X_2, respectivamente (WHITE, 1973). Em Orthoptera, exemplos de mecanismos cromossômicos de determinação sexual neo-X_1X_2Y foram observados em *Paratylotropidia brunneri* (WHITE, 1941), *P. morsei* (WHITE, 1953), *Ronderosia dubius* (MESA e MESA, 1967; CASTILLO et al., 2010b) e em espécies do gênero *Dichromatos* (CASTILLO et al., 2010a; FERREIRA e MESA, 2010).

Comparado com outras regiões do mundo, em ortópteros Neotropicais é comum a ocorrência de espécies com mecanismos cromossômicos sexuais derivados, e as modificações morfológicas que eles sofrem têm sido bem discutidas por vários autores (ver por exemplo, WHITE, 1973; HEWITT, 1979; MESA et al., 1982; CASTILLO et al., 2010a; CASTILLO et al., 2010b). Porém, a origem, estrutura e evolucão de neo-sistemas cromossômicos de determinação do sexo têm sido estudados apenas do ponto de vista citogenético clássico, como por exemplo em

Xyleus laevipes (SÁEZ e DÍAZ, 1960), *Neuquenina fictor* (MESA, 1961), *Pachyosa signata* (MESA, 1964), *Dichroplus vitattus* (BIDAU e MARTÍ, 2001), *Atrachelacris unicolor, Ronderosia malloi*, *R. forcipatus*, *R. bergi* (CASTILLO et al., 2010a) e *Mariacris viridipes* (CASTILLO et al., 2010b). Em *N. fictor* a região de formação de quiasmas ocupa os dois terços distais do neo-Y sem observação de regiões heterocromáticas e com a posibilidade de formar até quatro quiamas entre o neo-X e neo-Y (SÁEZ e DÍAZ, 1960; MESA, 1961), enquanto que em *R. bergi*, a diferenciação entre os cromossomos neo-sexuais é notável, onde uma inversão pericêntrica foi estabelecida no neo-Y, além do mesmo ser completamente heterocromático (MESA, 1962; SÁEZ, 1963; CASTILLO et al., 2010a).

Com base nestas evidências, a divergência morfológica progressiva do neo-Y em Orthoptera é o resultado da perda de homologia, redução da recombinação, heterocomatinização, além de rearranjos estruturais como ocorre no neo-Y de *R. bergi*. Estas evidências também mostram que o neo-Y do tipo diferenciado acumula características de cromossomo sexual com heteropicnose positiva durante os primeiros estágios meióticos (SÁEZ, 1963; MESA, 1964; WHITE, 1973; HEWITT, 1979; MESA et al., 2001; CASTILLO et al., 2010a). Porém, a diferença de outros organismos como humanos (SKALESTKY et al., 2003; CARVALHO e CLARK, 2013), *Drosophila* (CARVALHO, 2002; STEINEMANN e STEINEMANN, 2005; CARVALHO e CLARK, 2013) e plantas (NICOLAS et al., 2003; NAVAJAZ-PÉREZ et al.; 2005; MATSUNAGA, 2009; KEJNOVSKY et al., 2009; KEJNOVSKY et al., 2013), as modificações moleculares que levam a diferenciação molecular do neo-Y de Orthoptera são até o momento desconhecidas.

1.6 Antecedentes citogenéticos e neo-sistemas em Melanoplinae

Dentro da família Acrididae, embora o número cromossômico mais frequente seja 2n=23♂/24♀ com um sistema cromossômico de determinação sexual do tipo XX♂/X0♀, existem diversas espécies com mecanismos cromossômicos sexuais derivados, sendo estas modificações mais frequentes na região Neotropical. Por exemplo, dentro da subfamília Melanoplinae os quais são mais diversos e abundantes na região acima mencionada, existem pelo menos 50 espécies com mecanismos cromossômicos de determinação sexual neo-XY♂/XX♀ e neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀ (MESA et al., 1982; CASTILLO et al., 2010b). Como mencionado anteriormente a alta frequência destes neo-sistemas cromossômicos de determinação sexual está

relacionada com fusões Rb e translocações entre autossomos e cromossomos sexuais, além de ocorrência de inversões e perda da recombinação. Um exemplo da extrema divergência do número diploide neste grupo foi descrito na espécie *Dichroplus silveiraguidoi* que possui 2n=8,XY com NF= 13♂/14♀ (CARDOS et al., 1974; SÁEZ e PÉREZ-MOSQUERA, 1977).

Sem dúvida a situação de diversificação cromossômica na subfamília Melanoplinae parece ser mais complexa devido as frequentes variações em relação ao cariótipo comunmente observado em Acrididae. Dentro do taxon *Chlorus* se conhece apenas o número cromossômico de quatro espécies, todas com um mecanismo cromossômico de determinação sexual de origem simples X0♂/XX♀ (MESA et al., 1982). Porém, *Ch. borellii* e *Ch. bolivianus* provavelmente apresentem polimorfismos para fusões Rb, por causa da redução do número cromossômico observada nestas espécies (MESA et al., 1982). Da mesma forma dentro de *Eurotettix* composto de doze espécies, se conhece apenas o cariótipo de *E. minor* com 2n=22♂/22♀-neo-XY♂/XX♀, no qual foi proposto que a origem do sistema cromossômico de determinação sexual envolveria uma fusão Rb X-A (MESA et al., 1982; FERREIRA e MESA, 2010). Por outro lado, os cariótipos das quatro espécies pertenecentes ao gênero *Dichromatos* são conhecidos, com 2n=21♂/22♀ e um sistema de determinação do sexo neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀, distinto do observado para *Eurotettix* e *Chlorus*. Estes sistemas cromossômicos de determinação sexual múltiplos em *Dicromatos* se originaram por fusões Rb envolvendo autossomos e X (MESA et al., 1982; CASTILLO et al., 2010a; FERREIRA e MESA, 2010).

O uso do mapeamento de DNAs repetitivos através da hibridização *in situ* fluorescente tem se mostrado uma excelente ferramenta para entendimento dos padrões de diversificação cariotípica e genômica em distintos grupos de eucariotos, assim como na análise de origem e evolução de cromossomos sexuais. Em ortópteros embora se tenham realizado alguns estudos relativos a estrutura e organização de famílias multigênicas (DNAr e genes de histona) e poucos DNAs satélites, os estudos nunca enfocaram o papel destas sequências na origem e evolução dos sistemas sexuais neste grupo. As análises utilizando ferramentas de citogenética molecular em *R. bergi* e nas espécies dos gêneros filogeneticamente relacionados *Chlorus*, *Eurotettix* e *Dichromatos* com sistemas sexuais simples e derivados permitirão um melhor conhecimento e teste de hipóteses quanto a origem e padrões de evolução

destes sistemas em Ortópteros, além de seu possível papel no processo de especiação do grupo.

2. OBJETIVOS

2.1 Objetivo geral

Auxiliar no entendimento dos padrões de origem e diversificação de sistemas cromossômicos de determinacão sexual (neo-sistemas cromossômicos sexuais) em espécies de gafanhotos, utilizando como modelo espécies dos gêneros *Chlorus*, *Eurotettix, Dichromatos* e *Ronderosia*.

2.2 Objetivos específicos e metas

- Caracterizar a macro-estrutura cromossômica de distintas populações das espécies modelo, descrevendo as características gerais de seus cariótipos, presença de polimorfismos e estrutura dos neo-sistemas cromossômicos sexuais;
- Analisar a distribuição e riqueza de pares de base dos blocos de heterocromatina constitutiva e sua relação com os neo-sistemas cromossômicos sexuais;
- Analisar os padrões de diversificação de famílias multigênicas (incluindo genes de RNAr, histonas e de RNAsn U1 e U2), sequência telomérica e fração de DNA C_0t-1 nos cariótipos das espécies de *Chlorus*, *Eurotettix, Dichromatos* elucidando o possível envolvimento dos cromossomos portadores destas sequências na origem dos sistemas sexuais derivados, buscando traçar o caminho evolutivo do mesmo nas distintas espécies;
- Mapear distintos DNAs repetitivos (famílias multigênicas, fração DNA C_0t-1, microssatélites e sequências teloméricas) e cromossomos obtidos por microdissecção da espécie *Ronderosia bergi*, buscando elucidar a possível diferenciação entre os cromossomos sexuais neo-X e neo-Y da espécie.

- Utilizar o DNA dos cromossomos sexuais microdissectados como molde para amplificação de famílias multigênicas em *R. bergi*. As análises comparativas entre estas sequências ocorrentes no genoma de fêmeas serão úteis no entendimento dos padrões de diversificação dos DNAs repetitivos ocorrentes

nos sistemas cromossômicos sexuais e mecanismos de evolução destes cromossomos;

- Utilizar anticorpos para acetilação, metilação e fosforilação dos resíduos dos aminoácidos de proteínas histônicas: H3K4me2, H4K5ac, H3K9me2 e H3S10ph, identificando as modificações dos autossomos e do sistema neo-XY nas metáfases de *R. bergi*, comparando os resultados com os obtidos em espécies com sistema sexual simples (X0).

3. MATERIAIS E MÉTODOS

3.1 Animais, preparações cromossômicas e extração de DNA

Machos e fêmeas adultos de *Chlorus vittatus, Ch. chiquitensis*; *Eurotettix brevicerci, E. minor*; *Dichromatos lilloanus, D. schrottkyi* e *Ronderosia bergi* foram coletados em diferentes localidades de Paraguai, Argentina e Brasil. Os testículos dos machos foram removidos e fixados em una solução de etanol: acético (3:1), enquanto que os cecos gástricos das fêmeas foram removidos e fixados seguindo o protocolo descrito por Castillo et al. (2011). Para *R. bergi* animais coletados foram mantidos em cativeiro até a oviposição das fêmeas para obtenção de cromossomos mitóticos a partir de embriões, seguindo o protocolo proposto por Webb et al (1978). Todos os espécimes foram estocados em etanol e armazenados em freezer a -20°C para posterior extração de DNA.

Para visualizar as características cromossômicas gerais de cada espécie utilizamos à coloração convencional com Giemsa 5%. O bandeamento C e a coloração com fluorocromos base-específicos (CMA_3/DA/DAPI) seguiram os protocolos descritos por Sumner (1972) e Schweizer et al. (1983), respectivamente. A extração de DNA genômico foi realizado utilizando o protocolo de Fenol: Clorofórmio (SAMBROOK e RUSSEL, 2001).

3.2 Isolamento de DNAs repetitivos através de PCR

A sequência parcial de RNAr 5S e do gene de histona H3 foram obtidas diretamente por PCR usando o DNA genômico de *Abracris flavolineata* e primers descritos por Loreto et al. (2008) e Cabral de Mello et al. (2010) para DNAr 5S, e Colgam et al. (1998) para histona H3. As sequências para DNAsn U1 e U2 foram obtidas usando o DNA genômico de *Rhammatocerus brasiliensis* com os primers descritos por Cabral de Mello et al. (2012) e Bueno et al. (2013). Estes fragmentos

foram sequenciados para confirmação da obtenção das sequências de interesse, números de acesso a o GeneBank KC896792 (histona H3), KC936996 (DNAr 5S), KC896793 (DNAsn U1) e KC896794 (DNAsn U2).

A sequência de DNAr 18S foi obtida a partir de um fragmento previamente clonado e isolado do genoma de *Dichotomius semisquamosus* (número de acesso do GeneBank GQ443313, CABRAL-DE-MELLO et al., 2010). A sonda telomérica foi obtida por PCR usando os primers $(TTAGG)_5$ e $(CCTAA)_5$ como descrito por Ijdo et al. (1991).

3.3 Isolamento de DNA alta e moderadamente repetitivo: fração de DNA C_0t-1.

Amostras enriquecidas com DNAs repetitivos de cada uma das espécies foram obtidas baseadas na cinética de renaturação da fração de DNA C_0t-1 (DNA enriquecido com sequências alta e moderadamente repetitivas) de acordo com o protocolo descrito por Zwick et al. (1997), com modificações. Brevemente, amostras de DNA (200 µL de 100-500-ng/µL de DNA genômico em 0,3 M de NaCl) foram digeridas com deoxyribonuclease I (Sigma, St Louis, MO, USA) a 0,01 U/µL durante 80 a 105 sec, dependendo da concentração da amostra. Em seguida, os fragmentos de DNA foram separados em gel de electroforese em 1% de agarose. Os fragmentos de DNA esperados variaram em tamanho de 100 a 1.000 pb. Para cada uma das espécies, 50 µL da amostra de DNA fragmentado foram desnaturadas a 95°C por 10 min, colocadas em gelo por 10 sec e transferidas a banho maria a 65°C para reanelamento por 25 min. Subseqüentemente, as amostras foram incubadas a 37°C por 8 min com 1 U de nuclease S1 para digerir o DNA de cadeia única. Os fragmentos foram purificados e extraídos usando o protocolo tradicional de Fenol: Clrorofórmio (SAMBROOK e RUSSEL, 2001).

3.4 Microdissecção dos cromossomos sexuais e amplificação de famílias multigênica em *Ronderosia bergi*

A microdissecção dos cromossomos neo-X e neo-Y foram realizadas de células meióticas obtidas de machos. Antes da microdissecção foi gerada uma suspensão celular de quatro folículos testiculares em 100 µl de ácido acético. Em seguida a suspensão celular foi espalhada em uma lamínula e colocada sobre uma placa quente a ~ 50 ºC. Os neo-cromossomos sexuais foram facilmente reconhecidos

devido a sua configuração em C nas metáfases I (CASTILLO et al., 2010b) e a segregação precoce de estes elementos evitou a contaminação cruzada.

A microdissecção foi realizada em um micromanipulador Eppendorf 5171 acoplado a um microscópio invertido Nikon Axiphot. Dez cromossomos neo-Y e oito cromossomos neo-X foram microdissectados separadamente e, em seguida, amplificados através do kit GenomePlex Single Cell Whole Genomic Amplification WGA4 (Sigma) seguido por reamplificação utilizando o kit GenomePlex WGA3 (Sigma). Os DNA amplificados por o kit GenomePlex WGA3 foram utilizados para produzir sondas dos cromosomos neo-X (μX-DNA) e neo-Y (μY-DNA).

A fim de verificar a presença de famílias multigênicas como DNAr, DNAsn U1 e U2 nos neo-cromossomos sexuais, os DNAs dos cromossomos microdissectados foram utilizados como molde para realização PCRs. Além disso, para análises comparativas as mesmas repetições foram também amplificadas a partir do DNA genômico da fêmea (♀gDNA). Os primers usados estão descritos em Cabral-de-Mello et al. (2010) para DNAr 18S e 5S, Colgan et al. (1998) para histona H3, Cabral-de-Mello et al. (2012) para DNAsn U1 e Bueno et al. (2013) para DNAsn U2. As PCRs foram realizadas em 10 x PCR de Buffer Rnx, 0,2 mM de Cl_2Mg, 0,16 mM de dNTPs, 2 mM de cada par de primers, 1 U de DNA Polymerase *Taq* Platinum (Invitrogen, San Diego, CA, USA) e 50-100 ng/μl de DNA dependendo da amostra. As condições das PCRs incluíram uma desnaturação inicial a 94ºC por 5 min seguido de 30 ciclos a 94ºC (30 sec), 55º (30 sec) e 72ºC (1 min e 20 sec), mais uma extensão final a 72ºC por 5 min.

3.5 Clonagem, sequenciamento e análise das sequências

Os produtos das PCRs foram visualizados em gel de agarose 1% e as bandas foram isoladas e purificadas usando o kit Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., The Epigenetics Company, USA) comforme as recomendações do fabricante. Para comparação das sequências todos os fragmentos de DNA isolados e purificados foram inseridos em vetores plasmidiais e utilizados para transformar bactérias competentes de *Escherichia coli* DH5α (Promega, Madison, WI, USA).

Os plasmídios recombinantes foram submentidos à sequenciamento nucleotídico através do sequenciador automático ABI 3100 (Aplied Biosystems). A qualidade das sequências foram verificadas e alinhadas usando o software Geneious

4.8.5 (DRUMMOND et al., 2009). Todas as sequências consenso foram submetidos a pesquisa BLAST (ALTSCHUL et al., 1990) do National Center for Biotechnology Information (NCBI), website (http://www.ncbi.nlm.nih.gov/blast).

Além disso, análises estatísticas das sequências foram realizadas no programa DnaSP v.5.10.01 (LIBRADO e ROZAS, 2009), enquanto que os análises filogenéticas e moleculares foram realizadas utilizando o programa MEGA versão 3.1 (TAMURA et al., 2011).

3.6 Hibridização *in situ* fluorescente

As sequências de DNAs repetitivos de DNAr 5S, DNAsn U (U1 e U2) e repetição telomérica foram marcadas com Digoxigenina 11-dUTP (Roche, Mannheim, Germany) através da PCR, enquanto que o plasmídeo contendo o gene de DNAr 18S, os produtos de PCR do gene de histona H3, a fração de DNA C_0t-1 e, os produtos dos μX-DNA e μY-DNA foram marcadas com Biotina 14-dATP (Invitrogen) através da *Nick translation*, seguindo recomendações do fabricante. Além disso, foram usados dezesseis sondas de microsátelites marcadas diretamente com biotina-14 dATP na própia sínteses no extremo 5': $(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(CG)_{15}$, $(TA)_{15}$, $(AG)_{10}$, $(CAA)_{10}$, $(CAC)_{10}$, $(TAA)_{10}$, $(GAA)_{10}$, $(CGG)_{10}$, $(GAC)_{10}$, $(CAT)_{10}$, $(GAG)_{10}$, $(GACA)_4$ e $(GATA)_8$ (Sigma).

A hibridização *in situ* fluorescente foi basicamente realizada usando o protocolo descrito por Pinkel et al. (1986), com modificações propostas por Cabral-de-Mello et al. (2010). As sondas foram detectadas usando anti-digoxigenin-Rhodamine (Roche) ou streptavidina alexa fluor-488 (Invitrogen). Todas as preparações foram contracoradas com DAPI e montadas com meio de montagem Vectashield (Vector, Burlingame, CA, USA). As fotografias e sinais foram observadas utilizando um microscópio Olympus BX61 equipado com uma lâmpada fluorescente e filtros adequados. As imagens foram fotografadas usando uma câmera digital DP70 em escala de cinza. Adicionalemte, as imagens foram pseudo-coradas em azul (cromossomos), vermelho ou verde (sinais), sobrepostas e otimizadas em brilho e contraste usando Adobe Photoshop CS2.

3.7 Imunodetecção

Testículos de machos adultos foram removidos e fixados em uma solução fresca de paraformaldeído 2% em PBS (Phosphate-Buffered Saline) contendo 0,05% de Tween por 15 min. Em seguida, os testículos foram imersos em uma pequena gota deste fixador sobre uma lâmina e suavemente esmagado depois de cubrir com uma lamínula, o qual foi removida depois da imersão em nitrogênio líquido e inmediatamente transferida para PBS gelado. A inmunodetecção foi basicamente realizada seguindo o protocolo descrito por Cabrero et al. (2007).

Anticorpos primários (rabbit polyclonal IgG, Upstate Biotechnology, USA) anti-H3K4me2, anti-H3K9me2, anti-H4K5ac, and anti-H3S10ph foram diluídos 1:600 em 1% de BSA (bovine-serum albumin) em PBS. As lâminas foram incubadas durante a noite a 4ºC e depois das lavagem foram detectadas com anti-rabbit IgG FITC-conjugado (Sigma) diluído 1:60 em PBS, 1% de BSA por 60 min. Após a lavagem final em PBT (1x PBS, 0.01% de Tween 20), as preparações foram contracoradas com DAPI e montadas em Vectashield (Vector). Finalmente, as imagens cromossômicas foram obtidas usando os mesmos equipamentos mencionados acima para FISH.

4. RESULTADOS E DISCUSSÂO

Os resultados da presente dissertação estão apresentados em dois manuscritos a seguir, sendo um publicado na revista ***BMC Evolutionary Biology*** (ISSN 1471-2148) e um a ser submetido a ***Heredity*** (ISSN: 0018-067X).

4.1 Capítulo 1:

Tracking the evolution of sex chromosome systems in Melanoplinae grasshoppers through chromosomal mapping of repetitive DNA sequences

Octavio M Palacios-Gimenez, Elio R Castillo, Dardo A Martí and Diogo C Cabral-de-Mello

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Tracking the evolution of sex chromosome systems in Melanoplinae grasshoppers through chromosomal mapping of repetitive DNA sequences

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Abstract

Background: The accumulation of repetitive DNA during sex chromosome differentiation is a common feature of many eukaryotes and becomes more evident after recombination has been restricted or abolished. The accumulated repetitive sequences include multigene families, microsatellites, satellite DNAs and mobile elements, all of which are important for the structural remodeling of heterochromatin. In grasshoppers, derived sex chromosome systems, such as neo-XY♂/XX♀ and neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀, are frequently observed in the Melanoplinae subfamily. However, no studies concerning the evolution of sex chromosomes in Melanoplinae have addressed the role of the repetitive DNA sequences. To further investigate the evolution of sex chromosomes in grasshoppers, we used classical cytogenetic and FISH analyses to examine the repetitive DNA sequences in six phylogenetically related Melanoplinae species with X0♂/XX♀, neo-XY♂/XX♀ and neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀ sex chromosome systems.

Results: Our data indicate a non-spreading of heterochromatic blocks and pool of repetitive DNAs (C_0t-1 DNA) in the sex chromosomes; however, the spreading of multigene families among the neo-sex chromosomes of *Eurotettix* and *Dichromatos* was remarkable, particularly for 5S rDNA. In autosomes, FISH mapping of multigene families revealed distinct patterns of chromosomal organization at the intra- and intergenomic levels.

Conclusions: These results suggest a common origin and subsequent differential accumulation of repetitive DNAs in the sex chromosomes of *Dichromatos* and an independent origin of the sex chromosomes of the neo-XY and neo-X_1X_2Y systems. Our data indicate a possible role for repetitive DNAs in the diversification of sex chromosome systems in grasshoppers.

Background

For more than a century, the evolution of the sex chromosomes and the genetics of sex determination have been the source of some of the most intriguing questions in evolutionary biology and have been the focus of many genetic and cytological studies (see for example [1-12]). Sex chromosomes evolve from a pair of homologous autosomes [13], and the restriction or absence of recombination and the further accumulation of repetitive sequences on chromosomes Y or W are important events in the differentiation of these elements [14-16].

Based on evidence obtained from molecular studies in different taxa, DNA sequence restructuring occurs within new sex chromosome regions (Y or W) during the early evolution of the sex chromosomes, and this process involves modifications to the chromatin structure and the insertion of repetitive DNA sequences. These morphological and genetic changes are consistent with the abolition of recombination, which precedes the genetic degeneration of neo-Y or neo-W chromosomes with unknown fates [1,15-21].

Among the inserted repetitive DNA sequences, a remarkable preponderance of mobile elements, satellite

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DNAs, microsatellites and multigene families, which can remodel euchromatic structures into heterochromatin, has been observed [17,22-25]. Non-recombining regions of the Y chromosome containing accumulated repetitive DNAs have been well documented in, for example, mammalian species [11,26] and *Drosophila melanogaster* [27], in which the sex chromosome systems are evolutionarily ancient [8,28]. The accumulation of repetitive sequences, even in young sex chromosomes, has also been observed in other organisms, such as *Drosophila miranda* [19], *Silene latifolia* [29-31] and *Rumex acetosa* [23].

Grasshopper species are characterized by a high frequency of 2n=23♂/24♀ karyotypes comprising acro-telocentric chromosomes and X0♂/XX♀ sex chromosome determination system. According to White [32] and Hewitt [4], this karyotype is considered atavistic, at least for Caelifera. Although grasshoppers within Acrididae have this form of karyotypic stability and X0♂/XX♀ sex chromosome system, the Melanoplinae subfamily shows an unusually high frequency of derived neo-sex chromosome systems, which have been observed in at least 50 species [33-35]. This sex chromosome variability primarily reflects the occurrence of Robertsonian fusions (Rb-fusions), which generate complex neo-XY♂/XX♀ and neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀ sex chromosome systems [32,35-37].

In contrast with other insect orders such as Lepidoptera [38-41] and Diptera [6,7,21] in which the evolution of the sex chromosomes has been studied by mapping distinct classes of DNAs, there is a complete lack of knowledge at the molecular level concerning the evolution of the neo-sex chromosomes in grasshoppers and the mechanisms that underlie the degeneration of the neo-Y chromosome. The great diversity of the sex chromosome systems observed in Melanoplinae suggests that this group represents an excellent experimental model to analyze any changes in patterns of linked gene groups within the sex chromosomes. With the aim of a better understanding of the evolution of sex chromosomes in grasshoppers we used classical cytogenetic techniques and fluorescence *in situ* hybridization (FISH) to analyze five multigene families, telomeric repeats and repetitive DNA fractions (C_0t-1 DNA fraction) in six phylogenetically related Melanoplinae species: *Chlorus vittatus* and *Ch. chiquitensis*; *Eurotettix minor* and *E. brevicerci*; and *Dichromatos lilloanus* and *D. schrottkyi* [42,43]. These species present different sex chromosomes, including X0, neo-XY and neo-X_1X_2Y in males ([33,34], this work). We focused mainly on the dynamics of repetitive DNA incorporation into new sex chromosomes as an evolutionary force that contributes to the chromosomal diversification of this group, and we examined the evidence for independent or common origins of the neo-sex chromosome systems in the analyzed species.

Results

Meiosis and karyotypes

Different diploid numbers were observed in the six species studied: 2n=23♂/24♀ in *Chlorus vittatus* and *Eurotettix brevicerci*, 2n=19♂/20♀ in *Ch. chiquitensis*, 2n=22♂/22♀ in *E. minor* and 2n=21♂/22♀ in *Dichromatos lilloanus* and *D. schrottkyi* (Figure 1; Table 1). The autosomes were, in general, acro-telocentric; however, in *Ch. chiquitensis*, pair 5 was submetacentric. Three types of sex chromosome systems were observed: X0♂/XX♀ in *Ch. vittatus*, *Ch. chiquitensis* and *E. brevicerci*; neo-XY♂/XX♀ in *E. minor* and neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀ in *D. lilloanus* and *D. schrottkyi* (Figure 1; Table 1).

The X sex chromosome in the X0♂/XX♀ system was acro-telocentric, showing negative heteropycnotic behavior during metaphase I and variability in size among the species (Figure 1). In *E. minor*, the neo-XY♂/XX♀ sex pair was formed by a metacentric neo-X, the product of Rb-fusion between the ancestral X and an autosome, whose homologue has become a telocentric neo-Y. The neo-sex chromosomes showed distal contact during metaphase I, and adopted the typical L-shaped configuration (Figure 1). Finally, in the *Dichromatos* species, neo-sex chromosomes were formed from the metacentric neo-X_1, the acro-telocentric neo-X_2 and the metacentric neo-Y, being the neo-Y chromosome the largest element. At metaphase I, the neo-sex chromosomes were observed in the typical convergent orientation of a Robertsonian trivalent, with the XR arm distally associated with the YL arm of the neo-Y chromosome and the YR arm of the neo-Y chromosome distally associated with the neo-X_2 chromosome (Figure 1).

Heterochromatin, C_0t-1 DNA and telomeric mapping

In all of the species analyzed here, C-positive blocks were observed in the pericentromeric region of all complements, including the sex chromosomes (Figure 2). These C-positive regions were labeled by the C_0t-1 DNA fractions obtained from each species, except the pericentromeric region of the neo-Y chromosome in *D. lilloanus*. Additionally, terminal blocks were detected in the *Ch. chiquitensis*, *Eurotettix* and *D. lilloanus* chromosomes. In *E. brevicerci*, interstitial blocks were also observed in pairs 1, 3 and 9 (Figure 2). In the *Dichromatos* species, the specimens used to perform the FISH analysis with the C_0t-1 DNA probes carried B chromosomes that presented pericentromeric, interstitial or terminal blocks (Figure 2).

The CMA_3/DAPI fluorochrome staining revealed homogeneous DAPI staining (results not shown) and distinct patterns of G+C-rich blocks (CMA_3 positive) as follows: *Ch. vittatus*, all pericentromeric regions; *Ch. chiquitensis*, pericentromeric regions of pairs 3, 5 and the X chromosome; *E. brevicerci*, interstitial region

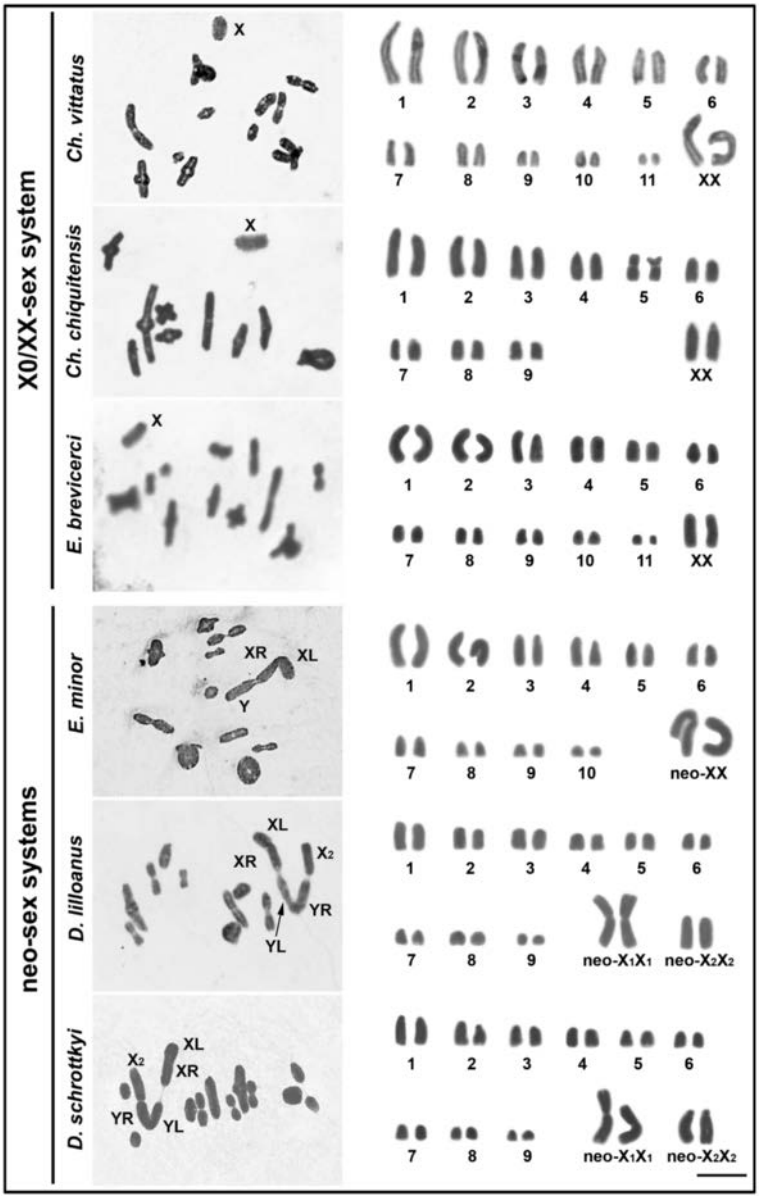

Figure 1 Conventional staining of male metaphase I (left panel) and female mitotic karyotypes (right panel). The sex-chromosome system types and the species names are shown directly in the figure. The sex chromosomes and chromosome arms of the neo-sex chromosomes involved in Rb-fusions are indicated. XL: arm derived from the original X chromosome fused to an autosome; XR: autosomal arm of the neo-X that shares homology with the neo-Y; YL: arm that shares homology with the XR arm; YR: arm that shares homology with the neo-X_2 chromosome. Bar = 5 μm.

of pair 6 and pericentromeric regions of the X chromosome and pair 9 (heteromorphic); *E. minor*, pericentromeric region of pair 5 and the neo-X chromosome and the distal region of pair 7; *D. lilloanus*, pericentromeric regions of pair 5 and the neo-X_1 chromosome; *D. schrottkyi*, pericentromeric regions of pair 4 and the neo-X_1 and neo-X_2 chromosomes (Figure 3).

In all species with X0♂/XX♀ sex system and those with Rb-fusion-derived sex chromosomes (neo-XY♂/XX♀, neo-X_1X_2Y♂/$X_1X_1X_2X_2$♀), only terminal sites were observed with a telomeric probe in both the autosomes (result not shown) and the sex chromosomes (Figure 3, insets).

Cytogenetic mapping of multigene families

FISH analysis with an 18S rDNA probe revealed signals in two autosomal pairs in *Ch. vittatus,* the X chromosome of *Ch. chiquitensis* and one pair of autosomes in *E. brevicerci* and *E. minor*; an additional cluster in the X chromosome of *E. brevicerci* was also observed (Table 1;

Table 1 Species, locality, number of males and females (M/F), diploid numbers and chromosomal positions of multigene families in grasshoppers from the *Chlorus*, *Eurotettix* and *Dichromatos* genera studied in this paper

Species	Locality	Number of individuals (M/F)	2n	18S rDNA	H3 histone gene	5S rDNA	U1 snDNA	U2 snDNA
Ch. vittatus	Parque Nacional Ybycuí (Paraguay)	15/2	♂23/X0 ♀24/XX	3 pc; 6 d	7 i	3 i; 4 d; 6 i	4 d	1 i; 2 i; 9 i
Ch. chquitensis	Corumba (Brazil)	9/2	♂19/X0 ♀20/XX	X pc	7 pc	6 i	4 d	1 i; 2 i
E. brevicerci	Botucatu (Brazil)	15/9	♂23/X0 ♀24/XX	X pc; 3 pc	7 pr	3 i	4 d	1 i; 9 pc*
E. minor	Paraguarí (Paraguay)	16/4	♂22/XY ♀22/XX	3 pc	5 d	3 pc; 5 pr; XR i	4 d; XR i; Y i	1 i; 2 i
	Atyra (Paraguay)	2/0						
	Altos (Paraguay)	1/0						
	Parque Nacional Ybycuí (Paraguay)	10/1						
	Ybycuí (Paraguay)	7/0						
D. lilloanus	Reserva Provincial Yaguaroundí (Argentina) Eldorado (Argentina)	30/35	♂21/ X_1X_2Y	X_1 pc; 5 pr	1-9 pc; X_1 pc; X_2 pc	3 i; YL pr, i, d; YR pr	2 i	1 i; 6 pr; YL pr
		0/2	♀22/ $X_1X_1X_2X_2$					
D. schrottkyi	Eldorado (Argentina)	2/4	♂21/ X_1X_2Y	X_1 pc; 4 pr	5 pr	2 i; YL i; YR pr, i	3 d	1 i; 5 pr *
			♀22/ $X_1X_1X_2X_2$					

pc=pericentromeric; *pr*=proximal; *i*=intersticial; *d*=distal. * indicate the occurrence of heteromorphism.

Figure 4). In the *Dichromatos* species, signals were detected in the neo-X_1 chromosome and in one autosomal pair (*D. lilloanus* pair 5 and *D. schrottkyi* pair 4) (Table 1; Figure 4).

Hybridization signals of the 5S rDNA probe were observed in three autosomal pairs of *Ch. vittatus*, but only in one pair of autosomes in *Ch. chiquitensis* and *E. brevicerci* (Table 1; Figure 4). *Eurotettix minor* showed clusters of the 5S rDNA genes in two autosomal pairs and in the XR arm of the neo-X chromosome, whereas *D. lillonaus* and *D. schrottkyi* each showed one cluster in a pair of autosomes and multiple 5S rDNA sites in the neo-Y chromosome (Table 1; Figure 4).

In four species, *Ch. vittatus*, *Ch. chiquitensis*, *E. brevicerci* and *E. minor*, the U1 snRNA gene was distally located in pair 4. Additionally, U1 snRNA was present at interstitial sites in the XR and neo-Y chromosomes of *E. minor*. *Dichromatos lilloanus* and *D. schrottkyi* showed U1 snDNA clusters only in one bivalent (Table 1; Figure 5). U2 snDNA clusters were located interstitially in two autosomal pairs in the *Chlorus* species, and in *Ch. vittatus,* U2 snDNA was detected in an additional autosomal pair. In the *Eurotettix* species, these sequences were observed in two autosomal pairs. *Dichromatos* showed hybridization signals in two autosomal pairs; however, this gene cluster was also located on the YL arm in *D. lilloanus* (Table 1; Figure 5).

Finally, FISH analysis of histone H3 revealed conserved hybridization signals in pair 7 of *Ch. vittatus*, *Ch. chiquitensis* and *E. brevicerci*. *Eurotettix minor* and *D. schrottkyi* presented the histone H3 cluster in the distal and interstitial regions of pair 5, respectively; whereas in *D. lilloanus*, this gene was spread throughout the pericentromeric regions of all chromosomes, except for the neo-Y chromosome (Table 1; Figure 6).

The FISH results showing the chromosomal locations of the multigene families are summarized in Table 1, and the FISH results for the sex chromosomes are summarized in Figure 7, except for those obtained using the telomeric probe.

Discussion

General organization of repetitive DNAs in autosomes

The general distribution patterns of the C-positive blocks found in the studied species were similar to those reported for other grasshopper species and occurred as pericentromeric blocks in the autosomal complements [34,44,45]. However, other repetitive DNA rich regions were detected using the C_0t-1 DNA fraction, including telomeres and interstitial areas.

For the multigene families, intra- and intergenomic variability were observed for the distinct sequences and species. Our findings revealed remarkable variability in the number and location of major rDNA genes; this is

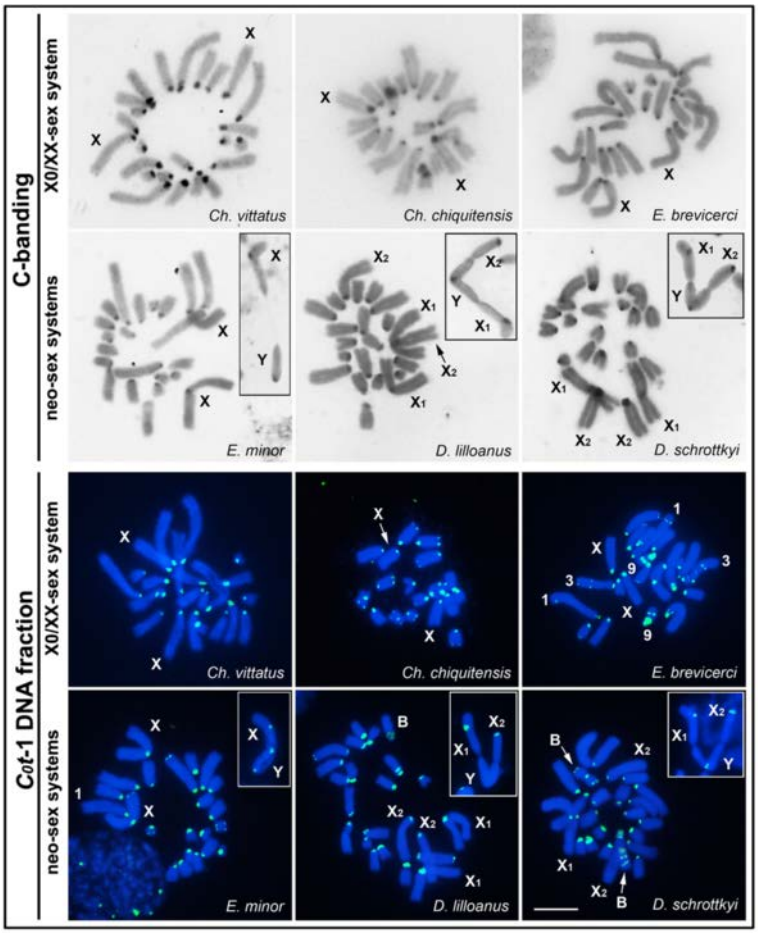

Figure 2 C-banding and C_0t-1 DNA fractions in female mitotic chromosomes. The species names and the types of sex chromosome determination systems are indicated in each figure. Inserts show the locations of C-banding and C_0t-1 DNA fractions in the neo-sex chromosomes during meiosis. Note the absence of the C_0t-1 DNA fraction in the neo-Y chromosome of *D. lilloanus*. Bar = 5 μm.

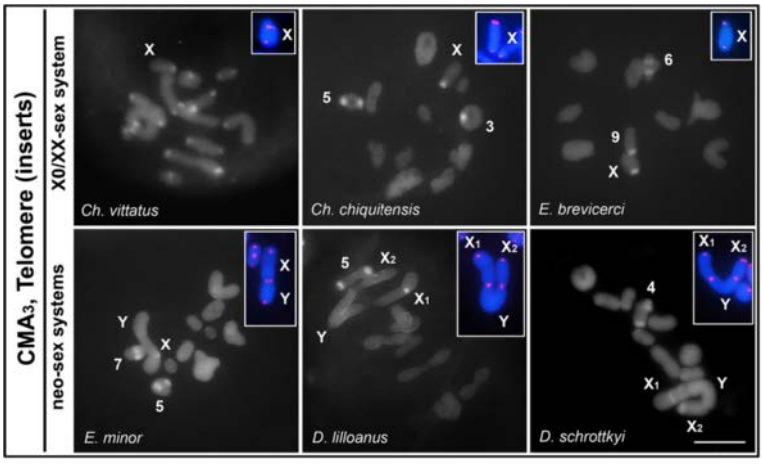

Figure 3 CMA_3 fluorochrome staining and FISH with a telomeric probe (insert) in male meiotic cells. The species names and sex chromosome system types are indicated in each figure. Additionally, autosomes with CMA_3-positive blocks are indicated. Note the absence of interstitial telomeric sites in the neo-sex chromosomes produced by Rb-fusions. Bar = 5 μm.

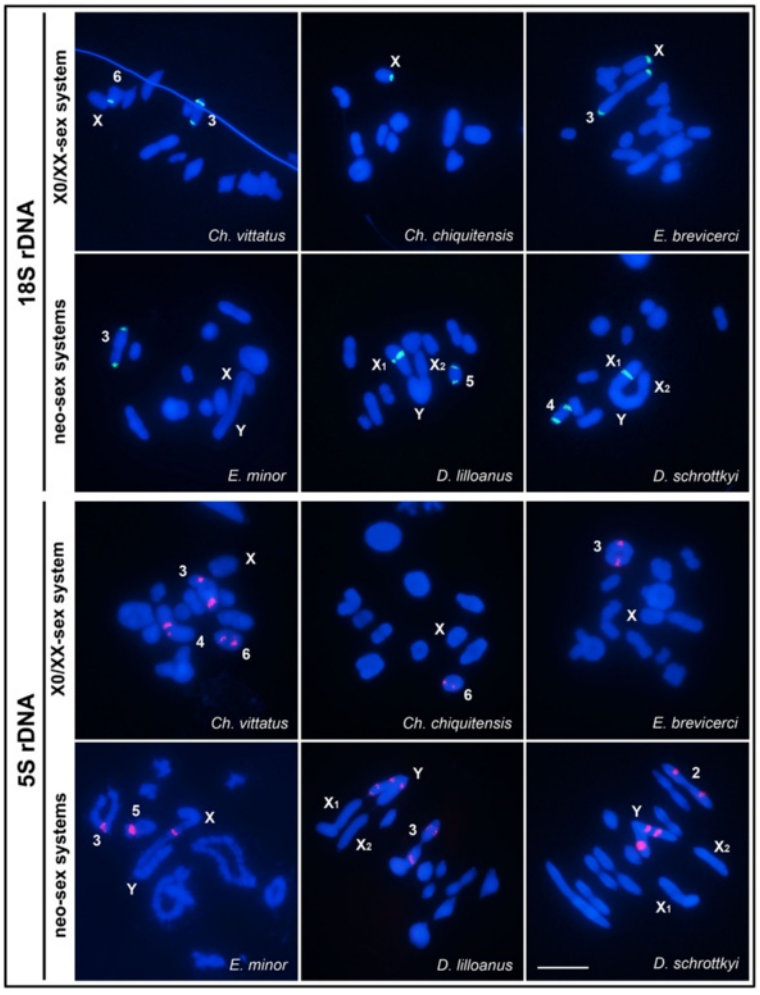

Figure 4 FISH with 18S and 5S rDNA probes in meiotic cells from males. The probe, type of sex-chromosome system and name of species are indicated in each figure. Chromosomes with positive signals and sex chromosomes are indicated. Note the presence of pericentromeric sites for 18S rDNA on the X-chromosomes of *Ch. chiquitensis* and *E. brevicerci* and on the neo-X_1 chromosomes of *D. lilloanus* and *D. schrottkyi*, as well as the 5S rDNA on the sex bivalent neo-XY chromosome of *E. minor* and in multiple sites on the neo-Y chromosome in *D. lilloanus* and *D. schrottkyi*. Bar = 5 μm.

consistent with previous studies in which similar patterns were observed in grasshopper species [45-47] and in other insects, such as Lepidoptera [48], Coleoptera [49] and Heteroptera [50]. The variability for 5S rDNA also reflects common patterns seen in grasshoppers [47]. In contrast with the rDNAs, the Melanoplinae species analyzed here showed less variability in the U1 snRNA genes; this stability of the U1 snDNA clusters has been previously documented in other biological models, such as in cichlid fishes [51]. Although an additional U snRNA gene, U2 snRNA, showed more variability than U1 snRNA, it was also conserved in the interstitial position of pair 1, potentially reflecting the ancestral placement in these species.

According to Cabrero et al. [52], the occurrence of one autosomal cluster of histone H3 genes represents the ancestral placement for Acrididae. This location was observed in our study for five of the species analyzed. However, it is possible that the unusual dispersion observed for the histone H3 genes in *D. lilloanus*, also observed for example in *Abracris flavolineata* [53], could be the result of multiple mechanisms, such as association with mobile elements, ectopic recombination or extrachromosomal circular DNA (eccDNA), as has been postulated for rDNAs [46,54-56].

Diversification of the sex chromosomes

As we mentioned above, the organization of different repetitive DNA sequences has been described in grasshoppers, mainly for multigene families [46,47,52]. However, there are no records of studies focusing on the possible role of such genomic elements in the diversification of sex chromosomes.

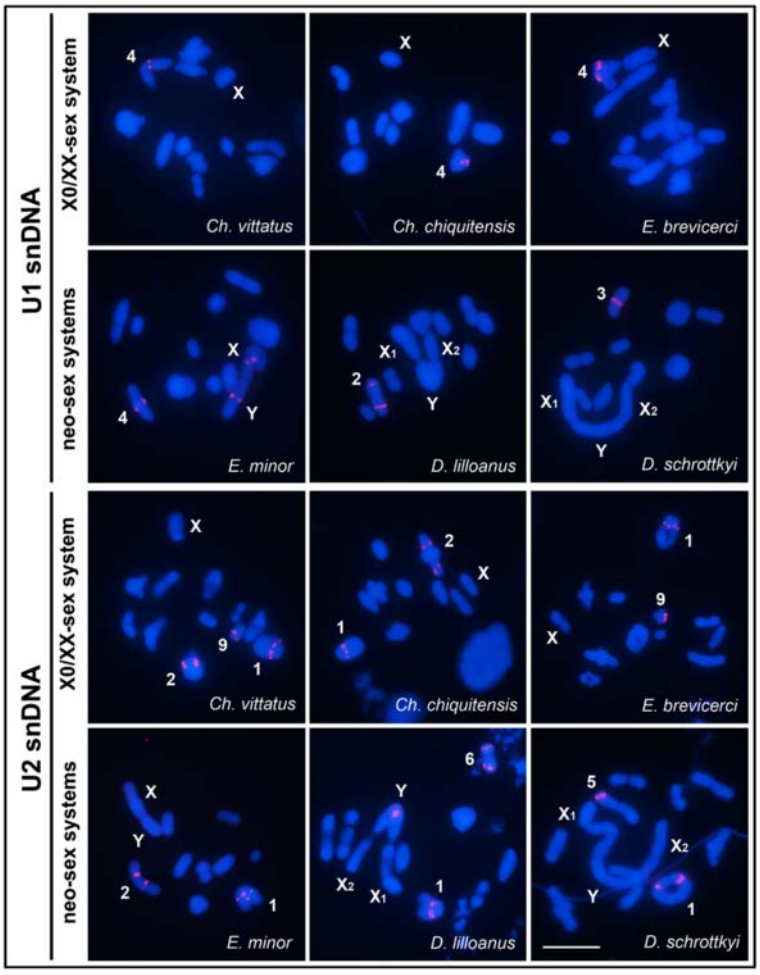

Figure 5 Chromosomal mapping of the U1 and U2 snRNA genes in meiotic cells from males. The probe type, sex chromosome system and name of species are shown for each cell. Chromosomes with positive hybridization signals and sex chromosomes are indicated in the images. Note the presence of U1 snDNA clusters in the interstitial region of the neo-XY chromosomes of *E. minor*, and the U2 snDNA clusters in the proximal region of the neo-Y chromosome of *D. lilloanus*. Bar = 5 μm.

C-positive blocks in the pericentromeric regions observed in the three different sex chromosome systems indicate non-spreading of heterochromatic segments after their origin. Additionally, the mapping of C_0t-1 DNA fractions reinforced the non-massive spreading of repetitive DNA pools in these sex chromosomes, which contrasts with the repetitive DNA accumulation expected on sex chromosomes after recombination restriction [19,25,31,57]. An alternative hypothesis is that these chromosomes possess variable repetitive DNAs not isolated in the C_0t-1 DNA fraction.

For *E. minor*, *D. schrottkyi* and *D. lilloanus*, the mapping of the C_0t-1 DNA fraction suggested different evolutionary scenarios for the divergence of the neo-Y sex chromosomes. The results could be interpreted as evidence of the loss of selection pressure in the non-recombining regions during their differentiation, leading to a high rate of genetic diversification in the neo-Y chromosome. In the *D. lilloanus* neo-Y chromosome, we observed the absence of a C_0t-1 DNA block compared with the *E. minor* and *D. schrottkyi* neo-Y chromosomes. Different accumulation/diversification patterns of repetitive DNAs in sex chromosomes were also documented for example in plants from the *Rumex* genus [23] and Parodontidae fish [58].

Considering the presence of all of the multigene families mapped in the sex chromosomes, we propose that these sequences could be involved in the diversification of the sex-chromosome determining mechanisms found in Melanoplinae. The 18S rDNA mapping results indicate the independent evolution of the neo-XY and neo-X_1X_2Y sex systems in the related genera *Eurotettix* and *Dichromatos*, due to the absence and presence of this marker in the X chromosomes, respectively. However, we could not rule out the possibility of transposition in these derived sex chromosomes. The noticeable

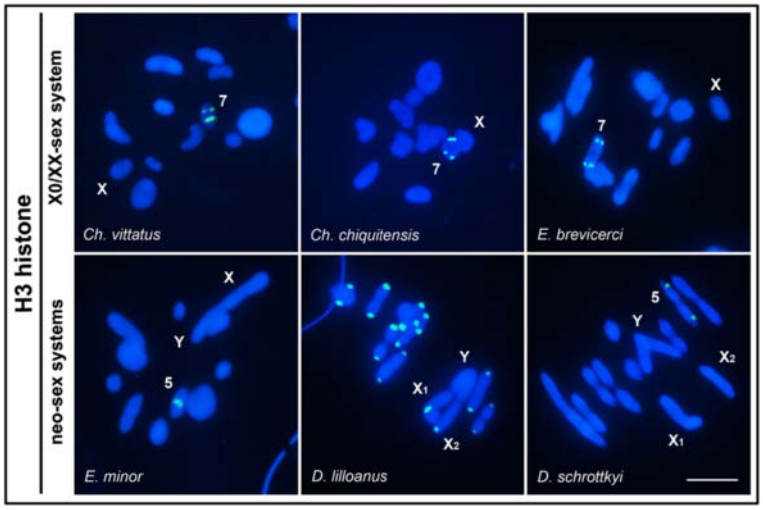

Figure 6 FISH for the histone H3 gene in male meiotic cells. Divergent sex chromosomes, names of species and the chromosomes with hybridization signals are indicated in the figure. Remarkably, the histone H3 gene cluster was found in pericentromeric regions of all chromosomes, except in the neo-Y in *D. lilloanus*. Bar = 5 μm.

accumulation of 5S rDNA in the XR arm of *E. minor* and the neo-Y chromosome of *D. lilloanus* and *D. schrottkyi* could initially be attributed to Rb-fusion X-A and also due to the absence of recombination between sex chromosomes, with the gene cluster localized on the autosome involved in the rearrangement. Moreover, the presence of multiple sites containing 5S rDNA on the neo-Y chromosome of *D. lilloanus* and *D. schrottkyi* suggests the strong accumulation of these sequences after chromosomal rearrangement or the potential action of intrachromosomal recombination, followed by amplification and transposition. The multiple sites observed for these sequences could make this region less likely to undergo recombination and allow it to play an important role in chromatin remodeling, as has been observed for other repetitive DNAs. The rDNA locus, located on sex chromosomes in salmonid fishes, for example, has been suggested to be involved in the restriction of crossing-over near the sex-determining locus [59].

The U1 snRNA gene did not show a strong relationship with sex chromosome diversification, occurring only in the neo-XY chromosome of *E. minor*; this result supports the existence of divergent evolutionary pathways from the *Dichromatos* neo-sex chromosomes. For *D. lilloanus,* the presence of U2 snDNA in the neo-Y chromosome demonstrates the diversification of this chromosome relative to the other congeneric species, *D. schrottkyi*. Although the histone H3 genes were present in the neo-X_1 and neo-X_2 chromosomes of *D. lilloanus*, this sequence was not apparently consistent with sex chromosome diversification; this phenomenon could be associated with the intrinsic mechanism of histone H3

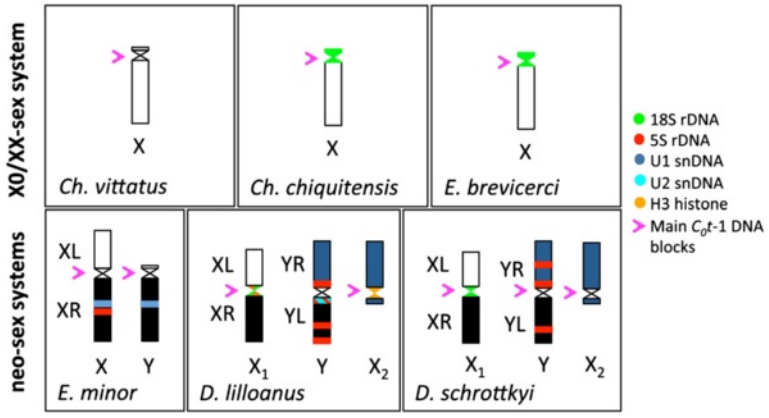

Figure 7 FISH signals with six probes in the sex chromosomes of all the species analyzed in this study. The probes and its position on the sex chromosomes are shown. Colors on the sex chromosomal arms represent the ancestral regions of homology.

dispersion in the *D. lilloanus* genome after the divergence of the *Dichromatos* species.

Phylogenetic analyses suggest that *Chlorus*, *Eurotettix* and *Dichromatos* are monophyletic groups with uncertain evolutionary relationships to the rest of the Dichroplini tribe [42,43]. Considering the morphological characteristics of these species with all brachypterous species, and consequently low vagility, it is possible that the neo-sex chromosome systems might have played a significant role in the divergence and isolation between populations, leading to the restriction of gene flow and speciation. After isolation, the sex chromosomes could undergo molecular differentiation, as observed for the species studied here. Similar models of phenotypic divergence, reproductive isolation and speciation through neo-sex chromosomes have been proposed, for example, for closely related species of fishes [12].

Notably, neo-sex chromosome systems derived from autosome-sex chromosome fusion have been frequently reported in animals [3,12,60-62]. Such rearrangement results in specific intrinsic properties, such as recombination-free regions, due to chiasmata shifts that lead to low intra-chromosomal recombination between involved chromosomes, and the reduction of linkage groups, resulting in lower rates of inter-chromosomal recombination [3,15,21]. According to Charlesworth et al. [20], these factors create strong linkage between the genes on evolving sex chromosomes, which is favorable in the heterogametic sex. These mechanisms might potentially be involved in sex chromosome diversification among Melanoplinae grasshoppers undergoing Rb-fusions that result in reduced chromosome numbers. Indeed, we demonstrated in this study that the presence of telomeric sequences occurred only in current telomeres, which confirms a previous hypothesis that Rb-fusions [32] originate from double chromosome breaks with the loss of telomeric sequences. Although we cannot rule out completely the occurrence of interstitial telomeric sites not detected by FISH.

Conclusions

Different organization of repetitive sequences in the sex chromosomes indicates independent diversification of the sex chromosome systems in Melanoplinae grasshoppers of the *Chlorus*, *Eurotettix* and *Dichromatos* genera. However, the localization of 18S and 5S rDNA on the neo-X_1 and neo-Y chromosomes of *Dichromatos* species suggests that the neo-X_1X_2Y sex determination systems share a common origin, but these chromosomes have also undergone distinct modifications that led to their differentiation. In addition, the presence of structural genes (like 5S rRNA, U1 snRNA and U2 snRNA) mapped to the neo-Y chromosome of *E. minor* and *Dichromatos* species would prevent the complete degeneration and loss of these chromosomes (X0 reversion). The results presented in this paper provide an initial characterization of the derived sex chromosomes in grasshoppers at a molecular level, focusing on the presence of repetitive DNA sequences. To obtain a more detailed picture of sex chromosome evolution in grasshoppers, future studies should be performed using cross-species chromosome painting and the isolation of different repetitive DNAs, such as transposable elements and satellite DNAs.

Methods

Animals, DNA samples and chromosome spreading

Male and female adult grasshoppers from the species *Chlorus vittatus*, *Ch. chiquitensis*, *Eurotettix brevicerci*, *E. minor*, *Dichromatos lilloanus* and *D. schrottkyi* were sampled from distinct localities in Paraguay, Argentina and Brazil (Table 1). Male testes were fixed in a 3:1 ethanol: acetic acid solution, and female gastric caeca were removed and fixed as described by Castillo et al. [63]. All specimens were stored in 100% ethanol until subsequent DNA extraction.

We used conventional staining with 5% Giemsa to visualize the general chromosomal characteristics present in the individuals of each species. C-banding was performed according to Sumner [64], and fluorochrome staining (CMA_3/DA/DAPI) was performed according to Schweizer et al. [65]. Genomic DNA extraction was performed using the phenol-chloroform protocol [66].

The nomenclature proposed by White [3] was used to describe the neo-sex chromosome arms in simple neo-XY systems; the arms of neo-X chromosomes were designated XL, which is the ancestral X, and XR, which shares homology with the neo-Y. In multiple neo-X_1X_2Y systems, the neo-X_1 chromosome was designated as described for the neo-XY type; the metacentric neo-Y chromosome is formed from the YL and YR arms, which share homology with the XR and neo-X_2 chromosome, respectively.

Isolation of multigene families and telomeric repeats

The partial sequences of the 5S rRNA and histone H3 genes were amplified by polymerase chain reaction (PCR) using genomic DNA obtained from *Abracris flavolineata* and the primers described by Loreto et al. [67] and Cabral-de-Mello et al. [68] for 5S rDNA and Colgan et al. [69] for histone H3. The sequences for the U snDNAs were obtained from the *Rhammatocerus brasiliensis* genome using primers described by Cabral-de-Mello et al. [51] for U1 snDNA and Bueno et al. [53] for U2 snDNA. The amplified fragments were sequenced and deposited in GenBank under the accession numbers KC936996 (5S rDNA), KC896792 (histone H3 gene), KC896793 (U1 snDNA) and KC896794 (U2 snDNA).

The 18S rDNA sequence was obtained from a cloned fragment previously isolated from the *Dichotomius se misquamosus* genome (GenBank accession number GQ443313, Cabral-de Mello et al. [68]), and the telomeric probes were obtained by PCR using the complementary primers $(TTAGG)_5$ and $(CCTAA)_5$ [70].

C_0t-1 DNA isolation

Repetitive DNA-enriched samples from each species were obtained based on the renaturation kinetics of C_0t-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences), according to the protocol described by Zwick et al. [71] with modifications [68]. Briefly, the DNA samples (200 μL of 100–500-ng/μL genomic DNA in 0.3 M NaCl) were digested with deoxyribonuclease I (Sigma, St Louis, MO, USA) at 0.01 U/μL for 80 to 105 sec, depending on the sample concentration, and the fragmented DNA was separated using 1% agarose gel electrophoresis. The expected DNA fragments ranged in size from 100 to 1,000 base pairs (bp). For each species, 50 μL samples of the fragmented DNA were denatured at 95°C for 10 min, placed on ice for 10 sec and transferred to a 65°C water bath to reanneal for 25 min. Subsequently, the samples were incubated at 37°C for 8 min with 1 U of S1 nuclease to digest the single-stranded DNA. The DNA was purified and extracted using a traditional phenol-chloroform protocol [66].

Fluorescence in situ *hybridization*

The plasmid containing the 18S rRNA gene, the PCR products from the histone H3 gene and the C_0t-1 DNA fraction were labeled by nick translation using biotin-14-dATP (Invitrogen, San Diego, CA, USA). The 5S rDNA, U snDNAs (U1, U2) and telomeric probes were PCR labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany).

Single- or two-color FISH was performed according to Pinkel et al. [72], with modifications [68] using distinct mitotic and meiotic cells. Although some two-color FISH assays were performed, the same metaphase is shown separately for each probe. Probes labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin-rhodamine (Roche), and probes labeled with biotin-14-dATP were detected using streptavidin, alexa fluor 488 conjugate (Invitrogen). The preparations were counterstained using 4′, 6-diamidine-2′-phenylindole dihydrochloride (DAPI) and mounted using Vectashield (Vector, Burlingame, CA, USA). The chromosomes and FISH signals were observed using an Olympus microscope BX61 equipped with a fluorescence lamp and appropriate filters. The photographs were recorded using a DP70 cooled digital camera. The images were merged and optimized for brightness and contrast using Adobe Photoshop CS2 software.

Abbreviations

2n: diploid number; Bp: Base pairs; CMA_3: Chromomycin A_3; C_0t: C_0 is the initial concentration of single-stranded DNA in mol/l and t is the reannealing time in seconds; DA: Distamicyn; DAPI: 4′, 6-Diamidino-2-phenylindole; FISH: Fluorescence *in situ* hybridization; PCR: Polymerase chain reaction; Rb-fusion: Robertsonian fusion; rDNA: Ribosomal DNA; rRNA: Ribosomal RNA; snRNA: Small nuclear RNA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

OMPG conducted the chromosome preparations and the molecular cytogenetic experiments, interpreted the data, and drafted of the manuscript. ERC and DAM interpreted the data and drafted the manuscript. DCCM conceived the study, participated in its design and coordination, interpreted the data and assisted in drafting the manuscript. All authors have read and approved the final manuscript.

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4.2 Capítulo 2:

Neo-sex chromosome of *Ronderosia bergi*: Insights on molecular evolution of sex chromosomes in grasshoppers

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Neo-sex chromosome of *Ronderosia bergi*: Insights on molecular evolution of sex chromosomes in grasshoppers

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Short running title: Neo-sex chromosome in *Ronderosia bergi*

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Abstract

Sex chromosomes have evolved several times from morphologically identical autosome pairs, presenting gradual constraint and accumulation of repetitive DNAs. In Orthoptera, the X0 sex chromosome systems is observed in most species studied; however, in the subfamily Melanoplinae, derived variants (neo-XY or neo-X_1X_2Y) evolved several times. Aiming the understanding of neo-sex chromosomes evolution in grasshoppers here we used as model the species *Ronderosia bergi* that presents a neo-XY/XX system. For this propose it was integrated (i) classical cytogenetic analysis; (ii) cytogenetic mapping for some repetitive DNAs and microdisssected sex chromosomes; and (iii) immunolocalization for distinct histone modifications. Moreover the microdissected chromosomes were used as source for specific amplification of multigene families in order to address the specific variation of these sequences in the sex chromosomes. Our data supports the argument that the *R. bergi* neo-Y is an element highly differentiated and degenerates (including for repetitive genes), reflecting the potential role of the Rb-fusions and inversions as first step to create new sex chromosomes in grasshoppers. The sequence analysis for three multigene families revealed homogenization among autosomes and neo-Y for U2 snRNA, divergence for U1 snRNA between the neo-Y and autosomes and mixed (diversification/homogenization) among neo-X, neo-Y and autosomes for 5S rDNA. However, and although the *R. bergi* neo-XY sex-chromosome are well differentiated the ex-autosomes incorporate at the new systems retain autosomal post-translational histone modifications, suggesting that (i) the molecular content do not influence the post-translational modification patterns for histones; (ii) that these regions could retain some important autosomal genes that follow similar modifications to actual autosomes; and (iii) that the establishment of functional modifications in neo-sex chromosomes are

slower than the molecular diversification. These results provide new information regarding chromosomal variability for repetitive DNAs in grasshoppers and the specific molecular composition of neo-XY sex chromosome.

Keywords: Rb- fusions, inversion, neo-sex chromosomes, FISH, repetitive DNAs, histone modification.

Introduction

Sex chromosomes have evolved several times from morphologically identical autosome pairs within of a wide range of organisms, including plants and animals (Ohno 1967, Bull 1983). It is widely assumed that the gradual constraint of recombination between sex chromosomes leads to morphological and genetic divergence of the between X and Y or Z and W chromosomes (Rice 1996, Filatov 2000). The ultimate fate of this process is the genetic degeneration and loss of many genes on the Y or W chromosomes, due to the accumulation of repetitive DNAs which are strong after the abolition of recombination and that sometimes can lead to complete disappearance of these chromosomes (Charlesworth et al. 1994; Rice 1996, Steineman and Steinemann 1997, 2005; Navajaz-Pérez et al. 2005, 2009; Bachtrog 2006; Kejnovsky et al. 2009; Pokorná et al. 2011; Palacios-Gimenez et al. 2013).

There are some well-documented examples of the role of repetitive DNAs accumulation and gene degeneration as important requirements for the generation of morphological and genetic differences between sex chromosomes. Thus for example, the human Y chromosome carries just 27 distinct protein coding genes and only 16 genes have homologs on the X chromosome. About half of the human Y chromosome is entirely heterochromatic, while the euchromatic portion is a mosaic of X degenerated, X transposed and ampliconic sequences (Skalestky et al. 2003). Similarly the *Drosophila melanogaster* Y chromosome is almost completely heterochromatic and distinct from the X chromosome, carrying just a total of ~ 15 genes (Carvalho 2002). These Y chromosomes are relatively ancient and have evolved from autosomes ~ 300 millon years ago (m.y.a) and actually containing low gene density (Skaletsky et al. 2003; Carvalho 2002). In plants, the *Silene latifolia* XY sex chromosomes evolved recently from autosomes through chromosomal inversion ~ 20-30 m.y.a, but a strong

accumulation of repetitive DNAs has been also reported (Nicolas et al. 2003; Matsunaga 2009).

Among insects it is assumed that the sex systems X0/XX and Z0/ZZ in certain groups, like Orthoptera, Blattodea, Mantodea, Phasmatodea and Lepidoptera evolved from an ancestral XY/XX or ZW/ZZ sex chromosomes, suggesting that the reversion of the X0 or Z0 state occurred in the common ancestors of these lineages (White 1973; Hewitt 1979; Castillo et al. 20010a; Charlesworth and Mank 2010; Kaiser and Bachtrog 2010; Yoshido et al. 2013). In grasshoppers the sex system X0/XX is observed in most part of species studied, which male are the heterogametic sex (X0); however, in the subfamily Melanoplinae, derived variants neo-XY or neo-X_1X_2Y evolved several times by repeated autosome-sex chromosome Robtersonian fusions (Rb-fusions), that besides the emergence of neo-sex systems also resulted in reduced chromosome numbers (White 1973; Hewitt 1979; Bidau and Martí 2001; Castillo et al 2010b; Palacios-Gimenez et al. 2013).

The data concerning sex chromosomes evolution in grasshoppers is concentrated in classical descriptions (Castillo et al. 2010a) and the molecular content and patterns of evolution for neo-sex chromosomes at molecular point to view still poorly known (see Palacios-Gimenez et al. 2013). In order to an increase the understanding of molecular content and patterns of evolution for neo-XY sex-chromosome in grasshoppers here we used as model for analysis of sex chromosome evolution the species *Ronderosia bergi* (Acrididae, Melanoplinae) that harbor a neo-XY sex bivalent, 2n=22,neo-XY♂ (Castillo et al. 2010b). For this propose it was integrated (i) classical cytogenetic analysis; (ii) cytogenetic mapping through fluorescent *in situ* hybridization (FISH) for distinct probes, such five multigene families, sixteen microsatellites, telomeric probe, C_0t-1 DNA fraction, and paint for microdisected X and Y chromosomes; and (iii)

immunolocalization for distinct histone methylations, acetylation and phosphorylation. Moreover the microdissected chromosomes were used as source for specific amplification of five multigene families in order to address the specific variation of these sequences in the sex chromosomes.

Materials and methods

Animals, DNA samples and chromosome spreading

Adult males and females of *R. bergi* were collected at the campus of the Univ Estadual Paulista - UNESP (Rio Claro, São Paulo State, Brazil) from December 2012 to March 2013. Male testes were dissected and fixed in a 3:1 ethanol: acetic acid solution. For immunolabeling the fixation was performed using 2% paraformaldehyde (see below). In addition, some individuals were kept in captivity until females oviposition occurred. The cytological preparations of embryos were done as Webb et al. (1978) with slight modifications. All specimens were stored in 100% ethanol for posterior DNA extraction.

In order to describe the general chromosomes aspects we used conventional staining with 5% Giemsa for each individual. The C-banding procedure was conducted according to Sumner (1972. The female and male genomic DNA extractions were performed using the phenol-chloroform procedure (Sambrook and Russel 2001).

Probes for multigene families and telomeric repeat

DNA probes for four multigene families were obtained through Polymerase Chain Reaction (PCR) from the genomes of *Abracris flavolineata* (5S rDNA and H3 histone gene) and *Rhammatocerus brasiliensis* (U1 and U2 snDNA) using primers designated by Cabral-de-Mello et al. (2010, 2012), Colgan et al. (1998) and Bueno et al.

(2013). The fragments amplified were previously sequenced to confirm the sequence of interest and they are deposited in GeneBank under the accession numbers KC936996 (5S rDNA), KC896792 (H3 histone gene), KC896793 (U1 snDNA) and KC896794 (U2 snDNA). For 18S rDNA the probe was obtained from a cloned fragment previously isolated from the *Dichotomius semisquamosus* genome (Cabral-de Mello et al. 2010, GeneBank accession number GQ443313). The telomeric probe was obtained through PCR using the self complementary primers $(TTAGG)_5$ and $(CCTAA)_5$ following the indications described by Ijdo et al. (1999).

C_0t-*1 fraction obtaining*

The obtaining of repetitive DNA-enriched sample was based on the renaturation kinetics of *C_0t*-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences), according to the protocol of Zwick et al. (1997), with modifications (Cabral-de-Mello et al. 2010). The DNA samples were digested (200 µl of 100–500-ng/µl genomic DNA in 0.3 M NaCl) with Deoxyribonuclease I (Sigma, St Louis, MO, USA) at 0.01 U/µl for 50 s and the fragmented DNA was checked in 1% agarose gel electrophoresis. The expected DNA fragment ranged in size from 100 to 1,000 bases pairs (bp). 50 µl samples of the DNA fragments was denatured at 95°C for 10 min, placed on ice for 10 s and transferred into a 65°C water bath for reannealing for 25 min, and subsequently, incubated at 37°C for 8 min with 1 U of S1 nuclease to digest the single-stranded DNA. The DNA was purified/extracted using a traditional phenol-chloroform procedure.

Sex chromosomes microdissection and multigene families amplification

Microdissection of neo-X and neo-Y chromosomes was performed from male meiotic cells. Before microdissection it was generated a cell suspension of four testicular follicles in 100 µl of 50% acetic acid which was spread onto a coverslip on a warm plate at ~ 50 ºC. The neo-sex chromosomes were easily recognized due to their C-shape configuration at metaphase I (Castillo et al. 2010b) and the early segregation of these elements avoided the cross contamination.

Microdissection was conducted in an Eppendorf 5171 micromanipulator coupled to a Nokon Axiphot inverted microscope. Ten neo-Y chromosomes and eighth neo-X chromosomes were microdissected separately and then amplified by means of GenomePlex Single Cell Whole Genomic Amplification kit WGA4 (Sigma, St Louis, MO, USA) followed by reamplification using GenomePlex WGA3 kit (Sigma). We used the DNAs amplified by GenomePlex WGA3 kit for generate probes of the neo-X (µX-DNA) and neo-Y (µY-DNA) chromosomes.

In order to check the presence of multigene families for rDNAs, U1 and U2 snDNAs and H3 histone gene in the neo-sex chromosomes PCRs were employed using as model the microdissected chromosomes separately. Moreover for comparative analysis the same repeats were also amplified from genomic DNA from female (♀gDNA). The primers used are described in Cabral-de-Mello et al. (2010) for 18S and 5S rDNAs, Colgan et al. (1998) for H3 histone, Cabral-de-Mello et al. (2012) for U1 snDNA and Bueno et al. (2013) for U2 snDNA. PCR was performed in 10 x PCR Rnx Buffer, 0.2 mM Cl_2Mg, 0.16 mM dNTPs, 2 mM of every primer, 1 U of *Taq* Platinum DNA Polymerase (Invitrogen, San Diego, CA, USA) and 50-100 ng/µl of DNA depending of the samples. PCR condition included an initial denaturation at 94ºC for 5 min and then 30 cycles at 94ºC (30 s), 55º (30 s), and 72ºC (1min and 20 s) plus a final

extension at 72ºC for 5 min. The PCR products were visualized in 1% agarose gel and the bands were isolated and purified using the kit Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., The Epigenetics Company, USA) according to manufacture recommendations.

For sequence comparison the purified PCR products were cloned using the cloning kit pGEM-T (Promega, Madison, WI, USA) and DH5α *Escherichia coli* competent cells. A total of 120 recombinant colonies were chosen for DNA sequencing using the service of Macrogen Inc. (Korea), being 33 clones of U1 snDNA, 35 clones of U2 snDNA and 52 clones of 5S rDNA.

Sequence analysis

The quality of the sequences was checked using Geneious 4.8.5. Software (Drummond et al. 2009). All consensus sequences were subjected to BLAST (Altschul et al. 1990) searches in the NCBI website (http//: www.ncbi.nlm.nih.gov/blast) and as expected were recognized as 5S rRNA, U1 and U2 snRNAs genes. The sequences are deposited in the NCBI database under the following accesses numbers (XXXX).

For DNA sequences analyses it was computed basic sequences statistics with the program DnaSP v.5.10.01 (Librado and Rozas 2009). Phylogenetic and analysis molecular evolutionary were conducted using MEGA v.5 (Tamura et al. 2011).

Fluorescence in situ hybridization (FISH)

The plasmid containing the 18S rRNA gene, the PCR products from the H3 histone gene, the C_0t-1 DNA fraction and the μX-DNA and μY-DNA chromosomes were labeled through nick translation using biotin-14-dATP (Invitrogen). Additionally, we used 16 synthetic oligonucleotide probes directly labeled with biotin-14 dATP

during their synthesis at 5' end: $(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(CG)_{15}$, $(TA)_{15}$, $(AG)_{10}$, $(CAA)_{10}$, $(CAC)_{10}$, $(TAA)_{10}$, $(GAA)_{10}$, $(CGG)_{10}$, $(GAC)_{10}$, $(CAT)_{10}$, $(GAG)_{10}$, $(GACA)_4$ and $(GATA)_8$ (Sigma). The 5S rDNA, U snDNAs (U1 and U2) and telomeric probes were PCR-labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany).

Single or two color FISH was performed according to Pinkel et al. (1986), with modifications (Cabral-de-Mello et al. 2010) using meiotic cells. Probes labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin rhodamine (Roche), and probes labeled with biotin-14-dATP were detected using streptavidin, alexa fluor 488 conjugated (Invitrogen).

The preparations were counterstained using 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) and mounted in Vectashield (Vector, Burlingame, CA, USA). The chromosomes and signals were observed using an Olympus microscope BX61 equipped with a fluorescence lamp and appropriate filters. The photographs were recorded using a DP70 cooled digital camera in gray scale. The images were pseudo-colored in blue (chromosomes) and red or green (signals), merged and optimized for brightness and contrast using Adobe Photoshop CS2.

Inmunolabeling

Testes from two adult males were removed and fixed in freshly prepared 2% paraformaldehyde in PBS (Phosphate-Buffered Saline) containing 0.05% Tween for 15 min. Subsequently, the testes were immersed in a small droplet of the fixative on a glass slide and gently squashed after setting a coverslip, which was them removed after immersing the preparation on the liquid nitrogen, and the slides were immediately transferred to cold PBS. Inmunulabeling followed the technique described by Cabrero et al. (2007). Primary antibodies (rabbit polyclonal IgG, Upstate Biotechnology, USA)

anti-H3K4me2, anti-H3K9me2, anti-H4K5ac, and anti-H3S10ph were diluted 1:600 in 1% BSA (bovine-serum albumin) in PBS. The slides were incubated overnight at 4ºC and after washed were detected with FITC-conjugated anti-rabbit IgG (Sigma) diluted 1:60 in PBS, 1% BSA for 60 min. After final washing in PBT (1x PBS, 0.01% Tween 20), the preparations were counterstained using DAPI and mounted in Vectashield (Vector). Finally, the chromosomal images were recorded using the same equipment mentioned above for FISH.

Results

Conventional staining, heterochromatin and C_0t-1 DNA fraction

As described by Castillo et al. (2010b) all individuals of *Ronderosia bergi* presented karyotype composed by 2n=22♂/22♀ with ten pairs of acrocentric autosomes and a neo-XY♂/XX♀ sex-chromosome mechanism. The sexual pair of this species is formed by a metacentric neo-X, product of the Roberstonian fusion (Rb-fusion) between the ancestral X and an autosome, while the homologue of the translocate autosome becomes the acrocentric neo-Y. The neo-Y chromosome is easily recognizable because its short arm is larger than those observed in autosomes (Figure 1a).

At the meiosis and using White's (1973) terminology, the arms of the neo-X chromosome will be referred as XL the arm derived from the original X-chromosome and XR the arm that shares homology with the neo-Y. Due to the position of both centromeres the sexual pair adopts a C-shape configuration at metaphase I. Therefore, the sex bivalent oriented with the neo-Y parallel to the XL arm of the neo-X, at right angle with XR (Figure 1a, insert).

C-positive blocks were observed throughout the neo-Y chromosome, while only centromeric and terminals blocks were observed in the neo-X chromosome; concerning to autosomes just centromeric blocks were observed (figure 1b). The C_0t-1 DNA fraction obtained of a male generated positive hybridization signals in the centromeric region of the neo-X chromosome and in some centromeric and proximal regions of the autosomes; no signals on the neo-Y chromosome were detected (Figure 1c).

Multigene families, microsatellites and telomeric mapping

FISH analysis indicated that the 18S rRNA is located in the pairs 7 and 8, with centromeric and interstitial location, respectively (Figure 1d), while multiple sites of 5S rRNA were identified in pairs 3 (centromeric and interstitial), 4 (proximal and subterminal), and 7 (proximal) (Figure 1e). Like 5S rRNA, multiples sites for H3 histone gene were observed, i.e. pairs 1, 3-5, 9, neo-X (centromeric) and pair 2 (centromeric and proximal) (Figure 1e). The U1 snRNA gene presented positive hybridization signals in the proximal region of the pair 4 (Figure 1f), while the U2 snDNA clusters were observed in the interstitial region of the pair 1 and in the neo-Y (Figure 1g). Finally, telomeric DNA was located on both ends at each chromosome, including the metacentric neo-X chromosome, being remarkable the intensity of labeling in the long arm of neo-Y chromosome (Figure 1h).

Microsatellite arrays like $(A)_{30}$, $(C)_{30}$, $(CG)_{15}$, $(CGG)_{10}$, $(GAA)_{10}$, $(TAA)_{10}$, $(GACA)_4$ and $(GATA)_8$ were spread along whole chromosomes including both the neo-X and the neo-Y sex-chromosomes, moreover for some of these repeats enrichment in some regions of autosomes were observed (result not shown). On the other hand, other microsatellites arrays like $(CA)_{15}$, $(CAC)_{10}$, $(CAA)_{10}$, $(GAC)_{10}$ and $(GAG)_{10}$ also showed specific and scattered signals, including the sex chromosomes (Figure 2).

Finally, some specific and differential accumulation for $(AG)_{10}$, $(TA)_{15}$ and $(CAT)_{10}$ on the autosomes and neo-X chromosome were observed (Figure 2).

Chromosome painting of the neo-sex chromosomes

The probe obtained by chromosome microdissection from neo-Y probe painted exclusively the entire neo-Y chromosome (Figure 3a, c), while the neo-X probe revealed positive signals in the centromeric regions of pairs 4, 5 and neo-X chromosome (Figure 3b, d).

Inmunolabeling of H4K5ac, H3K9me2, H3K4me2, and H3S10ph

In autosomes, the inmunostaining against H3K4me2 begins at early diplotene, but at metaphase I the pericentromeric regions were hypomethylated. Regarding the neo-sex chromosomes this modification also starts in early diplotene, although the XL arm of the neo-X chromosome (ancestral X chromosome) revealed not signals for this marker (Figure 4a), being this characteristic maintained throughout meiotic cycle. Additionally, although the neo-Y chromosome always exhibited a staining patterns similar to autosomes it was noticed that at metaphase I this chromosome presents stronger signals in the centromeric region (Figure 4a). The inmunostaining against H3K9me2 also beginning at early diplotene reaching its maximum peak during metaphase I (Figure 4b), being similar to autosomes and sex chromosomes.

For acetylation the H4K5 revealed hypoacetylation at prophase I and at metaphase I the XL arm of the neo-X chromosome remained hypoacetylated, while the remaining chromosomes were strongly acetylated, including the neo-Y and the XR arm of the neo-X chromosome (Figure 4c). For phosphorylation the onset of H3S10

modification was observed at early diplotene (results not shown) being more intense during metaphase I (Figure 4d).

In the ideogram of the figure 5 shown the chromosomal location of markers applied in FISH analysis and the histone post-translational modifications (at metaphase) observed in the neo-XY sex chromosomes.

Comparative analysis for multigene families in autosomes and sex chromosomes

For all five multigene families mapped through FISH only the 5S rDNA and U1 and U2 snDNA were successfully amplified through PCR from the DNA of microdisected chromosomes. Coincident with FISH results the U2 snDNA was amplified through PCR in the neo-Y, while the U1 snDNA and 5S rDNA although not detected under FISH mapping were obtained in the neo-Y and neo-X/neo-Y through PCR, respectively. For the sequence analysis we eliminated the primers regions and used as outgroup sequences from *Drosophila virilis*.

From the partial U1 snDNA sequence were analyzed 33 clones: 17 clones of ♀gDNA and 16 of µY-DNA. The sequences analyzed presented 102 nucleotides containing an average of 57.45 % G+C. When they were analyzed separately, both the µY-DNA and the ♀gDNA sequences presented 2 and 3 haplotypes, respectively (see Table 1); and when the whole set of sequences was pooled and analyzed they showed five different haplotypes, indicating not overlapping among the haplotypes between ♀gDNA and µY-DNA. Nucleotide diversity per site (Pi) in the ♀gDNA sequences (0.00332) was almost three times higher than in the µY-DNA sequences (0.00123), and the mean number of nucleotides substitution per site (K) of the ♀gDNA sequences (0.33824) was also almost three times higher than the µY-DNA sequences (0.125). The phylogenetic analysis using the whole set of U1 snDNA sequences and the *Drosophila*

349 *virilis* resulted in a neighbor-joining tree showing the existence of two clades separated,
350 one clade including only ♀gDNA, while the other included only the μY-DNA,
351 suggesting non homogenization and distinct mechanisms of molecular evolution
352 between these sequences (Figure 6a).

353 Regarding to the partial U2 snDNA sequences it was analyzed 34 sequences, 18
354 clones of ♀gDNA and 16 clones of μY-DNA. These DNA sequences have a size of 141
355 nucleotides and G+C content 48.3%. When these sequences were analyzed separately
356 showed 2 (♀gDNA) and 4 (μY-DNA) haplotypes, respectively. Thus, the nucleotide
357 diversity per site of the μY-DNA (0.00485) was four-fold higher than the ♀gDNA
358 (0.00158), and the mean number of nucleotides substitution per site of the μY-DNA
359 (0.683) was also three-fold higher than the ♀gDNA (0.222) (Table 2). Differentially
360 from observed for U1 snDNA the phylogenetic analysis using the whole set of U2
361 snDNA sequences retrieved a neighbor-joining tree showing just one clade, suggesting
362 homogenization and similar mechanisms of molecular evolution between autosomes
363 and μY-DNA sequences (Figure 6b).

364 In the case of 5S rDNA 52 sequences were analyzed: 14 clones of ♀gDNA, 19
365 clones of μX-DNA and 19 clones of μY-DNA. The length of 5S rDNA sequences was
366 93 nucleotides and these sequences contained overall mean in G+C of 56.9%. In the
367 three types of samples different haplotypes were found, i.e, 4 in the ♀gDNA, 7 in the
368 μX-DNA and 7 in the μY-DNA. Remarkably, high polymorphic sites were presented in
369 the neo-sex chromosomes in contrast with the ♀gDNA (Table 3), and when whole set
370 of sequences was analyzed showed that 18 haplotypes were different among themselves.
371 Nucleotide diversity per site was 0.01037 in the ♀gDNA, 0.17418 in the μX-DNA and
372 0.09666 in the μY-DNA. The mean number of nucleotides substitution per site in the
373 ♀gDNA (0.54945) was comparatively lower than both the μX-DNA (5.12281) and the

μY-DNA (8.88304). Thus, the phylogenetic analysis using the whole set of 5S rDNA sequences revealed a neighbor-joining tree showing two distinct clades, the first clade including a mix of ♀gDNA, μY-DNA, μX-DNA and *D. vrilis* 5S rDNA sequences, and the second containing only sequences from sex chromosomes. In the clade formed by sex chromosomes the sequences from neo-X and from neo-Y were also separated. This date suggest that 5S rRNA gene have undergone distinct patterns of molecular evolution (homogeneization/diversification) after the origin of neo-sex chromosomes (Figure 6c). As the 5S rDNA was amplified from μX-DNA, when used the ♀gDNA as template some repeats from neo-X could also be obtained.

Discussion

Molecular diversification of sex chromosomes in R. bergi

The macro-chromosomal structure and the C-shaped orientation of the *R. bergi* neo-XY sex bivalent is similar to previous description to Castillo et al. (2010b), evidencing the complex origin of the system, involving a Rb-fusion X-A followed by a pericentric inversion in the neo-Y and similarity among distinct populations. The FISH for telomere repeat confirms this notion and the absence of internal telomeric repeats indicate that this sequence was lost with the rearrangement or it was eliminated along sex chromosomes differentiation; however, the possibility of internal telomeric sites not detected by FISH could be also conceivable. As in other well studied species, such as for example *Drosophila melanogaster* (Carvalho 2002), *D. miranda* (Zhou et al. 2013), and humans (Skalestky et al. 2003) the neo-Y chromosome of *R. bergi* presents widespread heterochromatin, although the C_0t-1 DNA fraction indicate the non-massive scattering of highly/moderately repetitive DNA in its chromosome, indicating a variable composition of repetitive DNAs not sufficient to be isolated in the C_0t-1 DNA fraction,

399 which contrast with the awaited buildup of repetitive DNAs after constraint of
400 recombination (Steinemann and Steinemann 1997, Charlesworth et al. 1994,
401 Charlesworth et al. 2005).

402 The wide expansion/distribution of microsatellite repeats throughout the *R. bergi*
403 genome, also observed in *Abracris flavolineata* (Milani and Cabral-de-Mello *in*
404 *preparation*), may be related to the substantial increase of genome size in grasshoppers
405 (Westerman et al. 1987, Ruiz-Ruano et al. 2011), similar to dispersion of other
406 repetitive element as in *Drosophila*, humans and plants (San Miguel et al. 1996; Vieira
407 et al. 1999; Matula and Kypr 1999; Hurst and Werren 2001). Although the evolutionary
408 dynamics of microsatellites at chromosomal point to view is little understood there is
409 one well-document example in the large and young non-recombining Y chromosomes
410 of *Rumex acetosa* (Kejnovsky et al. 2013). As well differential accumulation of
411 microsatellites were also observed in *R. bergi* with more evident bands for $(AG)_{10}$,
412 (CA)15, $(TA)_{15}$, $(CAC)_{10}$, $(CAT)_{10}$, $(CAA)_{10}$, $(GAC)_{10}$ and $(GAG)_{10}$ in both the
413 autosomes and the neo-X, which reinforces mainly the differentiation among the neo-X
414 and neo-Y chromosome. In *Rumex acetosa*, the abundance of microsatellites are
415 preferentially localized in the vicinity of transposable elements (TEs), suggesting that
416 microsatellites arrays are probably target for TEs insertions and in which both repetitive
417 elements could also spread via ectopic recombination (Kejnovsky et al. 2009;
418 Kejnovsky et al. 2013). Although in grasshoppers, we have no evidence that these
419 mechanisms could be operating during the propagation of microsatellites arrays, there is
420 sufficient evidence in other taxa that TEs accumulate preferentially in regions with high
421 rates of recombination and possibly recombination machinery use for dispersion
422 (Rizzon et al. 2002), while other repetitive elements are accumulated in regions less
423 prevalent or absent recombination (Charlesworth et al. 1994). Hence, the wide

distribution of microsatellites in the *R. bergi* genome is probably the result of combining multiple poorly understood processes, such as ectopic recombination, replication slippage and transposition, which contribute the dynamism of oligonucleotides in regions of high and low recombination, as well as observed here to autosomes and neo-XY sexual system.

The molecular differentiation between the neo-sex chromosomes of *R. bergi*, suggested by the heterochromatin, C_0t-1 DNA, two microsatellites and U2 snDNA could be definitely corroborated by the use of probes from microdissected neo-X and neo-Y. The use of neo-Y as probe indicated that the neo-Y is a highly differentiated element and share few or no repetitive sequences with the ex-autosomal homologous (XR arm) of the neo-X involved at the Rb-fusion. The neo-X probe also led to similar conclusion for this differentiation, although some similarity with centromeres of some autosomes was observed, that could indicate centromeric sequences conserved in these elements.

The chromosomal mapping of multigene families, i.e. rDNAs, U1 and U2 snDNAs and H3 histone genes, revealed that these sequences, at least large clusters revealed by FISH, are almost exclusively autosomal, except for U2 snDNA that is amplified in the neo-Y chromosome, and is involved with sex chromosome diversification in *R. bergi*. Although though PCR amplification using microdissected chromosomes as template we also identified the presence of repeated for U1 snDNA and 5S rDNA in the sex chromosomes, indicating that at least some repetitions for these sequences, not detected by FISH, are present in these elements, and are also involved in the process sex chromosome differentiation in the species studied.

In insects as general, the evolution of snDNA is ruled primarily by purifying selection, unequal crossing over, gene conversion and evolution of birth and death

449 (Mount et al. 2007). In *R. bergi*, comparative analysis of U1 snDNA between both the
450 ♀gDNA and the μY-DNA did not shown the high intragenome homogeneity
451 characteristic of concerted evolution, indicating non-homogenization and divergence of
452 sequences, possibly consequence of relaxed purifying selection of U1 snDNA on the
453 neo-Y. Another alternative is that this gene may has transposed recently to the neo-Y
454 after the origin of the neo-sex chromosomes following divergent evolutionary ways, or
455 this gene is being continuously degraded due the heterochromatic and non recombinant
456 nature of the neo-Y. These hypotheses are supported by the absence of U1 snRNA gene
457 on the neo-X chromosome, and also by presence of distinct haplotype between both the
458 ♀gDNA and the neo-Y chromosome. Concerning the U2 snDNA, the presence this
459 sequence in the neo-Y and absence in the neo-X chromosome could also indicate recent
460 transposition for this chromosome, which in this case was followed by amplification,
461 allowing detection of large cluster through FISH. This amplification could be also
462 responsible by the low variability for this sequence in the neo-Y chromosome probably
463 caused by homogenization along this process. In addition, the U2 snRNA sequences
464 analysis shows strong homogenization among the autosomal repeats (detected by FISH
465 in the pair 1) and the neo-Y, which is in accordance with the mechanisms of concerted
466 evolution proposed for this gene (Nei and Rooney 2005). The occurrence of these
467 repeated for U1 and U2 snDNA in the autosome involved in the Rb-fusion with the
468 ancestral X is also conceivable, and these repeats could be eliminated from the neo-X
469 along sex chromosome differentiation.

470 The most complex picture for molecular evolution between sex chromosomes
471 and autosomes was observed for the 5S rDNA, and this variability could be attributed to
472 distinct factors, (i) the multiplicity of sites for 5S rDNA, corroborated by FISH analysis,
473 (ii) the occurrence of sites in both sex chromosomes that apparently after their origins

474 have been experienced distinct evolutionary fates. The evolution of divergent variants of
475 5S rRNA within of the genome have been reported in several organisms including
476 *Drosophila melanogaster* (Lohe and Roberts 1990), *Salmo trutta* (Castro et al. 2001),
477 *Paracentrotus lividus* (Caradonna et al. 2007), *Lens* species (Fernandez et al. 2005) and
478 fungi (Rooney and Ward 2005), although in most case this variation is attributed to non-
479 transcribed spacer region (Williams et al. 1987; Martins and Wasko 2004; Rooney and
480 Ward 2005; Vierna et al. 2011; Vizoso et al. 2011). The occurrence of neo-X and neo-Y
481 haplotypes together with ♀gDNA haplotypes in the same grouping indicate that initially
482 the germ sequence for 5S rDNA present in the neo-sex chromosomes could be
483 autosomal, suggesting that the ancestor autosome involved in the X-A Rb-fusion carried
484 5S rDNA repeats. Some of these repeats were maintained in the neo-XY and are
485 experiencing specific modifications, higher for the neo-Y, generating exclusive
486 sequences for these elements that not appear to be fully deleterious, but they are
487 constitutively inactive repeats or pseudogenes. These results also suggest inefficient
488 purifying selection and faster molecular diversification for this genome content. The
489 mechanisms of molecular evolution for 5S rDNA in *R. bergi* is not clear, but based on
490 our evidence we conjecture that mechanisms of evolution concerted and birth and death
491 are actually occurring. In fact, this idea is reflected by a mix of
492 homogenization/diversification among sequences present in both the autosomes and the
493 neo-sex chromosomes.
494
495 ***Post-translational modifications in chromosomes of* R. bergi**
496 In order to better understand of the differentiation of sex chromosomes in
497 comparison to autosomes the patterns of post-translational modifications were also
498 analyzed, mainly in the metaphase I, in which the sex chromosomes are well

499 recognized. The methylation of H3 histone is a modification linked to the
500 transcriptionally inactive euchromatin or heterochromatin, being the H3K4me2 highly
501 conserved euchromatic modification among eukaryotes (see for example Turner 2000;
502 Kouzarides 2007; Fuchs and Schubert 2012; Houben et al. 2013). In *R. bergi* autosomes
503 the pattern for this modification was similar to those reported, for example in plants,
504 occurring at high levels in euchromatic arms and just absent in C-positives
505 heterochromatic regions, that could be related the centromeric activity, which are
506 important for proper meiotic segregation (Oliver et al. 2013; Fuchs and Schubert 2012).
507 Regarding to neo-sex chromosomes, the absence of signals for H3K4me2 in the XL arm
508 may be due to its facultative heterochromatin nature. The XR and neo-Y conserve the
509 marks associated to autosomes even after the involvement in the origin of the neo-sex
510 system.

511 H3K9me2 is commonly associated with heterochromatin and has been observed
512 also in the unsynapsed heterochromatin of sex chromosomes (Fuchs and Schubert 2012;
513 Page et al. 2012). No differences between autosomes and sex-chromosomes were
514 observed for this modification and the strong marks for H3K9me2 suggests that this
515 modification is not heterochromatin-specific, at least in *R. bergi* and another
516 grasshopper *Abracris flavolineata* (Palacios-Gimenez et al., *in preparation*). A possible
517 explanation could be the abundance of microsatellites arrays and TEs interposed in the
518 euchromatin, which could be silenced by heterochromatinization involving
519 heterochromatin-specific methylation marks. Similar observations to strong H3K9me2
520 along whole nucleus have been observed in some plants (Houben et al. 2003).

521 The H4K5ac modification pattern observed in *R. bergi* are apparently constant
522 throughout the genome and independent of the compaction degree of chromatin, a
523 different scenario to those described in plants like *Aegilops* and *Secale cereale* (Oliver

et al. 2013). High acetylation levels similar to those observed in *R. bergi* have been reported by several authors, which are connected with decondensation of the nucleosomal structure facilitating the binding of proteins associated with transcription and DNA replication, recombination, and repair (Lee et al. 1993; Grunstein 1997; Ikura et al. 2000; McBlane and Boyes 2000; McMurry and Krangel 2000; Bird et al. 2002). However, some differences were seen in the neo-sex chromosomes, mainly in the XL arm which remains hipoacetylated throughout meiosis. These results contrast with the pattern observed for H3K4me2 and could be also related with the facultative nature of this part of neo-X chromosome. Additionally as for H3K4me2 the XR and neo-Y conserve the marks associated when they were autosomes.

The H3S10ph is a post-translational modification highly conserved among eukaryotes and primarily associated with chromatin condensation during mitosis and meiosis (Cobb et al. 1999a, b; Manzanero et al. 2000, 2002; Fuchs and Schubert 2012 Houben et al. 2013). The onset for H3S10ph in early diplotene are in agreement with previously reported in *E. plorans* (Manzanero et al. 2000), *Mus musculus* (Cobb et al. 1999a, b) and some other insects (Sotero-Caio et al. 2011). Considering the sex chromosomes the patterns observed for the XL (that correspond to the ancestral X) with strong signals is similar to described by Manzanero and colleagues (2000) in *E. plorans*, but differ to observed for two other grasshopper species (Sotero-Caio et al. 2011), indicating that the H3S10ph could be highly dynamic for sex chromosomes in grasshoppers.

Conclusions

Our data supports the argument that the neo-Y in *R. bergi* is an element highly differentiated and degenerates (including for repetitive genes), reflecting the potential

role of chromosomal rearrangements (Rb-fusions and inversions) as first step to create new linkage groups and sex chromosomes in grasshoppers. As consequence, as also observed in other groups, these recombination-free regions accumulate repetitive DNAs and heterochromatic regions are established, followed by structural, functional and morphological differentiation of neo-sex chromosomes. Although the neo-X and neo-Y chromosomes are well differentiated in *R. bergi* the regions that correspond to the autosome involved in the Rb-fusion retain autosomal post-translational histone modifications, suggesting that (i) the molecular content do not influence the post-translational modification patterns for histones; (ii) that these regions could retain some important autosomal genes that follow similar modifications to actual autosomes; and (iii) that the establishment of functional modifications in neo-sex chromosomes are slower than the molecular diversification.

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783

784 **Figure captions**

785 **Figure 1.** Classical cytogenetic (a,b) analysis and repetitive DNA mapping (c-h) at
786 mitotic cells of *R. bergi*. Each technique and probe type used are indicated directly on
787 the images using colors. The neo-XY sex-chromosomes and some autosomes that
788 carries positive signals are indicated. The insert in (a) shows the sex bivalent neo-XY at

789 the meiosis (metaphase I); the chromosome arms of the neo-sex chromosomes involved
790 in Rb-fusions are indicated. XL: arm derived from the original X-chromosome fused to
791 an autosome; XR: autosomal arm of the neo-X that shares homology with the neo-Y
792 chromosome. Note in (b) the neo-Y entirely heterochomatic and in (e) the multiples
793 sites for 5S rRNA and H3 histone gene.
794
795 **Figure 2.** FISH for microsatellite probes in male mitotic cells of *R. bergi*. Each probe
796 used is indicated directly on the images. Observe the specific signals for microsatellites
797 arrays in (a), (c) and (e), and specific and dispersed signals in (b), (d), (f-h). The sex
798 chromosomes are indicated.
799
800 **Figure 3.** Chromosome painting of the neo-Y (μY-DNA) and neo-X (μX-DNA)
801 chromosomes obtained from microdissection in *R. bergi*. Each probe used is indicated
802 directly on the images. The cells in (a) correspond to metaphase I and (b) metaphase II,
803 while (c) and (d) are the neo-XY sex bivalent selected from meiotic metaphase I.
804
805 **Figure 4.** Chromatin post-translational modification on the neo-XY bivalent of *R. bergi*
806 selected from metaphase I. Each type of histone modification is indicated directly in the
807 images.
808
809 **Figure 5.** FISH signals and histone modification in the *R. bergi* neo-XY sex
810 chromosomes analyzed in this paper (upper panel). The probes, histone modification
811 and its relative position on the neo-XY bivalent are indicated using lines and colors. The
812 down panel shows selected sex chromosomes evidencing the position of some marks
813 obtained through FISH.

814

815 **Figure 6.** Relationship among sequences obtained from genomic DNA female
816 (♀gDNA) and mocrodissected neo-X (μX-DNA) and neo-Y (μY-DNA) chromosomes
817 inferred by neighbor-joining method. (a) Relationship derived of U1 snDNA sequences
818 between the ♀gDNA and the μY-DNA, (b) relationship among the ♀gDNA and μY-
819 DNA for U2 snDNA sequences and (c) relationship between the ♀gDNA, μY-DNA and
820 μX-DNA for the 5S rDNA sequences. The haplotypes of the ♀gDNA, μY-DNA and
821 μX-DNA are shows on the image in blue, red and green, respectively. The trees are
822 drawn to scale, with branch lengths expressed in number of substitutions.

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824

825 **Table 1.** Polymorphism, nucleotide diversity and distribution of substitutions in the U1
826 snDNA sequence from female genomic DNA (♀gDNA) and Y-chromosome DNA
827 obtained by microdissecction (μY-DNA). * indicates overall sum and # average values.

	♀gDNA	**μY-DNA**	**All**
Number of sequences	17	16	33*
Number of haplotypes	3	2	5*
Number of sites	102	102	204*
Number of variable sites	2	1	3*
Number of mutations	2	1	3*
G+C	0.570	0.579	0.5745#
Nucleotide diversity (Pi)	0.00332	0.00123	0.002275#
Average numbers of nucleotide differences (K)	0.33824	0.125	0.23162#
Haplotypes diversity (Hd)	0.324	0.125	0.22455#

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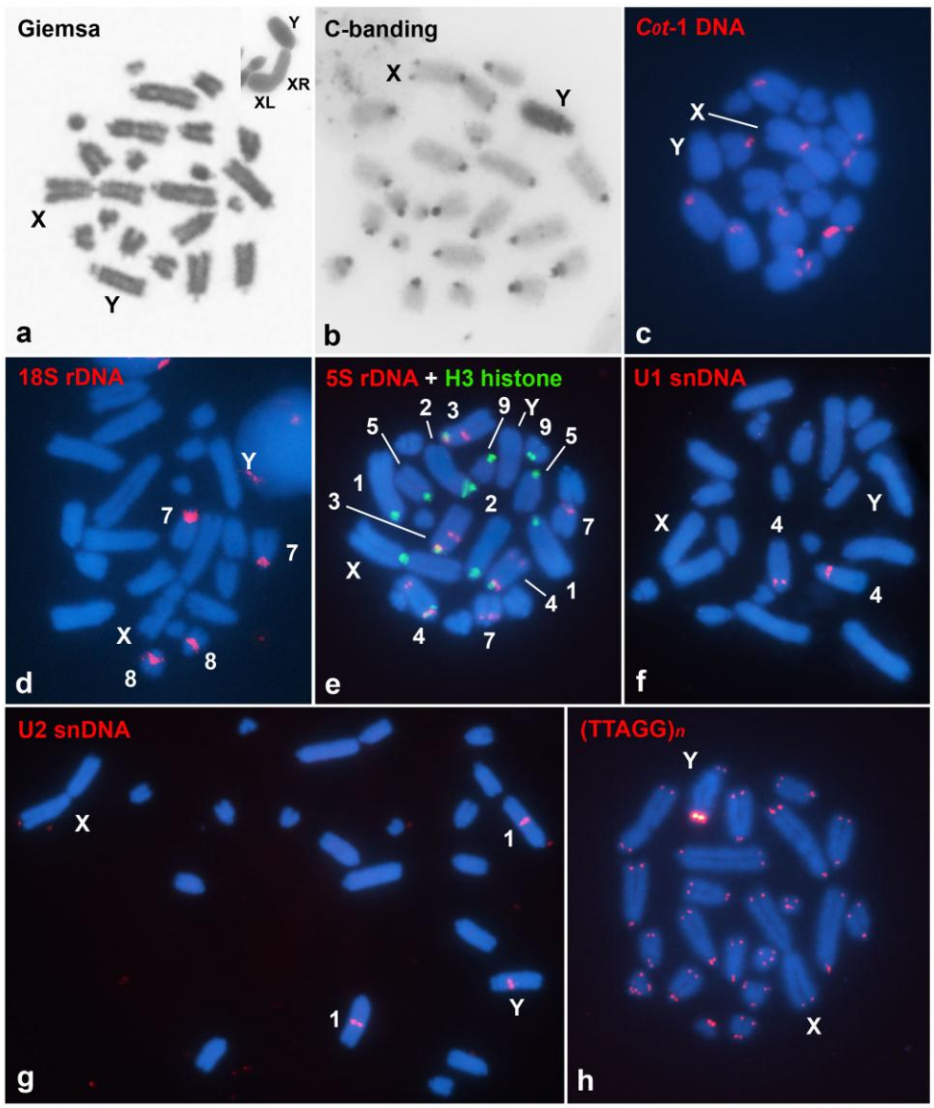
Table 2. Polymorphism, nucleotide diversity and distribution of substitutions in the U2 snDNA sequence from female genomic DNA (♀gDNA) and Y-chromosome DNA obtained by microdissecction (μY-DNA). * indicates overall sum and # average values.

	♀gDNA	**μY-DNA**	**All**
Number of sequences	18	16	34*
Number of haplotypes	2	4	6*
Number of sites	141	141	282*
Number of variable sites	2	3	5*
Number of mutations	2	3	5*
G+C	0.483	0.483	0.483#
Nucleotide diversity (Pi)	0.00158	0.00485	0.00321 5#
Average numbers of nucleotide differences (K)	0.222	0.683	0.4525#
Haplotypes diversity (Hd)	0.111	0.442	0.2765#

866 **Table 3.** Polymorphism, nucleotide diversity and distribution of substitutions in the 5S
867 rDNA sequence from female genomic DNA (♀gDNA) and Y-chromosome DNA (μY-
868 DNA) and X-chromosome (μX-DNA) obtained by microdissecction. * indicates overall
869 sum and # average values.

	♀gDNA	**μY-DNA**	**μX-DNA**	**All**
Number of sequences	14	19	19	52*
Number of haplotypes	4	7	7	18*
Number of sites	53	53	53	159*
Number of variable sites	3	19	17	39*
Number of mutations	3	20	18	41*
G+C	0.566	0.534	0.608	0.569#
Nucleotide diversity (Pi)	0.01037	0.17418	0.09666	0.09373 #
Average numbers of nucleotide differences (K)	0.54945	8.88304	5.12281	4.8517#
Haplotypes diversity (Hd)	0.396	0.749	0.608	0.584#

870


Giemsa
Y
XR
XL
X
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C-banding
X
Y
b
Cot-1 DNA
X
Y
c
18S rDNA
Y
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7
X
8
8
d
5S rDNA + H3 histone
2 3
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Y
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1
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2
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X
4
1
4
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e
U1 snDNA
X
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U2 snDNA
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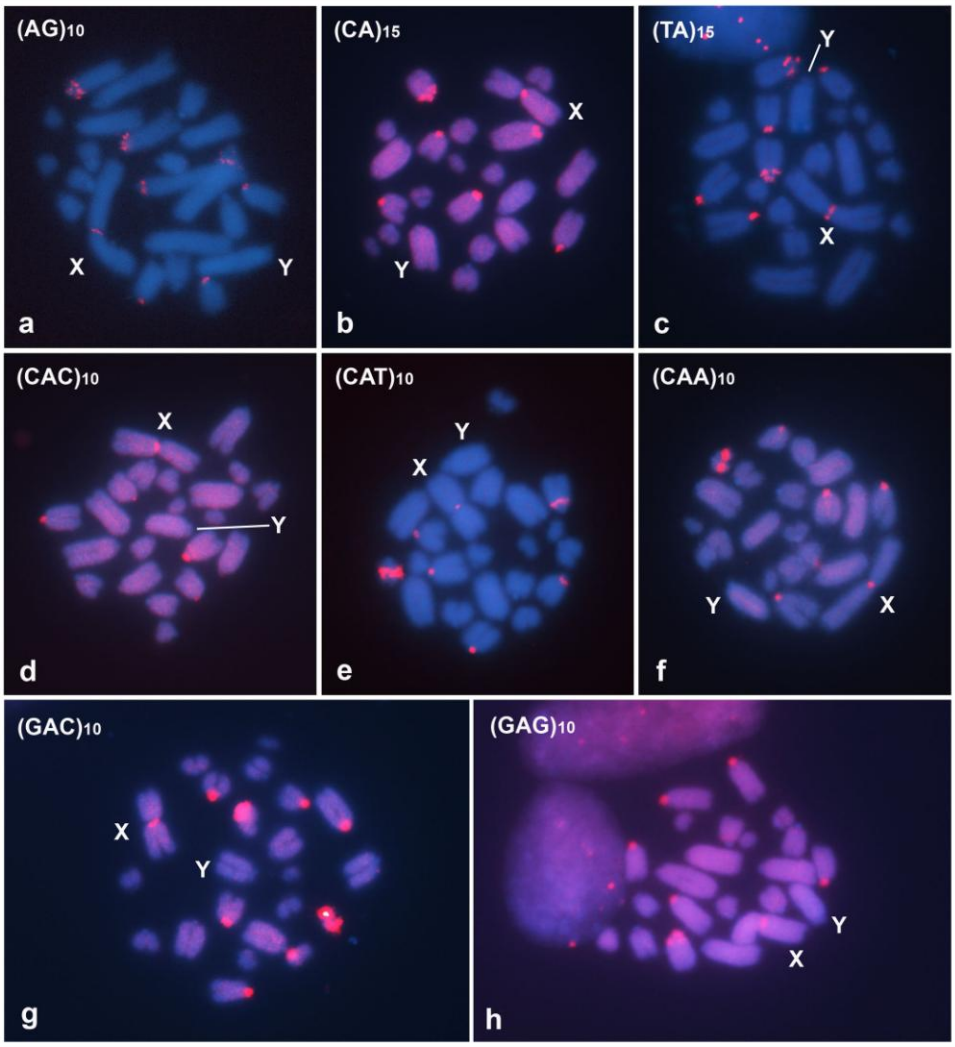
Figure 1


(AG)10
X
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a
(CA)15
X
Y
b
(TA)15
Y
X
c
(CAC)10
X
Y
d
(CAT)10
Y
X
e
(CAA)10
Y
X
f
(GAC)10
X
Y
g
(GAG)10
Y
X
h

Figure 2

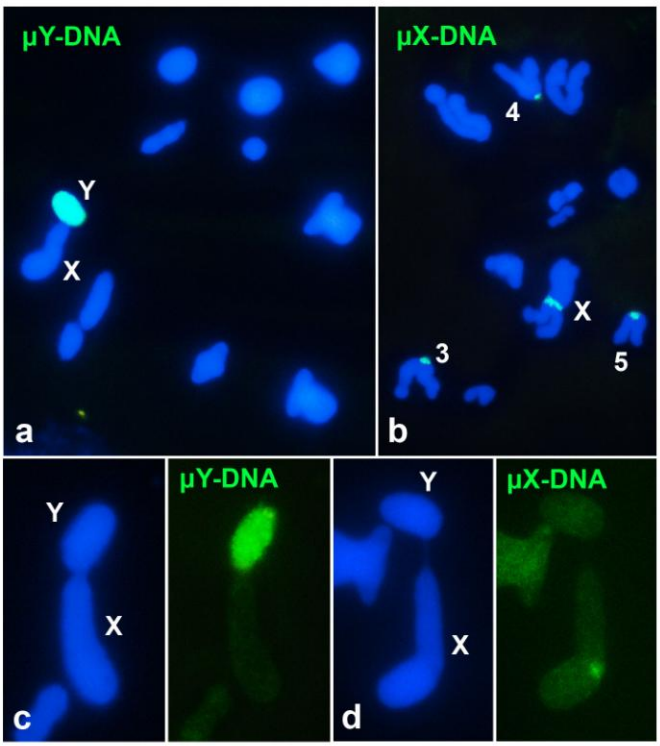
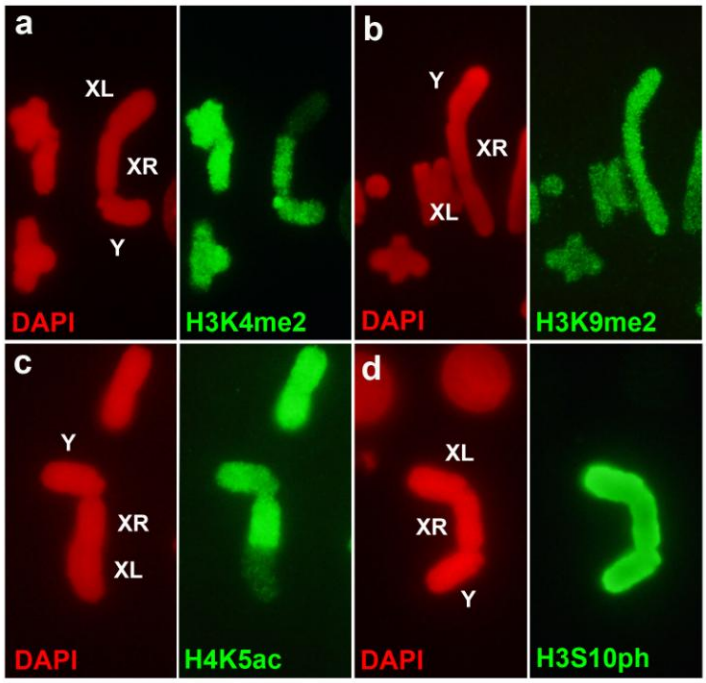


Figure 3


a
XL
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b
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XR
XL
DAPI
H3K9me2
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H4K5ac
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H3S10ph

Figure 4

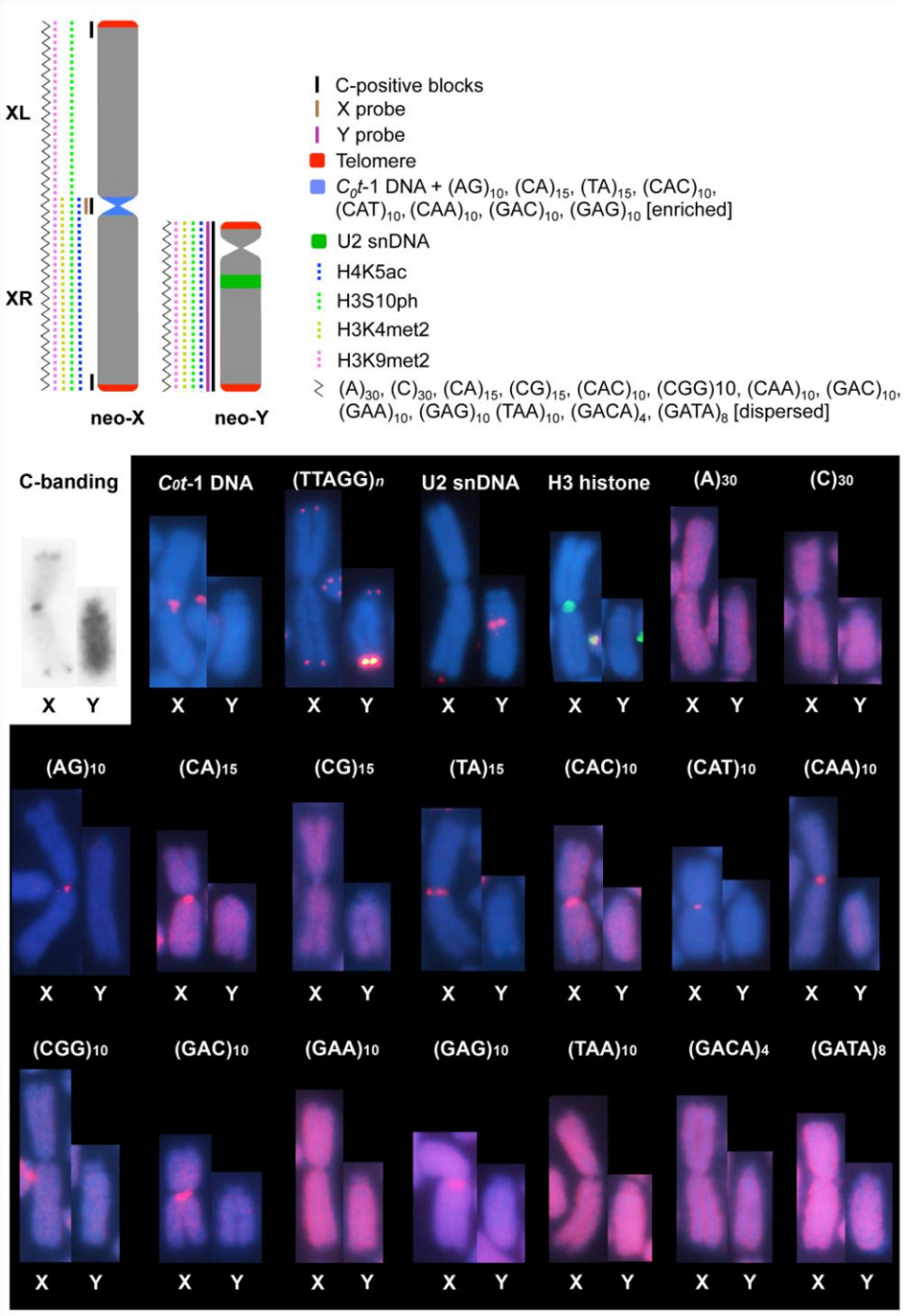
XL
XR
neo-X
neo-Y
C-positive blocks
X probe
Y probe
Telomere
C_0t-1 DNA + $(AG)_{10}$, $(CA)_{15}$, $(TA)_{15}$, $(CAC)_{10}$, $(CAT)_{10}$, $(CAA)_{10}$, $(GAC)_{10}$, $(GAG)_{10}$ [enriched]
U2 snDNA
H4K5ac
H3S10ph
H3K4met2
H3K9met2
$(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(CG)_{15}$, $(CAC)_{10}$, (CGG)10, $(CAA)_{10}$, $(GAC)_{10}$, $(GAA)_{10}$, $(GAG)_{10}$ $(TAA)_{10}$, $(GACA)_4$, $(GATA)_8$ [dispersed]
C-banding
Cot-1 DNA
(TTAGG)n
U2 snDNA
H3 histone
(A)30
(C)30
(AG)10
(CA)15
(CG)15
(TA)15
(CAC)10
(CAT)10
(CAA)10
(CGG)10
(GAC)10
(GAA)10
(GAG)10
(TAA)10
(GACA)4
(GATA)8
X Y

Figure 5

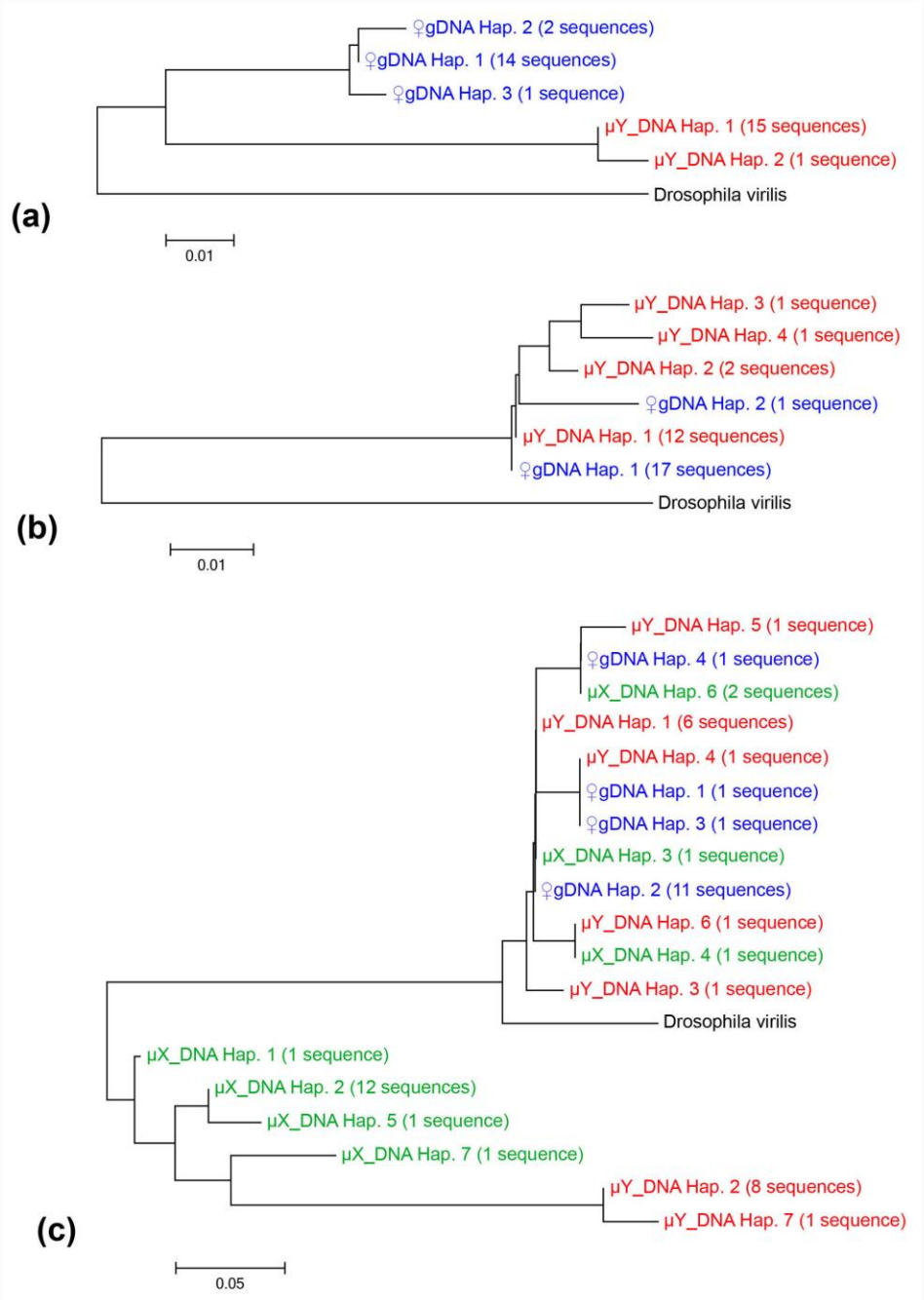
♀gDNA Hap. 2 (2 sequences)
♀gDNA Hap. 1 (14 sequences)
♀gDNA Hap. 3 (1 sequence)
μY_DNA Hap. 1 (15 sequences)
μY_DNA Hap. 2 (1 sequence)
Drosophila virilis
(a)
0.01
μY_DNA Hap. 3 (1 sequence)
μY_DNA Hap. 4 (1 sequence)
μY_DNA Hap. 2 (2 sequences)
♀gDNA Hap. 2 (1 sequence)
μY_DNA Hap. 1 (12 sequences)
♀gDNA Hap. 1 (17 sequences)
Drosophila virilis
(b)
0.01
μY_DNA Hap. 5 (1 sequence)
♀gDNA Hap. 4 (1 sequence)
μX_DNA Hap. 6 (2 sequences)
μY_DNA Hap. 1 (6 sequences)
μY_DNA Hap. 4 (1 sequence)
♀gDNA Hap. 1 (1 sequence)
♀gDNA Hap. 3 (1 sequence)
μX_DNA Hap. 3 (1 sequence)
♀gDNA Hap. 2 (11 sequences)
μY_DNA Hap. 6 (1 sequence)
μX_DNA Hap. 4 (1 sequence)
μY_DNA Hap. 3 (1 sequence)
Drosophila virilis
μX_DNA Hap. 1 (1 sequence)
μX_DNA Hap. 2 (12 sequences)
μX_DNA Hap. 5 (1 sequence)
μX_DNA Hap. 7 (1 sequence)
μY_DNA Hap. 2 (8 sequences)
μY_DNA Hap. 7 (1 sequence)
(c)
0.05

Figure 6

5. CONCLUSÕES FINAIS

1. A organização diferencial de DNAs repetitivos nos sistemas cromossômicos de determinação sexual dos gêneros relacionados *Chlorus*, *Eurotettix* e *Dichromatos* indicam uma origem independente dos mencionados sistemas cromossômicos sexuais intergenericamente. Por outro lado, a localização de genes de DNAr nos cromossomos sexuais das espécies de *Dichromatos* refletem possivelmente uma origem comun para estes cromossomos sexuais seguido de distintas modificações que levaram a sua diferenciação;

2. A presença de genes, tais como para RNAr 18S, 5S e RNAsn U1 e U2 mapeados nos cromossomos neo-Y de distintas espécies estudadas poderiam prevenir a completa degeneração e perda destes cromossomos (reversão X0);

3. O cromossomo neo-Y de *R. bergi* é um elemento diferenciado e degenerado (incluindo genes de DNA repetitivos), refletindo o papel potencial das fusões-Rb e inversões para gerar novos grupos de ligação e novos sistemas cromossômicos de determinação sexual em gafanhotos.

4. Embora o neo-X e neo-Y de *R. bergi* sejam bem diferenciados, as regiões correspondentes ao autossomo envolvido na fusão-Rb (braço XR) e ao neo-Y conservam as modificações post-translacionais de histonas similares aos autossomos. Estes dados indicam que o conteúdo molecular pode não influenciar nestas modificações e que o estabelecimento de regiões funcionais diferenciadas é mais lento em relação a divergência molecular no neo-XY.

5. A ampla diversidade observada para os distintos sistemas sexuais nas espécies estudadas indicam que assim como em outros grupos de organismos os padrões de evolução de sistemas sexuais em Orthoptera é complexo e ocorreu distintas vezes ao longo de sua história evolutiva.

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