ELIANE MARIZA DORTAS MAFFEI

CARACTERIZAÇÃO CITOGENÉTICA DE ESPÉCIES DE COCCINELLIDAE (COLEOPTERA) OCORRENTES EM VIÇOSA-MINAS GERAIS.

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de "*Doctor Scientiae*".

VIÇOSA
MINAS GERAIS - BRASIL
2001

Ao meu esposo Norberto, filhos Lígia e Luciano e à minha mãe Surya.

Dedico.

Aos meus irmãos, sogra, sobrinhos, tios, primos, e enteados, especialmente

Rodrigo.

Ofereço.

AGRADECIMENTOS

À Universidade Federal de Viçosa, pela oportunidade de realização deste Programa.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), pelo apoio financeiro.

A CAPES pela concessão da bolsa.

A professora Silvia das Graças Pompolo pela orientação, apoio e ensinamentos.

Aos meus conselheiros Professores Lucio Antonio de Oliveira Campos e Jorge Abdala Dergam dos Santos, pelos bons momentos de convivência, pela amizade, pelas valiosas sugestões, contínuos conhecimentos transmitidos e indispensável participação durante o curso.

A Maria A. Marin-Morales, Paulo Ruas, Claudete Ruas e Marcelo Guerra pelos conhecimentos transmitidos continuamente, apoio e amizade.

Aos amigos do laboratório de Citogenética de Insetos especialmente Cléa Mariano, Paula Soares, Ana Paula, Juvenal e do laboratório de Citogenética Vegetal especialmente Eveline, Lyderson, Cristina, Jane, Bárbara e Juliana pela amizade, incentivo, apoio e pelo alegres momentos que passamos juntos.

As amigas Maria Rita Campos, Andréa Raposo e Elaine Gasparino pela amizade, apoio, convívio e bons momentos que passamos juntas.

A todos os professores que contribuíram de alguma forma para o meu crescimento profissional e humano.

Aos funcionários José Francisco e José Estevão pela amizade, colaboração e convívio.

CONTEÚDO

RESUMO

MAFFEI, Eliane Mariza Dortas, Universidade Federal de Viçosa, março de 2001. **Caracterização citogenética de espécies de Coccinellidae (Coleoptera) ocorrentes em Viçosa-Minas Gerais.** Orientadora: Silvia das Graças Pompolo. Conselheiros: Lucio Antonio de Oliveira Campos e Jorge Abdala Dergam dos Santos.

A família Coccinellidae (Coleoptera) apresenta, aproximadamente, 4.000 espécies. Muitas delas têm sido utilizadas com eficiência tanto em programas de manejo de pragas quanto em estudos relacionados com impacto ambiental, causado por plantas transgênicas. As análises citogenéticas, na família Coccinellidae, foram realizadas somente em, aproximadamente, 4,3% das espécies, sendo que a maioria restringiu-se à descrição do número de cromossomos. Neste trabalho, foram realizadas análises citogenéticas em *Eriopis connexa*, *Olla v-nigrum* e *Cycloneda sanguinea*, visando: 1. adaptar a técnica para obtenção de cromossomos em células mitóticas e meióticas; 2. determinar o número e morfologia dos cromossomos metafásicos por coloração convencional; 3. avaliar o comportamento dos cromossomos durante a meiose, descrevendo o sistema de determinação sexual; 4. caracterizar a distribuição da heterocromatina constitutiva utilizando banda-C, banda HSS (Hot Saline Solution), enzimas de restrição (*Msp*-I e *Hae*-III) e fluorocromo (CMA_3); 5. aplicar metodologia de banda Ag-NOR para localizar a atividade gênica da região organizadora de nucléolo;

6. mapear, por meio de FISH, os cromossomos carreadores de genes rDNA. O número cromossômico foi 2n=18+XX para fêmeas e 2n=18+Xy para os machos, em todas as espécies. A única espécie que apresentou um cromossomo B eucromático restrito a fêmeas foi *E. connexa*. A meiose dos machos revelou a meio-fórmula n=9+Xy_p, e o modo de associação dos cromossomos sexuais foram do tipo pára-quedas, em todas as espécies. Em *C. sanguinea*, durante a prófase I (paquíteno), a associação dos cromossomos sexuais ocorreu de maneira ponta-a-ponta linearmente até formar um pseudo-anel. Os resultados das metáfases mitóticas avaliadas por banda NOR, tanto em *E. connexa* quanto em *C.sanguinea*, mapearam a região organizadora de nucléolo ativa em um par autossômico. A análise com fluorocromo CMA_3, realizada em *E. connexa*, foi consistente com a banda NOR sendo positiva nesta região. Em células meióticas de *O. v-nigrum*, a região NOR ativa esteve presente na vesícula sexual, sendo confirmada por FISH com genes rDNA. Entretanto, os resultados das análises da mitose e meiose de *C. sanguinea*, por banda NOR e coloração seqüencial FISH/AgNOR, foram controversos. Os genes ativos localizaram-se em um par autossômico (metáfase espermatogonial), porém a vesícula sexual (prófase I) e o lúmen do pára-quedas (metáfase I) ficaram fortemente impregnados por prata. O FISH mapeou os genes rDNA fora da vesícula sexual. Com base nestes resultados sugeriu-se que essas substâncias sejam proteínas nucleolares, sintetizadas pelo par autossômico e importadas durante a associação dos cromossomos sexuais (prófase I). Tanto em *C. sanguinea* quanto em *E. connexa*, a heterocromatina constitutiva avaliada por banda-C, localizou-se principalmente na região pericentromérica de todos os cromossomos. Esta região foi rica em pares de base GC (digerida pela *Hae* III). Entretanto, alguns blocos de heterocromatina constitutiva (telômeros) não foram digeridos. A banda HSS (Hot Saline Solution) extraiu toda a heterocromatina, sugerindo-se que a heterocromatina seja rica, também, em seqüências repetitivas de bases AT, conferindo, portanto, uma natureza molecular heterogênea. A utilização da enzima *Msp*-I forneceu resultado negativo.

ABSTRACT

MAFFEI, Eliane Mariza Dortas, Universidade Federal de Viçosa, March 2001. **Cytogenetic characterization of some Coccinellidae (Coleoptera) species from Viçosa, Minas Gerais**. Adviser: Silvia das Graças Pompolo. Commitee members: Lucio Antonio de Oliveira Campos and Jorge Abdala Dergam dos Santos.

The family Coccinellidae (Coleoptera) comprises about 4,000 species in which some of them are used in pest management programs and in environmental disturbance studies caused by transgenic plants. Cytogenetic analyses of *Eriopis connexa*, *Olla v-nigrum* and *Cycloneda sanguinea* were made with the following purposes: 1. Modification of methodology with the purpose of obtaining best mitotic and meiotic chromosome preparations; 2. Establishment of number and morphology of mitotic chromosomes obtained by conventional staining; 3. Evaluation of meiotic chromosome behavior and description of sexual determination system; 4. Characterization of constitutive heterochromatin by C-band, HSS-band (Hot Saline Solution), restriction enzymes (*Msp*-I and *Hae* III), and CMA_3 fluorochrome; 5. Determination of genetic activity of NOR-region by Ag-NOR band; 6. Application of FISH methodology with the purpose of locating the chromosomes with rDNA genes. All the species studied showed 2n = 18 + XX for females and 2n = 18 + Xy for males. *E. connexa* was the only species with an euchromatic B-chromosome in female individuals. The

male meiosis showed n= 9 + Xy_p, with a parachute association of sexual chromosomes in all species studied. In prophase I of *C. sanguinea*, the association of sexual chromosomes were initially end-to-end, finishing in a pseudo-ring formation. The NOR-band technique in *E. connexa* and *C. sanguinea* showed the nucleolar organizing region in a pair of autosomic chromosomes. These chromosomes in *E. connexa* were also stained by CMA_3 fluorochrome. Both NOR and CMA_3 bands stained the same chromosome region. In meiotic cells of *O. v-nigrum*, an active NOR-region was observed in sexual vesicle, which was confirmed by FISH/rDNA probe. However, the results of C-band and sequential staining by FISH/agNOR were controversial. The functional genes were located in an autosomic pair (spermatogonial metaphase); however, the sexual vesicle (prophase I) and the parachute lumen (metaphase I) were staining strongly with Ag. The FISH methodology showed the rDNA genes out of the sexual vesicle. These results suggested that nucleolar proteins made in the autosomic pair were imported during the association of sexual chromosomes (prophase I). Both in *C. sanguinea* and *E. connexa*, constitutive heterochromatin showed by C-band was found mainly in pericentromeric regions of all chromosomes. These regions were GC-rich (digested by *Hae* III), but some heterochromatic blocks (telomere) were not digested by *Hae* III). The HSS band extracted all the heterochromatin, suggesting that this chromosome region was also AT-rich with a heterogeneous molecular nature. A negative result was seen with digestion by *Msp* I restriction enzyme.

1. INTRODUÇÃO GERAL

1.1. Considerações gerais

A Ordem Coleoptera é dividida taxonomicamente em quatro subordens: Adephaga, Polyphaga, Archostemata e Myxophaga (CROWSON, 1981; HENNIG, 1981) e apresenta, aproximadamente, 350.000 espécies descritas (LAWRENCE, 1982). Entretanto, os conhecimentos citogenéticos para este grupo são escassos. Com base na literatura e em seus resultados, SMITH e VIRKKI (1978) fizeram um levantamento citogenético de 2.160 espécies desse grupo de insetos, sendo que a maioria se concentrou na subordem Polyphaga (aproximadamente 2.000) e os demais na subordem Adephaga. Nas outras subordens, as pesquisas foram quase que inexistentes, sendo descrito para *Micromalthus debilis* (Archostemata) 2n=20 (Scott, 1936 citado em SMITTH e VIRKKI, 1978) e para *Ytu zeus* (Myxophaga) o número cromossômico de 2n=18+ Xy (MESA e FONTANETTI 1985).

Segundo PETITPIERRE (1996), aproximadamente 3.000 espécies de besouros haviam sido examinadas citogeneticamente, porém os estudos permaneceram concentrados na subordem Polyphaga.

O número cromossômico 2n=20, com meio-fórmula 9+ Xy_p, tem sido sugerido ser o ancestral (cariótipo básico) em coleópteros, pois foi descrito para a maioria das espécies avaliadas (SMITH e VIRKKI, 1978). Deve-se ressaltar que as análises citogenéticas têm sido, em grande parte, realizadas na meiose de machos, devido à dificuldade de obterem cromossomos mitóticos metafásicos (PETITPIERRE, 1996).

Entretanto, as análises dos cromossomos meióticos podem fornecer informações importantes, como observado nas ordens Coleoptera e Megalopteran, em que várias famílias destas subordens apresentaram a associação dos cromossomos sexuais do tipo pára-quedas (Xy_p), sugerindo uma possível relação filogenética entre elas (HUGHES-SCHRADER, 1980).

O cromossomo y é representado pela letra minúscula, por ser de tamanho muito reduzido em relação ao cromossomo X, sendo, portanto, o "Xy" adotado para designar o mecanismo cromossômico heterogamético de determinação sexual e o "$_p$" representa o tipo de associação dos cromossomos bivalentes sexuais que apresentam a configuração em pára-quedas na metáfase I (SMITH, 1950).

As pesquisas realizadas em coleópteros têm demonstrado que existe uma grande variabilidade no número de cromossomos, desde 2n=4, em *Chalcolepidius zonatus* (FERREIRA et al., 1984), até 2n=69, em *Ditomus capito* (SERRANO, 1981), sendo necessário, portanto, o estudo de maior número de espécies para melhor compreender esta variabilidade (FERREIRA e MESA, 1977; MARTINS, 1994).

Em relação ao número cromossômico, algumas famílias são mais conservadas, como Scarabaeidae, Coccinellidae, Tenebrionidae e Cerambycidae, as quais apresentam número cromossômico típico de Polyphaga, n=10, indicando que a tendência foi manter o número cromossômico próximo do original (SMITH e VIRKKI, 1978; PETITPIERRE, 1987).

1.2. Subordem Adephaga

Na subordem Adephaga, as análises se concentram nas famílias Cicindelidae e Carabidae, e a variabilidade no número cromossômico detectada é grande, com o número de cromossomos variando de 2n=12 *Craspedophorus angulatus*, até 2n=80 em *Graphipterus serrator* (SMITH e VIRKKI, 1978).

Nesta subordem, diferentes sistemas cromossômicos de determinação sexual foram caracterizados como observado na subfamília Cicindelinae, em que o cariótipo de maneira geral apresentou 9 a 12 cromossomos autossômicos e o sistema Xn_y, o n variando entre dois a quatro (SERRANO, 1980; SERRANO et al., 1986; SERRANO e COLARES-PEREIRA, 1989, 1992; GALIÁN et al., 1990).

Recentemente, foram caracterizadas citogeneticamente duas espécies de *Cicindela* da Austrália, em que o número de cromossomos para *Cicindela cardinalba* foi $n=10+X_1X_2X_3y$ e *C. gillesensis*, $n=11+X_1X_2X_3y$ (GALIÁN e HUDSON, 1999).

Outros sistemas de determinação sexual foram descritos, mas em menor freqüência, como o sistema X0 em *Megacephala euphratica* (tribo Megacephalini) e *Cicindela paludosa* (tribo Cicindelini) (SERRANO et al., 1986). Segundo VIRKKI (1984), em Adephaga primitivos, o cariótipo pode ter sido derivado do tipo básico $9II+Xy_p$ e no decorrer da evolução ocorreu aumento no número de cromossomos, por meio de fissões cêntricas autossômicas.

1.3. Subordem Polyphaga

A grande maioria das espécies da subordem Polyphaga apresenta o cariótipo 9+Xy_p, sendo a associação dos cromossomos sexuais em pára-quedas, sugerindo ser o ancestral para esse taxon (SMITH e VIRKKI, 1978; VIRKKI et al., 1991). Entretanto, em algumas superfamílias, observou-se grande variação no número de cromossomos, como em Curculionoidea, Cerambycoidea e Scarabaeoidea (TAKENOUCHI, 1974a, b).

FERREIRA et al. (1993) descreveram a meio-fórmula de 16 espécies de Cerambycidae, em que sete pertenciam à subfamília Cerambycinae, oito à Lamiinae e uma a Prioninae. Apenas duas espécies de Cerambycinae e uma de Lamiinae apresentaram o cariótipo considerado primitivo para Polyphaga, 9+Xy_p. O número cromossômico das outras espécies variou de 5+ Xy_p (*Pachypeza teres*) a 17 + Xy_p (*Pirodes nitidus*). Segundo os autores, outros cariótipos intermediários foram evidenciados 10+Xy_p, 11+Xy_p, 12+Xy_p, 14+Xy_p e 15+Xy_p, os quais originaram a partir de fusões e fissões cêntricas.

1.4. Determinação sexual em coleópteros

Com base no modo de pareamento dos cromossomos sexuais, SMITH e VIRKKI (1978) classificaram estes mecanismos em sistemas ortodoxos (aquiasmáticos e quiasmáticos) e não-ortodoxos.

1.4.1. Sistemas ortodoxos aquiasmáticos

Sistemas : Xy_p, Xny_p, nXy_p, Xy_c , XO e X_1X_2.

Em muitos insetos, tem sido descrito que a meiose dos machos comumente apresenta variações adaptativas. Algumas destas variações se

fixaram em alguns organismos, como a meiose aquiasmática (atípica) em um dos sexos, geralmente nos machos (PERONDINI et al., 1981).

1.4.1.1. Pára-quedas simples : Xy_p

A associação aquiasmática característica é a que predomina, portanto sugere-se ser ancestral "Xy_p" em Coleoptera, apresentando maior ocorrência na subordem Polyphaga e esta associação tem sido descrita em todas as famílias de Polyphaga (SMITH e VIRKKI, 1978).

O cromossomo X deste sistema é freqüentemente do tipo metacêntrico grande, e o y normalmente é muito pequeno, sendo evidenciados pela primeira vez por Stevens (1906), que os comparou com "pára-quedas", considerando o cariótipo básico da ordem . Esta associação dos cromossomos sexuais vem sendo exemplificada como o "pára-quedas" formado pelo cromossomo X e o "pára-quedista" pelo cromossomo y, pela localização nestes cromossomos da região organizadora de nucléolo (FERREIRA e MESA, 1977; SMITH e VIRKKI, 1978; SMITH, 1950). SMITH (1950) introduziu o símbolo Xy_p para designar esta associação peculiar.

Existem polêmicas na tentativa de explicar o pareamento entre o Xy_p, que varia entre associações nucleolares e quiasmáticas. Segundo WHITE (1973), não existe evidência clara de quiasma no Xy_p, provavelmente pelo grau de compactação e tamanho reduzido do bivalente sexual em besouros, o que descarta as associações quiasmáticas.

Em *Epilachna paenulata* (Coleoptera, Coccinellidae), as análises de banda-C realizadas por DRETS et al., (1983) revelaram que a formação do bivalente sexual diferia das outras espécies de besouros avaliadas, e que segmentos da heterocromatina constitutiva desempenharam importante papel na formação do

Xy_p. Os autores propuseram um modelo em que os cromossomos sexuais se associavam ponta-a-ponta pelo braço longo no início da meiose. O "pára-quedas" é composto por três segmentos distintos: dois corpúsculos intensamente heterocromatinizados, quando avaliados por banda-C, e o outro segmento eucromático formando o pára-quedista (forma toda em V). Estes resultados foram interpretados como: os segmentos eucromáticos seriam o braço longo do cromossomo X (pára-quedista) e a formação do "pára-quedas", a associação dos cromossomos X e y.

Alguns autores sugeriram que a forma de pára-quedas do bivalente sexual pode ser uma característica primitiva, que, quando o organismo sofre alteração cromossômica estrutural, perde este tipo de associação que não pode ser readquirida (CROWSON, 1981; VIRKKI et al., 1991). Por outro lado, análises citogenéticas realizadas em *Dendroctomus* (Curculionoidea-Scolytidae), sugeriram que a condição Xy_p parece ter sido readquirida por rearranjos estruturais (LANIER, 1981).

Informações sobre a localização das NORs em Coleoptera podem constituir dados importantes para estudar a evolução dos cariótipos (PETITPIERRE, 1996). Embora escassos, os resultados têm evidenciado que um par autossômico organizador de nucléolo pode estar amplamente distribuído na ordem. A atividade de NORs nos autossomos em machos desta ordem de insetos parece ser comum, no entanto, as pesquisas têm demonstrado que estas possuem vida curta, desaparecendo no diplóteno mediano (WEBER, 1971; VIRKKI, 1984; POSTIGLIONI e BRUM-ZORRILLA, 1988).

Localização da atividade gênica nos núcleos meióticos em coleópteros no gênero *Cicindela* (Cicindelinae) com cromossomos sexuais múltiplos revelaram a presença de NORs na cromatina sexual. Os resultados evidenciaram na espécie *C. melancholica*, tanto por impregnação pela prata quanto por hibridização "in situ" (FISH) fluorescente, com sonda de genes de RNA ribossômico, que as NORs se localizaram em um dos três cromossomos sexuais X e no cromossomo $_Y$. A coloração pela prata e FISH mostrou que a NOR na espécie *C. paludosa*, com sistema de determinação sexual XO (macho), estava localizada em um par

autossômico e no cromossomo X. Em *Megacephala euphratica*, estas técnicas indicaram que as NORs estavam localizadas nos três pares de autossomos e não nos cromossomos sexuais. As mudanças na localização das NORs dos cromossomos heterossomos para os autossomos ocorreram entre as espécies deste gênero, mostrando um novo padrão cariotípico (GALIÁN et al., 1994).

Contudo, existe muita controvérsia em relação a associação do Xy_p, por meio do nucléolo (teoria nucleolar), pois tem sido detectado durante a prófase I, em várias espécies, a presença de material argirofílico, o que sugere ser de origem protéica, mas a região organizadora de nucléolo pode não estar localizada nestes cromossomos (VIRKKI et al., 1991).

A teoria de associação nucleolar do sistema de determinação sexual Xy_p foi rejeitada por POSTIGLIONI e BRUM-ZORRILLA (1988) e POSTIGLIONI et al (1991), pois estes verificaram em *Chelymorpha variabilis* que o nucléolo não estava relacionado aos cromossomos sexuais e sim a um autossomo, como evidenciado pela reação positiva ao fluorocromo acridina orange e à coloração com prata. DRETS et al. (1983) obtiveram resultados semelhantes em *Epilachna paenulata*. Segundo DRETS e STOLL (1974), os segmentos de heterocromatina constitutiva desempenham um papel importante nas associações cromossômicas e na formação Xy_p.

1.4.1.2. Sistema XO

O sistema X0 é o segundo de maior ocorrência dentre os mecanismos de determinação sexual de Coleoptera, e podem ser de dois tipos: os de origem pós-reducional e os pré-reducional. Tem sido descrito, por análises citogenéticas, que neste sistema o cromossomo X passa por uma divisão pós-reducional, ou seja, separa as cromátides irmãs na primeira divisão meiótica (SMITH e VIRKKI, 1978).

Entretanto, tem sido relatado em algumas famílias como Curculionidae e Chrysomelidae a ocorrência do cromossomo X com

comportamento pós-reducional. Em Curculionidae, na espécie *Gelus californicus*, foram descritos o cromossomo X univalente e mais um a três cromossomos B, todos com comportamento pós-reducional e tendências a permanecer estreitamente agrupados, sugerindo-se que esses cromossomos B seriam cromossomos y que persistiram após o X ter perdido a capacidade de organizar o nucléolo (ENNIS, 1972).

1.4.2. Sistemas quiasmáticos

Sistemas simples **Neo XY, Xy, Xyr** e múltiplos (X_1X_2Y, XY_1Y_2, nXnY, $X_p$neoXneoY_p).

Sistema simples Neo XY

MARTINS (1994), avaliando cinco espécies do gênero Scarabeidae (Poliphaga, Coleoptera) por meio de análises meióticas, relatou que somente a espécie *Strategus validus* apresentou o cariótipo típico da subordem Polyphaga, 9 + Xy_p. As outras espécies apresentaram variações nos cariótipos, como redução no número de cromossomos e, ou, alterações no mecanismo de determinação sexual, apresentando o sistema "neo-Xy", considerados cariótipos derivados.

Em Coccinellidae, foi descrita a ocorrência deste sistema, como relatado nos gêneros *Hyperaspis* (6 ou 7 autossomos +X+y assinápticos), *Brachyacantha* com sistema neoXY, sugerindo-se que os cromossomos sexuais Xy de *Hyperaspis* tenham derivado do neo-XY, seguido de elevada condensação nos segmentos de pareamentos, com subseqüente falha na formação de quiasmas (SMITH e VIRKKI, 1978).

Em *Dermestes maculatus* (Polyphaga-Dermestidae), descreveu-se que o mecanismo de determinação sexual normalmente é do tipo Xy_p e a segregação ocorre devido à presença de nucléolo localizado no cromossomo X. Entretanto, em uma população originária da Índia, foi encontrada variação onde vários indivíduos apresentaram cromossomo y supernumerário (de um a quatro), e o produto desta meiose irregular levou a formação de gametas inviáveis (erosão do y). Antes ou depois da "erosão" do y ser completada, pode ter ocorrido a incorporação de novo

material autossômico ao sistema sexual por fusão cêntrica e isso leva a redução do número cromossômico e marca o início de um novo ciclo de condensação do elemento neo-Y. Desta forma, sugeriu-se um possível caminho de evolução cíclica dos cromossomos sexuais de Coleoptera, por meio de erosão e redistribuição do material cromossômico. A perda do y resulta no sistema XO, e o único bivalente sexual derivado deste mecanismo é o neo-Xy (Shaw, 1968, citado em BIONE, 1999).

1.4.3. Sistemas não-ortodoxos

Sistemas aquiasmáticos incluem os cromossomos sexuais que perderam a capacidade de serem pareados convencionalmente (via nucléolo ou quiasma). O comportamento destes cromossomos sexuais durante a meiose pode ser como univalentes, separando-se tardiamente na anáfase I (pós-reducional), ou podem se manter distantes entre si e em relação aos autossomos. Estas formas de segregação ocorrem raramente em coleópteros, e foram descritas em poucos gêneros. Na subtribo Oedionychina, a fórmula cariotípica que predomina é 10 II +X+Y, o tamanho dos cromossomos autossomos são pequenos e os sexuais muito grandes, conseqüentemente a meiose é irregular (atípica) (SMITH e VIRKKI, 1978).

1.5. Importância da citogenética em Coccinellidae.

As análises citogenéticas realizadas em Coccinellidae são muito escassas, abrangendo aproximadamente 174 espécies, sendo que em 42%

foi descrito o cariótipo típico ancestral $9+Xy_p$. Outros sistemas foram

relatados, mas com menor ocorrência, como o neoXy, X+y, Xyr, XXy, X0, Xy macho:XXXX fêmea (SMITH e VIRKKI, 1978).

Análises comparativas dos cariótipos foram realizadas em Coccinellidae visando diferenciar todos os cromossomos do complemento. A tribo Chilochorini não é muito indicada para este propósito, mas se torna importante pelo fato de que as homologias no cariótipo propostas podem ser testadas por meio de análises de híbridos F1. SMITH e VIRKKI (1978) trabalharam extensivamente e conseguiram obter resultados muito importantes. Por meio de análises de meiose e mitose, em 35 espécies e subespécies, foram identificados marcadores citológicos, que auxiliaram na identificação de cada par cromossômico.

O número de cromossomos no gênero *Chilocorus* foi caracterizado por apresentar 2n=22, usualmente representados na metáfase I por dois bivalentes em anéis biaquiasmáticos, 8 bivalentes em "roda" com somente um quiasma e o par sexual "neo Xy". Este tipo de cariótipo ocorreu em todas as espécies avaliadas da América do Norte, sendo descrito também na Europa e Índia, e foi considerado, portanto o cariótipo básico do gênero (ENNIS, 1974).

Chilocorus stigma da América do Norte é o único Coccinellidae que apresenta o sistema de determinação $X_1X_1X_2X_2/X_1X_2Y$ e extremo polimorfismo cromossômico. Os machos apresentam número diplóide que variaram de 25 a 19, indicando uma variabilidade consideravelmente grande, por adição de cromossomos B. Foi estabelecida, com a exclusão dos Bs, a média do número de cromossomos por região geográfica, e verificou-se que esta diminuía de 25.0 na Flórida a 20.2 em Alberta (carioclina), sugerindo que a redução no número cromossômico ocorreu por fusões Robertsonianas. Sugeriram-se dois tipos principais de mudanças que contribuíram para a evolução do cariótipo de *C. stigma*: Dissociação (e, ou, fissão cêntrica) seguida por acréscimo de heterocromatina nos produtos resultantes acrocêntricos originando os metacêntricos. O processo de dissociação gerou perda de cromatina,

ocorrendo fusão e compactação dos derivados eucromáticos, o que originou os cromossomos B (SMITH e VIRKKI, 1978).

Segundo WHITE (1973) , SMITH e VIRKKI (1978), em seis espécies de *Chilochorus* endêmicas da América do Norte, também foram realizados cruzamentos testes, e, por meio destes, puderam ser separadas as espécies em dois grupos distintos. Um grupo compreendendo *C. cacti* e *C. fraternus*, com o número cromossômico 2n=22 (Xy_p) e o outro formado por *C. orbus* (2n=22), *C. tricyclus* (2n=20), *C. hexacyclus* (2n=14) e *C. stigma*, todos "neo Xy". Deve-se ressaltar que, dos cruzamentos realizados com *C. stigma* X *C. hexacyclus*, os híbridos que apresentaram neoX e X2 não exibiam pareamento entre esses cromossomos, o que se pode concluir sem dúvida que o sistema X_1X_2y foi derivado do básico neoXy por fusão cêntrica.

1.6. Objetivos

Os objetivos deste trabalho foram caracterizar as espécies *Eriopis connexa***,** *Olla v-nigrum***,** *Cycloneda sanguinea* por meio de análises citogenéticas, as quais foram desenvolvidas visando:

1. Adaptar uma metodologia para análise de cromossomos em células mitóticas.
2. Determinar o número e morfologia cromossômica por coloração convencional**.**
3. Verificar o comportamento dos cromossomos durante a meiose, descrevendo o sistema de determinação sexual.
4. Caracterizar a distribuição da heterocromatina utilizando técnicas de bandamento-C, banda HSS, enzima de restrição e fluorocromo.
5. Aplicar a metodologia de banda-Ag-NOR para avaliar a atividade gênica desta região.

6. Adaptar a metodologia de FISH e mapear os genes rDNA.

REFERÊNCIAS BIBLIOGRÁFICAS

BIONE, E.G. (1999). Citogenética de Coleópteros das Subfamílias Dynastinae e Rutelinae (Polyphaga, Sacarabaeoidea). Dissertação de Mestrado Universidade Federal de Pernambuco.

CROWSON, R.A. (1981). The biology of Coleoptera. London (Academic Press) 802 pp.

DRETS, M.E., STOLL, M. (1974). C-banding and non-homolous association in *Gryllus argentinus*. Chromosoma, 48-367-390.

DRETS, M.E., CORBELLA, E., PANZERA, F., FOLLE, G.A. (1983). C-banding and non-homologous associations. II The "parachute" Xy_p sex bivalente and behavior of heterochromatic segments in *Epilachna paenulata*. Chromosoma, 88:249-255.

ENNIS, T.J. (1972). Low chromosome number and post-reducional XO in *Gelus californicus* (Lec) (Coleoptera: Curculionidae). Can. J. Genet. Cytol., 14:851-857.

FERREIRA, A., MESA, A. (1977). Estudos citológicos em três espécies brasileiras de Coleópteros (Chrysomelidae, Cerambycidae e Meloidae). Rev. Bras. Biol., 37:61-64.

FERREIRA, A., CELLA, D., TARDIVO, J.R., VIRKKI, N. (1984). Two pairs of chromosomes: A new low record for Coleoptera. Rev. Brasil. Genet. 7:231-39.

FERREIRA, A. ; CONDUTA, V. and MARTINS, V.G. (1993). Cytogenetic survey of some brazilian Cerambycidae (Coleoptera, Polyphaga, Chrysomelidae). Rev. Brasil. Genet., 16:51-57.

GALIÁN, J., ORTIZ, A.S., SERRANO, J. (1990). Karyotypes of nine species of nine species of Cicindelini and cytotaxonomic notes on Cicindelinae. Genetica, 82:17-24.

GALIÁN, J., SERRANO, J., de La RUÁ, P., PETITPIERRE, JUAN, C. (1994). Localization and activity of rDNA genes in tiger beetles (Coleoptera: Cincidelinae). Heredity, 74:524-530.

GALIÁN, J., HUDSON, P. (1999). Cytogenetic analysis of Australian tiger beetles (Coleoptera: Cicindelidae) chromosome number sex-determinig system and localization of rDNA genes. Journal of Zoological Systematics and Evolutionary Research, 37(1)1-6.

HENNIG, W., 1981. *Insect Phylogeny.* John Wiley & Sons, Chinchester.

HUGHES-SCHRADER, S. (1980). Segregational mechanism of sex chromosomes in Megaloptera (Neuropteroidea). Chromosoma, 81:307-314.

LAWRENCE, C. (1982). Coleoptera, pp. 482-553. In: J. A. Parker (ed.) Synopsis and Classification of Living Organisms, v. 2. McGraw-Hill, New York.

LANIER, G.N. (1981). Cytotaxonomy of *Dendroctomus*. In:Application of Genetics and Cytology in Insects Systematics and Evolution (Stock M.W. ed.) Proc. Symp. Entomol. Soc. Am. p 33-65.

MARTINS, V.G. (1994). The chromosomes of five species of Scarabaeidae (Polyphaga, Coleoptera). Naturalia, 19:89-96.

MESA, A., FONTANETTI, C.S. (1984). Multiple sex chromosomes, autosomal polymorphism and a higth number of chromosomes in *Euchroma gigantea* L 1735 (Coleoptera, Buprestidae). Rev. Bras. Genet. , 4:629-637.

MESA, A., FONTANETTI, C.S. (1985). The chromosomes of a primitive species of beetle: *Ytu zeus* (Coleoptera, Myxophaga, Torridincilidae). Proc. Acad. Nat. Sci. Philadelphia, 137:102-105.

PERONDINI, A.L.P., BASILE, R., MORI, L. (1981). Meioses atípicas nos insetos. Ciência e Cultura, 33(7).

PETITPIERRE, E. (1996). Molecular cytogenetics and taxonomy of insects, with particular reference to the Coleoptera. Int. J. Insect Morphol. and Embryol., 25:115-133.

PETITPIERRE, E. (1987). Why beetles have strikingly different rates of chromosomal evolution ? Elytron 1:25:-32.

POSTIGLIONI, A., BRUM-ZORRILLA, N. (1988). Non-relationship between nucleolus and sex chromosomes system Xy_p in *Chelymorpha variabilis* Boheman (Coleoptera: Chrysomelidae). Genetica, 77:134-141.

POSTIGLIONI, A., STOLL, M., BRUM-ZORRILLA, N. (1991). Haploid karyotype analysis of *Chelymorpha variabilis* Boheman (Coleoptera, Chrysomelidae) with microspreading techniques. Rev. Brasil. Genet. 14(3):653-660.

SERRANO, J. (1981). Chromosome numbers and karyotype evolution of Caraboidea. Genetica 55:51-60.

SERRANO, J. (1980). Diferencias cariotípicas entre *Cicindela maroccana pseudomaroccana* Y. C. campestris (Coleoptera Cicindelidae) Bol. Asoc. Esp. Entom., 4:65-68.

SERRANO, J., GALIÁN, J., ORTIZ, A. (1986). Cicndelid beetles without multiple sex chromosomes (Coleoptera, Carabidea). Can.J.Genet. Cytol., 28:235-239.

SERRANO, A.R.M., COLARES-PEREIRA, M.J. (1989). Cytotaxonomic study of *Cephalota hispanica* (Gory, 1933) and *Spiralia maura* (Linnaeus, 1758) two cicindelids from Portugal (Coleptera). Genetica, 79:69-75.

SERRANO, A.R.M., COLARES-PEREIRA, M.J. (1992). Further analysis of the cytotaxonomy of tiger beetles (Coleoptera: Cicindelidae) from South Portugal. Nucleous, 35:19-24.

SMITH, S.G. (1950). The cytotaxonomy of Coleoptera. Can. Entomol., 82:58-68.

SMITH, S.G., VIRKKI, N. (1978). Animal cytogenetics. Coleoptera. Berlin:Gebruder Borntraeger, 1-365.

VIRKKI, N. (1984). Chromosomes in evolution of Coleoptera In: Chromosomes in evolution of eukaryotic grroups. (Sharma, AK & Sharma, A eds) CRS Press, Florida p. 260.

VIRKKI, N., MAZZELLA, C., DENTON, A. (1991). Silver staining of the colepteran Xy_p sex bivalent. Cytobios, 67:45-63.

TAKENOUCHI, Y. (1974a). A study of the chromosomes of thirty-four species of japanese weevils (Coleoptera: Curculionidae). Genética, 45:91-110.

TAKENOUCHI, Y. (1974b). A study of the chromosomes of twenty-two species of japanese weevils (Coleoptera: Curculionidae). Jap. J. Genet., 49:147-157.

WEBER, F. (1971). Koerreliert formersanderungen von nukleolus und nukleoluzassozii ertertem heterochromatin bei der Gattung Carabus (Coleoptera). Chromosoma, 34:261-273.

WHITE, M.J.D. (1973). Animal cytology and evolution. Third edition. Cambridge University. London, pp.961.

KARYOTYPIC CHARACTERIZATION BY MITOSIS, MEIOSIS AND C-BANDING OF *ERIOPIS CONNEXA* MULSANT (COCCINELLIDAE: COLEOPTERA: POLYPHAGA), A PREDATOR OF INSECT PESTS

Artigo publicado na revista Hereditas 132:79-85 (2000).

ABSTRACT

Eriopis connexa presents a chromosome number of 2n=18+XX for most females analyzed and a meioformula of n=9+Xy_p for all males. A small metacentric B chromosome restricted to females occurred in 10% of our sample and, when submitted to C-banding, it was shown to be almost completely euchromatic. Chromosome pairs 2 and 3 had satellites and probably contained the nucleolar organizer regions (NORs). C-band analysis also revealed that the constitutive heterochromatin was localized in the centromeres of all chromosomes in the complement.

INTRODUCTION

Since the end of the last century, many Coccinellids have been found to be efficient predators of aphids, cochineals and lepidopteran eggs (De BACH, 1964; GORDON, 1985). They have been used efficiently in Pest Management Programs and there is great interest in more detailed studies (SMITH and REYNOLDS, 1966; De BACH, 1964; LEVINS and WILSON, 1980). While there are large number of ladybug species native to Brazil, few studies about this group are available. The Order Coleoptera comprises approximately 350,000 described species (LAWRENCE, 1982), but cytologic information about the families are highly fragmented, and covers some 3000 species (SMITH and VIRKKI, 1978; SHARMA et al., 1980; SERRANO and YADAV, 1984; PETITPIERRE et al., 1988; GILL et al., 1990; GALIÁN and MOORE, 1994). The chromosome numbers vary widely ranging from 2n=4 in *Chalcolepidius zonatus* (FERREIRA et al., 1984) to 2n=69 in *Ditomus capito* (SERRANO, 1981). There is certainly a need to study a larger number of species for a better understanding of this variability (FERREIRA and MESA, 1977; MARTINS, 1994). The basic karyotype of Coleoptera, probably the ancestral one, has been reported to consist of nine pairs of autosomes and the X and y_p sex chromosomes, which associate in a "parachute" configuration during metaphase I, with chromosome X being relatively much larger than chromosome y, which is a very small chromosome (SMITH, 1950).

Cytogenetic analyses of Coleoptera have been mostly performed during male meiosis because of the difficulty in obtaining mitotic metaphase chromosomes (PETITPIERRE, 1996). In the family Coccinellidae, few analyses have been made, but available data have shown that the basic karyotype $n=9+Xy_p$ described for Coleoptera is the most frequently (SMITH and VIRKKI, 1978). No cytogenetic analyses have been performed thus far on any species of the genus *Eriopis*.

We describe here the number and morphology of mitotic metaphase chromosomes, evaluate their behavior during meiosis, and describe the distribution of heterochromatin in the genome of *Eriopis connexa*.

MATERIAL AND METHODS

Specimens of *Eriopis connexa* were collected on the Campus of the Universidade Federal de Viçosa, Minas Gerais, Brazil, where they occur naturally, and were reared in the laboratory for reproduction. Adults and larvae were fed with aphids and their eggs were separated from the adults, hatching on average after 5 days. Fifteen larvae in the prepupal stage were used to obtain mitotic metaphase chromosomes. Twenty five adult males were used for analysis of meiosis. Ten mitotic metaphases per individual were analyzed, on average, during the larval phase.

Cytogenetic analysis of mitotic metaphase chromosomes was performed according to the method of IMAI et al. (1988). Cerebral ganglions were dissected from prepupal larvae into hypotonic solution-colchicine (1 % sodium citrate plus 0.005% colchicine) and left in this solution for 1:30 hours. After this time, each ganglion was transferred to a slide and several drops of fixative 1 (1:3:4, water: ethanol:acetic acid) were added. The ganglion was dissociated with a pair of dissecting needles and two drops of fixative 2 (1:1, ethanol:acetic acid) were added. Three drops of fixative 3 (100% acetic acid) were then added and 24 hours later the slides were stained with Giemsa in Sorensen buffer (0.06 M buffer, pH 6.8, at the proportion of 1 ml Giemsa:30 ml buffer) for 10 minutes. The chromosomes were classified according to the nomenclature of LEVAN et al. (1964). For meiotic analysis, males testes were removed in Ringer and slides were prepared by the method of IMAI et al. (1988) without using colchicine.

C-Banding

C-banding was performed by the technique of SUMNER (1972), modified by POMPOLO and TAKAHASHI (1990). The slides were submitted to the following treatments: a) hydrolysis with 0.2 N HCl at room temperature for 4 minutes; b) a quick wash in distilled water and incubation with 5% barium hydroxide in a water bath at 60°C for 8 minutes; c) a wash in 0.2 N HCl for about 30 seconds; d) incubation with 2xSSC solution (0.03 mol/L sodium citrate and

0.3 mol/L sodium chloride, pH 7.0) at 60°C for 10 minutes; e) Giemsa staining (2 ml Giemsa: 30 ml 0.06 M Sorensen buffer, pH 6.8) for 50 minutes.

RESULTS

Mitotic chromosomes of 12 *Eriopis connexa* females were analyzed by standard staining. Eight of these females presented a diploid number of 2n=20 and four showed a small metacentric B-chromosome in all cells, resulting in a karyotype of the 2n=18+XX+B type (Fig. 1 and 2A, B and C). The autosomes of this species were grouped into 4 metacentric (M) pairs, 4 submetacentric (SM) pairs and 1 subtelocentric (ST) pair. The X chromosome was of the M type. Pairs 2 and 3 presented secondary constrictions located on the short arms of the chromosomes. C-banding analysis revealed that constitutive heterochromatin is located in the centromeric region of all chromosomes in the complement. The chromosome B, in turn, was almost completely euchromatic (Fig. 2E).

The three male larvae evaluated for mitosis presented 2n=18+Xy with a very small y chromosome (Fig. 2D). The 25 adult males evaluated for meiosis had a chromosome number of n=9+Xy_p, with all prophase I stages being visible. More intensely stained regions (heteropycnotic) were observed in leptotene (Fig. 3A), while they were less intensely stained by C banding (Fig. 4A). A larger heterochromatic region and other smaller heterochromatic regions were observed during zygotene both by standard staining (Fig. 3B and C) and by C banding (Fig. 4B). At pachytene, the bivalents were individualized and it was possible to visualize the chromomeres (Fig. 3D). Chiasmata and some bivalents were observed at diplotene (Fig. 3E), and at diakinesis the bivalents were more condensed and uniformly distributed (Fig. 3 F and G).

Metaphase I was characterized by the presence of 9 bivalents and by an associated sex pair forming a "parachute" figure in all cells evaluated (Fig. 5A and B). At anaphase I, chromosome segregation was normal.

DISCUSSION

Eriopis connexa presented a chromosome number of 2n=18+XX for most females analyzed and a meioformula of $n=9+Xy_p$ for males. The chromosome number and the "parachute" configuration during metaphase I agree with the descriptions for most Coleoptera species, probably representing the typical (ancestral) karyotype, especially in the Polyphaga suborder (SMITH, 1950; SMITH and VIRKKI, 1978).

Chromosome pairs 2 and 3 presented satellites and probably contained the nucleolar organizer regions (NORs). The chromosomes presenting these regions are frequently seen associated with the nucleoli during prophase (GUERRA, 1988).

B chromosomes are frequently fully heterochromatic (JONES and REES, 1982; JONES, 1995). However, some euchromatic B chromosomes have been reported, e.g., in *Allium fava* (VOSA, 1973), *Najas marina* (VIINIKKA, 1975) and *Allium schoenoprasum* (STEVENS and BOUGOURD, 1994). In some populations of *Allium ericetorum* a B chromosome occurred and no correlation between B chromosomes and particular C-banding patterns was observed (WETSCHNIG, 1995).

The function and composition of B chromosomes is still a controversial question. Reports on the occurrence of B chromosomes in the lizard *Nothobachia ablephara* showed the Bs were not clearly distinguishable from the autossomes in Giemsa-stained metaphases and C-banding, but showed late replication after R-banding. One widespread heterochromatin feature is it late-replication, the Bs could be a specific class of heterochromatin undetected by rotine C-banding procedures (PELLEGRINO et al., 1999).

Recently, SILVA and YONENAGA-YASSUDA (1998) reported a conspicuous heterogeneity of size, morphology, constitutive heterochromatin patterns and localization of telomeric sequences of B chromosomes for the rodent *Nectomys*, which allowed them to suggest differences in the composition of these chromosomes.

Different types of repetitive sequences of DNA from a B chromosomes have been characterized by molecular analyses, in some species of plants (rye *Secale cereale* HOUBEN et al., 1996; daisy *Brachycome dichromosomatica* JOHN et al., 1991; LEACH et al., 1995; among others). In animals the parasitic wasp *Nasonia vitripennis*, presents males that carry a B chromosome, called PSR (paternal sex ratio), which causes the compaction and subsequent loss of the paternal chromosomes in fertilized eggs. Three families of related tandemly repetitive DNAs (PSR2, PSR18, PSR22) were shown to be present only on the PSR chromosome (EICKBUSH et al., 1992). A species specific satellite DNA family (pSsP216) of *Drosophila subsilvestris* appears predominantly in B chromosomes. The pSsP216 family consists of tandemly arranged 216 bp repetitive units (GUTKNECHT et al., 1995).

According to SMITH and VIRKKI (1978), supernumerary chromosomes have been reported in approximately 50 species and subspecies of Coleoptera (suborder Polyphaga), in which they are restricted to only a few individuals, occurring in small numbers.

A correlation between frequency of B-chromosomes and sex proportion has been reported for *Exochomus quadripulsatus*, and probable factors influencing the sex proportion have been suggested to occur, affecting the frequency of B chromosomes in different populations (HENDERSON, 1988).

B-chromosomes restricted to one sex have also been reported in fish, e.g., *Astyanax scabripinnis*, which presented a B chromosome limited to males (STANGE and ALMEIDA-TOLEDO, 1993). Also a casual relationship has been reported to occur in fish between sex proportion and presence of B-chromosomes (VICENTE et al., 1996).

The hypothesis that explains the absence of supernumerary chromosomes in a given sex may be the existence of a mechanism of elimination of these chromosomes in the somatic tissues of these individuals. The elimination of B-chromosomes in somatic tissues has been reported, as observed in grasshoppers (LOPEZ-LEON et al., 1991) and fish (KOHNO et al., 1986;

NAKAI and KOHNO, 1987). However, the causes of these mechanisms are still unclear.

C-band analysis revealed that the constitutive heterochromatin of *Eriopis connexa* was localized in the centromeres of all chromosomes in the complement. Evaluation of meiosis by C-banding revealed the presence of small chromocenters which were more numerous in leptotene than at the end of zygotene. These chromocenters increased in size and decreased in number, with the presence of a much larger heterochromatic region suggesting that the pairing of homologous chromosomes had occurred. The larger heterochromatic region is due to the association of the sex chromosomes. Reductions in the number of chromocenters at the beginning of prophase I (leptotene) have also been described for *Epilachna paenulata* (Coccinellidae) (DRETS et al., 1983).

A small-sized supernumerary chromosome restricted to females occurred in 10% of our sample. When submitted to C-banding it presented staining (an intermediate positive block) in a small region of the chromosome, with the remainder being euchromatic.

ACKNOWLEDGEMENTS

We are grateful to Dra. Maria José de Souza Lopes (Universidade Federal de Pernambuco, Recife, Brasil) for manuscript review and suggestions and Dra. Lúcia Massutti de Almeida (Departamento de Zoologia, Universidade Federal do Paraná, Curitiba-Paraná), who provided the identification the species used in the present study. Research was suported by "Fundação de Amparo à Pesquisa do Estado de Minas Gerais" (FAPEMIG) and "Universidade Federal de Viçosa" (UFV) Viçosa, MG, Brasil.

Figure 1 - Metaphase and karyotype of an *E. connexa* female with 2n=18+XX+1B. The arrow indicates the B chromosome. Bar=5 µm.

Figure 2- Karyotypes of various *E. connexa* individuals. (A) Karyotype of a female with 2n+XX+1B. (B and C) Females with 2n=18+XX. (D) Male with 2n+18+Xy. (E) C band in a female with an almost completely euchromatic B-chromosome. Bar=5 µm.

Figure 3- Meiosis of an *E. connexa* male. (A) Leptotene. (B and C) Zygotene. (D) Pachytene. (E) Diplotene. (F and G) Diakinesis. Bar=5 μm.

Figure 4- Meiosis of an *E. connexa* male. (A and B) Metaphase I of a male. **(C)** Anaphase I. The arrow indicates "parachute"-shaped sex chromosomes. Bar=5 µm.

Figure 5- C-band of an *E. connexa* male. (A) Leptotene. (B) Zygotene. The arrow indicates the association of sex chromosomes after C-banding. Bar=5 μm.

REFERENCES

De Bach P, (1964). Biological control of insects pests and weeds. London, Chapman and Hall.

Drets ME, Corbella E, Panzera F and Folle GA, (1983). C-banding and nonhomologous associations. II The "parachute" Xy_p sex bivalente and behavior of heterochromatic segments in Epilachna paenulata. Chromosoma 88:249:255.

Eickbush DG, Eickbush, TH, Werren, JH (1992). Molecular characterization of repetitive DNA sequences from a B chromosome. Chromosoma 101:575-583.

Ferreira A and Mesa A, (1977). Estudos citológicos em três espécies brasileiras de Coleópteros (Chrysomelidae, Cerambycidae e Meloidae). Rev. Bras. Bio. 37(1):61-64.

Ferreira A, Cella D, Tardiv, JR. and Virkki N, (1984). Two pairs of chromosomes: A new low record for Coleoptera. Rev. Brasil. Genet. 7:231-39.

Galián J and Moore BP, (1994). Chromosome numbers and sex-determining mechanism in Australian Carabidae (Coleoptera).Coleop.Bull. 48:226-35.

Gill TK., Gulati M and Pajni HR, (1990). Chromosome numbers in Indian weevils (Coleoptera:Curculionoidea). Coleop. Bull. 44:437-41.

Gordon RD, (1985). Coccinellidae (Coleoptera) of America North of México. Journal of New York Entomological Society, New York, 93(1):1-912.

Guerra M, (1988) Introdução àCitogenética Geral. Editora Guanabara.

Gutknecht, J.; Sperlich, D.; Bachmann, L, (1995). A species specific satellite DNA family of Drosophila subsilvestris appearing predominantly in B chromosomes. Chromosoma 103:539-544.

Henderson AS, (1988). A correlation between B chromosome frequency and Sex ratio in Exochomus quadripustulatus . Chromosoma 96:376-381.

Houben A, Kynast RG, Heim U, Hermann H, Jones RN and Forster JW, (1996). Molecular cytogenetic characterization of the terminal

heterochromatic segment of the B-chromosome of rye (Secale cereale). Chromosoma 105:97-103.

Imai H, Taylor, RW, Crosland, MWJ and Crozier RH, (1988). Modes of spontaneous evolution in ants with reference to the minimum interation hipothesis. Jpn. J.Genet. 63:159-185.

Jones RN and Rees H, (1982) B chromosomes. London: Academic Press.

Jones RN, (1995). B chromosomes in plants. New Phyt. 131:411-435.

John UP, Leach CR and Timmis JN, (1991). A sequence specifc to B chromosomes of Brachycome dichromosomatica. Genome 34:739-744.

Kohno S, Nakai Y, Satoh S, Yoshida M and Kobayashi H, (1986). Chromosome elimination in the Japanese hagfish Eptatretus burgeri. Agnatha, Cyclostomata). Cytogenet. Cell Genet. 41:209-214.

Lawrence C, (1982). Coleoptera, In: J. A. Parker (ed.) Synopsis and Classification of Living Organisms, Vol.2 McGraw-Hill, New York, pp. 482-553.

Leach CR, Donald TM, Franks TK, Spiniello SS, Hanrahan CF and Timmis JN, (1995). Organisation and origin of a B chromosome centromeric sequence from Brachycome dichromosomatica. Chromosoma 103:708-714.

Levan A, Fredga K, Sandberg AA, (1964). Nomenclature for centromeric position on chromosomes. Heredity 52:201-220.

Levins R and Wilson M (1980). Ecological theory and pest management. Annual Rewiew of Entomology 25:287-308.

Lopez-Leon MD, Cabrero J and Camacho JPM (1991). Meiotic drive against an autosomal supernumerary segment promoted by the presence of a B chromosome in females of the grasshopper Eyprepocnemis plorans. Chromosoma 100:282-287.

Martins VG, (1994). The chromosomes of five species of Scarabaidae (Polyphaga, Coleoptera). Naturalia 19:89-96.

Nakai Y and Kohno S, (1987). Elimination of the largest chromosome pair during differentiation into somatic cells in the Japanese hagfish, Myxine garmani (Cyclostomata, Agnatha). Cytogenet. Cell Genet. 45:80-83.

Pellegrino KCM, Rodrigues MT and Yonenaga-Yassuda Y, (1999). Chromosomal polymorphisms due to supernumerary chromosomes and pericentric inversions in the eyelidless microteiid lizard Nothobachia ablephara (Squamata, Gymnophthalmidae). Chromosome Research 7:247-254.

Petitpierre E, Segarra C, Yadav JS and Virkki N, (1988). Chromosome numbers and meioformulae of Chrysomelidae. In P. Jolivet, E. Petitpierre and T. H. Hsiao (eds) Biology of Chrysomelidae. Kluwer Academic, Dordrecht, pp. 161-86.

Petitpierre E, (1996). Molecular cytogenetics and taxonomy of insects, with particular reference to the Coleoptera. Int. .J. Morphol. and Embryol. vol.25, nº 1/2.

Pompolo SG and Takahashi CS, (1990). Chromosome numbers and C-banding in two wasps species of the genus Polistes (Hymenoptera, Polistinae, Polistini). Insectes Sociaux, 37:251-257.

Serrano J, (1981). Chromosome numbers and karyotype evolution of Caraboidea. Genetica 55:51-60.

Serrano J and Yadav JS, (1984). Chromosome numbers and sex-determining mechanisms in Adephagan Coleoptera. Coleopt. Bull. 38:335-57.

Sharma GP, Gill TK and Pal V, (1980). Chromosomes in the curculionid beetles (Coleoptera, Curculionidae). Coleopt. Bull. 34:361-67.

Silva MJJ and Yonenaga-Yassuda Y, (1998) Heterogeneity and meiotic behaviour of B and sex chromosomes, banding patterns and localization of (TTAGGG)n sequences by FISH, in the Neotropical water rat Nectomys (Rodentia, Cricetidae). Chromosome Research 6:455-462.

Smith SG, (1950). The cytotaxonomy of Coleoptera. Can. Entomol. 82:58-68.

Smith SG and Virkki N, (1978). Animal Cytogenetics. Coleoptera. Berlin:Gebruder Borntraeger.

Smith RF and Reynolds HT, (1966). Principles, definitions and scope of integrated pest control. In: FAO. Symposium on Integated Pest Control. Proceedings. Rome, P.11-17.

Stange EAR and Almeida-Toledo LF, (1993). Supernumerary B chromosomes restricted to males in Astyanax scabripinnis (Pisces, Characidae). Rev. Bras. Genet. 16,3,601-615.

Stevens JP and Bougourd SM, (1994). Unstable B-chromosomes in a European population of Allium schoenoprasum L. (Liliaceae). Biological Journal of the Linnean Society 52:357-363.

Sumner AT, (1972). A simple technique for demonstrating centromeric heterochromatin. Exp. Cell. Res. 75:304-306.

Vicente VE, Moreira-Filho O and Camacho JPM, (1996). Sex-ratio distortion associated with the presence of a B chromosome in Astyanax scabripinnis (Teleostei, Characidae). Cytogenet. Cell Genet. 74:70-75.

Viinikka Y, (1975). Allocyclic regions and banding patterns in the chromosomes of Najas marina. Hereditas 81:47-54.

Vosa CG, (1973). The enhanced and reduced quinacrine fluorescence bands and their relationship to the Giemsa patterns in Allium flavum. In: Casperson T, Zech L, eds. Nobel Symposium 23 (Medicine and Natural Science). Chromosome Identification. 156-158.

Wetschnig W, (1995). Giemsa C-banded karyotypes of Allium ericetorum and Allium kermesinum (Alliaceae). Plant Systematics and Evolution 195:1-2, 45-59.

SILVER STAINING OF NUCLEOLAR ORGANIZER REGIONS (NORS) IN SOME SPECIES OF HYMENOPTERA (BEES AND PARASITIC WASP) AND COLEOPTERA (LADY-BEETLE)

Artigo publicado pela revista Cytobios 104, 119-125, 2001.

Ressalva: Este artigo foi incluído por se tratar de uma adaptação da metodologia para o bandeamento NOR, que foi realizado em Hymenoptera e Coleoptera.

Abstract

Adaptations of the nucleolar organizer regions (NOR) banding technique using precipitation of silver salts significantly improved the NOR characterization of some species of hymenopterans and coleopteran. The bee *Melipona marginata marginata* (2n=18) showed one metacentric pair of chromosomes with a NOR in pericentromeric position. The parasitic wasp *Mellitobia australica* (2n=12) also showed one metacentric pair with a strongly Ag-positive NOR. The male lady-beetle *Cycloneda sanguinea* L. (2n=18+Xy_p) displayed a NOR on a pair of acrocentric autosomes. In *Euglossa sp* males (a haplodiploid species) (n=21) NOR were multiple, and occurred in five chromosomes. In the bee *Plebeia sp*1 (2n=34) the NOR seemed restricted to one of the homologues of a metacentric pair. We indicate the systematic advances brought out by this technique in the context of current theories of karyotypic evolution of these taxa.

Introduction

The nucleolar organizer region (NOR) is one of the most frequently studied cytogenetic characters due to its important function and to structural particularities of this region (Esponda and Giménez-Martín, 1975). The use of Ag-staining methodology to detect active NORs, as originally proposed by Howell and Black (1980) to human chromosomes, has been successfully applied to many organisms, furnishing important data for mitotic and meiotic chromosomes, such as, for example the characterization of a hierarchy of activation of nucleolar organizer regions in *Citrus sinensis* (Pedrosa *et al*., 1997).

Silver salts also stain components of interphase nucleoli (Ploton *et al.*, 1987). It has been suggested that the NOR technique highlights the presence and amount of the phosphoprotein C23 (nucleolin), but other proteins, including factors necessary for rDNA transcription and early rRNA processing, seem to contribute to the Ag-NOR staining behavior (Schwarzacher and Wachtler, 1993).

Among the Coleoptera, this methodology has been particularly useful to understand chromosome evolution and architecture (Petitpierre, 1996). Several hypotheses have been forwarded to explain Xy_p parachute configuration, typical of many species of this order. John and Lewis (1960) advocated for nucleolar association, but this view was challenged by the lack of evidence for the existence of NORs regions in the sex chromosomes of beetles (Drets *et al*., 1983; Postiglioni and Brum-Zorrilla, 1981, 1988; Wettstein, 1981). These authors interpreted the Xy_p

association as composed of chromosomal end contacts of heterochromatic nature, without further discussion of the possible role of the composition of the Xy_p.

In Hymenoptera, the Ag-NOR technique has been historically difficult to develop, although it has been successfully applied in some ants (Palomeque *et al*., 1988; Palomeque *et al*., 1990; Lorite *et al*., 1997). These authors stained metaphase chromosome with $AgNO_3$ and identified NORs in pericentromeric heterochromatin. Imai and Taylor (1989) made a similar observation in the ant *Myrmecia croslandi* (2n=3), but the results were considered controversial (Imai *et al*., 1992). By using a *Drosophila* rDNA probe for fluorescent *in situ* hybridization (FISH), these authors demonstrated that the silver-stained bodies corresponded to the kinetochores. This has been recently confirmed by FISH using an ant 28S rDNA probe (Hirai *et al*., 1994).

In this study we have successfully applied NOR banding protocol to some species of the bees, parasitic wasps and lady-beetles. We were able to identify the number and location of NORs in metaphase mitotic chromosomes and the modal number of nucleoli per interphase nucleus in some species of hymenopterans and one species of coleopteran.

Material and methods

We collected wild specimens of *Cycloneda sanguinea* (Coleoptera, Coccinellidae), *Mellitobia australica* (Hymenoptera, Eulophidae) and *Euglossa sp* (Hymenoptera, Apinae, Euglossini). Also, specimens of *Plebeia sp*1 (Hymenoptera, Apinae, Meliponini) and *Melipona marginata marginata* (Hymenoptera, Apinae, Meliponini) were obtained from colonies of the Apiário Central of the Universidade Federal de Viçosa, Minas Gerais state, southeastern Brazil.

Metaphases were obtained from cerebral ganglia at prepupal phase, according to Imai *et al.* (1988). On average, we analyzed five individuals per species.

Modifications of the Ag-staining of NOR (Howell and Black 1980).

Two parts of developer (2g of gelatine dissolved in 100 ml of distilled water and 1 ml of formic acid) and four drops of $AgNO_3$ (Merck) in 50% aqueous solution were mixed on slides. Then, the material was covered with a coverslip, transferred to petri dishes, overlaid with a moist filter paper and incubated at 38ºC for 25 to 30 minutes, until the staining solution turned golden brown. The coverslip was rinsed off first with tap water and then the slide was squirted vigorously with a 20 ml syringe. After the drying process the slide was mounted on Entellan (Merck). After placing slides in a warm oven (37ºC) for 24 hours, metaphases were photographed with light microscopy.

Results

This protocol was effective for detection of the nucleolar organizer regions on the metaphase mitotic chromosomes and for nucleolus in the interphase nucleous. *Melipona marginata marginata* females (2n=18) showed NOR-bearing metacentric pair (Fig.1A). Parasitic waps *Mellitobia australica* (2n=12) and one strongly Ag-positive metacentric pair (Fig.1B). The lady-beetle *Cycloneda sanguinea* ($2n=18+Xy_p$), the nucleolar organizer region was located on a acrocentric autosome pair (Fig.1C). In *Euglossa sp* males (haplodiploid) (n=21), five chromosomes bore NORs (Fig.1D). Finally, in the females bee *Plebeia* sp_1 (2n=34), the expression of the NORs was always restricted to one of the homologues of a chromosome metacentric pair (active NOR heteromorphism) (Fig.1E). All species usually showed two conspicuous nucleoli in the interphase nucleus, and sometimes a single, relatively larger nucleolus.

Discussion

The results indicate that this protocol is versatile and effective to many species of hymenopterans and coleopteran. It seem good quality of banding on mitotic metaphases has only been possible by vigorous rinsing of the slide, as it reduces non-specific silver precipitation. This simple modification has worked out a long standing problem of applying this banding to hymenopterans. Because of unsuccessful results in ants, Imai *et al.* (1992) suggested that failure to obtain NOR banding might be explained by an unusual non-histonic protein associated with r-DNA.

Our results, however, demonstrate that silver nitrate stains NORs of mitotic cells that were functionally active during the preceding interphase, as has been reported for plants and animals (Sessions, 1996). In Orthoptera data obtained with the Ag-staining technique has been instrumental to analyse the NORs in many species of grasshoppers (Gosálvez, *et al.* 1981; Cabrero and Camacho, 1986; López-León *et al*., 1995).

The relevance of patterns of NORs for systematic studies in Hymenoptera is evidenced by the great variation in number, size and location reported in this study. For instance, in *Melipona marginata marginata* we observed only one pair of active, pericentromeric NOR, while in *Mellitobia australica* the NOR position was telomeric. In males of *Euglossa sp* was observed five chromosomes bearing relatively small NORs regions. Recently, growing data on ant karyotypes (more 500 species) indicate that this is a chromosomally highly diversified taxon,

ranging from 2n=2 up to 2n=94 (Crosland and Crozier, 1986; Imai *et al*., 1988, 1990). Imai *et al*., (1988) divided ants into two groups, (1) species having low numbers of chromosomes (n< 12) and (2) species with high numbers (n>12), based on the cytological evidence that translocation polymorphisms appear preferentially in the former, while Robertsonian polymorphisms are predominant in the latter. If this classification system is applicable to hymenopterans as a whole (Hoshiba *et al*., 1989), then the species analyzed here would all be members of the second group.

In *Plebeia sp*1 we observed an apparent heteromorphism of the expression of NORs, which occurs exclusively in one the homologues of a chromosome pair. In other species with NORs expression restricted to one the homologues of a chromosome pair, the phenomenon is often associated with structural heterozigosity of that locus (Jamilena *et al*., 1990; Yabuya *et al* , 1995). Kinetochores as well as active NORs are stained by the silver method in many eukaryotes (Sumner, 1990).

In the lady-beetle *Cycloneda sanguinea* the nucleolar organizer region is located on a acrocentric autosome pair. Postiglioni and Brum-Zorrilla (1988) and Postiglioni *et al.* (1991), described one pair of NORs with autosomal location in the chrysomelid *Chelymorpha variabilis* by Ag-NOR staining, orange acridine fluorescence, and microspreading techniques. This pattern led, these authors to reject John and Lewis (1960)'s hypothesis of a nucleolar origin for the Xy_p association. Observations of nucleolus hypothesis have been reported also in beetles, such as *Tenebrio molitor*, were FISH data support the classical hypothesis of a nucleolar origin for the Xy_p association (Juan *et al*., 1993).

Acknowledgements

This work was supported by FAPEMIG, UFV, CNPq and CAPES

Figure 1 Silver staining. (A) Bee *Melipona marginata marginata* female (2n=18). (B) Parasitic wasp *Melittobia australica* female (2n=12). (C) Lady-beetle *Cycloneda sanguinea* male (2n=20). (D) Bee *Euglossa sp*. male (n=21). (E) Bee *Plebeia sp*1 female (2n=34). Arrows showing expression of NORs. Bar=5µm.

References

CABRERO J. and Camacho J.P.M. 1986. Cytogenetic studies in gomphocerine grasshoppers. II. Chromosomal location of active nucleolar organizing regions. *Can. J. Genet. Cytol*. **28** 540-544.

CROSLAND M.W.J. and Crozier R.H. 1986. *Myrmercia pilosula*, an ant with only one pair of chromosomes. *Science* **231** 1278.

DRETS M.E., Corbella E., Panzera F. and Folle G.A. 1983. C-banding and non-homologous associations. II The "parachute" Xy_p sex bivalent and the behavior of heterochromatic segments in *Epilachna paenulata*. *Chromosoma* **88** 249-255.

ESPONDA P. and Giménez-Martín G. 1975. Nucleolar organizer ultrastructure in *Allium cepa*. *Chromosoma* **52** 73-87.

GOSÁLVEZ J., López Fernandez C. and Morales Agacino E. 1981. The chromosome system in three species of the genus *Arcyptera* (Orthoptera: Acrididae). I. Heterochromatin variation, DNA content and NOR activity. *Acrida* **10** 191-203.

HIRAI H., Yamamoto M-T., Ogura K., Satta Y., Yamada M., Taylor R.W.and Imai H.T. 1994. Multiplication of 28S rDNA and NOR activity in chromosome evolution among ants of the *Myrmecia pilosula* species complex. *Chromosoma* **103** 171-178.

HOSHIBA H., Matsuura M. and Imai H.T. 1989. Karyotype evolution in the social wasps (Hymenoptera, Vespidae). *Jpn. J. Genet*. **64** 209-222.

HOWELL W.M., and Black, D.A. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* **36** 1014-1015.

IMAI H.T. and Taylor R.W. 1989. Chromosomal polymorphisms involving telomere fusion, centromeric inactivation and centromere shift in the ant *Myrmecia* (*pilosula*) n=1. *Chromosoma* **98** 456-460.

IMAI H.T., Taylor, R.W., Crosland, M.W.J., and Crozier, R.H. 1988. Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis. *Jpn. J. Genet*. **63** 159-185.

IMAI H.T., Taylor R.W, Kubota M., Ogata K. and Wada M.Y. 1990. Notes on the remarkable karyology of primitive ant *Northomyrmecia macrops*, and of the related genus Myrmecia (Hymenoptera: Formicidae). *Psyche* **97** 133-140.

IMAI H.T., Hirai, H., Satta, Y., Shiroishi, T., Yamada, M., and Taylor, R.W., 1992. Phase specific Ag-staining of nucleolar organizer region (NORs) and kinetochores in the Australian ant *Myrmecia croslandi*. *Jpn. J. Genet*. **67** 437-447.

JAMILENA M., Ruiz Rejón C. and Ruiz Rejón M. 1990. Variation in the heterochromatin and nucleolar organizing regions of *Allium subvillosum* L. (Lilliaceae). *Genome* **33** 779-84.

JOHN B. and Lewis K.R. 1960. Nucleolar controlled segregation of the sex chromosomes in beetles. *Heredity* **15** 431-39.

JUAN C., Pons J. and Petitpierre E. 1993. Localization of tandemly repeated DNA sequences in beetle chromosomes by fluorescent *in situ* hibridization. *Chrom. Res.* **1** 167-74.

LÓPEZ-LEÓN M.D., Cabrero J., and Camacho, J.P.M. 1995. Changes in DNA methylation during development in the B chromosome NOR of the grasshopper *Eyprepocnemis plorans*. *Heredity* **74** 296-302.

LORITE P., Aránega A.E., Luque F., and Palomeque T. 1997. Analysis of the nucleolar organizing regions in the ant *Tapinoma nigerrimum* (Hymenoptera, Formicidae). *Heredity* **78** 578-582.

PALOMEQUE T., Chica E., Cano M.A. and Díaz de la Guardia R. 1988. Karyotypes, C-banding, and chromosomal location of active nucleolar organizing regions in *Tapinoma* (Hymenoptera, Formicidae). *Genome* **30** 277-280.

PALOMEQUE T., Chica E., Cano M.A. and Díaz de la Guardia R. 1990. Development of silver stained structures during spermatogenesis in different genera of Formicidae. *Genetica* **81** 51-58.

PEDROSA A, Guerra M. and Soares Filho S. 1997. An hierarchy of activation of nucleolar organizer regions in *Citrus sinensis* (L.) Osbeck. *Cytobios* **92** 43-51.

PETITPIERRE E. 1996. Molecular cytogenetics and taxonomy of insects, with particular reference to the coleoptera. *Int. J. Insect Morphol. & Embryol.* **25** 115-133.

PLOTON D., Thiry M., Menager M., Lepoint A., Adnet, J.J. and Goessens G. 1987. Behaviour of nucleolus during mitosis. A comparative ultrastructural study of various canceruos cell lines using the Ag-NOR staining procedure. *Chromosoma* **95** 95-107.

POSTIGLIONI A. and. Brum-Zorrilla N. 1981. Localización de regiones organizadoras nucleolares (NORs) en otra especie con sistema sexual Xy_p *Calligrapha polyspila* (Coleoptera, Chrysomelidae, Chrysomelinae). *Resúmenes Comum. Jornadas Cienc. Nat.* Montevideo 2.

POSTIGLIONI A. and. Brum-Zorrilla N. 1988. Non-relationship between nucleolus and sex chromosome system Xy_p in *Chelymorpha variabilis* Boheman (Coleoptera: Chrysomelidae). *Genetica* **77** 137-41.

POSTIGLIONI A., Stoll M. and Brum-Zorrilla N. 1991. Haploid karyotype analysis of *Chelymorpha variabilis* Boheman (Coleoptera, Chrysomelidae) with microspreading techniques. *Rev. Brasil. Genet*. **14** 653-60.

SCHWARZACHER H.G. and Wachtler F. 1993. The nucleolus. *Anat. Embryol.* **188** 515-536.

SESSIONS S. K. 1996. Chromosomes: molecular cytogenetics. *In: Molecular Systematics.* Second Edition. pp 121-168 Edited by D.M. Hillis, M. Craig, and B.K. Mable, Sinauer Associates, Sunderland, Massachusetts, U.S.A.

SUMNER A.T. 1990. Chromosome banding. Unwin Hyman Ltd, London, UK.

WETTSTEIN R. 1981. *Unusual mechanisms of chromosome pairing in arthropods.* International Cell Biology 1980-81. pp 187-94. Edited by H.G. Schweizer , Springer-Verglag.

YABUYA T., Kihara S., Yoshino H. and Ohba A. 1995. Variation in the nucleolar organizing regions in Japanese garden iris and its wild forms (*Iris ensata* Thunb.). *Cytologia* **60** 383-7.

MORPHOLOGICAL AND CYTOGENETICAL STUDIES
ON THE FEMALE AND MALE REPRODUCTIVE TRACTS OF
***ERIOPIS CONNEXA* MULSANT**
(COLEOPTERA, POLYPHAGA, COCCINELLIDAE)

Artigo enviado para revista The Netherlands Journal of Zoological.

ABSTRACT

The present paper reports information on the anatomic, histological and cytogenetic on sex organs of sexually mature specimens of *Eriopis connexa*. The male reproductive organs are composed of a pair of testes, two vas deferens and a single ejaculatory duct. In 21% of males examined the testis were fused. In both cases each testes is formed by a large numbers of droplike follicles, which contained spermatocytes at different developmental stages. In females each ovary is formed by four bunchy ovarioles, joined by short terminal filaments at their upper portions. There is a single spermatheca and the ovarioles are the type meroistic telotrophic. Females presented a diploid number of 2n=18+XX. Chromosome pairs 2 and 3 presented secondary constrictions located on the short arms, and fluorochrome staining showed only one pair CMA_3^+ (one block in each chromosome). NOR-banding showed only one pair Ag-positive. In the male meiosis the chromosome number was $n=9+Xy_p$. In the prophase I the NOR-banding showed one pair of autosomes with active region and the sexua1 vesicle stained strongly with silver nitrate suggesting association of these chromosomes.

INTRODUCTION

Beetles (Coleoptera) are an enormously diverse group and the largest of insect orders. To date, more than 350,000 species have been described (LAWRENCE, 1982), which are divided into 4 subgroups: Archostemata, Adephaga, Myxophaga and Polyphaga (CROWSON, 1981; HENNING, 1981; LAWRENCE & NEWTON, 1982). The species *Eriopis connexa* belongs to the family Coccinellidae and it is widely distributed in South America. This species is a polyphagous lady-beetle that preys on soft-bodied insects including aphids (GORDON, 1985; MILLER, 1995). Recently, it has been imported in the United States for biological control of the aphid *Diuraphis noxia* (QUIROGA *et al.*, 1991; MILLER, 1995; MICHELS *et al.*, 1997). Research on the role of coccinellids in Brazil as a pest control agent is still developing (LEVINS & WILSON, 1980; CHAVES, 1991). The characterization of male and female reproductive systems can be useful for taxonomic and phylogenetic studies, as well as for understanding the life cycle and adaptations of the species (UENO, 1994; JAGLARZ & BILINSKI, 1998). As already indicate by BONHAG (1958) beetle ovaries (ovarioles) are of meroistic type. The division of Coleoptera into two majors groups: Adephaga and Polyphaga corresponds well with the distribution of the variations of the meroistic ovary (CROWSON, 1981; KING & BUNING, 1985). The former group is characterized by the occurrence of polytrophic ovarioles (JAGLARZ, 1988; JAGLARZ, 1992; JAGLARZ & BILINSKI, 1998) whereas the latter has telotrophic ovarioles (MATUSZEWSKI *et*

al. 1985; STEBBINGS, 1981; ULLMANN, 1973). Is not much known about the reproductive organs of Coccinellidae. Only a few detailed studies are available, e.g. in 22 species of *Epilachna* from Asia (KATACURA *et al*., 1994) using the bursa copulatrix and spermathecal morphology found that these species form three phylogenetic groups. Cytological information about the families of the Coleoptera is highly fragmented, and includes some 3000 species (SMITH & VIRKKI, 1978; SHARMA *et al*., 1980; SERRANO & YADAV, 1984; PETITPIERRE *et al*., 1988; GILL *et al*., 1990; GÁLIAN & MOORE, 1994). The chromosome numbers vary widely ranging from 2n=4 in *Chalcolepidius zonatus* (FERREIRA *et al*., 1984) to 2n=69 in *Ditomus capito* (SERRANO, 1981). A large number of species must be analyzed cytogenetically for a better understanding of this pattern of variability (FERREIRA & MESA, 1977; MARTINS, 1994). The basic karyotype of Coleoptera, and possibly the ancestral one, has been reported as composed of nine pairs of autosomes and the X and y_p sex chromosomes, which associate in a "parachute" configuration during metaphase I, with chromosome X being relatively larger than chromosome y, which is typically represented by a very small chromosome (SMITH, 1950). Cytogenetic analyses of Coleoptera have been mostly performed during male meiosis because of the difficulty in obtaining mitotic metaphase chromosomes (PETITPIERRE, 1996). In the family Coccinellidae, few analyses have been made, but available data have shown that the basic karyotype $n=9+Xy_p$ described for Coleoptera is the most frequently (SMITH & VIRKKI, 1978). The description of chromosome number in *E. connexa* male was made by Vidal (1984) that found $n=9+Xy_p$. The karyotypic characterization (mitosis, meiosis and constitutive heterochromatin) of both sexes of *E. connexa* was made by MAFFEI *et al*. (2000), in a population of *Eriopis connexa* from Viçosa, MG Brazil. These authors found one small-size supernumerary chromosome restricted to females, with a frequency of 10% in the sample. When submitted to C-banding this chromosome presented an intermediate positive block restricted to a small region of the chromosome, while the remaining portions were euchromatic suggesting the Bs could be a specific class of heterochromatin undetected by routine C-banding procedures.

The present paper provides the anatomical, histological and cytogenetical, information on the female and male organs sexually mature.

MATERIALS AND METHODS

Materials

Twenty three specimens (nine female and fourteen male) of *Eriopis connexa***,** were obtained in the field in Amoras neighborhood (city Viçosa, Southeastern Brazil).

Methods

Anatomy and Histology

The reproductive tracts were dissected in presence of saline solution for insects. The pieces were transferred to 4% paraformaldehyde at phosphate buffer 0.1M, pH 7.2 for 72 h. After the pieces were examined and photographed under stereo microscope Olympus. For histological analyses, after fixation, the pieces were dehydrated in ethanol series and embedded in historesin JB-4 (Polysciences). The 4 μm sections were stained with haematoxilyn and eosin. Some sections were submitted to Feulgen reaction as described by PEARSE (1986).

Cytogenetic analysis

Cytogenetic analysis of mitotic metaphase chromosomes was performed according to MAFFEI *et al*. (2000). Brains were dissected from prepupae into hypotonic solution-colchicine (1 % sodium citrate plus 0.005% colchicine) and left in this solution for 1:30 hours. Afterwards the ganglia were transferred to a slide and several drops of fixative 1 (4:3:3,

water: ethanol: acetic acid) were added. Each ganglion was dissociated with a pair of dissecting needles and two drops of fixative 2 (1:1, ethanol:acetic acid) were added. Three drops of fixative 3 (100% acetic acid) were then added and 24 hours later the slides were stained with Giemsa in Sörensen buffer (0.06 M pH 6.8, at the proportion of 1 ml Giemsa: 30 ml buffer) for 10 minutes. The chromosomes were classified according to LEVAN *et al*. (1964). For meiotic analysis, testes were removed to a Ringer solution and slides were prepared by the method of MAFFEI *et al.* (2000) described above, without colchicine.

The NOR banding was performed according to MAFFEI *et al* (2001)

Two parts of developer (2g of gelatin dissolved in 100 ml of distilled water and 1 ml of formic acid) and four drops of $AgNO_3$ (Merck) in 50% aqueous solution were mixed on slides. Then, the material was covered with a coverslip, transferred to petri dishes, overlaid with a moist filter paper and incubated at 38ºC for 25 to 30 minutes, until the staining solution turned golden brown. The coverslip was rinsed off first with tap water and then the slide was squirted vigorously with a 20 ml syringe. After the drying process the slide was mounted on Entellan (Merck). After storing slides at 37ºC for 24 h, metaphases were photographed under light microscopy.

Fluorescent bands

Fluorescent bands were studied using the base-specific DNA-binding fluorochrome Chromomycin A_3 (CMA_3) as suggested by SCHWEIZER (1980).

RESULTS

Morphology of reproductive tracts

The male reproductive organs of *E. connexa* (Fig. 1A) showed a pair of testes, two vas deferens and a single ejaculatory duct. Interesting were not see accessory glands neither a differentiated seminal vesicle. In 21% of males examined the testes were fused (Fig. 1B), but not the vas deferens. In both cases each testis is formed by a large number of droplike follicles, which presented different stages of spermatocytes formation (Figs. 2A, 2B). In females each ovary is formed by 4 bunchy ovarioles, which are joined in the upper portion by short terminal filaments. An ovary envelope is missing, but each ovariole is surrounded by a thin peritoneal sheath. The lateral and common oviducts as well as the vagina are very short (Fig. 3). There is a single spermatheca, which consists of a relatively small reservoir for sperm storage and a short convoluted spermathecal duct between the vagina and the common oviduct. In each ovariole one can see mature oocytes (Fig. 3). The ovarioles are of meroistic telothrophic type. Just below the terminal filament there is an enlarged region, the germarium, which consists of trophic tissue as well as oogonia and prefollicular tissues (Fig. 4A). When the oocyte leaves the germarium follicle cells (Fig. 4B) clothe it.

Cytogenetic analysis

Mitotic chromosomes females presented a diploid number of 2n=18+XX (Fig. 5A). The autosomes of this species were grouped into 4 metacentric (M) pairs, 4 submetacentric (SM) pairs and 1 subtelocentric (ST) pair. The X chromosome was of the M type. Pairs 2 and 3 presented secondary constrictions located on the short arms of the chromosomes. Analysis by CMA_3 showed only one block in one pair CMA_3 positive (Fig. 5B). Females examined by NOR banding showed only one pair Ag-positive (Fig. 5C). In the male meiosis (prophase I), the results obtained with Ag-NOR banding in males showed one pair of autosomes with an active region and the sexual vesicle strongly silver stained. In the early prophase (pachytene) there is the association of these chromosomes, which become strongly stained (argentophilic) (Fig. 6 A-D). The metaphase I was characterized by the presence of 9 bivalent and by an associated sex pair forming a "parachute" figure in all cells examined ($n=9+Xy_p$), with a very small y chromosome (Fig. 6E and F). At anaphase I, chromosome segregation was normal.

DISCUSSION

The meroistic telotrophic ovaries, characterized by the presence of trophic tissue, as well as oogonia in the germarium, is widely distributed among Coleoptera, especially in Polyphaga as reported by BÜNING (1994). The presence of various mature oocytes inside each ovariole suggests that a greater number of eggs can be laid down in a single posture, which agrees with the observations of SWEETMAN (1958) and HAGEN (1974). Usually, a morphological gradient exists along the germarium: its apical parts contain small, mitotically active cells while the basal part comprises large and polyploid nurse cells (SIMICZYJEW *et al*., 1998). However, mitotically active cells were not observed in the *E. connexa* female reproductive tract. We suggest that mitotic activity of these cells in this species is confined to immature stages. This is an expected result, because unlike panoistic and polytrophic ovarioles germ cell divisions stop after oocyte growth begins in telotrophic ovarioles. The structure of the male reproductive tract is basically alike the ones described for other insects. A striking contrast with other

insects, however, is provided by the presence of fused testes in 21% of the specimens. Our results are also unambigous, and it not is the case of a false fusion of the tunica propria, as reported by CHAPMAM (1998).

Cytogenetic data

The karyological pattern observed in this population of *E. connexa* of Amoras, was consistent with the results obtained found in population of Universidade Federal de Viçosa (UFV) (MAFFEI *et al*. 2000). However, no B chromosomes were found in females, such as it was observed in UFV population, although this pattern may be an artefact of our small sample size. Meiotic analysis of indicated an $n=9+Xy_p$ and in the anaphase I chromosomes segregation was normal. The fluorochrome CMA_3 is mostly used for characterization of heterochromatin (LEEMAN & RUCH 1983; 1984), and its molecular action is exerted by binding to the minor groove of double-stranded DNA, at GC-rich sites (SUMNER, 1990). Silver staining (Ag-NOR staining) is most frequently used, because of its high specificity for NORs (GOODPASTURE & BLOOM, 1975; HOWELL & BLACK, 1980). This methodology has been particularly useful to understand chromosome evolution and architecture (PETITPIERRE, 1996). The nucleolar organizer region was detected on a submetacentric satellite pair. However, the results of Ag-NOR staining in male meiosis, showed unexpectedly one pair of autosome and the sexual vesicle as active regions. During the zygotene and pachytene it was observed an association between these chromosomes. Several hypotheses have been forwarded to explain Xy_p parachute configuration, typical of many species of this order. JOHN & LEWIS (1960) advocated for nucleolar association, but this view was challenged by the lack of evidence for the existence of NORs regions in the sex chromosomes of beetles (DRETS *et al.*, 1983; POSTIGLIONI & BRUM-ZORRILLA, 1981, 1988; WETTSTEIN, 1981). These authors interpreted the Xy_p association as composed of chromosomal end-to-end contacts of heterochromatic nature, without further discussion of the possible role of the composition of the Xy_p. In the lady-beetle *Cycloneda sanguinea* NOR is located

on a acrocentric autosome pair (in preparation). POSTGLIONI & BRUM-ZORRILLA (1988) and POSTIGLIONI *et al*. (1991) described one pair of NORs with autosomal location in the chrysomelid *Chelymorpha variabilis* by Ag-NOR staining, orange acridine fluorescence, and microspreading techniques. This pattern led, these authors to reject JOHN & LEWIS (1960)'s hypothesis of a nucleolar origin for the Xy_p association. Observations supporting the nucleolus hypothesis have been reported in beetles, such as *Tenebrio molitor*, were FISH data are consistent with the classical hypothesis of a nucleolar origin for the Xy_p association (JUAN *et al*., 1993). Unfortunately, our results in *E. connexa* were unconclusive in this respect, and that further studies will be necessary, with the application of others techniques, such as FISH with rDNA probes.

Acknowledgements

We are grateful to Dr. Jorge A. Dergam (Universidade Federal de Viçosa, Minas Gerais, Brasil) for manuscript review and suggestions and Dra. Lúcia Massutti de Almeida (Departamento de Zoologia, Universidade Federal do Paraná, Curitiba-Pr). This work was supported by FAPEMIG, UFV, CNPq and CAPES.

Figure 1 - Male reproductive organs of *E. connexa*. A) Male with two testes, T: Testis; VD: vas deferens; DE: ejaculatory duct; F: folicle. B) Male with fused testis.

Figure 2 - Section of the testis shows the follicles with different stages of spermatocytes formations. A) 400x e B) 200X.

Figure 3 - Female reproductive organs of sexually mature adult *E. connexa*. CO: Common oviduct; SC: Spermathecal capsule; Oo: Oocyte; V: Vagina.

Figure 4 - Section of the ovarioles of *E. connexa* showing the oocytes (Oo) and the germarium (G). A=400x.

Figure 5 - Karyotype of *E. connexa* A) Female with 2n=18+XX. B) One pair chromosomes showing CMA_3^+ staining. C) Expression of NORs in one pair presented secondary constrictions. Bar=5 μm.

Figure 6 - Silver staining meiosis of an *E. connexa* male. A) Zygotene. The arrow indicates the vesicle sexual AG-NOR positive. B) Late zygotene showing two regions strongly silvers stained (arrowhead). C) Early pachytene shows the approximation of the AG-NORs positive regions. D) Pachytene shows the junction of these regions. E) Metaphase I. Note the "parachute" shaped sex chromosomes (arrows) (phase contrast). F) Metaphase I showing chromosome aspects after NOR banding. Bar = 5 µm.

REFERENCES

BONHAG, P.F., 1958. Ovarian structure and vitellogenis in insects. Ann. Rev. Entomol.: 3:137-160.

BÜNINGF, J., 1994. The insect ovary. Ultrastructure, previtellogenic growth and evolution. Chapman & Hall, p.400.

CHAVES, L.E.L., 1991. Levantamento e identificação de Coccinellidae (Coleoptera) em frutíferas na região de Jundiaí, SP. Piracicaba, (Mestrado:Escola Sup. de Agricultura "Luiz de Queiroz")/USP.

CHAPMAM, R.F., 1998 . *The insects: Struture and Function*. American Elsevier Publishing Company, INC.

CROWSON, R. A, 1981. *The Biology of the Coleoptera*. Academic Press, London.

DRETS, M.E., E. CORBELLA, F. PANZERA & G.A. FOLLE 1983. C-banding and nonhomologous associations. II The "parachute" Xy_p sex bivalente and behavior of heterochromatic segments in *Epilachna paenulata*. Chromosoma: 88:249:255.

FERREIRA, A. & A. MESA, 1977. Estudos citológicos em três espécies brasileiras de Coleópteros (Chrysomelidae, Cerambycidae e Meloidae). Rev. Bras. Biol.: 37:61-64.

FERREIRA, A., D. CELLA, J.R. TARDIVO & N. VIRKKI, 1984 Two pairs of chromosomes: A new low record for Coleoptera. Rev. Brasil. Genet.:
7:231-39.

GALIÁN, J. & B.P. MOORE, 1994. Chromosome numbers and sex-determining mechanism in Australian Carabidae (Coleoptera). Coleop. Bull.: 48:226-35.

GILL, T.K., M. GULATI & H.R. PAJNI, 1990. Chromosome numbers in Indian weevils (Coleoptera:Curculionoidea). Coleop. Bull.: 44:437-41.

GOODPASTURE, C. & S.E. BLOOM, 1975. Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma: 53:37-50.

GORDON, R.D., 1985. Coccinellidae (Coleoptera) of America North of México. J.New York Entomol. Soc.: 93(1):1-912.

HAGEN, K.S.,1974 The significance of predaceous Coccinellidae in Biological and pest Control of insects. *Entomophaga:* **7**:25-44, 1974.

HENNING, W., 1981. *Insect Phylogeny.* John Wiley & Sons, Chinchester.

JAGLARZ, M.K.& S.M. BILINSKI, 1998. Organization of the egg chambers in Hydradephaga (Insecta, Coleoptera-Adephaga) and its phylogenetic significance. Folia Biol.: 46:55-60.

JAGLARZ, M., 1988. Oogenesis in the common tiger beetle *Cicindela campestris* L. (Coleoptera). I. The structure of the ovary and organization of the egg chamber. Acta Biol Cracov Ser Zool.: 30:99-107.

JAGLARZ, M., 1992. Peculiarities of the organization of egg chambers in carabid ground and their phylogenetic implications. Tissue Cell :24:397-409.

JOHN, B. & K.R. LEWIS, 1960. Nucleolar controlled segregation of the sex chromosomes in beetles. Heredity: 15: 431-39.

JUAN, C.; J. PONS & E. PETITPIERRE, 1993. Localization of tandemly repeated DNA sequences in beetle chromosomes by fluorescent *in situ* hibridization. Chrom. Res.: 1:167-74.

KATAKURA, H.; S. NAKANO, T. HOSOGAI & S. KAHONO, 1994. Female internal reproductive organs, modes of sperm transfer, and phylogeny of Asian Epilachninae (Coleoptera:Coccinellidae). J. Nat. Hist.: 28:577-583.

KING, R.C. & J. BÜNING, 1985. *The origin and functioning of insect oocytes and nurse cells. In: Comprehensive Insect Physiology, Biochemistry, and Pharmacology,* Vol.1: Embryogenesis and Reproduction. Kerkut GA, Gilbert LJ (Eds) Pergamon Press, Oxford: 37-82.

LAWRENCW, C., 1982. *Coleoptera*, In: Synopsis and Classification of Living Organisms, vol.2. J. A. Parker (ed.) McGraw-Hill, New York. p. 482-553.

LAWRENCE, JF & A. F. NEWTON, 1982. Evolution and classification of beetles. Annu. Rev. Ecol. Syst:. 13:261-90.

LEEMAN, U. & F. RUCH, 1983. DNA and base content in the nuclei and the sex chromatin of *Rumex acetosa*. Bot. Helvetica: 93:77-83.

LEEMAN, U. & F. RUCH, 1984. Cytofluorometry and visualisation of DNA base composition in the chromosomes of *Drosophila melanogaster*. Chromosoma: 90:6-15.

LEVAN, A, K. FREDGA. & A.A SANDBERG, 1964. Nomenclature for centromeric position on chromosomes. Hereditas: 52:201-220.

LEVINS, R. & WILSON, M., 1980. Ecological theory and pest management. Annu. Rew. Entomol., Palo Alto: 25:287-308.

MATUSZEWSKI, B., K. CIECHOMSKI; J. NURKOWSKA. & M. KLOC, 1985. The linear cluster of oogonial cells in the development of telotrophic ovarioles in polyphage Coleptera. Roux 's Arch Dev Biol: 194:462-469.

MAFFEI, E.M.D.; E. GAAPARINO & S.G. POMPOLO, 2000. Karyotipic characterization by mitosis, meiosis and C-banding of *Eriopis connexa* Mulsant (Coccinellidae: Polyphaga), a predator of insect pests. Hereditas:132:79-85.

MAFFEI, E.M.D.; S.G. POMPOLO; J.C., SILVA-JUNIOR; A.P.A,CAIXEIRO; M.P., ROCHA & DERGAM, J.A., 2001. Silver staining of of nucleolar regions (NORs) in some species of Hymenptera (bees and parasitic wasp) and Coleoptera (lady-beetles). Cytobios: 104:119-125.

MICHELS, G.L.JR., R.V. FLANDERS & J.B. BIBLE, 1997. Overwintering survival of seven imported coccinellids in the Texas high plains. Southwestern-Entomologist: 22:157-166.

MILLER, J.C., 1995. A comparison of techniques for laboratory propagation of a South American ladybeetle, *Eriopis connexa* (Coleoptera: Coccinellidae). Academic Press, Inc. Sept.: 5:462-465.

MARTINS, V.G., 1994. The chromosomes of five species of Scarabaidae (Polyphaga, Coleoptera). Naturalia:19:89-96.

PETITPIERRE, E.; C. SEGARRA, J.S. YADAV & N. VIRKKI, 1988. *Chromosome numbers and meioformulae of Chrysomelidae, In Biology of Chrysomelidae.* P. Jolivet, E. Petitpierre and T. H. Hsiao (eds) Kluwer Academic, Dordrecht., pp. 161-86.

PETITPIERRE, E., 1996. Molecular cytogenetics and taxonomy of insects, with particular reference to the Coleoptera. Int.J. Morphol. & Embryol.: .25:115-133.

PEARSE, A.G.E., 1986. *Histochemistry. Theoretical and Applied.* J. & A.Churchill Ltd. London p.530.

POSTIGLIONI, A.; M. STOLL & N. BRUM-ZORRILLA, 1991. Haploid karyotype analysis of *Chelymorpha variabilis* Boheman (Coleoptera, Chrysomelidae) with microspreading techniques. Rev. Bras. Genet.: 14 653-60.

POSTIGLIONI, A. &. N. BRUM-ZORRILLA, 1981. Localización de regiones organizadoras nucleolares (NORs) en otra especie con sistema sexual Xy_p *Calligrapha polyspila* (Coleoptera, Chrysomelidae, Chrysomelinae). Res. Comum. Jornadas Cienc. Nat. Montevideo: 2.

POSTIGLIONI, A. & N. BRUM-ZORRILLA, 1988. Non-relationship between nucleolus and sex chromosome system Xy_p in *Chelymorpha variabilis* Boheman (Coleoptera: Chrysomelidae). Genetica: 77 137-41.

QUIROGA, D., P. ARRETZ & J.E. ARAYA, 1991. *Sucking insects damaging jojoba, Simmondsia chinensis (Link) Schneider, and their natural enemies, in the North Central and Central regions of Chile.* Crop-Prot. Oxford: Butterworths-Heinemann. LTD: 10:469-479.

SERRANO, J., 1981. Chromosome numbers and karyotype evolution of Caraboidea. Genetica: 55:51-60.

SERRANO, J & J.S. YADAV, 1984. Chromosome numbers and sex-determining mechanisms in Adephagan Coleoptera. Coleopt. Bull.: 38:335-57.

SHARMA, G..P., T.K. GILL & V. PAL, 1980. Chromosomes in the curculionid beetles (Coleoptera, Curculionidae). Coleopt. Bull.: 34:361-67.

SIMICZYJEW, B., A. OGORZALEK & P. STYS, 1998. Heteropteran ovaries: variations on the theme. Folia Histochemica et Cytobiologica: 36:147-156.

SMITH, S.G., 1950. The cytotaxonomy of Coleoptera. Can Entomol: 82:58-68.

SMITH, S.G. & N. VIRKKI, 1978. *Animal cytogenetics*. Coleoptera. Berlin:Gebruder Borntraeger:1-365.

STEBBINGS, H., 1981. Observations on cytoplasmic transport along ovarian nutritive tubes of polyphagous coleopterans. Cell Tissue Res .: 200:153-161.

SCHWEIZER, D., 1980. Simultaneous fluorescent staining of R bands and specific heterochromatic regions (DA-DAPI bands) in human chromosomes. Cytogenet. Cell Genet.: 27:190-3.

SWEETMAN, H.L., 1958. *The principles of biological control.* Dubuque, W.M.C. Brown, 560p.

SUMNER, A.T., 1990. *Chromosome Banding*. Unwin Hyman, London

UENO, H., 1994. Fluctuating asymmetry in relation to two fitness components, adult longevity and male mating success in a ladybird beetle, *Harmonia axyridis* (Coleoptera:Cocconellidae). Ecol. Entomol.: 19:87-88.

ULLMANN, S.L, 1973. Oogenesis in *Tenebrio molitor*: Histological and autoradiographical observations on pupal and adult ovaries. J Embryol. Exp. Morphol.: 30:179-217.

VIDAL, O.R., 1984. Chromosome numbers of Coleoptera from Argentina. Genetica: 65:235-239.

WETTSTEIN, R., 1981. *Unusual mechanisms of chromosome pairing in arthropods*. International Cell Biology 1980-81. Edited by H.G. Schweizer , Springer-Verglag. p. 187-94.

SEQUENTIAL FISH ANALYSIS WITH RDNA GENES AND AG-NOR BANDING OF THE MELANIC FORM OF *OLLA V-NIGRUM* (LADY BEETLE)

Artigo enviado para revista Hereditas.

ABSTRACT

The objective of the present study was to characterize the meiosis of *Olla v-nigrum* by standard analysis, to perform a NOR study using NOR banding, FISH of rDNA genes and sequential FISH/AgNOR analysis, and to describe the adaptation of the FISH methodology to Coccinellidae. The chromosome number determined at metaphase I was $n=9+Xy_p$. At zygotene it was possible to identify the sex vesicle which presented a deeply stained heteropycnotic block. Chromosome X was much larger than the y and the two combined, forming a "parachute" in metaphase I. FISH analysis using a probe of rDNA genes 18S, 28S and 5.8S of *D. melanogaster* was used to map the genes in the sex vesicle. The NOR band presented high gene activity in this region and sequential FISH/Ag NOR analysis confirmed these results. The data obtained for *Olla v-nigrum* agree with the classical hypothesis raised to explain the type of sex chromosome association in a parachute format (Xy_p) as being due to the presence of nucleolar material. The chromosome number and parachute configuration during metaphase I in this species agree with the description for most Coleoptera species (basic karyotype).

INTRODUCTION

The melanic form of the lady beetle *Olla v-nigrum* originating from the New World belongs to the order Coleoptera, family Coccinellidae, which comprises approximately 4000 species, most of them being effective predators mainly of aphids (De Bach, 1964; Gordon, 1985). Many species have been efficiently used in programs of pest management and there is great interest in a better study and evaluation of these organisms (Smith & Reynolds, 1966; De Bach, 1964; Levins & Wilson, 1980; Chaves, 1991). Particularly important is the fact that these insects can be used in studies on the environmental impact of transgenic plants. A recent study conducted in Switzerland showed that lady beetles fed on aphids, which in turn had fed on transgenic potatoes, reduced their ovipositing and suffered a 50% reduction in life span (Birch *et al*, 1997).

The method of fluorescent *in situ* hybridization (FISH) first developed by Langer-Safer *et al*. (1982) has become an efficient and versatile technique for both the localization and mapping of specific sequences and for the identification of chromosomal DNA alterations in different organisms (Maluszynska & Heslop-Harrison, 1991). The efficiency of the technique is high even when the chromosomes are of small size (Moyzis *et al*., 1988; Traski 1991; Hamilton *et al*., 1992), as is usually the case for Coleoptera (Smith & Virkki 1978; Juan *et al*., 1993). There are relatively few reports of FISH applications in insects (Petitpierre, 1996). The FISH technique with rDNA genes differs from NOR banding since it reveals the number of chromosome pairs of a given taxon that carry rDNA genes, while silver staining reveals active NORs in a given stage of the cell cycle (Galián *et al*., 1995). However, in ant *Tapinoma nigerrimum* NOR-banding results have shown that several chromosomes were stained with silver and the FISH technique mapped the rDNA genes on a single chromosome pair (Lorite *et al.* 1997).

The application of both methods is of particular interest for the study of beetles of the suborder Polyphaga, especially to clarify the non-chiasmatic associations of sex chromosomes such as the sex system of the XY_p type in Coleoptera (Petitpierre, 1996). John and Lewis (1960) proposed the nucleolar theory, suggesting that the association of sex chromosomes was due to the presence of nucleoli associated with these chromosomes and several authors agree with this theory (Smith and Virkki, 1978; Virkki and Sepúlveda, 1990; Virkki *et al*., 1990). However, the nucleolar theory was questioned by other investigators who found nucleoli in an autosomal chromosome pair (Drets *et al.*, 1983; Virkki 1983; Weber 1971 Postiglioni and Brum Zorrilla 1988; Postiglioni *et al*. 1991). The subject is controversial because in both the cases described above the sex vesicle forms during meiosis (prophase I) in Coleoptera males with the Xy_p, sex system and is stained with silver when NOR banding is performed. In the Coccinellidae family, the FISH method has not been applied to any species thus far. The objectives of the present study were to map the rDNA genes 18, 28 and 5.8S by FISH during meiosis in *Olla v-nigrum*, and to compare this localization with those of silver-stained NORs.

MATERIAL AND METHODS

Fifteen *Olla v-nigrum* males were collected in Viçosa-MG where they occur naturally. Slides to be submitted to FISH were prepared by the method of MAFFEI *et al.* (2000) without using colchicine**.** The testes were removed in Ringer and transferred to a slide to which several drops of fixative I (4 water:3 ethanol:3 acetic acid) was applied. The testes were dissociated with a stylet and two drops of fixative 2 (1 ethanol:1 acetic acid) were added, followed by three drops of fixative 3 (100% acetic acid).

The *in situ* hybridization technique (FISH) was based on the methods of Viégas-Péquignot (1992) and Juan et al. (1993), with adaptations.

rDNA probes (pDm 238) containing ribosomal genes 28S, 18S and 5.8S of *Drosophila melanogaster* were labelled with biotin by nick translation according to the instruction manual of the BIONICK kit (GIBCO). Slides with the fixed meiocytes were incubated with RNase (100 µg/ml in 2xSSC) for 1 h in a chamber humidified with water at 37ºC, treated with 0.005% pepsin in 10 mM HCL at 37ºC for 10 minutes, and then treated with three 2xSSC baths for 3 minutes per bath. The material was dehydrated in 50%, 75% and 100% alcohol for 3 minutes per treatment.

Simultaneous hybridization and denaturation.

Five 5 µl of the probe (1 µl probe diluted in 4 µl of hybridization mixture) was added to each hybridization area and the slide was covered with a

20 x 20 mm coverslip and incubated at 80ºC for 3 minutes. Overnight incubation was then performed at 37ºC.

After incubation, the slides were treated with two 50% formamide baths of 2 minutes each at 37ºC and then with two PBT baths of 2 minutes each.

Immunological detection.

The slides were treated with anti-biotin (VECTOR:SP3000) and anti-goat IgG-FITC (VECTOR:FI5000). DNA was counterstained with 100 µl propide iodide, the slides were washed with PBS for 1 minute and mounted with 13 µl Vectashied. The material was photographed with an Olympus BX60 photomicroscope using an appropriate fluorescence filter.

NOR banding was performed as described Maffei *et al*. (2001).

Two parts of developer (2 g of gelatine dissolved in 100 ml of distilled water and 1 ml of formic acid) and four drops of $AgNO_3$ (Merck) in 50% aqueous solution were mixed on slides. Then, the material was covered with a coverslip, transferred to Petri dishes, overlaid with a moist filter paper and incubated at 38ºC for 25 to 30 minutes, until the staining solution turned golden brown. The coverslip was rinsed off first with tap water and then the slide was squirted vigorously with a 20 ml syringe. After the drying process the slide was mounted on Entellan (Merck). After placing slides in a warm oven (37ºC) for 24 hours, metaphases were photographed with a light microscope.

RESULTS

The specimens of the melanic form of *Olla v-nigrum* submitted to standard staining (meiosis) showed the $n=9+Xy_p$ meio formula in metaphase I. During prophase I, two more intensely stained points (heteropycnotic) were obserevd in leptotene (Fig. 1A). In zygotene it was possible to identify the sex vesicle which presented a deeply stained large heteropycnotic block (Fig.1B). In pachytene the chromosomes were already individualized and chromosome association was observed in the heteropycnotic block (Fig. 1C). Chromosome X was much larger than the y and the two combined to form a parachute in metaphase I (Fig. 1D and E). Few cells with anaphase bridges were observed (Fig. 1F). FISH analysis using a probe of rDNA genes of *D. melanogaster* mapped the genes in the sex vesicle (Fig. 2A and B). After sequential FISH/Ag NOR analysis, the sex vesicle was stained with silver, indicating gene activity. We observed that in the region where the probe hybridized completely there was little or no silver staining. (Fig. 2C and D). Figure 3A illustrates a block of rDNA genes mapped in the sex vesicle (FISH). NOR banding alone revealed larger silver-stained blocks which combined to form the sex vesicle (Fig.3. B,C and D) and a large single block was strongly stained with silver in pachytene (Fig.3E).

DISCUSSION

Olla v-nigrum males are cytogenetically characterized by the presence of a chromosome number $n=9+Xy_p$. The chromosome number and parachute configuration during metaphase I observed in this species agree with the

description of most Coleoptera species. In the family Coccinellidae, approximately 42% of the karyotypes are of this basic type (Smith and Virkki, 1978). Recently, our group (MAFFEI *et al*., 2000) characterized the karyotype (mitosis, meiosis and constitutive heterochromatin) of both sexes of *Eriopis connexa* in a population from Viçosa, MG, Brazil, and detected n=9+Xyp in male meiosis and one small-sized supernumerary chromosome restricted to females.

The analysis of meiosis (prophase I) using only NOR banding indicated that the meiocytes of *Olla v-nigrum* had an active NOR region in the sex vesicle FISH of rDNA genes mapped this region in the sex vesicle (prophase I) and sequential AG-NOR staining confirmed gene activity in the same region. However, silver staining was reduced due to the use of pepsin. The results described for *Olla v-nigrum* agree with the classical hypothesis suggested by some investigators to explain that the association of the sex chromosomes (Xy_p) may be due to the presence of nucleolar material (John and Lewis 1960; Smith and Virkki, 1978).

The FISH technique using a rDNA probe has been little applied to Coleoptera. Juan *et al*. (1993) applied FISH with rDNA probes to *Tenebrio molitor* and *Misolampus goudoti*, both with 2n= 20 and an Xy_p sex determination system. Analysis of mitotic metaphase of *T. molitor* showed that the NORs are located in two autosomal chromosome pairs and in the sex pair, supporting the classical hypothesis of a nucleolar origin of the Xy_p association. However, in *M. goudoti* the rDNA genes were mapped only on an autosome pair. FISH of rDNA genes was applied to *Cicindela melancholica* and the results revealed that the rDNA genes are located in one of the three X chromosomes and in the y (multiple sex system). In contrast, in *Cicindela paludosa* (a related species) which presents an XO sex system, the rDNA genes were mapped on a pair of autosomal chromosomes (Galián *et al*., 1995). FISH of the rDNA genes 18S-28S was applied to other *Cicindela* species (Cicindelini tribe) and in three species, *Cicindela cardinalba*, *Cicindela* sp. (saetigera group) and *Cicindela gillesensis*, the genes were mapped on two of the four sex chromosomes that formed the sex vesicle. However, in *Megacephala whelani,* hybridization during meiosis and

mitosis revealed that the rDNA genes were localized in three autosome pairs (Galián & Hudson, 1999). 18S rDNA genes were detected in the largest autosome pair in 12 species of *Carabus* and two of *Calosoma* (Carabini tribe) and in three related species of *Ceroglossus chilensis* (Ceroglossini tribe). The results suggested the existence of a conservative pattern in these genera. However, in *Cychrus* carabids (Cychrini tribe), the ribosomal cistrons were localized in two medium-sized autosome pairs (De la Rua *et al.* 1996). FISH data obtained for seven species of chrysomelids of the genus *Timarcha* revealed that in all species except one with the neoXy system, the rDNA genes were in an autosomal pair, even though Xy_p was strongly silver stained (Gómez-Zurita & Petitpierre, personal communication).

The major adaptation of the methodology was simultaneous denaturation and hybridization, as first proposed by Juan *et al.* (1993), which permitted the preservation of chromosome morphology, an essential factor especially when the chromosomes are of small size, as is usually the case for Coleoptera (Smith & Virkki 1978; Juan *et al*., 1993). Usually, mitotic metaphase chromosomes are employed for FISH analysis. However, other phases can be used, such as prophase I of meiosis which has the following advantages: 1) cells free of cell debris which might interfere with probe penetration, 2) chromatin that is little condensed in this phase and is highly accessible to the probe, and 3) considerably voluminous cells showing a ten-fold increase in size, permitting high resolution mapping by FISH (Peterson *et al*. 1999).

These rDNA and others, used as probes, could probably identify marker chromosomes for evolutionary studies, and for the larger rDNA loci, the level of correspondence between the results of FISH and Ag-NOR staining is also pertinent for measuring gene activity at NOR sites and possible shifts during evolution (Petitpierre, 1996). This was reported, for example, for the Australian primitive ant group of *Myrmecia pilosula* complex, in which great changes in diploid numbers are paralleled by increases in the number of NORs (Hirai *et al*., 1994). Numerical and structural polymorphism in rDNA genes was reported by Vitturi (et al. 1999) for the coleopteran *Thorectes intermeditus* using FISH. The

results showed numerical and structural inter- and intraindividual polymorphism in NOR number and size. FISH was recently used in Coleoptera to map the loci of the rDNA gene 18S in 19 taxa of the genus *Zabrus* (2n=47-63). Variations were detected both in the number of chromosomes bearing rDNA gene clusters (2-12) and in the signals of hybridization, which varied from small points up to the entire chromosome arm (Sanchez-Gea *et al*. 2000).

Acknowledgements

We are very grateful to Dr. Shirlei M. Recco-Pimentel, Klélia Santos, Luciana B. Lourenço for providing the labeled *Drosophila* rDNA probe and technical assistance, and to Dr. Lúcia Massutti de Almeida (Departamento de Zoologia, Universidade Federal do Paraná, Curitiba-Paraná) for identifying the species used in the present study. Research supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) and Universidade Federal de Viçosa (UFV) Viçosa, MG, Brasil. E. P. has been funded with the project BOS2000-0822, Ministry of Science and Technology of Spain.

Figure 1) Meiosis of *Olla v-nigrum* males. A) Prophase I (leptotene). B) Zygotene. C) Pachytene. D and E) Chromosome X was much larger than chromosome Y and the two combined to form a parachute in metaphase I. F) Few cells formed anaphase bridges. The arrowheads indicate the sex vesicle and the arrow indicates the parachute. Bar=5µm

Figure 2. *Olla v.nigrum.* A and B) FISH rDNA genes in the sex vesicle. C and D) Sequential FISH/Ag NOR, with the sex vesicle being stained with silver. Bar=5µm.

Figure 3. *Olla v.nigrum.* A) FISH of rDNA genes at zygotene. B, C and D) NOR banding (alone) revealed large silver-stained blocks which associated to form the sex vesicle. E) Pachytene showing a large strongly silver-stained single block. Bar=5µm.

REFERENCES

Birch ANE, Geoghegan I.E., Majerus MEN, Hackett C and Allen J, (1997). Interactions between plant resistance genes, pests, aphid populations and beneficial predators. Scottish Crop Research Institute, Annual Report 1995/97 pp.68-72 SCRI:Dundee.

Chaves LEL, (1991). Levantamento e identificação de Coccinellidae (Coleoptera) em frutíferas na região de Jundiaí (Master's Thesis, Escola Superior de Agricultura "Luiz de Queiroz")/USP.

De Bach P, (1964). Biological control of insects pests and weeds. London, Chapman and Hall.

De la Rua P, Serrano J, Hewitt GM and Galián J, (1996). Physical mapping of rDNA genes in the ground beetle *Carabus* and related genera (Carabidae: Coleoptera). J. Zool. Syst. Evol. Res. 34:95-101.

Drets ME, Corbella E, Panzera F and Folle GA, (1983). C-banding and nonhomologous associations. II The "parachute" Xy_p sex bivalent and behavior of heterochromatic segments in *Epilachna paenulata*. Chromosoma 88:249:255.

Galián J, Serrano J, La Rúa P, Petitpierre E and Juan C, (1995). Localization and activity of rDNA genes in tiger beetles (Coleoptera: Cicindelinae). Heredity 74:524-530.

Galián J and Hudson P, (1999). Cytogenetic analysis of Australian tiger beetles (Coleoptera: Cicindelidae): chromosome number, sex-determining system and localisation of rDNA. J. Zool. Syst. Evol. Res. 37:1-6.

Gordon RD, (1985). Coccinellidae (Coleoptera) of America North of México. Journal of the New York Entomological Society 93(1):1-912.

Hamilton M.J, Hong G and Wichman HA, (1992). Intragenomic movement and concerted evolution of satellite DNA in *Peromyscus*: evidence from *in situ* hybridization. Cytogenet. Cell Genet. 60:40.

Hirai H, Yamamoto MT, Ogura K, Satta Y, Yamada M, Taylor RW and Imai HT, (1994). Multiplication of 28S rDNA and NOR activity in chromosome evolution among ants of the *Myrmecia pilosula* species complex. Chromosoma 103:171-78.

John B and Lewis KR, (1960). Nucleolar controlled segregation of the sex chromosome in beetles. Heredity 15:431-439.

Juan C, Pons J and Petitpierre E, (1993). Localization of tandemly repeated DNA sequences in beetle chromosomes by fluorescent *in situ* hybridization. Chrom. Research 1:167-174.

Langer-Safer PR, Levine M and Ward DC, (1982). Immunological method for mapping genes on *Drosophila polytene* chromosomes. Proc. Natl. Acad. Sci. 79:4381-4385.

Levins R and Wilson M, (1980). Ecological theory and pest management. Annual Rewiew of Entomology 25:287-308.

Lorite P, Aránega AE, Luque F and Palomeque T, (1997). Analysis of the nucleolar organizing regions in the ant *Tapinoma nigerrimum* (Hymenoptera, Formicidae). Heredity 78:578-582.

Maluszynska J and Heslop-Harrison JS, (1991). Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. Plant J. 1:159-166.

Maffei EMD, Gasparino E and Pompolo SG, (2000). Karyotypic characterization by mitosis, meiosis and C-banding of *Eriopis connexa* Mulsant (Coccinellidae: Coleoptera: Polyphaga), a predator of insect pests. Hereditas 132:79-85.

Maffei EMD, Pompolo SG, Silva-Junior JC, Caixeiro APA., Rocha MP and Dergam JA, (2001). Silver staining of nucleolar organizer regions (NORs) in some species of Hymenoptera (bees and parasitic wasps) and Coleoptera (lady-beetle). Cytobios 104: 119-125.

Moyzis RK, Buckingham JM and Cram LS, (1988). A highly conserved repetitive DNA sequence (TTAGGG)n present at the telomeres of human chromosomes. Proc. Natl. Acad. Sci. 85:6622-6626.

Peterson DG, Lapitan NLV and Stack SM, (1999). Localization of single and low copy sequences on tomato synaptonemal complex spreads using fluorescence in situ hybridization (FISH). Genetics 152:427-439.

Petitpierre E, (1996). Molecular cytogenetics and taxonomy of insects, with particular reference to the Coleoptera. Int. J. Insect Morphol. and Embryol. 25:115-133.

Postiglioni A and Brum-Zorrilla N, (1988). Non-relationship between nucleolus and sex chromosome system Xy_p in *Chelymorpha variabilis* Boheman (Coleoptera: Chrysomelidae). Genetica 77:137-41.

Postiglioni A., Stoll M and Brum-Zorrilla N, (1991). Haploid karyotype analysis of *Chelymorpha variabilis* Boheman (Coleoptera, Chrysomelidae) with microspreading techniques. Rev. Bras. Genet. 14:653-60.

Sanchez-Gea JF, Serrano J and Galián J, (2000). Variability in rDNA loci in Iberian species of the genus *Zabrus* (Coleoptera: Carabidae) detected by fluorescence in situ hybridization. Genome 43:22-28.

Smith SG and Virkki N, (1978). Animal cytogenetics. Coleoptera. Berlin:Gebruder Borntraeger p.1-365.

Smith RF and Reynolds HT, (1966). Principles, definitions and scope of integrated pest control. In: FAO Symposium on Integrated Pest Control. Proceedings. Rome p.11-17.

Trask BJ, (1991). Fluorescent in situ hybridization: applications in cytogenetics and gene mapping. Trends Genet 7:149-154.

Viégas-Péquignot E, (1992). In situ hybridization to chromosomes with biotinyled probes In: In situ hybridization: a practical approach (Willernson, D ed.). Oxford University Press, IRL, Press pp137-158.

Vitturi R., Colomba MS, Barbieri R and Zunino M, (1999). Ribosomal DNA location in the scarab beetle *Thorectes intemedius* (Costa) (Coleoptera: Geotrupidae) using banding and fluorescent *in-situ* hybridization. Chromosome Res. 7:255-60.

CARACTERIZAÇÃO CARIOTÍPICA, ANÁLISE DA HETEROCROMATINA CONSTITUTIVA E DA REGIÃO ORGANIZADORA DE NUCLEÓLO EM *CYCLONEDA SANGUINEA* LINNEU (COLEOPTERA:COCCINELLIDAE) OCORRENTE EM VIÇOSA (MG)

Manuscrito a ser enviado à revista Hereditas. Periódico pubicado pela Lund, Sweden ISSN 0018-0661.

RESUMO

Análises citogenéticas foram realizadas em *Cycloneda sanguinea,* utilizando células mitóticas (gânglio cerebral) e meióticas (gônadas de machos), caracterizando o número e a morfologia dos cromossomos, a distribuição e a constituição da heterocromatina constitutiva , a localização da região organizadora de nucléolos por meio de banda NOR (Ag-NOR), genes rDNA (FISH) e coloração seqüencial FISH/Ag-NOR. As fêmeas apresentaram 2n=18+XX e os machos 2n=18+Xy. Os cromossomos foram agrupados em 2 pares M (metacêntrico), 4 pares SM (submetacêntrico) e 3 pares ST (subtelocêntrico). O cromossomo X foi do tipo M e o y com tamanho muito reduzido. Na meiose, a meio-fórmula cariotípica foi n=9+Xyp, sendo a associação dos cromossomos sexuais em pára-quedas. Na prófase I no início do paquíteno, os bivalentes mostraram-se individualizados e os cromossomos sexuais se associaram ponta-a-ponta linearmente, pela região mais heteropicnótica do cromososmo X. Com a progressão do paquíteno, ocorreu uma maior compactação, formando um pseudo-anel, no bivalente sexual. Na metáfase I, os cromossomos sexuais formaram o pára-quedas. A heterocromatina constitutiva mostrou distribuição pericentromérica em todos os cromossomos, e a utilização de enzima de restrição *Hae* III revelou DNA rico em seqüências GC. Entretanto, em algumas regiões cromossômicas (telômeros) não houve digestão. A utilização da enzima *Msp*I forneceu resultado negativo. A banda HSS (Hot Saline Solution) extraiu a heterocromatina, sugerindo que a mesma seja constituída em grande parte por DNA rico em pares de bases AT. Com base nestes resultados, pode-se inferir que a heterocromatina do genoma de *C. sanguinea* possa ser de natureza molecular heterogênea. Os resultados das análises da região organizadora de nucléolo, por Ag-NOR, FISH e coloração seqüencial FISH/AgNOR, mostraram os genes ativos em um par autossômico (metáfase espermatogonial). Na meiose, os genes foram mapeados por FISH fora da vesícula sexual, apesar do Xy_p se impregnar fortemente pela prata. Assim, nestes resultados pode-se inferir que essas substâncias argirofílicas sejam de

natureza protéica nucleolar, sendo produzidas pelo par autossômico e importadas na meiose (prófase I) durante a associação dos cromossomos sexuais.

INTRODUÇÃO

A joaninha, *Cycloneda sanguinea*, pertence à ordem Coleoptera, família Coccinellidae que apresenta aproximadamente 4.000 espécies. Muitas espécies têm sido utilizadas com eficiência, em programas de manejo de pragas, por serem eficazes predadores principalmente de afídeos (BORROR e De LONG, 1988; De BACH, 1964; GORDON, 1985; SMITH e REYNOLDS, 1966; De BACH, 1964; LEVINS e WILSON, 1980). Outra grande importância destes insetos benéficos, é a sua utilização em estudos relacionados com o impacto ambiental, causado por plantas transgênicas. Recentemente, descobriu-se que joaninhas alimentadas com pulgões, que, por sua vez, foram alimentados com batatas transgênicas, ocorreu diminuição na postura e viveram a metade do tempo de vida (BIRCH *et al.*, 1997).

Hibridização "in situ" fluorescente (FISH) tem se tornado uma técnica poderosa e versátil para localização e mapeamento de seqüências específicas do DNA em cromossomos de diferentes organismos, mesmo quando estes são de tamanho pequeno (MALUSZYNSKA e HESLOP-HARRISON, 1991; MOYZIS *et al*., 1988; TRASKI 1991; HAMILTON *et al*., 1992), como são geralmente os de coleópteros (SMITH e VIRKKI 1978; JUAN *et al*., 1993).

A metodologia de banda Ag-NOR vem fornecendo resultados importantes e úteis para avaliar a atividade gênica desta região, principalmente em besouros, pois fornecem informações sobre a arquitetura dos cromossomos e contribuem para o entendimento da evolução dos cariótipos (PETITPIERRE, 1996).

Vários autores formularam a hipótese clássica da teoria nucleolar, para explicar o tipo peculiar de associação dos cromososmos sexuais Xy_p. Esta teoria vem sendo confirmada com utilização do FISH com sonda de genes rDNA, como descrito em algumas espécies de *Cicindelas* australianas (GALIÁN e HUDSON, 1999). Por outro lado, em algumas espécies, têm sido encontrados os genes rDNA somente nos cromossomos autossômicos, como em crisomelídeos do gênero *Timarcha* (GOMEZ-ZURITA e PETITPIERRE, comunicação pessoal).

Na família Coccinellidae, as análises citogenéticas são escassas, foram realizadas aproximadamente em 3% das espécies e mostram que o cariótipo básico n=9+Xy_p descrito é de grande ocorrência (aproximadamente 42%). As análises citogenéticas em *Cycloneda sanguinea* são quase que inexistentes, sendo descrito somente o número cromossômico na meiose de machos n=9+Xy_p (SMITH e VIRKKI, 1978).

Neste trabalho, descreveram-se o número e a morfologia dos cromossomos mitóticos metafásicos, avaliou-se o comportamento dos cromossomos durante a meiose, descreveram-se a distribuição e constituição molecular da heterocromatina constitutiva e realizou-se um estudo detalhado da região organizadora de nucléolo no genoma de *Cycloneda sanguinea* L.

MATERIAL E MÉTODOS

Espécimes de *Cycloneda sanguinea* L. foram coletadas no Campus da UFV (Universidade Federal de Viçosa), onde ocorrem naturalmente. Foram analisadas por mitose 12 pré-pupas e meiose 40 machos adultos, avaliando-se em média 10 metáfases mitóticas por indivíduo.

As preparações citogenéticas de cromossomos mitóticos metafásicos (gânglio cerebral) e análise da meiose foram conforme MAFFEI *et al*. (2000).

Bandeamento C foi realizado segundo os procedimentos de MAFFEI *et al.* (2000)

As lâminas foram submetidas aos seguintes tratamentos: a) Hidrólise com HCl 0.2 N por 4 minutos na temperatura ambiente; b) Lavadas com água destilada e incubadas com hidróxido de bário 5% a 60ºC por 8 minutos; c) Lavadas à temperatura ambiente com HCl 0.2 N por 30 segundos; d) Incubadas com 2xSSC a 60ºC por 10 minutos; e e) Coradas com Giemsa (2 ml Giemsa: 30 ml tampão Sorensen 0.06M pH 6.8) por 50 minutos.

Digestão com Enzimas de Restrição (GOSÁLVEZ *et al.*, 1987)

Foram utilizadas as enzimas *Msp-I* que cliva as regiões do DNA rico em seqüências de bases C/GGC; e a enzima *Hae* III que cliva CC/GG. Estas foram suspensas em tampões apropriados. Sobre o material fixado foram adicionados 30µl de solução, contendo 1µl da enzima (10U), 9µl de solução tampão apropriados à enzima e 90 µl de água milli-Q. As lâminas foram incubadas a 37ºC por 12 horas, lavadas com água destilada e coradas com Giemsa por 40 minutos.

Bandeamento por Tratamento a Quente com Solução Salina (Banda HSS) (VERMA e BABU, 1989)

As lâminas, após seis dias de preparação convencional, foram imersas em tampão fosfato Sorënsen, pH 6.8, a 85ºC, por 5 minutos, lavadas com água destilada e coradas com Giemsa a 5%, por 7 minutos.

Para detectar as regiões organizadoras de nucléolos ativas foi utilizada a técnica de impregnação por prata conforme descrita por Maffei *et al*. (2001)

As lâminas foram preparadas com um dia de antecedência sem corar, e, posteriormente, adicionou-se uma gota de gelatina (2 g, diluída em 100 ml de água destilada e 0,5 ml de ácido fórmico) e quatro gotas de solução aquosa de Ag-NO_3 a 50% (Merck). O material foi coberto com lamínula e incubado em câmara úmida a 38% por 25 minutos. As lâminas foram lavadas com jatos fortes por, aproximadamente, 5 minutos, montadas permanentes com etellan (Merck) e as melhores metáfases foram fotografadas.

O mapeamento dos genes rDNA foi realizado por meio da técnica de hibridização "in situ" fluorescente (FISH) (Viégas-Péquignot, 1992)

Sondas de rDNA (pDm 238), que continham os genes 18S, 28S e 5.8S, foram marcadas com biotina por "nick translation", seguindo o kit BIONICK – GIBCO.

As lâminas com os meiócitos já fixados foram incubadas com RNase (100µg/ml) por 1h em câmara úmida de formamida 70%, a 37ºC. A seguir foram incubadas em três banhos de 2xSSC por 3 minutos cada e desidratadas em álcool 50%, 75% e 100% por 3 minutos cada. Após a desnaturação do DNA cromossômico em formamida 70% por 2 minutos a 70ºC, as lâminas foram desidratadas em 2xSSC gelado por 2 minutos e transferidas para álcool 50%, 75% e absoluto por 2 minutos cada. Paralelamente, a sonda foi incubada a 100ºC por 2 minutos e mantida no gelo. A seguir, adicionaram-se, na lâmina, 10 µl de sonda (diluída em mistura de hibridação) por área de hibridação, e foram incubadas em câmara úmida de formamida 70% a 37ºC por 36 h. Após o período

de incubação, as lâminas foram tratadas com 2 banhos de formamida a 50% 37ºC por 2 minutos cada, lavadas com 2xSSC a 37ºC por 2 minutos cada. Em seguida, as lâminas foram tratadas com 2 banhos de PBT por dois minutos cada.

Detecção Imunológica

As lâminas foram tratadas com antibiotina (VECTOR:SP3000) e anti-goat IgG-FITC (VECTOR:F I5000). O DNA foi contracorado com 100 µl de Iodeto de Propídeo por 1 minuto, lavados com PBS e as lâminas foram montadas com 13 µl de Vectashied. As fotografias foram feitas, usando o fotomicroscópio Olimpus BX60 como filtro para fluorescência apropriado.

RESULTADOS

Meiose e Mitose: Coloração Convencional.

Os espécimens de *Cycloneda sanguinea* analisados por meiose apresentaram na prófase I (zigóteno) um grande cromocentro heteropicnótico regular único (Fig. 1A e 1B). Durante o início do paquíteno, os bivalentes estavam individualizados e os cromossomos sexuais se associaram ponta-a-ponta, linearmente. Com a progressão do paquíteno, ocorreu condensação do DNA e formou-se um pseudo-anel, pelos bivalentes sexuais (Fig.1C). Na metáfase I, a meio-fórmula foi n=9+Xyp (Fig. 1D), e na anáfase I a segregação dos cromossomos foi normal.

Cromossomos mitóticos de dez fêmeas de *Cycloneda sanguinea* analisados por coloração convencional apresentaram 2n=18+XX e os dois machos 2n=18+Xy. Os cromossomos dessa espécie foram na maioria SM (submetacêntricos) e sexual y muito pequeno (Fig. 2A e 2B).

Análise da Heterocromatina Constitutiva por Banda C, Banda HSS e Enzimas de Restrição

A análise de banda C revelou que a heterocromatina constitutiva se localizou nas regiões pericentroméricas de todos os cromossomos, incluindo os braços curtos (Fig. 2C). A banda HSS foi positiva na região da heterocromatina, o que deixou essa região pouco corada em todos os cromossomos (Fig. 3A). O tratamento das metáfases espermatogoniais com a enzima de restrição *Msp*I foi negativo, para todos os cromossomos do complemento (Fig. 3B). O tratamento com a enzima *Hae* III foi positivo, principalmente em alguns pares autossômicos e no cromossomo y, digerindo estas regiões (Fig. 3C).

Localização de genes para rDNA e Banda Ag-NOR

A técnica de FISH de genes de rDNA mapeou um grande bloco bem evidente na prófase I (zigóteno) (Fig. 4A). A análise em seqüuência de FISH/AgNOR mostrou que os genes de rDNA se localizaram fora da vesícula sexual (Fig. 4B) que ficou fortemente impregnada pela prata (Fig. 4C). O núcleo interfásico mostrou duas grandes regiões Ag-NOR positivas (Fig. 4D). Na metáfase espermatogonial, foram mapeados genes ativos em um par de autossomos, resultado publicado anteriormente por Maffei *et al*. (2001).

Os resultados da metáfase I, submetida somente ao tratamento de banda NOR, não evidenciaram substâncias argirofílicas no lúmen do pára-quedas (Fig. 5A). Entretanto, quando se realizou coloração seqüencial FISH/AgNOR, o lúmen dos cromossomos sexuais associados em pára-quedas ficou fortemente impregnado pela prata (Fig. 5B).

A prófase I (zigóteno), quando avaliada somente por banda NOR, quase não ocorreu impregnação pela prata (Fig. 6A). Entretanto, quando foi realizado o tratamento seqüencial FISH/AgNOR, a vesícula sexual ficou fortemente impregnada pela prata (Fig. 6B).

FIGURA 1. Meiose em macho de *Cycloneda sanguinea*. A) Zigóteno. B) Final de Zigóteno. C) Paquíteno, mostrando os cromossomos X e y unidos ponta a ponta (1), com maior grau de condensação (2) e formação de um anel (3). D) Metáfase I (seta indica associação dos cromossomos sexuais formando pára-quedas). Barra=5μm.

FIGURA 2. Cariótipo de *Cycloneda sanguinea.* A) Fêmea com 2n=18+XX.
B) Macho com 2n=18+Xy_p. C) Banda C em fêmea. Barra=5μm.

FIGURA 3. Metáfases espermatogoniais de *Cycloneda sanguinea*.(A) Banda HSS. (B) Banda com Enzima de Restrição *Msp* I. (C). Banda com Enzima de Restrição *Hae* III. Setas indicam cromossomo y. Barra=5μm.

FIGURA 4. *Cycloneda sanguinea.* A) Localização dos genes rDNA em zigóteno. Sequencial FISH e banda Ag-NOR (B e C). Setas indicam que os genes de rDNA se localizaram fora da vesícula sexual. Barra=5μm.

FIGURA 5. Células de *Cycloneda sanguinea* em metáfase I. (A) Banda Ag-NOR. (B) Seqüencial FISH/Ag-NOR. Setas indicam a impregnação por prata no lúmem do “pára-quedas”. Barra=5μm

FIGURA 6. Células de *Cycloneda sanguinea* em zigóteno. (A) Banda NOR. Sequencial FISH/AgNOR (B). Barra=5µm.

DISCUSSÃO

Mitose e Meiose: Coloração Convencional

Cycloneda sanguinea apresentou número cromossômico 2n=18+XX para as fêmeas e a meio-fórmula n=9+Xy_p para os machos. O número cromossômico e a associação do Xy_p, em configuração de "pára-quedas" (metáfase I), estão de acordo com a descrição que vem sendo feita para a maioria das espécies de coleópteros, sendo provavelmente o cariótipo básico (ancestral), principalmente na subordem Polyphaga (SMITH, 1950; SMITH e VIRKKI, 1978).

Na família, embora as análises citogenéticas sejam escassas, este cariótipo tem sido descrito com bastante freqüência (42%), como recentemente para *Eriopis connexa* (MAFFEI *et al.*, 2000).

Na meiose de machos durante a prófase I (paquíteno), associação dos cromossomos sexuais ocorreu ponta-a-ponta, onde se visualizou as regiões do braço menor do cromossomo X mais condensadas unidas com o y, linearmente. A seguir, formou-se um pseudo-anel e, finalmente, o pára-quedas na metáfase I, sugerindo que a cromatina mais distendida do braço longo do cromossomo X formou o pára-quedista. Estes resultados estão de acordo com o modelo de associação dos cromossomos sexuais Xy_p, proposto por DRETS *et al.,* (1983) em *Epilachna paenulata* (Coleoptera: Coccinellidae). As análises de banda C realizadas por estes autores revelaram que a associação dos cromossomos sexuais diferia das outras espécies de coleópteros avaliadas, e que segmentos da heterocromatina constitutiva desempenharam importante papel, tanto na associação cromossômica quanto na formação do Xy_p. Os autores propuseram um modelo em

que os cromossomos sexuais se associavam ponta-a-ponta pelo braço longo, no início da meiose. O "pára-quedas" compõe-se de três segmentos distintos: dois corpúsculos intensamente heterocromatinizados, quando avaliados por banda-C, e o outro segmento eucromático formando o pára-quedista (forma toda em V). Os resultados foram interpretados como: o segmento eucromático seria o braço longo do cromossomo X (pára-quedista) e a formação do "pára-quedas" seria devido à associação do X e y. Foi proposto que essa associação não seria devido à região organizadora de nucléolos, por não ter sido detectado material nucleolar associado aos cromossomos sexuais.

VIRKKI *et al*. (1991), investigando o comportamento da associação Xy_p, por impregnação pela prata em seis espécies de coleópteros, relataram que a maioria dos seus resultados aceitava a interpretação de DRETS *et al*. (1983).

Análise da heterocromatina constitutiva

A heterocromatina em *C. sanguinea* localizou-se principalmente na região pericentromérica em todos os cromossomos e nos braços curtos, como tem sido descrito para a maioria das espécies de Coleoptera avaliadas por banda C (ENNIS, 1974; ANGUS, 1982, 1983; DRETS *et al.,* 1983; JUAN e PETITPIERRE, 1989).

A banda HSS extraiu toda a heterocromatina C mapeada anteriormente por banda C. Segundo a literatura, essa metodologia pode extrair parcialmente ou desnaturar o DNA rico em AT, sendo que o rico em CG permanece nos cromossomos (COMINGS, 1978).

Entretanto, a análise com enzima de restrição *Hae* III digeriu grande parte da heterocromatina, mostrando que esta é constituída por DNA rico em GC. Os telômeros, após tratamento com enzima de restrição, ficaram corados mais fortemente, indicando que não ocorreu digestão. Considerando os resultados obtidos com banda HSS e enzima de restrição *Hae* III, pode-se sugerir que a heterocromatina constitutiva nesta espécie possa ser de natureza molecular heterogênea. Em várias espécies de insetos, vem sendo descrita heterogeneidade da heterocromatina, como em *Drosophila melanogaster* e *D. virilis* e algumas

espécies de abelhas do gênero *Melipona* (MEZZANOTTE, 1986; MEZZANOTTE *et al*., 1986; ROCHA, 2000). Em gafanhotos, as enzimas de restrição apropriadas demonstraram heterogeneidade molecular em *Caledia captiva*, *Arcyptera tornosi* (ARNOLD e SHAW, 1985; ARNOLD *et al*., 1986; GOSÁLVEZ *et al*., 1987), *Oedipoda germanica* (LÓPEZ-FERNANDES *et al*., 1989). Em Coleoptera, algumas espécies vêm sendo extensivamente pesquisadas com enzimas de restrição, como em *Tenebrio molitor*, que apresenta aproximadamente, 50% do genoma constituído de DNA satélite heterogêneo, rico em AT e GC (JUAN *et al*., 1991a, b; JUAN *et al*., 1993). Na espécie *Tenebrio obscurus*, descreveram-se duas famílias de DNA satélites, sendo uma parcialmente similar (80%) ao de *T. molitor* e a outra completamente diferente na seqüência, mas ambas ricas em seqüência AT (PLOHL e UGARKOVIC, 1994, UGARKOVIC *et al*., 1994). Em *Ceroglossus chilensis* (Carabidae), foram identificadas duas famílas de DNA satélite, usando-se enzimas de restrição, o que indica que a heterocromatina nesta espécie apresenta vários sítios de restrições (GALIÁN *et al*,. 1996). A enzima de restrição *Msp*I não produziu bandas, sugerindo, assim, que o genoma de *C. sanguinea* não apresentou sítios de clivagem para a atuação da enzima.

Localização de genes rDNA (FISH) e atividade gênica.

As análises da meiose (Prófase I), por meio de banda NOR, indicaram que os meiócitos de *C. sanguinea* apresentaram marcação positiva por prata na vesícula sexual e na metáfase espermatogonial, resultado publicado anteriormente por MAFFEI et al. (2001), em que foi localizada a região ativa em um par autossômico. Os resultados de FISH de genes rDNA mapearam a região fora da vesícula sexual (prófase I). O tratamento seqüencial FISH/Ag-NOR confirmou a marcação por prata, no bivalente Xy_p. Os resultados descritos nesta espécie não estão de acordo com a hipótese clássica da teoria nucleolar (JOHN e LEWIS 1960; SMITH e VIRKKI, 1978). Estes resultados do FISH e banda NOR na mitose e meiose de *C. sanguinea* em relação a substâncias

argirofílicas, detectadas após o tratamento seqüencial FISH/Ag-NOR, levam a sugerir que essas substâncias sejam proteínas nucleolares, sintetizadas pelo par autossômico e importadas durante a prófase I, quando da associação dos cromossomos sexuais.

A técnica de hibridização *in situ* fluorescente (FISH), em que se utiliza sonda de rDNA, tem sido pouco aplicada em Coleoptera. Em *Tenebrio molitor* e *Misolampus goudoti*, ambos com 2n= 20 e sistema de determinação sexual Xy_p, o FISH com sondas de genes rDNA foi realizado por JUAN *et al*. (1993). Os resultados obtidos com a análise das metáfases mitóticas em *T. molitor* foram que as NORs se localizaram em dois pares de cromossomos autossômicos e no par sexual, apoiando na hipótese clássica de origem nucleolar para a associação Xy_p. Entretanto, em *M. goudoti*, os genes rDNA foram mapeados somente em um par autossômico. Em *Cicindela melancholica*, o FISH mapeou genes de rDNA em um dos três cromossomos X e no y (sistema sexual múltiplo). No entanto, em *Cicindela paludosa* (espécie relacionada) que apresenta sistema sexual X0, os genes de rDNA foram mapeados em um par de cromossomos autossômicos (GALIÁN *et al*., 1995).

REFERÊNCIAS BIBLIOGRÁFICAS

Angus RB, (1982). Separation of two species standing as *Helophorus aquaticos* (L.) (Coleoptera), Hydrophilidae) by banded chromosome analysis. Syst. Entomol. 7:256-281.

Angus RB, (1983). Separation of *Helophorus grandis maritimus* and *occidentalis* sp. (Coleotera, Hydrophilidae) by banded chromosome analysis. Syst. Entomol. 8:1-13.

Arnold ML and Schaw DD, (1985). The heterochromatin of grasshoppers from *Caledia captiva* species complex. Chromosoma 93:183-90.

Arnold ML, Appels R and Schaw DD, (1986). The heterocromatin of grasshoppers from the *Caledia captiva* species complex I Sequence evolution and conservation in a highly repeated DNA family. Mol. Biol. Evol. 3:29-43.

Birch ANE, Georghegan IE, Majirus MEN, Hackett C and Allen J, (1997). Interactions between plant resistence genes, pests, aphid populations and beneficial predators. Scottish Crop Research Institute. Annual Report 1995/97, 68-72. SCRI: Dundee.

Borror DJ and Delong DM, (1988). Introdução ao estudo dos insetos. São Paulo, Blucher 653p.

Comings DE, (1978). Mechanisms of chromosome banding and implication fo r chromosome structure. Ann. Rev. Genet. 12:23-46.

De Bach P, (1964). Biological control of insects pests and weeds. London, Chapman & Hall 844p.

Drets ME, Corbella E, Panzera F and Folle GA, (1983). C-banding and nonhomologous associations. II The "parachute" Xy_p sex bivalente and behavior of heterochromatic segments in *Epilachna paenulata*. Chromosoma 88:249:255, 1983.

Ennis TJ, (1974). Chromosome structure in *Chilocorus* (Coleoptera:Coccinellidae). In: Fluorescent and Giemsa banding patterns. Can. J. Genet. Cytol. 16:651-661.

Galián J, Serrano J, De La Rúa P, Petitpierre E and Juan C (1995). Localization and activity of rDNA genes in tiger beetles (Coleoptera, Cicindelinae). Heredity 74:524-530.

Galián J, Pruser F and Dela Rúa P, Serrano J and Mossakowsky D, (1996). Cytological and molecular differences in *Ceroglossus chilensis* species complex (Coleoptera: Carabidae). Annales Zoologici Fennici 33:23-30.

Galián J and Hudson P, (1999). Cytogenetic analysis of Australian tiger beetles (Coleoptera: Cicindelidae). Chromosome number, sex-determining system and localization of rDNA genes. J. Zool. Syst. and Evol. Res. 37:1-6.

Gosálvez J, Bella JL, López-Fernandez C and Mezzanotte R, (1987). Correlation between constitutive heterochromatin and restriction enzyme resistant chromatin in *Arcyptera tornosi* (Orthoptera). Heredity 59:173-180.

Gordon RD, (1985). Coccinellidae (Coleoptera) of America North of México. J. New York Entomol. Society 93:1-912.

Hamilton MJ, Hong G and Wichman HA, (1992). Intragenomic movement and concerted evolution of satellite DNA in *Peromyscus*: evidence from *in situ* hybridization. Cytogenet. Cell Genet. 60:40.

John B and Lewis KR, (1960). Nucleolar controlled segregation of the sex chromosomes in beetles. Heredity 15: 431-39.

Juan C and Petitpierre E, (1989). C-banding and DNA content in seven species of Tenebrionidae (Coleoptera). Genome 32:834-839.

Juan C and Petitpierre E, (1991a). Evolution of genome size in darkling beetles. (Coleoptera, Tenebrionidae). Genome 34:169-73.

Juan C and Petitpierre E, (1991b). Chromosome numbers and sex-determining mechanisms in Tenebrionidae (Coleoptera). In: M. Zuninio, Bellés and M Blas (eds). Advances in Coleopterology, European Association of Coleopterology p.167-76.

Juan C, Pons J and Petitpierre E, (1993). Localization of tandemly repeated DNA sequences in beetle chromosomes by fluorescent *in situ* hibridization. Chrom. Research 1:167-174.

Levins R and Wilson M, (1980). Ecological theory and pest management. Ann. Rev Entomol. 25:287-308.

López-Fernandes C, Gosálvez J and Mezzanotte R, (1989). Heterochromatin heterogeneity in *Oedipoda germanica* (Orthoptera) detected by "in situ" digestion with restriction endonulceases. Heredity 62:269-77.

Maluszynska J and Heslop-Harrison JS, (1991). Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*. Plant J. 1:159-166.

Maffei EMD, Gasparino E and Pompolo SG, (2000). Karyotypic characterization by mitosis, meiosis and C-banding of *Eriopis connexa* Mulsant (Coccinellidae: Coleoptera: Polyphaga) a predator of insect pests. Hereditas 132:79-85.

Maffei EMD, Pompolo SG, Silva-Junior JC, Caixeiro APA, Rocha MP and Dergam JA, (2001). Silver staining of nucleolar organizer regions (NORs) in some species of Hymenoptera (bees and parasitic wasp) and Coleoptera (lady-beetles). Cytobios 104:119-125.

Mezzanotte R, (1986). The selective digestion of polytene and mitotic chromosomes of *Drosophila melanogaster* by *Alu* I and *Hae* III restriction endonucleases. Chromosoma 93:249-55.

Mezzanotte R, Mancini PE and Ferrucci R, (1986). On the possibility of localizing *in situ Mus musculus* and *Drosophila vitris* satellite DNAs by *Alu* I and *Eco* RI restriction endonucleases. Genetica 70:107-11.

Moyzis RK, Buckingham JM and Cram LS, (1988). A highly conserved repetitive DNA sequence (TTAGGG) present at the telomeres of human chromosomes. Proc. Nat. Acad. Sci. 85:6622-6626.

Petitpierre E, (1996). Molecular cytogenetics and taxonomy of insects, with particular reference to the Coleoptera. Int. J. Morphol. & Embryol. 115-133.

Plohl M and Ugarkovic D, (1994). Characterization of two abundant satellite DNAs from the mealworn *Tenebrio obscurs*, J. Mol. Evol. 39:389-95.

Rocha MP, (2000). Análise cariotípica de dez espécies de abelhas do gênero *Melipona* Illiger, 1806 (Hymenoptera, Apidae), baseada em padrões de heterocromatina. Dissertação de Mestrado, UFV.

Smith SG, (1950). The cytotaxonomy of Coleoptera. Can Entomol. 82:58-68.

Smith SG and Virkki N, (1978). Animal cytogenetics. Coleoptera 1-365.

Smith RF and Reynolds HT, (1966). Principles, definitions and scope of integrated pest control. In: FAO. Symposium on Integrated Pest Control. Proceedings. Rome P.11-17.

Traski BJ, (1991). Fluorescent *in situ* hybridization: applications in cytogenetics and gene mapping. Trends Genet. 7:149-154.

Ugarkovic D, Plohl M., Petitpierre E, Lucijanic-Justic V and Juan C, (1994). *Tenebrio obscurus* satellite DNA is restriction endonucleases *in situ*. Chromosome Res. 2:217-23.

Verma RS and Babu A, (1989). Human Chromosomes. Principles and Techniques. McGraw-Hill, New York.

Viégas-Péquinot E, (1992). In situ hibridization to chromosomes with biotinyled probes. In: In situ hibridization: a practical approach (Willernson, D. ed.) Oxford University Press, IRL, Press, p.137-158.

Virkki N, Mazzella C and Denton A, (1991). Silver staining of the colepteran Xy_p sex bivalent. Cytobios 67:45-63.

2. CONCLUSÕES GERAIS

1. A adaptação da técnica, principalmente para a obtenção de cromossomos mitóticos metafásicos, permitiu detectar alteração numérica (cromossomos B) em fêmeas de *E. connexa*, evidenciando que se as análises fossem realizadas somente na meiose de machos, a variabilidade intraespecífica seria subestimada.
2. O número cromossômico 2n=18+XX para fêmeas e 2n=18+Xy nos machos (n=9+Xy_p), ocorreu em todas as espécies, estando de acordo com o cariótipo básico sugerido para a ordem.
3. Os resultados das bandas com Enzima de Restrição, Banda-C e Banda HSS em *C. sanguinea*, localizou a heterocromatina na região pericentromérica de todos os cromossomos, sendo esta região rica em pares de base GC (digerida pela *Hae* III). Entretanto, alguns blocos de heterocromatina constitutiva (telômeros) não foram digeridos por esta enzima e a banda HSS (Hot Saline Solution) extraiu toda a heterocromatina, sugerindo que esta seja rica também em DNA rico em seqüências AT conferindo, portanto, natureza molecular heterogênea.
4. A banda Ag-NOR em células meióticas de *O. v-nigrum*, revelou atividade gênica na vesícula sexual e foi confirmada por FISH com genes de rDNA. Estes resultados estão de acordo com a teoria nucleolar.

5. A banda Ag-NOR em metáfases mitóticas de *E. connexa* e de *C. sanguinea* mapeou a região organizadora de nucléolo ativa em um par de autossomos. A utilização do fluorocromo CMA_3 em *E. connexa* revelou que essa região é rica em pares de bases GC.
6. As análises da mitose e meiose em *C. sanguinea* por banda NOR e o tratamento seqüencial FISH/AgNOR, localizaram os genes ativos em um par autossômico (metáfase espermatogonial). Porém, a vesícula sexual (prófase I) e o lúmen do pára-quedas (metáfase I) ficaram fortemente impregnados pela prata. O FISH mapeou os genes rDNA fora da vesícula sexual. Baseado nestes resultados, sugeriu-se que essas substâncias sejam proteínas nucleolares, sintetizadas pelo par autossômico e importadas durante a associação dos cromossomos sexuais (prófase I).