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Kaio Cesar Chaboli Alevi

Citotaxonomia de triatomíneos: a citogenética como ferramenta no estudo do complexo *Triatoma brasiliensis* e do subcomplexo Brasiliensis

São José do Rio Preto
2014

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, Área de Concentração – Biologia Estrutural, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de São José do Rio Preto.

Orientador: Prof^ª. Dr^ª. Maria Tercília Vilela de Azeredo Oliveira

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Comissão Examinadora

Prof^a. Dr^a. Maria Tercília Vilela Azeredo Oliveira
Professor Titular
UNESP – São José do Rio Preto
Orientadora

Prof^a. Dr^a. Grasiela de Campos Dias Severi Aguiar
Professor Adjunto
Centro Universitário Hermínio Ometto - UNIARARAS

Prof^a. Dr^a. Hermione Elly Melara de Campos Bicudo
Professor Titular
UNESP – São José do Rio Preto

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“Descobrir consiste em olhar para o que todo mundo está vendo e pensar uma coisa diferente”

Roger Von Oech

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Resumo

RESUMO

As análises citogenéticas têm se mostrado como importantes ferramentas para o estudo da taxonomia dos triatomíneos. Dessa forma, a utilização de técnicas citogenéticas poderá esclarecer a problemática existente na classificação atual das espécies que compõem o subcomplexo *Brasiliensis*. Essas espécies foram agrupadas apenas por dados morfológicos e pela distribuição geográfica e diferem das espécies agrupadas no complexo *Triatoma brasiliensis*, com base em diferentes abordagens que levaram à indicação da monofilia. O presente trabalho descreve aspectos citogenéticos de *T. lenti*, *T. sherlocki*, *T. melanocephala* e *T. vitticeps*, com o intuito de avaliar a posição desses vetores no subcomplexo *Brasiliensis* e analisar uma possível relação de *T. lenti*, *T. melanocephala* e *T. vitticeps* com o complexo *T. brasiliensis*. Análises citogenéticas clássicas (Orceína Lacto-Acética, Impregnação por íons prata e Bandamento C) e moleculares (Hibridização *in situ* – FISH) foram realizadas em machos adultos. *T. lenti* e *T. sherlocki*, que apresentaram muitas características cromossômicas em comum, como o número de cromossomos ($2n = 20A + XY$), disposição da heterocromatina constitutiva nos autossomos (uma ou ambas as extremidades dos cromossomos) e na disposição das Regiões Organizadoras Nucleolares (RONs) (um par de autossomos). Esses insetos foram diferenciados por meio de marcações observadas nas espermatídes, o que foi proposto como ferramenta citotaxonômica diagnóstica. *T. melanocephala* e *T. vitticeps* apresentaram o mesmo número de cromossomos ($2n = 20A + X_1X_2X_3Y$) e as mesmas características durante a espermatogênese. No entanto, esses vetores puderam ser diferenciados pela disposição dos blocos heterocromáticos, pela disposição das RONs e pela análise da espermiogênese. Assim, com base nessas características, corroboramos a posição de *T. lenti* e *T. sherlocki* no subcomplexo *Brasiliensis* e propomos a exclusão de *T. melanocephala*, *T. vitticeps* e *T. tibiamaculata*, por apresentarem fragmentação do cromossomo sexual X e se aproximarem dos triatomíneos da América do Norte. Além disso, embora análises moleculares devam ser realizadas, os dados citotaxonômicos suportam o agrupamento de *T. lenti* como sexto membro do complexo *T. brasiliensis*.

Palavras-chave: *Triatoma lenti*. *Triatoma melanocephala*. Triatominae.

Abstract

ABSTRACT

Cytogenetic analyzes have shown to be important tools to study the taxonomy of the triatomines. Thus, the use of cytogenetic techniques may clarify the problems existing in the current classification of species that form the Brasiliensis subcomplex. The species in the subcomplex were grouped only by morphological data and geographical distribution and differs from the species in the *Triatoma brasiliensis* complex, on the basis of on different approaches that indicate the monophyly. The present paper describes cytogenetically *T. lenti*, *T. sherlocki*, *T. melanocephala* and *T. vitticeps*, in order to evaluate the position of these vectors in the Brasiliensis subcomplex and analyze a possible relationship of *T. lenti*, *T. melanocephala* and *T. vitticeps* with the *T. brasiliensis* complex. Classical cytogenetic analysis (Lacto-acetic orcein, silver nitrate staining and C banding) and molecular (in situ hybridization - FISH) were performed in adult males. *T. lenti* and *T. sherlocki* showed several chromosomal characteristics in common, such as the number of chromosomes ($2n = 20A + XY$), disposition of constitutive heterochromatin in the autosomes (one or both ends of the autosomes) and the staining of nucleolar organizer regions (NORs) (a pair of autosomes). These insects were able to be differentiated by markings observed in spermatids, which were proposed as a diagnostic tool cytotaxonomic. *T. melanocephala* and *T. vitticeps* shared the chromosome number ($2n = 20A + X_1X_2X_3Y$) and the same characteristics during spermatogenesis. However, these vectors were able to be differentiated by the disposition of heterochromatic blocks, by the arrangement of NORs and the examination of spermiogenesis. Thus, these characteristics corroborate the position of *T. lenti* and *T. sherlocki* on Brasiliensis subcomplex and we propose the exclusion of *T. melanocephala*, *T. vitticeps* and *T. tibiamaculata* because they present fragmentation of the sex chromosome X and are closer to of the insects of North America. Furthermore, although molecular analysis should be performed, the cytotaxonomic data support the group of *T. lenti* as the sixth member of *T. brasiliensis* complex.

Keywords: *Triatoma lenti*. *Triatoma melanocephala*. Triatominae.

Introdução

I. INTRODUÇÃO

Os triatomíneos, taxonomicamente, pertencem à Ordem Hemiptera, Subordem Heteroptera, Família Reduviidae e Subfamília Triatominae (LENT e WYGODZINSKY, 1979). A subfamília Triatominae é composta por 146 espécies distribuídas em 18 gêneros e seis tribos (ALEVI et al., 2013a; JURBERG et al., 2013). Todas as espécies de triatomíneos são suscetíveis à infecção pelo protozoário *Trypanossoma cruzi* Chagas, 1909 (Kinetoplastida, Trypanosomatidae) e, conseqüentemente, são potenciais vetoras da doença de Chagas. A infecção ocorre a partir da alimentação com sangue de mamífero infectado com o protozoário, sendo que todos os estádios ninfais (N1, N2, N3, N4, N5) estão sujeitos a ingerirem o parasita, uma vez que, após a eclosão, a hematofagia é obrigatória em todas as fases de vida dos triatomíneos (NOIREAU et al., 2009).

A identificação específica e a sistemática dos triatomíneos, por muito tempo, pautaram-se, fundamentalmente, em observações morfológicas e em variações cromáticas (SHERLOCK e SERAFIM, 1967; LUCENA, 1970; LENT e WYGODZINSKY, 1979). Com o surgimento e aprimoramento de novas técnicas que permitiram que outros parâmetros fossem avaliados, como genéticos, evolutivos e ecológicos, muitos problemas taxonômicos foram esclarecidos.

Lucena (1970) propôs, pela primeira vez, um arranjo sistemático denominado complexo *brasiliensis*, composto, principalmente, por espécies encontradas na região Nordeste do Brasil. Esse complexo foi descrito, inicialmente, incluindo as espécies *Triatoma brasiliensis* Neiva, 1911, *T. petrochii* Pinto e Barreto, 1925, *T. pessoai* Sherlock e Serafim, 1967, *T. lenti* Sherlock e Serafim, 1967 e *T. bahiensis* Sherlock e Serafim, 1967 e pelas subespécies *T. b. melanica* Neiva e Lent, 1941, *T. b. macromelanosoma* Galvão, 1955.

A espécie *T. brasiliensis* é uma das principais espécies vetorais da região Nordeste do Brasil, pois está diretamente relacionada com o clima semi-árido dessa região (LUCENA, 1970). Essa espécie habita seis Estados brasileiros, a saber, Maranhão, Paraíba, Pernambuco, Piauí, Rio Grande do Norte e Tocantins (GURGUEL-GONÇALVES et al., 2012).

A espécie *T. petrochii* foi descrita por Pinto e Barreto, em 1925, baseada em apenas um exemplar proveniente do Rio Grande do Norte. Os autores nomearam a espécie homenageando a Dra. Juana Petrochi e a descreveram como idêntica a *T. brasiliensis*, diferenciando apenas por não ter manchas claras nos fêmures. Carcavallo e Martínez (1985) corrigiram o nome científico de *T. petrochii* para *T. petrochiae*, por homenagear uma mulher. Lent e Wygodzinsky (1979) confirmaram a similaridade existente entre as duas espécies, contudo ressaltaram que apresentam algumas diferenças morfológicas, tais como: ausência de fosseta esponjosa nas tíbias dos machos de *T. petrochiae*, rostro praticamente glabro e primeiro segmento antenal mais curto. Espínola (1971), por meio de cruzamento experimental e Monteiro et al. (1998), por meio de parâmetros genéticos, confirmaram o *status* específico de *T. petrochiae*.

As espécies *T. lenti*, *T. pessoai* e *T. bahiensis* foram descritas, em 1967, como formas melânicas de *T. brasiliensis* (SHERLOCK e SERAFIM, 1967). As principais diferenças descritas, pelos autores, para separar as três espécies foram: a espécie *T. pessoai* com manchas grandes e de cor vermelha no conexivo; a espécie *T. lenti* com manchas pequenas e amarelas no conexivo e a espécie *T. bahiensis* com manchas de tamanho intermediário no conexivo e presença de duas manchas no cório. Lent e Wygodzinsky, em 1979, sinonimizaram *T. pessoai* e *T. bahiensis* com *T. lenti*.

A subespécie *T. b. melanica*, por meio de parâmetros morfológicos, biológicos, ecológicos, cruzamentos experimentais, enzimáticos e moleculares, teve seu *status* elevado

para o nível de espécie (COSTA et al., 2006). Os autores ressaltam que *T. melanica* é a forma evolutiva mais diferenciada dentro do complexo, uma vez que apresenta incompatibilidade genética e híbridos inviáveis com os outros membros do complexo *brasiliensis*. Já a subespécie *T. b. macromelanosoma* foi descrita com base em formas melânicas de *T. brasiliensis* encontradas no domicílio (GALVÃO, 1955). Essa subespécie foi recentemente redescrita e revalidada por Costa et al. (2013).

Costa e colaboradores, por meio de análises isoenzimáticas (COSTA et al., 1997a), morfológicas (estruturas das genitálias) (COSTA et al., 1997b), da superfície exocorial do ovo (COSTA et al., 1997b), biológicas (COSTA et al., 1998a), ecológicas (COSTA et al., 1998b), genéticas (MONTEIRO et al., 1999), citogenéticas (PANZERA et al., 2000), biogeográficas (COSTA et al., 2002), de cruzamentos experimentais (COSTA et al., 2003), moleculares (MONTEIRO et al., 2004) e da morfometria dos testículos (FREITAS et al., 2008) propuseram que o complexo *T. brasiliensis* seria formado pelas espécies *T. brasiliensis*, *T. b. macromelanosoma*, *T. melanica* e uma população de *T. brasiliensis* de Juazeiro/Bahia. Em 2007 Costa e Felix descreveram essa população de Juazeiro como uma nova espécie, *T. juazeirensis*. Em 2009, Mendonça e colaboradores, por meio de reconstrução filogenética com dois genes mitocondriais (CytB e 16S), sugeriram que *T. sherlocki* deveria fazer parte do complexo *T. brasiliensis*. Assim, baseados em todos os estudos relacionados com o complexo *T. brasiliensis*, Costa et al. (2013) propuseram que esse complexo de espécies é constituído por quatro espécies (*T. brasiliensis*, *T. melanica*, *T. juazeirensis* e *T. sherlocki*) e uma subespécie (*T.b. macromelanosoma*). *T. petrochiae* não é considerado como membro do complexo pelos autores. Argola et al. (2008) ressaltaram que esse inseto pode ser diferenciado dos demais agrupados no complexo pelas seguintes características: primeiro segmento antenal incomumente curto, atingindo pouco mais da metade da distância entre a base e o ápice da cabeça e fosseta esponjosa ausente

em machos e fêmeas.

Schofield e Galvão (2009) agruparam os triatomíneos em complexo e subcomplexos específicos. Os insetos classificados no complexo *T. brasiliensis* por Costa et al. (2013) foram agrupados, por Schofield e Galvão (2009) no complexo Infestans e no subcomplexo Brasiliensis. Contudo, além das espécies *T. brasiliensis*, *T. melanica*, *T. juazeirensis* e *T. sherlocki*, os autores agruparam no subcomplexo *T. lenti*, *T. petrochiae*, *T. tibiamaculata*, *T. vitticeps* e *T. melanocephala*. Como o número de informações disponíveis na literatura sobre as cinco “novas” espécies eram escassas, os autores utilizaram, principalmente, dados morfológicos e a disposição geográfica das espécies como parâmetros. Ressaltamos que Lucena, em 1970, já agrupava *T. lenti* e *T. petrochiae* no complexo *brasiliensis*.

Sendo assim, o presente trabalho analisou citogeneticamente e citotaxonomicamente as espécies *T. lenti*, *T. sherlocki*, *T. vitticeps* e *T. melanocephala*, com o objetivo de ampliar o conhecimento sobre informações biológicas dessas espécies e, principalmente, para avaliar a posição desses triatomíneos no complexo *T. brasiliensis* e no subcomplexo Brasiliensis.

Objetivos

II. OBJETIVOS

II.1 Objetivos gerais

a) Analisar detalhadamente a espermatogênese, a espermiogênese e o cariótipo das espécies *T. lenti*, *T. sherlocki*, *T. vitticeps* e *T. melanocephala*, por meio do esmagamento celular e após coloração com Orceína Lacto-Acética;

b) Obter informações sobre a atividade nucleolar durante a espermatogênese, espermiogênese e a localização das Regiões Organizadoras Nucleolares (RONs) ativas nos cromossomos holocêntricos das espécies *T. lenti* e *T. melanocephala*, por meio da técnica de impregnação por íons prata;

c) Caracterizar e localizar as regiões de heterocromatina constitutiva na cromatina e nos cromossomos holocêntricos das espécies *T. lenti*, *T. sherlocki* e *T. melanocephala*, por meio da técnica de Bandamento-C;

d) Analisar molecularmente a disposição das Regiões Organizadoras Nucleolares (RONs) nos cromossomos de *T. lenti* e *T. melanocephala*, por meio da hibridização *in situ* (FISH);

II.2 Objetivos específicos

- a) Comparar citogeneticamente as espécies *T. lenti* e *T. sherlocki*, com o objetivo de estabelecer um parâmetro citogenético que auxilie na diagnose das espécies;
- b) Comparar citogeneticamente as espécies *T. melanocephala* e *T. vitticeps*, para estabelecer um parâmetro citogenético que auxilie na diagnose das espécies;
- c) Analisar a posição inicial de todas as espécies no subcomplexo Brasiliensis;
- d) Analisar uma possível relação de *T. melanocephala*, *T. vitticeps* e *T. lenti* com as espécies que constituem o complexo *T. brasiliensis*.

Material e Métodos

III. MATERIAL E MÉTODOS

III.1 Preparação das lâminas

Foram analisados os túbulos seminíferos de, no mínimo, 10 machos de triatomíneos das quatro espécies. Após a dissecação, os testículos foram transportados para a solução fisiológica (Demerec), onde foi realizada a limpeza e a individualização dos túbulos seminíferos. Posteriormente, cada túbulo foi colocado em um recipiente de vidro contendo três partes de metanol para uma de ácido acético e conservado no freezer à -20°C. Para preparação das lâminas, os túbulos seminíferos foram retirados da solução fixadora e colocados em uma lâmina limpa, onde foram realizados dois banhos de água destilada, por cinco minutos cada e, posteriormente, um banho com ácido acético 45%, durante 10 minutos. Após esse período, o túbulo seminífero foi dilacerado e sobre esse material foi colocada uma lamínula para a realização da técnica de esmagamento celular. A remoção da lamínula ocorreu em nitrogênio líquido.

III.2 Técnicas citogenéticas

As técnicas citogenéticas utilizadas foram:

- ✓ **Orceína Lacto-Acética** (DE VAIO et al., 1985, com modificações de acordo com ALEVI et al., 2012a) (descrição dos cariótipos, da espermatogênese e espermiogênese)
- ✓ **Bandamento-C** (SUMNER, 1972) (descrição do padrão de heterocromatina constitutiva no material genético das espécies)
- ✓ **Impregnação por íons prata** (HOWELL e BLACK, 1980) (descrição do comportamento nucleolar durante a meiose dos insetos)

- ✓ **Hibridização *in situ* (FISH)** (PANZERA et al., 2012) (localização das Regiões Organizadoras Nucleolares (RONs), por meio de sondas específicas, nos cromossomos holocêntricos dos triatomíneos)

III.3 Análise dos resultados

O material submetido às técnicas citogenéticas convencionais foi analisado ao microscópio de luz *Jenaval* (Zeiss), acoplado à câmera digital e ao sistema analisador de imagens *Axio Vision LE 4.8* (Copyright ©2006-2009 Carl Zeiss Imaging Solutions GmbH), com aumento de 1000 vezes. Já o material submetido à técnica citogenética molecular (FISH) foi analisado em microscopia de fluorescência (*Nikon Eclipse 80i*).

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Resultados

V.1 ANEXO 1 (Publicado na revista *Infection, Genetics and Evolution*, FI: 3,1)

ALEVI, K.C.C.; MENDONÇA, P.P.; SUCI, M.; PEREIRA, N.P.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Karyotype and spermatogenesis in *Triatoma melanocephala* Neiva and Pinto (1923). Does this species fit in the Brasiliensis subcomplex? **Infect Gen Evol** v. 12, p. 1652-1653, 2012.

Karyotype of *Triatoma melanocephala* Neiva and Pinto (1923). Does this species fit in the Brasiliensis subcomplex?

Kaio Cesar Chaboli Alevi, Priscila Pasquetto Mendonça, Nathália Paiva Pereira, João Aristeu da Rosa and Maria Tercília Vilela de Azeredo Oliveira

ABSTRACT

Triatoma melanocephala is a rare species of Hemiptera that belongs to the Brasiliensis subcomplex. This subcomplex is composed of cryptic species, and the basic number of chromosomes for triatomines of this subcomplex is $2n = 22$; however, *T. melanocephala* showed a karyotype of $2n = 24$ ($20A + X_1X_2X_3Y$). Thus, this study allowed us to describe the karyotype of the species and, more specifically, to propose the exclusion of *T. melanocephala*, as well as *T. vitticeps* and *T. tibiamaculata*, which also has fragmentation of the X chromosome, from the Brasiliensis subcomplex.

SHORT COMMUNICATION

The species *Triatoma melanocephala*, described by Neiva and Pinto (1923), is a rare species of Hemiptera that is found exclusively in the Brazilian states of Bahia, Pernambuco, Paraíba, Rio Grande do Norte and Sergipe (Gurgel-Gonçalves et al., 2012). It was found to be infected by *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), the

causative agent of Chagas disease. The species is likely an important link of sylvatic and domestic transmission cycles that transmits the parasite of mice and marsupials to humans (Sherlock and Guitton, 1980).

The species belongs to the *Brasiliensis* subcomplex (Schofield and Galvão, 2009). This subcomplex is present in South America and consists of nine species: *T. brasiliensis*, *T. juazeirensis*, *T. melanica*, *T. melanocephala*, *T. petrochiae*, *T. lenti*, *T. sherlocki*, *T. tibiamaculata* and *T. vitticeps* (Schofield and Galvão, 2009).

According to Monteiro et al. (2001), it is difficult to explain the *Brasiliensis* subcomplex classification by morphological parameters alone, because these species are cryptic; i.e., morphologically similar. Thus, cytogenetic studies can be considered an important tool for the differentiation of these species (Pérez et al., 1992).

Most of the cytogenetic results on triatomines were obtained based on chromosome comparisons that relied on conventional staining. In these studies, the comparison criteria were often the number, the morphology, and the disposition of chromosomes in the metaphase plate. The data obtained from these investigations gave the first indications of the processes that occurred during chromosomal evolution in these insects (Pérez et al., 1992; Panzera et al., 1996; Tavares and Azeredo-Oliveira, 1997).

The basic number of chromosomes for triatomines is $2n = 22$, with ten pairs of autosomes and one pair of sex chromosomes (Ueshima, 1966). However, there are species with multiple sex chromosomes (XY, X_1X_2Y , $X_1X_2X_3Y$), caused by fragmentation of the original X (Manna, 1950; Ueshima, 1966).

Seminiferous tubules of fifteen adult males of *T. menalocephala* were first shredded, smashed, and set the slide in liquid nitrogen. Then were stained with the lacto-acetic orcein cytogenetic technique of De Vaio et al. (1985), with modifications to the biological material, which is no longer the whole testicle, but instead only a seminiferous tubule, and to the duration in acetic acid, which is now only ten minutes. Based on the

analysis of meiotic metaphases (Figure 1), we observed that males of *T. melanocephala* have the karyotype $2n = 24$ ($20A + X_1X_2X_3Y$), demonstrating that this organism does not have the modal chromosome set found in triatomines ($2n = 22$) (Ueshima, 1966).

The species *T. tibiamaculata* and *T. vitticeps* share some morphological similarities with the species of the Brasiliensis subcomplex, but when analyzed cytogenetically and molecularly, they are related more closely to the Triatominae of North America, which have multiple sex chromosomes (Panzera et al., 2010). The karyotype of *T. tibiamaculata* is $2n = 20A + X_1X_2Y$ (Panzera et al., 1996) and the karotype of *T. vitticeps* is $2n = 20A + X_1X_2X_3Y$ (Schreiber and Pellegrino, 1950). Mitochondrial DNA sequences also confirm the close phylogenetic relationship between these Hemiptera and the ones present in North America (Schofield and Galvão, 2009).

Only *T. eratyrusiformis* and *T. vitticeps* karyotypes have been described previously as $20A + X_1X_2X_3Y$ (Panzera et al., 2010). This similarity highlights the importance of karyotype studies in combination with other techniques for understanding the classification of insects in a subcomplex, because until this study was developed, the inclusion of only *T. vitticeps* and *T. tibiamaculata* in this subcomplex was questioned (Schofield and Galvão, 2009).

The present study showed a direct karyotypic relationship between *T. melanocephala*, *T. vitticeps*, and *T. tibiamaculata*. All species presented fragmentation of the X chromosome and, thus, are similar to North American species. Therefore, we propose the exclusion of *T. melanocephala*, *T. vitticeps*, and *T. tibiamaculata* from the Brasiliensis subcomplex. This study underscores the importance of cytogenetics in the correct classification of triatomines, vectors of Chagas disease across Brazil.

The correct classification of the triatomines enables differentiate species of primary importance for species of minor importance, since, currently, some species in the process

of domiciliation; i.e., leaving the natural habitat and began living in urban environments such as indoors and peridomicile (Dias and Scholfield, 1998). Thus, through the correct classification is possible to distinguish the main species vectors of Chagas disease and thereby allow greater attention of vector control programs for these species.

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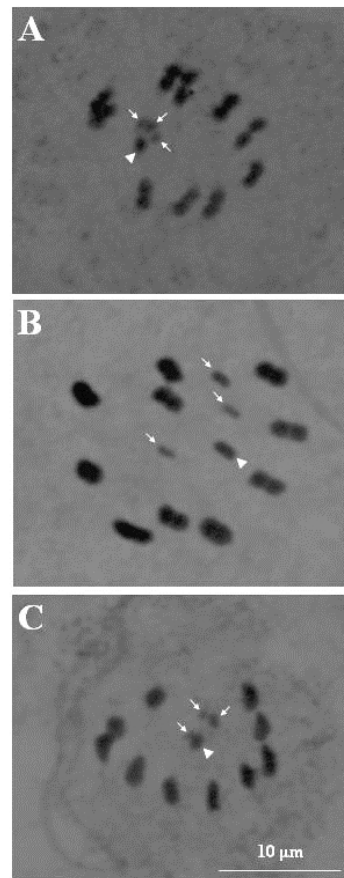


Fig. 1: Seminiferous tubule of *Triatoma melanocephala* stained by lacto-acetic orcein.
A,B: Metaphase I with ten bivalent autosomes and three sex chromosomes. C: Metaphase
II. Arrows: X chromosome fragmentation (X₁X₂X₃). Arrowhead: Y chromosome.

V.2 ANEXO 2 (Publicado na revista *Genetics and Molecular Research*, FI: 1,1)

ALEVI, K.C.C.; MENDONÇA, P.P.; PEREIRA, N.P.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Spermatogenesis in *Triatoma melanocephala* (Hemiptera, Triatominae). **Gen Mol Res** v. 12, p. 4944-4947, 2013.

Spermatogenesis in *Triatoma melanocephala* (Hemiptera: Triatominae)

Kaio Cesar Chaboli Alevi, Priscila Pasqüetto Mendonça, Nathália Paiva Pereira, João Aristeu Rosa and Maria Tercília Vilela de Azeredo-Oliveira

ABSTRACT

Triatoma melanocephala is a rare species of Hemiptera. It belonged to the Brasiliensis subcomplex and presents morphological characteristics very close to those of *Triatoma vitticeps*. We investigated spermatogenesis of *T. melanocephala* and compared it with that of *T. vitticeps* in order to determine whether these organisms have similar cytogenetic characteristics. Lacto-acetic orcein staining was used to observe all stages of meiosis. These two species were found to have the same karyotype ($2n = 20A + X_1X_2X_3Y$), heteropycnotic corpuscles in the polyploid spermatogonial cells, interfasic and profasic nucleus, and chromocenter with four sex chromosomes during prophase. Thus, we conclude that besides the morphologic similarity of *T. melanocephala* with *T. vitticeps*, they also have similar spermatogenesis and cytogenetics.

SHORT COMMUNICATION

The species *Triatoma melanocephala*, described by Neiva and Pinto (1923), is a rare species of Hemiptera that is found exclusively in the Brazilian States of Bahia,

Pernambuco, Paraíba, Rio Grande do Norte, and Sergipe (Gurgel-Gonçalves et al., 2012). It is known to be infected by *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), the causative agent of Chagas disease. The species is likely an important link of sylvatic and domestic transmission cycles, which transmits the parasite of mice and marsupials to humans (Sherlock and Guitton, 1980).

Sherlock and Guitton (1980), from their morphological description of *T. melanocephala*, proposed that the hemipteran has morphologic characteristics very close to those of *Triatoma vitticeps*, where the only distinguishing features are the absence of range testacea in the head and the presence of two brown spots on the thorax.

The species belonged to the Brasiliensis subcomplex (Schofield and Galvão, 2009). This subcomplex is present in South America and consisted of 9 species: *T. brasiliensis*, *T. juazeirensis*, *T. melanica*, *T. melanocephala*, *T. petrochiae*, *T. lenti*, *T. sherlocki*, *T. tibiamaculata*, and *T. vitticeps*. However, recently, by means of karyotype analysis, it was proposed to exclude *T. melanocephala*, *T. vitticeps*, and *T. tibiamaculata* from the Brasiliensis subcomplex (Alevi et al., 2012a). Thus, cytogenetic studies can be considered an important tool for the classification of this subcomplex (Alevi et al., 2012a,b; 2013a,b).

Most of the cytogenetic results on triatomines have been obtained on the basis of chromosome comparisons relying on conventional staining. In these studies, the comparison criteria were often the number, morphology, and arrangement of chromosomes in the metaphase plate. The data obtained from these investigations gave the first indications of the processes that occurred during chromosomal evolution in these insects (Pérez et al., 1992; Panzera et al., 1996; Tavares and Azeredo-Oliveira, 1997).

This paper describes the spermatogenesis of *T. melanocephala* and compares it with that of *T. vitticeps* to find out whether these organisms have convergent cytogenetic characteristics.

Seminiferous tubules of 15 adult males of *T. menalocephala* were first shredded and smashed on a slide, which was then placed in liquid nitrogen. The preparation was stained using the lacto-acetic orcein cytogenetic technique of De Vaio et al. (1985), with modifications according to Alevi et al. (2012a). The slides were analyzed using a light microscope (Jenaval-Zeiss), which was coupled to a digital camera and an AxioVision LE 4.8 image analyzer (Copyright © 2006-2009 Carl Zeiss Imaging Solutions Gmb H). The images were magnified 1000X.

It was possible to observe all stages of meiosis during spermatogenesis (Figure 1). The polyploid nucleus, which is a cell of the wall of the seminiferous tubule responsible for nutrition of the cells during the meiotic division, showed a heteropycnotic body (Figure 1A, arrow). We observed the interphase nucleus (Figure 1B), the prophase nucleus (Figure 1C) and the spermatogonial metaphase nucleus (Figure 1D). Interphase and mitotic prophase also displayed one heteropycnotic body (arrow). The initial meiotic prophase demonstrated the four individual sex chromosomes (Figure 1E, arrows). This chromocenter formed by sex chromosomes remained throughout prophase I (Figure 1F and G, arrows). The nucleus in metaphase I (Figure 1H) allowed us to observe the diploid chromosome set of the species ($2n = 20A + X_1X_2X_3Y$). Note that the Y chromosome is larger and more heteropycnotic than the X chromosomes. Furthermore, it was possible to observe two larger and more heteropycnotic autosomes (arrows). It was also possible to observe metaphases in the lateral view (Figure 1I), late anaphase (Figure 1J) and telophase (Figure 1K).

The basic number of chromosomes for triatomines is $2n = 22$, with 10 pairs of autosomes and 1 pair of sex chromosomes (Ueshima, 1966). However, there are species

with multiple sex chromosomes (XY, X1X2Y, X1X2X3Y), caused by fragmentation of the original X (Manna, 1950; Ueshima, 1966), as in *T. tibiamaculata* (X1X2Y) (Panzera et al., 1996) and *T. vitticeps* (X1X2X3Y) (Schreiber and Pellegrino, 1950). The karyotype of *T. melanocephala* has been recently described (Alevi et al., 2012a) and approaches that of *T. vitticeps* and *T. eratyrusiformis*, because among the 140 species of triatomines described, only the males of these species have a diploid chromosome number $2n = 24$ (20A + X1X2X3Y) (Panzera et al., 2010).

In addition to karyotype, *T. melanocephala* has some characteristics during spermatogenesis that resembles those of *T. vitticeps*, such as heteropycnotic bodies present in the polyploid, interphase and prophase nucleus (Severi-Aguiar et al., 2006). Moreover, Severi-Aguiar et al. (2006) also observed a chromocenter with four individual sex chromosomes during prophase in *T. vitticeps*.

Thus, on the basis of comparative analysis of spermatogenesis of *T. melanocephala*, we propose that beyond morphologic characteristics, this species has similar cytogenetic characteristics as *T. vitticeps*.

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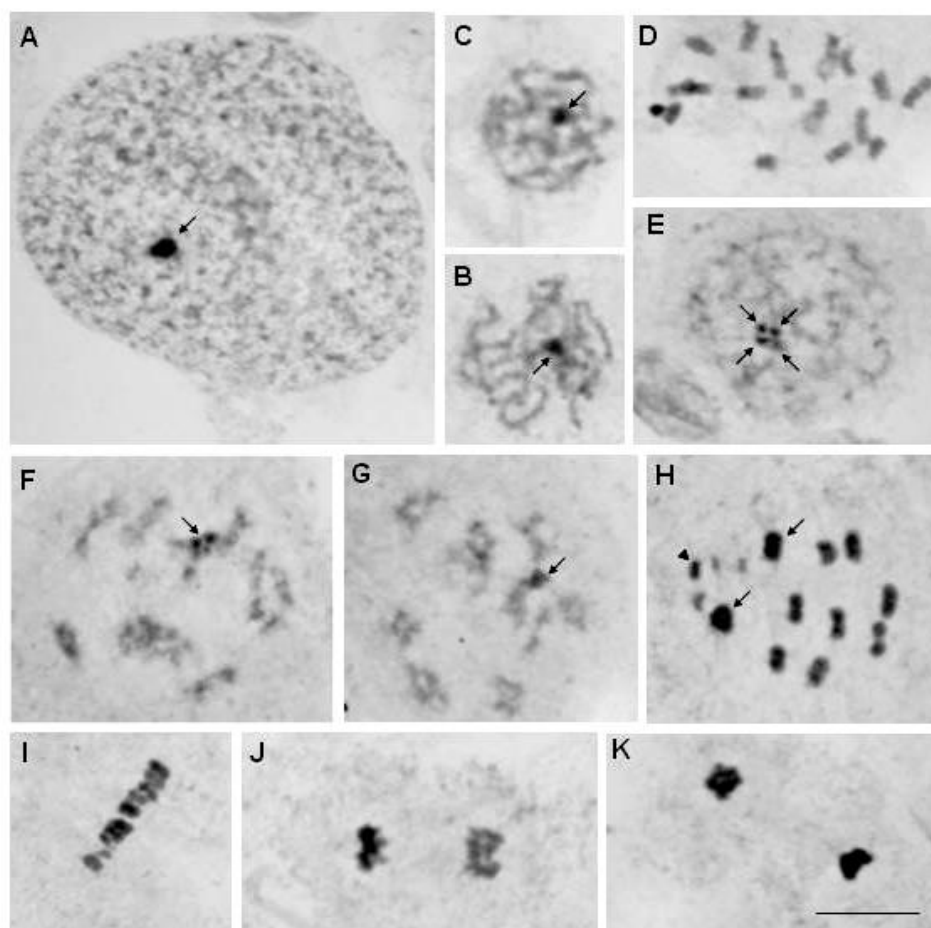


Figure 1. Seminiferous tubule of *Triatoma melanocephala* stained by lacto-acetic orcein. (A) Polyloid nucleus. Note the heteropycnotic corpuscle (arrow). (B) Interfacic nucleus. (C) Spermatogonial prophase. (D) Spermatogonial metaphase. (E) Initial prophase demonstrated the four individualized sex chromosomes (arrows). (F, G) Prophase I. Note the persistence of chromocenter. (H) Metaphase I ($2n= 20 A + X_1X_2X_3Y$). (I) Metaphases in lateral view. (J) Anaphase. (K) Telophase.

V.3 ANEXO 3 (Publicado na revista *Invertebrate Reproduction & Development*, FI: 0,52)

Heteropyknotic filament in spermatids of *Triatoma melanocephala* and *T. vitticeps* (Hemiptera, Triatominae)

ALEVI, K.C.C.; MENDONÇA, P.P.; PEREIRA, N.P.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Heteropyknotic filament in spermatids of *Triatoma melanocephala* and *T. vitticeps* (Hemiptera, Triatominae). **Inv Rep Dev** v. 58, p. 9-12, 2013.

Kaio Cesar Chaboli Alevi, Priscila Pasqüetto Mendonça, Nathália Paiva Pereira, João Aristeu da Rosa and Maria Tercília Vilela de Azeredo Oliveira

ABSTRACT

The species *Triatoma melanocephala* and *T. vitticeps* are vectors of Chagas disease in Brazil. An analysis of the number of chromosomes has led to the proposal of the exclusion of both these species and *T. tibiamaculata* from the *Brasiliensis* subcomplex because of their similarities to the triatomines of North America. *T. melanocephala* has been found to have morphologic characteristics that are very similar to those of *T. vitticeps*, and these species have been considered synonymous. Thus, this paper analyzes the spermiogenesis of *T. melanocephala* and *T. vitticeps* in order to compare the two and to evaluate another possible relationship between the species. This study describes spermatogenesis and observes the morphology, cell size and cytogenetic characteristics of triatomines. In all *T. melanocephala* cells, a slightly prominent peripheral heteropyknotic filament was observed. In *T. vitticeps*, an extensive peripheral heteropycnotic filament was observed in spermatids. This filament was observed in either the periphery or the middle of the cell. Thus, this paper describes the spermiogenesis stage of two cryptic species of triatomines

and suggests that the central heteropyknotic filament can be used as a tool in the diagnosis of *T. vitticeps*, a vector of Chagas disease.

INTRODUCTION

Triatomines are insects that are taxonomically included in the Hemiptera order and the Heteroptera suborder within the Reduviidae family and in the Triatominae subfamily (Lent and Wygodzinsky 1979). These organisms are of great medical importance, because all of the species that belong to the Triatominae subfamily are both bloodsucking and susceptible to infection from the protozoan *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae) and are therefore potential vectors of Chagas disease (Noireau et al. 2009).

The species *Triatoma melanocephala*, as described by Neiva and Pinto (1923), is a rare species of Hemiptera that is found exclusively in the Brazilian states of Bahia, Pernambuco, Paraíba, Rio Grande do Norte, and Sergipe (Gurgel-Gonçalves et al. 2012). It was found to be infected by *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), the causative agent of Chagas disease. The species is likely an important link of sylvatic and domestic transmission cycles that transmit the parasite from mice and marsupials to humans (Sherlock and Guitton 1980).

T. vitticeps, as described by Stal (1859), is a species found in the Brazilian states of Bahia, Espírito Santo, Minas Gerais and Rio de Janeiro (Gurgel-Gonçalves et al. 2012). This species is considered to be of secondary importance in the transmission of Chagas disease (Gonçalves et al. 1998), since it defecates only a few minutes after practicing hematophagy (Coura 2003). This vector is predominant in Espírito Santo state, and was found to have infected some homes in Rio de Janeiro (Gonçalves et al. 1998).

When they morphologically described *T. melanocephala*, Sherlock and Guitton (1980) proposed that this hemipteran possessed morphological characteristics that are very similar to those of *T. vitticeps*, and that the only features that distinguished them were the absence of the shell-like region on the head and the presence of two brown stains on the thorax. Additionally, *T. melanocephala* has long been considered to be a synonym of *T. vitticeps* (Lent and Wygodzinsky 1979).

These two species belong to the *Brasiliensis* subcomplex. This subcomplex is present in South America and consists of nine cryptic species: *T. brasiliensis*, *T. juazeirensis*, *T. melanica*, *T. melanocephala*, *T. petrochiae*, *T. lenti*, *T. sherlocki*, *T. tibiamaculata* and *T. vitticeps* (Schofield and Galvão 2009). However, an analysis of the number of chromosomes has led to the proposal of the exclusion of *T. melanocephala*, *T. vitticeps*, and *T. tibiamaculata* from the complex because of their similarities to the triatomines of North America (Alevi et al. 2012).

In addition to their morphological similarities, the species *T. melanocephala* and *T. vitticeps* share the same number of chromosomes. These organisms, as well as *T. eratyrsiformis*, are an irregularity among the 140 species of triatomines (Schofield and Galvão 2009), since they have the diploid chromosome set $2n = 24$ ($20A + X_1X_2X_3Y$) (Schreiber and Pellegrino 1950; Panzera et al. 2010; Alevi et al. 2012). Furthermore, they share the same cytogenetic pattern during spermatogenesis (Alevi et al. 2013).

Thus, this paper analyzes the spermatids of *T. melanocephala* and *T. vitticeps* in order to compare them and to evaluate another possible relationship between the species.

MATERIAL AND METHODS

In this study, five males from each species were used. They had been assigned by

the Triatominae Insectarium within the Department of Biological Sciences, in the College of Pharmaceutical Sciences, at Sao Paulo State University's Araraquara campus (Insetário de Triatominae, UNESP – Araraquara). The seminiferous tubules of adult males were torn and fixed to a cover slip. They then underwent the cytogenetic technique of lacto-acetic orcein (De Vaio et al., 1985). The biological material was analyzed using a Jenaval light microscope (Zeiss) attached to a digital camera and an Axio Vision LE 4.8 image analyzer (Copyright © 2006-2009 Carl Zeiss Imaging Solutions Gmb H). The images were magnified by a factor of 1000.

RESULTS

T. melanocaphala

The cytogenetic technique of lacto-acetic orcein revealed the elongation of the spermatids (Fig. 1A-I). In all cells, a slightly prominent peripheral heteropyknotic filament was observed (Fig. 1A-I, arrow). Note that the spermatids are relatively larger.

T. vitticeps

Spermiogenesis was observed through the use of the cytogenetic technique of lacto-acetic orcein (Fig. 2A-G). An extensive peripheral heteropycnotic filament was observed in the spermatids (Fig. 2A-F, arrows). This filament was observed in either the periphery (Figure 4A, B, D) or the middle of the cell (Fig. 2C, E, F). During the spermatozoid analysis, the heteropycnotic filament was not found to be present. (Fig. 2G).

DISCUSSION

Spermiogenesis is a period of spermatogenesis in triatomines that is rarely addressed in the literature. It mainly consists of the differentiation of the spermatid into the spermatozoid.

When compared, *T. melanocephala* and *T. vitticeps* showed differences in their degrees of heteropycnose and in their arrangements of the filaments. The morphology and cell size also differed between the species: *T. melanocephala* cells were found to be relatively larger than those of *T. vitticeps*.

The peripheral heteropyknotic filament was found in both species, as it had been in *T. pseudomaculata* (Mendonça, 2010). However, the thick filament core observed in some *T. vitticeps* spermatids may be considered an initial characteristic that can be used in the differentiation of this species from other triatomines, because few organisms whose spermiogenesis have been studied have only a rounded heteropyknotic corpuscle during spermiogenesis, a characteristic which was also observed in *T. maculata* (Mendonça 2010), *T. klugi* (Costa and Azeredo-Oliveira, 2008) and *T. melanosoma* (Bardela et al. 2008).

Thus, this paper describes the spermiogenesis of two cryptic species of triatomines and suggests that central heteropyknotic filament can be used as a tool in the diagnosis of *T. vitticeps*, a vector of Chagas disease.

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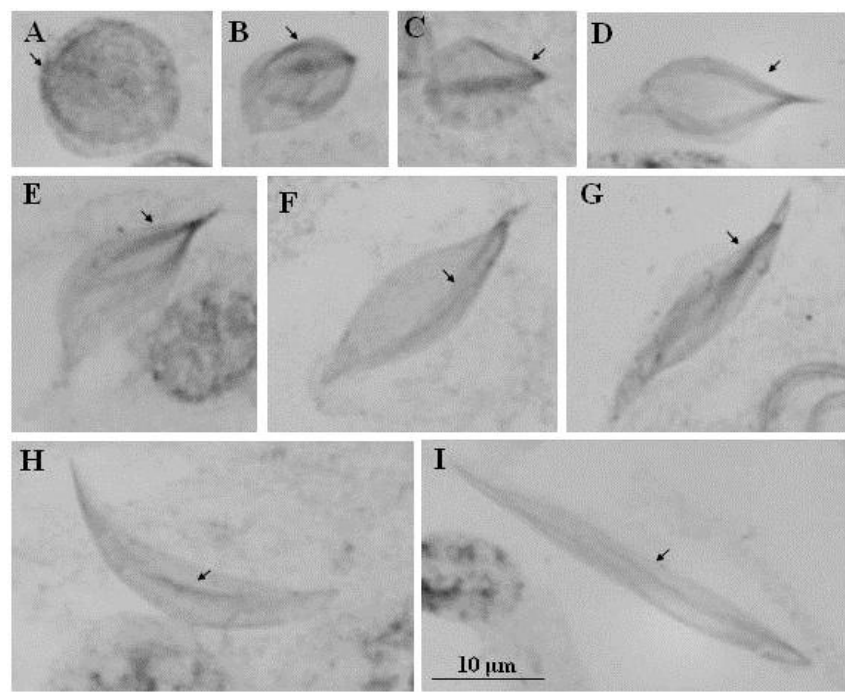


Fig. 1: Spermiogenesis of *T. melanocephala* (A-I). Note the filament heteropyknotic peripheral little prominent (arrow).

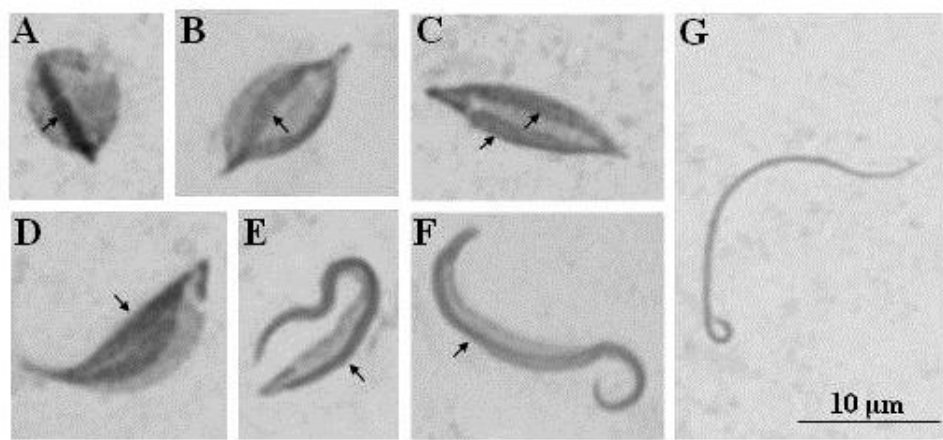


Fig. 2: Spermiogenesis of *T. vitticeps* (A-G). Note the filament extensive peripheral heteropyknotic (A, B, D, arrow) and middle of the cell (C, E, F, arrow).

V.4 ANEXO 4 (Aceito para publicação na revista *Genetics and Molecular Research*, FI: 1,1)

Distribution of constitutive heterochromatin in *Triatoma melanocephala*: would *T. melanocephala* a species evolutionarily related to *Panstrongylus lutzi*?

ALEVI, K.C.C.; MENDONÇA, P.P.; PEREIRA, N.P.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Distribution of constitutive heterochromatin in *Triatoma melanocephala*: would *T. melanocephala* a species evolutionarily related to *Panstrongylus lutzi*? **Gen Mol Res**, *in press*, 2014.

Kaio Cesar Chaboli Alevi, Priscila Pasquetto Mendonça, Nathália Paiva Pereira, João Aristeu da Rosa and Maria Tercília Vilela de Azeredo Oliveira

ABSTRACT

Triatoma melanocephala, in principle, by means of morphological parameters and the geographical layout was included Brasiliensis subcomplex, since there was no other data available in the literature this. By describing karyotype proposed exclusion of *T. melanocephala* as well as of *T. vitticeps* and *T. tibiamaculata* the subcomplex, which present fragmentation of sexual chromosomes X and thereby approaching the species of North America. Therefore, in order to provide new data on cytotaxonomic this vector of Chagas disease, and especially, try to relate this species with some group, complex or subcomplex of triatomine species, aiding in their classification, the present work describes the pattern constitutive heterochromatin of *T. melanocephala*. This species shows no constitutive heterochromatin in autosomes and X sex chromosome, only the Y sex

chromosome. Already banding pattern of heterochromatic the *T. melanocephala* when compared to that described in *P. lutzi*, proved to be identical. Thus, through the present work we demonstrate a relationship between initial *T. melanocephala* and *P. lutzi*. However, we emphasize that other comparative studies should be conducted between these species as experimental crosses, molecular analyzes, enzymatic, morphological and morphometric analysis in order to evaluate whether these species are actually evolutionarily related or if the number of chromosomes and the heterochromatic pattern emerged as homoplasies in *T. melanocephala* and *P. lutzi*.

SHORT COMMUNICATION

Triatomines are insects that are taxonomically included in the Hemiptera order and the Heteroptera suborder within the Reduviidae family and in the Triatominae subfamily (Lent and Wygodzinsky, 1979). These organisms are of great medical importance, because all of the species that belong to the Triatominae subfamily are both bloodsucking and susceptible to infection from the protozoan *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae) and are therefore potential vectors of Chagas disease (Noireau et al., 2009).

Specific identification and systematics of triatomines have been based primarily on morphological observations, mainly by light microscopy (Lent and Wygodzinsky, 1979). However, molecular data (Mendonça et al., 2009), enzymatic (Costa et al., 1997) and cytogenetic techniques (Panzera et al., 2010; Alevi et al., 2012a; Alevi et al., 2013a) has been used as important tools to aid in the classification of species.

Cytogenetic tools were used to differentiate *Triatoma garciabesi* of *T. sordida* and elevates it to the level of species (Panzera et al., 1997). Cytogenetic data, as the number of chromosomes and the pattern of constitutive heterochromatin, were also used to group the

species of *Brasiliensis* subcomplex (Panzera et al., 2010; Alevi et al., 2012a, Alevi et al., 2012b; Alevi et al. 2013a). So cytotaxonomy shows up as an important tool in systematics of triatomines.

T. melanocephala is a species endemic to Brazil, specifically the States of Bahia, Paraíba, Pernambuco, Rio Grande do Norte and Sergipe (Gurguel-Gonçalves et al., 2012). Recently, this organism was found infected with *T. cruzi* in intradomiciliary environment in the State of Pernambuco (Silva et al., 2012).

This species, in principle, by means of morphological parameters and the geographical layout was included *Brasiliensis* subcomplex (Schofield and Galvão, 2009), since there was no other data available in the literature. Alevi et al. (2012a), by describing karyotype proposed exclusion of *T. melanocephala* as well as of *T. vitticeps* and *T. tibiamaculata* the subcomplex, which present fragmentation of sexual chromosomes X and thereby approaching the species of North America.

Therefore, in order to provide new data on cytotaxonomic this vector of Chagas disease, and especially, try to relate this species with some group, complex or subcomplex of triatomine species, aiding in their classification, the present work describes the pattern constitutive heterochromatin of *T. melanocephala*.

Preparation of seminiferous tubules of at least ten adult male *T. melanocephala*, from the "Insectarium of Triatominae" installed in Araraquara, Sao Paulo, after being shredded, crushed and fixed in liquid nitrogen on glass slide, were stained by C-banding (Sumner, 1972).

The initial prophase showed a large chromocenter (Figure 1A, arrowhead) constituted by sex chromosomes and some heterochromatic blocks dispersed in the nucleus (Figure 1 A, arrows). Based on the analysis of metaphases I (Figure 1B, C), we confirmed that males of *T. melanocephala* have the karyotype $2n = 24$ ($20A + X_1X_2X_3Y$). Moreover,

by analyzing the metaphase I in polar view (Figure 1B, C), and side (Figure 1D) we observed that this species shows no constitutive heterochromatin in autosomes and X sex chromosome (arrows), only the Y sex chromosome (arrowheads).

The fragmentation of the sex chromosome X is a characteristic derived, since according Ueshima (1966), sexual system of sex determination in ancestral Triatominae subfamily is XY. This peculiarity has been described in the literature in only four species, out more, *T. melanocephala* (Alevi et al., 2012a), *T. vitticeps* (Schreiber and Pellegrino, 1950), *T. eratyrusiformis* (Panzer et al., 2010) and *Panstrongylus lutzi* (Santos et al., 2010). Of these species, only *T. vitticeps* and *P. lutzi* present the pattern heterochromatic described. Both as observed in *T. melanocephala* show no constitutive heterochromatin in autosomes. However, *T. vitticeps* presents constitutive heterochromatin in the sex chromosome X (Severi-Aguiar et al., 2006).

T. melanocephala and *T. vitticeps* species were considered synonymous for Lent and Wygodzinsky (1979). The unique morphological characteristics that differentiate these species are the absence of testacea range in the head and the presence of two brown stains in thorax (Sherlock and Guitton, 1980). Alevi et al. (2013b), by analyzing the pattern heteropycnotic observed a direct correlation between the species, since *T. melanocephala* showed the same characteristics described by Severi-Aguiar et al. (2006) for *T. vitticeps*. Thus, although the number of chromosomes and the pattern heteropyknotic are identical, the provision of constitutive heterochromatin in the sex chromosomes X can be considered as a diagnostic characteristic for *T. vitticeps*, since *T. melanocephala* has no sex chromosome heterochromatic blocks in X.

Already banding pattern of heterochromatic the *T. melanocephala* when compared to that described in *P. lutzi*, proved to be identical. Both showed heterochromatic blocks in prophase, the same number of chromosomes and the same disposition of constitutive

heterochromatin in the chromosomes, i.e., only the Y sex chromosome appeared heterochromatic. Santos et al. (2010) believe that possibly the heterochromatic bands observe in prophase were not seen during metaphase due to the high degree of condensation of chromosomes. However, we believe that the blocks do not really exist during the metaphase, since the C-banding technique is specific constitutive heterochromatin and was totally clear that single chromosome heterochromatin the sex chromosome Y.

Thus, through the present work we demonstrate a initial relationship between *T. melanocephala* and *P. lutzi*. However, we emphasize that other comparative studies should be conducted between these species as experimental crosses, molecular analyzes, enzymatic, morphological and morphometric analysis in order to evaluate whether these species are actually evolutionarily related or if the number of chromosomes and the heterochromatic pattern emerged as homoplasies in *T. melanocephala* and *P. lutzi*.

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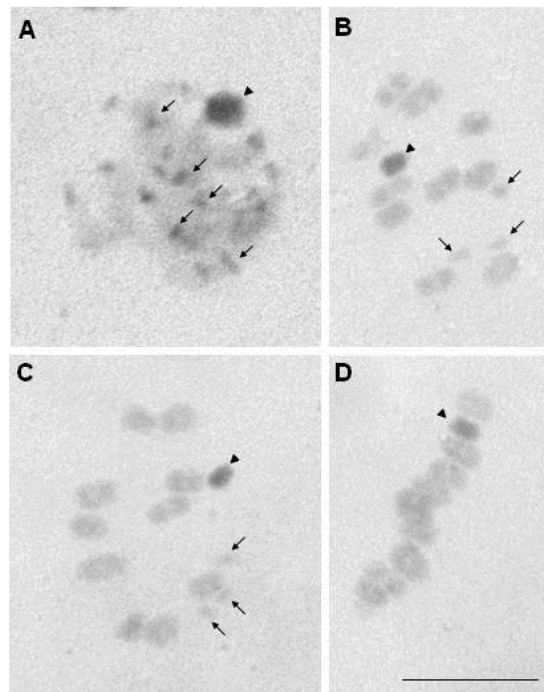


Figure 1: Seminiferous tubule of *Triatoma melanocephala* stained by C-Banding. (A) Initial Prophase. Note a large chromocenter (arrowhead) and heterochromatic blocks (arrows) in the nucleus. (B, C, D) Metaphase I. Note the Y sex chromosome heterochromatic (arrowhead) and the X sex chromosomes shows no constitutive heterochromatin (arrow). Bar: 10 μ m.

V.5 ANEXO 5 (Publicado na revista *Revista de Ciências Farmacêuticas Básica e Aplicada*)

ALEVI, K.C.C.; MENDONÇA, P.P.; PEREIRA, N.P.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Análise das possíveis Regiões Organizadoras Nucleolares e da atividade nucleolar em *Triatoma melanocephala* e *T. lenti*, importantes vetores da doença de Chagas. *Rev Ciên Farm Básica Apl* v. 34, p. 417-421, 2013e.

Análise das possíveis Regiões Organizadoras Nucleolares e da atividade nucleolar em *Triatoma melanocephala* e *T. lenti*, importantes vetores da doença de Chagas

Kaio Cesar Chaboli Alevi¹, Priscila Pasqüetto Mendonça, Nathália Paiva Pereira, João Aristeu da Rosa e Maria Tercília Vilela de Azeredo-Oliveira

RESUMO

Triatoma melanocephala e *T. lenti* são espécies crípticas de triatomíneos pertencentes ao subcomplexo *Brasiliensis*. Esses organismos foram agrupados no subcomplexo apenas por caracteres morfológicos e pela disposição geográfica. Sendo assim, estudos citogenéticos são considerados como importantes ferramentas na classificação dos triatomíneos e, com isso, podem auxiliar na criação de um plano de profilaxia da doença. Por meio da técnica citogenética de impregnação por íons prata, foi possível visualizar o comportamento nucleolar e as Regiões Organizadoras Nucleolares (RONs) desses insetos. *T. melanocephala* apresentou três RONs ativas nos autossomos durante a prófase I. *T. lenti* apresentou duas RONs ativas nos autossomos durante a prófase I e a metáfase I. Ambas as espécies apresentaram o fenômeno de persistência do material nucleolar encontrado em triatomíneos. Sendo assim, por meio da análise das RONs, foi possível observar que *T.*

lenti, quando comparado com os outros organismos do subcomplexo previamente analisadas, apresentou marcações semelhantes à *T. tibiamaculata* e que *T. melanocephala* não apresenta semelhanças com as demais espécies analisadas do subcomplexo, quanto a esse aspecto.

INTRODUÇÃO

Os triatomíneos pertencem a Ordem Hemiptera, subordem Heteroptera, família Reduviidae e subfamília Triatominae (Lent & Wygodzinsky, 1979). Essa ordem é caracterizada por insetos com um par de asas anteriores do tipo hemiélitro, um par de asas posteriores membranosas e um aparelho bucal do tipo picador-sugador (Schofield, 1994).

A subfamília Triatominae é composta por 145 espécies distribuídas em 18 gêneros e seis tribos (Alevi et al., 2013a). Todas as espécies de triatomíneos são suscetíveis à infecção pelo *Trypanossoma cruzi* (Kinetoplastida, Trypanosomatidae) e, conseqüentemente, potenciais vetores da doença de Chagas. A infecção ocorre por meio da alimentação com sangue de mamífero infectado, sendo todos os estádios ninfais sujeitos a ingerirem o parasito, uma vez que, após a eclosão, a hematofagia é obrigatória em todas as fases de vida dos triatomíneos (Noireau et al., 2009).

A identificação específica e a sistemática dos triatomíneos têm se pautado fundamentalmente em observações morfológicas, principalmente por meio de microscopia de luz (Lent & Wygodzinsky, 1979) e de varredura (Rosa et al., 2000). Schofield & Galvão (2009) propuseram a classificação destes organismos em subcomplexos específicos. Contudo, para agrupar os insetos utilizaram apenas a disposição geográfica e os caracteres morfológicos.

Triatoma melanocephala é uma espécie rara encontrada principalmente nos estados da Bahia, Paraíba, Sergipe, Rio Grande do Norte e Pernambuco (Gurgel-Gonçalves et al.,

2012). Embora tenha sido encontrado em áreas domiciliares, esse organismo é incapaz de nele colonizar, pois é considerado essencialmente silvestre. Esse triatomíneo habita principalmente regiões de Caatinga e, em seu ecótono natural, foi encontrado infectado pelo protozoário *T. cruzi*. Provavelmente, a espécie é um importante elo dos ciclos de transmissão silvestre e domiciliar, levando o protozoário dos marsupiais e ratos para o homem (Sherlock & Guitton, 1980).

T. lenti é uma espécie endêmica dos estados da Bahia, Goiás e Roraima (Gurgel-Gonçalves et al., 2012). Esse triatomíneo é considerado como um potencial vetor da doença de Chagas de importância secundária e, de acordo com Sherlock & Guitton (1974), foi encontrado infectado pelo protozoário *T. cruzi*.

Esses hemípteros pertencem ao grupo Infestans, complexo Infestans e subcomplexo Brasiliensis (Schofield & Galvão, 2009). Esse subcomplexo está presente na América do Sul e é composto por nove espécies: *T. brasiliensis*, *T. juazeirensis*, *T. melanica*, *T. melanocephala*, *T. petrochiae*, *T. lenti*, *T. sherlocki*, *T. tibiamaculata* e *T. vitticeps*.

Monteiro et al. (2001) relatam que os táxons do subcomplexo Brasiliensis são dificilmente esclarecidos apenas por parâmetros morfológicos, pois as espécies são crípticas. Assim, os estudos citogenéticos que abrangem estes organismos são considerados como importantes ferramentas para a diferenciação e sistemática destes insetos (Pérez et al., 1992; Panzera et al., 2000; Alevi et al., 2012a; Alevi et al., 2012b; Alevi et al., 2013b, Alevi et al. 2013c, Alevi et al., 2013d).

As Regiões Organizadoras Nucleolares (RONs) correspondem a alças do DNA contendo genes responsáveis pela transcrição do RNA ribossômico (RNAr), 18S; 5,8S e 28S, situados no nucléolo da célula que, durante a meiose, se organizam nos cromossomos, e quando ativas expressam as proteínas B23 e C23 que, por possuírem reatividade com a prata, são chamadas de AgNORs (Hsu et al., 1975).

Durante a divisão celular, as células cessam a transcrição de RNAr na prófase, ocorre fragmentação nucleolar e a maior parte das proteínas migram para o citoplasma, sendo que algumas permanecem associadas às RONS dos cromossomos. Apenas na anáfase a transcrição de RNAr é reativada e gradualmente ocorre reorganização do nucléolo (González-Garcia & Rufas, 1995). Contudo, Tartarotti & Azeredo-Oliveira (1999) observaram que durante a meiose de triatomíneos ocorria persistência do material nucleolar, ou seja, resquícios desse material eram visualizados durante todas as fases da divisão meiótica.

A profilaxia da doença de Chagas pode ser otimizada quando associada a dados biológicos desses insetos, que informam sobre a distinção entre espécies silvestres de baixo risco e espécies domiciliares nocivas, hábitos e horários de hematofagia, capacidade reprodutiva, resistência a inseticidas, ciclo biológico, reservatórios domésticos e silvestres do agente etiológico. Bem como as inter-relações entre espécies e gêneros estudados no contexto evolutivo, por meio de ensaios citogenéticos que comparam padrões cromossômicos os quais podem ser aplicados à filogenia dos triatomíneos (Tavares & Azeredo-Oliveira, 1996).

Assim, o presente trabalho tem como objetivo analisar a atividade nucleolar e as possíveis RONS ativas durante a meiose desses importantes vetores da doença de Chagas no Brasil.

MATERIAL E MÉTODOS

No estudo, foram utilizados dez indivíduos machos da espécie *T. melanocephala* e dez da espécie *T. lenti*, cedidos pelo “Insetário de Triatominae”, instalado no Departamento de Ciências Biológicas, da Faculdade de Ciências Farmacêuticas, Câmpus de Araraquara. Os túbulos seminíferos dos machos adultos, depois de dilacerados e fixados

na lâmina, foram submetidos à técnica citoquímica de impregnação por íons prata (Howell & Black, 1980). O material biológico foi analisado ao microscópio de luz *Jenaval* (Zeiss), acoplado à câmera digital e ao sistema analisador de imagens *Axio Vision LE 4.8* (Copyright ©2006-2009 Carl Zeiss Imaging Solutions Gmb H), com aumento de 1000 vezes.

RESULTADOS

Por meio da técnica citogenética de impregnação por íons prata foi possível observar a atividade nucleolar e estudar as RONS durante a espermatogênese de *T. melanocephala*. No núcleo poliploide (Figura 1I), célula da parede do túbulo seminífero responsável pela nutrição das células em divisão meiótica, foi possível a visualização de um corpúsculo impregnado com os íons prata (seta). A análise da célula interfásica (Figura 1II) possibilitou a visualização de um nucléolo (seta). Foi possível observar a fragmentação nucleolar durante a prófase I (Figura 1III-VI, setas) que persistiu durante a metáfase (Figura 1VII), onde foi possível encontrar resquícios do material nucleolar (seta). Durante a prófase I, também foi visível três RONS ativas nos autossomos (ponta de seta). No final da anáfase, mais próximo ao início da telófase, foi possível visualizar a reativação nucleolar (Figura 1VIII). A espermátide inicial permitiu a visualização do nucléolo reativado (Figura 1IX).

Por meio da técnica citogenética de impregnação por íons prata foi possível observar a atividade nucleolar e estudar as RONS durante a espermatogênese de *T. lenti*. No núcleo poliploide (Figura 2I), foi possível visualizar muitos corpúsculos impregnados pelos íons prata. A análise da célula interfásica (Figura 2II) possibilitou a visualização de dois nucléolos (ponta de seta). Durante a prófase I, foi possível observar a fragmentação nucleolar (Figura 2III-V, ponta de seta) que persistiu durante a metáfase (Figura 1VI, VII).

Tanto na prófase I (Figura 2IV), como na metáfase I (Figura 2VI), foi possível observar duas RONS ativas nos autossomos (setas). A metáfase em visão lateral permitiu observar apenas uma RON ativa (Figura 2VII, seta). Na espermatíde inicial (Figura 2VIII) foi possível visualizar o nucléolo reativado.

DISCUSSÃO

Acredita-se que a persistência do material nucleolar é muito importante para a espermiogênese, pois esta fase consiste na diferenciação morfológica das espermatídes em espermatozoides, e não há nenhuma atividade transcricional de RNAr (Olivieri & Olivieri, 1965).

A ideia de ausência de síntese nucleolar durante a espermiogênese foi proposta inicialmente por Olivieri & Olivieri (1965), por meio de técnicas autorradiografia. Nesse trabalho não foi relatada nenhuma atividade transcricional de RNAr durante a espermiogênese, que foi corroborada por Rickards et al. (2007), após descobrirem que durante essa fase da espermatogênese há baixa atividade da nucleolina, proteína que desempenha importante papel na transcrição de RNAr. Todas as proteínas necessárias para a espermiogênese são produzidas a partir de RNAm transcritos durante a meiose e armazenados no citoplasma (Schäfer et al., 1995). Dessa forma, a persistência nucleolar observada nos triatomíneos é um evento muito importante, pois esse material em conjunto com as RONS são os principais fatores transcricionais que permitem que a espermiogênese ocorra.

A análise dos núcleos poliploides e das células interfásicas de *T. melanocephala* possibilita ressaltar que essas células não apresentam alta atividade sintética, pois ambas apresentam um pequeno corpúsculo nucleolar. No entanto, o núcleo poliploide de *T. lenti* apresentou grandes marcações pelos íons pratas que, em conjunto com os dois nucléolos

encontrados na célula interfásica, permitem ressaltar que essa espécie apresenta alta atividade sintética. O tamanho e o número de nucléolos e corpos pré-nucleolares dependem das características funcionais das células e pode refletir, então, em diferenças metabólicas e funcionais (Tavares & Azeredo-Oliveira, 1997).

Apesar do conhecimento sobre a importância das RONS na formação do nucléolo durante a interfase e, principalmente, na biogênese dos ribossomos, esses estudos são pouco abordados nos triatomíneos. A quantidade de RONS nos núcleos refletem o nível de transcrição de DNAr e, com isso, quantifica a síntese proteica nos organismos. Esses dados também podem auxiliar no estudo evolutivo das espécies, pois as RONS são fatores atuantes na evolução dos organismos.

A espécie *T. melanocephala* apresentou três RONS ativas em três pares de autossomos durante a divisão meiótica, mais especificamente no final da prófase I. Em *T. lenti* foram observadas duas RONS ativas na prófase I e na metáfase I. O estudo das RONS permite o estabelecimento de graus de parentesco e um possível estabelecimento da ou sexuais) são comparados.

A análise da metáfase de *T. lenti* em visão lateral permitiu a observação de apenas uma RON. Isso ressalta a importância da análise das células metafásicas também em visão polar nos estudos citogenéticos, uma vez que, em visão lateral pode ocorrer sobreposição do material cromossômico e, com isso, marcações importantes deixam de ser observadas.

Entre as nove espécies que compõem o subcomplexo Brasiliensis, em apenas três foram descritos os números de RONS, a saber, *T. brasiliensis*, *T. tibiamaculata* e *T. vitticeps*. Bardella et al. (2010) relataram que *T. brasiliensis* apresenta RONS ativas em um par de autossomos. Segundo Severi-Aguiar et al. (2005), *T. tibiamaculata* apresenta marcações AgNORs em apenas dois autossomos, assim como foi observado em *T. lenti*. Já a espécie *T. vitticeps* não apresenta marcações nos autossomos, mais sim em dois

cromossomos sexuais (X1X2) (Severi-Aguiar et al., 2006). Contudo, a técnica de impregnação por íons prata só marca as RONS que foram ativadas na interfase anterior, seria necessário o emprego da técnica citogenética molecular de FISH em *T. melanocephala* e *T. lenti* para que análises comparativas mais específicas fossem realizadas.

Assim, o presente trabalho possibilitou descrever o comportamento nucleolar de *T. melanocephala* e *T. lenti* e, assim, corroborar a hipótese de que os triatomíneos apresentam persistência do material nucleolar durante a meiose. Além disso, por meio do estudo das RONS ativas durante a espermatogênese, foi possível observar similaridade no número e na localização entre *T. lenti* e *T. tibiamaculata* e, ainda, relatar que *T. melanocephala* não apresentou nenhum grau de similaridade com os organismos do subcomplexo Brasiliensis. Dessa forma ressaltamos a importância das análises citogenéticas como uma ferramenta no estudo de espécies crípticas, bem como no entendimento da biologia e evolução desses insetos, importantes vetores da doença de Chagas.

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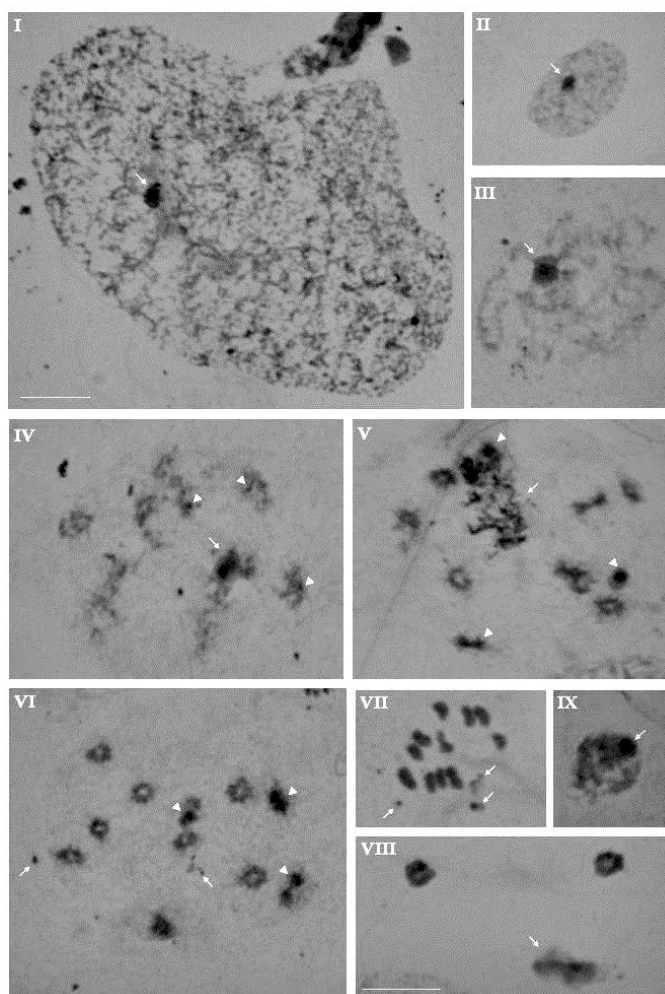


Figura 1: Espermatogênese de *Triatoma melanocephala* corada com a técnica citogenética de impregnação por íons prata. I: Núcleo poliploide com apenas uma marcação nucleolar (seta). II: Núcleo interfásico. Notar a presença do nucléolo (seta). III-VI: Prófase I. Notar a fragmentação nucleolar (setas) e as RONS (pontas de seta). VII: Metáfase I. Notar a persistência nucleolar (setas). VIII: Começo de Telófase. Note a reativação nucleolar (seta). IX: Espermátide inicial com o nucléolo reativado. Bar: 10 um.

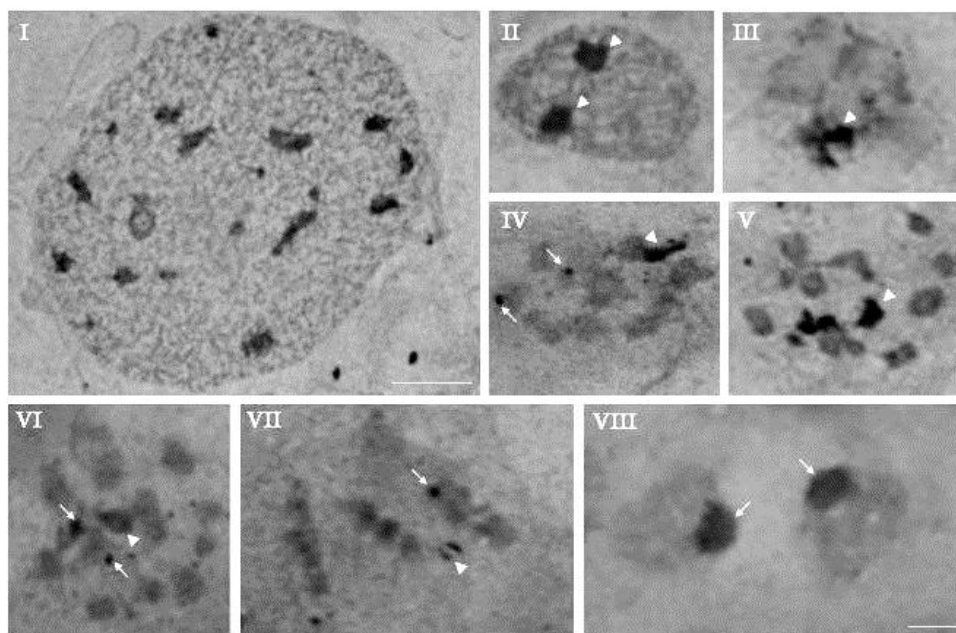


Figura 2: Espermatogênese de *Triatoma lenti* corada com a técnica citogenética de impregnação por íons prata. I: Núcleo poliplóide. Notar vários corpúsculos impregnados com AgNOR. II: Núcleo interfásico. Notar a presença de dois nucléolos (pontas de seta). III-V: Prófase I. Notar a fragmentação nucleolar (setas) e as RONS (pontas de seta). VI Metáfase I em visão polar. Notar as RONS (setas) e a persistência do material nucleolar (ponta de seta). VII: Metáfase em visão lateral. Notar uma RONS (seta) e a persistência do material nucleolar (pontas de seta). VIII: Espermátides iniciais com o nucleolo reativado (setas). Bar: 10 um.

V.6 ANEXO 6 (Resumo apresentado no IV Simpósio Latino-Americano de Citogenética e Evolução, com modificações)

Location of the 45S ribosomal DNA clusters in *Triatoma lenti* and *T. melanocephala* (Hemiptera, Triatominae)

Kaio Cesar Chaboli Alevi, Sebastián Pita, Yanina Panzera, Maria Tercília Vilela de Azeredo Oliveira and Francisco Panzera

Triatoma lenti and *T. melanocephala* are endemic Brazilian species of triatomines infected by the protozoan *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), causative agent of Chagas disease. Based on morphological characters and their geographical distribution, both species were included in the brasiliensis subcomplex (SCHOFIELD and GALVÃO, 2009). Recently, the description of different chromosomal traits supported the inclusion of *T. lenti* in the brasiliensis subcomplex while that the exclusion of *T. melanocephala* of this group (ALEVI et al., 2012a, 2012b, 2013, 2014). However, we emphasized the importance to apply other genetic approaches to confirm the taxonomic grouping of both species. Herein, by means of fluorescent in situ hybridization (FISH), we analyzed the chromosomal location of the 45S ribosomal DNA (rDNA) clusters in both species using a triatomine rDNA probe (PANZERA et al., 2012). We observed that in *T. lenti* ($2n= 20$ autosomes plus XY/XX, ALEVI et al., 2012a), the rDNA signals are located in one autosomal pair, similar as observed in other two species of the brasiliensis subcomplex: *T. sherlocki* (PANZERA et al., 2012) and *T. brasiliensis* (BARDELLA et al., 2010). However in *T. melanocephala* ($2n= 20$ autosomes plus $X_1X_2X_3Y/X_1X_1X_2X_2X_3X_3$, ALEVI et al., 2012b), the rDNA clusters is located in one X chromosome. Our FISH results confirm

previous cytogenetic data about the taxonomic grouping of the both species here analyzed. We suggested that the autosomal chromosome location of the 45S rDNA may be a common cytogenetic characteristic of the species that compose the brasiliensis subcomplex.

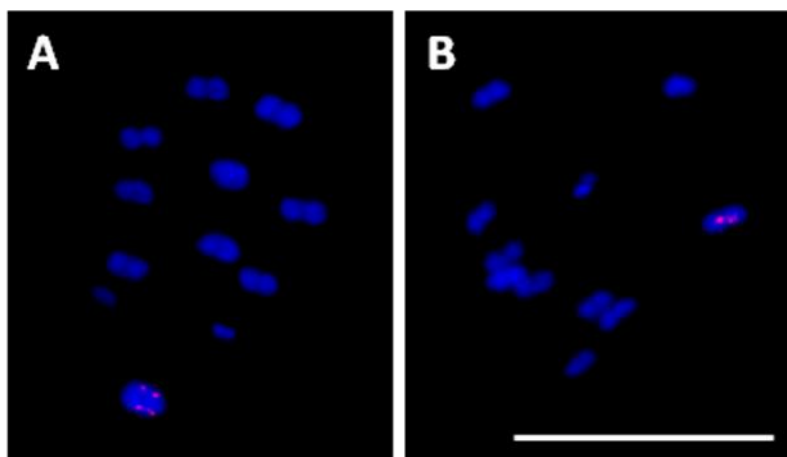


Figure 1: Metaphases I (A) e II (B) of *T. lenti*. Note the rDNA signals in one autosomal pair Bar: 10 μ m.

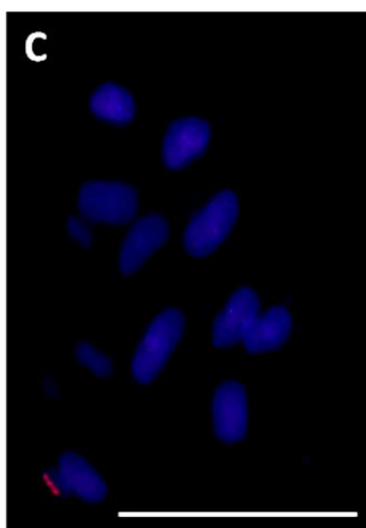


Figura 2: Metaphase I (A) of *T. melanocephala*. Note the rDNA clusters in one X chromosome. Bar: 10 μ m.

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V.7 ANEXO 7 (Artigo publicado na revista *Genetics and Molecular Research*, FI: 1,1)

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Karyotype and spermatogenesis in *Triatoma lenti* (Hemiptera: Triatominae), a potential Chagas vector

ABSTRACT

All species of Triatominae are susceptible to infection by *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae) and consequently, potential insect vectors of Chagas disease. Currently there are 140 known species of triatomine bugs, which can be grouped into specific species complexes. The species *Triatoma lenti* (Hemiptera: Triatominae) is found only in Brazil and is considered a potential vector of Chagas disease. We karyotyped male *T. lenti* and examined its spermatogenesis in detail. The karyotype was found to be $2n = 20A + XY$, demonstrating that this organism has the modal chromosome set found in triatomines. This new information concerning males of this species contributes biological data that will be useful for understanding this potentially important Chagas disease vector.

INTRODUCTION

All species of Triatominae are susceptible to infection by *Trypanosoma cruzi* and consequently, are potential insect vectors of Chagas disease. Infection occurs from feeding on mammalian infected blood, with all instars capable of parasite ingestion since blood meal is required at all stages of life in triatomines (Noireau et al., 2009). Currently, there

are 140 known species of Triatominae, which is divided into 5 tribes and distributed across 15 genera (Schofield and Galvão, 2009), with the genera *Triatoma*, *Panstrongylus*, and *Rhodnius* being epidemiologically the most important.

Triatominae species can be grouped into specific complexes according to morphological similarities, geographical distribution, possibility of hybridization, and molecular proximity. Because of several possible approaches on the basis of the parameters evaluated, up to now there is no consensus about the standardization of the group into complexes and subcomplexes (Schofield and Galvão, 2009).

The systematic and specific identification of the insects has been based primarily on morphological observations, mainly with light microscopy (Lent and Wygodzinsky, 1979). However, analysis of morphological parameters alone cannot always answer all taxonomic questions, as in the case of species such as the Brasiliensis subcomplex and *Rhodnius* genus (Monteiro et al., 2001; Jurberg, 2003). In order to resolve these issues, more sensitive cytogenetic approaches are needed to distinguish among these species.

In cytogenetics, biological models of triatomine bugs are important because they have holocentric chromosomes, which have diffuse kinetochores distributed along the chromosomes and not a single kinetochore located in the centromeric region. These insects also perform an unusual meiosis in which the segregation of sex chromosomes is post-reductional (Barth, 1956; Ueshima, 1966).

Most of the cytogenetic results for triatomines were obtained on the basis of chromosome comparisons that relied on conventional staining. In these studies, the comparison criteria were often the number, morphology, and disposition of chromosomes in the metaphase plate. The data obtained from these investigations provided the first indications of the processes that occurred during chromosomal evolution in these insects (Pérez et al., 1992; Panzera et al., 2010).

According to Monteiro et al. (2001), it is difficult to explain the Brasiliensis subcomplex classification by using morphological parameters alone, because these species are cryptic. Thus, cytogenetic studies can be considered an important tool for the differentiation of these species (Pérez et al., 1992).

The species *Triatoma lenti*, described by Sherlock and Serafim in 1967, is a Hemiptera that belongs to the Infestans group, Infestans complex, and Brasiliensis subcomplex (Schofield and Galvão, 2009). This subcomplex is present in South America and consists of 9 species: *Triatoma brasiliensis*, *Triatoma juazeirensis*, *Triatoma melanica*, *Triatoma melanocephala*, *Triatoma petrochiae*, *T. lenti*, *Triatoma sherlocki*, *Triatoma tibiamaculata*, and *Triatoma vitticeps* (Schofield and Galvão, 2009).

T. lenti is found only in Brazil and is endemic to Bahia and Goiás (Carcavallo et al., 1999), and according to Sherlock and Guitton (1974), was found infected with the protozoan *T. cruzi*.

To elucidate the mechanisms involved in the cytogenetic characteristic of these insects during cell division and to understand and differentiate the organisms of the Brasiliensis subcomplex, we have described for the first time the karyotype of *T. lenti* and examined in detail its spermatogenesis. These data will aid in the evolutionary study of the insects, and thus, help us understand this important insect vector of Chagas disease and contribute to the control of the populations.

MATERIAL AND METHODS

The species analyzed was *T. lenti*. For the study, 15 specimens were used. The insects were provided by Triatominae Insectarium of São Paulo State University (UNESP, city of Araraquara, Brazil). The seminiferous tubules of the adult males underwent lacto-acetic orcein staining (De Vaio et al., 1985, with modifications). The slides were analyzed

using a light microscope (Jenaval-Zeiss), which was coupled to a digital camera and an AxioVision LE 4.8 image analyzer (Copyright© 2006–2009 Carl Zeiss Imaging Solutions Gmb H). The images were magnified by 1000X.

RESULTS

Through the cytogenetic technique of lacto-acetic orcein staining, it was possible to determine the diploid chromosome set of *T. lenti*. On the basis of the meiotic metaphases, the studied species showed the karyotype $2n = 22$ (20A + XY), i.e., 10 pairs of autosomes and 2 sex chromosomes in metaphase I and 10 autosomes and 1 pair of sex chromosomes in metaphase II (Figure 1). Karyotype analysis showed that some autosomes were larger than the others. The sex chromosomes were the smallest in the chromosome set and differed in size and coloration, being the largest and most heteropycnotic. The polyploid nuclei of the nutritive cells of the seminiferous tubules showed a higher degree of heteropycnosis and a more evident corpuscle (Figure 2A). In relation to the spermatogenesis of *T. lenti*, the following was observed. During meiotic division, and more specifically during the diffuse stage of prophase I, it was possible to monitor chromatin condensation. In the initial diffuse stage (Figure 2B-C), the condensed chromatin presented some regions as more heteropycnotic than others. An individualization of chromosomes was noted in nuclei in the intermediate diffuse stage (Figure 2D) and final diffuse stage (Figure 2E). In metaphase I, 2 univalent sex chromosomes were located in the center of the ring formed by autosomal bivalents (Figure 2F). In this phase, the condensed chromosomes showed varying degrees of heteropycnosis among them, with some autosomes being more heteropycnotic than the sex chromosomes. In metaphase II, chromosomes showed the same ring disposition (Figure 2G). Different degrees of heteropycnosis were observed on chromosomes during metaphases analyzed in lateral view (Figure 2H). In the meiotic

metaphase, the difference in size among the heterochromosomes was evident. Cells in the telophase did not show the late migration phenomenon (Figure 2I).

DISCUSSION

Cytogenetic studies have significantly contributed to our understanding of the taxonomy and systematics of insects, despite the small size of the chromosomes and their holocentric nature, which hinder in the detection of their rearrangement or changes in the structures (Camargo et al., 2006).

Cytogenetic analysis of *T. lenti* permitted us to observe that these organisms have holocentric chromosomes, supporting the hypothesis that all Heteroptera have holocentric chromosomes (Souza and Itoyama, 2006). The Heteroptera holocentric chromosomes are an excellent material for study and are used as models in investigations, since they have peculiar and interesting characteristics such as kinetic activity restricted to the chromosome ends, which is presumed to occur during chiasma finalization. These insects also undergo inverted meiosis for sex chromosomes, since the first division is reductional for autosomes and equational for the sex chromosomes, while the second division is equational for autosomes and reductional for sex chromosomes (Ueshima, 1979).

Through this technique, it was possible to identify the condensed chromatin, since this conventional cytogenetic technique enables a refined differentiation between euchromatin and heterochromatin. This is because of the stronger staining of euchromatin than heterochromatin, since heterochromatin has higher compression and more proteins than euchromatin (Azeredo-Oliveira, 1990).

The basic number of chromosomes for triatomines is $2n = 22$, with 10 pairs of autosomes and 1 pair of sex chromosomes. However, there are species with multiple sex

chromosomes (XY, X_1X_2Y , $X_1X_2X_3Y$), caused by fragmentation of the original X (Ueshima, 1966).

The analysis of metaphases in frontal view allows the description of the karyotype and analysis of chromosomes in Heteroptera, for in this phase of cell division, chromosomes are highly compressed, meaning that they are in their highest degree of condensation. Thus, on the basis of the analyzed metaphases, we observed that males of *T. lenti* have the karyotype $2n = 22$ ($20A + XY$), demonstrating thereby that this organism has the modal chromosome set found in triatomines. It is believed that 60% of the analyzed species of triatomine bugs have a diploid number of chromosomes: $2n = 20A + XY$ (Tavares and Azeredo-Oliveira, 1997a).

Among the triatomines, there is little variation in the size of the chromosomes (De Vaio et al., 1985). However, analysis of the karyotype of *T. lenti* demonstrates that the chromosomes show variation in size and degree of heteropycnosis. De Vaio et al. (1985) performed cytogenetic analysis of 3 species of insects and noted that these organisms had 1 to 3 larger and more heteropycnotic chromosomes.

The Brasiliensis subcomplex is composed of 9 species (Schofield and Galvão, 2009), and the karyotypes of 7 species have already been described: *T. brasiliensis*, *T. juazeirensis*, *T. melanica*, *T. petrochiae*, *T. tibiamaculata*, *T. vitticeps* (Panzera et al., 2010) and *T. melanocephala* (Alevi et al., 2012). Here, on the basis of the analysis of the meiotic metaphase, the karyotype of *T. lenti* has been described for the first time. This organism, like *T. brasiliensis*, *T. juazeirensis*, *T. melanica*, and *T. petrochiae*, has a diploid chromosome set that consists of $2n = 22$ chromosomes ($2n = 20A + XY$), demonstrating that *T. lenti*, with respect to karyotype analysis, is close to most species that have already been analyzed cytogenetically in this subcomplex.

The species *T. tibiamaculata* and *T. vitticeps* share some morphological similarities with the species of the Brasiliensis subcomplex, but when analyzed cytogenetically and on a molecular basis, they are related to a greater extent to the Triatominae of North America, such as *T. tibiamaculata* and *T. vitticeps*, which have a system of multiple sex determination because of multiple sex chromosomes. In other words, the karyotype of male *T. tibiamaculata* is $2n = 20A + X_1X_2Y$ (Panzer et al., 1996) and the karyotype *T. vitticeps* is $2n = 20A + X_1X_2X_3Y$ (Schreiber and Pellegrino, 1950). Mitochondrial DNA sequences also confirm the close phylogenetic relationship between these Hemiptera and the ones present in North America (Schofield and Galvão, 2009). Alevi et al. (2012) recently described the karyotype of *T. melanocephala* ($2n = 20A + X_1X_2X_3Y$) and suggested the exclusion of *T. melanocephala*, *T. vitticeps* and *T. tibiamaculata* the subcomplex by similarity with the triatomines in North America.

Analysis of the seminiferous tubule cells of *T. lenti*, allowed the observation of chromosome characteristics and the meiotic behavior of this insect. The polyploid nuclei showed a high degree of heteropycnosis and a more evident corpuscle. Other species of Heteroptera analyzed cytogenetically also demonstrated these characteristics in the polyploid nucleus (Tavares and Azeredo-Oliveira, 1997b).

The meiotic process of holocentric chromosomes was corroborated by the analysis of the spermatogenesis of *T. lenti*. The chromosomes showed normal patterns found in triatomines, such as the arrangement of sex chromosomes in the center of the ring formed by autosomes (De Vaio et al., 1985, Pérez et al., 1992). The inverted meiosis phenomenon for heterochromosomes was also confirmed in the present study.

This study contributed to knowledge on Triatominae. This data allow the Brasiliensis subcomplex to be understood better, since it is composed of cryptic species. Therefore, accumulation of more biological data will make it easier to understand the

evolution and biology of these insects, which are important insect vectors of Chagas disease.

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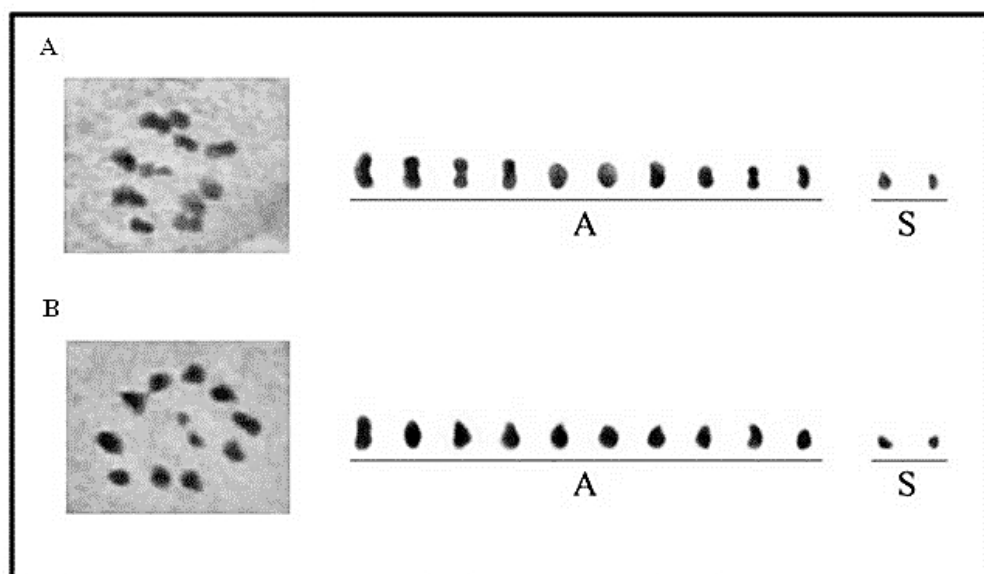


Figure 1. Karyotype of *Triatoma lenti*. (A) Circular metaphase of first meiotic division and respective karyotype with ten bivalent autosomes and two sex chromosomes (XY). (B) Circular metaphase of second meiotic division and respective karyotype $n = (10A + XY)$. A = Autosomes; S = Sex chromosomes.

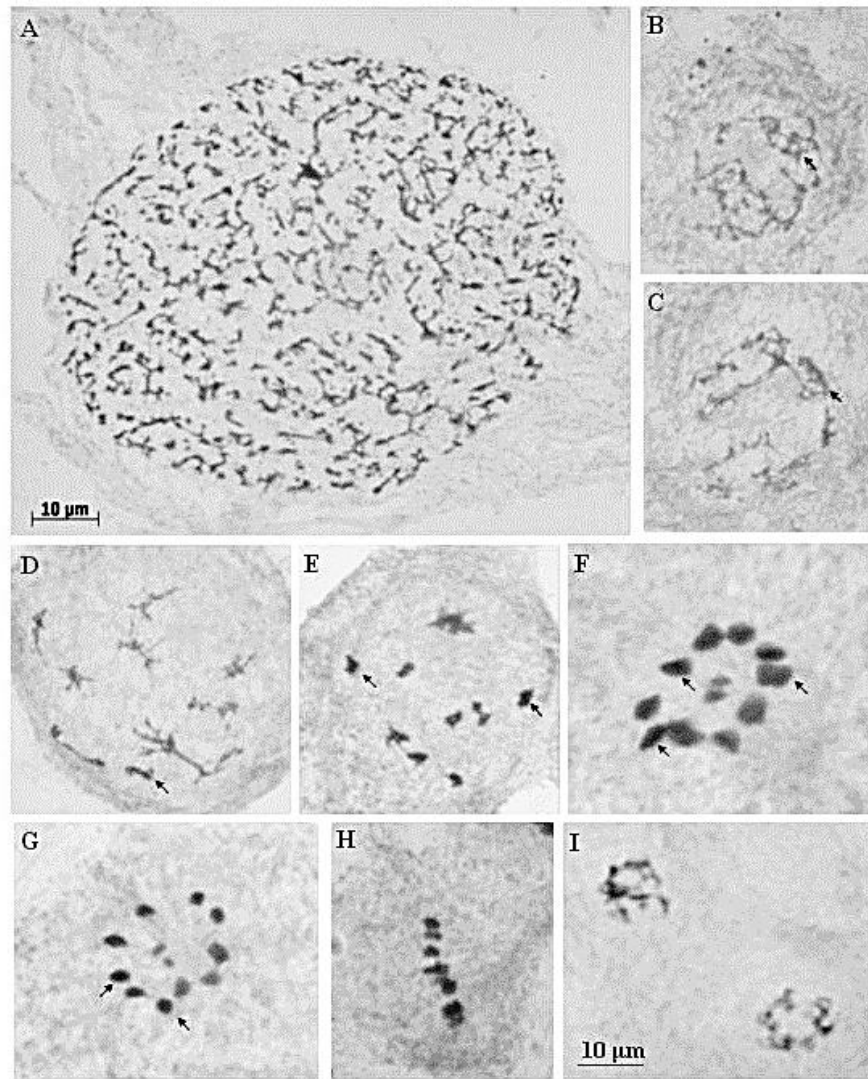


Figure 2. Seminiferous tubule of *Triatoma lenti* stained by lacto-acetic orcein. (A) Polyploidy nuclei with a high degree of heteropycnose and a evident corpuscle. (B, C) Diffuse stages. Note the heteropycnotic regions (arrows). (D) Intermediate diffuse stages. (E) Final diffuse stage with the chromosomes more condensed, showing different degrees of heteropycnose (arrows). (F) Metaphase I with ten bivalent autosomes and two sex chromosomes. Some autosomes (arrow) were more heteropycnotic than the sex chromosome. (G) Metaphase II in ring configuration. (H) Metaphases in lateral view. (I) Telophase.

V.8 ANEXO 8 (Artigo publicado na revista *Infection, Genetics and Evolution*, FI: 3,1)

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Distribution of constitutive heterochromatin in two species of triatomines: *Triatoma lenti* Sherlock and Serafim (1967) and *Triatoma sherlocki* Papa, Jurberg, Carcavallo, Cerqueira & Barata (2002)

Kaio Cesar Chaboli Alevi, Priscila Pasquetto Mendonça, Nathália Paiva Pereira, Ana Letícia Guerra, Camila Helena Facina, João Aristeu da Rosa and Maria Tercília Vilela de Azeredo Oliveira

ABSTRACT

Triatoma lenti and *Triatoma sherlocki* are hemipterans that belong to the brasiliensis subcomplex. In triatomines, the constitutive heterochromatin pattern is species-specific and allows, in many cases, for the grouping of species. Thus, we cytogenetically analyzed *T. sherlocki* and *T. lenti* using C-banding, and we compared the results with previous ones obtained in other species of the brasiliensis subcomplex. Both species were found to have a male diploid chromosome number of 22 chromosomes ($2n = 20A + XY$) with heterochromatic blocks at one or both chromosomal ends of all autosomal pairs. During

early meiotic prophase, they showed a large heteropycnotic chromocenter constituted by the association of both sex chromosomes plus two autosomal pairs and many heterochromatic blocks dispersed inside the nucleus. All of these cytogenetic characteristics are similar to those observed in other species of *brasiliensis* subcomplex, results which confirm the grouping of *T. sherlocki* and *T. lenti* within this subcomplex. However, we emphasize the importance of other approaches, such as molecular analysis, to confirm the placement of *T. lenti* within the *brasiliensis* subcomplex.

SHORT COMMUNICATION

Triatoma lenti is an endemic species of triatomine present only in the Brazilian states of Bahia, Goiás, and Roraima (Gurgel-Gonçalves et al., 2012) and it was found to be infected by the protozoan *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae) (Sherlock and Guitton, 1974). This triatomine is considered to be a potential vector of Chagas disease of secondary importance (Salvatella et al., 1998). *Triatoma sherlocki* is an endemic species of triatomine present only in the Brazilian state of Bahia (Gurgel-Gonçalves et al., 2012). Specimens in all bionomic stages were found in human dwellings and were infected by the protozoan *T. cruzi*, a finding which indicates a process of domestication of this vector (Almeida et al., 2009). This species has some morphological characteristics that are similar to those of *T. lenti*, such as reduced hemelytra, reddish orange-colored rings on the femur, and spots in the connective (Papa et al., 2002). Although *T. sherlocki* lacks the ability to fly, it has developed legs that allow for its dispersion (Almeida et al., 2012).

The *brasiliensis* subcomplex is present in South America and includes nine species: *Triatoma brasiliensis*, *Triatoma juazeirensis*, *Triatoma melanica*, *Triatoma melanocephala*, *Triatoma petrochiae*, *T. lenti*, *T. sherlocki*, *Triatoma tibiamaculata*, and

Triatoma vitticeps, which are grouped by molecular and morphometric comparisons (Schofield and Galvão, 2009). The inclusion of *T. lenti* and *T. sherlocki* in this group was based only on morphological characters and geographical distribution, because no genetic analyses were performed on these two species. Recent studies based on mitochondrial sequences suggest that *T. sherlocki* is included in this subcomplex as a sister species of *T. melanica* (Mendonça et al., 2009), and their chromosome number and meiotic processes were described (Panzera et al., 2010). Through the use of cytogenetic data, Alevi et al. (2012) proposed the exclusion of *T. melanocephala*, *T. tibiamaculata*, and *T. vitticeps* from the brasiliensis subcomplex because of their cytogenetic similarities to triatomine species from North America.

Ueshima (1966) proposed that the chromosome number and male meiotic process are useful cytogenetic parameters to clarify evolutionary relationships among different triatomine groups. Furthermore, Pérez et al. (1992) reported the importance of the C-banding technique to reveal several cytogenetic traits that are useful in the characterization and differentiation of triatomine species. In triatomines, the C-heterochromatin pattern in autosomes and sex chromosomes is species-specific, and their diagnosis allows for species identification and the grouping of species (Panzera et al., 1998, 2000, 2010). Thus, we have cytogenetically analyzed *T. sherlocki* and *T. lenti* using C-banding, and we have compared them to the other species of the brasiliensis subcomplex.

Seminiferous tubules of ten adult males of *T. lenti* and *T. sherlocki* from the Triatominae Insectarium located in the city of Araraquara, Sao Paulo, Brazil were shredded, crushed and fixed in liquid nitrogen on a glass slide. They were then stained using Cbanding (Sumner, 1972).

The analysis of the prophase I (diffuse stage) of *T. sherlocki* (Fig. 1A, arrowhead) and *T. lenti* (Fig. 2A, arrowhead) revealed a large chromocenter made up of the association of both sex chromosomes plus two autosomal pairs and many heterochromatic blocks arranged inside the nucleus. Both species were found to have heterochromatic blocks in one or both chromosomal ends of autosomes during final diplotene (Figs. 1 and 2B). Diakinesis in *T. sherlocki* (Fig. 1C) and *T. lenti* (Fig. 2C) revealed large heterochromatic blocks in the autosomes and showed a diploid chromosomal set of $2n = 22$ (20A + XY). Note that the Y sex chromosome is greater and more heterochromatic than the X (arrowhead).

All species included in the brasiliensis subcomplex except *T. vitticeps*, *T. melanocephala*, and *T. tibiamaculata* share some cytogenetic characteristics that allow for species distinction between this complex and other triatomine groups (Pérez et al., 1992; Panzera et al., 1998, 2010). The first characteristic is the diploid chromosome number consisting of 20 autosomes plus two sex chromosomes (XY in males and XX in females). The second is the amount of autosomal C-heterochromatin (25–32%). The third is the location of the autosomal C-blocks (at one or both chromosomal ends in all autosomal pairs), and the fourth is the chromosome configuration during early meiotic prophase (a large chromocenter made up of the association of both sex chromosomes plus two autosomal pairs, and multiple C-dots spread in the nucleus) (Panzera et al., 2000, 2010). These cytogenetic similarities help to group the species within the brasiliensis subcomplex, but it also makes it difficult to differentiate between them (Panzera et al., 2000). However, *T. vitticeps*, *T. melanocephala*, and *T. tibiamaculata* have been found to have multiple sex systems and different patterns of C-heterochromatin (Panzera et al., 2010; Alevi et al., 2012). *T. tibiamaculata* possesses terminal C-dots in some autosomal pairs; *T. vitticeps*

does not have autosomal C-heterochromatin (Panzera et al., 2012), while in *T. melanocephala*, the heterochromatic distribution is unknown.

Cytogenetic approaches have been used to clarify the taxonomic status of several triatomine groups, such as those of the *infestans* (*Triatoma infestans*, *Triatoma platensis*, and *Triatoma delpontei*) and *sordida* (*Triatoma sordida*, *Triatoma patagonica*, and *Triatoma guasayana*) subcomplexes (Panzera et al., 1995, 1997, respectively). These studies were confirmed using isozymes that demonstrated the efficiency of cytogenetics in taxonomic and evolutionary problem solving (Pereira et al., 1996). However, though it is possible to observe that *T. lenti* possesses cytogenetic characteristics that are similar to those of the *brasiliensis* subcomplex, more studies should be conducted so that the organism can be properly grouped into this subcomplex. As for *T. petrochiae*, it is considered to be a species that is closely related to the organisms of the complex because it possesses cytogenetic and morphologic characteristics similar to those of the triatomines complex (Panzera et al., 2000); however, isozyme studies confirmed the specific status of *T. petrochiae* and suggested that this organism evolved independently (Monteiro et al., 1998). Thus, this study confirms the current grouping of *T. sherlocki* and *T. lenti* because both species were found to have the same chromosomal traits observed in the other species of the *brasiliensis* subcomplex. However, we emphasize the importance of other approaches, such as molecular analysis, so that the position of *T. lenti* relative to the *brasiliensis* subcomplex can be better clarified.

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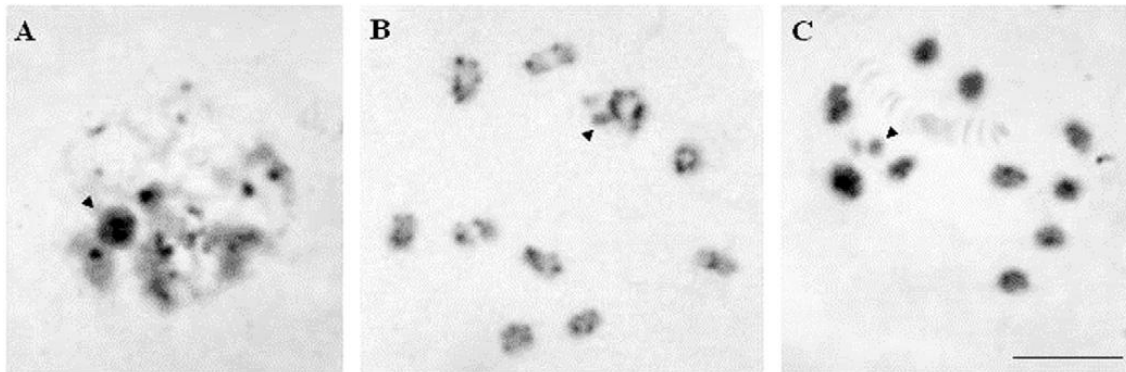


Fig. 1. Male meiotic in *Triatoma sherlocki* $2n = 20A + XY$. (A) Prophase I (diffuse stage). Note a large chromocenter (arrowhead). (B) Final diplotene. Note the C-heterochromatic blocks in one or both chromosomal ends of autosomes and on the Y chromosome (arrowhead). (C) Diakinesis with large heterochromatic blocks in the autosomes. Bar: 10 μ m.

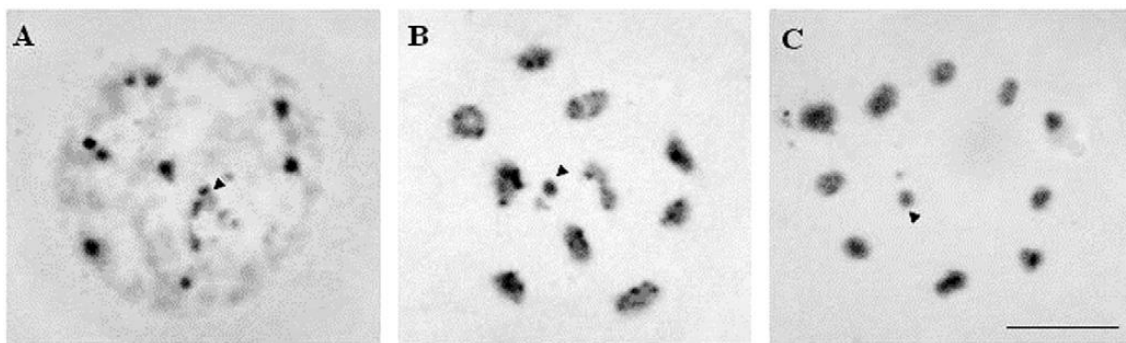


Fig. 2. Male meiotic in *Triatoma lenti* $2n = 20A + XY$. (A) Prophase I (diffuse stage). Note a large chromocenter (arrowhead). (B) Final diplotene. Note the heterochromatic blocks in one or both ends of autosomes and the Y chromosome (arrowhead). (C) Diakinesis with large heterochromatic blocks in the autosomes. Bar: 10 μ m.

V.9 ANEXO 9 (Artigo publicação na revista *Comptes Rendus Biologies*, FI: 1,8)

ALEVI, K.C.C.; MENDONÇA, P.P.; PEREIRA, N.P.; FERNANDES, A.L.V.Z.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Analysis of spermiogenesis like a tool in the study of the triatomines of the Brasiliensis subcomplex. **Comp R Biol** v. 336, p. 46-50, 2013.

Analysis of spermiogenesis like a tool in the study of the triatomines of the Brasiliensis subcomplex

Kaio Cesar Chaboli Alevi; Priscila Pasquetto Mendonça; Nathália Paiva Pereira; André Luiz Vieira Zorzetto Fernandes; João Aristeu da Rosa; Maria Tercília Vilela de Azeredo Oliveira

ABSTRACT

The specific identification and systematic of triatomines have been based fundamentally on morphological observations. These organisms are classified into complexes and specific subcomplexes, principally for morphological parameters and geographical disposition. The use of cytogenetic analyzes has been represented as a tool in systematic and taxonomy of triatomines. Thus, the present work, through the analysis of spermiogenesis, aims to characterize this stage of spermatogenesis in triatomines little studied, and especially to compare it among the species *Triatoma lenti* and *T. sherlocki*, to assist in the diagnosis of differentiation of these insects. The presence of the heteropyknotic corpuscle is shown as a diagnostic tool to differentiate *T. sherlocki* and *T. lenti*, since it is absent in *T. lenti*. The analysis of the spermiogenesis in *T. sherlocki* also allowed to address morphological differences between elongating cells, which were relatively smaller and more filamentous

when compared to *T. lenti*. Furthermore, the flagellum was observed in all stages of cell differentiation and elongation. This structure, which helps in the locomotion of the sperm, is hardly observed in cytogenetic analysis, especially throughout spermiogenesis. Thus, although other comparative approaches should be taken, this paper allowed emphasize the analysis of spermiogenesis as an important cytotaxonomic tool that assists in the differentiation of morphologically related species, such as *T. lenti* and *T. sherlocki*.

INTRODUCTION

The specific identification and systematics of triatomines have been based fundamentally on morphological observations, mainly by means of optical [1] and electron scanning microscopy [2, 3]. These organisms are classified into complexes and specific subcomplexes [4].

Triatoma lenti and *T. sherlocki* are hemipterans that belongs to the Brasiliensis subcomplex [4]. This subcomplex is present in South America and consists of nine species, out more, *T. brasiliensis*, *T. juazeirensis*, *T. melanica*, *T. melanocephala*, *T. petrochiae*, *T. lenti*, *T. sherlocki*, *T. tibiamaculata* and *T. vitticeps* [4]. However, the parameters used to group the subcomplex in triatomines were principally morphological and geographical disposition.

Costa and colleagues proposed the *T. brasiliensis* complex using egg morphology [3], morphological data [3], biological [5], isoenzymes [6] and ecological [5]. This complex comprises the subspecies *T. b. brasiliensis* and *T. b. macromelanosoma* and species, *T. juazeirensis* and *T. melanica*. Mendonça et al. (2009), by means of phylogenetic reconstruction proposed the recent inclusion of the *T. sherlocki* to complex [7]. Thus, the complex *T. brasiliensis* shows up as a monophyletic group. It is possible that *T. brasiliensis* species complex may encompass other species such *T. lenti*. However, new

approaches, such as molecular analysis, should be performed such as in *T. sherlocki*, to propose the grouping of this species in the complex [7, 8].

T. lenti is an endemic triatomine of the States of Bahia, Goias and Roraima [9]. The species is considered a potential vector for Chagas disease of secondary importance and it was found infected with the etiologic agent, the protozoan *Trypanosoma cruzi* [10]. *T. sherlocki* is an endemic species of Bahia [9]. It presents unique morphological characteristics that resembles *T. lenti* as a reduced hemelytra, reddish orange colored rings on the femur, and spots in the connective [11]. It was also reported that these organisms live in sympatry in some regions [12]. *T. sherlocki* has been found in human habitations, infected with *T. cruzi*, indicating a process of domiciliation of this vector [13]. Although both species were found infected with *T. cruzi*, only *T. sherlocki* presents epidemiological importance, because *T. lenti* is specie exclusively sylvatic [14].

The use of cytogenetic analyzes has been represented as a tool in systematic and taxonomy of cryptic species of triatomine [15, 16]. In addition, it has been showed recently that cytogenetic is very important in the study of subcomplexes, since karyotype analysis were used to propose the exclusion of *T. melanocephala*, *T. vitticeps* and *T. tibiamaculata* from the Brasiliensis subcomplex for approaching the triatomines in South America [17].

Thus, the present work, through the analysis of spermiogenesis, aims to characterize this stage of spermatogenesis in triatomines little studied, and especially to compare it among the species *T. lenti* and *T. sherlocki*, to assist in the diagnosis of differentiation of these insects.

MATERIAL AND METHODS

In this study, it was used five males of each species, assigned by the "Triatominae Insectarium" installed at the Department of Biological Sciences, Faculty of Pharmaceutical

Sciences, Araraquara campus. The seminiferous tubules of adult males, after being torn and fixated on cover slip, underwent cytogenetic technique of lacto-acetic orcein [18]. The biological material was analyzed by Jenaval light microscope (Zeiss) coupled to a digital camera and an image analyzer Axio Vision LE 4.8 (Copyright © 2006-2009 Carl Zeiss Imaging Solutions Gmb H). The images were magnified by a factor of 1000.

RESULTS

By using the cytogenetic technique of orcein lacto-acetic it was observed the elongation of spermatid until the differentiation into spermatozoa (Figure 1A-F) and (Figure 2A-G). During spermiogenesis of *T. lenti*, it was possible to detect a peripheral heteropcnotic filament in all cells (arrows). Already in *T. sherlocki* the early spermatids showed a heteropyknotic corpuscle (Figure 2A, arrowhead) that took the cell periphery during cell elongation (Figure 2B-G, arrowheads). During cell elongation of *T. sherlocki*, it was possible to observe the flagellum (Figure 2B-G, arrows). Note that the cells this species are smaller and more tapered.

DICUSSION

Spermatogenesis occurs in the seminiferous tubules and consists, respectively, of three major divisions, out more, spermatocitogenesis, meiosis and spermiogenesis, which results in the production of sperm [19]. Data on spermatogenesis in triatomines is commonly discussed in the literature, but focus mainly on meiosis, more specifically on the behavior of chromosomes.

By analyzing the spermiogenesis on *T. lenti* and *T. sherlocki*, we observed that both have a heteropyknotic filament on the periphery of the cells. This characteristic was also

observed in *T. pseudomaculata* [20]. These organisms also share characteristics of egg morphology [24] and karyotypic, since all have 22 chromosomes [20, 21, 22].

The analysis of the initial spermatid of *T. sherlocki* allowed the visualization of a heteropyknotic corpuscle. This characteristic was also observed in *T. klugi* [23], *T. maculata* [20] and *T. melanosoma* [25]. *T. maculata*, *T. sherlocki* and *T. melanosoma* also share characteristics of egg morphology [24]. Furthermore, all species have the diploid chromosome set $2n = 22A (20A + XY)$. [20, 21, 25].

The presence of the corpuscle is shown as a diagnostic tool to differentiate *T. sherlocki* and *T. lenti*, since it is absent in *T. lenti*. The analysis of the spermiogenesis in *T. sherlocki* also allowed to address morphological differences between elongating cells, which were relatively smaller and more filamentous when compared to *T. lenti*. Furthermore, the flagellum was observed in all stages of cell differentiation and elongation. This structure, which helps in the locomotion of the sperm, is hardly observed in cytogenetic analysis, especially throughout spermiogenesis.

Thus, although other comparative approaches should be taken, this paper allowed emphasize the analysis of spermiogenesis as an important cytotaxonomic tool that assists in the differentiation of morphologically related species, such as *T. lenti* and *T. sherlocki*.

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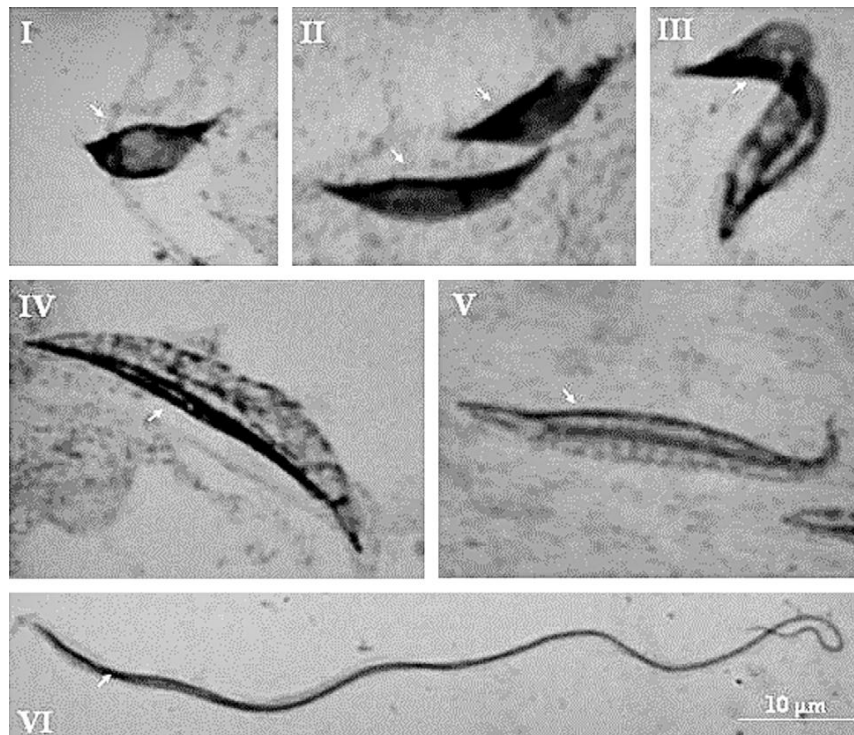


Figure 1- Spermiogenesis of *Triatoma lenti* (I-VI). Note a peripheral heteropcnotic filament in all cells (arrows).

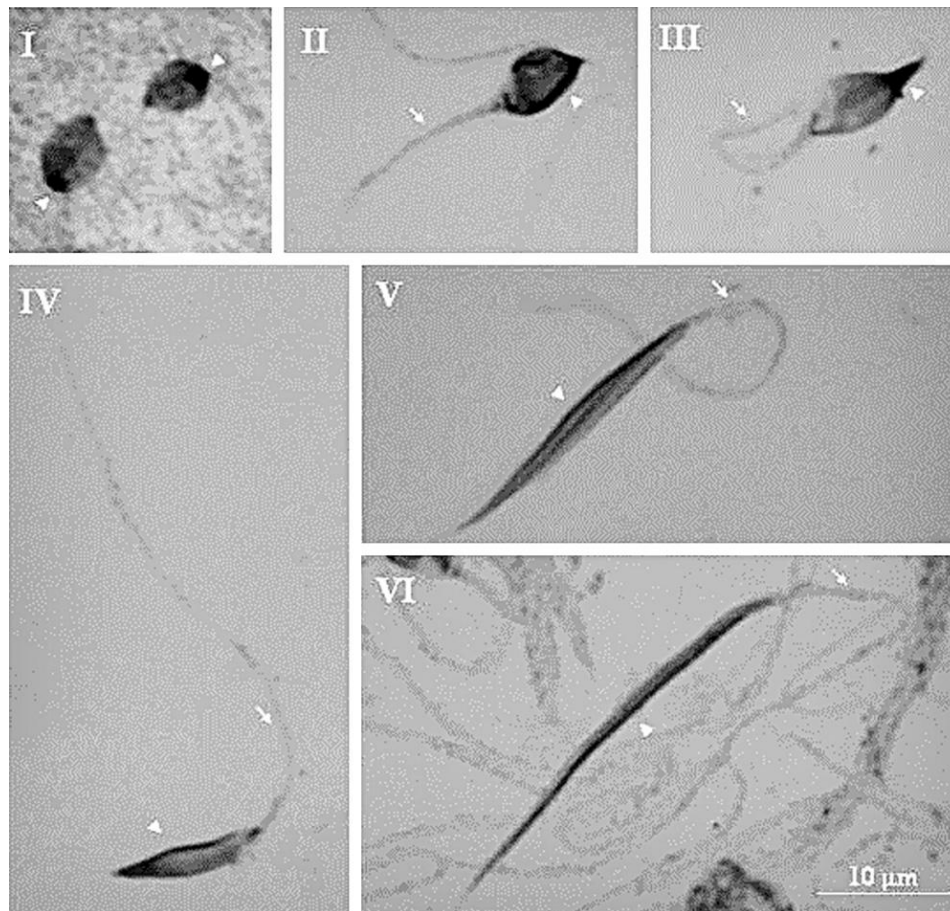


Figure 2- Spermiogenesis of *Triatoma sherlocki* (I-VI). I: Note the heteropyknotic corpuscle (arrowheads). II-VI: Note a peripheral heteropyknotic filament in all cells (arrowheads) and the flagellum (arrows).

V.10 ANEXO 10 (Artigo publicado na revista *Invertebrate Reproduction & Development*, FI: 0,52)

ALEVI, K.C.C.; MENDONÇA, P.P.; PEREIRA, N.P.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Is There Post-Meiotic Transcriptional Activity during Hemipteran Spermiogenesis? **Inv Rep Dev**, *in press*, 2014b, DOI: 10.1080/07924259.2014.889767

Is There Post-Meiotic Transcriptional Activity during Hemipteran Spermiogenesis?

Kaio Cesar Chaboli Alevi, Priscila Pasquetto Mendonça, Nathália Paiva Pereira, João Aristeu da Rosa and Maria Tercília Vilela de Azeredo-Oliveira

ABSTRACT

Different techniques have been used to evaluate post-meiotic nucleolar activity during spermatogenesis. However, the results have not been consistent, and transcriptional activity during spermiogenesis is still a highly debated issue. Through the use of cytogenetic tools, we evaluated whether the nucleolus is active during spermiogenesis in triatomines, more specifically, in *Triatoma lenti*. During spermatid differentiation, the nucleolus was observed using the cytogenetic technique of silver staining. Through the Feulgen reaction, we found that these markings by silver ions referred to the ribosomal DNA (rDNA). Using molecular cytogenetic techniques (DAPI and FISH) we were able to highlight the nucleolus and the Nucleolus Organizing Region (NOR) in spermatid images. Using the C-banding technique, we found rDNA and the NOR to be completely heterochromatic. Since this nuclear structure is present throughout the length of the spermatids stage, we believe that the rDNA and NOR are inactivated by epigenetic factors,

because we found a direct relationship between the nucleolus, rDNA, the NOR and heterochromatin. Because the heterochromatization of DNA is an important way to inactivate genetic material, we believe that the entire transcriptional activity required for cellular differentiation during spermiogenesis is mediated by the chromatoid body (CB).

INTRODUCTION

Spermatogenesis is the process of division and differentiation by which spermatozoa are produced in the seminiferous tubules. It consists of three different phases: spermatocytogenesis, which is a phase of proliferation; meiosis, which is the multiplication phase; and spermiogenesis, which is the differentiation phase (Johnson et al. 1997).

The nucleolus is a nuclear structure that has an important function in protein synthesis, and one that is therefore indispensable for cells (González-Garcia and Rufas 1995; Lewis et al. 2007). Nucleoli are responsible for ribosome biogenesis, which occurs through many events that involve the transcription of ribosomal RNA (rRNA) genes, the processing of pre-rRNA and the assembly of pre-ribosomal particles (Scheer et al. 1997). During meiosis, the cells interrupt the transcription of rRNA. Generally, the nucleolus becomes disorganized in prophase, and some of its components, such as nucleolar proteins, disperse into the nucleoplasm. However, some of these proteins can be associated with chromosomal Nucleolus Organizer Regions (NORs) and/or found at the chromosomal periphery during metaphase and anaphase. rRNA transcription is only reactivated at the end of meiosis, when the reorganization of the nucleolus gradually takes place (González-Garcia and Rufas 1995).

In most cases, nucleolar reorganization starts during late anaphase or early telophase, when the nucleolar material located on the surface of the chromosomes begins to aggregate into numerous small structures called prenucleolar bodies (PNBs). However, in some heteropteran species, the persistence of nucleolar material during meiosis has been

found. In heteropteran phytophagous, this persistence occurs only in metaphase I (Fossey and Liebenberg 1995), which is why it is called semi-persistence. Hematophagous species differ from these insects – their nucleolar corpuscles are found during both metaphase I and metaphase II, and nucleolar fragments are present even in advanced stages of division such as anaphase and telophase (Tartarotti and Azeredo-Oliveira 1999). This persistent material may carry a primer for new nucleolar material or represent a source of nucleolar RNA for the daughter cells while new nucleoli are being organized (Brinkley 1965; Mello 1995).

Medina et al. (1995) utilized mammalian cells and state that the resumption of the pre-ribosomal RNA synthesis in daughter cells during telophase is necessary for the aggregation of PNBs that are previously formed in the NOR, so that the nucleolus is reorganized. However, although the nucleolus is reorganized and persistent throughout spermiogenesis, questions still remain regarding post-meiotic transcriptional activity. In 1965, Monesi analyzed meiosis in male mouse and proposed that the transcription of messenger RNA (mRNA) falls to very low levels or ceases completely in initial spermatids. Monesi was able to confirm this phenomenon because the study showed that the final spermatids analyzed showed no sign of RNA in the nucleus. However, in meiotic prophase, the cytoplasm was found to have significant RNA values. This RNA had been synthesized at least one week before the experiment. Therefore, the author concluded that protein synthesis is supported by “templates” that are produced during meiotic division and conserved in the cell to make the cellular differentiation possible during spermiogenesis. In another study, Olivieri and Olivieri (1965) used radiographic studies to show that there is no post-meiotic transcriptional activity in *Drosophila melanogaster* spermatids.

Based on this data and other similar studies, it can be accepted that no transcriptional activity occurs during the post-meiotic stage, and that the proteins necessary

after these stages are translated from mRNA that is produced in meiosis and then stored in the cytoplasm (Schäfer et al. 1995).

In 2008, Morielle-Souza and Azeredo-Oliveira analyzed the triatomine species *Panstrongylus megistus*, *Rodnius prolixus* and *Triatoma infestans*. They used cytogenetic techniques such as acridine orange (a technique that differentiates between DNA (green) and RNA (orange)), and a variant of critical electrolytic concentration (CEC) (a method that differentiates between DNA (green) and metachromatic RNA (blue or violet)). Using these techniques, they corroborated the hypothesis regarding post-meiotic activity – in both cases, they observed the persistence of rRNA in spermatids. However, the published images are not convincing.

According to Arakaki et al. (2010), *de novo* rRNA synthesis occurs during spermatogenesis of the phytophagous hemipteran *Chariesterus armatus*. However, the only method used in this study was the silver impregnation technique. Using this cytogenetic technique, which can reveal the nucleolus or nucleolares corpuscles, the authors noticed the persistence of nucleolar material during spermatogenesis. However, the presence of this material does not support the hypothesis of post-meiotic transcriptional activity, since the nucleolus can be present but inactivated.

The post-meiotic transcription hypothesis has been challenged by two studies with *Drosophila*: one that demonstrates mRNA accumulation in post-meiotic spermatids of (Barreau et al. 2008), and a microarray analysis that estimates genome-wide expression during post-meiosis (the latter study demonstrated that 20 to 30% of testis genes that are transcribed are over-expressed during post-meiosis when compared to gene transcription during meiosis (Vibranovski et al. 2009)). According to Vibranovski et al. (2010), neither study provided direct evidence for post-meiotic transcription because they quantitatively or qualitatively measured the amount of mRNA in post-meiotic cells and did not discover

evidence for the production of nascent RNA. Moreover, few genes were analyzed in the studies of Barreau et al. (2008), and Vibrationovski et al. (2009), the mRNA levels were measured from dissected tissues, meaning that the expression at the cellular level is still not clear.

According to Biggiogera et al. (1991), spermatogenesis of mouse is a process that is characterized by a progressive reduction of nucleolar activity. Their data were clearly supported in an RNA blot experiment in which the mRNA for nucleolin decreased during spermatogenesis, while the mRNA for numatrin increased in amount during the early phases. In this same study, immunoblotting revealed that both proteins were present during early spermatogenesis and absent in mature sperm. A study by Vibrationovski et al. (2010) on *Drosophila melanogaster* contradicted the studies by Biggiogera et al. (1991). In this study, the researchers directly visualized RNA transcripts in intact testes using 5-bromouridine (BrU), and they observed a strong BrU sign near the spermatids' nuclei. These findings supported the idea of *de novo* transcription of RNA during the post-meiotic phases. In isolated spermatids, the sign of BrU may be, as the authors believe, directly involved in post-meiotic RNA synthesis, since the sign was reduced in the presence of actinomycin D, which is a general inhibitor of RNA synthesis that was also used in the experiment. However, the study didn't consider the possible presence of the chromatoid body (CB), a cytoplasmic organelle of spermatogenic haploid cells that is involved in RNA and protein accumulation and/or reserves for spermiogenesis (Söderström and Parvinen 1976a, b; Saunders et al. 1992).

Therefore, new approaches should be developed, questions remain regarding nucleolar activity during this important phase of spermatogenesis. To resolve these issues, this study sought to analyze nucleolar activity during spermiogenesis in the hemipteran *Triatoma lenti* through the use of cytogenetic techniques.

MATERIAL AND METHODS

For the study, ten male *T. lenti* were used. The insects were provided by the Triatominae Insectarium of Sao Paulo State University (UNESP, in the city of Araraquara, Brazil). The seminiferous tubules of adult males were analyzed using silver staining (Howell and Black 1980) (a technique specific to nucleolar markers) the Feulgen reaction (Mello and Vidal 1978) (a technique specific to DNA), and C-banding (Sumner 1972) (a technique for constitutive heterochromatin). The tubules were also stained using DAPI. This technique highlighted the nucleolus. Finally, the samples were analyzed using *in situ* hybridization with a 45S rDNA probe to recognize the NOR (Panzer et al. 2012). The slides were analyzed using a Jenaval light microscope (Zeiss), coupled with a digital camera and an Axion Vision LE 4.8 image analyzer (Copyright ©2006-2009 Carl Zeiss Imaging Solutions GmbH). The images were magnified by a factor of 1000.

RESULTS

During spermatid differentiation, the nucleolus was observed using the cytogenetic technique of silver staining (Figure 1A, 2A). The Feulgen reaction revealed that these markings by silver ions referred to the ribosomal DNA (rDNA) (Figure 1B, 2B), because the markings with Feulgen that are specific for DNA were identical the markings with silver ions. Using molecular cytogenetic techniques (DAPI banding and *in situ* hybridization with 45S ribosomal probe of *T. infestans*), we were able to highlight the nucleolus and the NOR in the spermatid images (Figure 1D, 2D). Using the cytogenetic C-banding technique, we found the rDNA and the NOR to be completely heterochromatic (Figure 1C, 2C).

DISCUSSION

Cytologic data shows that the nucleolin (C23) and the numatrin (B23) proteins are responsible for silver ion impregnation (Ochs et al. 1983; Ochs and Busch 1984; Casseb-Hassan and Azeredo-Oliveira, 1999). This nucleolar material had been previously visualized in haploid cells from other species of triatomines (Severi-Aguiar et al. 2006; Costa et al. 2008; Bardella et al. 2008; Morielle-Souza and Azeredo-Oliveira 2004, 2008) as well as in in phytophagous hemipteran (Castanhole et al. 2008, 2010, 2012; Souza and Itoyama 2009, 2010; Souza et al. 2008, 2009).

To verify that the marks in the spermatids did actually refer to rDNA, the Feulgen reaction was used, which is a cytogenetic technique that is DNA specific (Mello and Vidal 1978). Both techniques marked the same region of the cell. No other cytogenetic study in the literature made the connection between nucleolar material and rDNA. As the marks for silver (nucleolar) and for Feulgen (DNA) were similar, we assume that the DNA marked by the Feulgen is rDNA. Furthermore, molecular cytogenetic techniques (DAPI and FISH) also revealed the nucleolus in early spermatids, and they also highlighted the presence of the NOR in nucleolar materials. The NOR consists of loops of DNA that transcribe RNA (rRNA).

The presence of rDNA and a NOR in spermatids may support the post-transcriptional activity hypothesis. However, to evaluate whether this material is activated, the C-banding technique (which can reveal the existence of constitutive heterochromatin regions (Sumner 1972)) was employed. Although some studies in the literature show that the nucleolus is associated with heterochromatic corpuscles in somatic and meiotic cells in some species of triatomines (Azeredo-Oliveira and Mello 1986; Mello and Recco-Pimentel 1987), there is no other study that details this relationship during spermiogenesis in these insects.

The relative positions of constitutive heterochromatin and the nucleolar structures during spermiogenesis was observed by Czaker (1987), who noticed a relationship between the nucleolus and the heterochromatic chromocenter; however, this author reported that the nucleolus starts to disintegrate during the spermatid elongation stage. However, the author pointed out that this persistence of nucleolar material represents continuous transcriptional activity, and therefore supports the post-meiotic transcriptional activity hypothesis. The C-banding revealed a strong relationship among rDNA, the NOR and constitutive heterochromatin. The demarcations for the four cytogenetic techniques that represented the nucleolus, rDNA, the NOR and constitutive heterochromatin were identical. This relationship supports the argument that there is no post-meiotic transcriptional activity, since epigenetic factors are involved in the silenced or inactive form of the genetic material, which results in the formation of constitutive heterochromatin (Richards and Elgin 2002; Baulcombe 2004).

The Nucleolar Remodeling Complex (NORC) is a novel chromatin remodeling complex that results in the formation of heterochromatin which, in turn, causes the silencing of the ribosomal genes and then represses rDNA transcription. This silenced state is caused by epigenetic factors. According to Santoro et al. (2002), the NORC silences rDNA recruited DNA methyltransferase and histone deacetylase activity to the rDNA promoter, thereby establishing structural characteristics of heterochromatin such as DNA methylation, histone hypoacetylation and methylation of the Lys9 residue of histone H3 and DNA. This process suggests that both active and inactive rRNA genes could be demarcated by their associated proteins, and could also link chromatin remodeling to DNA methylation and specific histone modifications.

In addition, we would like to suggest that the CB (which was recently described in triatomines (Silistino-Souza et al. 2012)) supports the inactive form of rDNA and therefore

a lack of post-meiotic transcriptional activity in the spermatids, because this cytoplasmatic structure sustains all transcriptional activity necessary for cellular differentiation during spermiogenesis (Kotaja and Sassone-Corsi 2007). The CB originates from the fragmentation of nucleolar material in the early stages of spermatogenesis. A fraction of these fragments migrate to the cytoplasm, forming small “nuages,” a ribonucleoprotein complex derived from the nucleolus that is linked to the spermatid cytoplasm. These nuages help to create the structure known as the CB (Paniagua et al., 1986; Peruquetti et al. 2008, 2010, 2011, 2012). In triatomines, the CB also originates from nucleolar fragmentation and possesses fibrilarin in its molecular composition (Silistino-Souza et al. 2012).

Through this study, we corroborated the hypothesis that transcriptional activity does not occur during spermiogenesis. Although the nucleolus may be present in this stage of spermatogenesis, we believe that rDNA is inactivated through epigenetic factors. Moreover, cellular differentiation can be supported by the transcriptional activity performed by the CB.

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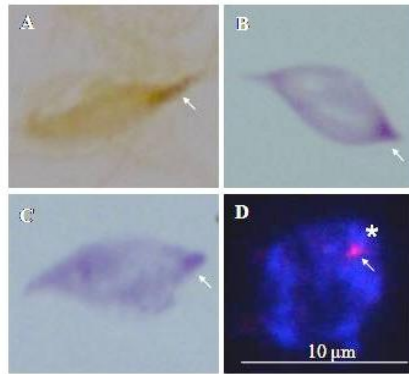


Fig. 1. Seminiferous tubules of *Triatoma lenti* stained using silver staining (A), Feulgen reaction (B), C-banding (C), and DAPI staining and in situ hybridization (D). In the early spermatid, note the presence of a prominent nucleolus (A, arrow), constitutive heterochromatin (B, arrow), heterochromatic DAPI regions (D, asterisk) and the NOR highlighted during hybridization (D, arrow).

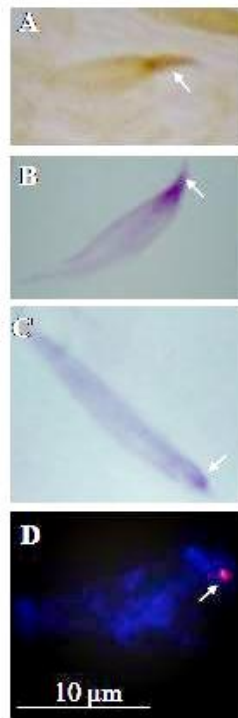


Fig. 2. Seminiferous tubules of *Triatoma lenti* stained using silver staining (A), Feulgen reaction (B), C-banding (C), and DAPI banding and in situ hybridization (D). In the late spermatid, note the presence of a prominent nucleolus (A, arrow), constitutive heterochromatin (B, arrow) and the NOR highlighted during hybridization (D, arrow).

V.11 ANEXO 11 (Artigo publicado na revista *Entomology, Ornithology & Herpetology*)

ALEVI, K.C.C.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Mini review: Karyotypic survey in Triatominae subfamily (Hemiptera, Heteroptera). **Entomol Ornithol Herpetol**, v.2, p. 106, 2013.

Mini review: Karyotypic survey in Triatominae subfamily (Hemiptera, Heteroptera)

Kaio Cesar Chaboli Alevi; João Aristeu da Rosa and Maria Tercília Vilela de Azeredo Oliveira

Abstract

The Triatominae subfamily consists of 145 species distributed in 18 genera and grouped in six tribes. Currently, there are 86 karyotypes described in the literature, distributed in 11 genera. There are five chromosomal complements described for these bloodsucking insects, out more, 22 ($20A + XY$), 23 ($20A + X_1X_2Y$), 24 ($20A + X_1X_2X_3Y$), 21 ($18A + X_1X_2Y$), 25 ($22A + X_1X_2Y$). Thus, we review all triatomine species with the number of chromosomes described in the literature. Through these data highlight the importance of further analysis cytogenetic with karyotype description in Triatominae subfamily, since it can help as an important tool cytotaxonomy and mainly allows the understanding of the evolution of this important group of insect vectors of Chagas disease.

MINI REVIEW

Triatomines are insects that are taxonomically included in the Hemiptera order and the Heteroptera suborder within the Reduviidae family and in the Triatominae subfamily [1]. These organisms are of great medical importance, because all of the species that belong to the Triatominae subfamily are both bloodsucking and susceptible to infection from the protozoan *Trypanosoma cruzi* Chagas, 1909 (Kinetoplastida, Trypanosomatidae) and are therefore potential vectors of Chagas disease [2].

The Triatominae subfamily consists of 145 species [3-8] distributed in 18 genera and grouped in six tribes [9, 10] (Table I).

In cytogenetics, biological models of triatomine bugs are important because they have holocentric chromosomes, which have diffuse kinetochores distributed along the chromosomes and not a single kinetochore located in the centromeric region. These insects also perform an unusual meiosis in which the segregation of sex chromosomes is post-reductional [11, 12].

The description of the diploid chromosome set in Triatominae subfamily was initiated in 1909 with the description of the karyotype of *Triatoma sanguisugus* Le Conte, 1856 [13]. In 1950, the karyotypic studies were resumed and new karyotypes were described in the literature [14].

Ueshima, in 1966, described the karyotype of twenty new species and proposed cytogenetic studies as a tool in the taxonomy of these vectors (cytotaxonomy). Moreover, the author analyzes the genetic viability during meiosis of experimental hybrids resulting from crosses between species of triatomines [12].

Currently, there are 86 karyotypes described in the literature, distributed in 11 genera (Table I). Three tribes and seven genera show no cytogenetic studies maybe due to the limited geographic location which difficulty the granting of specimens of the species.

The three principal genera known and studied maybe by large epidemiological importance are *Triatoma*, *Rhodnius* and *Panstrongylus*. However, even in these three genera, we emphasize the importance of new karyotypic studies, since, according to Table I, we are aware of approximately 65%, 57% and 78%, the number of chromosomes in their respective genera.

Ueshima, in 1996, propose that the type number of chromosomes of Triatominae subfamily is 22 ($20A + XY$). However, he points out that there are five chromosomal complements described for these bloodsucking insects, out more, 22 ($20A + XY$), 23 ($20A + X_1X_2Y$), 24 ($20A + X_1X_2X_3Y$), 21 ($18A + X_1X_2Y$), 25 ($22A + X_1X_2Y$). Thus, we review the literature and grouped species according to the number of chromosomes (Table II).

As can be observed in Table II, some genera are poorly described derivatives as compared to the common ancestor, if we consider that the common ancestor of these bloodsucking insects presented 22 chromosomes (type number). On the other hand, if we analyze the tribe Triatomini, we observed that different chromosomal complements can be found, corroborating the hypothesis that the tribe is polyphyletic Triatomini.

Recently, through karyosystematic, Alevi and collaborated proposed a subcomplex of species were reorganized. The authors described the karyotype of the species *Triatoma lenti* Sherlock and Serafim, 1967 [15] and *Triatoma melanocephala* Neiva and Pinto, 1923 [16], and observed that *Triatoma melanocephala* Neiva and Pinto, 1923, as well as *Triatoma vitticeps* Stal, 1859 and *Triatoma tibiamaculata* Pinto, 1926 showed fragmentation of the sex chromosome X, different from other species also grouped in Brasiliensis subcomplex. Thus, as the initial proposal of the grouping of species in the subcomplex was performed using basically morphological data and geographical distribution [5], the number of chromosomes showed to be an important tool

cytotaxonomic and, therefore, was proposed the exclusion of the three species with fragmented sex chromosome X of *Brasiliensis* subcomplex [16].

Thus, we review all triatomine species with the number of chromosomes described in the literature. Through these data highlight the importance of further analysis cytogenetic with karyotype description in Triatominae subfamily, since it can help as an important tool cytotaxonomy and mainly allows the understanding of the evolution of this important group of insect vectors of Chagas disease.

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Table I- Revision of the Triatominae subfamily (species and karyotypes)

TRIBE	GENUS	NUMBER OF SPECIES	KARYOTYPE DESCRIBED
Alberproseniini	<i>Alberprosenia</i>	2	0
Bolboderini	<i>Belminus</i>	8	2
	<i>Bolbodera</i>	1	0
	<i>Microtriatoma</i>	2	0
	<i>Parabelminus</i>	2	0
Cavernicolini	<i>Cavernicola</i>	2	0
Linshcosteini	<i>Linshcosteus</i>	6	0
Rhodniini	<i>Psammolestes</i>	3	2
	<i>Rhodnius</i>	18	14
Triatomini	<i>Dipetalogaster</i>	1	1
	<i>Eratyrus</i>	2	2
	<i>Hermanlenticia</i>	1	0
	<i>Meccus</i>	6	6
	<i>Mepraia</i>	3	3
	<i>Nesotriatoma</i>	3	2
	<i>Panstrongylus</i>	14	8
	<i>Paratriatoma</i>	1	1
	<i>Triatoma</i>	70	45
Total		145	86

Table II – Chromosome number of 86 species of triatomines with karyotypic analysis. A: autosomes.

Karyotype (2n)	Genus: species
21 = 18A + X ₁ X ₂ Y	<i>Panstrongylus</i> : <i>megistus</i> <i>Triatoma</i> : <i>nitida</i>
22 = 20A + XY	<i>Psammolestes</i> : <i>coreodes</i> , <i>tertius</i> <i>Rhodnius</i> : <i>brethesi</i> , <i>colombiensis</i> , <i>domesticus</i> , <i>ecuadoriensis</i> , <i>milesi</i> , <i>montenegrensis</i> , <i>nasustus</i> , <i>neglectus</i> , <i>neivai</i> , <i>pallenscens</i> , <i>pictipes</i> , <i>prolixus</i> , <i>robustus</i> , <i>stali</i> <i>Dipetalogaster</i> : <i>maximus</i> <i>Paratriatoma</i> : <i>hirsuta</i> <i>Triatoma</i> : <i>arthurneivai</i> , <i>baratai</i> , <i>brasiliensis</i> (<i>b. brasiliensis</i> , <i>b. macromelanosoma</i>), <i>carcavalloei</i> , <i>circummaculata</i> , <i>costalimai</i> , <i>delponteii</i> , <i>garciabesi</i> , <i>guasayana</i> , <i>guazu</i> , <i>infestans</i> (<i>i. melanosoma</i>), <i>juazeirensis</i> , <i>jurbergi</i> , <i>klugi</i> , <i>lectularia</i> , <i>lenti</i> , <i>maculata</i> , <i>matogrossensis</i> , <i>melanica</i> , <i>patagonica</i> , <i>petrochiae</i> , <i>platensis</i> , <i>pseudomaculata</i> , <i>rubrovaria</i> , <i>sherlocki</i> , <i>sordida</i> , <i>vandae</i> , <i>williami</i>
23 = 20A + X ₁ X ₂ Y	<i>Belminus</i> : <i>herrerii</i> , <i>corredori</i> <i>Eratyrus</i> : <i>cuspidatus</i> , <i>mucronatus</i> <i>Meccus</i> : <i>bassolsae</i> , <i>longipennis</i> , <i>mazzottii</i> , <i>pallidipennis</i> , <i>phyllosoma</i> , <i>picturatus</i> <i>Mepraia</i> : <i>gajardoi</i> , <i>parapatrica</i> , <i>spinolai</i> <i>Nesotriatoma</i> : <i>bruneri</i> , <i>flavida</i> <i>Panstrongylus</i> : <i>chinai</i> , <i>geniculatus</i> , <i>howardi</i> , <i>lignarius</i> , <i>rufotuberculatus</i> , <i>tupynambai</i> <i>Triatoma</i> : <i>barberi</i> , <i>dimidiata</i> (<i>d. capita</i> , <i>d. dimidiata</i> , <i>d. maculipennis</i>), <i>gerstaeckeri</i> , <i>hegneri</i> , <i>mexicana</i> , <i>peninsularis</i> , <i>protracta</i> , <i>rubida</i> , <i>ryckmani</i> , <i>sanguisuga</i> , <i>sinaloensis</i> , <i>tibiamaculata</i>
24 = 20A + X ₁ X ₂ X ₃ Y	<i>Panstrongylus</i> : <i>lutzi</i> <i>Triatoma</i> : <i>eratyrsiformis</i> , <i>melanocephala</i> , <i>vitticeps</i>
25 = 22A + X ₁ X ₂ Y	<i>Triatoma</i> : <i>rubrofasciata</i>

Discussão

VI. DISCUSSÃO GERAL

VI.1 *T. melanocephala* e *T. vitticeps*

As análises citogenéticas da espécie *T. melanocephala* mostraram-se de grande importância para a taxonomia desse triatomíneo, por disponibilizarem potenciais características diagnósticas, quando comparadas com características de espécies morfológicamente semelhantes, bem como para a avaliação da sua posição inicial no subcomplexo Brasiliensis.

Por meio da descrição do cariótipo de *T. melanocephala*, Alevi et al. (2012a) propuseram que essa espécie, bem como as espécies *T. vitticeps* e *T. tibiamaculata* deveriam ser excluídas do subcomplexo Brasiliensis, uma vez que diferem do número de cromossomos observado nas espécies do subcomplexo ($2n = 20$ autossomos + XY), por apresentarem fragmentação do cromossomo sexual X. As espécies *T. vitticeps* e *T. melanocephala* possuem um sistema de determinação do sexo do tipo $X_1X_2X_3Y$ (SCHREIBER e PELLEGRINO, 1950; SEVERI-AGUIAR et al., 2006; ALEVI et al., 2012a) e *T. tibiamaculata* apresenta o sistema X_1X_2Y (PANZERA et al., 1996), aproximando-se, dessa forma, das espécies de triatomíneos da América do Norte.

Por meio da descrição da espermatogênese de *T. melanocephala* foi possível comparar características cromatínicas e cromossômicas com as descritas por Severi-Aguiar et al. (2006) para *T. vitticeps*, pois Sherlock e Guitton (1980), quando descreveram morfológicamente *T. melanocephala*, propuseram que esse hemíptero apresentava características morfológicas muito próximas de *T. vitticeps*, sendo que as únicas características que os diversificavam era a ausência de faixa testácea na cabeça e a presença de duas manchas castanhas no tórax. Assim, além da proximidade morfológica, observamos que esses organismos também são idênticos quanto aos parâmetros

citogenéticos. Alevi et al. (2013b) observaram que ambas as espécies apresentavam o mesmo número de cromossomos e as mesmas características citogenéticas, como corpúsculos heteropicnóticos nos núcleos poliploides, nas células prófásicas e interfásicas. Ressaltamos que durante a prófase inicial foi possível observar um cromocentro formado pelos quatro cromossomos sexuais. Essa característica também foi observada em *T. vitticeps* por Severi-Aguiar et al. (2006).

Contudo, realizamos novas análises comparativas com *T. melanocephala* e *T. vitticeps*, com o intuito de encontrar alguma característica que pudesse ser utilizada como diagnóstica para auxiliar na diferenciação desses triatomíneos. Por meio da análise do padrão heteropicnótico durante a espermiogênese dessas espécies, observamos que as espermatídes de *T. melanocephala* apresentaram um filamento heteropicnótico periférico pouco proeminente, além de células relativamente maiores quando comparadas com *T. vitticeps*. A espécie *T. vitticeps* apresentou um extenso filamento heteropicnótico ora periférico, ora central nas células haplóides durante a espermiogênese. Assim, pode ser sugerido que o filamento heteropicnótico central, observado em *T. vitticeps*, pode representar uma característica diagnóstica utilizada para diferenciar essas espécies morfologicamente relacionadas (ALEVI et al., 2013c).

O padrão heterocromático de *T. melanocephala* também foi descrito, a fim de comparar com as outras espécies do subcomplexo Brasiliensis (ALEVI et al., 2014a). Por meio desse resultado, foi possível observar que essa espécie só apresenta heterocromatina constitutiva no cromossomo sexual Y. Essa característica permitiu que a exclusão dessa espécie do subcomplexo fosse corroborada, uma vez que o subcomplexo Brasiliensis apresenta como característica diagnóstica blocos heterocromáticos em uma ou ambas as extremidades de todos os autossomos (PANZERA et al., 2000). Além disso, por meio do padrão heterocromático também foi possível diferenciar *T. melanocephala* de *T. vitticeps*,

uma vez que *T. vitticeps* apresenta o cromossomo sexual Y e um dos cromossomos sexuais X heterocromáticos (SEVERI-AGUIAR et al., 2006). Além disso, Alevi et al. (2014a) também propuseram uma relação inicial entre *T. melanocephala* e *Panstrogylus lutzi*, uma vez que ambas espécies apresentam 24 cromossomos ($20A + X_1X_2X_3Y$) e o mesmo padrão de heterocromatina constitutiva (ALEVI et al., 2012a, 2013d, 2014a). Ressaltamos que dentro da subfamília Triatominae, apenas quatro espécies apresentam 24 cromossomos (ALEVI et al., 2013a). Sendo assim, questionamos se essas espécies seriam evolutivamente relacionadas, por apresentarem o mesmo número de cromossomos que, no caso é incomum dentro da subfamília, e o mesmo padrão heterocromático ou se essas características surgiram como homoplasias nessas espécies. Concluimos, assim, que novas abordagens comparativas devem ser realizadas, como: morfométricas, moleculares, enzimáticas e cruzamentos experimentais. Esses dados permitirão que o *status* de *T. melanocephala* dentro do gênero *Triatoma* seja avaliado.

Por meio da técnica citogenética de impregnação por íons prata foi possível estudar o comportamento nucleolar e a atividade transcricional durante a espermatogênese de *T. melanocephala* e *T. lenti*. Alevi et al. (2013e) observaram o fenômeno de persistência nucleolar durante a meiose em ambas as espécies de triatomíneos. Esse fenômeno foi descrito, na subfamília Triatominae, por Tartarotti e Azeredo-Oliveira (1999) e é caracterizado por material nucleolar durante a metáfase, diferindo, assim, do ciclo nucleolar observado nos outros eucariotos onde o nucléolo se fragmenta e desaparece na prófase e é reorganizado no final da anáfase (GONZÁLEZ-GARCIA e RUFAS, 1995). Também foi possível analisar a atividade transcricional de ambas as espécies, por meio das marcações com íons prata no núcleo poliplóide e nas células interfásicas, pois de acordo com Tavares e Azeredo-Oliveira (1997), o tamanho e o número de nucléolos e de corpos pré-nucleolares dependem das características funcionais das células e podem refletir, então,

em diferenças metabólicas e funcionais. Por meio das marcações, foi possível aferir que a espécie *T. lenti* tem maior atividade que *T. melanocephala*, uma vez que apresentou muitos corpúsculos impregnados no núcleo poliplóide e dois nucléolos nos núcleos interfásicos. Já a espécie *T. melanocephala* apresentou poucas marcações no núcleo poliplóide e apenas um nucléolo no núcleo interfásico.

A técnica citogenética molecular de Hibridização *in situ* (FISH) foi realizada na Universidad de La República (UDELAR), Uruguai/Montevidéo, em colaboração com o Dr. Francisco Panzera. As marcações específicas para as Regiões Organizadoras Nucleolares (RONs) foram realizadas com sondas 45S de *T. infestans*. Em *T. melanocephala*, a marcação ocorreu apenas em um dos cromossomos sexuais X, diferente do encontrado em outras duas espécies do subcomplexo Brasiliensis com as RONs descritas, *T. brasiliensis* (BARDELLA et al., 2010) e *T. sherlocki* (PANZERA et al., 2012), corroborando, mais uma vez a exclusão dessa espécie do subcomplexo.

VI.2 *T. lenti* e *T. sherlocki*

Por meio da descrição do conjunto cromossômico diploide de *T. lenti*, Alevi et al. (2012b) confirmaram a classificação inicial desse organismo no subcomplexo Brasiliensis, pois *T. lenti*, assim como todas as espécies do subcomplexo Brasiliensis, após a exclusão proposta por Alevi et al. (2012a) para *T. melanocephala*, *T. vitticeps* e *T. tibiamaculata*, apresentam 22 cromossomos (20 autossomos e XY).

Por meio da descrição do padrão de heterocromatina constitutiva de *T. lenti* e *T. sherlocki*, Alevi et al. (2013f) também puderam confirmar a classificação dessas espécies no subcomplexo Brasiliensis, pois, assim como *T. brasiliensis*, *T. melanica*, *T. juazeirensis*, *T. b. macromelanosoma* e *T. petrochiae*, esses triatomíneos apresentaram as características diagnósticas descritas por Panzera et al. (2000) para as espécies do

subcomplexo, a saber: blocos heterocromáticos em uma ou ambas as extremidades de todos os pares de autossomos; grande cromocentro na prófase inicial constituído pela associação dos cromossomos sexuais e de dois pares de autossomos e muitos blocos heterocromáticos dispersos no interior do núcleo. A relação de *T. sherlocki* com o complexo *T. brasiliensis* também foi recentemente comprovada por cruzamento experimental da espécie com triatomíneos que constituem o complexo (CORREIRA et al., 2013).

T. lenti e *T. sherlocki* apresentam semelhanças morfológicas, como hemiélitros reduzidos, anéis de cor laranja avermelhado no fêmur e manchas no conexivo (PAPA, et al., 2002). Dessa forma, foi realizado uma análise comparativa da espermiogênese desses insetos, a fim de, possivelmente, destacar uma característica diagnóstica, à semelhança do trabalho de Alevi et al. (2013c) em *T. melanocephala* e *T. vitticeps*. Por meio da análise das espermátides iniciais de *T. sherlocki*, observamos a presença de um curpúsculo heteropicnótico. Essa característica, bem como o tamanho e a morfologia das células haplóides de *T. sherlocki* (células menores e mais filamentosas) e a presença do flagelo durante toda a espermiogênese, mostraram-se como características importantes para diferenciar essa espécie de *T. lenti* (ALEVI et al., 2013g).

Análises citogenéticas da espermiogênese de *T. lenti* foram realizadas com o objetivo de estudar a atividade nucleolar durante essa fase de diferenciação celular. Na literatura, existem trabalhos, em diferentes modelos de estudo, que corroboram o fenômeno de reativação da atividade nucleolar pós-meiótica. Contudo, outros grupos de pesquisadores supõem que não existe atividade nucleolar pós-meiotica. Sendo assim, foi avaliado o comportamento nucleolar durante a espermiogênese de *T. lenti* com o objetivo de avaliar se, em triatomíneos, o nucléolo apresenta ou não atividade transcricional pós-meiótica. Por meio da técnica de Impregnação por íons prata, foi possível observar o

nucléolo nas espermátides. Comprovamos que o corpúsculo impregnado DNA ribossômico (DNAr) pela técnica de Reação de Feulgen, uma vez que essa técnica é específica para DNA. As marcações argirófiras foram idênticas as de Feulgen, indicando que o DNA marcado era o DNAr. A técnica de FISH foi realizada, permitindo a observação da presença da RON próxima ao nucléolo. Contudo, a técnica de Bandamento C, indicou que a marcação positiva também correspondia às outras técnicas. Portanto, foi sugerido que embora o nucléolo e as RONS estejam presentes durante toda a espermiogênese, estão inativados, pois, de acordo com Baulcombe (2004), o processo de heterocromatização do material genético está diretamente envolvido com sua atividade transcricional, uma vez que a formação de heterocromatina constitutiva é um importante inativador do DNA. Sendo assim, foi sugerido que embora o nucléolo tenha persistido durante toda a espermiogênese, esse não apresentava atividade transcricional, pois o DNAr poderia ter sido inativado por fatores epigenéticos, resultando em heterocromatina constitutiva (ALEVI et al., 2014b). Merece, ainda, ser ressaltado que toda a atividade transcricional necessária para a diferenciação das células haplóides é suportada pelo corpo cromatóide, organela citoplasmática descrita recentemente por Silistino-Souza et al. (2012) na subamília Triatominae.

A técnica citogenética molecular de FISH também foi realizada na UDELAR (Uruguai, Montevideo). Por meio das sondas 45S específicas de *T. infestans*, foi possível observar que *T. lenti*, assim como *T. brasiliensis* (BARDELLA et al., 2010) e *T. sherlocki* (PANZERA et al., 2012), apresentaram as RONS na mesma localização durante a metáfase, especificamente, em um grande par de autossomos. Embora novas análises devam ser realizadas nos outros integrantes do subcomplexo, os resultados sugerem que essa possa ser uma característica evolutiva diagnóstica do subcomplexo Brasiliensis.

Além disso, com o objetivo de compilar todas as espécies descritas até o momento, bem como ressaltar a importância da citogenética na classificação dos triatomíneos, foi realizada uma revisão de todas as espécies descritas na literatura, agrupando-as nas seis tribos e nos 18 gêneros, propostos por Galvão et al. (2003), assim como do número total de cariótipos descritos na literatura até o momento (Alevi et al., 2013d).

Os trabalhos originados da presente dissertação de mestrado contribuíram para reavaliar a classificação das espécies de triatomíneos do complexo *T. brasiliensis* e do subcomplexo Brasiliensis.

Conclusões

VI. CONCLUSÕES GERAIS

- ✓ *T. lenti* e *T. sherlocki* apresentaram muitas características cromossômicas que as aproximam citogeneticamente, como número de cromossomos, padrão de heterocromatina constitutiva e localização das RONS. No entanto, a análise das espermatídes possibilitou a descrição de uma ferramenta citotaxonômica diagnóstica diferencial para esses vetores.
- ✓ *T. melanocephala* e *T. vitticeps* apresentaram o mesmo número de cromossomos, assim como as mesmas características durante a espermatogênese. No entanto, esses vetores puderam ser diferenciados pela disposição dos blocos heterocromáticos, pela disposição das RONS e pelas características citotaxonômicas das espermatídes.
- ✓ As análises citotaxonômicas permitiram corroborar o enquadramento de *T. lenti* e *T. sherlocki*, assim como excluir *T. melanocephala*, *T. vitticipes* e *T. tibiamaculata* do subcomplexo Brasiliensis.
- ✓ Embora análises moleculares devam ser realizadas, os dados citotaxonômicos suportam o enquadramento de *T. lenti* como o sexto membro do complexo *T. brasiliensis*.

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