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Kaio Cesar Chaboli Alevi

Citotaxonomia e evolução cromossômica na subfamília Triatominae

São José do Rio Preto
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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biociências, área de concentração Genética, junto ao Programa de Pós-Graduação em Biociências, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

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Agradecimentos

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

Roger Von Oech

Resumo

RESUMO

A taxonomia e a sistemática dos triatomíneos baseiam-se, principalmente, em caracteres morfológicos. No entanto, existem espécies muito semelhantes ou até mesmo idênticas (espécies crípticas) do ponto de vista morfológico. Dessa forma, novas abordagens são necessárias para caracterizar um táxon e análises citogenéticas têm se mostrado como importantes ferramentas para a caracterização taxonômica e o conhecimento evolução desses vetores. Assim, o presente trabalho teve como objetivos principais: caracterizar os subcomplexos Brasiliensis, Rubrovaria, Matogrossensis, Maculata e Rubrofasciata; auxiliar na revalidação de *Triatoma bahiensis*; avaliar o *status* específico de *Rhodnius montenegrensis*, *Rhodnius* sp. (afim de *R. neglectus*), *T. pintodiasi*, *T. lenti* e *T. sherlocki*; analisar a relação evolutiva da tribo Rhodniini; caracterizar o fenômeno de persistência nucleolar na subfamília Triatominae e analisar a variabilidade genética intraespecífica de *T. sordida* proveniente do Brasil. Os resultados obtidos permitiram caracterizar citogeneticamente as espécies do subcomplexo Brasiliensis; revalidar *T. bahiensis* e corroborar que essa espécie, bem como *T. lenti* e *T. petrocchiae* são membros do subcomplexo Brasiliensis; corroborar o *status* específico de *T. lenti*, *T. sherlocki* e *T. bahiensis* pela presença de barreira de isolamento reprodutivo pós-zigótica (desmoronamento do híbrido); sugerir que durante a muda imaginal, os triatomíneos cessam a meiose e estimulam a espermiogênese para diminuir os gastos energéticos e garantir a chance de paternidade; caracterizar citogeneticamente os subcomplexos Matogrossensis e Rubrovaria e distingui-los dos outros subcomplexos da América do Sul; reagrupar as espécies dos subcomplexos Matogrossensis, Sordida, Maculata e Rubrovaria, com base na disposição do DNAr 45S; propor a criação de um novo subcomplexo (subcomplexo *T. vitticeps*), com base em dados fenotípicos e genotípicos; caracterizar citogeneticamente o subcomplexo Maculata e diferenciar *T. wygodzinskyi* e *T. arthurneivai*; diferenciar *T. rubrofasciata* de todas as outras espécies de triatomíneos pela citotaxonomia e cariossistemática; corroborar, por meio da análise cariotípica e das espermátides, que a tribo Rhodniini é um grupo monofilético; caracterizar a evolução cromossômica no grupo pallescens e relacionar a perda de

heterocromatina com a ocupação de diferentes ambientes; corroborar o *status* específico de *R. montenegrensis* (com base na posição do DNAr 45S) e de *T. pintodiasi* (com base no comportamento meiótico e na distância genética para o gene mitocondrial 16S); demonstrar que *R. neglectus* não apresenta variação cromossômica intraespecífica e descrever uma nova espécies do gênero *Rhodnius*, a saber, *R. taquarussuensis* sp. n.; discorrer sobre a evolução cariotípica nas tribos Rhodniini, Triatomini e Cavernicolini; confirmar o fenômeno de persistência nucleolar como uma sinapomorfia de Triatominae e, por fim, demonstrar que *T. sordida* do Brasil não apresenta variação cromossômica intraespecífica e corroborar o *status* desses vetores como *T. sordida sensu strito*. Esses resultados mostraram-se de extrema importância para elucidar várias questões sobre a biologia, taxonomia e evolução dos triatomíneos.

Palavras-chave: Citogenética. Taxonomia. Evolução, Triatomíneos. Doença de Chagas.

Abstract

ABSTRACT

The taxonomy and systematics of triatomines are mainly based on morphological characters. However, there are very similar or even identical species (cryptic species) from the morphological point of view. Thus, new approaches are necessary to characterize a taxon and cytogenetic analyzes have been shown as important tools for the taxonomic characterization and the evolutionary knowledge of these vectors. Thus, the main objectives of this study were: characterize the Brasiliensis, Rubrovaria, Matogrossensis, Maculata and Rubrofasciata subcomplexes; assist in the revalidation of Triatoma bahiensis; evaluate the specific status of Rhodnius montenegrensis, Rhodnius sp., T. pintodiasi, T. lenti and T. sherlocki; analyze the evolutionary relationship of the Rhodniini tribe; characterize the nucleolar persistence phenomenon in the Triatominae subfamily and analyze the intraspecific genetic variability of T. sordida from Brazil. The results obtained allowed characterize cytogenetically the species of the Brasiliensis subcomplex; revalidate T. bahiensis and corroborate that this species, as well as T. lenti and T. petrocchiae are members of the Brasiliensis subcomplex; corroborate the specific status of T. lenti, T. sherlocki and T. bahiensis by the presence of a post-zygotic reproductive isolation barrier (collapse of the hybrid); suggest that during the imaginal molt, cell division is disrupted (reduce energy costs) and the differentiation into sperm is stimulated (ensure the paternity of the adult male); characterize cytogenetically the Matogrossensis and Rubrovaria subcomplexes and distinguish them from other subcomplexes of South American; regroup the species of the Matogrossensis, Sordida, Maculata and Rubrovaria subcomplexes, based on the arrangement of the 45S rDNA; propose the creation of a new subcomplex (T. vitticeps subcomplex), based on phenotypic and genotypic data; characterized cytogenetically the Maculata subcomplex and differentiate the T. arthurneivai and T. wygodzinskyi; differentiate T. rubrofasciata from all other triatomine species by cytotaxonomy and karyosystematics; corroborate, by means of karyotypic and spermatids analysis, the monophyly of the Rhodniini tribe; characterize the chromosomal evolution in the pallescens group and relate the loss of heterochromatin with the occupation of different

environments; corroborate the specific status of R. montenegrensis (based on the position of the 45 rDNA) and T. pintodiasi (based on meiotic behavior and genetic distance for the 16S mitochondrial gene); demonstrate that R. neglectus does not present intraspecific chromosomal variation and describe a new species of the genus Rhodnius, namely, R. taquarussuensis sp. n.; discuss karyotype evolution in the Rhodniini, Triatomini and Cavernicolini tribes; confirming the nucleolar persistence phenomenon as a Triatominae synapomorphy, and, finally, demonstrate that T. sordida from Brazil does not present intraspecific chromosome variation and corroborate the status of these vectors as T. sordida sensu strito. These results were extremely important to elucidate several questions about the biology, taxonomy and evolution of triatomines.

Keywords: *Citogenetics. Taxonomy. Evolution. Triatomines. Chagas Disease.*

Sumário

SUMÁRIO

Introdução

1. INTRODUÇÃO

A doença de Chagas é um problema de saúde pública na América Latina e está se espalhando, cada vez mais, para novas regiões geográficas, como a Europa, a América do Norte, o Japão e a Austrália, associada, principalmente, à migração de pessoas infectadas pelo protozoário *Trypanosoma cruzi* (Chagas 1909) (Kinetoplastida, Trypanosomatidae), agente etiológico da doença de Chagas (GASCON; BERN; PINAZO, 2010; JACKSON; PINTO; PETT, 2014; WHO, 2017). Embora estima-se que oito milhões de pessoas estejam infectadas por *T. cruzi* e dez mil pacientes chagásicos morram anualmente, aproximadamente, 70 milhões de pessoas vivem em risco de contrair a doença de Chagas, tornando-a a principal causa de morte por doença parasitária na América Latina, bem como a principal causa da miocardiopatia infecciosa no mundo (MARTINS-MELO et al., 2012; CUCUNUBÁ et al., 2016; DNDi, 2017; WHO, 2017).

A principal forma de transmissão do *T. cruzi* é vetorial, por meio das fezes de triatomíneos contaminados com o parasito, pois esses insetos são hematófagos e possuem o hábito de defecar durante o repasto sanguíneo (CHAGAS, 1909; WHO, 2017). Os triatomíneos pertencem à ordem Hemiptera, subordem Heteroptera, família Reduviidae e subfamília Triatominae (LENT; WYGODZINSKY, 1979). Atualmente, são admitidas 152 espécies na subfamília Triatominae, agrupadas em 18 gêneros e cinco tribos (Tabela 1) (GALVÃO, 2014; ALEVI et al., 2015a; MENDONÇA et al., 2016; SOUZA et al., 2016; ROSA et al., 2017). O Brasil apresenta grande diversidade de espécies (GALVÃO, 2014) que, em consequência de ações antrópicas (como o desmatamento e as queimadas), estão migrando para o ambiente domiciliar, processo conhecido como domiciliação (DIAS; SCHOFIELD, 1998; ALMEIDA et al., 2009).

Embora existam espécies com maior ou menor grau de importância na transmissão da doença de Chagas [com destaque para *Triatoma infestans*, *Rhodnius prolixus*, *T. dimidiata*, *Panstrongylus megistus* e *T. brasiliensis*, que possuem importância mundial na transmissão da doença, assim como para *T. infestans*, *T. brasiliensis*, *T. pseudomaculata*, *T. sordida* e *P. megistus*, que apresentam maiores competências vetoriais no Brasil (GALVÃO, 2014)], todos os triatomíneos, de ambos os sexos e em qualquer fase do desenvolvimento, são considerados como potenciais vetores dessa doença.

Tabela 1 – Revisão do número de espécies da subfamília Triatominae.

TRIBO	GÊNERO	NÚMERO DE ESPÉCIES
Alberproseniini	*Alberprosenia*	2
Bolboderini	*Belminus*	8
	Bolbodera	1
	Microtriatoma	2
	Parabelminus	2
Cavernicolini	*Cavernicola*	2
Rhodniini	*Psammolestes*	3
	Rhodnius	21
Triatomini	*Dipetalogaster*	1
	Eratyrus	2
	Hermanlentia	1
	Linshcosteus	6
	Meccus	6
	Mepraia	3
	Nesotriatoma	3
	Panstrongylus	15
	Paratriatoma	1
	Triatoma	73
Total		**152**

Além da grande importância epidemiológica, os triatomíneos destacam-se, também, por constituírem um excepcional modelo para estudos celulares, pois apresentam características cromossômicas peculiares, como cromossomos holocêntricos (com cinetócoro difuso) (UESHIMA, 1966), meiose invertida para os cromossomos sexuais (em que a primeira fase da divisão meiótica é equacional, ou seja, separa as cromátides irmãs) (UESHIMA, 1966) e persistência nucleolar durante a meiose (TARTAROTTI; AZEREDO-OLIVEIRA, 1999).

O fenômeno de persistência nucleolar é caracterizado pela presença do nucléolo ou de corpúsculos nucleolares durante todas as fases da meiose (TARTAROTTI; AZEREDO-OLIVEIRA, 1999). Esse comportamento nucleolar é incomum quando comparado aos outros eucariontes, uma vez que o nucléolo geralmente se fragmenta no final da prófase e só é reorganizado no final da anáfase/começo da telófase (GONZÁEZ-GARCIA et al., 1995). Até o momento, a nucleologênese de 22 espécies de triatomíneos (distribuídas nos gêneros *Triatoma*, *Rhodnius* e *Panstrongylus*) foi descrita (TARTAROTTI; AZEREDO-OLIVEIRA,

1999; MORIELLE; AZEREDO-OLIVEIRA, 2004; SEVERI-AGUIAR; AZEREDO-OLIVEIRA, 2005a; SEVERI-AGUIAR et al., 2006; MORIELLE; AZEREDO-OLIVEIRA, 2007; COSTA; AZEREDO-OLIVEIRA; TARTAROTTI, 2008; ALEVI et al., 2013a, 2014a; PEREIRA et al., 2015). Alevi et al. (2014a) sugerem que novas espécies e, principalmente, novos gêneros de triatomíneos sejam analisados para avaliar se esse fenômeno é uma sinapomorfia da subfamília Triatominae.

A taxonomia e a sistemática dos triatomíneos baseiam-se, principalmente, em caracteres morfológicos (LENT; WYGODZINSKY, 1979; GALVÃO et al., 2003, 2014; ROSA et al., 2010, 2014). No entanto, existem espécies muito semelhantes ou até mesmo idênticas (espécies crípticas) do ponto de vista morfológico (MONTEIRO et al., 2000, 2003; PANZERA et al., 2006, 2015). Dessa forma, a utilização de novas abordagens é necessária para caracterizar um táxon e as análises citogenéticas têm se mostrado como importantes ferramentas para diferenciar esses insetos hematófagos (PÉREZ et al., 1992; PANZERA et al., 1995, 1007; ALEVI et al., 2012a, 2013b, 2015a, b; ALEVI; ROSA; AZEREDO-OLIVEIRA, 2014b).

Os estudos citogenéticos na subfamília Triatominae foram iniciados com a descrição do cariótipo de *Triatoma sanguisuga* (Leconte, 1855) (PAYNE, 1909). Todavia, apenas em 1950 os estudos citogenéticos foram retomados e novos cariótipos foram descritos (SCHREIBER; PELLEGRINO, 1950). Ueshima, em 1966, descreveu o conjunto cromossômico diplóide e o comportamento meiótico de vinte novas espécies, propôs que 22 cromossomos (20 autossomos + XY) é o número tipo para a subfamília Triatominae e ressaltou que os estudos citogenéticas são importantes ferramentas taxonômicas para esses vetores, iniciando, assim, a citotaxonomia dos triatomíneos. Atualmente, 88 espécies apresentam o cariótipo descrito que varia de 21 a 25 cromossomos (Tabela 2) (ALEVI et al., 2015a).

Schofield; Galvão (2009) propuseram o agrupamento dos triatomíneos em complexos e subcomplexos específicos (Tabela 3). Esses autores compilaram diferentes informações de várias espécies, como, por exemplo, caracteres morfológicos, distribuição geográfica e dados genéticos. No entanto, foram utilizados os dados disponíveis na literatura até 2009 e, com a publicação de novos resultados, alguns agrupamentos foram questionados (ALEVI et al., 2012a; GARDIM et al., 2014; JUSTI et al., 2014).

Tabela 2 – Espécies da subfamília Triatominae com o cariótipo descrito.

Cariótipo (2n)	**Gênero: espécie (s)**
21 = 18A + X_1X_2Y	***Panstrongylus****: megistus* ***Triatoma****: nitida*
22 = 20A + XY	***Psammolestes****: coreodes, tertius* ***Rhodnius****: brethesi, colombiensis, domesticus, ecuadoriensis, milesi, montenegrensis, nasutus, neglectus, neivai, pallescens, pictipes, prolixus, robustus, stali* ***Dipetalogaster****: maximus* ***Paratriatoma****: hirsuta* ***Triatoma****: arthurneivai, baratai, brasiliensis (b. brasiliensis, b. macromelanosoma), carcavalloi, circummaculata, costalimai, delpontei, garciabesi, guasayana, guazu, infestans, juazeirensis, jurbergi, klugi, lectularia, lenti, maculata, matogrossensis, melanica, patagonica, petrocchiae, pintodiasi, platensis, pseudomaculata, rubrovaria, sherlocki, sordida, vandae, williami, wygodzinskyi*
23 = 20A + X_1X_2Y	***Belminus****: herreri, corredori* ***Eratyrus****: cuspidatus, mucronatus* ***Meccus****: bassolsae, longipennis, mazzottii, pallidipennis, phyllosoma, picturatus* ***Mepraia****: gajardoi, parapatrica, spinolai* ***Nesotriatoma****: bruneri, flavida* ***Panstrongylus****: chinai, geniculatus, howardi, lignarius, rufotuberculatus, tupynambai* ***Triatoma****: barberi, dimidiata (d. capita, d. dimidiata, d. maculipennis), gerstaeckeri, hegneri, mexicana, peninsularis, protracta, rubida, ryckmani, sanguisuga, sinaloensis, tibiamaculata*
24 = 20A + $X_1X_2X_3Y$	***Panstrongylus****: lutzi* ***Triatoma****: eratyrusiformis, melanocephala, vitticeps*
25 = 22A + X_1X_2Y	***Triatoma****: rubrofasciata*

Tabela 3 – Complexos e subcomplexos de triatomíneos.

COMPLEXOS	SUBCOMPLEXOS
Phyllosoma	
	Dimidiata
	Phyllosoma (=*Meccus*)
Flavida (=*Nesotriatoma*)	
Rubrofasciata	
Protracta	
Lecticularia	
Dispar	
Infestans	
	Brasiliensis
	Infestans
	Maculata
	Matogrossensis
	Rubrovaria
	Sordida
Spinolai (=*Mepraia*)	

O subcomplexo Brasiliensis foi, inicialmente, proposto por Schofield; Galvão (2009) com nove espécies, a saber, *T. brasiliensis* Neiva, 1911, *T. juazeirensis* Costa & Félix, 2007, *T. melanica* Neiva & Lent, 1941, *T melanocephala* Neiva & Pinto, 1923, *T. petrocchiae* Pinto & Barreto, 1925, *T. lenti* Sherlock & Serafim, 1967, *T. sherlocki* Papa et al., 2002, *T. tibiamaculata* (Pinto, 1926) e *T. vitticeps* (Stål, 1859). No entanto, Alevi et al. (2012a), por meio de dados cariossistemáticos, propuseram a exclusão de *T. tibiamaculata*, *T. melanocephala* e *T. vitticeps* do subcomplexo Brasiliensis, por apresentarem cariótipos diferentes dos observados para os outros membros do subcomplexo (derivado da fragmentação do cromossomo sexual X). Esses resultados foram corroborados por análises citogenéticas (ALEVI; ROSA; AZEREDO-OLIVEIRA, 2014b; ALEVI et al., 2014c), morfométricas (OLIVEIRA et al., 2017) e filogenéticas (GARDIM et al., 2014; OLIVEIRA et al., 2017).

Costa e colaboradores, com base em análises isoenzimáticas (COSTA et al., 1997a), morfológicas (COSTA et al., 1997b; COSTA et al., 2013), da superfície exocorial do ovo (COSTA et al., 1997b), biológicas (COSTA; SILVA, 1998a), ecológicas (COSTA et al., 1998b), genéticas (MONTEIRO; COSTA; BEARD, 1999), citogenéticas (PANZERA et al., 2000), biogeográficas (COSTA; PETERSON; BEARD, 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* é um grupo monofilético

formado pelas espécies *T. brasiliensis*, *T. melanica* e *T. juazeirensis* e pela subespécie *T. b. macromelanosoma* Galvão, 1965. Mendonça et al. (2009), por meio de reconstrução filogenética com dois genes mitocrondriais (CytB e 16S), sugeriram que *T. sherlocki* deveria fazer parte do complexo *T. brasiliensis*. A inclusão foi confirmada, posteriormente, por análises citogenéticas (ALEVI et al., 2013b) e cruzamento híbrido experimental (CORREIA et al., 2013). Além disso, foi sugerido que *T. lenti* venha a ser o sexto membro do complexo (ALEVI et al., 2013b) e, recentemente, com base em dados moleculares (genes mitocondriais 12S, 16S, COI e Cytb) e dados morfométricos (morfométrica geométrica de asas e cabeças), *T. petrocchiae* foi agrupado ao complexo *T. brasiliensis* (OLIVEIRA et al., 2017).

Espécies do subcomplexos Matogrossensis (*T. baratai* Carcavallo & Jurberg, 2000, *T. costalimai* Verano & Galvão, 1958, *T. deaneorum* Galvão, Souza & Lima, 1967, *T. guazu* Lent & Wygodzinsky, 1979, *T. jurbergi* Carcavallo, Galvão & Lent, 1998, *T. matogrossensis* Leite & Barbosa, 1953, *T. vandae* Carcavallo et al., 2002 e *T. williami* Galvão, Souza & Lima, 1965) e Rubrovaria [*T. carcavalloi* Jurberg, Rocha & Lent, 1998, *T. circummaculata* (Stål, 1859) , *T. klugi* Carcavallo et al., 2001, *T. limai* Del Ponte, 1929, *T. oliverai* (Neiva, Pinto & Lent, 1939) e *T. rubrovaria* (Blanchard, 1843)] (SCHOFIELD; GALVÃO, 2009) foram, inicialmente, agrupadas no complexo *oliverai* (NOIREAU et al., 2002). Dados moleculares demonstram que apenas o subcomplexo Rubrovaria forma um grupo monofilético (JUSTI et al., 2014), ressaltando a necessidade de novos estudos para as espécies do subcomplexo Matogrossensis. Além disso, duas espécies foram descritas e agrupadas nesses complexos, a saber, *T. jatai* Gonçalves et al., 2013 (subcomplexo Matogrossensis) (GONÇALVES et al., 2013) e *T. pintodiasi* Jurberg et al., 2013 (subcomplexo Rubrovaria) (JURBERG et al., 2013).

O subcomplexo Maculata é composto pelas espécies *T. maculata* (Erichson, 1848), *T. pseudomaculata* Corrêa & Espínola, 1964, *T. arthurneivai* Lent & Martins, 1940 e *T. wygodzinskyi* Lent 1951 (SCHOFIELD; GALVÃO, 2009). Por meio de morfometria da cabeça e do tórax, Santos et al. (2007) alertaram para um possível problema taxonômico envolvendo *T. arthurneivai* e *T. wygodzinskyi*. Recentes estudos de morfometria geométrica demonstraram que os insetos capturados fora da região da Serra do Cipó são espécimes de *T. wygodzinskyi* (CARBAJAL-DE-LA-FUENTE et al., 2010). Os autores relatam que as populações de *T. arthurneivai* de São Paulo, estudadas por mais de quarenta anos por muitos autores (CORRÊA; ALVES; PASCALE, 1962; CORRÊA; PINTO ALVES; NODA, 1965; PINTO ALVES; NODA, 1964; FORATTINI; JUAREZ; RABELLO, 1968; FORATTINI;

RABELLO; PATTOLI, 1972; JUAREZ et al., 1970; BARRETTO; RIBEIRO, 1981; HYPSA et al., 2002; PAULA; DIOTAIUTI; SCHOFIELD, 2005; ROSA et al., 2005; SANTOS et al., 2007; BARGUES et al., 2008), correspondem a *T. wygodzinskyi*.

A citotaxonomia dos triatomíneos é uma importante ferramenta para resolver problemas sistemáticos e taxonômicos: Jurberg et al. (1998) e Frías-Lasserre (2010), por exemplo, utilizaram dados citogenéticos para revalidar a espécie *T. garciabesi* Carcavallo et al., 1967 e descrever *Mepraia parapatrica* Frías-Lasserre, 2010; Panzera et al. (1995, 1997), Santos et al. (2007), Alevi; Rosa; Azeredo-Oliveira (2014b) e Alevi et al. (2015b), por meio da análise citogenética de prófases iniciais, caracterizaram as espécies dos subcomplexos Infestans, Sordida, Maculata, Matogrossensis, Rubrovaria e Brasiliensis, respectivamente. Além disso, a citotaxonomia também pode auxiliar na diferenciação de espécies morfologicamente relacionadas (ALEVI et al., 2013c, d, 2015b) e é importante ferramenta para estudar variação intraespecífica nos triatomíneos (Tabela 4).

Tabela 4 – Variação cromossômica intraespecífica na subfamília Triatominae.

ESPÉCIES	VARIAÇÃO CROMOSSÔMICA INTRAESPECÍFICA	REFERÊNCIAS
R. ecudoriensis	Presente	Pita et al. (2013)
R. pallescens	Presente	Gómez-Palacio et al. (2008)
P. geniculatus	Presente	Crossa et al. (2002)
T. dimidiata	Presente	Panzera et al. (2006)
T. infestans	Presente	Panzera et al. (2004, 2012)
T. sordida	Presente	Panzera et al. (1997, 2015)
R. neglectus	Ausente	Alevi et al. (2015c)
R. prolixus	Ausente	Ravazi et al. (2017)
R. nasutus	Ausente	Ravazi et al. (2017)
P. megistus	Ausente	Alevi et al. (2015d)
T. brasiliensis	Ausente	Panzera et al. (2000)
T. pseudomaculata	Ausente	Imperador et al. (2016)

A tribo Rhodniini é composta por 23 espécies, sendo 20 do gênero *Rhodnius* e três do gênero *Psammolestes* (ALEVI et al., 2015a; SOUZA et al., 2016). Esses insetos apresentam homogeneidade cariotípica (PITA et al., 2013, ALEVI et al., 2015a) e apenas quatro espécies

apresentam heterocromatina constitutiva nos autossomos (PITA et al., 2013). Além disso, *Rhodnius* spp. são muito semelhantes do ponto de vista morfológico (MONTEIRO et al., 2000) e, com base em análises moleculares, foram agrupados em grupos ou complexos monofiléticos (MONTEIRO et al., 2003; ROSA et al., 2012; JUSTI; GALVÃO, 2017). O grupo pallescens, por exemplo, é formado pelas espécies *R. colombiensis* Mejia, Galvão & Jurberg, 1999, *R. pallescens* Barber, 1932 e *R. ecuadoriensis* Lent & León, 1958 (SCHOFIELD; DUJARDIN, 1999) e Abad-Franch; Monteiro (2007) sugerem que análises citogenéticas podem auxiliar na compreensão da taxonomia e evolução desse importante grupo de vetores.

Análises citogenéticas moleculares também são importantes ferramentas que auxiliam no entendimento evolutivo dos triatomíneos. Panzera et al. (2012), por meio da técnica de hibridização *in situ* (FISH) com sonda de DNA ribossômico (DNAr) 45S, apresentaram o padrão evolutivo das Regiões Organizadoras Nucleolares (RONs) para 38 espécies de triatomíneos. Até então, poucos estudos com FISH tinham sido realizados na subfamília Triatominae, todos comandados por Azeredo-Oliveira (SEVERI-AGUIAR; AZEREDO-OLIVEIRA, 2005b; SEVERI-AGUIAR et al., 2006; MORIELLE-SOUZA; AZEREDO-OLIVEIRA, 2007; BARDELLA et al., 2010). Pita et al. (2013) analisaram a disposição das RONs em 10 espécies da tribo Rhodniini e observaram que em *Rhodnius* e *Psammolestes* as RONs ficam restritas apenas aos cromossomos sexuais (X, Y ou X e Y), diferente, por exemplo, das espécies do gênero *Triatoma* (tribo Triatomini) em que as RONs podem ser observadas nos cromossomos sexuais e/ou nos autossomos (PANZERA et al., 2012).

Apesar dos conhecimentos relatados sobre as características citogenéticas desses insetos hematófagos de importância medico-sanitária, ainda é necessário ampliar os estudos citotaxonômicos na subfamília Triatominae. Dessa forma, os dados apresentados mostram-se importantes, em diferentes aspectos, para auxiliar na taxonomia e sistemática, assim como no entendimento da história evolutiva desses vetores, pois a doença de Chagas, embora descrita há mais de 100 anos, é a doença parasitária com o maior índice de mortalidade nas Américas e o controle vetorial é a principal forma de minimizar a incidência dessa enfermidade. Assim, a caracterização citotaxonômica desses vetores pode gerar subsídios para auxiliar na atividade dos agentes de saúde, uma vez que a correta classificação dos triatomíneos permite que as principais espécies vetoras sejam foco específico dos programas de controle de vetores.

Objetivos

2. OBJETIVOS

a) Caracterizar a espermatogênese das espécies dos subcomplexos Brasiliensis, Rubrovaria, Matogrossensis, Maculata e Rubrofasciata, por meio de técnicas citogenéticas clássicas e moleculares, com ênfase citotaxonômica.

b) Descrever citogeneticamente uma população de *T. lenti* com diferença no padrão cromático, a fim de auxiliar na possível revalidação de *T. bahiensis.*

c) Descrever citogeneticamente a espécie *R. montenegrensis*, por meio de técnicas citogenéticas clássicas e moleculares, a fim de contribuir com a taxonomia da tribo Rhodniini.

d) Avaliar a relação evolutiva das espécies do grupo pallescens (*R. pallescens*, *R. colombiensis* e *R. ecuadoriensis*), por meio de análises citogenéticas.

e) Descrever a espermiogênese das espécies da tribo Rhodniini, com foco citotaxonômico.

f) Descrever citogeneticamente *Rhodnius* sp. e comparar com diferentes populações de *R. neglectus* (espécie afim), com o intuito de auxiliar na descrição da nova espécie.

g) Descrever o comportamento nucleolar de triatomíneos, com ênfase no fenômeno de persistência nucleolar.

h) Estudar a variabilidade cromossômica intraespecífica em populações de *T. sordida* do Brasil, a fim de avaliar o fenômeno de especiação críptica.

Material e Métodos

3. MATERIAL E MÉTODOS

3.1. Obtenção e procedência das espécies

As espécies de triatomíneos utilizadas são provenientes do "Insetário de Triatominae", instalado na Faculdade de Ciências Farmacêuticas (FCFAR/UNESP), Câmpus de Araraquara, São Paulo, sob coordenação do Prof. Dr. João Aristeu Rosa; do Insetário do Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos, instalado na FIOCRUZ, Rio de Janeiro, coordenado pelo Dr. José Jurberg; e do Insetário do Laboratório de Triatomíneos e Epidemiologia da Doença de Chagas, instalado no CPqRR/FIOCRUZ, Minas Gerais, coordenado pela Dra. Liléia Diotaiuti.

3.2 Órgão analisado

Foram analisados os túbulos seminíferos de, pelo menos, três machos adultos de cada espécie de triatomíneo, pois a espermatogênese em Hemiptera é contínua na fase adulta, permitindo que os diferentes estágios da espermatogênese (espermatocitogênenese, meiose e espermiogênese) sejam estudados.

3.3 Fixação dos túbulos seminíferos

Após a dissecção, os testículos foram transportados para uma solução fisiológica (Demerec), onde foi realizada a limpeza e individualização dos túbulos seminíferos. Posteriormente, cada túbulo foi colocado em um recipiente de vidro contendo três partes de metanol para uma de ácido acético (3:1), e conservado no freezer à -20°C.

3.4 Preparação das lâminas

Os túbulos seminíferos foram retirados da solução fixadora e colocados em uma lâmina, onde foram realizados dois banhos de água destilada, por cinco minutos cada. Posteriormente, foi acrescentada uma gota de 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 do esmagamento celular. A remoção da lamínula ocorreu em nitrogênio líquido.

3.5 Técnicas citogenéticas convencionais

3.5.1 Orceína Lacto-Acética (DE VAIO et al., 1985, com modificações de acordo com ALEVI et al., 2012a): Estudo do cariótipo, da espermatogênese e da espermiogênese.

3.5.2 Bandamento C (SUMNER, 1972): Estudo do padrão de heterocromatina constitutiva.

3.5.3 Impregnação por íons prata (HOWELL e BLACK, 1980): Estudo do comportamento nucleolar.

3.6 Técnica citogenética molecular

3.6.1 Hibridização *in situ* (FISH) (PANZERA et al., 2012): Estudo da disposição das RONs.

3.6.2 CMA_3/DAPI (SCHIMID, 1980, com modificações de acordo com SEVERI-AGUIAR et al., 2006): Estudo da riqueza de AT e CG no material genético.

3.7 Forma de 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 Gmb H*). O material submetido à técnica citogenética molecular foi analisado em microscopia de fluorescência *Olympus BX-FLA*.

Resultados

4. RESULTADOS (CAPÍTULOS)

4.1 Capítulo 1 (Artigo científico publicado na revista *Journal of Vector Ecology* FI: 1,47)

ALEVI, K. C. C.; ROSA, J. A.; AZEREDO-OLIVEIRA, M. T. V. Spermatogenesis in *Triatoma melanica* Neiva and Lent, 1941 (Hemiptera, Triatominae). **Journal of Vector Ecology**, v. 39, p. 231-233, 2014.

Spermatogenesis in *Triatoma melanica* Neiva and Lent, 1941 (Hemiptera, Triatominae)

SCIENTIFIC NOTE

Costa and collaborators proposed the *Triatoma brasiliensis* complex using many approaches, including egg morphology (Costa et al. 1997a), morphometry of the testis (Freitas et al. 2008), hybrid cross (Costa et al. 2003), isoenzymes (Costa et al. 1997b), molecular data (Monteiro et al. 2004), morphological data (Costa et al. 1997a), biological data, and ecological data (Costa et al. 1998). This complex is comprised of the subspecies *T. b. brasiliensis* and *T. b. macromelanosoma*, and also the species *T. juazeirensis* and *T. melanica*. By means of phylogenetic reconstruction, Mendonça et al. (2009) proposed the inclusion of *T. sherlocki* to this complex. This inclusion was recently confirmed through the use of both cytogenetic analysis (Alevi et al. 2013a) and cross-mating experiments (Correia et al. 2013).

Although the *T. brasiliensis* complex is a monophyletic group, *T. melanica* is considered an independent evolutionary unit and is thought to be the most differentiated form of the complex, with a genetic composition that is incompatible and hybrids that are inviable with other members of the *T. brasiliensis* complex (Costa et al. 2003). In 1941, this species was described by Neiva and Lent as a subspecies of *T. brasiliensis* (*T. b. melanica*). Using different studies such as morphology, biology, ecology, crossing experiments, allozymes, and mtDNA sequences, Costa et al. (2006) increased the specific status of *T. b. melanica* to *T. melanica* (Costa et al. 2006).

Because *T. melanica* has only been collected in natural ecotopes and was considered to be important in the maintenance of the wild cycle of *Trypanosoma cruzi* (an etiologic agent of Chagas disease) (Costa 1999), all approaches used to study biology and reproduction of this

vector are important. Thus, the present study aims to describe the spermatogenesis of *T. melanica*.

Seminiferous tubules of two adult males of *T. melanica* from the Triatominae Insectarium within the Department of Biological Sciences, in the College of Pharmaceutical Sciences, at Sao Paulo State University's Araraquara campus, Brazil (FCFAR/UNESP) were first shredded, smashed, and set on a slide in liquid nitrogen. They were then stained using the cytogenetic technique of lacto-acetic orcein (De Vaio et al. 1985, with modifications according to Alevi et al. 2012). Spermatogenesis 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).

Spermatocytogenesis was represented only by mitotic metaphase (Figure 1). Note all 20 autosomes and two sex chromosomes (arrows). All stages of meiosis were observed (Figure 2), including the diffuse stage (prophase) (Figure 2A), metaphase I in polar (Figure 2B) and lateral view (Figure 2C), anaphase I (Figure 2D) and II (Figure 2E) and telophase (Figure 2F). In addition, the elongation of haploid cells was observed during spermiogenesis (Figure 3).

Analyses of mitotic and meiotic metaphases made it possible to confirm the karyotype described for *T. melanica* (2n = 20A + XY) (Panzera et al. 2000). However, *T. melanica* presented a peculiar behavior during mitotic metaphase: the chromatids of sex chromosomes were visible.

All species of the *T. brasiliensis* complex have the same chromosomal characteristics: namely, 22 chromosomes (2n = 20A + XY) with heterochromatic blocks at one or both chromosomal ends of all autosomal pairs and a large heterochromatic chromocenter formed by the association of both sex chromosomes plus two autosomal pairs and many heterochromatic blocks dispersed inside the nucleus (Panzera et al. 2000; Alevi et al. 2013a). The analysis of prophase revealed the same results described when the C-banding technique was used by Panzera et al. (2000). Thus, our analysis confirms the association of *T. melanica* with the species of the complex.

Through molecular data (16S and Cytb), *T. melanica* was considered to be a sister to *T. sherlocki* (Mendonça et al. 2009). Alevi et al. (2013b, c) propose the analysis of spermatids as a cytotaxonomic tool that can be used to compare related species. During spermiogenesis of *T. melanica*, two heteropycnotic filaments were noted in each of the haploid cells. These characteristics are quite different from those described for *T. sherlocki*, which presents early

spermatids and which possesses a heteropyknotic corpuscle that becomes a the periphery filament during cell elongation (Alevi et al. 2013b).

Thus, this paper describes the spermatogenesis of *T. melanica*, confirms the relationship this species shares with members of the *T. brasiliensis* complex, and differentiates *T. melanica* from *T. sherlocki* (sister species). Although morphometric analyses were not performed, we noted that cells of *T. melanica* are relatively larger than those of other members of complex. In addition to genetic load, this phenomenon may be a factor that is related to reproductive incompatibility in experimental hybrid crosses, thus representing an important pre-zygotic barrier between these hematophagous insects.

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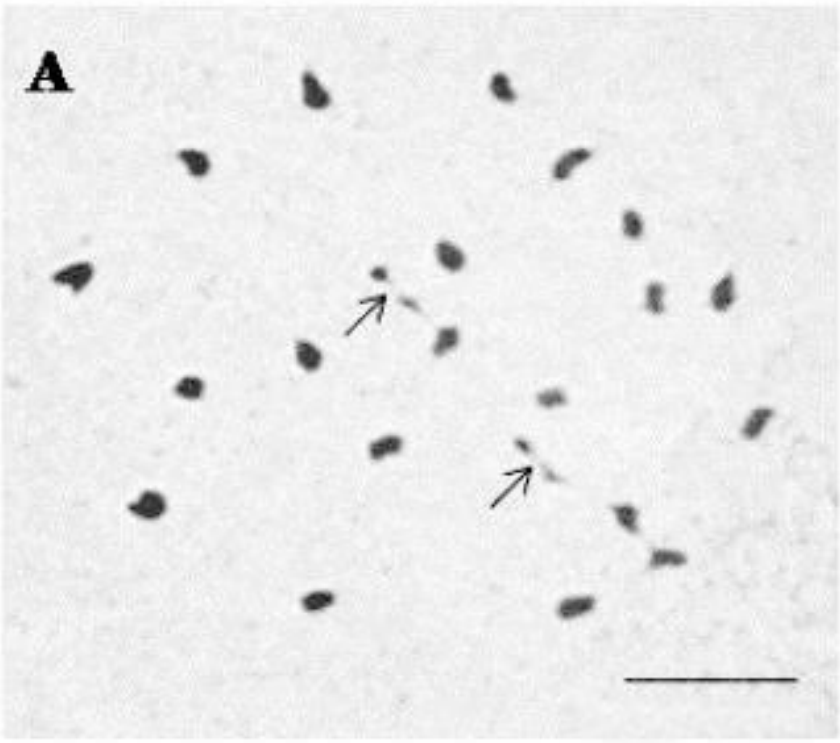

Figure 1. Mitotic metaphase of *Triatoma melanica*. (A) Note 20 autosomes and the two sex chromosomes (arrows). Bar: 10 µm.

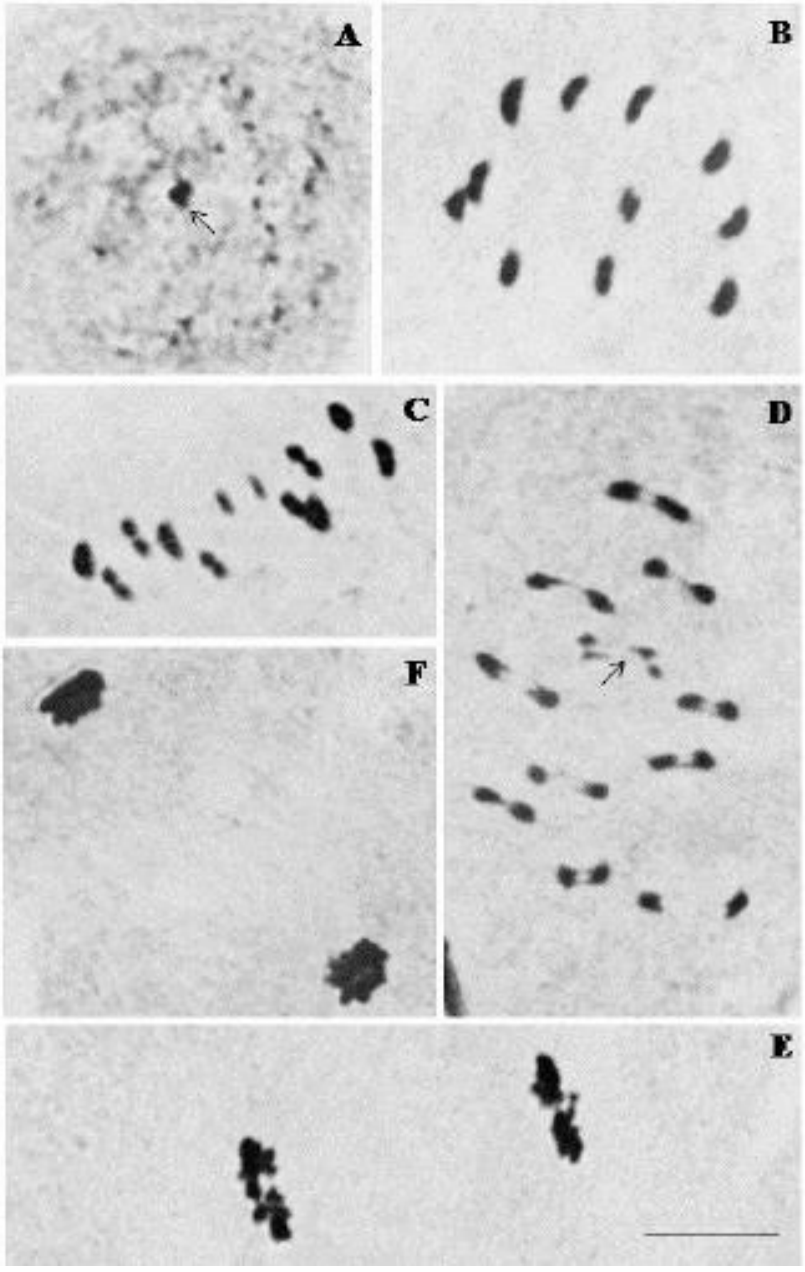

Figure 2. Stages of meiosis of *Triatoma melanica*. (A) Diffuse stage (prophase). Note the large heterochromatic chromocenter (arrow) and many heterochromatic blocks dispersed within the nucleus. (B, C) Metaphase I. Note the karyotype 2n = 20A + XY. (D) Anaphase I. Note the separation of homologous chromosomes in autosomes and the sister chromatids in sexual chromosomes (arrow). (E) Anaphase II. (F). Telophase. Bar: 10 µm.

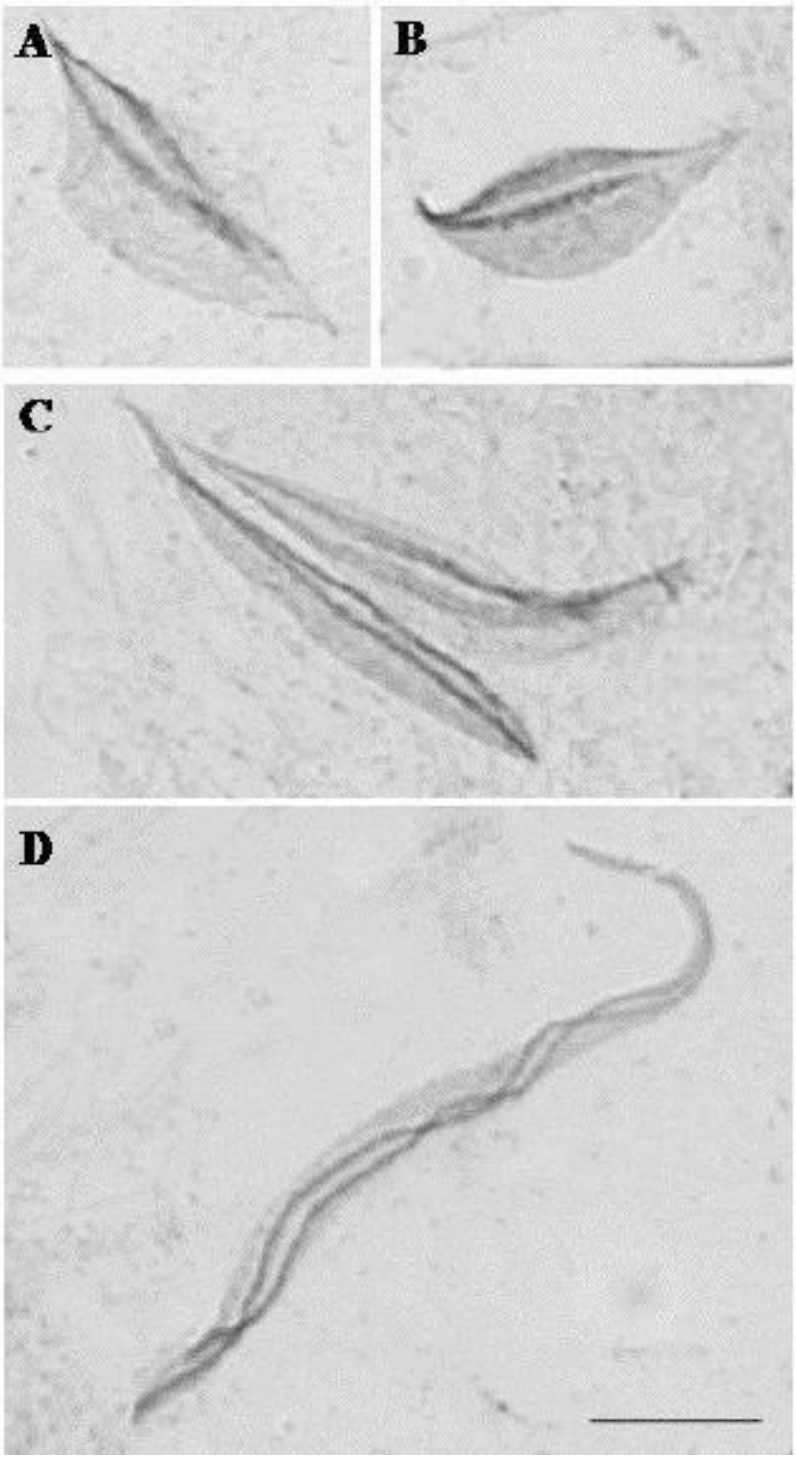

Figure 3. Spermiogenesis of *Triatoma melanica*. (A-D) Note the presence of two heteropycnotic filaments during the elongation of haploid cells. Bar: 10 µm.

4.2 Capítulo 2 (Artigo científico publicado na revista *Infection, Genetics and Evolution* FI 2,88)

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Cytogenetic and morphologic approaches of hybrids from experimental crosses between *Triatoma lenti* Sherlock & Serafim, 1967 and *T. sherlocki* Papa et al., 2002 (Hemiptera: Reduviidae)

Abstract

The reproductive capacity between *Triatoma lenti* and *T. sherlocki* was observed with the aim to verify the fertility and viability of the offspring, and by cytogenetic, morphologic and morphometric approaches to analyze the differences inherited. Experimental crosses were performed in both directions. The fertility rate of the eggs in crosses involving *T. sherlocki* females was 65% and 90% in F1 and F2 offspring, respectively, while in reciprocal crosses was 7% and 25% in F1 and F2 offspring, respectively. The cytogenetic analyses of the male meiotic process of the hybrids were performed using the lacto-acetic orcein, C-banding and Feulgen techniques. The male F1 offspring presented a normal chromosome behavior, similar as reported in parental species. However, cytogenetic analysis of F2 offspring showed errors in chromosome pairing. This post-zygotic isolation that prevents hybrid in nature may be collapse of the hybrid. This phenomenon is due to a genic deregulation occurred in the chromosomes of F1. The results were similar in the hybrids the both crosses. Morphological approaches, as color and size of connexive and presence of red rings on the legs were similar to *T. sherlocki*, while the wings size was similar to *T. lenti* in F1 offspring. The eggshells showed characteristics similar to species of origin, whereas the median process of the pygophore showed intermediate characteristics in the F1, and segregating pattern in F2 offspring. Geometric morphometric techniques of the wings showed that both F1 and F2 offspring were similar to *T. lenti*. Our studies about the reproductive capacity in experimental hybrids between *T. lenti* and *T. sherlocki* confirm that both species are evolutionarily closed

hence included in the brasiliensis subcomplex. The extremely reduced fertility observed in the F2 hybrids confirming the status specific of species analyzed.

1. Introduction

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted to mammalian hosts primarily through feces during the blood meal of insect of the Triatominae subfamily (Chagas, 1909; Lent and Wygodzinsky, 1979). It has been estimated that there are 7 to 8 million people infected by *T. cruzi* in Latin America and 25 million are exposed to infection (Moncayo and Silveira, 2009; Coura and Viñas, 2010; WHO, 2013).

Triatoma sherlocki was first studied by Cerqueira (1982) that performed experimental crosses between *T. sherlocki* with *T. brasiliensis*, *T. infestans* and *T. lenti*. Experimental crosses with *T. lenti* and *T. infestans* has not obtaining hybrids, whereas obtaining fertile hybrids with *T. brasiliensis* and *T. sherlocki*, classifying the latter primarily as a subspecies of *T. brasiliensis*, designated *T. b. santinacensis* (Cerqueira, 1982).

Morphological analysis of genital structures, pronotum and scutelum, suggest *T. sherlocki* related to *T. lenti* however peculiar characteristics as the reduced hemelytra, rings of color orange redish in the femur, allow the description of *T. sherlocki* as a valid species (Papa et al., 2002).

According to Schofield and Galvão (2009) the brasiliensis subcomplex comprises nine species distributed in South America: *T. brasiliensis*, *T. juazeirensis*, *T. lenti*, *T. melanica*, *T. melanocephala*, *T. petrocchiae*, *T. sherlocki*, *T. tibiamaculata* and *T. vitticeps*. The parameters used to group these species were principally morphological and geographical distribution. By means of cytogenetic data, Alevi et al. (2012a) proposes the exclusion of *T. melanocephala*, *T. tibiamaculata* and *T. vitticeps* of brasiliensis subcomplex and Alevi et al. (2012b, 2013a) confirm the inclusion of *T. lenti*. Mendonça et al. (2009), by analyses with mitochondrial genes and Alevi et al. (2013a), by cytogenetic analysis support the inclusion of the *T. sherlocki* in the brasiliensis subcomplex.

Experimental crosses between subcomplex brasiliensis members (as defined by Costa et al., 2013) showed hybridization potential and reproductive compatibility under laboratory conditions (Costa et al., 2003; Almeida et al., 2012; Correia et al., 2013).

Human modifications to ecological landscapes both may increase epidemiologic risks and facilitate endemic disease emergence and create new suitable environments for

integration and mating between species, potentially resulting in natural hybrids (e.g., *T. infestans* with *T. rubrovaria*) (Salvatella et al., 1990).

Considering that triatomine hybridization (a) allows formulation of hypotheses concerning origin and divergence of species, (b) may help understand the systematics of the group (Pérez et al., 2005), and (c) experimental hybridizations have enabled quantitative analyses of taxonomic relationships correlated with degrees of morphological similarities between species (Usinger, 1966), the present investigation was aimed to determine the reproductive compatibility between *T. sherlocki* and *T. lenti* and to analyze by cytogenetic and morphologic approaches the characteristics of the hybrids from crosses experimental of these species.

2. Methods

2.1 Insects

Specimens used in these crosses were obtained from colonies established for at least six generations with Triatominae from non-overlapping areas. *T. sherlocki* (Fig. 1A) samples used in this study were taken from a colony generated from 26 specimens collected in a wild and rock upland environment, near the village of Santo Inácio in the district of Gentio do Ouro, state of Bahia, Brazil, in 2003. *T. lenti* (Fig. 1B) samples are from a colony generated from 23 specimens adults and 5 nymphs collected in peridomestic environment in the rural village of Macaúbas/BA, state of Bahia, Brazil, in 2009. Both colonies are maintained at the Triatominae Insectarium of the São Paulo State University (UNESP, Araraquara/SP, Brazil).

2.2 Experimental crosses

T. sherlocki and *T. lenti* were crossed in both directions: *T. lenti* females × *T. sherlocki* males and *T. sherlocki* females × *T. lenti* males. The insects were sexed at the 5th instar nymphs and males and females were kept separately until they reach the adult stage, in order to get adults virgins (Martínez-Ibarra et al., 2011). For those crosses, three couples from each set were placed in plastic jars (5 cm diameter x 10 cm height) and maintained at room temperature.

The fertility rate and oviposition were calculated from each cross. The eggs were collected daily throughout the oviposition period of females. Viable F1 offspring were maintained to the adult stage, according to Belisário et al. (2007).

To determine whether the F1 offspring were fertile, crosses between F1 x F1, from the same couple, were performed to obtain F2 offspring. Likewise of experimental crosses of parental, the fertility rate and oviposition were calculated throughout the oviposition period of females, and to ascertain the fertility of the F2 offspring.

2.3 Morphologic and Morphometric Analysis

The phenotype of the offspring of each couple was described according to the major characteristics that species presented, as reduced hemelytra, rings of color orange redish in the femura and connexive (Papa et al., 2002).

Scanning Electron Microscopy (SEM) was used for the morphologic analysis of ten eggs and three median process of the pygophore of male genitalia for comparison among the parental and offspring. These images were examined by under a Topcon SM-300 microscope at the Instituto de Química (UNESP, Araraquara/SP, Brazil). The samples eggs and median process of the pygophore were washed, dehydrated in an alcohol series and oven-dried at 50°C, afterwards, sputtering metallization was performed for 2 minutes at 10 mA (Rosa et al., 2012).

Geometric morphometric techniques were applied to wings to evaluate whether the morphotype exhibited by offspring presented any differences in shape compared *T. lenti* and *T. sherlocki* (Almeida et al., 2012, Campos et al., 2011). Thirteen anatomical landmarks (Schachter-Brooide et al., 2004) were collected at intersections between venations and processed by the same researcher using modules available at the CLIC (Collection of Landmarks for Identification and Characterization, http://www.mpl.ird.fr/morphometrics/clic/index.html (Dujardin et al., 2010), and the COOWin software (Dujardin, 2004), as described by Dujardin (2008). We identified a total of 11 types I landmarks (venation intersections) and two type II landmarks (Bookstein, 1990).

Analyses were computed as nonuniform (partial warps) and uniform components, which describe regional and global deformations of the wing architecture (Bookstein, 1991). Prior to the generalized procrustes analysis, an isometric estimator of size variation (centroidsize) was calculated as the square root of the sum of the squared distances between the center of the configuration of landmarks and each individual landmark (Bookstein, 1991). A factorial map was built to illustrate the variation, which resulted from the first and second principal components of the analysis, representing 95% of the shape.

2.4 Cytogenetic approaches

Seminiferous tubules of ten F1 and F2 offspring adult males, from the crosses between *T. sherlocki* females × *T. lenti* males, after being shredded, crushed and fixed in liquid nitrogen on glass slide, using lacto-acetic orcein (De Vaio et al., 1985), C-banding (Sumner, 1972) and Feulgen reaction (Mello and Vidal, 1978). For the analysis of the affinity genomic hybrid methodology was used to Techio et al. (2005), with modifications, since besides diakinesis also analyze cells in meiosis I and meiosis II.

3. Results

3.1 Experimental crosses

The fertility rate of the eggs from couple involving *T. sherlocki* females × *T. lenti* males was 65%, while the reciprocal crosses revealed 7% of fertile eggs (Table 1). The mortality rate, before reaching adulthood, was 80% in the F1 offspring in both crosses. The fertility rate of the eggs from F1 offspring was greater in couple involving *T. sherlocki* females × *T. lenti* males than in couple involving *T. lenti* females × *T. sherlocki* males, 90% and 25%, respectively. In the crosses involving *T. lenti* females × *T. sherlocki* males, only one couple obtained viable eggs.

The fertility of the F2 offspring was only observed in couple involving *T. sherlocki* females × *T. lenti* males and the percentage of eggs viable was 2%, showing 100% of mortality rate before reaching adulthood.

3.2 Morphologic and Morphometric Analysis

Phenotypes of F1 offspring as size and color of connexive, presence or absence of rings color orange redish in the femura and size of hemelytra were identical in both directions of the couple (Fig. 2). The F1 offspring revealed morphological characteristics as color and size of the connexive and the presence of red rings on the legs similar to *T. sherlocki*, however, the size of the wings was intermediate (Fig. 2A, B). In the F2 offspring, these same characteristics showed patterns segregating in adults (Fig. 2C-G).

Under Scanning Electron Microscopy (1000X) the exochorion showed difference between *T. lenti* and *T. sherlocki* (Fig. 3A, C). In the F1 offspring, the exochorion showed similar characteristics that presented from the female of the original crossing (Fig. 3B, D).

The male genitalia, analyzed by median process of the pygophore revealed that the main difference between the two species is slender point in *T. sherlocki* and a wider base (Fig. 4A), whereas in *T. lenti* the rounded point and narrower base (Fig. 4B). Intermediate characteristics was observed in the F1 offspring that differentiated the parental species (Fig. 4C). In the F2 offspring, the median process of the pygophore was similar as *T. sherlokci* revealed a characteristics segregating (Fig. 4D). For this analysis were used hybrids from couple *T. sherlocki* female x *T. lenti* male.

For the wings geometric morphometrics the factorial map built with 40, 34, 43 and 15 specimens of *T. sherlocki*, *T. lenti*, F1 and F2, respectively, distinguished both species and hybrids in well-defined groups (Fig. 5). Considering the shape variation components, the contribution of the first principal (PC1) component accounted for 34% of the total variation, whereas the second principal component (PC2) accounted for 14%.

3.3 Cytogenetic approaches

We analyze the spermatogenesis of F1 (Fig. 6) through lacto-acetic orcein technique and F2 (Fig. 9) through Feulgen reaction, which show striking differences in their meiotic chromosome behavior. In the F1 offspring of both crosses, i.e., *T. sherlocki* females × *T. lenti* males and *T. lenti* females and *T. sherlocki* males, we observed the same cytogenetic characteristics (Figures 6-8).

During diffuse stage, a heteropycnotic corpuscle is observed formed by the association between both sex chromosomes and some autosomes (Fig. 6A, arrow). In diplotene, some of the ten bivalents showed chiasmata (Fig. 6B, asterisk). In metaphase I, the 10 autosomal bivalents and two sex chromosomes are clearly seen (Fig. 6C), while that the anaphase I are normal (Fig. 6D). The spermatids present a heteropycnotic filament on their periphery (Fig. 6E, F) and the spermatozoan it seen normal (Fig. 6G).

With C-banding, we observed that the hybrids showed the same diploid chromosome number and arrangement of constitutive heterochromatin than the parental species. During early meiotic prophase we observed a large chromocenter constituted by the association of both sex chromosomes plus two autosomal pairs (arrow), and multiple C-dots spread in the nucleus (Fig. 7A). In metaphase I, the ten autosomal bivalents showed C-blocks at one or both chromosomal ends (Fig. 7B).

By means of the Feulgen reaction analyzed 100 cells in diplotene (Fig. 8A) metaphase I (Fig. 8B) and metaphase II (Fig. 8C), in order to assess the compatibility between the

genomic parental species. It was observed that all cells showed 100% pairing. However, analysis of 50 F2 offspring cells diakinesis/metaphase I showed that in 90% of the cells showed some errors in pairing of autosomes, resulting in monovalent chromosomes (Fig. 9A-D). Not been possible view Metaphase II in F2 offspring. The spermatids present a heteropycnotic filament on their periphery (Fig. 10A-C, arrows).

4. Discussion

Morphological approaches has been an important tool in the characterization, identification and description of the species of the brasiliensis subcomplex (Costa et al, 2006, Costa and Felix, 2007) as well as the characterization of hybrid forms (Almeida et al., 2012, Costa et al., 2003, 2013). Based on morphological observations, Costa et al. (2009) propose that *T. b. macromelasoma* is a hybridization product between *T. b. brasiliensis* and *T. juazeirensis*.

Hybridization between closely related triatomine species is a well-known phenomenon detected in nature and in experimental laboratory cross-mating (Costa et al., 2003; Mas-Coma and Bargues, 2009; Perez et al., 2005; Usinger et al., 1966). The subspecies *T. b. brasiliensis* and *T. b. macromelasoma* and species *T. juazeirensis* presented genetic compatibility and generate fertile hybrids in the F1 and F2. However, the ability of hybridization may not be the only factor taken into account, as the species *T. melanica* presents genetic incompatibility and hybrid unviable with brasiliensis subcomplex members (Costa et al., 2003).

Experimental crosses were made between *T. sherlocki* and the species of *T. brasiliensis* complex to confirm *T. sherlocki* as a member of the *T. brasiliensis* complex (Correia et al., 2013). In this study all experimental combinations of *T. sherlocki* with members of the *T. brasiliensis* complex species produced viable eggs with variable percentages of survivor's index (52.3-73.5%), confirming the inclusion in brasiliensis subcomplex.

Crossing experiments already had been carried out to check reproductive compatibility in the *brasiliensis* subcomplex members (according to Schofield and Galvão, 2009) and *T. petrocchiae* and *T. lenti* failed to produce F1 offspring viable with *T. brasiliensis* (Espínola, 1971, Heitzmann-Fontenelle, 1984). Cerqueira (1982) in studies involving interspecific crosses between *T. lenti* and *T. sherlocki*, did not get viable hybrids which contradicts the results presented in this work.

T. sherlocki and *T. lenti* are closely related geographically and ecologically. According to ecological niche modeling, *T. sherlocki* showed distribution for the city of Ipupiara (Almeida et al., 2009), region known for the distribution of *T. lenti* (Sherlock and Serafim, 1967; 1972). Although the specimens used in the experiment were of different origin (peridomiciliary for *T. lenti* and wild for *T. sherlocki*), both species can be found in two types of ecotypes (Sherlock and Serafim, 1967; 1972; Almeida et al., 2009).

Since the ecological and geographic boundaries between *T. sherlocki* and *T. lenti* are not clear, the possible existence of natural hybrids would be expected. However, due to reproductive isolation pre-and post-zygotic described in this work, the formation of natural hybrids is unfeasible. According to Mas-Coma and Bargues (2009), *T. delpontei* x *T. infestans* and *T. platensis* x *T. infestans* hybrids have been detected in natural populations, it is still unclear if these represent accidental events or reflect the existence of large and stable hybrid populations.

Unlike the reproductive success of *T. sherlocki* females × *T. lenti* males, the reciprocal crosses did not show the same pattern. The crosses involving *T. lenti* females × *T. sherlocki* males showed high mortality and low fertility of eggs in the F1 and F2 offspring. This difference in reproductive success between pairs of couples was also reported by Sasabe et al. (2007) when analyzing the genetic basis of interspecific differences in genital morphology of closely related carabid beetles.

Almeida et al. (2012) showed that laboratory-bred hybrids of *T. sherloki* with *T. juazeirensis* possess intermediate morphological traits. Intermediate morphological traits were founded in the male genitalia, median process of the pygophore, in the F1 offspring in the crosses between *T. sherloki* and *T. lenti*. Furthermore, intermediate forms in nature have been observed between *T. b. brasiliensis*, *T. b. macromelasoma,* and *T. juazeirensis* in Pernambuco State (Costa et al., 2009).

Morphological approaches of the F1 offsprings, in both couples, as color and size of the connexive and the presence of red rings on the legs were similar to *T. sherlocki*. Phenotypes of F1 offspring of crosses between *M. phyllosomus* and *M. pallidipennis* showed that all F1 individuals were morphologically similar to *M. pallidipennis* (Martínez-Ibarra et al., 2011).

Hybrid fertility and fitness are key parameters determining the long-term outcome of the mixture of two species. The egg counts suggest that hybrid females are as fertile, at least as far as egg production, as parental populations, suggesting little or no prezygotic isolation

(Jiggins and Mallet, 2000, Barton and Cara, 2009). On the other hand, analysis of genotype frequencies of the different sexes and developmental stages strongly suggest the disappearance of hybrid females as they aged, possibly because of increased mortality. A lack of hybrid fitness may thus lead to a postzygotic barrier allowing for the maintenance of reproductive isolation of parental genotypes (Wiwegweaw et al., 2009). The presence of recombinants between parental genotypes suggests that gene flow does occur between sibling species but that selection processes act to maintain their distinctiveness.

The F1 offspring showed a normal meiotic behavior. Pérez et al. (2005) also observed that in hybrids of the species *T. infestans* and *T. platensis* meiotic division occurred normally. However, in experimental hybrids between *T. infestans* and *T. rubrovaria*, Pérez et al. (2005) assesses sterility of F1 offspring in pairing failures associated with the homeologus, which lead to the production of abnormal spermatids. By analyzing spermiogenesis was observed that the spermatids present the same characteristics described in *T. lenti*, by Alevi et al. (2013b), i.e., peripherals heteropycnotic filaments.

The parental species, *T. lenti* and *T. sherlocki*, show the same pattern of constitutive heterochromatin in the autosomes, as well as all other species that are part of brasiliensis subcomplex (Alevi et al., 2013a). Alevi et al. (2013c) by analyzing the pattern heterochromatic confirmed the relationship of *T. lenti* with the brasiliensis subcomplex. Experimental crosses are considered as a tool to evaluate the proximity found among species and, therefore, propose the group or not. However, we emphasize that to confirm the true position of *T. lenti* in the complex, many other approaches must be used, and since *T. melanica* presents genetic incompatibility with the triatomines of the complex and is still considered a member (Costa et al., 2003).

Pérez et al. (2005), when analyzed hybrid resulting from a cross between evolutionarily related species, proposed that differences in the pattern of constitutive heterochromatin between homologous chromosomes in parental species are not a barrier that influences synaptic recombination. So, as the parents do not show differences in heterochromatic pattern, F1 hybrids retained the same characteristics as *T. lenti* and *T. sherlocki*, out more, a large chromocenter made up of the association of both sex chromosomes plus two autosomal pairs, and multiple C-dots spread in the nucleus. The location of the autosomal C-blocks (at one or both chromosomal ends in all autosomal pairs), the diploid chromosome number consisting of 20 autosomes plus two sex chromosomes (XY

in males and XX in females) and the amount of autosomal C-heterochromatin (25-32%) (Panzera et al., 2010; Alevi et al., 2013a).

In the F1 offspring, as well as *T. lenti* (Alevi et al., 2013a) and *T. sherlocki* (Panzera et al., 2010) showed a diploid chromosome set of 2n = 20A + XY. Furthermore, we observed that homologous chromosomes showed 100% homeology. This result allows us to measure phylogenetic proximity between *T. lenti* and *T. sherlocki* since, according to Dewey (1982), the classical analysis of genomics, which involves evaluating the behavior of chromosomes in metaphase I in interspecific hybrids, allows establishing phylogenetic relationships in groups of different species, as well to be employed in defining taxonomic and evolutionary placements. Similar statements are made by Riley (1966) in holding that the two species have distinct genomes when their chromosomes are different in structure and gene content, so that no occurs pairing between one or more pairs of homeologus during meiosis of hybrids. This behavior leads to sterility, and consequently the genetic isolation between species.

Thus, if we analyzed only the F1 derived from crossing experiment, we propose an early homoploid speciation. Costa et al. (2009), suggest, through morphological analyses, that *T. b. macromelanosoma* is a species derived from crosses between the species *T. b. brasiliensis* and *T. juazeirensis*. However, when analyzing the F2, we found that possibly other evolutionary barriers to prevent hybridization.

Through the analysis in the F2 offspring were observed errors in chromosome pairing. These results possibly are related to one mechanism of post-zygotic isolation proposed by Dobzhansky (1970), out more, collapse of the hybrid. The collapse is a little known phenomenon that the F2 offspring or backcross has reduced viability or fertility, as observed in this work by the amount of F3 offspring. We believe that this phenomenon is due to a genic deregulation occurred in the chromosomes of F1. Probably, this imbalance is the result of crossing over that occurs in the F1 homeologus which results in lack of homology in euchromatic regions and prevents the normal pairing of chromosomes of some in F2.

Thus, in the F2 offspring, although some cells analyzed (10%) were normal (present 100% homeology between autosomes), most of the cells were abnormal, with an absence of homeology among autosomes, resulting in monovalent. This phenomenon results in the formation of inviable sperm. However, no significant changes in haploid cells during spermiogenesis, as observed by Schreiber et al. (1975).

Questions regarding the genetics and ecology of *T. sherlocki* and *T. lenti* are still in the initial studies, since the few published works on these two species. It would therefore be

reasonable to initiate quantitative trait locus (QTL) mapping, which provides fundamental information such as the number of loci involved in a certain trait (morphological or behavioral), locations on chromosomes, and magnitude of individual genetic effects. Thus, we believe that *T. lenti* is specie of *T. brasiliensis* complex, although new analysis to confirm it should be made to this matter. Furthermore, it can be seen that although *T. sherlocki* and *T. lenti* species are evolutionarily and citotaxonomically closed, there is a post-zygotic reproductive barrier that reduces the fertility in the F2 offspring, confirming the status specific of species analyzed.

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Table 1. Crosses involving *T. sherlocki* x *T. lenti* showing the fertile eggs and rate mortality.

	T. sherlocki females × *T. lenti* males		*T. lenti* females × *T. sherlocki* males	
	fertile eggs	mortality	fertile eggs	mortality
F1	65%	80%	7%	80%
F2	90%	90%	25%	40%
F3	2%*	-	-	-

*only three nymphs emerged

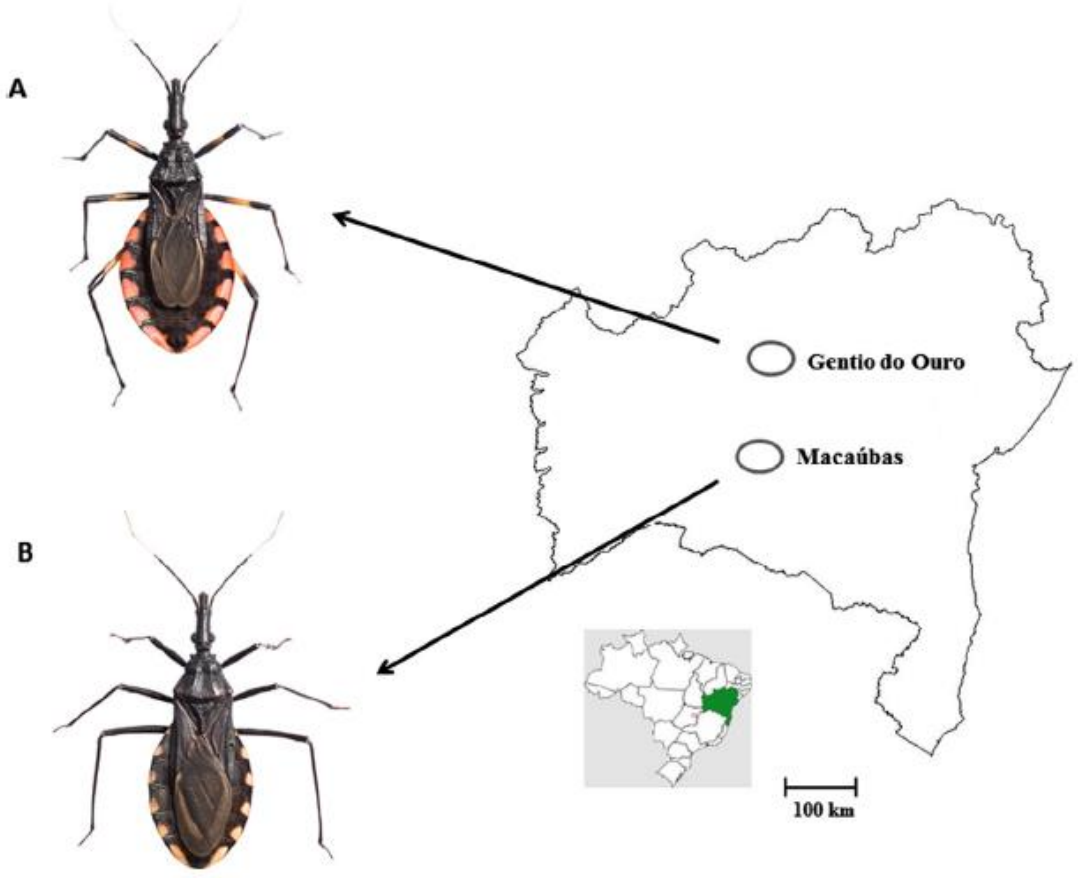

Figure 1. Distribution of *T. sherlocki* and *T. lenti* in the Bahia state, Brazil. *T. sherlocki* (A) is distributed in the region of Gentio do Ouro and *T. lenti* (B), in the region of Macaúbas.

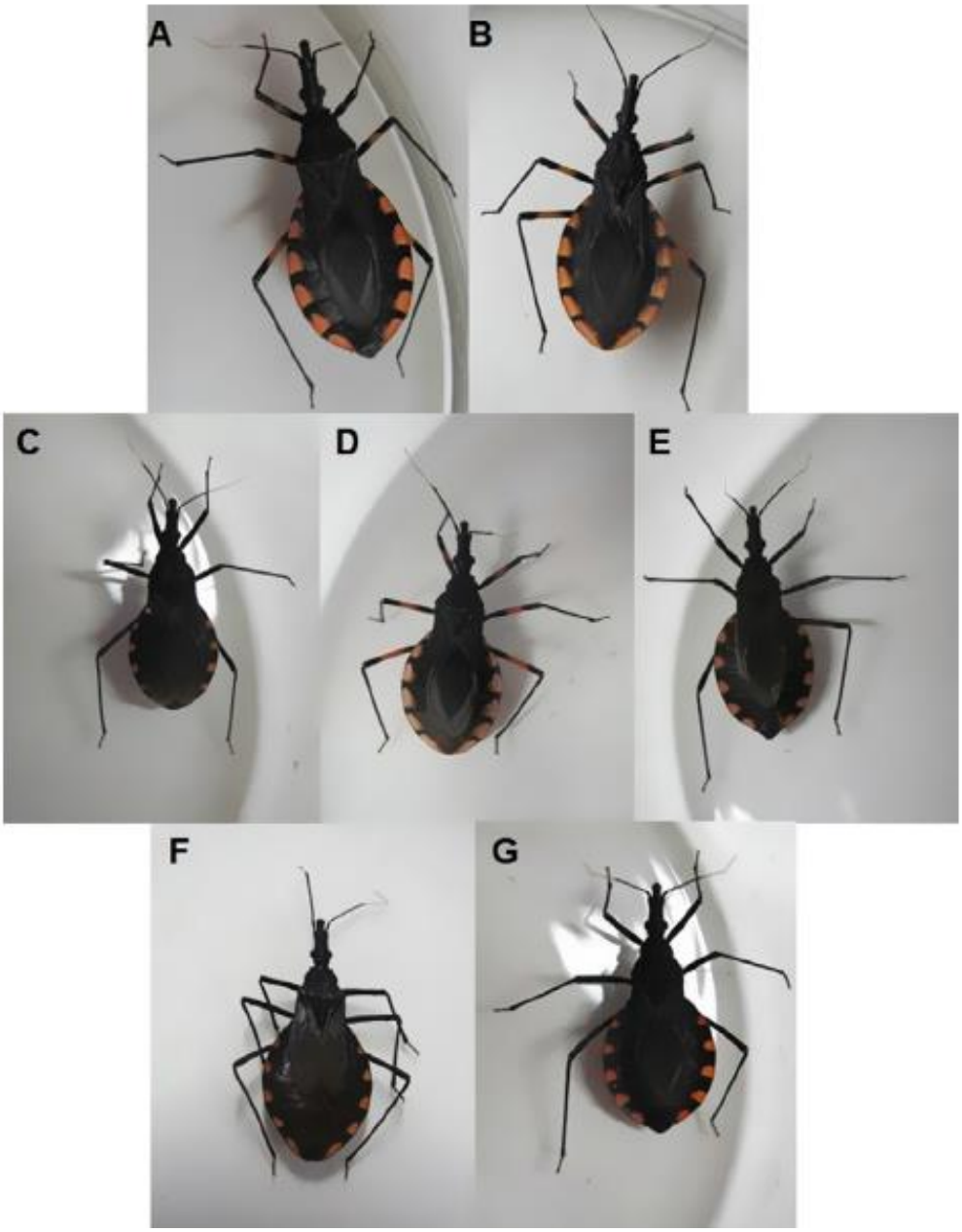

Figure 2. Phenotypes of F1 and F2 offspring from crosses involving *T. sherlocki* females × *T. lenti* males. A-B: female and male F1 offspring, respectively. C-G: F2 offspring adults C: similar to *T. lenti*; D: connexivum and rings of color orange redish in the femura similar to *T. sherlocki* and size of hemelytra similar to *T. lenti*; E-F: connexivum and absence rings of color orange redish in the femura similar to *T. lenti* and reduced hemelytra similar to *T. sherlocki*; G: connexivum and absence rings of color orange redish in the femura similar to *T. lenti* and color of connexivum and reduced hemelytra similar to *T. sherlocki*.

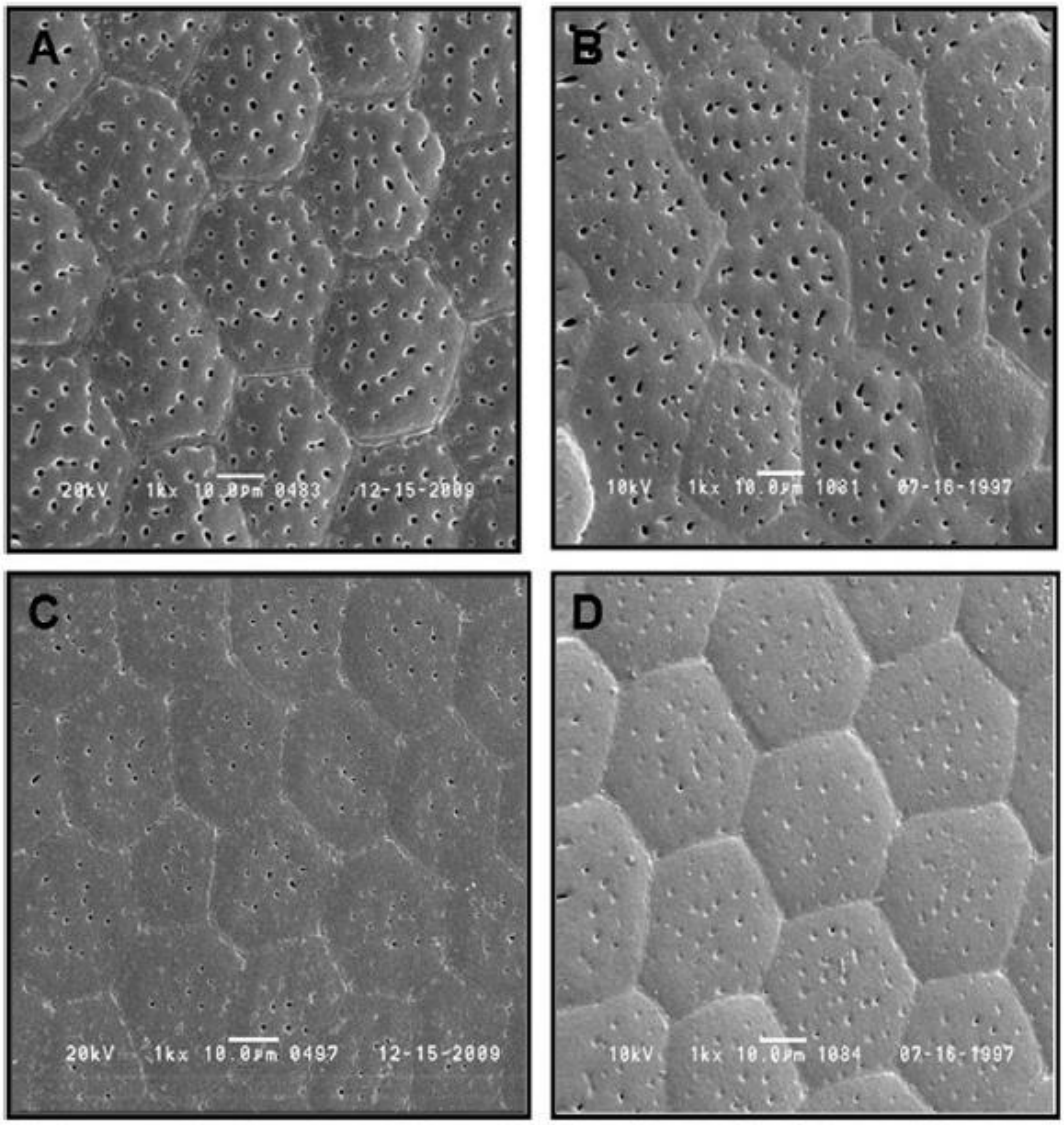

Figure 3. Egg exochorion detail by SEM (1000X). A: *Triatoma lenti*; B: F1 offspring from couple *T. lenti* females × *T. sherlocki* males; C: *Triatoma sherlocki*; D: F1 offspring from couple *T. sherlocki* females × *T. lenti* males.

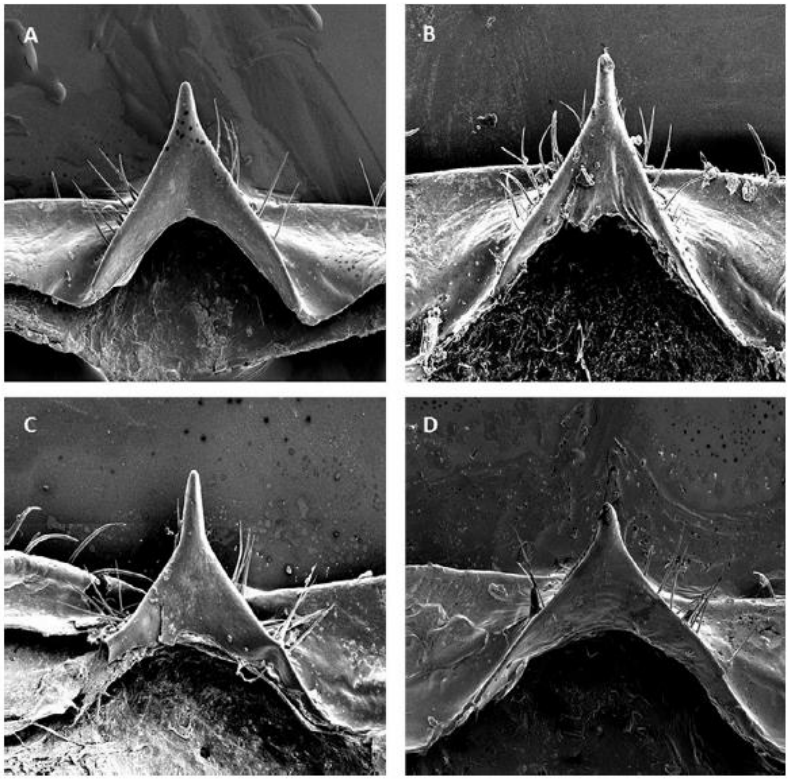

Figure 4. Median process of the pygophore by SEM (100X). A: *T. sherlocki*; B: *T. lenti*; C: F1 offspring; D: F2 offspring. (F1 and F2 offspring from couple *T. sherlocki* females × *T. lenti* males).

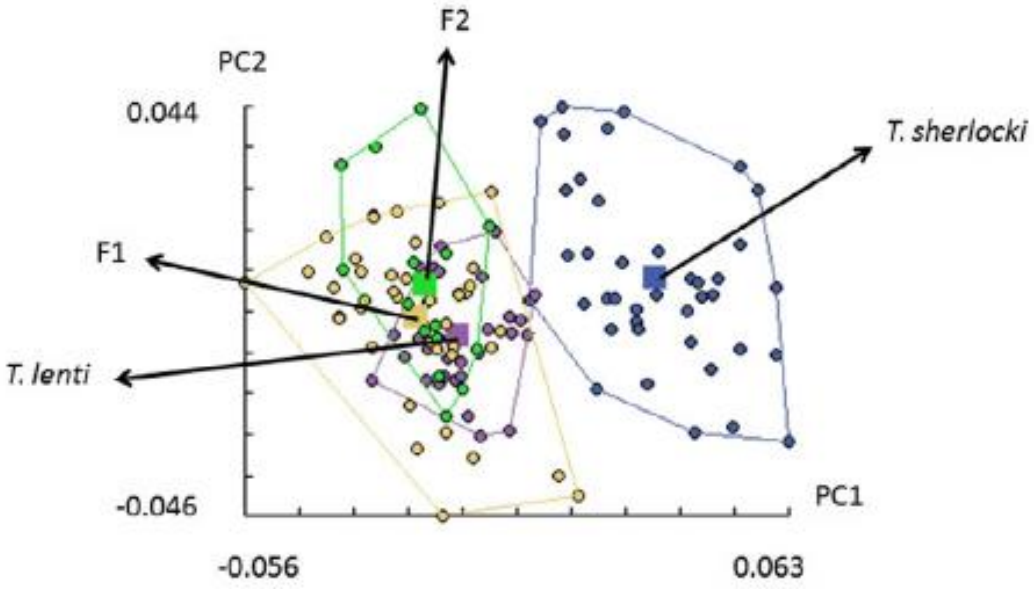

Figure 5. Factorial map of the wing shape. The factorial map of the wing shape for the species *T. sherlocki*, *T. lenti* and the F1 offspring and F2 offspring.

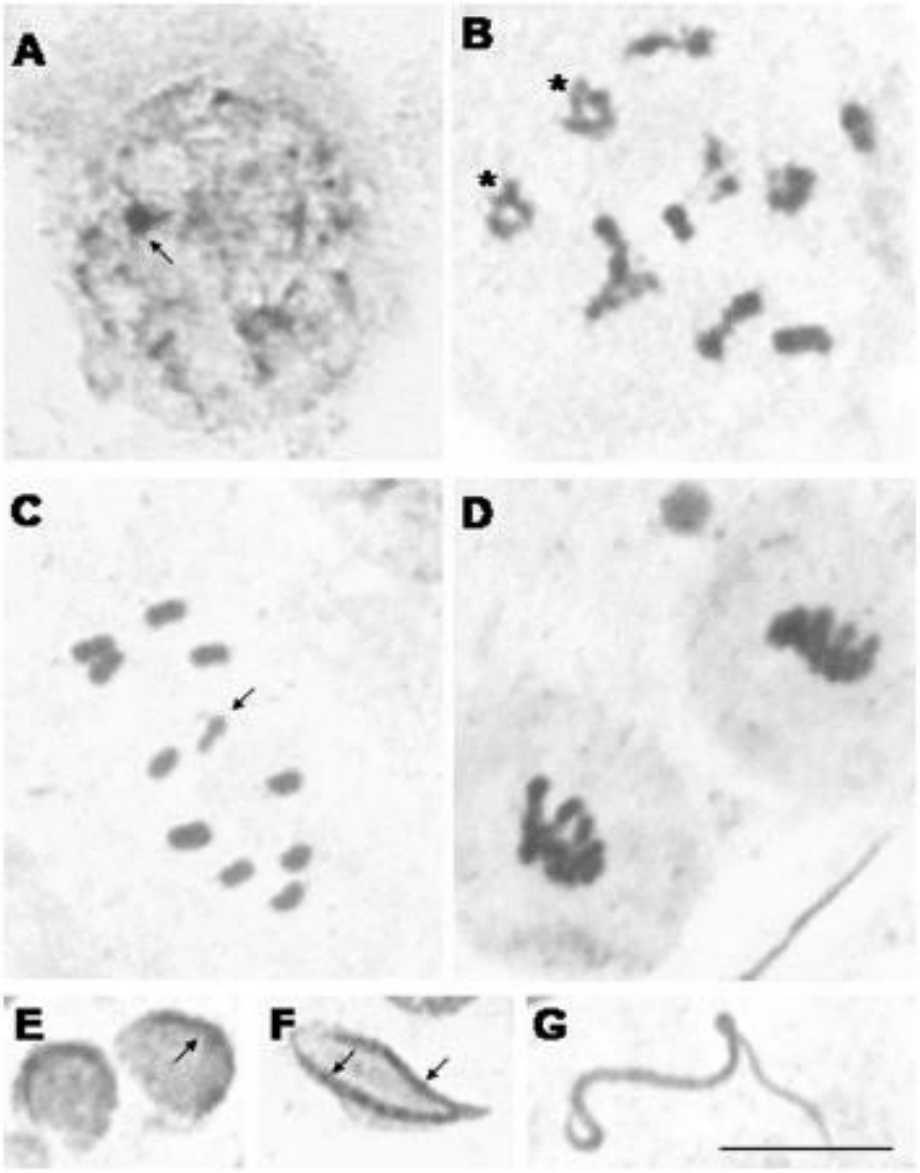

Figure 6. Spermatogenesis of F1 hybrids stained with lacto-acetic orcein. A-B: Prophase I. Note corpuscle heteropyknotic in the initial diffuse (A, arrow) and in diplotene was possible to visualize chiasmus (B, asterisk). C: Metaphase I. Note that it is possible to observe ten pairs of autosomes and the sex chromosomes, being the Y greater (arrow). D: Anaphase. E-F: Spermiogenesis. Note the peripheral heteropycnotic in spermatids (arrows). G: Spermatozoid. Bar: 10 um.

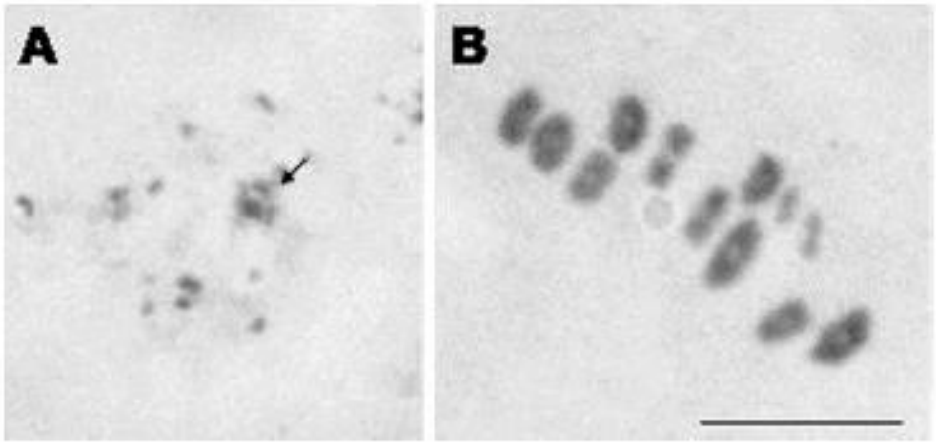

Figure 7. Constitutive heterochromatin pattern in F1 hybrids stained with C-banding. A-B: Constitutive heterochromatin pattern in hybrids. Note the chromosome configuration during early meiotic prophase (A), out more, a large chromocenter made up of the association of both sex chromosomes plus two autosomal pairs (arrow), and multiple C-dots spread in the nucleus. In metaphase I (B), note that diploid chromosome number consisting of 20 autosomes plus two sex chromosomes (XY in males and XX in females) and the amount of autosomal C-heterochromatin (25 - 32%). Bar: 10 um.

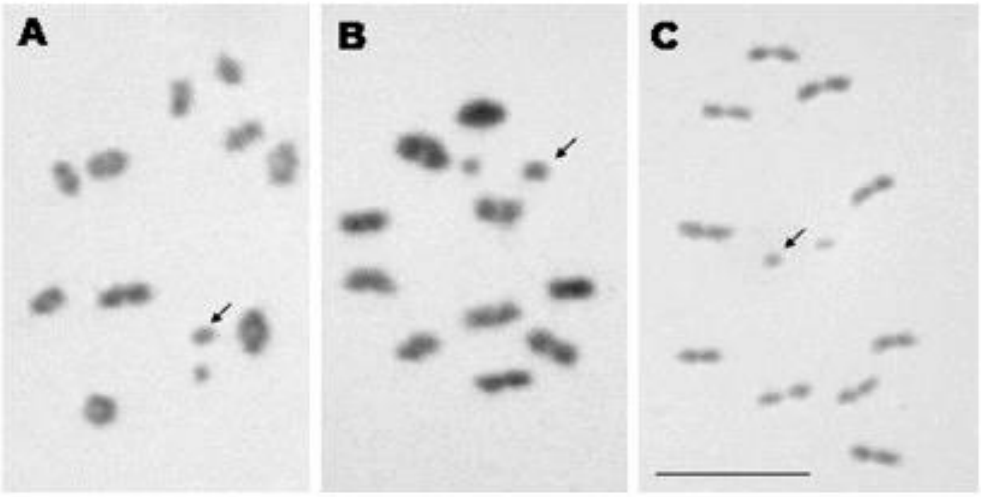

Figure 8. Cells meiotic of F1 hybrids stained with Feulgen reaction. A: Diplotene. B: Metaphase I. C: Metaphase II. Note the sex chromosome Y (arrow). Bar: 10 um.

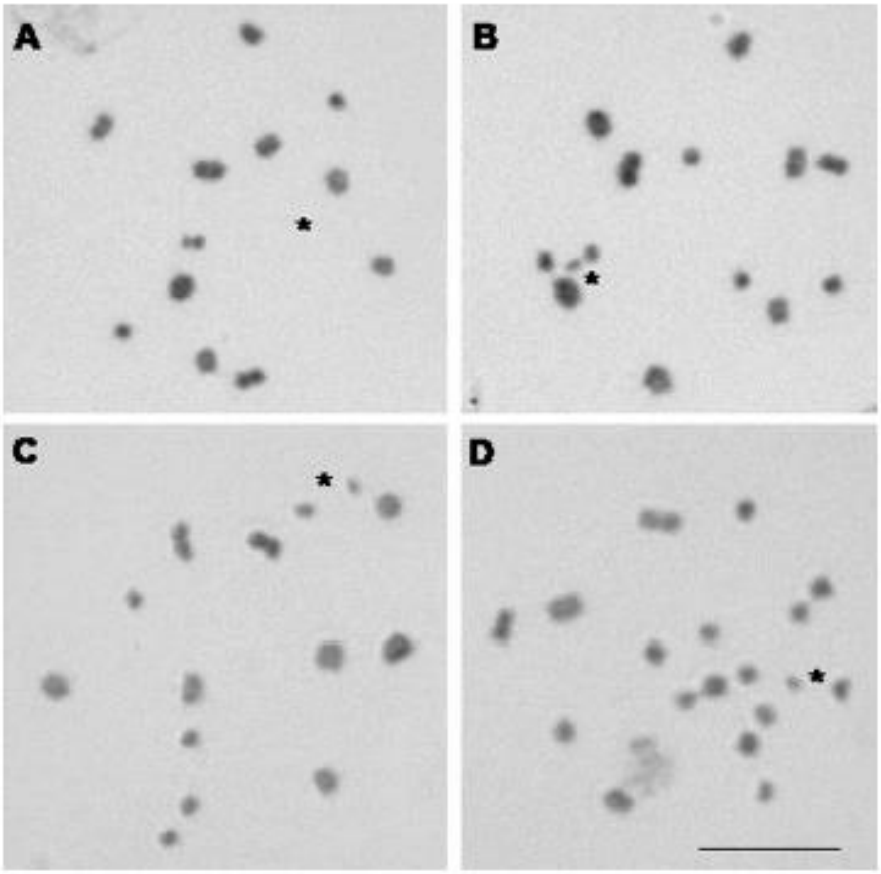

Figure 9. Diakinesis the F2 generation with Feulgen reaction. A-D: Diakinesis. Note autosomes monovalent by not the pairing among homeologous and sex chromosomes (asterisk). Bar: 10 um.

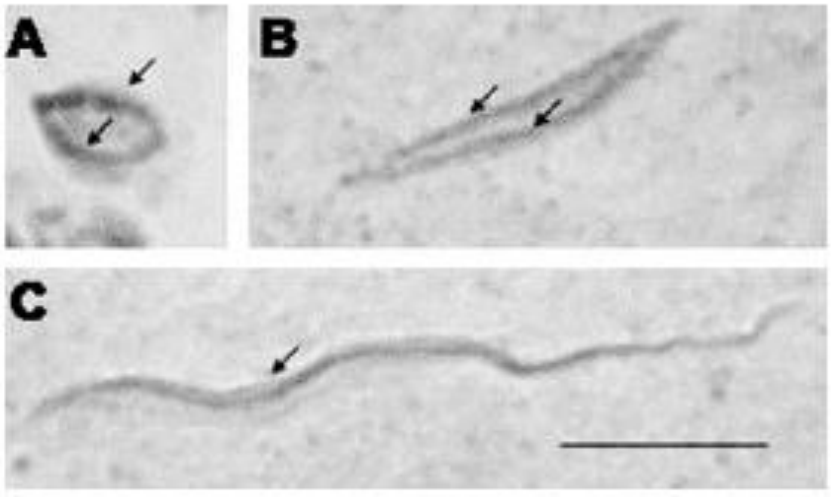

Figure 10. Spermiogenesis of F2 generation. A-C: Spermiogenesis. Note the peripheral heteropycnotic in spermatids (arrows). Bar: 10 um.

4.3 Capítulo 3 (Artigo científico publicado na revista *Zootaxa* FI 0,97)

ALEVI, K. C. C.; ROSA, J. A.; AZEREDO-OLIVEIRA, M. T. V. Cytotaxonomy of the Brasiliensis subcomplex and the *Triatoma brasiliensis* complex (Hemiptera: Reduviidae: Triatominae). **Zootaxa**, v. 3838, p. 583-589, 2014.

Cytotaxonomy of the Brasiliensis subcomplex and the *Triatoma brasiliensis* complex (Hemiptera, Triatominae)

Abstract

We analyzed the classical cytotaxonomy of the Brasiliensis subcomplex (*Triatoma brasiliensis* Neiva, *T. juazeirensis* Costa & Felix, *T. melanica* Costa, Argolo & Felix, *T. melanocephala* Neiva & Pinto, *T. petrocchiae* Pinto & Barreto, *T. lenti* Sherlock & Serafim, *T. sherlocki* Papa, Jurberg, Carcavallo, Cerqueira & Barata, *T. tibiamaculata* Pinto and *T. vitticeps* Stal) and the *T. brasiliensis* complex (*T. b. brasiliensis*, *T. b. macromelasoma* Neiva & Lent, *T. juazeirensis*, *T. melanica* and *T. sherlocki*). The five members of the *T. brasiliensis* complex share the same cytogenetic characteristics. Merely *T. sherlocki* show differences in spermatids, which confirms the status of more differentiated member of the complex. *T. lenti* also presented the same cytogenetic characteristics described for the species of the *T. brasiliensis* complex, which supports possible grouping of the species as sixth member of the complex, although further analysis as molecular and experimental crosses are needed to corroborate this hypothesis. *T. petrocchiae*, *T. vitticeps*, *T. tibiamaculata* and *T. melanocephala* presented one or more characteristics that allow questioning grouping in the proposed Brasiliensis subcomplex. Thus, we suggested that Brasiliensis subcomplex and *T. brasiliensis* complex should be constituted by the same triatomines (*T. b. brasiliensis*, *T. b. macromelasoma*, *T. juazeirensis*, *T. melanica* and *T. sherlocki*). However, we draw attention to *T. lenti* and suggest that although new analyzes should be performed, possibly this species is the sixth member of the *T. brasiliensis* complex.

Introduction

The Triatominae subfamily is composed of 148 species distributed in 18 genera and six tribes (Abad-Franch *et al.* 2013; Alevi *et al.* 2013a; Jurberg *et al.* 2013; Poinar *et al.* 2013). These insects present five nymphal stages (N1, N2, N3, N4, N5) and one adult. After

hatching, the triatomine are hematophagous strict and, once infected with the protozoan *Trypanosoma cruzi* Chagas (Kinetoplastida: Trypanosomatidae), can transmit the Chagas disease. Transmission occurs by the habit of defecating during the repast (Noireau *et al.* 2009).

The triatomines were grouped in complexes and subcomplexes specific by Schofield & Galvão (2009). The authors grouped in Infestans complex and the Brasiliensis subcomplex the species: *Triatoma brasiliensis* Neiva, *T. juazeirensis* Costa & Felix, *T. melanica* Costa, Argolo & Felix, *T. melanocephala* Neiva & Pinto, *T. petrochiae* Pinto & Barreto, *T. lenti* Sherlock & Serafim, *T. sherlocki* Papa, Jurberg, Carcavallo, Cerqueira & Barata, *T. tibiamaculata* Pinto and *T. vitticeps* Stal. However, to collate the species in the subcomplex, the authors used parameters as morphological characters and geographical disposition. This subcomplex species is endemic to Northeast Brazil and is of great epidemiological importance (Costa 2000; Almeida *et al.* 2009).

At first, it was believed that the species of the Brasiliensis subcomplex were only populations of *T. brasiliensis* with chromatic polymorphism (Lent & Wygodzinsky, 1979). However, Costa and collaborators, by means of different aspects, redescribed *T. melanica* (Costa et al. 2006) and *T. b. macromelasoma* Neiva & Lent (Costa *et al.* 2013), and described *T. juazeirensis* (Costa & Felix 2007). Furthermore, based on characteristics monophyletic, as egg morphology (Costa *et al.* 1997a), morphometry of the testis (Freitas *et al.* 2008), hybrid cross (Costa *et al.* 2003), isoenzymes (Costa *et al.* 1997b), molecular data (Monteiro *et al.* 2004), morphological data (Costa *et al.* 1997a), biological data, and ecological data (Costa *et al.* 1998), proposed that species should be grouped in *T. brasiliensis* complex, composed of two subspecies (*T. b. brasiliensis* and *T. b. macromelasoma*) and two species (*T. juazeirensis* and *T. melanica*). Mendonça *et al.* (2009) proposed the inclusion of *T. sherlocki* to this complex. This inclusion was recently confirmed through the use of both cytogenetic analysis (Alevi *et al.* 2013b) and cross-mating experiments (Correia *et al.* 2013).

Thus, we analyzed cytogenetically the species of the Brasiliensis subcomplex and the *T. brasiliensis* complex and grouped all the cytogenetic information described in the literature, with the objective of contributing to cytotaxonomy and evolution of this important group of vector species.

Material and methods

Seminiferous tubules of five adult males of *T. brasiliensis, T. b. macromelasoma, T. juazeirensis, T melanocephala, T. petrochiae, T. lenti, T. sherlocki, T. tibiamaculata* e *T. vitticeps* from the Triatominae Insectarium within the Department of Biological Sciences, in the College of Pharmaceutical Sciences, at Sao Paulo State University's Araraquara Campus, Brazil (FCFAR/UNESP) were first shredded, smashed, and set on a slide in liquid nitrogen. They were then stained using the cytogenetic technique of lacto-acetic orcein (De Vaio *et al.* 1985, with modifications according to Alevi *et al.* 2012a).

Results

The spermatogenesis of all of triatomines that make up the Brasiliensis subcomplex was analyzed. However, we present the cytogenetic description of the only species that did not have the heteropyknotic pattern described in the literature (Fig. 1). The subspecies *T. b. macromelasoma* (Fig. 1A) and the species *T. juazeirensis* (Fig. 1B) showed a large chromocenter (arrow) and blocks heteropycnotic dispersed in the nucleus in prophase. The species *T. petrocchiae* showed only a large chromocenter in the nucleus in prophase composed of the two sex chromosomes (Fig. 1C, arrow).

We also analyzed the heteropyknotic pattern present in spermatids of the species *T. brasiliensis, T. juazeirensis* e *T. petrocchiae* and of the subspecies *T. b. macromelasoma* and observed that these vectors have the same cytogenetic features: two heteropycnotic filaments in haploid cells (Fig. 2). Furthermore, grouped in two tables all data about the classical cytogenetic techniques described in the literature of the species that make up the Brasiliensis subcomplex and the *T. brasiliensis* complex (Table 1 and Table 2).

Discussion

The cytotaxonomy of the *T. brasiliensis* complex started when Panzera *et al.* (2000) analyzed cytogenetically different populations of *T. brasiliensis*. In this work, the authors proposed that the four populations (*T. b. brasiliensis, T. b. macromelasoma, T. juazeirensis* and *T. melanica*) presented chromosome homogeneity. Furthermore, the authors observed that *T. petrocchiae* also presents the same cytogenetic characteristics of populations of *T. brasiliensis*.

Panzera *et al.* (1998) described the chromosomal characteristics of *T. tibiamaculata* and observed that this was the first exclusive species of South America with system of sex determination X_1X_2Y. Panzera *et al.* (1998) and Severi-Aguiar *et al.* (2006) described the

cytogenetic characteristics of *T. vitticeps* and observed that this species also shows fragmentation of the sex chromosome X ($X_1X_2X_3Y$). More recently, Alevi *et al.* (2012a) resumed cytotaxonomic studies in Brasiliensis subcomplex and described the karyotype of the species *T. melanocephala*. In this work, the authors also observed fragmentation of the sex chromosome X ($X_1X_2X_3Y$) and, based on previous information, proposed the exclusion this species, as well as *T. vitticeps* and *T. tibiamaculata* of the Brasiliensis subcomplex. These results were recently confirmed by molecular data (cytochrome b, cytochrome oxidase I, and 16S rDNA) (Gardim *et al.* 2014).

The grouping of species in the Brasiliensis subcomplex, although initially occurred by morphological data and the geographic distribution should be reviewed, because the main function of grouping a set of species in a complex/subcomplex should be understand the evolution of this group. From this perspective, it is expected that the complex/subcomplex present monophyletic characteristics as well as the *T. brasiliensis* complex proposed by Costa and collaborators.

The five members of the *T. brasiliensis* complex share the same cytogenetic characteristics (Table 1), optimizing the grouping of these organisms in the complex. *T. lenti* also presents the same cytogenetic characteristics of the complex, which possible supports grouping of the species as sixth member of the complex, although molecular analyzes and experimental crosses are needed to corroborate this hypothesis. Have *T. petrocchiae, T. vitticeps, T. tibiamaculata* and *T. melanocephala* had one or more characteristics that allow for questioning the grouping proposed form Brasiliensis subcomplex (Table 1).

Although Panzera *et al.* (2000) reported that *T. petrocchiae* presents the same cytogenetic characteristics that *T. brasiliensis* species complex, we observed that the species has not heteropycnotic blocks dispersed in the nucleus and mainly the chromocenter is formed only by the sex chromosomes and not by association sex chromosomes with autosomes, as in other species of the complex (Panzera *et al.* 2000; Panzera *et al.* 2010; Alevi *et al.* 2013b).

Alevi and collaborators proposed the study of spermiogenesis as a tool to study the species Brasiliensis subcomplex (Alevi *et al.* 2013c) and to differentiate species morphologically related (Alevi *et al.* 2013d). With the exception of *T. sherlocki*, all the species of the *T. brasiliensis* complex presented the same cytogenetic characteristics during spermiogenesis, out more, the presence of two heteropycnotic filaments in spermatids (Table 2). However, we emphasize that *T. lenti* and *T. petrocchiae* also had the same disposition of filaments heteropycnotic described for members, demonstrating that more studies are needed

so that the actual position against the *T. brasiliensis* complex can to be clarified. *T. vitticeps*, *T. tibiamaculata* and *T. melanocephala* showed differences in the heteropyknotic pattern of spermatids, which helps to exclude these insects of subcomplex.

The peculiarity observed in spermatids *T. sherlocki* in relation to other members of *T. brasiliensis* complex (Table 2) only confirms the hypothesis that this species is the most differentiated of the complex (Almeida *et al.* 2009; Mendonça *et al.* 2009; Almeida *et al.* 2012). However, we draw attention to the fact that the differential characteristics observed in male gametes of this species is not a pre-zygotic barrier, as recently Correira *et al.* (2013) observed hybrid viability by experimental crossing between *T. sherlocki* with all species of the complex.

Thus, we suggest that Brasiliensis subcomplex and *T. brasiliensis* complex should be constituted by the same triatomines, out more, the subspecies *T. b. brasiliensis* and *T. b. macromelasoma*, and the species *T. juazeirensis*, *T. melanica* and *T. sherlocki*, as proposed by the studies of Costa and collaborators. However, we draw attention to *T. lenti* and suggest that although new analyzes should be performed, possibly this species is the sixth member of the *T. brasiliensis* complex.

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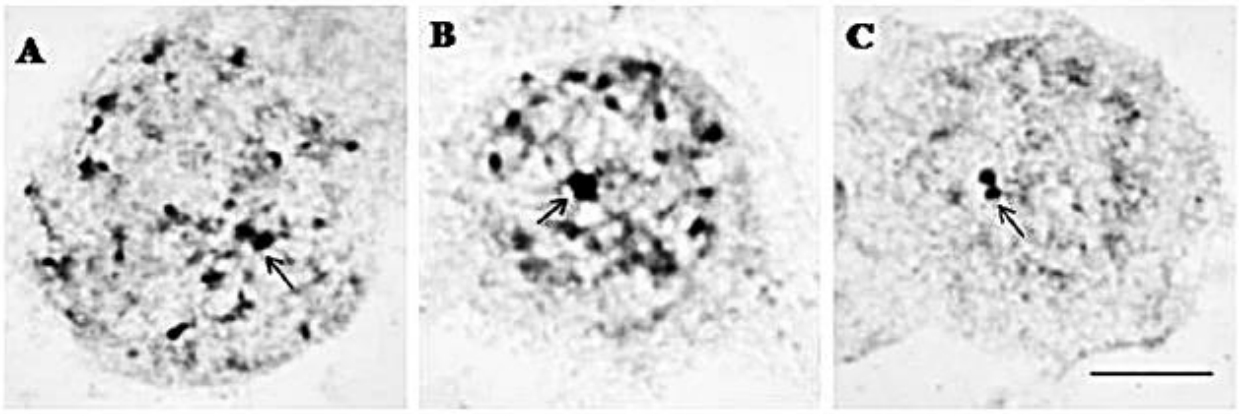

Figure 1. Prophase (diffuse stage) of *T. b. macromelasoma* (A), *T. juazeirensis* (B) and *T. petrocchiae* (C). Note one chromocenter (arrow) and heteropycnotic blocks dispersed in the nucleus in *T. b. macromelasoma* (A), *T. jauzeirensis* (B). Note one chromocenter formed by the sex chromosomes in *T. petrocchiae* (C, arrow). Bar: 10 µm.

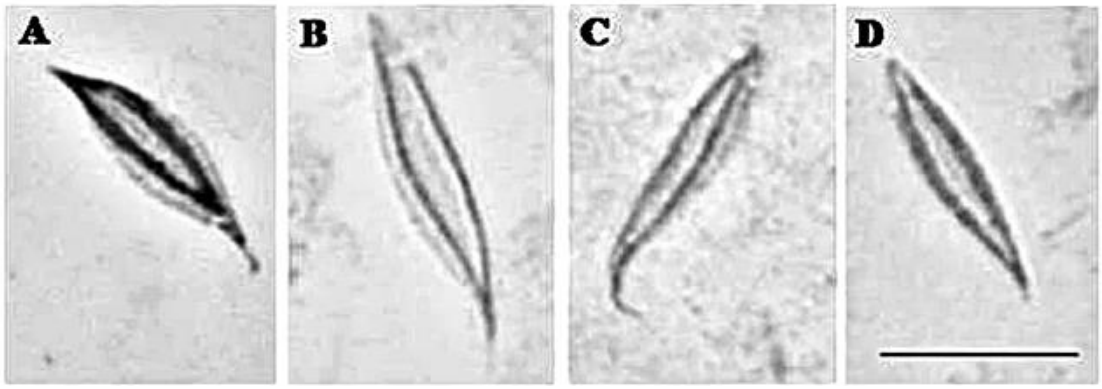

Figure 2. Early spermatids of *T. brasiliensis* (A), *T. petrocchiae* (B) *T. juazeirensis* (C) e *T. b. macromelasoma* (D) All species presented two heteropycnotic filaments in haploid cells analyzed. Bar: 10 µm.

Table 1. Cytogenetic characteristics of species of the Brasiliensis subcomplex and *T. brasiliensis* complex.

Species	Chromosome number	Sex mechanism	Relative autosomal size	Relative size of SC	HP in prophase	C-Banding		
						A	SC	
							X	Y
T. brasiliensis	2n = 22*[1]	XY	Small variation	Y>X	Large chromocenter and heteropycnotic blocks*[1]	Yes*[1]	No*[1]	Yes*[1]
T. b. macromelasoma	2n = 22*[1]	XY	Small variation	Y>X	Large chromocenter and heteropycnotic blocks	Yes*[1]	No*[1]	Yes*[1]
T. melanica	2n = 22*[1]	XY	Small variation	Y>X	Large chromocenter and heteropycnotic blocks*[9]	Yes*[1]	No*[1]	Yes*[1]
T. juazeirensis	2n = 22*[1]	XY	Small variation	Y>X	Large chromocenter and heteropycnotic blocks	Yes*[1]	No*[1]	Yes*[1]
T. sherlocki	2n = 22*[2]	XY	Small variation	Y>X	Large chromocenter and heteropycnotic blocks*[2]	Yes*[11]	No*[11]	Yes*[11]
T. lenti	2n = 22*[3]	XY	Small variation	Y>X	Large chromocenter and heteropycnotic blocks*[3]	Yes*[11]	No*[11]	Yes*[11]
T. petrocchiae	2n = 22*[1]	XY	Small variation	Y>X	Large chromocenter	Yes*[1]	No*[1]	Yes*[1]
T. tibiamaculata	2n = 23*[4]	X_1X_2Y	Small variation	Y> X_1, X_2	Large chromocenter and heteropycnotic blocks*[10]	Yes*[10]	Yes*[10]	Yes*[10]
T. vitticeps	2n = 24*[5]	$X_1X_2X_3Y$	Two larger autosomal pair*[7]	Y>X_1, X_2, X_3	Large chromocenter*[10]	No*[10]	Yes*[10]	Yes*[10]
T. melanocephala	2n = 24*[6]	$X_1X_2X_3Y$	Two larger autosomal pair*[8]	Y>X_1, X_2, X_3	Large chromocenter*[8]	No*[12]	No*[12]	Yes*[12]

*[1] Panzera et al. (2000); *[2] Panzera et al. (2010); *[3] Alevi et al. (2012b); *[4] Panzera et al. (1996); *[5] Schreiber and Pellegrino (1950); *[6] Alevi et al. (2012a); *[7] Severi-Aguiar et al. (2006); *[8] Alevi et al. (2013e); *[9] Alevi et al. (2014a); *[10] Panzera et al. (1998); *[11] Alevi et al. (2013b); *[12] Alevi et al. (2014b). SC: sex chromosomes; HP: heteropyknotic pattern; A: autosomes;

Table 2. Heteropyknotic pattern in spermatids of species of the Brasiliensis subcomplex and *T. brasiliensis* complex.

Species	Heteropyknotic pattern in spermatids	References
T. brasiliensis	Two heteropyknotic filament	Present paper
T. b. macromelasoma	Two heteropyknotic filament	Present paper
T. melanica	Two heteropyknotic filament	Present paper
T. juazeirensis	Two heteropyknotic filament	Present paper
T. sherlocki	Heteropyknotic corpuscle (early spermatids); Peripheral heteropyknotic filament during cell elongation	Alevi et al. (2013c)
T. lenti	Two heteropyknotic filament	Alevi et al. (2013c)
T. petrocchiae	Two heteropyknotic filament	Present paper
T. tibiamaculata	Peripheral heteropyknotic filament	Severi-Aguiar and Azeredo-Oliveira (2004)
T. vitticeps	Filament extensive peripheral heteropyknotic and middle of the cell	Alevi et al. (2013d)
T. melanocephala	Slightly prominent peripheral heteropyknotic filament	Alevi et al. (2013d)

4.4 Capítulo 4 (Artigo científico publicado na revista *Zootaxa* FI 0,97)

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Revalidation of *Triatoma bahiensis* Sherlock & Serafim, 1967 (Hemiptera: Reduviidae) and phylogeny of the *T. brasiliensis* species complex

ABSTRACT

Triatoma bahiensis Sherlock & Serafim, 1967, *T. lenti* Sherlock & Serafim, 1967, and *T. pessoai* Sherlock & Serafim, 1967 were described based on material collected in the Brazilian state of Bahia. These species were later included in the *T. brasiliensis* complex based on their geographic distribution. *Triatoma bahiensis* and *T. pessoai* were subsequently synonymized with *T. lenti*. However, the phylogenetic position of *T. lenti* within the *T. brasiliensis* complex has remained doubtful. This study aims to assess the taxonomic status of *T. bahiensis* and to infer the phylogenetic relationships between *T. lenti*, *T. bahiensis* and the other members of the *T. brasiliensis* species complex. The identities of the species in concern were confirmed by comparisons with high resolution photos of the respective type materials; lectotypes are designated for *T. pessoai* and *T. bahiensis*. Morphological, morphometric, molecular, and cytogenetic approaches as well as experimental crosses were used. The low viability of experimental crosses combined with morphological and morphometric data allow the differentiation of *T. bahiensis* and *T. lenti*. Pairwise cyt b sequence divergence between *T. lenti* and *T. bahiensis* was 2.5%. Cytogenetic and molecular analyses grouped *T. lenti* and *T. bahiensis* as members of the *T. brasiliensis* complex. These results revalidate the specific status of *T. bahiensis*.

Introduction

Trypanosoma cruzi (Chagas, 1909), the etiological agent of Chagas disease, is a parasite mainly transmitted by blood-sucking bugs of the subfamily Triatominae (Hemiptera: Reduviidae) (Chagas 1909, WHO 2013). To date, 150 valid species have been described as

part of this subfamily, including two fossils (Poinar Jr. 2005, 2013, Justi *et al*. 2014). Accurate vector identification is crucial for Chagas disease control and surveillance programs (Monteiro *et al*. 2001, Vinhaes *et al*. 2014).

In a triatomine survey carried out in the Brazilian state of Bahia between 1957 and 1971, Sherlock and Serafim (1967) described *Triatoma bahiensis* Sherlock & Serafim, 1967, *T. lenti* Sherlock & Serafim, 1967, and *T. pessoai* Sherlock & Serafim, 1967. The differences among them were based on the size and color of the connexivum: *T. pessoai* possesses large connexivum with red spots, *T. lenti* a small connexivum with yellow spots, and *T. bahiensis* a medium-sized connexivum with orange-red spots, a color in between that of the other two species. Furthermore, *T. bahiensis* presents two spots on each side of the corium, a pattern which is not present in *T. pessoai* or *T. lenti*.

The description of *T. bahiensis* was based in an uncertain number of specimens captured in the city of Seabra, while *T. lenti* and *T. pessoai* were found in intradomiciliary environments in the cities of Brotas de Macaúbas, Ipupiara, and Macaúbas. Based on their geographic distribution in northeastern Brazil, *T. lenti, T. pessoai* and *T. bahiensis* were included into the *T. brasiliensis* complex by Lucena (1970). A few years later, *T. bahiensis* was determined to be a variety of *T. pessoai* (Sherlock & Serafim 1972) and subsequently, morphological and morphometric analyses concluded that *T. pessoai* (= *T. pessoai* var. *bahiensis*) was a synonym of *T. lenti* (Lent & Wygodzinsky 1979). After the synonymy between them, Schofield and Galvão (2009) were the first to cite *T. lenti* as a member of the *T. brasiliensis* subcomplex. However, no study has been performed to confirm this phylogenetic position.

Based on morphological (Costa *et al*. 1997a, 2013) and biological data (Costa & Marchon-Silva 1998), experimental crosses (Costa *et al.* 2003b), ecological (Costa *et al.* 1998, 2002), genetic (Panzera *et al.* 2000), biogeographic (Costa *et al.* 2014), and molecular analyses (Costa *et al.* 1997b, Monteiro *et al.* 2004, Mendonça *et al.* 2009), the *T. brasiliensis* complex is considered to be a monophyletic group composed by *T. b. brasiliensis* Neiva, 1911, *T. b. macromelasoma* Galvão, 1956, *T. juazeirensis* Costa & Félix, 2007, *T. melanica* Costa *et al*. 2006, and *T. sherlocki* Papa *et al*. 2002. However, recent cytogenetic analyses (Alevi *et al.* 2012, 2013, 2014) and experimental crosses (Mendonça *et al.* 2014) suggest that *T. lenti* could also be a member of the *T. brasiliensis* complex.

In addition, Sherlock and Serafim (1967) did not designate holotypes for *T. lenti*, *T. pessoai* and *T. bahiensis*. They determined 20 “cotypes” for each of the first two species,

which should all be considered syntypes. As for *T. bahiensis*, the same authors did not mention any kind of "types". They described one male specimen, but presented drawings of the female genitalia, therefore the number of specimens originally studied is uncertain and they should also be collectively considered syntypes. Gonçalves *et al.* (1993) and Rodrigues *et al*. (2015) misinterpreted this and, based on specimens labels, listed a holotype of *T. lenti* and paratypes of the three species from the Oswaldo Cruz Institute, which is incorrect (International Code of Zoological Nomenclature, Recommendation 73F, Article 73.2).

A triatomine survey carried out in the south-central region of the Brazilian state of Bahia between 2008 and 2013 (Mendonça *et al.* 2015) revealed two specimens morphologically similar to *T. bahiensis* which were initially identified as *T. lenti*. These specimens have been kept in a colony and their descendants maintained the morphological characteristics of *T. bahiensis* initially described by Sherlock and Serafim (1967). Because *T. lenti* occurs in domiciliary units in Bahian municipalities and could represent a threat as a vector of *T. cruzi* (Costa *et al.* 2003, Mendonça *et al.* 2015), it is important to reassess its taxonomic status.

Thus, this study aims to i) check for the specific status of *T. bahiensis* using distinct taxonomic tools (morphological, morphometric, experimental crosses, and cytogenetic approaches) and ii) determine whether *T. lenti* and *T. bahiensis* are members of the *T. brasiliensis* species complex through phylogenetic analyses based on mtDNA sequencing.

Methods

Specimens

Collecting sites were located in the cities of Caturama (13°08.847'S; 42°21.284'W), Ibipitanga (12°58.039'S; 42°19.728'W), Macaúbas (12°58.698'S; 42°50.451'W), and Seabra (12°21.911'S; 42°13.415'W). These cities are within the physiographic region known as the Serra Geral, which is part of the Chapada Diamantina mountain rage in the south-central region of the Brazilian state of Bahia. Bugs were collected during manual searches in intradomiciliary, peridomiciliary, and sylvatic environments. Twenty-four sites (which included neighborhoods, rural areas, and villages) were surveyed in four municipalities (Mendonça *et al.* 2015).

The identity of the recently collected specimens was confirmed by comparisons with high resolution photos of type-specimens of *T. lenti*, *T. pessoai* and *T. bahiensis* (Fig. 1). These photos were obtained from the Triatomines Collection of the Oswaldo Cruz Institute

(CTIOC), where most of the material examined by Sherlock and Serafim (1967) was deposited. Considering the substandard type-handling by these authors, lectotypes have been selected among the syntypes of *T. pessoai* and *T. bahiensis* in order to enhance the stability of nomenclature. In the case of *T. lenti*, the wrong use of the term "holotype" by Gonçalves *et al*. (1993) fulfills the requirements of the International Code of Zoological Nomenclature, Article 74.5. Therefore, the specimen selected by them must be considered the lectotype of the species, and there is no need for a new designation.

Specimens morphologically similar to *T. bahiensis* were collected in rural areas within Caturama, Ibipitanga, and Macaúbas, but only two specimens from Macaúbas collected by our team in peridomiciliary environments in the rural area known as Cana Brava were analyzed, as well as 222 *T. lenti* specimens collected in the same area. Both species have been kept in the colony at the Triatomine Insectarium of the School of Pharmaceutical Sciences within São Paulo State University (FCFAR/UNESP) in the city of Araraquara, São Paulo, Brazil, since 2009. The specimens of these colonies were used in morphological, morphometric and experimental cross studies, as described below.

Morphological analysis

Scanning electron microscopy (SEM) was used for the morphological analyses of the eggs (n=10), scutellum (n=5), and thorax (n=1) from each species. Specimens were examined under a Topcon SM-300 microscope at the Chemical Institute of UNESP Araraquara. The samples were fixed on aluminum stubs, washed, dehydrated in an alcohol series, and oven dried at 50°C. Sputtering metallization was then performed for 2 minutes at 10 mA (Rosa *et al.* 2012).

Morphometric analysis

A Leica MZ APO stereomicroscope equipped with an imaging system (Motic Advanced 3.2 plus) was used for the measurement and description of morphological characters of *T. lenti* and *T. bahiensis*. Linear measurements (length of the head, thorax, abdomen, rostral segments, and inner distance between the eyes) were taken from 30 adult specimens (15 females and 15 males). Egg characteristics (length of eggshells, diameter and area of the opercular opening) were registered from 30 *T. lenti* and *T. bahiensis* eggshells (Frías-Lasserre 2010, Rosa *et al.* 2012, Gonçalves *et al.* 2013).

In addition, seven wing landmarks, which were venation intersections or type I landmarks according to Bookstein (1990), were used in geometric morphometric analyses of *T. bahiensis* (n=49) (Fig. 2) and *T. lenti* (n=46). Landmark coordinates were recorded using tpsDig Version 1.18 (Rohlf 1999a). Centroid size, an isometric size estimator derived from coordinate data (Bookstein 1990) was used to analyze wing size variation. The generalized procrustes analysis superimposition algorithm was used to obtain the shape variables (Rohlf 1996). A consensus configuration was derived from raw coordinate data based on least-squares iterative method, and 'partial warps' were computed as deformations of each individual structure compared to the consensus configuration. Deformation components (uniform and non-uniform) were computed and tested for variation using tpsRelw 1.18 (Rohlf 1999b). The variables, which were derived from partial warps, were used in multivariate discriminant function analysis. A factor map of the first two canonical factors was used to illustrate the main results. Size variation between species was explored by means of Student's t-tests. Finally, we measured the contribution of size to shape variation (allometry) using multiple regression of shape discriminant factors against centroid size values. Discriminant function analysis, multiple regression analysis and Student's t-tests were computed using Statistica® (StatSoft, Inc., Tulsa, OK, U.S.A.).

Experimental crosses

Triatoma lenti and *T. bahiensis* were crossed in both directions: *T. lenti* female × *T. bahiensis* male and *T. bahiensis* female × *T. lenti* male. The insects were sexed as nymphs in the fifth instar, and males and females were kept apart until they reached the adult stage to cross virgin adults. For those crosses, five couples from each set were placed in plastic jars (5 cm in diameter x 10 cm in height) and kept at room temperature.

The fertility rate and oviposition were calculated from each cross (Correia *et al*. 2013). The eggs were collected daily throughout the females' oviposition period. Viable F1 offspring was kept into the adult stage following Mendonça *et al* (2014). Parental lineages of the colonies of *T. lenti* and *T. bahiensis* kept in the Triatomine Insectarium of UNESP were observed as a parameter for qualitative comparisons.

Cytogenetic analysis

Seminiferous tubules of five adult males of *T. bahiensis* were shredded, squashed, and fixed onto a cover slip in liquid nitrogen. They were then stained using the C-banding

cytogenetic technique modified for triatomine chromosomes (Panzera *et al.* 1995). Microscope images were obtained under a Nikon Eclipse 80i microscope with a Nikon DS-5Mc-U2 digital cooled camera using Nikon Nis Elements 3.1 Advanced Research software.

Phylogenetic analysis

Total genomic DNA was extracted with standard phenol/chloroform technique (Sambrook *et al*. 1989) from three individuals of *T. lenti* and *T. bahiensis* collected from Macaúbas. A 690-bp fragment of the mitochondrial (mt) cytochrome b gene (*Cyt b*) was PCR-amplified (Lyman *et al.* 1999). Amplicons were purified using GFX PCR DNA and a Gel Band Purification Kit (Amersham Pharmacia Biotech, Inc.). Both strands were used in Sanger sequencing reactions (ABI Prism® BigDye® Terminator v.3.1 Cycle Sequencing Kit, Applied Biosystems) and run on an ABI 3730 sequencer. Consensus sequences were edited with the BioEdit program and aligned with the Clustal W program using default parameters (Thompson *et al.* 1994, Hall 1999).

The sequences of *T. lenti* (KT347299) and *T. bahiensis* (KT347298) were aligned with some fragments downloaded from GenBank from several species within the *T. brasiliensis* complex (*T. brasiliensis* AY494160, *T. b. macromelasoma* AY336526, *T. melanica* AY336527, *T. juazeirensis* AY494168, and *T. sherlocki* EU489058), and *T. dimidiata* (Latreille, 1811) JN585905 and *T. infestans* Klug, 1834 EF639038 were added to the sequence alignment as the outgroup to root the phylograms.

The dendrogram was constructed using Bayesian analysis and clade support was estimated using a Markov chain Monte Carlo algorithm (MCMC) performed in the MrBayes software (Huelsenbeck & Ronquist 2001). The first analysis was run for 5,000,000 generations, with sampling of every 1000 generations. Estimates of likelihood settings were calculated using jModelTest (Posada 2008), and the Akaike information criterion (AIC) was chosen to obtain the best model to be applied to the gene fragment. The general model of DNA substitution with gamma-distributed rate variation across sites (TIM2+ILsetnst=6 rates=equal) was used, with two substitution types that distinguish only between transitions and transversions (nst=2).

Results

***Triatoma bahiensis* Sherlock & Serafim, 1967, valid species**

Triatoma pessoai var. *bahiensis*: Sherlock & Serafim (1972).

Triatoma lenti: Lent & Wygodzinsky (1979) (synonym).

Specimens

Type material examined: *Triatoma pessoai*. Lectotype (present designation): BRAZIL, Bahia: Ipupiara, collector unknown, 1966, one male (antennae and legs partially broken off) (genitalia in slide, missing), *Triatoma pessoai* det. I. Sherlock III/1967 (CTIOC #3330, Fig. 1). ***Triatoma bahiensis*. Lectotype** (present designation): BRAZIL, Bahia: Seabra, collector unknown, 1960, one male (antennae and legs partially broken off) (genitalia in slide, missing), *Triatoma bahiensis* det. I. Sherlock III/1967 (CTIOC #3338, Fig. 1). ***Triatoma lenti*. Lectotype** (Gonçalves *et al*. 1993): BRAZIL, Bahia: Macaúbas, N. P. B. col., X/1966, one male (antennae and legs partially broken off) (genitalia in slide, missing), *Triatoma lenti* det. I. Sherlock X/1967 (CTIOC # 3334, Fig. 1).

Additional material examined: BRAZIL, Bahia: Macaúbas, J. G. Nogueira col., 08/IX/2009, one female and one male. Caturama, S. L. Costa col., 20/X/2012, one female. Ibipitanga, S. L. Costa col., 15/III/2013, one male.

Diagnosis. *Triatoma bahiensis* (Figs. 1, 3A-B) is black with orange-red spots on the connexivum that are greater than those of *T. lenti* (Figs. 1, 3C-D). The hemyelitrum of *T. bahiensis* presents discoidal cells black, does not reach the apex of the abdomen and possesses two orange-red spots on each side of the corium (Figs. 1, 3A-B), whereas the hemyelitrum of *T. lenti* is totally black without spots in the corium.

Morphological analysis

The exochorial cells of *T. bahiensis* eggs (Fig. 4A) revealed a higher quantity of pores than those of *T. lenti* (Fig. 4B). These pores are concentrated in the central region of *T. bahiensis* egg cells (Fig. 4A), while in *T. lenti* the pores are uniformly distributed and also found on egg cell borders (Fig. 4B).

The posterior portion of the central depression of the scutellum is rounded in *T. bahiensis* (Fig. 4C) and tapered in *T. lenti* (Fig. 4D). The first abdominal segment has two lateral prominences in *T. bahiensis* (Fig. 4C). These prominences are absent in *T. lenti* (Fig. 4D).

Morphological analysis of the ventral thorax showed that the anterior region of the prothorax (near the stridulatory sulcus) in *T. lenti* presents a depression (Fig. 5D), which is absent in *T. bahiensis* (Fig. 5A). Furthermore, the stridulatory sulcus of *T. bahiensis* (Fig. 5A) is narrower than that of *T. lenti* (Fig. 5D). In *T. bahiensis*, the posterior region of the stridulatory sulcus has rounded and well-defined edges (Fig. 5A), whereas in *T. lenti* this region has rough edges (Fig. 5D).

The mesothorax of *T. bahiensis* has a central longitudinal projection that is rectangular in shape (Fig. 5B). In *T. lenti,* however, the mesothorax is smooth and rounded (Fig. 5E). No distinctive features were observed between the metathoraces of *T. bahiensis* and of *T. lenti* (Figs. 5C, F).

Morphometric analysis

All of the morphometric characteristics analyzed in males and females of *T. bahiensis* were smaller than those of *T. lenti*, except for the third segment of the proboscis, which is the same size in females of both species and larger in *T. bahiensis* males (Tables 1 and 2).

Wings of *T. bahiensis* were smaller than those of *T. lenti* ($p<0.01$). The first two discriminant factors explained 76% and 21% of wing shape variation, respectively. *Triatoma lenti* and *T. bahiensis* were well separated in discriminant function analysis plots, and wing shape differences between species were evident, *T. lenti* wings being narrower than in *T. bahiensis* (Fig. 6). No allometric effects were observed on the regression analyses between shape variables and size of wings ($R^2 = 0.01$, $p = 0.94$).

Experimental crosses

Triatoma bahiensis female × *T. lenti* male: out of 5 couples crossed, four produced fertile eggs and only 4% reached adulthood (Table 3). The F1 egg hatching rate was 81.3%, but the coupling did not produce adults (F2 generation).

Triatoma lenti female × *T. bahiensis* male: only one of the five couples produced fertile eggs; only two eggs reached adulthood (Table 3). No eggs were generated from this offspring. Despite the low egg viability in both experimental crosses, no abnormalities were found in the hybrids.

Cytogenetic analysis

The analysis of the early first meiotic prophase I of *T. bahiensis* (Fig. 7A) revealed a large chromocenter made up of the association of both sex chromosomes plus two autosomal pairs (arrow) and many heterochromatic blocks arranged inside the nucleus (arrowheads). In the diplotene stage (Fig. 7B), *T. bahiensis* presented an association between two autosomal bivalents (arrowheads) with sex chromosomes (arrow). In diakinesis or in later diplotene (Fig. 7C), this species presented ten autosomal bivalents and two (XY) sex chromosomes with heterochromatic blocks in one or both chromosomal ends of the autosomes. Note that the Y sex chromosome (arrow) is larger and more heterochromatic that the X chromosome.

Phylogenetic analysis

The three specimens studied of each species did not present intraspecific variability. The comparison of *Cyt b* gene fragments between *T. lenti* and *T. bahiensis* revealed 17 variable sites, and pairwise sequence divergence was 2.5% (Table 4). This value is higher than that found for *T. b. brasiliensis* and *T. b. macromelasoma* (2.0%), and it is close to the value found for the *T. lenti* and *T. melanica* (2.7%). Bayesian analysis grouped *T. lenti*, *T. bahiensis*, and *T. melanica* into a monophyletic clade (Fig. 8). The other species of the *T. brasiliensis* complex are located in another clade. Phylogenetic analysis of *T. lenti* and *T. bahiensis* with *T. melanica* suggests that *T. bahiensis* and *T. lenti* represents two new members of the *T. brasiliensis* complex. Furthermore, the value of genetic distance between *T. lenti* and *T. bahiensis* support the revalidation of *T. bahiensis*.

The sequence analysis of *T. lenti* and *T. bahiensis* with other species of the *T. brasiliensis* complex revealed 75 variable sites and three autapomorphies for *T. bahiensis*. All branches of the clade had 100% supports.

Discussion

Revalidation of *T. bahiensis* was based on an integrative assessment that included morphology, mtDNA sequencing, morphometrics, experimental crosses, cytogenetics, and qualitative phenotype traits. Moreover, phylogenetic analyses revealed that both *T. bahiensis* and *T. lenti* should be included as members of the *T. brasiliensis* complex, which is a monophyletic group proposed by Costa *et al*. (2013). The *T. brasiliensis* complex is now represented by *T. b. brasiliensis*, *T. b macromelasoma*, *T. juazeirensis*, *T. melanica*, *T. sherlocki*, *T. lenti* and *T. bahiensis*.

Previous analyses of morphometric and morphological characters, including male genitalia, were unable to separate *T. bahiensis* (= *T. pessoai* var. *bahiensis*), *T. lenti*, and *T. pessoai* (Lent & Wygodzinsky 1979). Thus, Lent & Wygodzinsky (1979) concluded that *T. pessoai* and *T. bahiensis* were synonyms of *T. lenti*, because the only differences observed (size and color variation in the connexival spots and corium) were within the limits commonly accepted among *Triatoma* species.

Although analysis of chromatic patterns has been widely proposed for taxonomic definition of triatomine species (Lent & Wygodzinsky 1979), including some members of the *T. brasiliensis* complex and *T. phyllosoma* complex, integrative taxonomy, which includes biological and phylogenetic approaches, is recommended to support new species description (Costa *et al.* 2006, Martinez *et al*. 2006, Costa & Felix 2007, Martínez-Ibarra *et al*. 2009, Rosa *et al.* 2012, Abad-Franch *et al.* 2013). In addition, phenotypic variability was already observed and recorded in several triatomine species, as recently described in the *T. brasiliensis* complex (Costa *et al.* 2016), and also for *Triatoma rubrovaria*, *T. pseudomaculata*, *Panstrongylus geniculatus*, and several other species (Almeida *et al*. 2002, Lent & Wygodzinsky 1979). Therefore, the combination of independent taxonomic tools is crucial to avoid misidentifications.

Morphological and morphometric analyses are essential tools in species descriptions and validations (Rosa *et al.* 2012, Costa *et al.* 2013, Gonçalves *et al.* 2013, Jurberg *et al*. 2013). Although morphologically similar to *T. lenti*, *T. bahiensis* presents distinct characteristics. These include corium with two orange-red spots on each wing, a trait which can be used as diagnostic for species differentiation. The chromatic pattern was very important for initial studies on the *T. brasiliensis* complex, since the current species grouped by Costa *et al*. (2013) were previously considered to be only chromatic variations of *T. brasiliensis* (Lent & Wygodzinsky 1979, Costa *et al.* 2003). Later, Costa *et al*. (2006) raised the status of *T. b. melanica* to *T. melanica*. Costa & Felix (2007) then described *T. juazeirensis*, and Costa *et al*. (2013) redescribed *T. b. macromelasoma*.

Scanning electron microscopy revealed significant differences between *T. bahiensis* and *T. lenti*. Such differences could be observed in *T. bahiensis* egg exochorial cells which presented more pores than in *T. lenti*. The number of pores in exochorial cells of *T. brasiliensis* species complex is variable, being higher for *T. melanica* populations (Costa *et al*. 1997a, Costa *et al*. 2006). This species has pores distributed over the entire egg surface, similar to *T. lenti*, but with a higher number of pores. The posterior region of the central

depression of the scutelum is rounded in *T. bahiensis* and tapering in *T. lenti*. Both characters are important tools for taxonomy and were used to differentiate *R. montenegrensis* Rosa *et al*. 2012 from *R. robustus* Larrousse, 1927. They have also proven to be useful for the characterization of other triatomine species (Rosa *et al.* 2012).

The ventral thoracic region, specifically the prothorax and mesothorax, showed significant morphological differences between *T. bahiensis* and *T. lenti*. The mesothorax of *T. bahiensis* has a central longitudinal projection that is rectangular in shape, unlike that of *T. lenti*, which is smooth and rounded. The use of this structure as a tool for taxonomy was applied with satisfactory results for the first time in this study.

Geometric morphometric techniques also allowed the distinction between *T. bahiensis* and *T. lenti*, and the first two discriminant factors explained 97% of wing shape variation. These techniques have been applied previously to compare shape differences among populations (Campos *et al.* 2011, Almeida *et al.* 2012), to support species descriptions, as in the case of *T. jatai* (Gonçalves *et al*. 2013) and *R. montenegrensis* (Rosa *et al.* 2012), and also to characterize hybrid forms (Martínez-Ibarra *et al.* 2011, Mendonça *et al.* 2014).

Hybridization between closely related triatomine species is a well-known phenomenon that has been detected both in nature and in experimental cross-mating (Usinger *et al.* 1966, Costa *et al*. 2003b, Pérez *et al.* 2005, Mas-Coma & Bargues 2009, Mendonça *et al.* 2014). Experimental crosses offer a powerful tool for determining isolating mechanisms that limit gene flow between species and also for establishing taxonomic status among putative cryptic species (Arnold 1997). In triatomines, experimental crosses have been performed to validate the specific status of *T. petrochii* Pinto and Barreto 1925 (Espínola 1971), *T. sherlocki* (Correia *et al.* 2013), and other species of the *T. brasiliensis* complex (Costa *et al.* 2003b, 2009), the *T. phyllosoma* complex (Martínez-Ibarra *et al.* 2009, 2011), and the *T. dimidiata* complex (García *et al.* 2013). Pre-zygotic barriers, such as the incompatibility of genitalia (Mendonça *et al.* 2014) and gametes (Schreiber *et al.* 1974) as well as post-zygotic barriers, such as hybrid collapse (Mendonça *et al.* 2014) have been described in the subfamily Triatominae.

Though *T. bahiensis* females × *T. lenti* males resulted in limited reproductive success, the reciprocal crosses did not show the same pattern. The crosses between *T. lenti* females and *T. bahiensis* males resulted in high mortality before the offspring reached adulthood, and F2 generation did not produce eggs. Similar reproductive isolation was also reported between *T. sherlocki* and *T. lenti* (Mendonça *et al.* 2014). Although the experimental crosses have

occurred in F1 adults, the levels of 4% and 0.8% of adult hybrids are negligible. Mendonça *et al*. (2014) demonstrated through chromosome analysis that the collapse of the hybrid event happens from the F2 cross between *T. lenti* and *T. sherlocki*. Perhaps the same phenomenon is occurring between *T. lenti* and *T. bahiensis* crosses, which present post-zygotic reproductive barriers that are manifested in both the small percentage of individuals that reached adulthood in F1 and their sterility. This sterility is likely to be associated with failures in the pairing of homologous chromosomes, which leads to the production of abnormal gametes, similar to the results observed among other triatomine hybrids (Pérez *et al.* 2005).

Heitzmann-Fontenelle (1984) carried out an experimental cross between a *T. lenti* male and *T. brasiliensis* female. The author noted that, of the 153 eggs produced, only 17 reached adulthood in F1. The hybrid viability (11%) between these species is higher than that observed in crosses between *T. lenti* and *T. bahiensis*. It is noteworthy that parental lineages of these species were viable producing at least 10 generations with high oviposition and fertility rates at the FCFAR/UNESP, São Paulo, Brazil, since 2009.

Cytogenetic analysis suggests that *T. lenti* (Alevi *et al.* 2012, 2013, 2014) and *T. bahiensis* are members of the *T. brasiliensis* complex, since they have the same chromosomal characteristics as those used for diagnosing the other members: 22 chromosomes (20A+XY), meiotic prophase with a main chromocenter constituted of both sex chromosomes, plus two pairs of autosomes and heterochromatic chromocenters dispersed in the nucleus, as well as the presence of a heterochromatic block in one or both chromosomal ends of all autosomes (Panzera *et al.* 2000, Alevi *et al.* 2013).

The phylogenetic analysis of *T. bahiensis* and *T. lenti* included seven species of the *T. brasiliensis* complex, plus *T. dimidiata* and *T. infestans*. The low genetic distance between *T. bahiensis* and *T. lenti* (2.5%) showing them as sister-species, forming a monophyletic clade with *T. melanica* (distance 2.7–3.3%, Table 4). Although a low genetic distance was found between *T. lenti* and *T. melanica*, both are well-defined and phenotypically distinct species. Similar data was found by García *et al*. (2001) in their analysis of mitochondrial genes (12S and 16S) from *T. infestans*, *T. platensis* Neiva, 1913, and *T. delpontei* Romaña & Abalos, 1947, which showed genetic distances ranged between 0.0–0.8%. Cytogenetic data suggested that *T. infestans* and *T. platensis* would be the most closely related (Panzera *et al.* 1995). Furthermore, complete interfertility between *T. infestans* and *T. platensis* has been observed, whereas only partial sterility was reported between *T. platensis* and *T. delpontei* (Abalos 1948, Pérez *et al.* 2005). Thus, low viability of experimental crosses here reported, in

conjunction with morphological and morphometric data support the revalidation of the specific status *T. bahiensis*.

In the phylogeographic analysis of the *T. brasiliensis* complex, Monteiro *et al*. (2004) showed a greater genetic distance among the population "*melanica*" and the other members of that complex. Furthermore, Mendonça *et al*. (2009) included *T. sherlocki* in the complex and positioned it as a sister species of *T. melanica*, in a clade separated of the other members of the complex. After the inclusion of *T. lenti* and *T. bahiensis* the present phylogenetic analysis showed two main clades, one composed of *T. lenti*, *T. bahiensis*, and *T. melanica* and another by *T. sherlocki*, *T. b. brasiliensis*, *T. b. macromelasoma* and *T. juazeirensis*.

The cryptic speciation phenomenon is relatively common in Triatominae (Monteiro *et al.* 2003, Rosa *et al.* 2012, Gonçalves *et al.* 2013, Jurberg *et al.* 2013). This evolutionary event results in species that are almost identical morphologically, which often makes identification based on morphological parameters alone very difficult. Thus, other tools are needed to characterize these taxa. Therefore, the morphological similarity between *T. lenti* and *T. bahiensis*, combined with their similar geographic distribution, may lead to the misidentification of these taxa. Specimens morphologically similar to *T. bahiensis* have been collected in rural areas within three municipalities (Caturama, Ibipitanga, and Macaúbas) where *T. lenti* may also occur. Currently, *T. lenti* is distributed in at least two states, Goiás and Bahia, in Cerrado and Caatinga ecoregions, respectively (Costa *et al.* 2003, Gurgel-Gonçalves *et al.* 2012). Future studies should revise the geographic limits and the vectorial competence of *T. lenti* and *T. bahiensis*.

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Table 1. Measurements (mm) of *Triatoma lenti* and *T. bahiensis* females captured in the city of Macaúbas, Bahia State, Brazil, and eggs.

	Triatoma lenti					*Triatoma bahiensis*				
	Min	**Max**	**X**	**SD**	**S^2**	**Min**	**Max**	**X**	**SD**	**S^2**
Length of head*	4.83	5.48	5.30	0.23	0.05	4.70	5.14	4.98	0.12	0.01
Length of thorax*	5.79	6.80	6.33	0.31	0.01	5.00	5.86	5.48	0.33	0.11
Length of abdomen*	13.13	15.01	13.94	0.65	0.42	11.09	13.06	12.40	0.62	0.38
Length of 1st rostral segment	1.47	1.82	1.61	0.11	0.01	1.38	1.75	1.53	0.10	0.01
Length of 2nd rostral segment*	2.73	3.12	2.95	0.12	0.01	2.50	2.93	2.74	0.15	0.02
Length of 3rd rostral segment	1.20	1.31	1.24	0.03	0.00	1.17	1.35	1.24	0.06	0.00
Inner distance between eyes	1.33	1.43	1.37	0.03	0.00	1.27	1.44	1.34	0.05	0.00
Outer distance between eyes*	2.34	2.61	2.48	0.09	0.01	2.15	2.51	2.38	0.10	0.01
Length of eggshells*	2.27	2.73	2.50	0.11	0.01	1.88	2.17	2.04	0.08	0.00
Diameter of the opercular opening*	0.72	0.82	0.76	0.03	0.01	0.62	0.78	0.71	0.03	0.00
Area of opercular opening*	0.40	0.52	0.46	0.03	0.01	0.30	0.47	0.40	0.04	0.00

*differences between species are significant (analysis of variance - $p < 0.05$); Max: maximum; Min: minimum; SD: standard deviation; S^2: variance; X: average.

Table 2. Measurements (in mm) of *Triatoma lenti* and *T. bahiensis* males captured in the city of Macaúbas, Bahia State, Brazil.

	Triatoma lenti					*Triatoma bahiensis*				
	Min	**Max**	**X**	**SD**	**S^2**	**Min**	**Max**	**X**	**SD**	**S^2**
Length of head*	4.56	5.31	5.12	0.19	0.04	4.54	5.07	4.85	0.16	0.03
Length of thorax	5.73	6.20	5.94	0.19	0.02	5.24	6.19	5.84	0.31	0.09
Length of abdomen*	11.89	14.27	13.61	0.63	0.36	11.44	13.30	12.64	0.55	0.30
Length of 1st rostral segment*	1.39	1.73	1.59	0.08	0.00	1.42	1.66	1.52	0.08	0.01
Length of 2nd rostral segment	2.61	3.02	2.85	0.13	0.02	2.53	2.93	2.78	0.13	0.02
Length of 3rd rostral segment*	1.10	1.27	1.19	0.05	0.00	1.18	1.33	1.26	0.05	0.00
Inner distance between eyes	1.17	1.34	1.25	0.04	0.00	1.16	1.32	1.23	0.05	0.00
Outer distance between eyes	2.23	2.53	2.40	0.08	0.00	2.23	2.50	2.35	0.09	0.01

*differences between species are significant (analysis of variance - $p < 0.05$); Max: maximum; Min: minimum; SD: standard deviation; S^2: variance; X: average

Table 3. Results of experimental crosses between *T. bahiensis* and *T. lenti.*

Generation	*T. bahiensis* female x *T. lenti* male			*T. lenti* female x *T. bahiensis* male		
	Nº of eggs laid	Nº of eggs hatched (%)	Nº of adults (%)	Nº of eggs laid	Nº of eggs hatched (%)	Nº of adults (%)
F1	271	70 (25.8)	11 (4)	239	84 (35.1)	2 (0.8)
F2	107	87 (81.3)	-	-	-	-

Table 4. Pairwise distance genetic among the *T. brasiliensis* complex members and *T. dimidiata* and *T. infestans*.

Species	1	2	3	4	5	6	7	8
1. *T. bahiensis*								
2. *T. lenti*	0.0248							
3. *T. sherlocki*	0.0901	0.0856						
4. *T. melanica*	0.0338	0.0270	0.0901					
5. *T. b. brasiliensis*	0.1014	0.0991	0.1194	0.1036				
6. *T. b. macromelasoma*	0.1014	0.0991	0.1171	0.0991	0.0203			
7. *T. juazeirensis*	0.0991	0.1014	0.1081	0.1059	0.0878	0.0856		
8. *T. infestans*	0.1351	0.1216	0.1374	0.1329	0.1329	0.1351	0.1306	
9. *T. dimidiata*	0.1712	0.1689	0.1847	0.1779	0.1914	0.2005	0.2050	0.1667

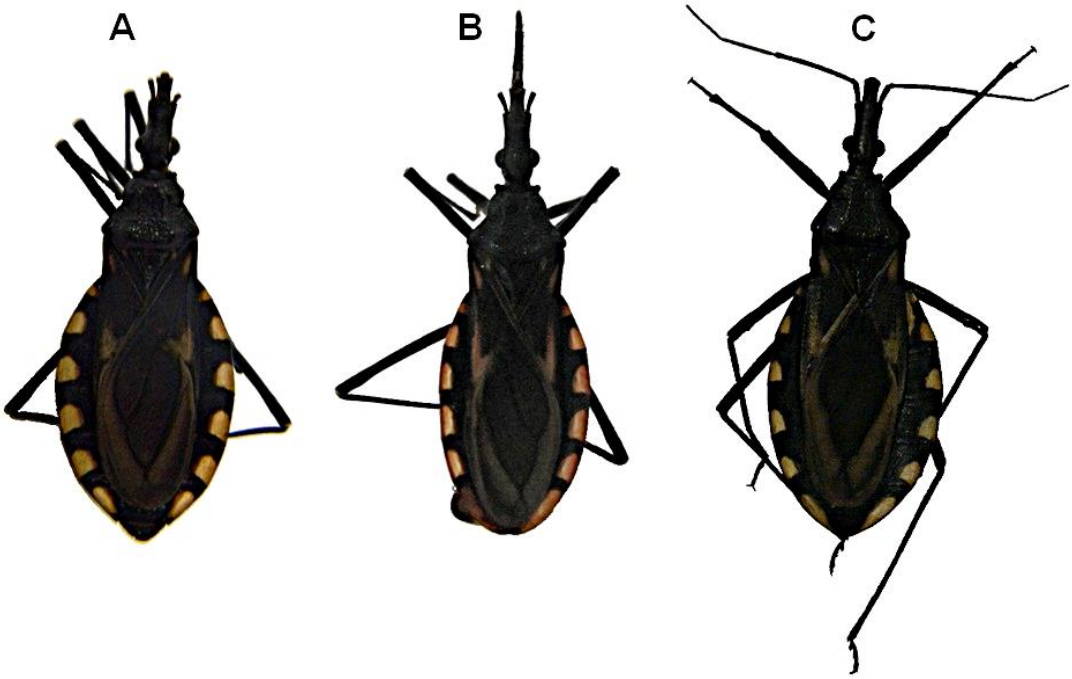

Figure 1. Type-material analyzed in the present study. A. *Triatoma bahiensis* Sherlock & Serafim, 1967, revalidated. B. *Triatoma pessoai* var. bahiensis Sherlock & Serafim (1972). C. *Triatoma lenti*.

Figure 2. Right wing of *Triatoma bahiensis* with the seven landmarks used in morphometric analysis. Following Bookstein (1990), all points correspond to type I landmarks (venation intersections).

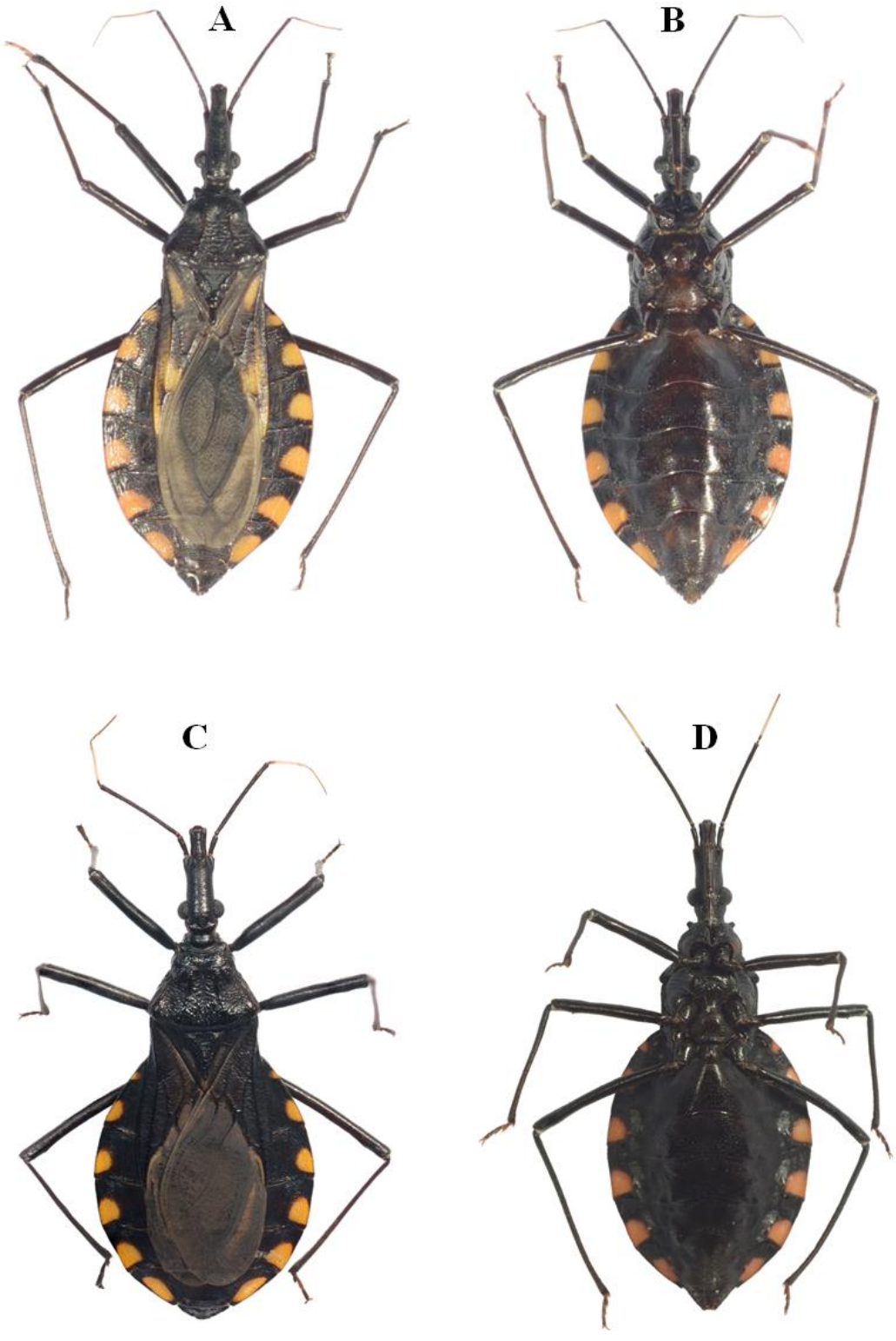

Figure 3. A - *Triatoma bahiensis* female, dorsal view; B - *Triatoma bahiensis* female, ventral view; C - *Triatoma lenti* female, dorsal view; D - *Triatoma lenti* female, ventral view.

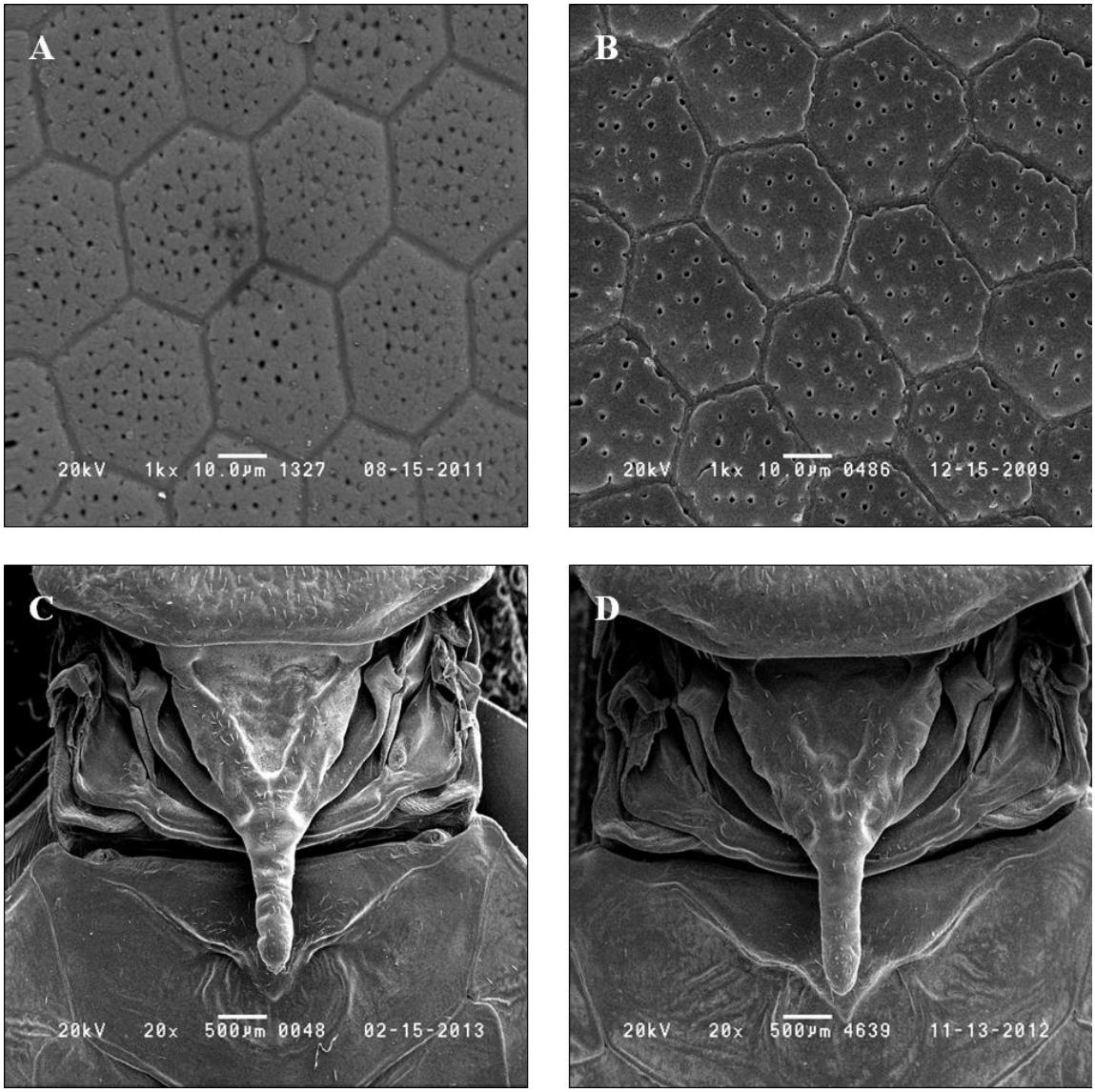

Figure 4. Egg exochorion detail via scanning electron microscopy in A - *T. bahiensis* and B - *T. lenti*. Scutellum detail via scanning electron microscopy in C - *T. bahiensis* and D - *T. lenti*.

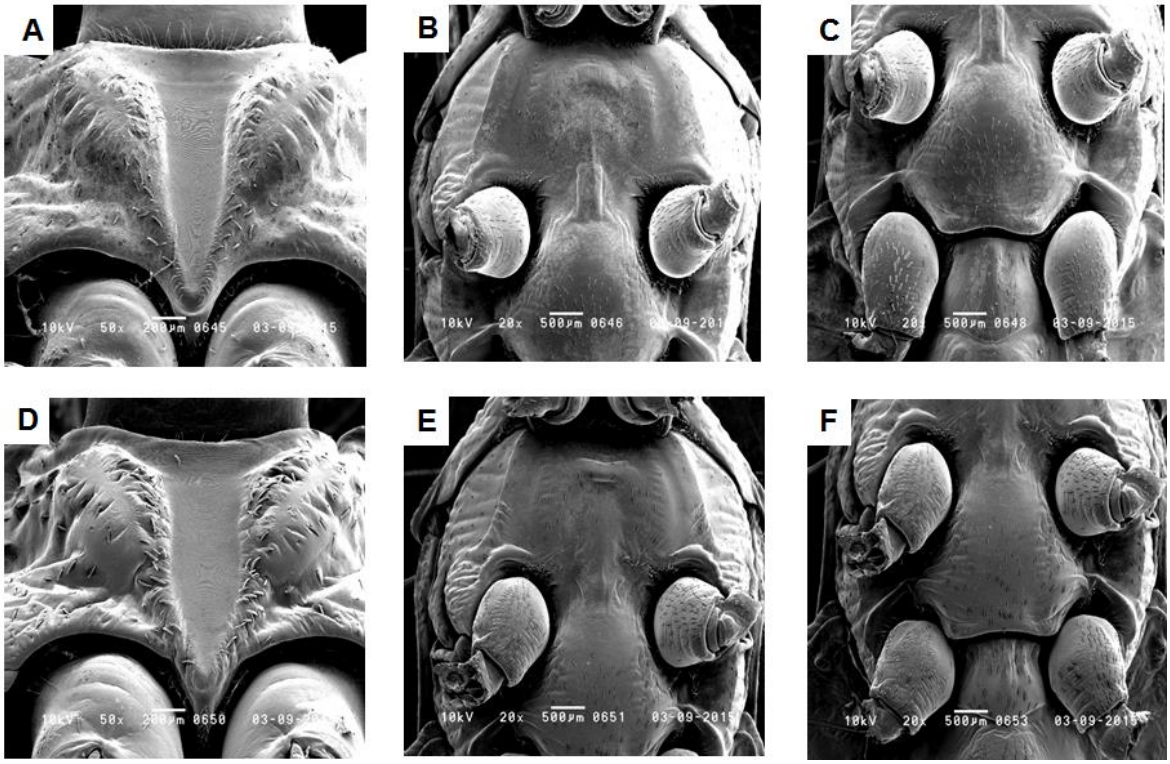

Figure 5. Ventral view of *T. bahiensis*: A – prothorax, B – mesothorax and C – metathorax; Ventral view of *T. lenti*: D – prothorax, E – mesothorax and F – metathorax

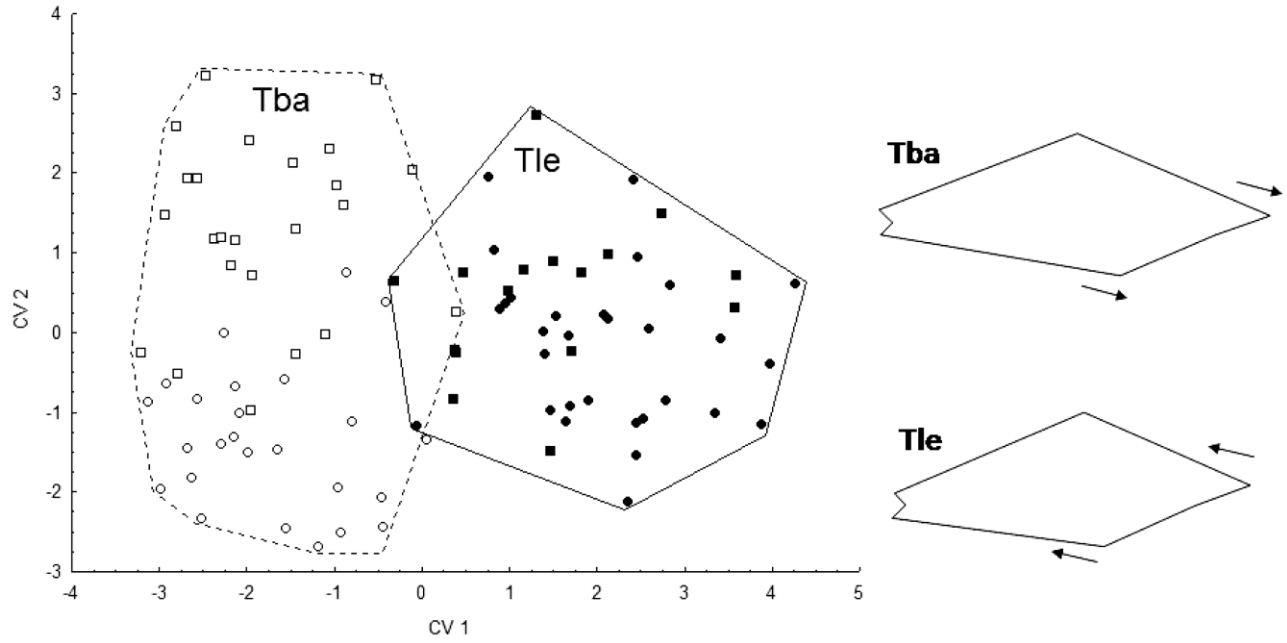

Figure 6. Factorial maps in the plane of the two discriminant factors of wing shape variation (canonical variables 1 and 2, or CV1 and CV2) presenting the distribution of specimens of *Triatoma bahiensis* (*Tba*, dashed polygon, open symbols) and *Triatoma lenti* (*Tle*, black polygon, closed symbols). Males are represented by squares and females are represented by circles. Drawings to the right of the plot show the consensus conformation of the wings of the species (see landmarks in Fig. 2). Arrows indicate the differences in wings of both species (see text for details).

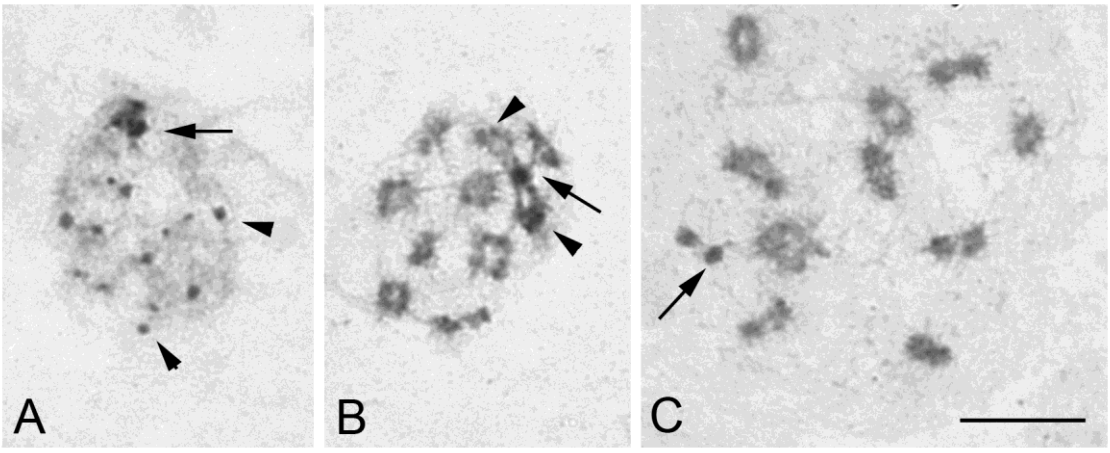

Figure 7. *Triatoma bahiensis* (2n= 22 chromosomes), male meiosis, C-banding. **(A)** Early first meiotic prophase: several heterochromatic chromocenters spread in the nucleus (arrowheads). A large chromocenter consists of the association of sex chromosomes plus autosomal bivalents (arrow). **(B)** Diplotene stage: the association between two autosomal bivalents (arrowheads) with associated sex chromosomes (arrow) is observed. **(C)** Diakinesis or later diplotene: ten bivalents and both sex chromosomes (XY) are clearly identified. The Y chromosome is entirely C-heterochromatic (arrow) and the X chromosome is euchromatic.

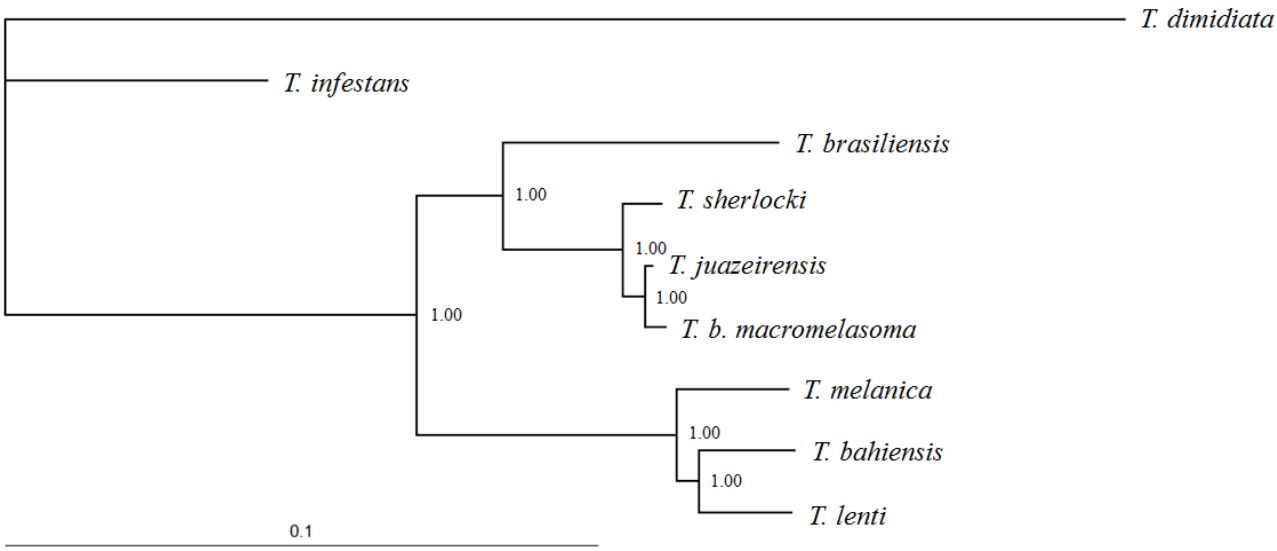

Figure 8. Bayesian inference consensus using a Markov chain Monte Carlo algorithm applied to mitochondrial sequences of *Cyt b* fragments of 510 bp. The values over the nodes refer to bootstrap value by maximum parsimony. *T. dimidiata* and *T. infestans* were used as outgroup. Accession code of GenBank in the text.

4.5 Capítulo 5 (Artigo científico aceito para publicação na revista *The American Journal of Tropical Medicine and Higiene* FI 2,54)

Hybrid collapse confirms the specific status of *Triatoma bahiensis* Sherlock & Serafim, 1967 (Hemiptera, Triatominae), an endemic species in Brazil

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Abstract

About 6 to 7 million people worldwide are estimated to be infected with *Trypanosoma cruzi*, etiological agent of Chagas disease transmitted mainly by contact with faeces/urine of triatomines. *Triatoma bahiensis* was recently collected in the state of Bahia and revalidated by different approaches. Whereas the main criterion that defines a good biological species is reproductive isolation, we evaluated the cytogenetics of the hybrids in first generation (F1) resulting from the experimental cross between *T. bahiensis* female × *T. lenti* male to characterize the post-zygotic isolation related with the hybrid breakdown. All cells analyzed presented a karyotype 2n = 22, with a pair of univalent autosomes. This behavior of the chromosomes represents the hybrid collapse and underscores the specific status of *T. bahiensis*. Thus, we characterize the presence of the hybrid collapse phenomenon for experimental cross and we confirm the specific status of *T. bahiensis*, contributing to the Triatominae taxonomy.

Short Report

About 6 million to 7 million people worldwide are estimated to be infected with *Trypanosoma cruzi* (Chagas, 1909), the parasite that causes Chagas disease.[1] In Latin America, *T. cruzi* parasites are mainly transmitted by contact with faeces/urine of infected blood-sucking triatomine bugs and the vector control is considered the most useful method to prevent Chagas disease.[1]

In Brazil, currently there are 68 triatomine species distributed throughout the country's 27 states.[2,3,4,5,6] In the state of Bahia, there are 25 species, distributed among the genres *Cavernicola* (1 species), *Eratyrus* (1 species), *Panstrongylus* (5 species), *Psammolestes* (1 species), *Rhodnius* (3 species), and *Triatoma* (14 species).[2,3,4]

Triatoma bahiensis Sherlock & Serafim, 1967 was recently collected in the south-central region of the Brazilian state of Bahia in an endemic area for *T. lenti*.[7] For more than three decades, *T. bahiensis* was considered as synonymous with *T. lenti* Sherlock & Serafim, 1967.[8] Mendonça et al.[3] used morphological, morphometric, molecular, cytogenetic, and experimental crosses to revalidate *T. bahiensis*. Although cytogenetic (same characteristics) and molecular (relatively low genetic distance) analysis did not generate enough support for the differentiation between *T. bahiensis* and *T. lenti*, the low viability of experimental crosses combined with morphological and morphometric data allow the differentiation of *T. bahiensis* and *T. lenti*.[3] These tools corroborated the phylogenetic relationship between *T. bahiensis* and *T. brasiliensis* complex.[3]

The main criterion that defines a good biological species is reproductive isolation, however this type of insulation must be considered under natural conditions.[9] Thus, although experimental crosses under laboratory condition are not conclusive, are important to knowledge about the evolutionary phenomena related to the biological species concept (reproductive isolation).

In Triatominae, several events of pre-zygotic (for example, mechanical isolation[10]) and reproductive isolation post-zygotic (sterility[11] and hybrid collapse[12]) were observed. Experimental crosses were important for the taxonomy of the *T. brasiliensis* complex: the specific status of *T. petrocchiae* was confirmed by reproductive isolation when crossed with *T. brasiliensis* (eggs did not hatch[13]); genetic incompatibility was found between melanica and brasiliensis populations[14] (important tool for the revalidation of *T. melanica* as species[15]); and more recently the collapse of the hybrid highlighted the specific status of *T. lenti* and *T. sherlocki*.[12]

As the experimental cross between *T. bahiensis* and *T. lenti* produced hybrids (which makes pre-zygotic isolation barriers unfeasible)[3], we evaluated the cytogenetics of the hybrids in first generation (F1) to characterize the possible post-zygotic isolation factors related with the hybrid breakdown observed by Mendonça et al.[3].

Three adult males hybrids resulting from the experimental cross between *T. bahiensis* female × *T. lenti* male were analyzed cytogenetically [the reciprocal crosses between *T. lenti* female × *T. bahiensis* male resulted in only two adults females and ovaries analysis is not feasible for cytogenetic studies because meiotic stages are not usually observed in their ovaries[16] – see more details of the conditions of experimental crosses at Mendonça et al.[3]]. The hybrids were dissected and the testicles were removed and fixed in methanol: acetic acid

(3:1). Seminiferous tubules were shredded, smashed, and the microscope slides were set in liquid nitrogen and coverslips were removed after freezing in liquid nitrogen. They were then stained with the lacto-acetic orcein cytogenetic technique[17,18] and the genetic affinity between the parental species was evaluated through the pairings between the homeologous chromosomes of the hybrids during prophase (diakinesis), and metaphase I and II (we observed at least 50 cells in different stages according to Campos-Soto et al.[16]).

All cells analyzed presented a karyotype 2n = 22 (20A + XY), with a pair of univalent autosomes (Figure 1A, B, arrow). This behavior of the chromosomes represents that the cross between *T. bahiensis* female × *T. lenti* male is unviable by the post-zygotic isolation barrier called hybrid collapse and underscores the specific status of *T. bahiensis*, since two species have distinct genomes when their chromosomes are different in structure and gene content, so that no pairing occurs between one or more pairs of homeologous during meiosis of hybrids.[19]

According to the information above, although *T. bahiensis* and *T. lenti* are considered morphologically related (differentiated initially by only two orange-red spots on each side of the corium[20]) these species underwent genomic reorganization during speciation, as suggested for the speciation of *Rhodnius colombiensis* by means of the experimental crossing with *R. pallescens*.[21] The presence of univalent chromosomes results in nonviable gametes, as already described for other hybrids[11,22].

Sherlock and Guitton[23] collected samples of *T. lenti* in Bahia and all of them were positive for *T. cruzi*. Recently, *T. cruzi* was again isolated from *T. lenti* from Bahia.[24] Although there is no report in the literature of *T. bahiensis* infected by *T. cruzi*, we call attention to the future entoepidemiological lifting for the possible presence of *T. bahiensis* among the triatomines collected in this State for evaluate the epidemiological importance of this vector.

Thus, we characterize the presence of the hybrid collapse phenomenon for experimental cross between *T. bahiensis* female × *T. lenti* male and we confirm the specific status of *T. bahiensis*, contributing to the Triatominae taxonomy.

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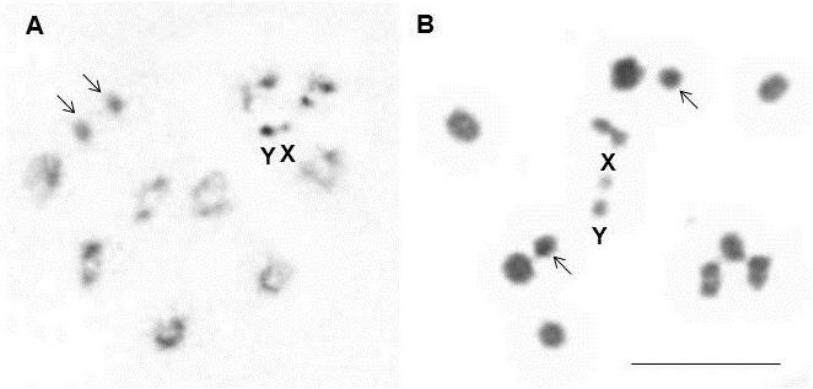

Figure 1. Diakinesis (A) and metaphase I (B) of hybrids resulting from the experimental cross between *T. bahiensis* female × *T. lenti* male. Note the univalent autosomes (arrow). Y: Y sex chromosome, X: X sex chromosome. Bar: 10 μm.

4.6 Capítulo 6 (Artigo científico publicado na revista *The American Journal of Tropical Medicine and Higiene* FI 2,54)

ALEVI, K. C. C.; GUERRA, A. L.; IMPERADOR, C. H. L.; ROSA, J. A.; AZEREDO-OLIVEIRA, M. T. V. Reproductive biology of *Triatoma brasiliensis* (Hemiptera, Triatominae) during the imaginal molt. **The American Journal of Tropical Medicine and Higiene**, v. 94, p. 689-690, 2016.

Reproductive biology of *Triatoma brasiliensis* (Hemiptera, Triatominae) during the imaginal molt

Abstract

The triatomines are vectors of the protozoan *Trypanosoma cruzi*, etiologic agent of Chagas disease. These insects are sexually active after the imaginal molt. Some aspects have been studied in *Triatoma brasiliensis* during the imaginal molt, such as autogeny in virgin females and the relationship between blood ingestion by N5 and the realization of the imaginal molt. Thus, in order to aid in the understanding of reproductive biology and developmental physiology of these vectors, this paper analyzes the spermatogenesis of *T. brasiliensis* during the imaginal molt. The analysis of the seminiferous tubules from males in the fifth instar during imaginal molt has demonstrated that *T. brasiliensis* has only a few spermatids and a plentiful quantity of sperm. Thus, we suggest that during imaginal molt the cell division is disrupted aiming to reduce energy costs and the differentiation into sperm is stimulated in order to ensure the paternity of the adult male.

Short Report

Chagas disease is a potentially life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi* and transmitted to humans by contact with faeces of triatomine bugs, known as 'kissing bugs'[1]. These vectors have a typical hemimetabolous life-cycle, from eggs through five nymphal instars (N1, N2, N3, N4, N5) to adult males and females. The transition from the fifth instar nymph to adult is named imaginal molt. During this process it occur some corporal changes, such as the emergence of wings[2], exocrine glands (metasternal and Brindley's glands)[3,4] and development of the reproductive system.[5, 6, 7, 8]

Some aspects have been studied in *Triatoma brasiliensis* Neiva, 1911 during the imaginal molt, such as autogeny in virgin females[8] and the relationship between blood ingestion by N5 and the realization of the imaginal molt.[9] This triatomine species is the most important Chagas disease vector in the Brazilian northeast.[10,11] Thus, in order to aid in the understanding of the reproductive biology and developmental physiology of these vectors, this paper analyzes the spermatogenesis of *T. brasiliensis* during the imaginal molt.

Five males in the fifth instar nymphs of *T. brasiliensis* were isolated and during imaginal molt their testicles were removed and fixed in methanol: acetic acid (3:1). 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 "Júlio de Mesquita Filho", Araraquara campus. The colony was formed from *T. brasiliensis* collected in intradomiciliary region of the municipality Olho d'Água, State of Paraiba, Brazil in the day April 17, 2008.

Seminiferous tubules were first shredded, smashed, and the microscope slides were set in liquid nitrogen. They were then stained with the lacto-acetic orcein cytogenetic technique.[11, 12] Based on the analysis of slides it was observed that the N5 nymphs, during imaginal molt, have only one of the phases of spermatogenesis, i.e., the spermiogenesis. This is represented by the presence of spermatids (Figure 1A-C) and sperm (Figure 1D).

Spermatogenesis is the process by which sperms are produced in the seminiferous tubules. It consists of three different phases: spermatocitogenesis, which is a phase of multiplication, meiosis, which is the division phase and spermiogenesis, which is the differentiation phase.[13]

Perez and collaborators,[14] reported that in some cases fifth instar nymph have mature gonads. Mello and collaborators,[15] analyzed fifth instar nymph of *T. infestans* and observed the presence of spermatogonia, spermatocytes (metaphase), spermatids and sperms. However, during imaginal molt of *T. brasiliensis* there are only a few spermatids and a plentiful quantity of sperm were observed, and we suggest that during imaginal molt the cell division is disrupted, aiming to reduce energy costs and the differentiation into sperm is stimulated in order to ensure the paternity of the adult male.

There are some offensive mechanisms that increase the chances to ensure the paternity, such as the characteristics of the genitalia,[16] the seminal fluid[17] and the courtship behavior.[18] Taking it into account, we suggest that the excessive increases of sperms during imaginal molt also increase the chances for the paternity.

Thus, during the imaginal molt it was observed that *T. brasiliensis* showed changes in the reproductive biology of development and physiology to decrease the energy cost, ensuring that the molt occur and mainly to increase the chance of paternity in adults. These results provide important information for understanding the biology of this important vector of the Chagas disease.

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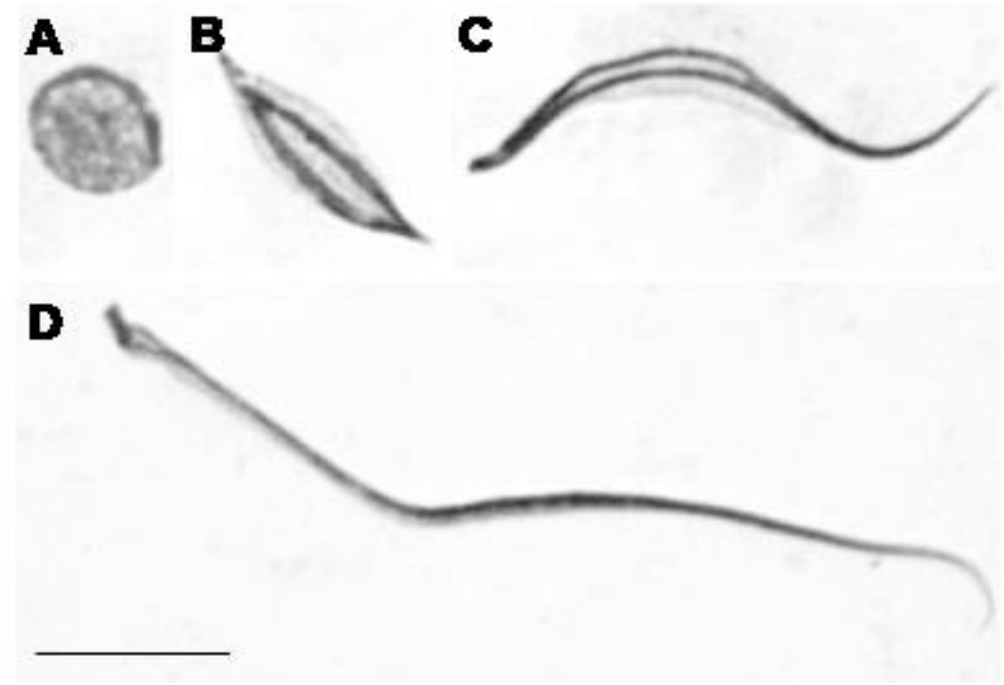

Figure 1: Spermiogenesis in *Triatoma brasiliensis*. Note the elongation of spermatids (A-C) and sperm (D). Bar: 10 μm.

4.7 Capítulo 7 (Artigo científico a ser submetido para publicação na revista *Parasites & Vectors* FI 3,03)

Tracking the chromosomal diversification in *Triatoma brasiliensis* complex (Hemiptera, Triatominae)

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Abstract

Background

Based mainly in morphological similarities and geographical distribution, the triatomines were grouped in complexes and specifics subcomplexes. *Triatoma brasiliensis* complex is a monophyletic group comprised of the species *T. brasiliensis*, *T. sherlocki*, *T. melanica*, *T. juazeirensis*, *T. lenti*, *T. bahiensis*, *T. petrocchiae* and the subspecie *T. b. macromelasoma*. *T. bahiensis* and *T. petrocchiae* were recently grouped in *T. brasiliensis* complex mainly by means of phylogenetic analyses. This way, this study has as objective tracking the chromosomal diversification in *T. brasiliensis* complex by means of cytogenomics analyses of the composition of DNA base pairs.

Findings

All species of the *T. brasiliensis* complex presented the same composition of base pairs: X sex chromosomes and ends of autosomes rich in CG and Y sex chromosome rich in AT. Furthermore, all species showed a chromocenter formed by the X and Y sex chromosomes plus a pair of autosomes, as well as initially suggested by C-banding.

Conclusions

Thus, we present a genomic characteristic that allows differentiate the species of the *T. brasiliensis* complex from all other complexes and subcomplexes of triatomines of the South America. Furthermore, we confirm the position of *T. bahiensis* and *T. petrocchiae* as member of the *T. brasiliensis* complex.

SHORT REPORT

Background

Chagas disease is caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909) and distributed in endemic areas of 21 Latin American countries, where it is mostly vector-borne

transmitted to humans by contact with faeces of triatomines [1]. There are 151 species of triatomines distributed in 18 genera and five tribes (all of the species are considered potential vectors of Chagas disease) [2, 3, 4, 5]. In Brazil, there are 67 species distributed throughout the country's 27 states, and 64% of them of endemic species [2, 4, 5].

Based on morphological similarities and geographical distribution these triatomines were grouped in complexes and subcomplexes [6]. Although these groupings are not formally recognized as taxonomic ranks and, thus, do not necessarily represent natural groups, Justi et al. [7] propose that they should be monophyletic, because once the relationships between vector species are known, information about a species may be reliably extrapolated to other closely related species [8].

The *T. brasiliensis* complex is a monophyletic group comprised of the species *T. brasiliensis*, *T. sherlocki*, *T. melanica*, *T. juazeirensis*, *T. lenti*, *T. bahiensis* and the subspecies *T. b. macromelasoma* [3]. Although there are many studies which support the groupings of the species at this complex, as for example, morphological relationships [9], and isoenzymes [10], the only study which takes into account *T. lenti* and *T. bahiensis* as members of the *T. brasiliensis* complex is Mendonça et al. [3], by means of phylogenetic and cytogenetic analyses. Moreover, *T petrocchiae* shares many morphological characteristics with *T brasiliensis* [11], presents the same cytogenetic characteristics (karyotype and heterochromatin pattern) of *T. brasiliensis* complex members [12] and was suggested as a member of this complex by morphometric and phylogenetic analyzes [13].

This way, this study has as objective tracking the chromosomal diversification in *T. brasiliensis* complex and evaluates the relationship between *T. bahiensis* and *T. petrocchiae* with the species of this complex, by means of cytogenomics analyses of the composition of DNA base pairs.

Methods

Were analyzed at least three adult male of each species [*T. brasiliensis* (10), *T. b. macromelasoma* (10), *T. juazeirensis* (8), *T. lenti* (10), *T. melanica* (5), *T. bahiensis* (5), *T. petrocchiae*, (10), *T. sherlocki* (10)] that were provided by the Insectarium of Triatominae, from FCFAR/UNESP, Araraquara, São Paulo, Brazil. The bugs were dissected, the seminiferous tubules were torn apart, crushed and fixed in the blades in liquid nitrogen. They then underwent the cytogenomic technique of CMA_3/DAPI banding [14] for differentiating

the regions of heterochromatin rich in AT (DAPI+) and CG (CMA+). The biological material was analyzed using in fluorescence microscopy Zeiss-Axioskop and Olympus BX-FLA.

Results and discussion

All species analyzed presented same composition of base pairs: X sex chromosomes and ends of autosomes rich in CG (Figure 1A) and Y sex chromosome rich in AT (Figure 1B). One of the characteristics cytogenetic considered as diagnostic for differentiating of the *T. brasiliensis* complex of all other complexes and subcomplexes of triatomines is the presence of a chromocenter formed by the X and Y sex chromosomes plus a pair of autosomes [12, 15, 16]. This characteristic is confirmed by our analysis who show the pair of autosomes and the X sex chromosome of the chromocenter rich in CG (Figure 1C, arrows and X, respectively) and the Y sex chromosome rich in AT (Figure 1C, Y).

The relation between *T. lenti* with the *T. brasiliensis* complex species has also been initially proposed based on geographic distribution [11], morphological data [6] and cytogenetic data [15, 16]. Only recently, with the realization of phylogenetic analysis is that the grouping of the species as the sixth member of the complex was confirmed [3]. *T. bahiensis* has recently been revalidated [3, 17] and DNA basis pair composition supports the grouping of the species as the seventh member of the complex. *T. petrocchiae* for a long time was considered synonym of *T. brasiliensis* [11], being the specific status of *T. petrocchiae* revalidated by experimental crosses [18] and DNA basis pair composition also supports he grouping of the species as the eighth member of the complex.

Conclusion

Thus, we present a genomic characteristic that allows differentiate the species of the *T. brasiliensis* complex from all other complexes and subcomplexes of triatomines of the South America. Furthermore, we confirm the position of *T. bahiensis* and *T. petrocchiae* as member of the *T. brasiliensis* complex.

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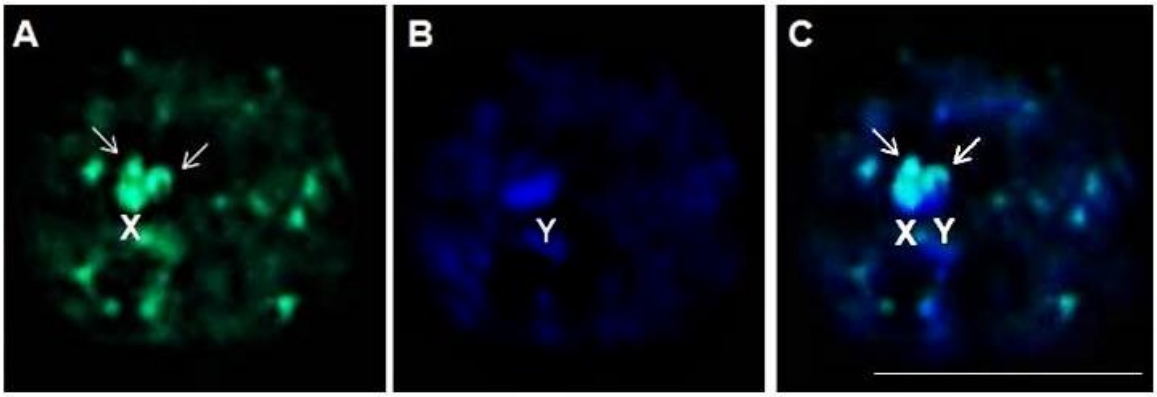

Fig. 1. Composition of DNA base pairs in *T. brasiliensis* complex represented by *T. bahiensis*. Note the X sex chromosomes and ends of autosomes rich in CG (A) and Y sex chromosome rich in AT (B). Furthermore, in the overlapping of the images (C), note that the chromocenter is formed by the X and Y sex chromosomes plus a pair of autosomes (arrows). Bar: 10 μm.

4.8 Capítulo 8 (Artigo científico publicado na revista *Genetics and Molecular Research* FI 0,98)

ALEVI, K.C.C.; REIS, Y.V.; BORGUETI, A.O.; MENDONÇA, V.J.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Diploid chromosome set of kissing bug *Triatoma baratai* (Hemiptera, Triatominae). **Genetics and Molecular Research**, v. 14, p. 1106-1110, 2015.

Diploid chromosome set of kissing bug *Triatoma baratai* (Hemiptera, Triatominae)

ABSTRACT

Triatomines are insects that are taxonomically included in the Hemiptera order and Triatominae subfamily. Based on phenotypic similarity, capacity hybridization, and genetic and ecological aspects, the triatomine species can be grouped into specific complexes and subcomplexes. However, these groupings have not been confirmed. Cytogenetic analyses are important cytotaxonomic tools for improving the taxonomic knowledge of triatomines. Thus, we examined the karyotype of *Triatoma baratai* and compared the results with those of other species in the Matogrossensis subcomplex in order increase the understanding of vector potential. We also examined the cytotaxonomic classification of this insect. *Triatoma baratai*, similarly to other species that currently compose the Matogrossensis subcomplex, contains 22 chromosomes (20A + XY). Here, we describe the diploid chromosome set of *T. baratai*. We confirmed their current classification in the Matogrossensis subcomplex and demonstrated that the species in this subcomplex present karyotype homogeneity.

INTRODUCTION

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

This illness is regarded as the second-most endemic disease in Latin America. In Brazil, approximately 2.5 million individuals are infected with *T. cruzi* (Neto and Pasternak,

2009). Because there is no specific treatment for Chagas disease, control of vector populations is considered as the main approach to minimizing the incidence of this disease (Hotez et al., 2012).

Deforestation and forest burning are important factors that influence the migration of vectors into urban regions. These organisms move to regions peridomiciliary and indoors in search of shelter and food, increasing the risk of disease infection (Dias and Schofield, 1998).

Based on phenotypic similarity, capacity hybridization, and genetic and ecological aspects, the triatomine species can be grouped into specific complexes and subcomplexes (Schofield and Galvão, 2009). However, new studies are still required to prove or disprove the groupings (Obara et al., 2012), because the correct classification of triatomines allows vector control programs focus on major vector species of Chagas disease (Alevi et al., 2012b).

Cytogenetic analyzes are important cytotaxonomic tools (Ueshima et al., 1966; Pérez et al., 1992) that can be used to increase the phylogenetic understanding of triatomines. Using cytogenetic data, we excluded *Triatoma melanocephala*, *T. tibiamaculata*, and *T. vitticeps*, confirmed the inclusion of *T. sherlocki* and suggest the inclusion of *T. lenti* from the Brasiliensis subcomplex (Alevi et al., 2012a, b; 2013a, b, c, d, e; 2014a, b). Moreover, the taxonomic classification of other subcomplexes was also verified through cytogenetic analysis, such as for the Infestans subcomplex (*T. infestans*, *T. platensis*, and *T. delpontei*) and Sordida subcomplex (*T. sordida*, *T. patagonica*, and *T. guasayana*) (Panzera et al., 1995; 1997).

The Matogrossensis subcomplex is the new designation for the *T. oliverai* complex as proposed by Carcavallo et al. (2000). This subcomplex includes 9 species groups (Table 1) (Schofield and Galvão, 2009; Gardim et al., 2013; Gonçalves et al., 2013) from the Midwest region of Brazil (Obara et al., 2012).

The species *T. baratai* is present in the State of Mato Grosso do Sul (Gurgel-Gonçalves et al., 2012) and although considered wild were found to inhabit homes (Obara et al., 2012).

Thus, the present study describes for the first time, the karyotype of *T. baratai* and compares with that of other species that make up the Matogrossensis subcomplex in order to enrich the knowledge about the biology of the vector potential and, mainly, auxiliary in the classification of this organism.

MATERIAL AND METHODS

Two *T. baratai* males, assigned by the "Triatominae Insectarium" installed at the Department of Biological Sciences, Faculty of Pharmaceutical Sciences, Araraquara campus, were included in this study. After removing and fixing seminiferous tubules of adult males on a cover slip, lacto-acetic-orcein was used for cytogenetic analysis (De Vaio et al., 1985), with modifications according to Alevi et al. (2012b). The biological material was analyzed using a Jenaval light microscope (Carl Zeiss AG, Jena, Germany) coupled to a digital camera and an image analyzer Axio Vision LE 4.8 (Carl Zeiss). The images were magnified by a factor of 100.

RESULTS

Based on the analysis of metaphase I (Figure 1) and II, male *T. baratai* presented a set diploid chromosome 2n = 22 (20A + XY). This chromosome number is common to all members of the Matogrossensis subcomplex (Table 1).

DISCUSSION

Currently, 86 karyotypes of triatomines have been described in the literature (Alevi et al., 2013f). All species of the tribe Rhodiniini contain 22 chromosomes (20A + XY) (Panzera et al., 2010; Alevi et al., 2013f; Pita et al., 2013; Alevi et al. 2015). The genus *Panstrongylus* show two karyotypic variations, *P. megistus* with 2n = 18A + X_1X_2Y (Schreiber and Pellegrino, 1950) and *P. lutzi* with 2n = 20A + $X_1X_2X_3Y$ (Santos, 2010). The genus *Triatoma* shows the most diverse karyotypes found in the Triatominae subfamily, including 2n = 21, 22, 23, 24, and 25 chromosomes (Alevi et al., 2013f).

The number of chromosomes, although initially described as homogeneous in the Triatominae subfamily (Ueshima, 1966), is an important tool in systematic triatomines. Phylogeny can be examined from a karyosystematic perspective because although it is thought that the common ancestor possessed 2n = 22 chromosomes, numerical variations exist between organisms classified based on morphological and geographical traits as closely related species. This was observed in the Brasiliensis subcomplex (Schofield and Galvão, 2009; Alevi et al., 2012b).

Although species complexes are not formally recognized as taxonomic ranks and, thus, do not necessarily represent natural groups, that they should be monophyletic (Just et al., 2014). This underscores the importance of using different tools to assess the initial classification of these organisms. *Triatoma baratai*, similarly to all other species that

currently compose the Matogrossensis subcomplex has 22 chromosomes (20 autosomes and 2 sex chromosomes) (Table 1). Therefore, in contrast to the Protracta, Lectularia, and Rubrofasciata complexes, the Matogrossensis subcomplex shows chromosomal homogeneity. Thus, the diploid chromosome set of *T. baratai*, confirms their current classification in the Matogrossensis subcomplex and demonstrates that the species in the subcomplex show karyotype homogeneity.

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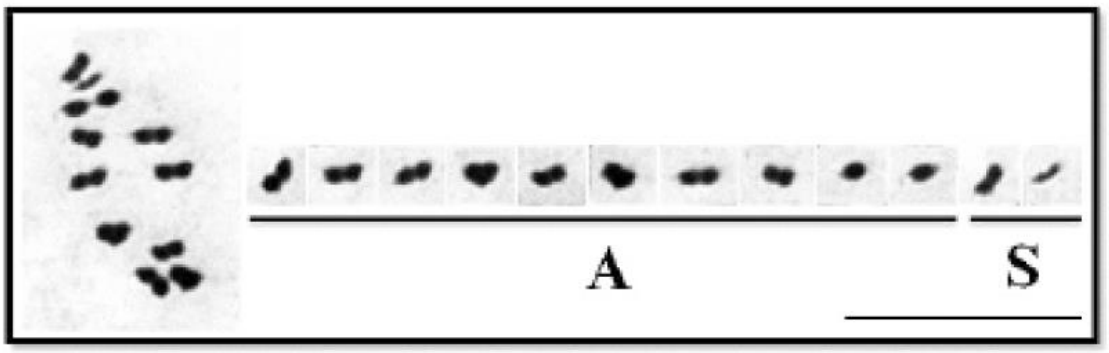

Figure 1. Karyotype of *Triatoma baratai* based on metaphase I with 22 chromosomes (20A + XY). A: autosomes. S: sex chromosomes. Bar: 10 µm.

Table I. Species grouped in Martogrossensis subcomplex and their respective karyotypes.

MATOGROSSENSIS SUBCOMPLEX	KARYOTYPE	DESCRIBED BY:
Triatoma baratai	2n= 22 (20A + XY)	This report
Triatoma costalimai	2n= 22 (20A + XY)	Dujardin et al. (2002)
Triatoma deaneorum	Not described	
Triatoma guazu	2n= 22 (20A + XY)	Dujardin et al. (2002)
Triatoma jatai	Not described	
Triatoma jurbergi	2n= 22 (20A + XY)	Dujardin et al. (2002)
Triatoma matogrossensis	2n= 22 (20A + XY)	Pérez et al. (1992)
Triatoma vandae	2n= 22 (20A + XY)	Panzera et al. (2010)
Triatoma williami	2n= 22 (20A + XY)	Dujardin et al. (2002)

4.9 Capítulo 9 (Artigo científico publicado na revista *Genetics and Molecular Research* FI 0,98)

ALEVI, K. C. C.; MOREIRA, F. F. F.; JURBERG, J.; AZEREDO-OLIVEIRA, M. T. V. Description of the diploid chromosome set of *Triatoma pintodiasi* (Hemiptera, Triatominae). **Genetics and Molecular Research**, v. 15, p. 01-10, 2016.

Description of the diploid chromosome set of *Triatoma pintodiasi* (Hemiptera, Triatominae)

ABSTRACT

Triatoma pintodiasi has been described and recently grouped in the Rubrovaria subcomplex. *T. pintodiasi* was initially compared to *T. carcavalloi* by staining and subsequently identified as *T. circummaculata*. However, after thorough examination, it was observed to be a cryptic species of *T. circummaculata*, and was described based on morphological features, morphometric data, and biochemical patterns of hemolymph. Thus, this paper aims to describe the karyotype of, and spermatogenesis in, *T. pintodiasi*, in order to elucidate the reproductive biology and taxonomy of the species. Sex chromosomes of *T. pintodiasi* formed a heteropyknotic chromocenter, and compaction of chromatin was observed during prophase. However, in contrast to observations in *T. carcavalloi* and *T. circummaculata*, in *T. pintodiasi* was observed individualization of the sex chromosomes. The diploid chromosome set of the species 2n = 22 (20A + XY) is described through analysis of metaphase I and II. Initial cytogenetic characteristics of *T. pintodiasi* are described and the observed differences in the chromocenter are suggested as a possible cytotaxonomic tool. To gain a better understanding of the specific status of this cryptic species, however, we emphasize the need for further cytogenetic, molecular, biological, and biogeographical analysis, in addition to experimental hybrid crosses with other species of the Rubrovaria subcomplex.

INTRODUCTION

The triatomines are insects of great epidemiological interest. The 148 species currently described (Abad-Franch et al., 2013; Alevi et al., 2013a; Jurberg et al., 2013; Poinar, 2013) are potential vectors of the protozoan *Trypanosoma cruzi*, the etiologic agent of Chagas

disease. Triatomines are also of biological interest because their cells have several peculiarities in comparison to cells of other eukaryotes, such as holocentric chromosomes (Panzera et al., 1996), inverted meiosis for sex chromosomes (Gómez-Palacio et al., 2008), and persistence of nucleolar material during meiosis (Tartarotti and Azeredo-Oliveira, 1999; Alevi et al., 2014a). In addition, triatomines are of evolutionary interest because their origins (monophyletic or polyphyletic) have not been conclusively determined (Tartarotti et al., 2006; Hwang and Weirauch, 2012).

For over 50 years, these hematophagous insects have been grouped into both complexes and specific subcomplexes (Lucena, 1970; Dujardin et al., 2002; Schofield and Galvão, 2009; Justi et al., 2014). Although species complexes and subcomplexes are not formally recognized as taxonomic ranks, they should be monophyletic (Justi et al., 2014).

Schofield and Galvão (2009) proposed that the Rubrovaria subcomplex is composed of the species *Triatoma carcavalloi*, *T. circummaculata*, *T. klugi*, *T. limai*, *T. oliveirai*, and *T. rubrovaria*. The species of the Rubrovaria subcomplex were initially grouped through morphological analyses: *T. rubrovaria* and *T. carcavalloi* categorized in the Rubrovaria subcomplex; *T. circummaculata* and *T. limai* in the Circummaculata complex; and *T. klugi* in the Oliverai complex (Dujardin et al., 2002). Almeida et al. (2009) grouped *T. circummaculata* in subcomplex Rubrovaria based on phylogenetic and molecular data, and Gardim et al. (2013) and Justi et al. (2014) identified the subcomplex Rubrovaria as monophyletic. However, new approaches such as experimental hybrid crosses and cytogenetic analyzes are important and necessary, to assist in understanding the evolutionary development of these vectors.

A new species, *T. pintodiasi*, has recently been described and grouped in Rubrovaria subcomplex (Jurberg et al., 2013). *T. pintodiasi* was initially compared to *T. carcavalloi* based on staining, but was found to be morphologically smaller. Subsequently, *T. pintodiasi* was classified as *T. circummaculata*. However, after thorough examination, it was observed to be a cryptic species of *T. circummaculata*, and was described based on morphological features, morphometric data, and biochemical patterns of hemolymph (Jurberg et al., 2013). The process of spermatogenesis and the karyotype of *T. pintodiasi* are described in the present study, with the aim of acquiring a detailed understanding of the reproductive biology and taxonomy of the species. In addition, a chromosomal review of all triatomine species is presented with karyotype descriptions.

MATERIAL AND METHODS

At least three adult males each of *T. pintodiasi*, *T. carcavalloi*, and *T. circummaculata* were analyzed. The specimens of *T. pintodiasi* were provided by the National Laboratory and International Reference on Taxonomy of Triatominae, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, and the species of *T. carcavalloi* and *T. circummaculata* were provided by Insectarium of Triatominae, FCFAR / UNESP, Araraquara, São Paulo, Brazil. Seminiferous tubules of *T. pintodiasi*, *T. carcavalloi*, and *T. circummaculata* were isolated, shredded, smashed, and set on a slide in liquid nitrogen. They were then stained by the cytogenetic technique using lacto-acetic orcein as outlined by De Vaio et al. (1985), with modifications according to Alevi et al. (2012a). The biological material was analyzed by a 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 GmbH). The images were subjected to 1000X magnification. All karyotypes described in the literature are presented in Table 1.

RESULTS

The early stages of meiosis in spermatogenesis were described by means of classical cytogenetic analysis. Compaction of chromatin (Figure 1A–C, 2A) was observed during prophase, with the formation of chiasma between autosomes (Figure 1C).

The sex chromosomes of *T. pintodiasi* formed a heteropyknotic chromocenter (Figure 1A–C, 2A) however, individualization remained throughout prophase. This observation was not consistent with that for *T. carcavalloi* (Figure 2B) and *T. circummaculata* (Figure 2C). The diploid chromosome set of the species 2n = 22 (20A + XY) (Figure 1C and D, respectively), was determined by analysis of metaphase I and II. In both metaphase I and II, the Y sex chromosomes of *T. pintodiasi* were larger and more heteropyknotic (Figure 1C, D; arrows).

Analysis of the species presented in Table 1, revealed that two species have 21 chromosomes (2n = 18 + X_1X_2Y); 51 species have 22 chromosomes (2n = 20 + XY); 30 species have 23 chromosomes (2n = 20 + X_1X_2Y); four species have 24 chromosomes (2n = 20 + $X_1X_2X_3Y$); and only one species has 25 chromosomes (2n = 22 + X_1X_2Y).

DISCUSSION

In cryptic speciation, the resulting species show great morphological similarity. Specific tools are necessary to differentiate between cryptic species. It was possible to

distinguish the sex chromosomes in a heteropyknotic chromocenter during meiotic prophase in *T. pintodiasi*. This feature is quite peculiar in the subfamily Triatominae, since most triatomines have only one heteropyknotic chromocenter, as observed in *T. carcavalloi* and *T. circummaculata* (Panzera et al., 1998).

T. pintodiasi presented a karyotype consisting of 22 chromosomes, consistent with that observed for all species of the Rubrovaria subcomplex (Ueshima, 1966; Panzera et al., 1996; Alevi et al., 2013a). Although this number can vary from 21 to 25 chromosomes, 22 has been described as the modal number of chromosomes for the Triatominae subfamily (Ueshima, 1966) (Table 1). Ueshima (1966) also proposed that the type number for Triatominae is 22 chromosomes (20A + XY). Ueshima (1979) suggested that the common ancestor of the triatomines showed 22 chromosomes. In addition, Nokkala and Nokkala (1983, 1984) believe that the common ancestor of the Hemiptera order had system sex determination of type XY. Thus, based on the principle that the common ancestor has 22 chromosomes, we suggest that major events that occurred during the karyotype evolution of these insects included mainly agmatoploidy (fission) and simploidy (fusion).

Cytogenetic analyses of triatomines are of great importance, because chromosome data, characteristics of spermatids, and meiotic features can be applied to determine cytotaxonomy of these vectors. Analysis of early prophase for example, can aid in differentiation between *T. sordida* and *T. guasayana* (Rebagliati et al., 1998). Cytogenetic data have also assisted in the revalidation of *T. garciabesi* (Jurberg et al., 1998); in the description of *Mepraia parapatrica* (Frías-Lasserre, 2010); and more recently, by analysis of spermatids, in the differentiation of morphologically related species (Alevi et al., 2013b, 2014b).

The present study describes initial cytogenetic characteristics of *T. pintodiasi* and suggests observed differences in the chromocenter, as a possible cytotaxonomic tool. The authors emphasize the need for further cytogenetic, molecular, biological, and biogeographical analysis, in addition to experimental hybrid crosses with other species of the Rubrovaria subcomplex.

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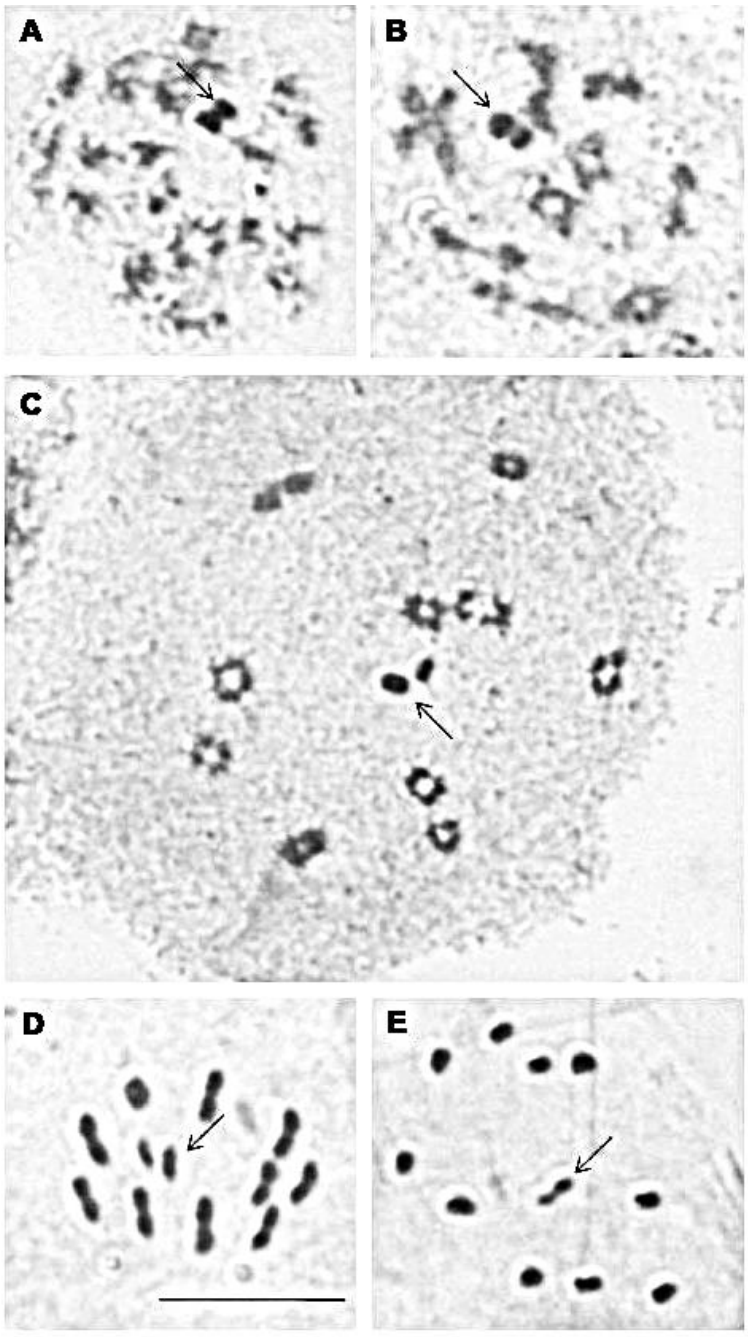

Figure 1. Meiosis of *T. pintodiasi*. Diffuse intermediate stage (zygotene/pachytene) (A, B) and diffuse final stage (diplotene) (C) with a chromocenter that made it possible to distinguish the two sex chromosomes (arrows). Metaphase I (C) and metaphase II (D) with 22 chromosomes (20 autosomes + XY). Note the Y sex chromosome more heteropycnotic (arrow). Bar: 10 μm.

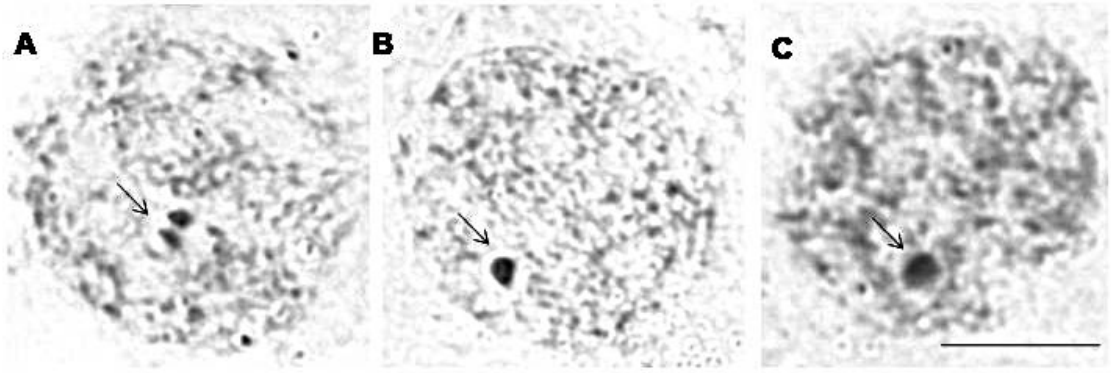

Figure 2. Prophase (stage diffuse initial) of the *T. pintodiasi* (A), *T. carcavalloi* (B) and *T. circummaculata* (C). Note that *T. pintodiasi* presented a chromocenter that made it possible to distinguish the two sex chromosomes (A, arrow) while *T. carcavalloi* (B, arrow) and *T. circummaculata* (C, arrow) showed a single chromocenter formed by the sex chromosomes. Bar: 10 μm.

Table I. Review of all species of triatomines with that described karyotype.

Nº	TRIATOMINES	KARYOTYPE	DESCRIBED BY:
	TRIBE ALBERPROSENIINI		
	Genus *Alberprosenia*		
1	*Alberprosenia goyovargasi*	Not described	
2	*Alberprosenia malheiroi*	Not described	
	TRIBE BOLBODERINI		
	Genus *Belminus*		
3	*Belminus corredori*	Not described	
4	*Belminus costaricensis*	Not described	
5	*Belminus ferroae*	Not described	
6	*Belminus herreri*	Not described	
7	*Belminus laportei*	Not described	
8	*Belminus peruvianus*	Not described	
9	*Belminus pittieri*	Not described	
10	*Belminus rugulosus*	Not described	

Genus *Bolbodera*

11	*Bolbodera scabrosa*	Not described

Genus *Microtriatoma*

12	*Microtriatoma borbai*	Not described
13	*Microtriatoma trinidadensis*	Not described

Genus *Parabelminus*

14	*Parabelminus carioca*	Not described
15	*Parabelminus yurupucu*	Not described

TRIBE CAVERNICOLINI

Genus *Cavernicola*

16	*Cavernicola lenti*	Not described
17	*Cavernicola pilosa*	Not described

TRIBE LINSHCOSTEINI

Genus *Linshcosteus*

18	*Linshcosteus carnifex*	Not described
19	*Linshcosteus chota*	Not described

20	*Linshcosteus confumus*	Not described	
21	*Linshcosteus costalis*	Not described	
22	*Linshcosteus kali*	Not described	
23	*Linshcosteus karupus*	Not described	
	TRIBE RHODNIINI		
	Genus *Psammolestes*		
24	*Psammolestes arthuri*	Not described	
25	*Psammolestes coreodes*	2n = 22 (20A + XY)	Schreiber and Pellegrino, 1950
26	*Psammolestes tertius*	2n = 22 (20A + XY)	Panzera et al., 1998
	Genus *Rhodnius*		
27	*Rhodnius amazonicus*	Not described	
28	*Rhodnius barretti*	Not described	
29	*Rhodnius brethesi*	2n = 22 (20A + XY)	Panzera et al., 1998
30	*Rhodnius colombiensis*	2n = 22 (20A + XY)	Dujardin et al., 2002
31	*Rhodnius dalessandroi*	Not described	
32	*Rhodnius domesticus*	2n = 22 (20A + XY)	Dujardin et al., 2002

33	*Rhodnius ecuadoriensis*	2n = 22 (20A + XY)	Scvortzoff et al., 1996
34	*Rhodnius milesi*	2n = 22 (20A + XY)	Panzera et al., 2010
35	*Rhodnius montenegrensis*	2n = 22 (20A + XY)	Alevi et al., 2015a
36	*Rhodnius nasutus*	2n = 22 (20A + XY)	Pérez et al., 1992
37	*Rhodnius. neglectus*	2n = 22 (20A + XY)	Barth, 1956
38	*Rhodnius neivai*	2n = 22 (20A + XY)	Koshy, 1979a
39	*Rhodnius pallescens*	2n = 22 (20A + XY)	Panzera et al., 1996
40	*Rhodnius paraensis*	Not described	
41	*Rhodnius pictipes*	2n = 22 (20A + XY)	Koshy, 1979b
42	*Rhodnius prolixus*	2n = 22 (20A + XY)	Schreiber and Pellegrino, 1950
43	*Rhodnius robustus*	2n = 22 (20A + XY)	Koshy, 1979b
44	*Rhodnius stali*	2n = 22 (20A + XY)	Dujardin et al., 2002
45	*Rhodnius zeledoni*	Not described	

TRIBE TRIATOMINI

Genus *Dipetalogaster*

46	*Dipetalogaster máxima*	2n = 22 (20A + XY)	Ueshima, 1966

Genus *Eratyrus*

47	*Eratyrus cuspidatus*	2n = 23 (20A + X_1X_2Y)	Dujardin et al., 2002
48	*Eratyrus mucronatus*	2n = 23 (20A + X_1X_2Y)	Dujardin et al., 2002

Genus *Hermanlentia*

49	*Hermanlentia matsunoi*	Not described	

Genus *Meccus*

50	*Meccus bassolsae*	2n = 23 (20A + X_1X_2Y)	Dujardin et al., 2002
51	*Meccus longipennis*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 1996
52	*Meccus mazzottii*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 1996
53	*Meccus pallidipennis*	2n = 23 (20A + X_1X_2Y)	Ueshima, 1966
54	*Meccus phyllosomus*	2n = 23 (20A + X_1X_2Y)	Dujardin et al., 2002
55	*Meccus picturatus*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 1996

Genus *Mepraia*

56	*Mepraia gajardoi*	2n = 23 (20A + X_1X_2Y)	Frías et al., 1998
57	*Mepraia parapatrica*	2n = 23 (20A + X_1X_2Y)	Frías-Lasserre, 2010
58	*Mepraia spinolai*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 1998

Genus *Nesotriatoma*

59	*Nesotriatoma bruneri*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 2010
60	*Nesotriatoma flavida*	2n = 23 (20A + X_1X_2Y)	Dujardin et al., 2002
61	*Nesotriatoma obscura*	Not described	

Genus *Paratriatoma*

62	*Paratriatoma hirsuta*	2n= 22 (20A + XY)	Ueshima, 1966

Genus *Panstrongylus*

63	*Panstrongylus chinai*	2n = 23 (20A + X_1X_2Y)	Pérez et al., 2002
64	*Panstrongylus diasi*	Not described	
65	*Panstrongylus geniculatus*	2n = 23 (20A + X_1X_2Y)	Pérez et al., 2002
66	*Panstrongylus guentheri*	Not described	
67	*Panstrongylus hispaniolae*	Not described	
68	*Panstrongylus howardi*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 2010
69	*Panstrongylus humeralis*	Not described	
70	*Panstrongylus lenti*	Not described	
71	*Panstrongylus lignarius*	2n = 23 (20A + X_1X_2Y)	Pérez et al., 2002

72	*Panstrongylus lutzi*	2n = 24 (20A + $X_1X_2X_3Y$)	Santos, 2010
73	*Panstrongylus megistus*	2n = 21 (18A + X_1X_2Y)	Schreiber and Pellegrino, 1950
74	*Panstrongylus mitarakaensis*	Not described	
75	*Panstrongylus rufotuberculatus*	2n = 23 (20A + X_1X_2Y)	Pérez et al., 2002
76	*Panstrongylus tupynambai*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 1998

Genus *Triatoma*

77	*Triatoma amicitiae*	Not described	
78	*Triatoma arthurneivai*	2n = 22 (20A + XY)	Dujardin et al., 2002
79	*Triatoma baratai*	2n = 22 (20A + XY)	Alevi et al., 2015b
80	*Triatoma barberi*	2n = 23 (20A + X_1X_2Y)	Ueshima, 1966
81	*Triatoma bolivari*	Not described	
82	*Triatoma boliviana*	Not described	
83	*Triatoma bouvieri*	Not described	
84	*Triatoma brailovskyi*	Not described	
85	*Triatoma brasiliensis*	2n = 22 (20A + XY)	Schreiber and Pellegrino, 1950
86	*Triatoma b. macromelanosoma*	2n = 22 (20A + XY)	Panzera et al., 2000

87	*Triatoma breyeri*	Not described	
88	*Triatoma carcavalloi*	2n = 22 (20A + XY)	Dujardin et al., 2002
89	*Triatoma carrioni*	Not described	
90	*Triatoma cavernicola*	Not described	
91	*Triatoma circummaculata*	2n = 22 (20A + XY)	Panzera et al., 1998
92	*Triatoma costalimai*	2n = 22 (20A + XY)	Dujardin et al., 2002
93	*Triatoma deaneorum*	Not described	
94	*Triatoma delpontei*	2n = 22 (20A + XY)	Ueshima, 1966
95	*Triatoma dimidiata*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 1994
96	*Triatoma dispar*	Not described	
97	*Triatoma eratyrusiformis*	2n = 24 (20A + $X_1X_2X_3Y$)	Ueshima, 1966
98	*Triatoma garciabesi*	2n = 22 (20A + XY)	Panzera et al., 1997
99	*Triatoma gerstaeckeri*	2n = 23 (20A + X_1X_2Y)	Ueshima, 1966
100	*Triatoma gomeznunezi*	Not described	
101	*Triatoma guasayana*	2n = 22 (20A + XY)	Panzera et al., 1996
102	*Triatoma guazu*	2n = 22 (20A + XY)	Dujardin et al., 2002

103	*Triatoma hegneri*	2n = 23 (20A + X_1X_2Y)	Dujardin et al., 2002
104	*Triatoma incrassata*	Not described	
105	*Triatoma indictiva*	Not described	
106	*Triatoma infestans*	2n= 22 (20A + XY)	Schreiber and Pellegrino, 1950
107	*Triatoma i. melanosoma*	2n= 22 (20A + XY)	Panzera et al., 1996
108	*Triatoma jataí*	Not described	
109	*Triatoma juazeirensis*	2n = 22 (20A + XY)	Panzera et al., 2000
110	*Triatoma jurbergi*	2n = 22 (20A + XY)	Dujardin et al., 2002
111	*Triatoma klugi*	2n = 22 (20A + XY)	Costa et al., 2008
112	*Triatoma lecticularia*	2n = 22 (20A + XY)	Ueshima, 1966
113	*Triatoma lenti*	2n = 22 (20A + XY)	Alevi et al., 2012a
114	*Triatoma leopoldi*	Not described	
115	*Triatoma limai*	Not described	
116	*Triatoma maculata*	2n = 22 (20A + XY)	Schreiber and Pellegrino, 1950
117	*Triatoma matogrossensis*	2n = 22 (20A + XY)	Pérez et al., 1992
118	*Triatoma melanica*	2n = 22 (20A + XY)	Panzera et al., 2000

119	*Triatoma melanocephala*	2n = 24 (20A + $X_1X_2X_3Y$)	Alevi et al., 2012b
120	*Triatoma mexicana*	2n = 23 (20A + X_1X_2Y)	Panzera et al., 2010
121	*Triatoma migrans*	Not described	
122	*Triatoma neotomae*	Not described	
123	*Triatoma nigromaculata*	Not described	
124	*Triatoma nitida*	2n = 21 (18A + X_1X_2Y)	Schreiber and Pellegrino, 1950
125	*Triatoma oliveirai*	Not described	
126	*Triatoma patagonica*	2n = 22 (20A + XY)	Ueshima, 1966
127	*Triatoma peninsularis*	2n = 23 (20A + X_1X_2Y)	Ueshima, 1966
128	*Triatoma petrochiae*	2n = 22 (20A + XY)	Panzera et al., 2000
129	*Triatoma pintodiasi*	2n = 22 (20A + XY)	This paper
130	*Triatoma platensis*	2n = 22 (20A + XY)	Schreiber and Pellegrino, 1950
131	*Triatoma protracta*	2n = 23 (20A + X_1X_2Y)	Ueshima, 1966
132	*Triatoma pugasi*	Not described	
133	*Triatoma pseudomaculata*	2n = 22 (20A + XY)	Schreiber et al., 1972
134	*Triatoma recurva*	Not described	

135	*Triatoma rubida*	2n = 23 (20A + X_1X_2Y)	Ueshima, 1966
136	*Triatoma rubrofasciata*	2n = 25 (22A + X_1X_2Y)	Manna, 1950
137	*Triatoma rubrovaria*	2n = 22 (20A + XY)	Schreiber and Pellegrino, 1950
138	*Triatoma ryckmani*	2n = 23 (20A + X_1X_2Y)	Dujardin et al., 2002
139	*Triatoma sanguisuga*	2n = 23 (20A + X_1X_2Y)	Payne, 1909
140	*Triatoma sherlocki*	2n = 22 (20A + XY)	Panzera et al., 2010
141	*Triatoma sinaloensis*	2n = 23 (20A + X_1X_2Y)	Ueshima, 1066
142	*Triatoma sinica*	Not described	
143	*Triatoma sordida*	2n = 22 (20A + XY)	Schreiber and Pellegrino, 1950
144	*Triatoma tibiamaculata*	2n = 22 (20A + XY)	Panzera et al., 1998
145	*Triatoma vandae*	2n = 22 (20A + XY)	Panzera et al., 2010
146	*Triatoma venosa*	Not described	
147	*Triatoma vitticeps*	2n = 24 (20A + $X_1X_2X_3Y$)	Schreiber and Pellegrino, 1950
148	*Triatoma williami*	2n = 22 (20A + XY)	Dujardin et al., 2002
149	*Triatoma wygodzinskyi*	2n = 22 (20A + XY)	Panzera et al., 2012
150	*Triatoma dominicana*	Not described	

4.10 Capítulo 10 (Artigo científico publicado na *The American Journal of Tropical Medicine & Hygiene* FI 2,54)

ALEVI, K. C. C.; GUERRA, A. L.; IMPERADOR, C. H. L.; JURBERG, J.; MOREIRA, F. F. F.; AZEREDO-OLIVEIRA, M. T. V. Mitochondrial gene confirms the specific status of *Triatoma pintodiasi* Jurberg, Cunha & Rocha, 2013 (Hemiptera, Triatominae), an endemic species in Brazil. **The American Journal of Tropical Medicine & Hygiene**, v. 96, p. 200-201, 2016.

Mitochondrial gene confirms the specific status of *Triatoma pintodiasi* Jurberg, Cunha & Rocha, 2013 (Hemiptera, Triatominae), an endemic species in Brazil

Abstract

Chagas disease is most frequently transmitted to humans through contact with feces of insects from the Triatominae subfamily. In Brazil, there are 65 species of triatomines distributed throughout the country's 27 states. Among the species in the state of Rio Grande do Sul, *Triatoma rubrovaria*, *T. oliveirai*, *T. pintodiasi*, *T. klugi*, *T. carcavalloi*, and *T. circummaculata* (with the addition *T. limai*, which is endemic to Argentina) form the *T. rubruvaria* subcomplex. The last species described and grouped into this subcomplex was *T. pintodiasi*. Thus, this study characterized the genetic distance between *T. pintodiasi* and of the other members of the *T. rubrovaria* subcomplex in order to evaluate the specific status of *T. pintodiasi*. The genetic distance observed between *T. pintodiasi* and the other species of the *T. rubrovaria* subcomplex was large, a finding which highlights the specific status of the species considered to be cryptic of *T. circummaculata*.

Short Report

Chagas disease is a potentially life-threatening illness caused by the protozoan *Trypanosoma cruzi* Chagas, 1909. It is mainly distributed in endemic areas in 21 Latin American countries, where it is mostly vector-borne and transmitted to humans through contact with feces of insects from the Triatominae subfamily. It is estimated that approximately 6 million to 7 million people are infected worldwide, most of whom are located in Latin America where Chagas disease is endemic.[1]

There are currently 150 known species of triatomines distributed across 18 genera. All of the species are considered potential vectors of Chagas disease.[2] In Brazil, there are 65 species distributed throughout the country's 27 states, and 64% of them of endemic species.[3] In the state of Rio Grande do Sul, there are 12 species of triatomines: *Triatoma rubrovaria, T. platensis, T. pintodiasi, T. oliveirai, T. klugi, T. delpontei, T. carcavalloi, T. circummaculata,* and *Panstrongylus tupynambai,* the latter of which has limited distribution in this Brazilian state.[3]

Among the species in Rio Grande do Sul, *T. rubrovaria, T. oliveirai, T. pintodiasi, T. klugi, T. carcavalloi,* and *T. circummaculata* (with the addition of *T. limai,* which is endemic to Argentina) [4] make up the *T. rubruvaria* subcomplex.[5] The last species described and grouped into this subcomplex was *T. pintodiasi*.[5] The description was based on chromatic, morphological, morphometric, and biochemical aspects of the species, which led the author to describe *T. pintodiasi* as a cryptic species of *T. circummaculata*.[5] Recent studies on meiotic behavior[2] and characterization of nymphs[6] showed significant differences between *T. pintodiasi* and other species of the *T. rubrovaria* subcomplex. However, new comparative analyzes were encouraged.[2,5]

Thus, this study characterized the genetic distance between the mitochondrial 16S rDNA gene of *T. pintodiasi* and that of the members of the *T. rubrovaria* subcomplex in order to evaluate the specific status of *T. pintodiasi*.

Three adult *T. pintodiasi* specimens were used in the study. The specimens were provided by the National and International Reference Laboratory of Taxonomy of Triatominae (LNIRTT) in Rio de Janeiro, Brazil. The triatomines were dissected, and genetic material was extracted from their legs using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions. PCR reactions were performed to amplify the 16S rDNA gene as described by Simon et al. (1994),[7] with the forward sequence 5´-CCGGTTTGAACTCAGATCATGT-3´ and the reverse sequence 5´-CGCCTGTTTAACAAAAACAT-3´. The conditions for the amplification reactions of this gene were as follows: an initial cycle at 94°C for 2 minutes, followed by 35 cycles comprised of denaturation (94°C, 30 seconds), annealing (50°C, 30 seconds), and extension (72°C, 1 minute), followed by a final single cycle at 72°C for 6 minutes. After electrophoresis, the amplified fragments were purified using the GFX PCR DNA & Gel Band kit (GE Healthcare and Life Technology) according to the manufacturer's instructions.

Direct sequencing was applied to the purified PCR products, and the samples were sent to the Human Genome and Stem Cell Research Center of the University of São Paulo (USP) in São Paulo, Brazil. The sequences generated were analyzed using the BioEdit software, version 7.0.5. A consensus sequence was obtained for each DNA segment, and the sequences were aligned using the ClustalW. Next, the MEGA 6.0 program[8] for phylogenetic analysis was employed using the maximum likelihood method as a distance criterion, and the bootstrapping resampling method was applied to assess support for the individual nodes (1,000 pseudoreplications). The Tamura 3-parameter model was used to calculate genetic distances pairwise between *T. pintodiasi* and the other species of the *T. rubrovaria* subcomplex in the MEGA 6.0 software,[8] and the sequences used for the *T. rubrovaria* subcomplex species were deposited into GenBank [Accession numbers: *T. rubrovaria* (KC249066); *T. klugi* (KC249028); *T. carcavalloi* (KC248991); and *T. circummaculata* (KC248992)].

The genetic distance observed between *T. pintodiasi* and the other species of the *T. rubrovaria* subcomplex was large: 1.195 in the case of *T. carcavalloi*, 1.123 in the case of *T. circummaculata*, 1.184 in the case of *T. klugi* and 1.194 in the case of *T. rubrovaria*. These results highlight the specific status of the species. They reflect the importance of the analysis used by Jurberg and collaborators[5] in the description of new species, particularly in the study of the phenomenon of cryptic speciation.

Although current phylogenetic analyses combine many different mitochondrial and nuclear genes,[9] the 16S has been used alone to assess the phylogenetic relationships among triatomines. It is important to note the utility of the 16S gene as molecular marker among triatomine species and its importance in systematic and taxonomy issues.[10] Furthermore, the extremely low genetic distance observed in this mitochondrial gene led to the suggestion that *Nesotriatoma flavida* and *N. bruneri* are the same species.[11]

Phylogenetic analyses have shown the *T. rubrovaria* subcomplex to be monophyletic[9] (*T. pintodiasi* has never been included in these analyses due to its recent description). Studies have also determined that this subcomplex diversified from the other subcomplexes approximately 14 million years ago as a result of climatic changes demonstrated a consequence of the rapid Andean uplift.[12] These triatomines share morphological[13] and cytogenetic[14] characteristics. Using chromosomal data, Pita and collaborators[15] recently proposed the grouping of *T. guasayana* and *T. patagonica* with the seven species of the *T. rubrovaria* subcomplex. This relationship was initially suggested by molecular data.[9,16]

Thus, we corroborate the specific status of *T. pintodiasi*. However, we suggest that different analyses should be combined in order to evaluate the evolutionary relationship between *T. guasayana, T. patagonica, T. pintodiasi* with *T. rubrovaria* subcomplex.

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4.11 Capítulo 11 (Artigo científico publicado na *Infection, Genetics and Evolution* FI 2,88)

Alevi, K. C. C.; OLIVEIRA, J.; MOREIRA, F. F. F.; JURBERG, J.; ROSA, J. A.; AZEREDO-OLIVEIRA, M. T. V. Chromosomal characteristics and distribution of constitutive heterochromatin in the Matogrossensis and Rubrovaria subcomplexes. **Infection, Genetics and Evolution**, v. 33, p. 158-162, 2015.

Chromosomal characteristics and distribution of constitutive heterochromatin in the Matogrossensis and Rubrovaria subcomplexes

ABSTRACT

Since 1966 the triatomines were grouped in complexes and specific subcomplexes. Although the complex and subcomplexes not have taxonomic importance, should be monophyletic groups and cytogenetic tools have proved to be of great importance to characterize these species groupings. Based on this, this paper aims to describe the chromosomal characteristics and heterochromatic pattern of Matogrossensis and Rubrovaria subcomplexes, in order to contribute to the taxonomic and evolutionary relationships of these vectors. In this study, at least three males from each species (*Triatoma baratai, Triatoma costalimai, Triatoma guazu, Triatoma jurbergi, Triatoma matogrossensis, Triatoma vandae, Triatoma williami, Triatoma carcavalloi, Triatoma circummaculata, Triatoma klugi, Triatoma pintodiasi* and *Triatoma rubrovaria*) were analyzed by means of cytogenetic techniques of C-banding. All species showed the same cytogenetic characteristics: 22 chromosomes, low variation in the size of autosomes, sex chromosome Y larger than X, initial prophase composed of only one heterochromatic chromocenter formed by the sex chromosomes X and Y (except for *T. pintodiasi* that presented the sex chromosomes individualized during all stages of prophase) and presence of constitutive heterochromatin restricted to sex chromosome Y. These characteristics, although common to Matogrossensis and Rubrovaria subcomplexes allow to distinguish these species of species grouped in most of South America subcomplexes, as Brasiliensis, Maculata, Sordida and Insfestans. Thus, the cytogenetic analysis was of extreme importance to differentiate both subcomplexes of the other subcomplexes of South America. However, probably due to evolutionary proximity existing between these subcomplexes was not possible to observar species differences that make up the Matogrossensis subcomplex of

the Rubrovaria subcomplex. Therefore, we emphasize that new comparative analyzes, as experimental hybrid crosses and molecular cytogenetic analysis are necessary to clarify the evolutionary relationship between these important subcomplexes of vectors.

SHORT COMMUNICATION

The triatomines are hematophagous insects vectors of the protozoan *Trypanosoma cruzi* Chagas, 1909, the etiologic agent of Chagas disease. These vectors are of great importance to public health, as about 7 million to 8 million people are estimated to be infected worldwide, mostly in Latin America and triatomines are considered as the main form of transmission (WHO, 2014).

Taxonomically, triatomines belong to the Hemiptera order, Heteroptera, suborder, Reduviidae family and Triatominae subfamily (Lent and Wygodzinsky, 1979). These vectors were clustered in complexes and specific subcomplexes (Usinger et al., 1966; Lent and Wygodzinsky, 1979; Carcavallo et al., 2000; Schofield and Galvão, 2009), based mainly of morphological and geographical distribution. However, Carcavallo et al. (2000) consider that ‘‘specific complex’’ is only the practical grouping of species and it does not exist as a taxonomic category in the International Code of Zoological Nomenclature.

Lent and Wygodzinsky (1979) based on morphology, proposed the division of triatomines the genus Triatoma in ten complexes and developed a dichotomous key to group the species in the respective complex. Recently, Schofield and Galvão (2009) based mainly of morphological and geographical distribution, grouped the triatomine in eight complexes and eight specific subcomplexes.

The Infestans complex is present in South America and consists of six subcomplexes: Brasiliensis, Infestans, Maculata, Matogrossensis, Rubrovaria and Sordida (Schofield and Galvão, 2009). Initially, some species of Matogrossensis (Table 1) and Rubrovaria subcomplexes (Table 2) were related in the same complex (Lent and Wygodzinsky, 1979). This relationship was maintained for long in oliveirai complex (Carcavallo et al., 2002).

Justi et al. (2014) emphasize that although the complex and subcomplexes not have taxonomic importance, should be monophyletic groups. The use of morphological analysis (mainly coloring) is not appropriate parameters for grouping species because intraspecific chromatic variation can exist (Costa, 2000; Almeida et al., 2002). Cytogenetic tools have proved to be of great importance to characterize these species groupings (Panzera et al., 1995, 1997; Santos et al., 2007; Alevi et al., 2012, 2014).

Based on this, this paper aims to describe the chromosomal characteristics and heterochromatic pattern of Matogrossensis and Rubrovaria subcomplexes (Almeida et al., 2009; Schofield and Galvão, 2009; Gonçalves et al., 2013; Jurberg et al., 2013), in order to contribute to the taxonomic and evolutionary relationships of these vectors.

In this study, at least three males from each species [*Triatoma baratai* (3), *Triatoma costalimai* (3), *Triatoma guazu* (3), *Triatoma jurbergi* (3), *Triatoma matogrossensis* (8), *Triatoma vandae* (3), *Triatoma williami* (3), *Triatoma carcavalloi* (3), *Triatoma circummaculata* (3), *Triatoma klugi* (5), *Triatoma pintodiasi* (3) and *Triatoma rubrovaria* (8)] were used. Males were utilized because spermatogenesis is continues in adulthood, allowing the visualization of all stages of meiosis. 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 (FCFAR/UNESP), São Paulo, Brazil and International and National Laboratory of Reference for Triatominae Taxonomy, Oswaldo Cruz Institute (FIOCRUZ) Rio de Janeiro, Rio de Janeiro, Brazil. The seminiferous tubules of adult males were torn and fixed to a cover slip. They then underwent the cytogenetic technique of C-banding (Sumner, 1972). At least 50 cells in prophase and 50 in metaphase of each species were analyzed. 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 1000x.

By means of the cytogenetic analyzes in Matogrossensis and Rubrovaria subcomplexes was possible to describe the chromosome number, sex mechanism, relative autosomal size, heterochromatic pattern in prophase and in chromosomes (Tables 1 and 2). All species analyzed showed the same cytogenetic characteristics: 22 chromosomes (Fig. 1C), low variation in the size of autosomes, sex chromosome Y larger than X, initial prophase composed of only one heterochromatic chromocenter formed by the sex chromosomes X and Y (Fig. 1A) (except for *T. pintodiasi* that presented the sex chromosomes individualized during all stages of prophase) and presence of constitutive heterochromatin restricted to sex chromosome Y (Fig. 1B and C).

Cytogenetic analysis in Matogrossensis subcomplex were initiated by Pérez et al. (1992) who described the chromosomal characteristics and distribution of constitutive heterochromatin of *T. matogrossensis*. Thereafter, Dujardin et al. (2002) analyzed cytogenetic characteristics of *T. guazu*, *T. williami*, *T. jurbergi* and *T. costalimai* as chromosome number

and pattern of heterochromatin and, more recently, Panzera et al. (2010) and Alevi et al. (2015) analyzed the *T. williami* (karyotype and heterochromatin) and *T. baratai* (karyotype), respectively.

Already cytogenetic analyzes in subcomplex Rubrovaria were initiated a long time with the description of the karyotype *T. rubrovaria* by Pellegrino and Schreiber (1950), followed by the description of chromosomal characteristics and heterochromatic pattern by Ueshima (1966) and Panzera et al. (1995). Subsequently, the karyotype and the heterochromatic pattern Triatoma curcummaculata was analyzed by Panzera et al. (1998) and Dujardin et al. (2002), and most recently, chromosomal analysis of *T. klugi* (Costa et al., 2008), karyotype analysis and heterochromatic pattern of *T. carcavalloi* (Panzera et al., 2010) and description of the karyotype of *T. pintodiasi* (Alevi et al., in press) were realized. All cytogenetic data for both subcomplexes described were confirmed by our results which brings together for, the first time, the complete description of chromosome analysis and heterochromatic pattern for most species of subcomplexes.

Analyses of heterochromatic pattern are extremely important for the characterization of taxon and optimization of molecular data, because generally these regions are specific to each species or monophyletic groups. This tool has helped in the characterization of Brasiliensis subcomplex, since the species that make up this complex have peculiarities as large chromocenter in prophase formed by sex chromosomes and two pairs of autosomes and heterochromatic blocks dispersed in the nucleus, as well as the presence of heterochromatin ends in autosomes (Alevi et al., 2012, 2014).

The species analyzed presented the same chromosomal characteristics and disposition of heterochromatin blocks (Tables 1 and 2). These characteristics, although common to Matogrossensis and Rubrovaria subcomplexes allow to distinguish these species of species grouped in most of South America subcomplexes, as Brasiliensis, Maculata, Sordida and Infestans (Panzera et al., 1995, 1997; Santos et al., 2007; Alevi et al., 2014) (Table 3).

Recent molecular analyzes have failed to rescue the monophyly of Matogrossensis subcomplex, because some species were grouped with the Sordida subcomplex (Gardim et al., 2013, 2014; Justi et al., 2014). However, cytogenetic data provided in this study with the results described for Sordida subcomplex (Panzera et al., 1997), allow to differentiate these subcomplexes of species, highlighting the importance of cytogenetics as taxonomic tool (Table 3).

Initially, as already mentioned, some species of Matogrossensis and Rubrovaria subcomplexes as T. costalimai, *T. matogrossensis, T. rubrovaria* and *T. williami* were related in Infestans complex (Lent and Wygodzinsky, 1979). This difficulty in separating these insects based on morphology is maintained in cytogenetic analysis because all species of both subcomplexes have the same characteristics (Table 1).

Thus, we characterize the chromosomes and the disposition of the heterochromatic pattern of the species grouped in Matogrossensis and Rubrovaria subcomplexes. The cytogenetic analysis was of extreme importance to differentiate both subcomplexes of the other subcomplexes of South America. However, probably due to evolutionary proximity existing between these subcomplexes was not possible to observar species differences that make up the Matogrossensis subcomplex of the Rubrovaria subcomplex. Therefore, we emphasize that new comparative analyzes, as experimental hybrid crosses and molecular cytogenetic analysis are necessary to clarify the evolutionary relationship between these important subcomplexes of vectors.

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Table 1. Cytogenetic characteristics of species of the Matogrossensis subcomplex.

Species	Chromosome number	Sex mechanism	Relative autosomal size	Relative size of sex chromosomes	Heteropycnotic pattern in prophase	C-Banding		
						Autosomes	Sex chromosomes	
							X	Y
T. baratai	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. costalimai	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. deaneorum	-	-	-	-	-	-	-	-
T. guazu	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. jatai	-	-	-	-	-	-	-	-
T. jurbergi	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. matogrossensis	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. vandae	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. williami	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes

Table 2. Cytogenetic characteristics of species of the Rubrovaria subcomplex.

Species	Chromosome number	Sex mechanism	Relative autosomal size	Relative size of sex chromosomes	Heteropycnotic pattern in prophase	C-Banding		
						Autosomes	Sex chromosomes	
							X	Y
T. carcavalloi	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. circummaculata	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. klugi	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes
T. limai	-	-	-	-	-	-	-	-
T. oliveirai	-	-	-	-	-	-	-	-
T. pintodiasi	2n = 22	XY	Small variation	Y>X	X and Y individualized	No	No	Yes
T. rubrovaria	2n = 22	XY	Small variation	Y>X	Large chromocenter (sex chromosomes)	No	No	Yes

Table 2. Cytogenetic characteristics of South America subcomplexes.

Subcomplexes	Constitution of chromocenter	Chromosomal characteristics	Heterochromatic pattern
Matogrossensis			
All species	X and Y	Small variation	Y
Rubrovaria			
All species	X and Y	Small variation	Y
Brasiliensis			
All species	X, Y and two autosomes	Small variation	Both ends of all autosomes and Y
Sordida			
T. sordida (Argentina)	X and Y	2 or 3 larger autosomal pairs	Y
T. sordida (Brasil)	X and Y plus autossomes	2 or 3 larger autosomal pairs	One end of 8-10 autosomes and Y
T. guasayana	X and Y	2 or 3 larger autosomal pairs	Y
T. patagonica	X and Y plus autossomes	One pair slightly larger	One or both ends and interstitial of all autosomes and Y
Maculata			
T. maculata	X and Y	Small variation	Most autosomes with terminal small dots and Y
T. pseudomaculata	X and Y and one autosome	Small variation	3-4 autosomes and Y
Infestans			
T. infestans (Andean)	X and Y plus autossomes	Three larger autosomal pairs	One or both ends of all autosomes and Y
T. infestans (Non Andean)	X and Y plus autossomes	Three larger autosomal pairs	One or both ends of 3-4 autosomes and Y
T. platensis	X and Y plus autossomes	Three larger autosomal pairs	One or both ends of 3-4 autosomes and Y
T. delpontei	X and Y plus autossomes	Small variation	One end of all autosomes and Y

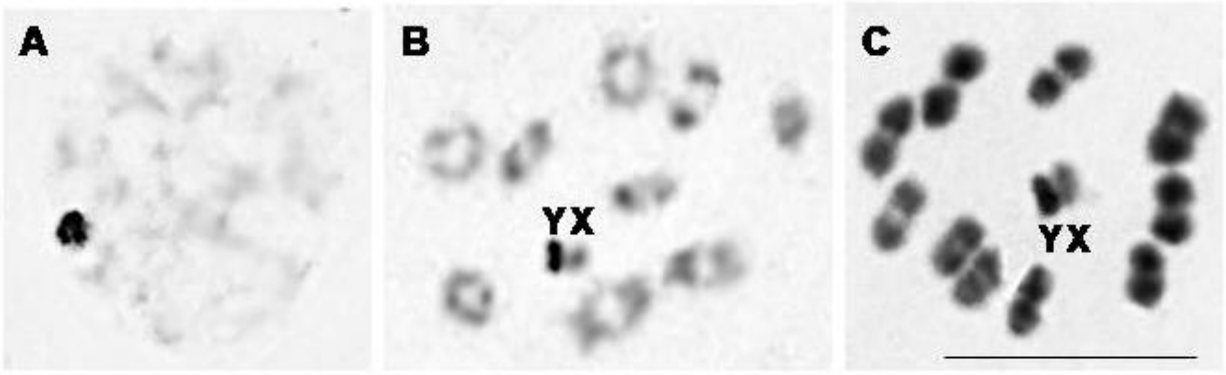

Fig. 1: Cytogenetics characteristics of the *T. baratai* (A) Prophase (initial diffuse stage). Note one heterochromatic chromocenter formed by the sex chromosomes X and Y. (B) Prophase (late diffuse stage). Note the heterochromatin restricted to only the sexual chromosome Y. (C) Metaphase I. Note the 22 chromosomes, small variation, sex chromosome Y larger of the X and heterochromatin restricted to only the sexual chromosome Y. Bar: 10 µm.

4.12 Capítulo 12 (Artigo científico publicado na revista científica *Infection, Genetics and Evolution* FI 2,88)

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New arrangements on several species subcomplexes of *Triatoma* genus based on the chromosomal position of ribosomal genes (Hemiptera - Triatominae)

Abstract

The hemipteran subfamily Triatominae includes 150 blood-sucking species, vectors of Chagas disease. By far the most specious genus is *Triatoma*, assembled in groups, complexes and subcomplexes based on morphological similarities, geographic distribution and genetic data. However, many molecular studies questioned the species integration of several subcomplexes as monophyletic units. In triatomines, chromosomal position of major ribosomal DNA (rDNA) loci is extremely variable but seems to be species-specific and an evolutionary conserved genetic trait, so that closely related species tend to have ribosomal clusters in the same chromosomal location. Considering that the autosomal position as the ancestral character for all heteropteran species, including triatomines, we suggest that the movement of rDNA loci from autosomes to sex chromosomes rapidly established reproductive barriers between divergent lineages. We proposed that the rDNA translocation from the autosomes to the sex chromosomes restrict reproductive compatibility and eventually promote speciation

processes. We analyzed the chromosomal position of 45S rDNA clusters in almost all species of the matogrossensis, rubrovaria, maculata and sordida subcomplexes. The fluorescent in situ hybridization results are discussed considering the available genetic data and we proposed new arrangements in the species that constitute each one of these subcomplexes.

Keywords: Triatominae**,** Chagas disease vectors**,** Holocentric chromosomes**,** Location changes of rDNA clusters**,** FISH

1. Introduction

Triatomines are blood-sucking insects which are well known for being vectors of Chagas disease or American trypanosomiasis. This illness is considered as the most serious human parasitic disease of Latin America with around 6–7 million infected people (WHO, 2016). In the absence of vaccines or adequate drugs for large-scale treatment, the reduction of disease burden critically depends on the control of vector transmission by triatomines. Given so, a correct taxonomic identification of species is extremely important for successful control campaigns.

The subfamily Triatominae includes 150 species in 15 to 18 genera, being by far the more frequent the *Triatoma* genus with 73 species (Galvão and Paula, 2014). Historically, several authors have assembled the *Triatoma* species in different groups and complexes based on their external characters and the genitalia of both sexes (Ryckman, 1962; Usinger et al., 1966; Lent and Wygodzinsky, 1979; Carcavallo et al., 2000). Currently, the most accepted grouping was proposed by Schofield and Galvão (2009), which subdivides *Triatoma* species in groups, complexes and subcomplexes based on morphological similarities, geographic distribution and genetic data. Although complex and subcomplexes are not considered as taxonomic categories for the International Code of Zoological Nomenclature (ICZN, 1999) its application on Triatominae attempt to reveal phylogenetic

relationships. This means that a subcomplex species represents a monophyletic unit including evolutionarily closed related species derived from a common ancestor. The establishment of monophyletic assemblages is very important in order to infer possible common attributes related to their biology, epidemiological significance and response to control interventions (Schofield and Galvão, 2009). Within *Triatoma* genus, these authors recognized 14 monophyletic units, most of them supported with different genetic analyses. However, new studies based on comparisons of DNA sequences question the species integration of several subcomplexes. These questions mainly involves almost all *Triatoma* subcomplexes from South America, such as brasiliensis (Gardim et al., 2014), maculata (Dos Santos et al., 2007; Carbajal de la Fuente et al., 2008), matogrossensis (Gardim et al., 2013; Teves et al., 2016) and rubrovaria (Noireau et al., 2002; Almeida et al., 2009; Justi et al., 2014).

Triatominae subfamily species, similar to other heteropterans have holocentric chromosomes, i.e. chromosomes with diffuse or non-localized centromeres (Hughes-Schrader and Schrader, 1961). The absence of a primary constriction and their homogeneity in their autosomal number limited comparative and evolutionary chromosomal studies (Panzera et al., 2010). As a taxonomic tool, banding techniques application has been a valuable for karyotypic differentiation and to detect cryptic species, particularly in sordida subcomplex (Panzera et al., 1997, 2015). Recent application of fluorescent in situ hybridization (FISH) to determine the chromosomal position of major ribosomal clusters shows that this trait is species-specific and also with a striking inter-specific variability, revealing an extraordinary dynamics of change in the genomes during the evolution in this insect group (Panzera et al., 2012). Ribosomal ribonucleic acid (rRNA) is the main structural and catalytic component of the ribosome, being essential for protein synthesis in all living organisms. It is indispensable for

cell viability and is one of only a few gene products present in all cells. In eukaryotes, the genes encoding ribosomal RNA (rDNA) are present in multiple copies, arranged as clusters and located in one or more chromosomes, named nucleolar chromosomes. Typically, each repeat unit of the major ribosomal cluster (45S rDNA) contains three regions encoding the 18S, 5.8S and 28S rRNAs.

In a wide range of organisms, including fungi, animals and plants, the location, size and degree of repetition of the basic repeat unit are highly variable. However, the nucleotide sequences of the coding regions are evolutionarily highly preserved by concerted evolution and they are frequently used to develop DNA probes that allow the chromosome location of the rDNA loci by FISH (reviewed in Richard et al., 2008). In different insect groups, such as Coleoptera, Diptera, Hymenoptera, Lepidoptera and Orthoptera, the distribution of these conserved rDNA clusters can be apply for the establishment of physical maps with phylogenetic and evolutionary goals (Hirai et al., 1996; Proença et al., 2005; Roy et al., 2005; Cabrero and Camacho, 2008; Cabral de Mello et al., 2011; Šíchová et al., 2013).

In Heteroptera order, FISH analyses of a hundred species from 38 genera showed that the rDNA clusters are restricted to one or two loci per haploid genome. The chromosomal position is extremely variable; on a pair of autosomes (exceptionally 2), on m-chromosomes, on one or two sex chromosomes or simultaneously on a pair of autosomes and the X chromosome. The most predominant location is on one autosomal pair, recorded in species with different chromosome numbers and sex chromosome systems, involving eight of ten studied families including the most ancient groups (Grozeva et al., 2015), being considered as an ancestral character of the Heteroptera order. In spite of that ribosomal loci are regularly inherited by Mendelian fashion, changes in the position of rDNA loci are often originated by chromosomal rearrangements such as fusions,

fissions or translocations. In some ants and heteropteran species, these changes may imply modifications in the chromosome number (Hirai et al., 1996; Bressa et al., 2009). However, in Triatominae, given that the number of autosomes remains almost unaffected (almost all species have 20 autosomes), we can rule out that these chromosomal rearrangements are responsible for variation of rDNA position. Inter-chromosomal mobility of rDNA by other mechanisms such as ectopic recombination and transposition is frequently reported in several insect groups (Cabrero and Camacho, 2008; Nguyen et al., 2010; Cabral de Mello et al., 2011) that could be also the origin of the rDNA location changes observed in Heteroptera (Panzera et al., 2012). In triatomines, chromosomal position of rDNA loci seems to be an evolutionary conserved genetic trait, so that closely related species tend to have the ribosomal genes in the same chromosomal location (Panzera et al., 2012, 2015).

In the current paper, we analyzed the 45S rDNA clusters chromosomal position in almost all species of the matogrossensis, rubrovaria, maculata and sordida subcomplexes, using an 18S rDNA probe isolated from *Triatoma infestans*. Our FISH results are discussed considering genetic data available and we proposed new arrangements in the species that constitute each one of these subcomplexes.

2. Materials and methods

In this paper we compared the 45S rDNA clusters chromosomal location in 21 of the 25 recognized species of matogrossensis, rubrovaria, maculata and sordida subcomplexes, ten of them described here for the first time (Table 1). About previously reported species, we improve our analysis including new populations. The four misplaced species (*Triatoma deanorum, Triatoma limai, Triatoma oliveirai, and Triatoma arthurneivai*) were not analyzed because of the great difficulty to collect and keep them

alive in insectariums (Noireau et al., 2002). Geographical origin, number of individuals analyzed and results about the chromosome location of ribosomal clusters are detailed on Table 1, including previous FISH data.

FISH was carried out using squashed gonad preparations. The gonads were extracted from living adult insects and fixed in ethanol-acetic acid (3:1). FISH procedure was applied using as probe an 18S rDNA fragment of 807-bp isolated from *T. infestans* from Uruguay as described by Panzera et al. (2012). Chromosome slides were examined under a Nikon Eclipse 80i microscope and the images were obtained with a DS-5McU2 digital camera. For each specimen, at least 20 cells in meiotic (metaphase I, II or diplotene) or mitotic divisions were analyzed to determine 45S rDNA clusters chromosomal location. Images were processed with the Adobe Photoshop® software.

3. Results

All species from matogrossensis, rubrovaria, maculata and sordida subcomplexes present the same diploid chromosome number of 22 chromosomes, constituted by 20 autosomes plus two sex chromosomes (XY in males and XX in females). In all species, the Y chromosome presents an intermediate size and always appears C-heterochromatic.

The 45S rDNA cluster has 1 or 2 chromosome loci per haploid genome, showing three location patterns: on one autosomal pair (14 species) (Fig. 1A, B and C), on the X and Y chromosomes (5 species) (Fig. 1D and E) and on the X chromosome (2 species) (Fig. 1F). Each analyzed species presented only one rDNA location pattern; intraspecific variation was not observed. In species that show the ribosomal clusters on both sex chromosomes, the X chromosome signal is much more intense than that observed in the Y chromosome (Fig. 1E), except in *T. jurbergi* which both sex chromosomes have similar signal intensity (Fig. 1D). In all cases, the

hybridization signals were located in a terminal or subterminal chromosomal position. FISH results are summarized in Table 1, including new and previous data.

Considering the subcomplexes until now recognized (Table 1), all rubrovaria subcomplex species are homogenous in their ribosomal clusters chromosomal location, presenting the rDNA signals on an autosomal pair (Fig. 1A). On the contrary, matogrossensis, maculata and sordida subcomplexes include species with rDNA clusters in different chromosomes. Matogrossensis and maculata subcomplex species present two ribosomal patterns: some species with 45S rDNA on an autosomal pair (Fig. 1B), while other carry them on both sex chromosomes (X and Y) (Fig. 1D). Sordida subcomplex species have three ribosomal patterns: on an autosomal pair (Fig. 1C), on both sex chromosomes (Fig. 1E) or on one sex chromosome (X chromosome) (Fig. 1F).

4. Discussion

4.1. Chromosomal location of rDNA clusters as taxonomic marker

In the 47 Triatomini tribe species currently studied, including ten species here described for the first time, the most frequent location of the rDNA is on one autosomal pair (30 species), usually the largest one, so it could be considered as ancestral for this group. The movement of the ribosomal clusters from autosomes to one (8 species) or both sex chromosomes (7 species) would be a secondary change, so the location of rDNA loci on sex chromosomes should be considered as an apomorphic character. Since the location of the rDNA loci on one or both sex chromosomes is observed in phylogenetically distant triatomini groups (*Dipetalogaster*, *Eratyrus*, *Mepraia* and several *Triatoma* species), it is likely that the transfer of rDNA loci from autosomes to sex chromosomes occurred several times during the evolution of this group. Analysis of rDNA

loci in Triatomini suggests that each species tend to fix its chromosomal position (character species-specific), so that groups with a common ancestry tend to have the same chromosomal location for rDNA loci.

4.2. Changes of location of rDNA clusters as an isolation mechanism and promoter of speciation

Such as observed in other insects, the movements of rDNA clusters from autosomal to sex chromosomal positions could alter the dynamics of gene recombination as well as the gene flow, genetic differentiation and speciation (Sætre et al., 2003). The genes residing on the X chromosome present a very different environment than autosomal genes in terms of gene expression and natural selection (Vicoso and Charlesworth, 2006). Recombination rates vary widely depending on the genomic position (autosomes or sex chromosomes) and the linkage with genes under selection. Since the sex chromosomes in male triatomines are asynaptic and achiasmatic (Solari, 1979), their rates of homologous recombination are reduced by half for the X chromosomes (which occurs only in females) and to virtually zero for the Y chromosome. In addition, the hemizygosity of the X chromosome in males will greatly increase the selection of recessive mutations, differentiating thus the rate of mutational changes between the autosomes and sex chromosomes. This can result in faster adaptive evolution of X chromosomes (the faster X effect) (Kaiser and Bachtrog, 2010). As the result of reduced recombination, genetic barriers to gene flow may arise rapidly between populations which fixed sex chromosomal variants. Similar as suggested in lepidopteran speciation (Šíchová et al., 2013), the reduced recombination of sex chromosomes enable the accumulation of genetic incompatibilities and leads to divergence and speciation in triatomines.

Another important consequence of the rDNA change from autosomes to sex chromosomes is the generation of new linkage groups in the X chromosomes. According to the available information in Diptera, controlling sex genes and reproduction-related traits genes are abundant on the X chromosomes, and many of them are involved in barriers to gene flow between diverging lineages (Noor and Feder, 2006). These genes, so-called "speciation" or "barrier" are related with reproductive isolation, including both pre-zygotic (such as pheromones) and postzygotic isolations (hybrid sterility and hybrid inviability) (for review see Qvarnström and Bailey, 2009). As a result of the insertion of the ribosomal genes it is likely that the formation of new linkage groups in the X chromosome establishes reproductive isolation among populations with different locations of the ribosomal genes. The translocation of ribosomal genes to sex chromosomes results in changes of the evolutionary dynamics and also has an effect on speciation. A hybrid resulting from a cross between two individuals with different localization of the rDNA loci (autosomes and sex chromosomes) produces unbalanced gametes in the number of rDNA loci resulting in reproductive disadvantage. Depending on the particular genotypes participating in a breeding, the combination of certain gametes could lead to a significant proportion of unviable zygotes (e.g., without rDNA clusters), selecting against heterozygotes and maintenance of polymorphisms in a population. These negative effects can be overcome if the two populations have fixed rDNA loci in both chromosomal positions. The simultaneous presence of rDNA loci in at least one sex chromosome and autosomes in reported in few species of Diptera (Roy et al., 2005), Coleoptera (Cabral de Mello et al., 2011) and Orthoptera (Cabrero and Camacho, 2008). In holocentric chromosomes, this double location is exceptional, having been reported only in two triatomine species: *T. delpontei* and *T. infestans* (Panzera et al., 2012, 2014). The last one exhibits an extensive rDNA

polymorphism involving more than one autosomal pair together with the X chromosome in their putative center of origin, but shows a strong tendency to fix the ribosomal clusters on the X chromosome during its dispersion process (Panzera et al., 2014). In conclusion, we propose that the rDNA translocation from the autosomes to the sex chromosomes limits reproductive compatibility and eventually promote speciation, similar as reported for other chromosomal rearrangements (Butlin, 2005). Perhaps the reverse movement of rDNA loci, i.e. from the sex chromosomes to autosomes is highly unlikely, given the new gene linkage relationships in the X chromosome and the establishment of isolation barriers to gene flow. Probably this mechanism may be acting on other insect groups, including with monocentric chromosomes such as Coleoptera, provided that the number of ribosomal clusters is present on one or two chromosomes per haploid complement.

4.3. Phylogenetic relationships in Triatominae subcomplexes

Triatominae species show high morphological plasticity which suggests that ecological factors may be the main force driving speciation in Triatominae (Dujardin et al., 1999). Very closely related species are able to develop rapid morphological changes in response to the adaptation to new environments. Conversely, similar morphs adapted to the same ecotope could be derived from different ancestors (Dujardin et al., 1999). Thus the existence of morphologically similar species could be reflecting both their evolution from a common ancestor or convergent adaptation to the same ecological niche. This phenotypic flexibility leads to misidentification of distinct genetic units by morphological convergence, arising taxonomic uncertainties in the description of new subspecies, species or even genera. Considering that the Triatominae species groupings in complexes and subcomplexes are mainly based on morphologically similarities (Schofield

and Galvão, 2009), the morphological plasticity confused both species identification and the establishment of evolutionarily related groups.

Phylogenetic origin of blood-feeding Triatominae has received considerable attention due to the epidemiological significance as vectors of Chagas disease. Conflicting hypotheses support Triatominae as a monophyletic (Hypša et al., 2002; Patterson and Gaunt, 2010; Weirauch and Munro, 2009), polyphyletic (Schofield, 1988; Paula et al., 2005; Schofield and Galvão, 2009) or paraphyletic group (Hwang and Weirauch, 2012). Although that the monophyly or polyphyly of *Triatoma* genus is unclear, there is unanimity in considering that the South American *Triatoma* species (except *T. melanocephala, T. tibiamaculata* and *T. vitticeps*) constitute a monophyletic group (Hypša et al., 2002; Schofield and Galvão, 2009; Gardim et al., 2014; Justi et al., 2014).

Chromosomal differentiation in triatomines is mainly restricted to the variation of different repeated sequences, particularly the C-heterochromatin and ribosomal clusters (for reviews see Panzera et al., 2010, 2012). Cytogenetic analyses of more than 80 species reveal that most species which constitute each subcomplex share similar chromosomal characteristics, such as autosomal heterochromatin localization and rDNA clusters chromosomal position. Chromosomal change rate is very different among subcomplexes, some of them where species change rapidly (e.g., infestans subcomplex) and others where the species remain completely undifferentiated (e.g., phyllosoma subcomplex). Our working hypothesis is that the chromosomal location of major rDNA clusters is a species-specific character and evolutionary conserved trait, so that closely related species tend to have the major ribosomal clusters in the same chromosomal location

In this paper, considering the ribosomal clusters chromosomal location by FISH and in the light of the available molecular data, we have evaluated the species integration of maculata, matogrossensis, rubrovaria

and sordida subcomplexes, and propose new arrangements that reflect their evolutionary relationships more accurately.

4.3.1. Rubrovaria subcomplex

It includes seven species that share morphological characteristics and geographical distribution (southern Brazil, Uruguay and Northwestern Argentina): *T. rubrovaria, T. carcavalloi, T. circummaculata, T. klugi, T. limai* and *T. oliveirai* (Schofield and Galvão, 2009). Recently, *T. pintodiasi* was described and incorporated in this subcomplex due to its close morphological, morphometric and biochemical (hemolymph proteins) similarities (Jurberg et al., 2013).

Several morphometric and molecular analyses show close evolutionary relationships among *T. carcavalloi, T. circummaculata, T. klugi* and *T. rubrovaria* (García et al., 2001; Hypša et al., 2002; Sainz et al., 2004; Paula et al., 2005; Almeida et al., 2009, Gardim et al., 2014). Morphometric similarities between *T. klugi* and *T. oliveirai* were reported by Noireau et al. (2002). Membership in this subcomplex of *T. limai, T. oliveirai* and *T. pintodiasi* must be confirmed as there are no molecular data on these 3 species.

Our FISH results show that all rubrovaria species analyzed hitherto present the ribosomal clusters on an autosomal pair (Tables 1 and 2), confirming their close phylogenetic relationships. Furthermore, two other species belonging to sordida subcomplex show the same chromosome location: *T. guasayana* and *T. patagonica* (Table 1). Analyses of several nuclear and mitochondrial fragments also cluster these two species in the same clade within rubrovaria subcomplex (García et al., 2001; Hypša et al., 2002; Sainz et al., 2004; Paula et al., 2005; Almeida et al., 2009; Gardim et al., 2014). The close association among *T. sordida* and *T. guasayana* observed in phylogenetic trees with COI and Cyt b fragments reported by

Gardim et al. (2013) and Justi et al. (2014) probably are due to an incorrect identification of the analyzed specimens since to their great morphological similarity, as has been suggested by Panzera et al. (2015).

In brief, considering all genetic data available and the similar ribosomal clusters location, we proposed that the rubrovaria subcomplex would be constituted by the following species: *T. carcavalloi, T. circummaculata, T. guasayana, T. klugi, T. limai, T. oliveirai, T. patagonica, T. pintodiasi* and *T. rubrovaria*.

4.3.2. Maculata subcomplex

Currently, this subcomplex is constituted by four species: *T. maculata, T. pseudomaculata, T. arthurneivai* and *T. wygodzinskyi* (Schofield and Galvão, 2009). These species are extremely similar and cannot be easily distinguished considering external characters alone. However, their ecological behavior is very different; the first two are arboreal while the latter are rupicolous (Lent and Wygodzinsky, 1979). Geometric morphometric analyses on wings suggested a misidentification between *T. arthurneivai* and *T. wygodzinskyi* (Carbajal de la Fuente et al., 2010). For this reason all published genetic data on *T. arthurneivai* must correspond to T. *wygodzinskyi. T. arthurneivai* would be restricted to Sierra do Cipó (Minas Gerais, Brazil) and no genetic data are available.

According to Schofield (1988), *T. maculata* and *T. pseudomaculata* resulted from the evolution of two geographic populations derived from a common ancestor. Our FISH results clearly splits the maculata subcomplex in two clades: species with the ribosomal genes in an autosomal pair (*T. pseudomaculata* and *T. wygodzinskyi*) and a species having the ribosomal genes in both sex chromosomes (*T. maculata*) (Table 1, Fig. 1). This clear division (*T. maculata* vs *T. pseudomaculata*/*T. wygodzinskyi*) and the close evolutionary relationship between *T. pseudomaculata* and *T. wygodzinskyi*

were also been reported by isoenzymes (Dos Santos et al., 2007), nuclear (Bargues et al., 2008; Justi et al., 2014) and mitochondrial sequences (Hypša et al., 2002; Paula et al., 2005; Justi et al., 2014). Genetic similarity between *T. maculata* and *T. pseudomaculata* is only reported by two papers (Sainz et al., 2004; Gardim et al., 2014). In the first paper the similarity between both species is due to a species misidentification, considering that *T. maculata* is not distributed in Sergipe (Brazil) (AF324512/AF324524). Same issue is probably happening with the specimens used by Gardim et al. (2014) which are from an unknown origin and at least 30 years old insectary colony.

In brief, unlike Schofield (1988) proposal, genetic data including our FISH results strongly suggest that *T. maculata* and *T. pseudomaculata*/*T. wygodzinskyi* not derived from a recent common ancestor and are evolutionarily distinct units. We propose the formation of a new subcomplex provisionally named Pseudomaculata including *T. pseudomaculata* and *T. wygodzinskyi* (Table 2) along with other species (see below). Molecular analyses on *T. arthurneivai* should determine whether this species belongs to maculata or pseudomaculata subcomplexes. In the latter case the new subcomplex will be called arthurneivai since as it would be the first species originally described. Considering the similar geographical distribution and habits of both species, probably *T. arthurneivai* is close to *T. wygodzinskyi*. On the other hand, we proposed that *T. maculata* constituted a separated subcomplex formed only by this species. Phylogenetic trees positioned this species alone and in a basal position within South American *Triatoma* (Hypša et al., 2002; Paula et al., 2005; Justi et al., 2014).

4.3.3. Matogrossensis subcomplex

Includes nine species which share morphological characteristics, all terrestrial and distributed throughout the Pantanal ecosystem in Central-

Western Brazil and Paraguay: *T. baratai, T. costalimai, T. deaneorum, T. guazu, T. jurbergi, T. matogrossensis, T. vandae* and *T. williami* (Schofield and Galvão, 2009). Recently, *T. jatai* was described and incorporated in the matogrossensis subcomplex due to its close morphological, morphometric and genetic similarities with *T. costalimai* (Gonçalves et al., 2013; Teves et al., 2016). Molecular data of *T. deaneorum* are not available.

None DNA sequences analysis succeeded to recover a clade formed by matogrossensis subcomplex species, reflecting a conflict between ecologic and genetic data (Hypša et al., 2002; Sainz et al., 2004; Paula et al., 2005; Gardim et al., 2013; Justi et al., 2014; Teves et al., 2016). Our FISH results clearly splits the matogrossensis subcomplex in two clusters: species with the ribosomal genes on an autosomal pair (*T. baratai, T. costalimai, T. guazu, T. jatai* and *T. williami*), and species bearing the ribosomal clusters on both sex chromosomes (*T. jurbergi, T. matogrossensis* and *T. vandae*) (Table 1, Fig. 1). A same dichotomy was also reported by morphometry (eight measurements of head and thorax) and isoenzyme (18 loci) analyses (Noireau et al., 2002) on the former called "*T. oliveirai* complex" (Carcavallo et al., 2001) currently matogrossensis subcomplex. Several analyses with mitochondrial genes (mainly 12S and 16S rDNA) have shown that *T. jurbergi, T. matogrossensis* and *T. vandae* are closely related among them and with sordida species (*T. sordida* and *T. garciabesi*) (García et al., 2001; Hypša et al., 2002; Sainz et al., 2004; Paula et al., 2005; Gardim et al., 2013; Justi et al., 2014; Teves et al., 2016). All these species showed the rDNA clusters on one or two sex chromosomes (Table 1). In conclusion, considering all available genetic data and the similar ribosomal clusters localization (all bearing ribosomal genes on sex chromosomes, either both or just the X chromosome), we proposed that the sordida subcomplex must be constituted by the following species: *T. garciabesi, T. sordida, T. sordida* Argentina (putative new species suggested by Panzera et al., 2015) plus *T.*

jurbergi, T. matogrossensis and *T. vandae* (from matogrossensis subcomplex). From this subcomplex we excluded *T. guasayana* and *T. patagonica* which has been proved to be related with the rubrovaria subcomplex (Table 2).

In the other matogrossensis subcomplex subdivision, constituted by *T. baratai, T. costalimai, T. guazu* and *T. williami*, their phylogenetic relationships among them are not clear, particularly the position of *T. costalimai*. In this species, molecular analyses of different mitochondrial genes (COI, COII and cyt b) performed on individuals from Brazil and Bolivia (numbers 35 and 42, respectively) show very large genetic distances (over 8.3%), which reveals an incorrect species identification in at least one of the specimens analyzed by Justi et al. (2014).

Isoenzyme and morphometric analyses clearly indicate a lack of differentiation between *T. guazu* and *T. williami* (Noireau et al., 2002). DNA sequence comparison between these two species of all mitochondrial fragments available in GenBank (12S, 16S, COI, COII and Cyt b) reveals nucleotide differences not exceeding 1.8% (data not showed), similar as observed among conspecific populations. All this information, along with a similar geographic distribution (Mato Grosso, Brazil), questions the existence of *T. guazu* and *T. williami* as two separate species.

Our FISH results in *T. baratai, T. costalimai, T. guazu, T. jatai* and *T. williami* (from matogrossensis subcomplex) as well as in *T. pseudomaculata* and *T. wygodzinskyi* (from maculata subcomplex) showed that the ribosomal clusters are localized on one autosomal pair. Evolutionary relationships between these two groups of species are not constant, and vary according to the specimens used and the molecular markers analyzed (Gardim et al., 2013). These inconsistencies clearly reveal improper sequencing or misidentification of species, such as above mentioned for *T. costalimai*. In spite of this, several phylogenetic trees, mainly with 12S and

16S fragments, show a close association among the two species groups afore mentioned (Hypša et al., 2002; Paula et al., 2005; Justi et al., 2014).

In summary, considering the available genetic data and the similar ribosomal clusters localization, we propose the inclusion of *T. baratai, T. costalimai, T. deaneorum, T. guazu, T. jatai* and *T. williami* within the same subcomplex that *T. pseudomaculata* and *T. wygodzinskyi* (Table 2).

5. Conclusions

We suggest that the movement of rDNA loci from autosomes to sex chromosomes rapidly established reproductive barriers between divergent lineages in triatomines. The same chromosomal location of the ribosomal genes reveals evolutionarily close species with a common ancestor. Since these changes can occur several times independently in distant triatomine groups, it is necessary to contrast the evolutionary relationships obtained with rDNA location with phylogenetic markers, such as the sequence comparisons of nuclear and mitochondrial genes. Based on these assumptions, we propose a reordering of species that composed several subcomplexes of *Triatoma* from South America.

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Table 1. Geographical origin of analyzed species and chromosomal location of 45S rDNA clusters by fluorescent in situ hybridization (FISH), according to the subcomplexes proposed by Schofield and Galvão (2009). Between brackets we included the number of individuals analyzed. LNIRTT = Laboratório Nacional e Internacional de Referencia em Taxonomia de Triatomíneos, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; LIVEDIH = Laboratório Interdisciplinar de Vigilância Entomológica em Diptera e Hemiptera, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil. CE = Ceará; GO = Goias, MG = Minas Gerais; MS = Mato Grosso do Sul; MT = Mato Grosso; ND = not determined RS = Rio Grande do Sul; RO = Roraima; SP = São Paulo, TO = Tocantins; D = domestic; P = peridomestic; S = sylvatic.

Species	**Geographical Origin**	**45S rDNA location**
Rubrovaria subcomplex		
T. carcavalloi	São Jerônimo, RGS, Brazil. LNIRTT.	One autosomal pair[b]
T. circummaculata	Cerro Largo, Uruguay. S. Vila São Jerónimo, RGS, Brazil. LNIRTT.	One autosomal pair[d]
T. klugi	Nova Petrópolis, RGS, Brazil. LNIRTT.	One autosomal pair[d]
T. rubrovaria	Artigas, Uruguay. S.	One autosomal pair[a,b]
T. pintodiasi	Rincão, Caçapava do Sul, RGS, Brazil. LNIRTT.	One autosomal pair[d]
Matogrossensis subcomplex		
T. baratai	Corumbá, MS, Brazil. LTL.	One autosomal pair[d]
T. costalimai	Posse, GO, Brazil. LNIRTT. Aurora de Tocantins, TO, Brazil.	One autosomal pair[d]
T. guazu	Barra do Garças, MG, Brazil. LNIRTT.	One autosomal pair[d]
T. jatai	Paranã, TO, Brazil. LTL.	One autosomal pair[d]
T. williami		
	Fazenda Nova, GO, Brazil. LNIRTT. Barra do Garças, MG, Brazil. LNIRTT.	One autosomal pair[d]
T. jurbergi	Alto Garças, MG, Brazil.	X & Y

	LNIRTT.	chromosomes[d]
T. matogrossensis	Serra das Arenas, MG, Brazil. LNIRTT.	X & Y chromosomes[a,b]
T. vandae	Rondonópolis, MG, Brazil. LNIRTT.	X & Y chromosomes[b]
Maculata subcomplex		
T. maculata	Boa Vista, RO, Brazil. Bolivar, RO, Venezuela. LNIRTT.	X & Y chromosomes[b,d]
T. pseudomaculata	Sobral, CE, Brazil. LNIRTT. Itadeim, MIG, Brazil. S.	One autosomal pair[b,d]
T. wygodzinsky	São João da Boa Vista, SP, Brazil. S. Espírito Santo do Pinhal, SP, Brazil. LNIRTT.	One autosomal pair[b,d]
Sordida subcomplex		
T. sordida Argentina	Populations from Argentina, Bolivia and Paraguay. D, P, S.	X & Y chromosomes[c]
T. sordida sensu stricto	Populations fromBrazil, Bolivia and Paraguay. D, P.	X chromosome[b,c]
T. garciabesi	Populations from Argentina, Bolivia and Paraguay. P., S.	X chromosome[b,c]
T. patagonica	San Martin, San Luis, Argentina. P.	One autosomal pair[d]
T. guasayana	Populations from Argentina and Bolivia	One autosomal pair[c]

FISH data from Bardella et al. (2010)[a], Panzera et al. (2012)[b,] Panzera et al. (2015)[c] and this paper[d].

Table 2. New proposal of South American *Triatoma* species involving maculata, matogrossensis, rubrovaria and sordida subcomplexes previously grouping by Schofield and Galvão (2009).

Subcomplex proposed by Schofield & Galvão (2009)	**Our new proposal**	**Chromosomal localization of ribosomal clusters**
Rubrovaria: *T. carcavalloi, T. circummaculata, T. klugi, T. limai, T. oliveirai, T. rubrovaria*	RUBROVARIA: *T. carcavalloi, T. circummaculata, T. klugi, T. limai*[ab], *T. oliveirai*[ab], *T. rubrovaria* plus *T. pintodiasi*[a] *T. guasayana, T. patagonica* (from sordida subcomplex)	One autosomal pai
Matogrossensis: *T. baratai, T. costalimai, T. deaneorum, T. guazu, T. jurbergi, T. matogrossensis, T. vandae, T. williami*	ELIMINATED	
Sordida: *T. garciabesi, T. guasayana, T. patagonica, T. sordida.*	SORDIDA: *T. garciabesi, T. sordida, T. sordida Argentina* (new species) plus *T. jurbergi, T. matogrossensis, T. vandae* (from matogrossensis subcomplex)	One (X) or two sex chromosomes (XY)
Maculata: *T. arthurneivai, T. maculata, T. pseudomaculata, T. wygodzinskyi*	MACULATA: *T. maculata*	Sex chromosomes (XY)
	NEW SUBCOMPLEX: PSEUDOMACULATA or ARTHURNEIVAI: *T. arthurneivai*[ab], *T. pseudomaculata, T. wygodzinskyi* plus *T. baratai, T. costalimai, T. deaneorum*[ab], *T. guazu, T. jatai, T. williami* (from matogrossensis subcomplex)	One autosomal pair

[a] Molecular data unknown.
[b] FISH data unknown

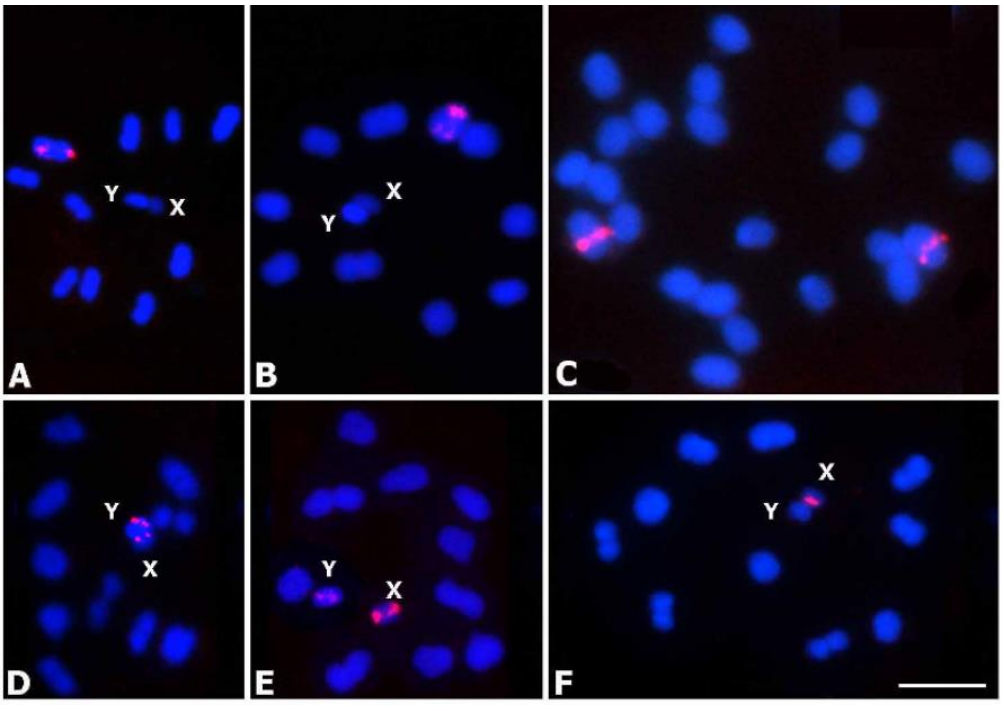

Fig. 1. Localization of 45S ribosomal DNA by fluorescent in situ hybridization (FISH) in male meiosis and spermatogonial prometaphase in different *Triatoma* species fromSouth America, using 18S rDNA as probe. All species showed a diploid chromosome number of 22 chromosomes (2n=20 autosomes plus XY inmales, XX in females). rDNA hybridization signals (in red) are located on one autosomal pair (A-B-C), on both XY sex chromosomes (D-E) or only on one X chromosome (F). (A): *T. klugi*. Second meiotic metaphase (MII). (B): *T. costalimai*. First meiotic metaphase (MI). (C): *T. patagonica*. Spermatogonial prometaphase. Hybridization signals are located in interstitial position on one pair of autosomes. (D): *T. jurbergi*. MI. Hybridization rDNA signals of similar intensities are located on both sex chromosomes. (E): *T. maculata*. MI. Ribosomal DNA signals on X chromosome are more intensity than the observed on the Y chromosome. (F): *T. sordida* sensu stricto. MI. Only one sex chromosome (X) shows rDNA clusters. Scale bar= 10 μm.

4.13 Capítulo 13 (Artigo científico publicado na revista científica *Genetics and Molecular Research* FI 0,98)

ALEVI, K. C. C.; IMPERADOR, C. H. L.; MOREIRA, F. F. F.; JURBERG, J.; AZEREDO-OLIVEIRA, M. T. V. Differentiation between *Triatoma arthurneivai* (Lent & Martins, 1940) and *T. wygodzinskyi* (Lent, 1951) (Hemiptera: Reduviidae: Triatominae) using cytotaxonomy. **Genetics and Molecular Research**, v. 15, p. 01-05, 2016.

Differentiation between *Triatoma arthurneivai* (Lent & Martins, 1940) and *T. wygodzinskyi* (Lent, 1951) (Hemiptera: Reduviidae: Triatominae) using cytotaxonomy

ABSTRACT

Using classic morphometric techniques to examine the head and thorax of *Triatoma* specimens, researchers identified a possible taxonomic problem involving *T. arthurneivai* Lent & Martins and *T. wygodzinskyi* Lent. A recent geometric morphometric study indicated that the insects captured outside the Serra do Cipó region, State of Minas Gerais, Brazil were *T. wygodzinskyi*. Them is identification of *T. arthurneivai* as *T. wygodzinskyi* could result in several problems associated with entoepidemiological lifting, the biological characterization of the species, and phylogenetic reconstruction. For the first time, we describe the use of cytogenetic analysis as a tool for differentiation between *T. arthurneivai* and *T. wygodzinskyi*. The results indicated that both species had the same number of chromosomes 2n = 22 (20A + XY). However, analyses of spermatocytes during early prophase indicated that it was possible to differentiate *T. arthurneivai* and *T. wygodzinskyi*, because only *T. arthurneivai* exhibited heteropycnotic blocks distributed in the chromatin. Therefore, we highlight

the analysis of spermatocytes as a taxonomic tool for the characterization of *T. arthurneivai* and *T. wygodzinskyi*, and suggest that the technique can be used for entoepidemiological lifting in vector control programs. Thus, the results presented here, in conjunction with morphometric analyses, are of utmost taxonomic and epidemiological importance for the identification of *T. arthurneivai* and *T. wygodzinskyi* specimens.

INTRODUCTION

The description of *Triatoma arthurneivai* Lent & Martins was based on a single female specimen collected in Serra do Cipó, State of Minas Gerais, Brazil (Lent and Martins, 1940). After approximately 10 years, Pellegrino (1951) collected specimens of this species in Santa Rita de Caldas, in southern Minas Gerais. Using the specimens collected by Pellegrino (1951), Lent (1951) described *T. wygodzinskyi* Lent. In addition to those collected in Minas Gerais, specimens first identified as *T. arthurneivai* were also collected in the states of São Paulo [Sorocaba (Corrêa etal., 1962) and Itupararanga/Votorantim (Forattini et al., 1968)] and Paraná (Stumpf et al., 1981).

Using classic morphometric techniques to examine the head and thorax, Dos Santos et al. (2007) suggested a possible taxonomic problem involving *T. arthurneivai* and *T. wygodzinskyi*. Indeed, a recent geometric morphometric study showed that the insects captured outside the Serra do Cipó region were *T. wygodzinskyi* specimens (Carbajal-de-La-Fuente et al., 2010). These authors reported that the populations of *T. arthurneivai* from São Paulo, which were studied for more than 40 years by several authors (Corrêa et al., 1962, 1965; Pinto Alves and Noda, 1964; Forattini et al., 1968, 1972; Juarez et al., 1970; Barretto and Ribeiro, 1981; Hypsa et al., 2002; de Paula et al., 2005; Rosa et al., 2005; Dos Santos et al., 2007; Bargues et al., 2008),were actually *T. wygodzinskyi* specimens.

The misidentification of *T. arthurneivai* as *T. wygodzinskyi* could result in several problems associated with entoepidemiological lifting (Pinto Alves and Noda, 1964), the biological characterization of the species (Forattini et.al., 1968; Juarez et al., 1970), and phylogenetic reconstruction (Hypsa et al., 2002; de Paula et al., 2005; Bargues et al., 2008). The identification of new inexpensive tools that aid the correct identification of the species is of great epidemiological importance. Therefore, we describe the use of cytogenetic analysis as a tool for differentiation of *T. arthurneivai* and *T. wygodzinskyi*.

MATERIAL and METHODS

At least two adult males from each species (*T. arthurneivai* and *T. wygodzinskyi*) were used, and the specimen were assigned by the insectariums of the National and International Laboratory of Reference for Triatominae Taxonomy, Oswaldo Cruz Institute (FIOCRUZ), Rio de Janeiro, Brazil (*T. arthurneivai*) and the Laboratory of Triatomines and Chagas Disease Epidemiology at the René Rachou Research Center (CPqRR/FICRUZ), Minas Gerais, Brazil (*T. wygodzinskyi*).

The biological material used to characterize cells was spermatocytes, which were easily obtained from testicular material by tearing the seminiferous tubules of adult males prior to fixation to a cover slip. The samples were then subjected to a cytogenetic technique that utilized lacto-acetic orcein (De Vaio et al., 1985 with modifications based on Alevi et al., 2012). At least 50 cells from each species were 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), and the obtained images were magnified by a factor of 1000X.

RESULTS

The results of the analyses indicated that both species had the same number of chromosomes 2n = 22 (20A + XY). However, analyses of spermatocytes during early prophase indicated that it was possible to differentiate *T. arthurneivai* and *T. wygodzinskyi,* because several heteropycnotic blocks distributed in the chromatin were present in *T. arthurneivai* (Figure 1A) but not *T. wygodzinskyi* (Figure 1B). In addition, both species have a chromocenter formed by the X and Y sex chromosomes (Figure 1A, B, arrows).

DISCUSSION

All species belonging to the Maculata subcomplex (*T. maculata*, *T. pseudomaculata*, *T. arthurneivai,* and *T. wygodzinskyi*) have the same number of chromosomes (2n = 22; 20A + XY) (Dos Santos et al., 2007). Dos Santos et al. (2007) also examined the meiotic prophase stages of *T. arthurneivai*, *T. maculata*, and *T. pseudomaculata*, and the authors found that the *T. arthurneivai* spermatocyte exhibited a heteropycnotic chromocenter formed by the sex chromosomes. However, our results confirmed that the insects analyzed by Dos Santos et al. (2007) were *T. wygodzinskyi* specimens, which corroborated the conclusion proposed by Carbajal-de-La-Fuente et al. (2010).

The cytogenetic characterization of triatomine spermatocytes was also important for the differentiation between *T. melanocephala* and other members of the Brasiliensis subcomplex (Alevi et al., 2013, 2014), between *T. maculata* and *T. pseudomaculata* (Dos Santos et al., 2007), between *T. guasayana* and *T. sordida* (Panzera et al., 1997), and between *T. rubrofasciata* and other 30 species of the triatomines (Alevi et al., 2016). The cellular analyses of spermatocytes allowed the characterization of many species-specific patterns, thereby ensuring that morphologically related

species were differentiated, so as observed for. *T. arthurneivai,* and *T. wygodzinskyi*.

The cytogenetic technique that utilized lacto-acetic orcein was cheaper and faster than molecular methods. This colorant has an affinity for basic structures, so histone and non-histone proteins involved in compaction of the genetic material (heteropycnotic blocks) are stained, and this characteristic was of great importance for the cytogenetic and cytotaxonomic studies of triatomines (Ueshima, 1966; De Vaio et al., 1985). The analysis of spermatocytes using orcein as a taxonomic tool for the characterization of *T. arthurneivai* and *T. wygodzinskyi* specimens was highlighted here, and we suggest that it may be used for entoepidemiological lifting in vector control programs.

Therefore, the combination of cytotaxonomic and morphometric analyses (Carbajal-de-La-Fuente et al., 2010) were proven to be of utmost taxonomic and epidemiological importance for the identification of *T. arthurneivai* and *T. wygodzinskyi* specimens.

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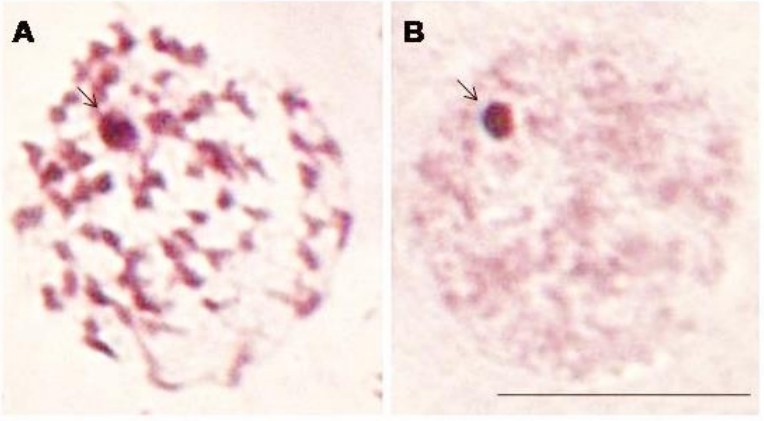

Figure 1. Early prophase of *Triatoma arthurneivai* (A) and *T. wygodzinskyi* (B). Note that T. arthurneivai exhibits several heteropycnotic blocks distributed in the chromatin, but these are not present in *T. wygodzinskyi*. Arrow: chromocenter formed by the X and Y sex chromosomes. Scale bar: 10 µm.

4.14 Capítulo 14 (Artigo científico publicado na revista *Brasilian Journal of Biology* FI 0,47)

Imperador, C. H. L.; Moreira, F. F. F.; Rosa, J. A.; Azeredo-Oliveira, M. T. V.; Alevi, K. C. C. Cytotaxonomy of the Maculata subcomplex (Hemiptera, Triatominae). **Brasilian Journal of Biology**, in press, 2017. DOI: http://dx.doi.org/10.1590/1519-6984.20915.

Cytotaxonomy of the Maculata subcomplex (Hemiptera, Triatominae)

SCIENTIFIC NOTE

Triatomines are hematophagous insects belonging to the Hemiptera order and Triatominae subfamily. All members of this subfamily are potential vectors of Chagas disease (Tartarotti et al., 2006). The Maculata subcomplex is composed of four species: *Triatoma maculata*, *T. pseudomaculata*, *T. arthurneivai* and *T. wygodzinskyi*. Recent phylogenetic analyzes showed conflicting results in relation to monophyly of this complex, because Justi et al. (2014) have failed to rescue the monophyly of the subcomplex and observed that *T. maculata* was more related to the species of the Brasiliensis subcomplex than the species of Maculata subcomplex (*T. arthurneivai* was not used in the study).

By means of cytogenetic characterization of the spermatocytes (prophase I), Santos et al. (2007) proposed that *T. maculata*, *T. pseudomaculata* and *T. arthurneivai* presented different heterochromatin patterns distribution and meiotic behavior. Panzera et al. (2010) describe the pattern heterochromatic of *T. wygodzinskyi* as identical to that observed for *T. arthurneivai*. However, Carbajal-de-La-Fuente et al. (2010) highlight that in many works of literature *T. wygodzinskyi* was erroneously classified as *T. arthurneivai*.

Thus, this work describes the heterochromatic pattern of all species of the Maculata subcomplex, with cytotaxonomic focus.

At least two males from each species were used. They had been assigned by the FCFAR/UNESP, Araraquara, São Paulo, Brazil, FIOCRUZ, Rio de Janeiro, Brazil and CPqRR-FIOCRUZ, Minas Gerais, Brazil. The seminiferous tubules of adult males were torn and fixed to a cover slip. They then underwent the cytogenetic technique of C-banding (Sumner, 1972) and analyzed using a Jenaval light microscope (Zeiss).

By analyzing of the distribution of the pattern of constitutive heterochromatin in spermatocytes in prophase I has been possible to differentiate all species of Maculata subcomplex: *T. maculata* (Figure 1A) showed a heterochromatic chromocenter formed by the sex chromosomes (arrow) and small heterochromatic blocks dispersed in nucleus; *T. pseudomaculata* (Figure 1B) showed a heterochromatic chromocenter formed by the sex chromosomes plus one pair of autosomes (arrow) and some heterochromatic blocks dispersed in the nucleus; *T. wygodzinskyi* (Figure 1C) showed only one heterochromatic chromocenter formed by the sex chromosomes (arrow) and *T. arthurneivai* (Figure 1D) showed a heterochromatic chromocenter formed by the sex chromosomes (arrow) and large heterochromatic blocks dispersed in the nucleus.

Schofield (1988) suggests that *T. maculata* and *T. pseudomaculata* species are derived from a common ancestor that was dispersed passively through the feathers of migratory birds and isolated geographically. These species are very similar from the morphological point of view (Corrêa and Espinola, 1964) and cytogenetic analysis described above confirm the data initially described by Santos et al. (2007) and corroborate the specific status of these triatomines. We suggest that during the speciation of *T. pseudomaculata*, heterochromatin loss events were involved in genomic

reorganization with loss of heterochromatin, as has been observed for the pallescens group (Alevi et al., 2015).

The phylogenetic relationship between *T. maculata* and Brasiliensis subcomplex reported by Justi et al. (2014) can be confirmed by standard heterochromatin of the species. However, the meiotic behavior of these vectors is different, since during prophase *T. maculata*, the chromocenter is formed by sex chromosomes, different of the species the Brasiliensis subcomplex which has the chromocenter form by sex chromosomes plus one pair of autosomes.

T. wygodzinskyi was described based on specimens collected in Santa Rita de Caldas, in southern Minas Gerais, Brazil and initially classified as *T. arthurneivai* (Lent, 1951). Different from the cytogenetic characteristics described initially, our results show that these species display large differences in heterochromatic pattern being the genetic material of *T. arthurneivai* rich in constitutive heterochromatin. Thus, we emphasize that the specimens analyzed by Santos et al. (2007) were *T. wygodzinskyi* and not *T. arthurneivai* as classified by the authors.

Thus, in the present study demonstrated that the analysis of heterochromatic pattern is a cytotaxonomic tool of utmost importance to characterize the species of Maculata subcomplex.

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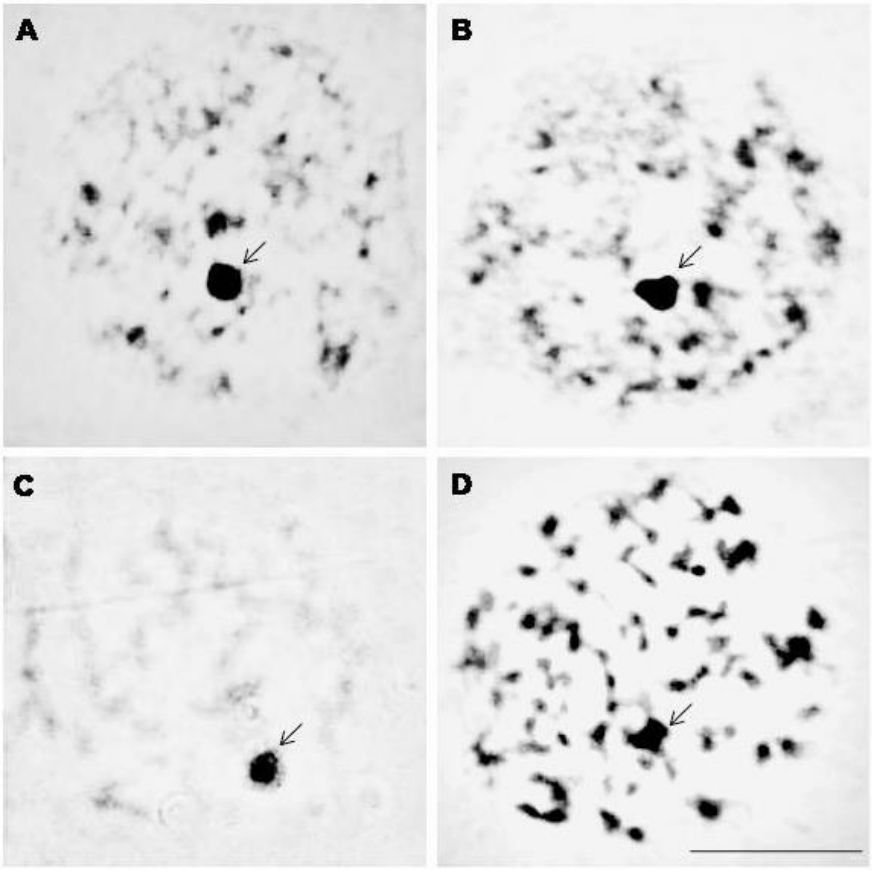

Figure 1. Constitutive heterochromatin in spermatocytes of all species of Maculata subcomplex. A) Note the heterochromatic chromocenter formed by the sex chromosomes (arrow) and small heterochromatic blocks dispersed in *T. maculata*; B) Note the heterochromatic chromocenter formed by the sex chromosomes plus one pair of autosomes (arrow) and some heterochromatic blocks dispersed in *T. pseudomaculata*; C) Note witch only one heterochromatic chromocenter formed by the sex chromosomes (arrow) in *T. wygodzinskyi*. D) Note the heterochromatic chromocenter formed by the sex chromosomes (arrow) and large heterochromatic blocks dispersed in *T. arthurneivai*. Scale bar: 10 µm.

4.15 Capítulo 15 (Artigo científico publicado na revista *Parasites & Vectors* FI 3,03)

ALEVI, K. C. C.; OLIVEIRA, J.; AZEREDO-OLIVEIRA, M. T. V.; ROSA, J. A. *Triatoma vitticeps* subcomplex (Hemiptera, Reduviidae, Triatominae): a new grouping of Chagas disease vectors from South America. Parasites & Vectors, v.10, p. 108, 2017.

Triatoma vitticeps subcomplex (Hemiptera, Reduviidae, Triatominae): a new grouping of Chagas disease vectors from South America

Abstract

Background: Triatomines have been grouped into complexes and subcomplexes based largely on morphological and geographical distribution. Although these groupings are not formally recognised as taxonomic ranks, they are likely monophyletic. However, recent studies have demonstrated that some subcomplexes from South America did not form monophyletic groups, and reorganisations have been suggested. One suggested reorganisation is to exclude *Triatoma vitticeps*, *T. melanocephala*, and *T. tibiamaculata* from the *T. brasiliensis* subcomplex. However, *T. vitticeps* and *T. melanocephala* exhibit several similar characteristics, including morphologic, cytogenetic, and phylogenetic features, a factor which supports the creation of a new subcomplex. Thus, this study aimed to describe the *T. vitticeps* subcomplex.

Results: *T. vitticeps* and *T. melanocephala* are sister species and share a phylogenetic relationship, several similar morphological characteristics, the same composition of constitutive heterochromatin (Xs CG-rich and Y AT-rich), the same karyotype (2n = 20A + $X_1X_2X_3Y$), and the same meiotic behaviour during spermatogenesis. Based on karyosystematics, for example, the *T. vitticeps* subcomplex may differ from all of the other subcomplexes from South America, as well as from the Rhodniini tribe and the genus *Panstrongylus*. We argue that the case of agmatoploidy involving the X chromosome was responsible for the karyotype divergence of this subcomplex in relation to the other South America subcomplexes.

Conclusions: Based on the phenotypic characteristics (morphology) and genotypes (cytogenetics and molecular features), we propose the creation of the monophyletic *T. vitticeps* subcomplex, which we believe is distinct from all other subcomplexes from South America.

Background

Chagas disease is a potentially life-threatening illness caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909), which is most commonly distributed in endemic areas of 21 Latin American countries. The disease is most frequently transmitted to humans through contact with faeces of triatomines. It is estimated that about 6 million to 7 million people are infected worldwide, most of whom reside in Latin America [1].

Chagas disease vectors belong to the order Hemiptera, the suborder Heteroptera, the family Reduviidae and, the subfamily Triatominae [2]. This subfamily is composed of 151 species distributed across 18 genera and five tribes [2–5], and all species (nymphs and adults of both sexes) are considered to be potential vectors of *T. cruzi*.

Based mainly on morphological and geographical distribution, these vectors have been grouped into complexes and subcomplexes [6–11]. Although these groupings are not formally recognized as taxonomic ranks and, thus do not necessarily represent natural groups, Justi et al. [12] propose that they are likely to be monophyletic: once the relationships between vector species are known, information about a species may be reliably extrapolated to other closely related species [13].

The species of the Triatomini tribe have been grouped into three groups, eight complexes, and eight subcomplexes [11]; the main species groups are *Triatoma rubrofasciata* (present mainly in North America and the Old World) and *T. infestans* (present in South America). South American triatomines were initially grouped into the *T. infestans* complex and the *T. brasiliensis*, *T. infestans*, *T. matogrossensis*, *T. maculata*, *T. rubrovaria*, and *T. sordida* subcomplexes [11]. However, several studies have demonstrated that the *T. brasiliensis* [12, 14–16], *T. matogrossensis* [17–19], *T. rubrovaria* [16, 17], and *T. sordida* subcomplexes [16, 19] do not form monophyletic groups, and reorganizations of the subcomplexes have been suggested [14–16, 20].

As part of these suggested reorganisations, Alevi et al. [14] and Gardim et al. [16] suggest that *T. vitticeps* (Stal, 1859), *T. melanocephala* Neiva and Pinto, 1923, and *T. tibiamaculata* (Pinto, 1926) should be excluded from the *T. brasiliensis* subcomplex. In some studies, it has been argued that these species lack a subcomplex [21, 22]. However, phylogenetic analyses detected a relationship between *T. tibiamaculata* and *Panstrongyus megistus* (Burmeister, 1835), which have been found to be sister species [12, 16, 23].

Recently, Justi et al. [19] argued that *T. tibamaculata* is a member of the clade *megistus*, along with other species of *Panstrongylus* [+ *Nesotriatoma bruneri* (Usinger 1944)].

Meanwhile, *T. vitticeps* and *T. melanocephala* were not grouped into any new subcomplexes, since these species do not share phenotypic and genotypic characteristics with the triatomine subcomplexes from South America [14, 15, 24–26]. However, these species exhibit several similar characteristics, including morphological [27], cytogenetic [14], and phylogenetic [19] features, similarities which support the creation of a new subcomplex. Thus, this study aimed to describe, for the first time, the *T. vitticeps* subcomplex, highlighting the main phenotypic and genotypic characteristics that support the grouping of these species and how this subcomplex is distinct from the others present in South America.

Methods

Ten adult males of each species of the new subcomplex were used for cytogenomic analysis. The species considered herein were *T. vitticeps* [geographic origin: Guarapari, Espírito Santo, Brazil (Coordinates: 20°39'01.41478"S, 40°30'25.29000"W)] and *T. melanocephala* [geographic origin: Bom Jesus da Serra, Bahia, Brazil (Coordinates: 14°22'04.46160"S, 40°30'52.55281"W), Jequié, Bahia, Brazil (Coordinates: 13°51'03.75834"S, 40°04'52.22281"W), and Poções, Bahia, Brazil (Coordinates: 14°31'01.94880"S, 40°22'43.37040"W)]. The specimens were provided by the Triatominae Insectarium within the Department of Biological Sciences in the College of Pharmaceutical Sciences at Sao Paulo State University's Araraquara campus (FCFAR/UNESP), São Paulo, Brazil. The seminiferous tubules were torn apart, crushed, and fixed on slides in liquid nitrogen. The cytogenomic technique of CMA_3/DAPI banding was then applied [28], with the modifications offered by Severi-Aguiar et al. [29] for differentiating the regions of heterochromatin rich in AT and CG. The biological material was analysed using an Olympus BX-FLA fluorescence microscope.

Results and Discussion

Both species presented the same composition of constitutive heterochromatin: X chromosomes rich in CG (Fig. 1a, c) and Y chromosome rich in AT (Fig. 1b, d), as initially observed by Severi-Aguiar et al. [29] in a study on *T. vitticeps* (initial prophases were used because the decondensed chromatin allows the labeling with fluorochromes to be more specific. Although Bardella et al. [30] have observed a small difference using CMA_3/DAPI in

T. vitticeps, we consider that size and compaction of the holocentric chromosomes in the metaphases may have made it difficult to interpret the results). In addition to this similarity, these species also share several morphological characteristics [27], and they exhibit the same 2n = 24 karyotype (20A + $X_1X_2X_3Y$) [14], the same meiotic behavior during spermatogenesis [29, 31], and the possible ability to produce natural hybrids (personal communication), all of which supports the grouping of these species into a *T. vitticeps* subcomplex (the *T. vitticeps* name was chosen based on *T. vitticeps* being the first species of the subcomplex described in the literature).

Cytotaxonomy and karyosystematics are important tools for determining the taxonomy of triatomines [14, 15, 32, 33]. For example, the karyotype analysis of the species within this *T. vitticeps* subcomplex is what distinguishes these species from all of the other South American subcomplexes, which have 2n = 22 (20A + XY) chromosomes [34, 35], from all of the species of the Rhodniini tribe (2n = 22) [36], and from the species of the genus *Panstrongylus* (2n = 21 or 23) [34].

In a recent phylogenetic study based on geological events, Justi et al. [19] suggested that *T. vitticeps* and *T. melanocephala* reached the Atlantic coast by dispersal and diversified prior to the Northern Andean uplift (23–10 Ma), an event which separated *T. maculata* from the other members of the *T. infestans* group. Based on this argument, and considering the fact that the ancestral karyotype of triatomines is 2n = 22 (20A + XY) [37, 38], we suggest that case of agmatoploidy involving the X chromosome was responsible for the karyotype divergence of this subcomplex in relation to the other South American subcomplexes (Fig. 2). Moreover, we argue that this was a unique event in the karyotype evolution of the *Triatoma* from South America, because, in addition to *T. melanocephala* and *T. vitticeps*, the only species that also presents fragmentation of the X chromosome is *T. tibiamaculata* 2n = 23 (20A + X_1X_2Y) [39]. However, the analysis provided by Justi et al. [19] allows us emphasise that this species inherited this number of chromosomes from the common ancestor shared with the *Panstrongylus* (a genus in which most of the species also have 23 chromosomes).

Conclusion

Based on the phenotypic characteristics (morphology) and genotypes (cytogenetics and molecular features) that define these species, we propose the creation of the monophyletic *T. vitticeps* subcomplex, one which believe is distinct from all other subcomplexes from South America.

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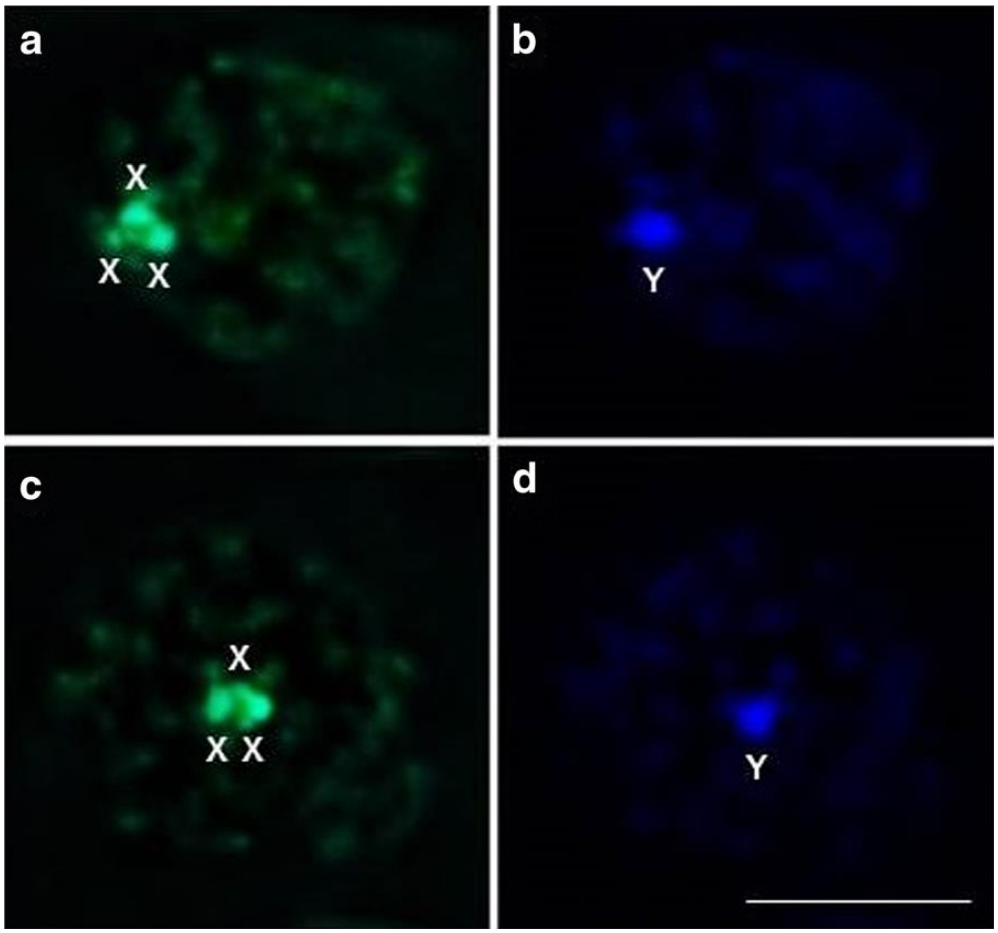

Fig. 1 Composition of constitutive heterochromatin in *T. vitticeps* (**a**, **b**) and *T. melanocephala* (**c**, **d**). Note that the X chromosomes are rich in CG (**a**, **c**) and the Y chromosome is rich in AT (**b**, **d**). X: X chromosomes, Y: Y chromosome. *Scale-bar*: 10 µm

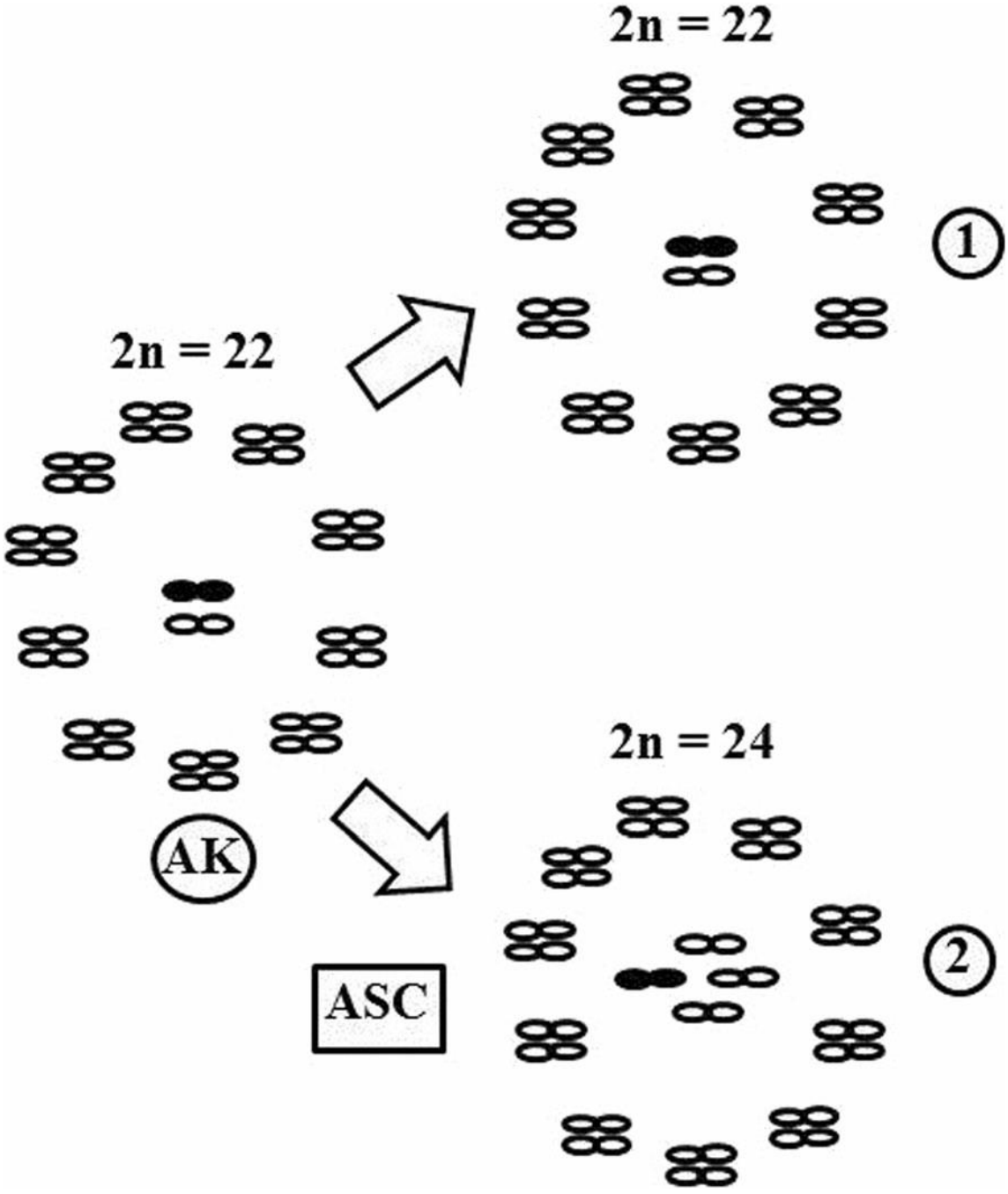

Fig. 2 Karyotype evolution of the South American subcomplexes. (1) Subcomplexes that remained with the same number of chromosomes from the ancestral karyotype (*T. brasiliensis*, *T. infestans*, *T. matogrossensis*, *T. maculata*, *T. rubrovaria* and *T. sordida*). (2) *T. vitticeps* subcomplex. Note that the species in this subcomplex have undergone two agmatoploidy events in the X chromosome. *Abbreviations*: AK, ancestral karyotype; ASC, agmatoploidy in sex chromosome.

4.16 Capítulo 16 (Artigo científico publicado na revista *Zootaxa* FI 0,92)

ALEVI, K. C. C.; BORSATTO, K. C.; MOREIRA, F. F. F.; JURBERG, J.; AZEREDO-OLIVEIRA, M. T. V. Karyosystematic of *Triatoma rubrofasciata* (De Geer, 1773) (Hemiptera, Reduviidae, Triatominae). Zootaxa, v. 3994, p. 433-438, 2015.

Karyosystematic of *Triatoma rubrofasciata* (De Geer, 1773) (Hemiptera, Reduviidae, Triatominae)

Abstract

Triatoma rubrofasciata (De Geer) is the first species of triatomine described, and little is known on its vector biology. Studies are restricted starvation resistance, interspecific morphometric variability, morphometry of testis follicles, coloration of the testicular peritoneal sheath, ultrastructure of the male accessory glands, phylogeny and cytogenetics. Thus, this study aims to address the karyosystematic of *T. rubrofasciata* and the possible events related to karyotype evolution of this species. Four adult males were analyzed cytogenetically. The analysis of meiotic metaphases of *T. rubrofasciata* allowed to confirm the karyotype of species, out more, 2n = 25 (22A + X_1X_2Y). This number is very important for taxonomic and evolutionary inferences on the species, because of the 88 triatomine species with that described karyotype, only *T. rubrofasciata* exhibits 25 chromosomes. Based on the hypothesis of the karyotype 2n = 22 (20A + XY) as ancestral for triatomines, we propose three evolutionary hypotheses for the emergence of the karyotype of *T rubrofasciata*, all supported by agmatoploidy events (fission). Basically the hypotheses are 1) fission for a pair of autosomes, resulting in 22 autosomes and later fission of sex chromosome X; 2) fission of pair of autosomes and the sex chromosome X concomitantly; 3) fission of sex chromosome X and subsequently fission of pair of autosomes. Thus, this study highlights for the first time the importance of the number of chromosomes of *T. rubrofasciata* as characteristic diagnosis in Triatominae subfamily and describes three evolutionary hypotheses that possibly led the emergence of karyotype of this insect of global importance.

Introduction

Triatoma rubrofasciata (De Geer) (Hemiptera, Reduviidae,Triatominae) is an insect of great importance for global public health, since it is pantropical, and has already been

captured in approximately 45 countries distributed in the Americas, Africa, Asia and Oceania (Diotaiut *et al.* 2000; Galvão *et al.* 2003). In addition, it is one of potential vectors of protozoan *Trypanosoma cruzi* (Chagas) (Kinetoplastida: Trypanosomatidae), the etiologic agent of Chagas disease, although *T. rubrofasciata* vector competence has not been yet fully explored.

The presence of *T. rubrofasciata* has been widely reported in the Americas (WHO 2011). There are reports of natural infection with *T. cruzi* (Sherlock & Serafim 1974; Brasil & Silva 1983) and adapting the domestic environment in Brazil (Fuentes *et al.* 1971; Brasil & Silva 1983). In the African continent, this species was reported in South Africa, Angola, Guinea, Tanzania, Sierra Leone and Congo (Galvão *et al.* 2003). Although there are few studies on the presence of these vectors in Africa, Asia and Western Pacific, it is believed that this triatomine was introduced through maritime routes (WHO 2011).

Entomoepidemiological studies for Chagas disease are extremely important in Africa, since the *T. cruzi* was isolated from African mammals (Hamilton *et al.* 2009). Knowing the general aspects of biology of *T. rubrofasciata* is fundamental to generate subsides that can assist in the prevention of Chagas' disease, because the main way to minimize the incidence of Chagas disease is by controlling populations of vectors (Dias & Schofield 1998; Alevi *et al.* 2012a).

Although *T. rubrofasciata* was the first species of triatomine described (in 1773 as *Cimex rubrofasciatus*), little is known on its vector biology. Studies are restricted starvation resistance (Cortéz & Gonçalves 1998), interspecifc morphometric variability (Patterson *et al.* 2001), morphometry of testis follicles (Freitas *et al.* 2007a), coloration of the testicular peritoneal sheath (Alevi *et al.* 2014), ultrastructure of the male accessory glands (Freitas *et al.* 2007b), phylogeny (Hypsa *et al.* 2002; Justi *et al.* 2014) and cytogenetics (Manna 1950).

The chromosomes of the triatomines are holocentric, i.e., not present diffuse kinetochore and, with this, the centromeric region is absent (Panzera *et al.* 1996). Based on holocentric nature, fusion and fission events are termed agmatoploidy and simploidy. In view of this, this paper aims to discuss about the karyosystematic of *T. rubrofasciata* and the possible events related to karyotype evolution of this species.

Material and methods

Four adult males coming from the National and International Reference Laboratory in Taxonomy of Triatominae, FIOCRUZ, Rio de Janeiro, Brazil, were analyzed cytogenetically.

Seminiferous tubules 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).

Results

The analysis of meiotic metaphases of *T. rubrofasciata* allowed to confirm the karyotype of species, out more, 2n = 25 (22A + X_1X_2Y) (Fig. 1). This number is very important for taxonomic and evolutionary inferences on the species, because from 88 species of triatomines with that described karyotype, only *T. rubrofasciata* has 25 chromosomes (Fig. 2).

Discussion

The number of chromosomes can be extremely important to characterize the species. However, in the Triatominae subfamily, the variation of the number of chromosomes between the species is very low because of the 88 species with karyotype described, 51 species have 22 chromosomes and 30 species have 23 chromosomes (Alevi et al. 2013, 2015a). Ueshima (1966), based on the large number of species with 22 chromosomes, with determined the type number and possibly the ancestor karyotype of these vectors is 2n = 22 (20A + XY). Our results confirm the large number of species with 22 chromosomes, with approximately 58% (Fig. 2). The variation in the number of chromosomes of the triatomines usually is associated only the fragmentation of the sex chromosome X [e.g. *T. tibiamaculata* with 2n = 23 (20A + X_1X_2Y) and *T. vitticeps* with 2n = 24 (20A + $X_1X_2X_3Y$) (Panzera *et al.* 1996)], although there is also cases of fusion [e.g. *Panstrongylus megistus* that has 18 pairs of autosomes, resulting from the fusion of two ancestral pairs (Crossa *et al*. 2002)].

The analysis of meiotic metaphases of *T. rubrofasciata* allowed to confirm the karyotype of species, out more, 2n = 25 (22A + X_1X_2Y) (Fig. 1). Taxonomically, the number of chromosomes is of utmost importance to *T. rubrofasciata* since it functions as diagnostic characteristic for the species. The number of chromosomes was recently used to assist in the taxonomy of the species that make up the Brasiliensis (Alevi et al. 2012a, b, 2014), Matogrossensis (Alevi et al., 2015b, c) and Rubrovaria subcomplexes (Alevi et al., 2015c).

By means of phylogenetic reconstruction with molecular analyzes, *T. rubrofasciata* form one monophyletic clade with the species of the genus *Linshcosteus* and it is believed that *T. rubrofasciata* might have originated *Linshcosteus* spp. (Hypsa et al. 2002). The description

of the karyotypes of six species of *Linshcosteus* would go certainly clarify the relationships described above. However, due to the difficulty in obtaining material of *Linshcosteus*, cytogenetic analyzes were never performed these insects. Indeed, *T. rubrofasciat*a is clustered with North American species of *Triatoma* genus, but also with *Dipetalogaster* spp., *Eratyrus* spp., and *Panstrongylus* spp. (Hypsa et al. 2002), highlighting the need of a revision in genera within Triatomini (Gardim et al. 2014).

Based on the hypothesis of the Ueshima (1966) which shows the karyotype 2n = 22 (20A + XY) as ancestral for the triatomines, we propose three evolutionary hypotheses for the emergence of the karyotype of *T rubrofasciata*, all supported by agmatoploidy events (Fig. 3). Basically the hypotheses are 1) fission for a pair of autosomes, results in 22 autosomes and later fission of sex chromosome X; 2) fission of pair of autosomes and the sex chromosome X concomitantly; 3) fission sex chromosome X and subsequently fission of pair of autosomes.

Taking into consideration that the causative phenomenon of agmatoploidy and the order of fission events cannot be defined, we consider the three hypotheses for the origin of the karyotype of *T. rubrofasciata*. The events of agmatoploidy are relatively most common during the evolution karyotype of holocentric chromosomes, because the holocentric nature with diffuse kinetochore facilitates the permanence of chromosomal fragments during cell division, resulting in the fixing of karyotypes with numerical variability. Based on that, we emphasize that the karyotype evolution in Triatominae subfamily mainly occurs by agmatoploidy events, followed by genomic reorganization. Due to a possible emerging public health issue regarding this vector, for further studies we recommend elucidating its capacity to disperse to human dwellings (see Almeida *et al.* 2012) and also on vector competence (see Almeida *et al.* 2003, 2003, 2005).

Conclusion

Thus, this study highlights the first time the importance of the number of chromosomes of *T. rubrofasciata* as characteristic diagnosis in Triatominae subfamily and describes three evolutionary hypotheses that possibly led the emergence of karyotype of this insect of global importance.

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Figure 1. Karyotype of *T. rubrofasciata*. Note the 25 chromosomes (22A + X_1X_2Y). A = autosomes, X = X sex chromosome, Y = Y sex chromosome. Bar: 10 µm.

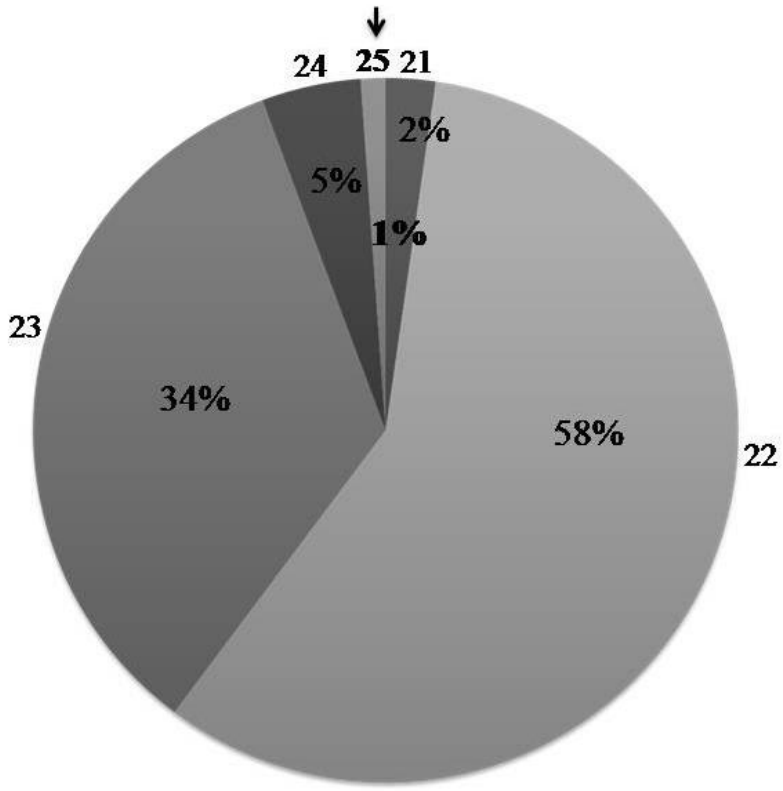

Figure 2. Graph representing the percentage of karyotypes of triatomines. 58% (2n = 22): tribe Rhodniini (*Rhodnius* and *Psammolestes*) and tribe Triatomini (*Dipetalogaster*, *Paratriatoma* and *Triatoma*); 34% (2n = 23): tribe Bolboderini (*Belminus*) and tribe Triatomini (*Eratyrus*, *Meccus*, *Mepraia*, *Nesotriatoma*, *Panstrongylus* and *Triatoma*); 5% (2n = 24): tribe Triatomini (*Panstrongylus* and *Triatoma*); 2% (2n = 21): tribe Triatomini (*Panstrongylus* and *Triatoma*); 1% (2n = 25): tribe Triatomini (*T. rubrofasciata*) (arrow).

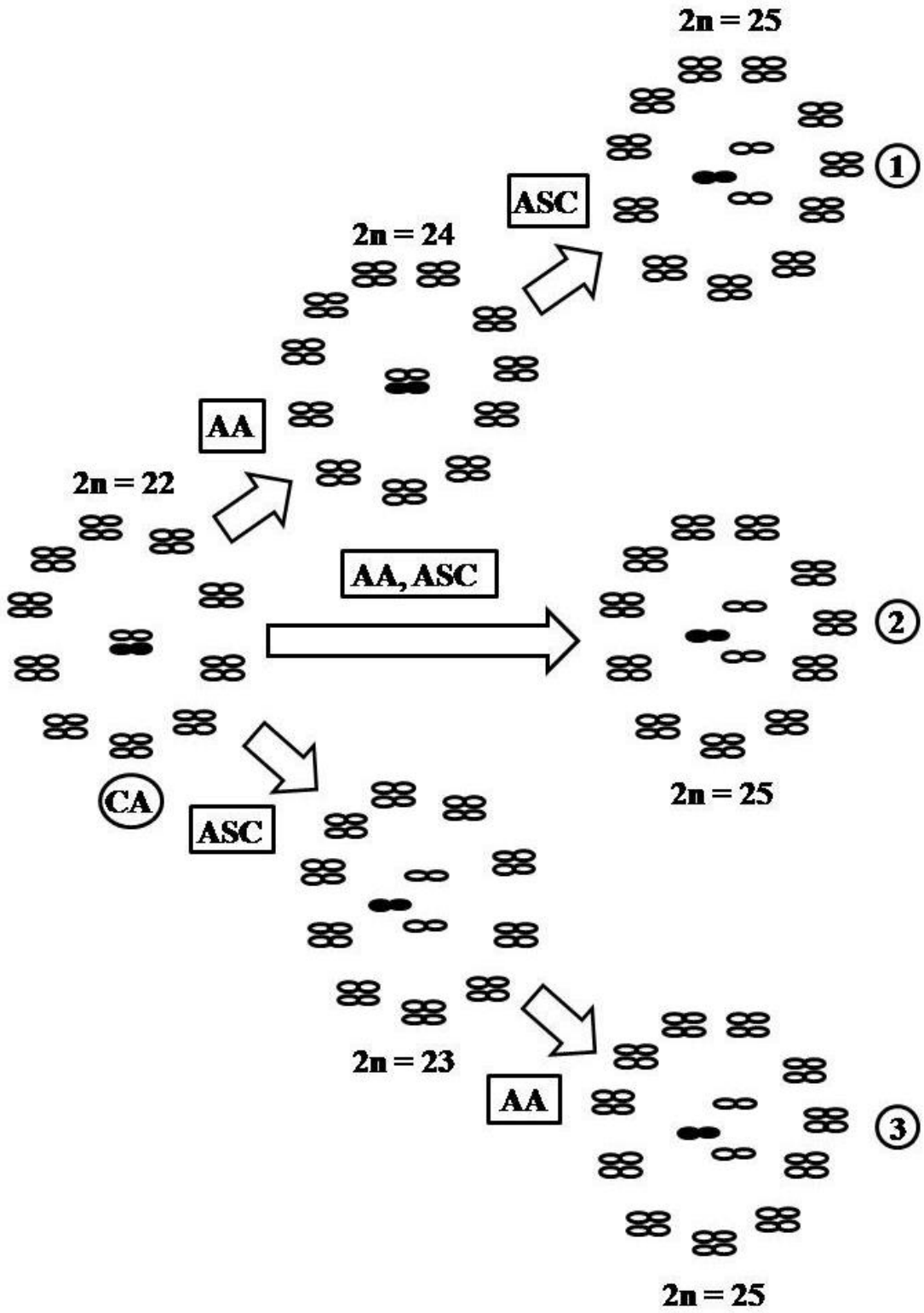

Figure 3. Diagram illustrating the three possible hypotheses for the karyotype evolution of *T. rubrofasciata*. CA: Ancestral common, AA: Agmetoploidy in pair of autosomes, ASC: Agmatoploidy in sex chromosome X.

IV.17 Capítulo 17 (Artigo científico publicado na revista *African Entomology* FI 0,81)

ALEVI, K. C. C.; NASCIMENTO, J. G. O.; MOREIRA, F. F. F.; JURBERG, J.; AZEREDO-OLIVEIRA, M. T. V. Cytogenetics characterization of *Triatoma rubrofasciata* (De Geer) (Hemiptera, Triatominae) spermatocytes and its cytotaxonomic application. **African Entomology**, v. 24, p. 257-260.

Cytogenetics characterization of *Triatoma rubrofasciata* (De Geer) (Hemiptera, Triatominae) spermatocytes and its cytotaxonomic application

SHORT COMMUNICATION

Triatoma rubrofasciata (De Geer) (Hemiptera, Triatominae) was the first Triatominae specie formally described, as *Cimex rubrofasciatus* De Geer, 1773. This insect presents anthropogenic habit (Galvão 2014) and is considered of global epidemiological importance, since it presents pantropical distribution and was found infected with *Trypanosoma cruzi* (Chagas, 1909) protozoan (Sherlock & Serafim 1974; Brazil & Silva 1983), the etiologic agent of Chagas disease.

This vector is found in approximately 45 countries (Galvao *et al.* 2003) and the Old World populations reached the New World probably by maritime transport (Patterson *et al.* 2001; Hypsa *et al.* 2002; Galvão 2014). In addition, this species had its dispersion favored by the interaction between the domiciliation event and human activities (Silveira & Rezende 1994).

Triatoma rubrofasciata is the only species of triatomine present in African continent reported in Angola, Congo (Katanga), Guinea (Conakry), Saudi Arabia, Sierra Leone, South Africa, Tanzania (Dujardin *et al.* 2015). Related studies regarding entomoepidemiological aspects of *T. cruzi* in Africa are scarce in the literature. However some exemplars of *T. cruzi* were identified from African mammals (Hamilton *et al.* 2009), which highlights the need for new epidemiological studies, including about the species of insect vector.

Biological studies about *T. rubrofasciata* are restricted to starvation resistance (Cortéz & Gonçalves 1998), interspecifc morphometric variability (Patterson *et al.* 2001), morphometry of testis follicles (Freitas *et al.* 2007a), coloration of the testicular peritoneal sheath (Alevi *et al.* 2014a), male accessory glands ultrastructure (Freitas *et al.* 2007b),

phylogeny (Hypsa *et al.* 2002; Justi *et al.* 2014) and karyotipic (Manna 1950; Alevi *et al.* 2015a).

The application of different cytogenetic markers has contributed substantially to triatomines taxonomy (Ueshima 1966; Pérez *et al.* 1992; Panzera *et al.* 1997, 2010, 2012; Alevi *et al.* 2012a, 2013a, 2014b, 2015a). Thus, this study aims to characterize cytogenetically the spermatocytes in early prophase of *T. rubofasciata* and compare with all the other species of the genus *Triatoma* already analyzed, with cytotaxonomic focus.

Four adult males coming from the National and International Reference Laboratory in Taxonomy of Triatominae, FIOCRUZ, Rio de Janeiro, Brazil, were analyzed cytogenetically. Seminiferous tubules 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 analysis of initial prophase of *T. rubrofasciata* allowed to observe that this species has a chromocenter formed by the sex chromosomes (Figure 1, arrow) and many heteropycnotic blocks dispersed in the nucleus. This result was compared with that described for 33 species of the genus *Triatoma* (Table I).

The comparative analysis of spermatocytes in early prophase were important taxonomic tools to assist in the differentiation of species *T. maculata* complex (Santos *et al.* 2007), assist in the grouping of *T. brasiliensis* subcomplex (Panzera *et al.* 2000, Alevi *et al.* 2012a, 2013a, 2014b) and demonstrate variation intraspecific in *T. sordida* (Panzera *et al.* 1997) and *T. dimidiata* (Panzera *et al.* 2006).

The comparative analysis of *T. rubrofasciata* with the species of the genus *Triatoma*, whether by chromocenter composition, whether by arrangement of heteropycnotic/heterochromatic blocks in the nucleus allowed the differentiation this vector of other 30 species (Table I). Only one of the *T. dimidiata* population, *T. protracta* and *T. tibiamaculata* showed the same characteristics observed for *T. rubrofasciata*. However, none of these species have evolutionary relationship with *T. rubrofasciata* (Justi *et al.* 2014) and by means of karyosystematic can be easily differentiated (Alevi *et al.* 2015b).

Therefore, the analysis of spermatocyte *T. rubrofasciata* showed to be an extremely important taxonomic tool, since it allows distinguish this species from other 30 species of triatomines of the genus *Triatoma*. The correct classification *T. rubrofasciata* is of global importance and can help vector control programs.

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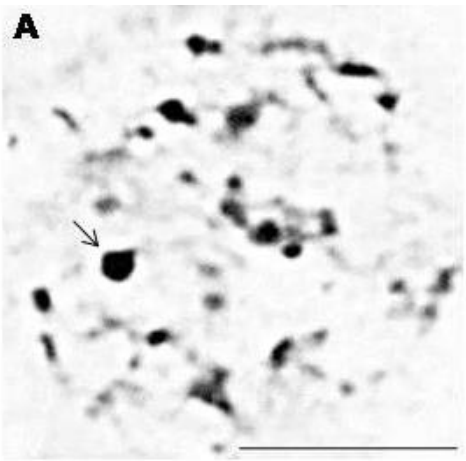

Fig. 1. Initial prophase of *T. rubrofasciata.* Note the presence of a chromocenter formed by the sex chromosomes (arrow) and blocks heteropycnotic dispersed in the nucleus. Bar: 10 μm.

Table 1. Cytogenetic characteristics in spermatocytes of *Triatoma*.

Species	Prophase		References
	chromocenter	chromatin	
Triatoma baratai	X and Y	-	Alevi et al. (2015a)
Triatoma brasiliensis	X, Y and two autosomes	dispersed blocks	Panzera et al. (2000)
Triatoma carcavalloi	X and Y	-	Alevi et al. (2015a)
Triatoma circummaculata	X and Y	-	Panzera et al. (1998)
Triatoma costalimai	X and Y	-	Alevi et al. (2015a)
Triatoma delpontei	X and Y plus autosomes	-	Panzera et al. (1995)
Triatoma dimidiata	X_1, X_2 and Y	dispersed blocks	Panzera et al. (2006)
Triatoma dimidiata	X_1, X_2 and Y	-	Panzera et al. (2006)
Triatoma garciabesi	X and Y	-	Jurberg et al. (1998)
Triatoma gerstaeckeri	X_1, X_2 and Y	-	Ueshima (1966)
Triatoma guasayana	X and Y	-	Panzera et al. (1997)
Triatoma guazu	X and Y	-	Alevi et al. (2015a)
Triatoma infestans	X and Y plus autosomes	-	Panzera et al. (1995)
Triatoma juazeirensis	X, Y and two autosomes	dispersed blocks	Panzera et al. (2000)
Triatoma jurbergi	X and Y	-	Alevi et al. (2015a)
Triatoma klugi	X and Y	-	Costa et al. (2008)
Triatoma lenti	X, Y and two autosomes	dispersed blocks	Alevi et al. (2012b)
Triatoma lecticularia	X and Y	-	Tavares and Azeredo-Oliveira (2007)
Triatoma maculata	X and Y	dispersed blocks	Santos et al. (2007)
Triatoma matogrossensis	X and Y	-	Pérez et al. (1992)
Triatoma melanica	X, Y and two autosomes	dispersed blocks	Alevi et al. (2014c)
Triatoma melanocephala	X_1, X_2, X_3 and Y	-	Alevi et al. (2013b)
Triatoma petrochiae	X, Y and two autosomes	dispersed blocks	Panzera et al. (2000)
Triatoma petrochiae	X and Y	-	Alevi et al. (2014b)
Triatoma pintodiasi	X and Y	-	Alevi et al. (2015c)
Triatoma platensis	X and Y plus autosomes	chromocenter (several autosomal)	Panzera et al. (1995)
Triatoma protracta	X_1, X_2 and Y	dispersed blocks	Ueshima (1966)
Triatoma pseudomaculata	X and Y plus autosomes	dispersed blocks	Santos et al. (2007)
Triatoma rubrofasciata	X_1, X_2 and Y	dispersed blocks	This paper
Triatoma rubrovaria	X and Y	-	Panzera et al. (1995)
Triatoma sherlocki	X, Y and two autosomes	dispersed blocks	Panzera et al. (2010)
Triatoma sordida	X and Y	-	Panzera et al. (1997)
Triatoma sordida	X and Y plus autosomes	dispersed blocks	Panzera et al. (1997)
Triatoma tibiamaculata	X_1,X_2 and Y	dispersed blocks	Panzera et al. (1998)
Triatoma vandae	X and Y	-	Panzera et al. (2010)
Triatoma vitticeps	X_1, X_2, X_3 and Y	-	Panzera et al. (1998)
Triatoma williami	X and Y	-	Succi et al. (2014)

4.18 Capítulo 18 (Artigo científico publicado na revista *Genetics and Molecular Research* FI 0,98)

ALEVI, K.C.C.; RAVAZI, A.; MENDONÇA, V.J.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Karyotype of *Rhodnius montenegrensis* (Hemiptera, Triatominae). Genetics and Molecular Research, v. 14, p. 222-226, 2015.

Karyotype of *Rhodnius montenegrensis* (Hemiptera, Triatominae)

ABSTRACT

The Triatominae subfamily comprises 6 tribes. The tribe Rhodniini comprises 2 genera and 22 nominal species. *Rhodnius montenegrensis* (Hemiptera, Triatominae) was recently described as evolutionarily related to *R. robustus*. Therefore, in order to contribute to karyosystematic study of the tribe Rhodniini, this report describes the number of chromosomes and compares the karyotype of *R. montenegrensis* to that of all other species in the tribe, in order to determine the karyotypic evolution of the tribe Rhodniini. The seminiferous tubules of adult males, after being removed and fixated on a cover slip, were processed with lacto-aceto-orcein for cytogenetic analysis. *R. montenegrensis*, as well as all other species of the tribe Rhodniini showed 22 chromosomes (20 autosomes + XY). Thus, we hereby describe the karyotype of the species *R. montenegrensis* and mainly highlight that the tribe Rhodniini displays karyotypic homogeneity, demonstrating itself as a derived group to a lesser extent when compared to the number of chromosomes of the common ancestors of the subfamily Triatominae.

INTRODUCTION

Triatomines are insects that are taxonomically included in the Order Hemiptera and Suborder Heteroptera, within the Family Reduviidae, and subfamily Triatominae (Lent and Wygodzinsky, 1979). The subfamily Triatominae consists of 148 species (Abad-Franch et al., 2013; Alevi et al., 2013f; Jurberg et al., 2013 Poinar, 2013). All triatomine species are susceptible to infection by the protozoan *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae) and therefore potential vectors of Chagas disease. Infection occurs through food with infected blood and all instars that are likely to ingest the parasite, since hematophagy is mandatory at all stages of the insect's lifecycle (Noireau et al., 2009).

The subfamily Triatominae comprises 6 tribes, namely, Alberproseniini, Bolboderini, Cavernicolini, Linshcosteusinii, Rhodniini, and Triatomini (Galvão et al., 2003; Tartarotti et al., 2006; Alevi et al., 2013f). The tribe Rhodniini comprises of 2 genera and 22 nominal species (Table 1). Recently, Rosa et al. (2012) described the species *Rhodnius montenegrensis* as related to *R. robustus* that belongs to *R. prolixus* complex (Rosa et al., 2012, 2014). This species was found to be infected by the protozoan *Trypanosoma rangeli* (Meneguetti et al., 2014).

Therefore, in order to contribute to karyosystematic study of the tribe Rhodniini, this report describes the number of chromosomes and compares the karyotype of the species *R. montenegrensis* with all other species of the tribe, in order to determine karyotypic evolution in the tribe Rhodniini.

MATERIAL AND METHODS

In this study, we used 10 males of the species *R. montenegrensis*, assigned by the "Triatominae Insectarium" at the Department of Biological Sciences, Faculty of Pharmaceutical Sciences, Araraquara campus. The seminiferous tubules of adult males, after being removed and fixated on a cover slip, were processed for cytogenetic analysis using lacto-acetic orcein technique (De Vaio et al., 1985, with modifications described by Alevi et al., 2012a). The biological material was analyzed using 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 GmbH). The images were magnified by a factor of 1000.

RESULTS

Analysis of metaphase I (Figure 1I) and metaphase II (Figure 1II) revealed that the species *R. montenegrensis* possesses a diploid chromosome set 2n = 22 (20A + XY). This chromosome number is shared across all members of the tribe Rhodniini (Table I).

DISCUSSION

The similarity between *Rhodnius* species is so great that one species is frequently mistaken for another, due to morphologic similarity. The classification of Rhodniini, as a monophyletic tribe, takes into account characteristics of the genus *Rhodnius* not shared with other triatomines, such as, apical antenna insertion, body forms, post-ocular callosities, male genital characteristics, egg-surface architecture, and presence of nitrophorin in the salivary

glands. Besides these characteristics, the genera *Rhodnius* and *Psammolestes* are primarily arboreal species in contrast to the terrestrial habits of most of the other triatomines (Schofield and Dujardin, 1999).

Using DNA sequence analysis of mitochondrial ribosomal RNA, mitochondrial cytochrome b, and nuclear RNA, Monteiro et al. (2000) indicated a paraphyletic nature of the genus *Rhodnius*, supporting the monophyly of the Rhodniini tribe. The Rhodniini, Cavernicolini, Bolboderini, Linshcosteini, and Alberproseniini tribes constitute monophyletic groups, while the Triatomini tribe is considered polyphyletic (Tartarotti et al., 2006). No reports are currently available regarding the number of chromosomes of representatives from Alberproseniini, Cavernicolini, Bolboderini, or Linshcosteini tribes.

Cytogenetic studies have focused mainly on Rhodniini and Triatomini tribes, where 86 karyotypes have been described to date (Alevi et al., 2013f). Cytogenetic data are important tools that assist with taxonomic and evolutionary knowledge of the triatomine bugs. (Ueshima, 1966; Pérez et al., 1992; Alevi et al., 2012a, b; 2013 a, b, c, d).

All species of the tribe Rhodniini with the karyotype described, including *R. montenegrensis*, had the same number of chromosomes, namely 22 (20 autosomes + XY). Besides the number of chromosomes, insects of the tribe Rhodniini share the location of the probe 45S of nucleolar organizing region (NOR) which is restricted to sex chromosomes (Pita et al., 2013). Ueshima (1966) indicated that the common ancestor of all organisms of the Triatominae subfamily presents the karyotype 2n = 20A + XY, as observed in the tribe Rhodniini. The tribe Triatomini is the most derivative, since all mechanisms of sex determination, derived from fragmentation of the sex chromosome X, are found in this group (XY, X_1X_2Y, $X_1X_2X_3Y$) (Alevi et al., 2013f).

Thus, we describe the karyotype of the species *R. montenegrensis* and highlight that the tribe possesses karyotypic homogeneity, demonstrating itself as a derived group to a lesser extent when compared to the number of chromosomes of the common ancestors of the subfamily Triatominae.

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Table I. Species of triatomines that constitute the tribe Rhodniini with their respectives karyotypes. P: genus *Psammolestes*, R: genus *Rhodnius*.

Tribe Rhodniini	Karyotype	Described by:
P. arthuri	Not described	
P. coreodes	2n = 20A + XY	Schereiber and Pellegrino (1950)
P. tertius	2n = 20A + XY	Panzera et al. (1998)
R. amazonicus	Not described	
R. barretti	Not described	
R. brethesi	2n = 20A + XY	Panzera et al. (1998)
R. colombiensis	2n = 20A + XY	Dujardin et al. (2002)
R. dalessandroi	Not described	
R. domesticus	2n = 20A + XY	Dujardin et al. (2002)
R. ecuadoriensis	2n = 20A + XY	Panzera et al. (1996)
R. milesi	2n = 20A + XY	Panzera et al. (2010)
R. montenegrensis	2n = 20A + XY	First described
R. nasutus	2n = 20A + XY	Pérez et al. (1992)
R. neglectus	2n = 20A + XY	Barth (1956)
R. neivai	2n = 20A + XY	Koshy (1979)
R. pallescens	2n = 20A + XY	Panzera et al. (1996)
R. paraensis	Not described	
R. pictipes	2n = 20A + XY	Koshy, (1979)
R. prolixus	2n = 20A + XY	Schereiber and Pellegrino (1950)
R. robustus	2n = 20A + XY	Koshy (1979)
R. stali	2n = 20A + XY	Dujardin et al. (2002)
R. zeledoni	Not described	

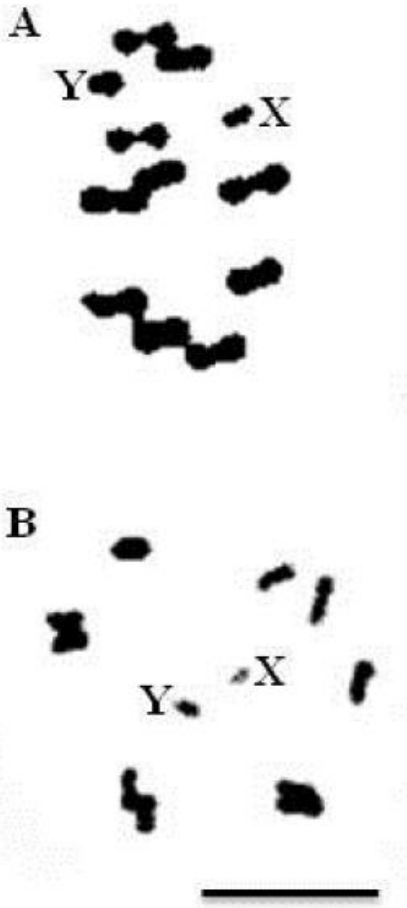

Figure 1. Seminiferous tubule of *Rhodnius montenegrensis* stained by lacto-aceto-orcein. (A) Metaphase I with 10 bivalent autosomes and sex chromosomes. (B) Metaphase II with 10 autosomes and sex chromosomes. X: X sex chromosome; Y: Y sex chromosome. Bar: 10 μm.

4.19 Capítulo 19 (Artigo científico publicado na revista *Genetics and Molecular Research* FI 0,98)

RAVAZI, A.; OLIVEIRA, J.; ROSA, J. A.; AZEREDO-OLIVEIRA, M. T. V.; ALEVI, K. C. C. ALEVI. Spermiotaxonomy of the tribe Rhodniini (Hemiptera, Triatominae). **Genetics and Molecular Research**, v. 15, p. 01-04, 2016.

Spermiotaxonomy of the tribe Rhodniini (Hemiptera, Triatominae)

ABSTRACT

The tribe Rhodniini is a monophyletic group composed of 22 species, with 19 in the *Rhodnius* genus and three in the *Psammolestes* genus. These insects are morphologically very similar (cryptic species), and new tools are important for investigating the taxonomy of these vectors. Spermiotaxonomy is an important tool in differentiating between related species, and this study analyzed the spermatids of Rhodniini species to elucidate their spermiotaxonomy. All of the Rhodniini species contained two heteropyknotic filaments in the extremities of their cells. Although spermiotaxonomy has been an important tool in differentiating between species of the *Triatoma* genus, all of the species in the *Rhodnius* genus exhibited the same characteristics in their male gametes. However, spermatids analysis made it possible to confirm the monophyly of the Rhodniini tribe, because *Psammolestes tertius* had the same pattern as that described for *Rhodnius*. The results of this study demonstrate that spermiotaxonomy, in addition to being an important tool for differentiating between related speciesof *Triatoma,* can be used as an optimization tool in phylogenetic analyses.

INTRODUCTION

The triatomines are insects that belong to the Hemiptera order, Heteroptera suborder, Reduviidae family, and Triatominae subfamily (Lent and Wygodzinsky, 1979). The Triatominae subfamily is composed of 150 species, grouped in 18 genera and six tribes (Alevi et al., 2015). All of the species of the subfamily are bloodsucking and potential vectors of the *Trypanosoma cruzi* protozoan, which is an etiological agent of Chagas disease (Noireau et al., 2009).

The tribe Rhodniini is a monophyletic group (Monteiro et al., 2000) composed of 22 species, with 19 in the *Rhodnius* genus and three in the *Psammolestes* genus (Rosa et al., 2012; Abad-Franch et al., 2013; Alevi et al., 2012). These insects are morphologically very similar (cryptic species) (Monteiro et al., 2000), and new tools are important for investigating the taxonomy of these vectors.

Recently, spermiotaxonomy was used in the *Triatoma* genus to differentiate between related species (Alevi et al., 2013a, 2014a). Cytogenetic analysis of male gametes of *Triatoma melanocephala* and *T. vitticeps*, which are considered sister species (Alevi et al., 2013b; Gardim et al., 2014), allowed the differentiation of these species (Alevi et al., 2014a). In addition, the same analysis also made it possible to differentiate *T. lenti* from *T. sherlocki* (Alevi et al., 2013a).Therefore, this study aimed to analyze spermatids of species of the Rhodniini tribe to elucidate the spermiotaxonomy of these vectors.

MATERIAL and METHODS

Three adult males of each species (Table 1) were cytogenetically analyzed. The insects were donated by "Insetário de Triatominae" of the Biological Sciences Department of the Faculty of Pharmaceutical Sciences, State University of São Paulo, Campus Araraquara, São Paulo, Brazil. Microscope slides with the biological material (seminiferous tubules) were prepared by the crushing technique and stained with lacto-acetic orcein (De Vaio et al., 1985) with modifications by Alevi et al. (2012). The slides were analyzed using a Jenavallight microscope (Zeiss) coupled to a digital camera and an AxioVision LE 4.8 image analyzer (Zeiss). The images were magnified by 1000X.

RESULTS

All of the species of the Rhodniini tribe exhibited the same spermatids characteristic, i.e., the presence of two heteropyknotic filaments in the extremities of their cells (Figure 1 and Table 1).

DISCUSSION

Abad-Franch et al. (2013) suggested that there may be many errors related to the taxonomy of the genus *Rhodnius*, mainly because of cryptic species. The authors propose that *R. milesi* is probably a *R. neglectus* variant from south-eastern Amazonia, *R. zeledoni* closely

resembles *R. domesticus*, and *R. montenegrensis* probably represents one of the *R. robustus* lineages (Monteiro et al., 2000).

Although spermiotaxonomy has been an important tool in differentiating between triatomines of the *Triatoma* genus (Alevi et al., 2013a, 2014a), all of the species in the *Rhodnius* genus exhibited the same male gamete characteristics. Therefore, the taxonomic issues raised by Abad-Franch et al. (2013) could not be assessed using this tool.

However, spermatids analysis made it possible to confirm the monophyly of the Rhodniini tribe, because *P. tertius* exhibited the same pattern as described for *Rhodnius*. This phylogenetic relationship was proposed by Lent and Wygodzinsky (1979) based on morphological characteristics, and by Monteiro et al. (2000) based on molecular analyses. This parameter was recently used to evaluate relationships between species of the Brasiliensis subcomplex, and it was possible to exclude *T. melanocephala*, *T. vitticeps*, and *T. tibiamaculata*, as well as suggest that *T. lenti* is the sixth subcomplex member (Alevi et al., 2014b). The results of this study demonstrate that spermiotaxonomy, in addition to being an important tool for differentiating between related species of *Triatoma*, can be used as an optimization tool in phylogenetic analyses.

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Table I. Heteropyknotic pattern in spermatids of species of the tribe Rhodniini.

Species	Heteropyknotic pattern in spermatids	References
Rhodnius colombiensis	Two heteropyknotic filament	Present paper
Rhodnius domesticus	Two heteropyknotic filament	Morielle et al. (2004)
Rhodnius montenegrensis	Two heteropyknotic filament	Present paper
Rhodnius nasutus	Two heteropyknotic filament	Present paper
Rhodnius neglectus	Two heteropyknotic filament	Present paper
Rhodnius neivai	Two heteropyknotic filament	Present paper
Rhodnius pictipes	Two heteropyknotic filament	Present paper
Rhodnius prolixus	Two heteropyknotic filament	Present paper
Rhodnius robustus	Two heteropyknotic filament	Present paper
Psammolestes tertius	Two heteropyknotic filament	Present paper

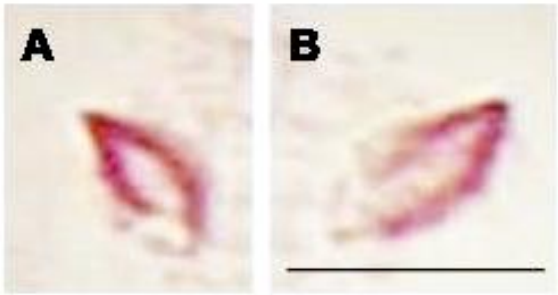

Figure 1. Early spermatids of *Psammolestes tertius* (A) and *Rhodnius montenegrensis* (B). All of the species analyzed had two heteropyknotic filaments in their haploid cells. Bar: 10 μm.

4.20 Capítulo 20 (Artigo científico publicado na revista *Genetics and Molecular Research* FI 0,98)

ALEVI, K.C.C.; RAVAZI, A.; FRANCO-BERNARDES, M.F.; ROSA, J.A.; AZEREDO-OLIVEIRA, M.T.V. Chromosomal evolution in the pallescens group (Hemiptera, Triatominae). **Genetics and Molecular Research**, v. 14, p. 12654-12659, 2015.

Chromosomal evolution in the pallescens group (Hemiptera, Triatominae)

ABSTRACT

Rhodnius colombiensis, in conjunction with *R. pallescens* and *R. ecuadoriensis*, forms the monophyletic pallescens group. Cytogenetic analyses of these closely related species would further our understanding of the taxonomy and evolution of this group. In this study, *R. colombiensis* was cytogenetically analyzed, and the results were compared with cytogenetic data from other species of the pallescens group, particularly their chromosomal evolution. We found that this triatomine has heteropycnotic blocks in five autosomal bivalents at both metaphase I and II. The derivation of *R. colombiensis* from *R. pallescens* led to significant loss of heteropycnotic and heterochromatic regions (approximately 50%). *R. ecuadoriensis* is the most differentiated of the group because it has lost all heterochromatin and heteropyknotic blocks in the autosomes. Based on the heteropyknotic and heterochromatic pattern of *R. colombiensis* and the chromosomal evolution analysis of the pallescens group, we suggest that the karyotype of *R. colombiensis* and *R. ecuadoriensis* lost its heteropycnotic and heterochromatic blocks during speciation. Furthermore, this loss could be related to adaptation to different environments.

INTRODUCTION

Triatomines are insects that are included in the Order Hemiptera and Suborder Heteroptera within the Family Reduviidae and subfamily Triatominae (Lent and Wygodzinsky, 1979). The subfamily Triatominae consists of 148 species distributed in 18 genera and 6 tribes (Abad-Franch et al., 2013; Alevi et al., 2013; Jurberg et al., 2013; Poinar Jr, 2013).

The tribe Rhodniini consists of 22 species, 19 of the genus *Rhodnius* and 3 of the genus *Psammolestes* (Abad-Franch et al., 2013; Alevi et al., 2013). Hemipterans of the genus

Rhodnius are divided into two lineages, Pictipes and Robustus (Table 1), and three species groups, namely, pallescens, prolixus, and pictipes (Abad-Franch et al., 2009).

Rhodnius colombiensis, in conjunction with *R. pallescens* and *R. ecuadoriensis*, form a monophyletic group known as pallescens (Mejia et al., 1999; Schofield and Dujardin, 1999; Abad-Franch et al., 2009; Díaz et al., 2014). Abad-Franch and Monteiro (2007) stated that the cytogenetic analysis of these closely related species would further our understanding of the taxonomy and evolution of this group.

Therefore, specimens of *R. colombiensis* were studied and the results were compared with cytogenetic data from the other species of the pallescens group, particularly their chromosomal evolution.

MATERIAL AND METHODS

We used five *R. colombiensis* males obtained from the Triatominae Insectarium, Department of Biological Sciences, Faculty of Pharmaceutical Sciences, Araraquara campus, Universidade Estadual Paulista, Brazil. The seminiferous tubules of adult males, after being removed and fixed onto a cover slip, were processed for cytogenetic analysis using the lacto-acetic orcein technique (De Vaio et al., 1985, with modifications described by Alevi et al., 2012). The biological material was analyzed using a Jenaval light microscope (Zeiss) coupled to a digital camera and an image analyzer (Axio Vision LE 4.8, Zeiss). The images were magnified by a factor of 1000.

RESULTS

R. colombiensis contains heteropyknotic blocks dispersed in the nucleus of the initial prophase (Figure1A), as well as at one or both ends of four or five autosomes in metaphase I (Figure1B) and II (Figure 1C). This is presented in an ideogram (Figure 2B), which can be compared with the arrangement of heteropycnotic/heterochromatic blocks in *R. pallescens* (Figure 2A) and *R. ecuadoriensis* (Figure 2C).

The results were then compared with those obtained by classical and molecular cytogenetic analyses performed in the pallescens group (Panzera et al., 1998, 2012; Dujardin et al., 2002; Morielle-Souza and Azeredo-Oliveira, 2007; Gómez-Palacio et al., 2008; Pita et al., 2013) (Table 2).

DISCUSSION

The divergence of *R. colombiensis* from *R. pallescens* is associated with the emergence of the Panama Isthmus (Díaz et al., 2014), and the time since the divergence of *R. ecuadoriensis* from its Colombian relatives (*R. pallescens* and *R. colombiensis*) roughly coincides with the uplift of the Andes in the Pliocene (Abad-Franch and Monteiro, 2007).

Pre-zygotic (infeasibility of the reproductive organs) and post-zygotic (errors in meiotic pairing) barriers have been observed in an experimental hybrid cross between the sister species *R. colombiensis* and *R. pallescens* (Díaz et al., 2014). We suggest that the divergence of *R. colombiensis* from *R. pallescens* led to a significant loss of heteropyknotic and heterochromatic regions in the former karyotype (approximately 50%). *R. ecuadoriensis* is the most cytogenetically differentiated species within the group, because it has lost all of the heterochromatin and heteropycnotic blocks in the autosomes.

Panzera et al. (2004) suggested that the loss of heterochromatin is related to adaptive genomic changes that contribute to the capacity to survive, reproduce, and disperse in different environments. Abad-Franch and Monteiro (2007) proposed that the current distribution of *Rhodnius* is related to the effects of adaptive radiation and vicariance. Schreiber and Pellegrino (1950) suggested that heteropyknotic pattern differences in the autosomes might be related to triatomine speciation. We are of the opinion that during speciation in the pallescens group the loss of heteropycnotic blocks and constitutive heterochromatin in the autosomes was related to adaptation to different environments.

By conducting cytogenetic and molecular analyses, Gómez-Palacio et al. (2008, 2012) detected polymorphisms in *R. pallescens*. Morielle-Souza and Azeredo-Oliveira (2007) and Pita et al. (2013) analyzed *R. pallescens* at different locations using the *in situ* hybridization (FISH) technique, which probed 45S and obtained variable results (Table 1). Pita et al. (2013) detected intraspecific variation in the location of 45S ribosomal DNA clusters in *R. ecuadoriensis* from Ecuador and Peru (Table 1). The great polymorphism detected for *R. pallescens* confirms the possible origin of the pallescens group from this species.

In conclusion, our results suggest that *R. colombiensis* and *R. ecuadoriensis* have lost heteropycnotic blocks and C-positive heterochromatin during speciation. Furthermore, this loss could be related to adaptation to different environments.

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Table 1. Lineages of the genus *Rhodnius*.

Robustus lineage	**Pictipes lineage**
R. barretti	*R. amazonicus*
R. dalessandroi	*R. brethesi*
R. domesticus	*R. colombiensis*
R. milesi	*R. ecuadoriensis*
R. montenegrensis	*R. pallescens*
R. nasutus	*R. paraensis*
R. neivai	*R. pictipes*
R. neglectus	*R. stali*
R. prolixus	*R. zeledoni*
R. robustus	

Table 2. Cytogenetic characteristics of the pallescens group.

Technique and cytogenetic characteristics	***Rhodnius colombiensis***	***Rhodnius ecuadoriensis***	***Rhodnius pallescens***
LACTO-ACETIC ORCEIN			
Karyotype (2n)	20A + XY	20A + XY	20A + XY
Heteropyknotic pattern in prophase	One chromocenter and heteropyknotic blocks in chromatin	One heteropyknotic chromocenter	One chromocenter and heteropyknotic blocks in chromatin
Heteropyknotic pattern in chromosomes	Heteropyknotic blocks in five autosomal bivalents	Absent	Heteropyknotic blocks in all autosomal bivalents
C-BANDING			
Heterochromatic pattern in prophase	One C-positive chromocenter and C-positive heterochromatic blocks in chromatin	One C-positive chromocenter	One chromocenter and C-positive heterochromatic blocks in chromatin
Heterochromatic pattern in chromosomes	C-positive heterochromatic blocks in five autosomal bivalents	Absent	C-positive heterochromatic blocks in one or both ends of almost all autosomes
FISH	X chromosome	X and Y or small signal in Y	X and Y or X chromosome

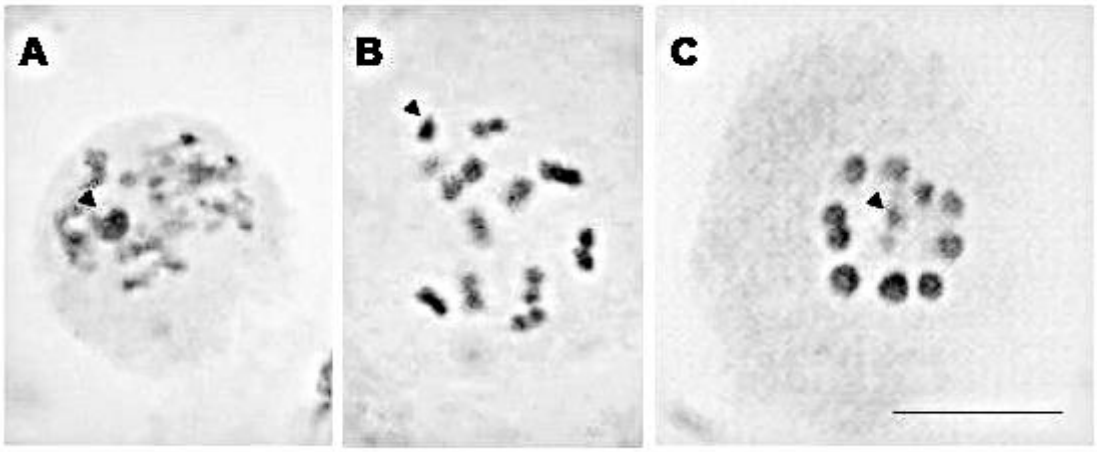

Figure 1. Seminiferous tubule of *Rhodnius colombiensis* stained by lacto-acetic orcein. (A) Prophase I. Note the chromocenter (arrowed) and the heteropycnotic blocks in chromatin. (B) Metaphase I. Note the heteropycnotic blocks in four or five autosomes and in the Y sex chromosome (arrowed). (C) Metaphase II. Note the heteropycnotic blocks in four or five autosomes and in the Y sex chromosome (arrowed). Bar = 10 μm.

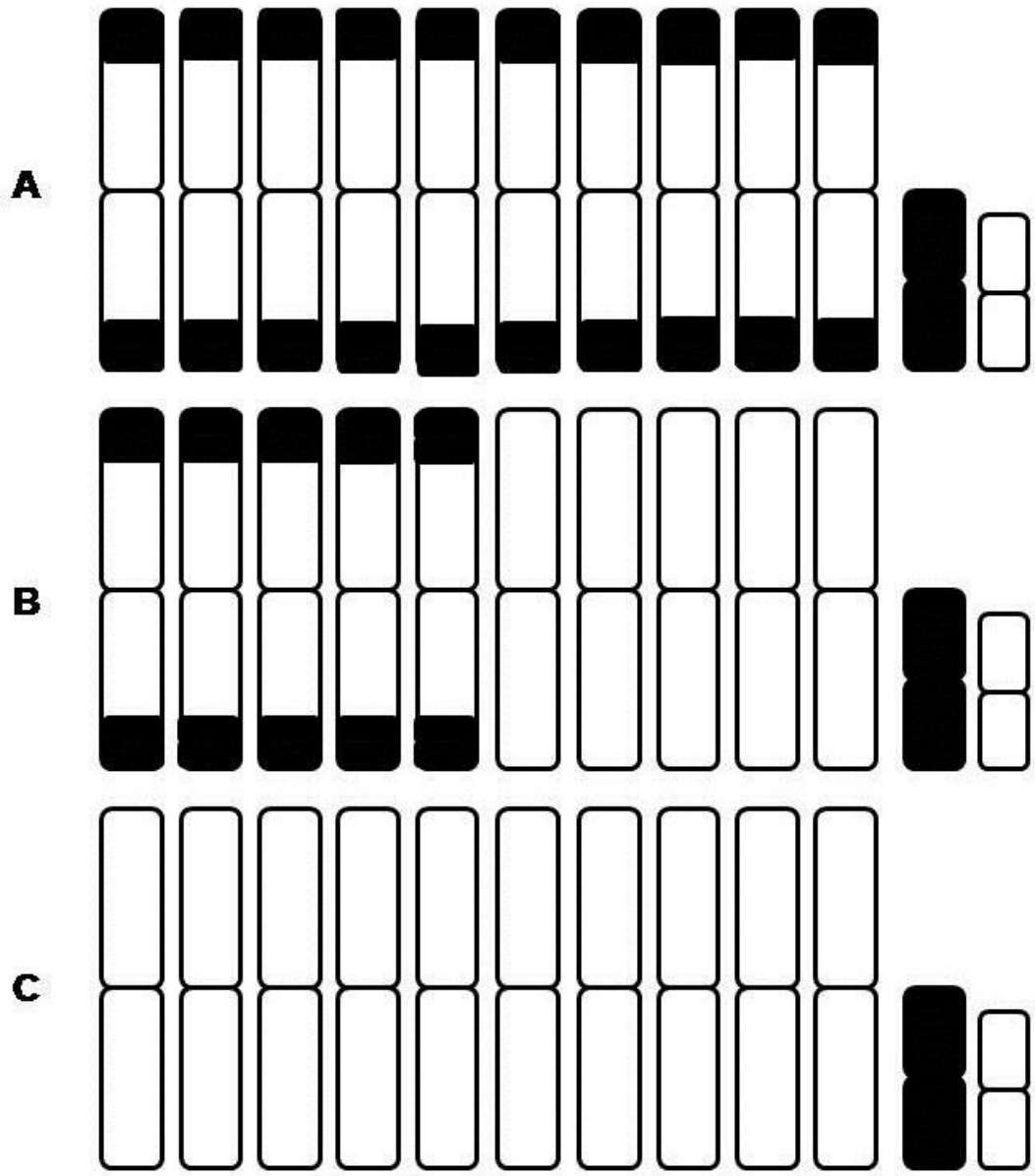

Figure 2. Ideogram of the male meiotic karyotype of *Rhodnius pallescens*, *R. colombiensis*, and *R. ecuadoriensis*, showing the distribution and evolution of the heteropyknotic and C-positive heterochromatin blocks in one or both ends of the autosomes. (A) *R. pallescens*. Note that all of the autosomes have heteropyknotic and C-positive heterochromatin. (B) *R. colombiensis*. There has been a significant loss of heteropyknotic and C-positive heterochromatin regions (approximately 50%). (C) *R. ecuadoriensis*. Note that none of the autosomes have heterochromatin or heteropycnotic blocks.

4.21 Capítulo 21

Chromosomal divergence between *Rhodnius montenegrensis* Rosa et al. (2012) and *R. robustus* Larrousse, 1927 (Hemiptera, Triatominae)

Abstract

Rhodnius robustus is a paraphyletic group formed by four different lineages, being suggested that *R. montenegrensis* is only one of lineages. Both the different lineages as *R. montenegrensis* have been found infected with *T. cruzi*, although *R. robustus* was observed infected invading houses, emphasizing that these species have different importance in the epidemiology of Chagas disease. Thus, this paper describes the disposition of the 45S rDNA clusters on chromosomes of *R. montenegrensis* and compares with *R. robustus*. Different from *R. robustus*, *R. montenegrensis* has marking on both sex chromosomes, confirming their specific status. Thus, assuming that *R. montenegrensis* is the most derived species, we suggest that possibly during the speciation it has occurred important performances of transposable elements (TEs) in genome evolution (exchange of ribosomal genes to the Y chromosome of the *R. montenegrensis* by TEs) and that this divergence has occurred a long time ago.

Short Report

The Chagas disease is a potentially life-threatening illness caused by the protozoan *Trypanosoma cruzi* Chagas, 1909, distributed mainly in endemic areas of 21 Latin American countries, where it is mostly vector-borne transmitted to humans by contact with faeces of triatomine, known as 'kissing bugs'. It is estimated that about 6 million to 7 million people are infected worldwide, mostly in Latin America where Chagas disease is endemic.[1]

As Chagas disease has no cure and treatment with benznidazole and nifurtimox is effective only in the acute phase of the disease (which is often asymptomatic), vector control is the most effective method of preventing this neglected disease.[1] Thus, all knowledge about these hematophagous insects is important and can generate subsidies to assist the vector control programs. The correct classification of the triatomines, for example, enables differentiates species of primary importance for species of minor importance, differentiating thereby the main species vectors of Chagas disease.

Currently, there are 152 species of triatomines, distributed in 18 genera and five tribes, being all species considered as potential vector of Chagas disease.[2] The Rhodniini tribe is

composed of 24 species grouped in two genera: *Rhodnius* (21 species) and *Psammolestes* (3 species). The taxonomy of the *Rhodnius* genus was initiated in 1859, with the description of *R. prolixus* and *R. nasutus*.[3] The species of *Rhodnius* genus are difficult to diagnose, since, morphologically are very similar.[4] Thus, these insects were grouped into specific monophyletic complex.[5,6]

Monteiro et al.[5] by means of molecular analysis with the Cyt b gene, observed that *R. robustus* is a paraphyletic group formed by four different lineages that occur in the north of South America. The lineage I of *R. robustus* occurs in the region of Orinoco (Venezuela) and lineages II, III and IV occur in the Amazon region, being that the line II occurs in Ecuador and Brazil, the III only in Brazil and IV in French Guiana and Brazil.

Rosa et al.[6] in describing of *R. montenegrensis* observed that this species is the sister of *R. robustus* and grouped as members of *prolixus* complex. However, Abad-Franch et al.[7] reported that due to cryptic speciation and phenotypic plasticity phenomenon in the *Rhodnius* genus, taxonomic errors can occur and suggested that *R. montenegrensis* were only one of lineages of *R. robustus* been described by Monteiro et al.[5]

The different lineages of *R. robustus* may have high rates of natural infection with *T. cruzi*. Recently, were detected *R. robustus* specimens infected by *T. cruzi* invading houses in the urban areas of Cochabamba, Bolivia.[8] On the other hand, although recently *T. cruzi* was also isolated from *R. montenegrensis* specimens in the State of Rondonia, Brazil,[9] this species has never been found invading homes, emphasizing that these species have different importance in the epidemiology of Chagas disease.

Recently, in order to better understand the evolutionary relationships between species of Rhodniini tribe, it analyzed the provision of 45S rDNA clusters in thirteen species of this tribe.[10] The authors observed chromosomal homogeneity (2n = 22) and restriction of the 45S gene to sex chromosomes, and, in all species analyzed marking have been restricted to the sex chromosome X (*R. nasutus, R. prolixus, R. robustus* and *R. colombiensis*) or the sex chromosomes X and Y (*R. domesticus, R. neglectus, R. neivai, R. milesi, P. tertius, R. pallescens, R. pictipes* and *R. stali*).

Pita et al.[10] analyzed the arrangement of 45S rDNA clusters of the lineage II of *R. robustus* and have noted that it shows restricted marking the sex chromosome X. This line of *R. robustus* is the same as specimens from Monte Negro, Brazil (lineage possibly described as *R. montenegrensis*[7]). Thus, in order to assess the specific status of *R. monetenegrensis* this

paper describes the layout of the 45S rDNA clusters on chromosomes of *R. montenegrensis* and compares the described for *R. robustus* II, with taxonomic and epidemiological focus.

Ten adult male specimens of *R. montenegrensis*, from the "Insectarium of Triatomine" FCFAR / UNESP, Araraquara, São Paulo, Brazil (insects initially captured in Monte Negro, Rio Grande do Sul, Brazil) were dissected and testis stored in methanol: acetic acid (3: 1). Subsequently, slides were prepared, FISH assays with the 45S probe were performed and the images captured according Pita et al.[10]

Analysis of meiotic metaphase from *R. montenegrensis* made possible to demonstrate that the species has a marking on both sex chromosomes (X and Y) (Figure 1). This provision, although it is most common in the genus *Rhodnius*[10], is different from that observed in *R. robustus* which presents marking with the 45S probe only on the X sex chromosome[10].

Pita et al.[10] consider the hypothesis that the presence of ribosomal genes in both sex chromosomes is the ancestral form and suggest that the species with the 45S probe only on the X chromosome lost ribosomal locus of chromosome Y. However, the authors did not discard the hypothesis of a possible partial transfer of the ribosomal genes of chromosome X to Y by means of transposable elements (TEs) or ectopic recombination.

The presence of 45S rDNA clusters in the sex chromosomes X and Y of *R. montenegrensis* compared with lineage II of *R. robustus*, supports the specific status of the species. Moreover, assuming that *R. montenegrensis* is the most derived species, we suggest that the evolutionary event that occurred during speciation *R. montenegrensis* was the exchange of ribosomal genes to the Y chromosomes by TEs.

TEs are sources of genetic variability that influence the evolutionary trajectory of the species. These mobile elements basically have the ability to copy and paste information from the genome and may accelerate the evolution of genes and trigger the rapid divergence between species.[11] However, to minimize the deleterious effects, TEs often are directed to regions of heterochromatin.[12,13] Thus, the heterochromatic nature of the sex chromosome Y of triatomines is a facilitator for occurrence of this event in Triatominae.

Taking into consideration that the TEs play an important role in genomic reorganization of species[14,15,16] and may be related to the process of speciation[17,18,19], we suggest that possibly during the divergence between *R. montenegrensis* and *R. robustus* has occurred important performances of TEs in genome evolution these vectors. Furthermore, although a molecular clock must be constructed, we also suggest that this divergence has

occurred a long time ago because the fixing of the genes takes millions of years as, for example, Hahn et al.[20] observed that fixing of 17 genes in flies took 1 million years.

Thus, we confirm the specific status of *R. montenegrensis*, we suggest that chromosomal divergence between *R. montenegrensis* and *R. robustus* was performed by TEs and we suggest that divergence between this species occurred a long time ago.

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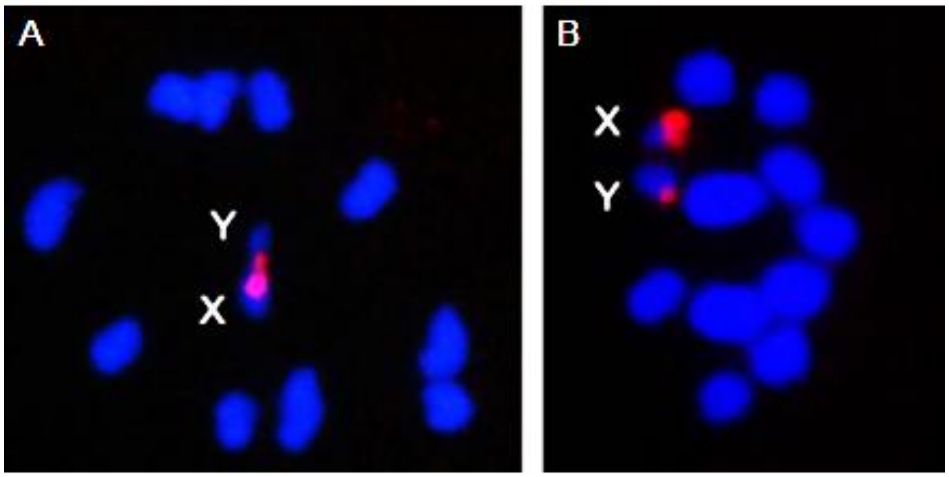

Figure 1: Metaphase I (B) and II (A) of *R. montenegrensis*. Note the presence of NORs in X and Y sex chromosomes.

4.22 Capítulo 22 (Artigo científico publicado na revista *Genetics and Molecular Research* FI 0,98)

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Entoepidemiology of Chagas disease in the Western region of the State of São Paulo from 2004 to 2008, and cytogenetic analysis in *Rhodnius neglectus* (Hemiptera, Triatominae)

ABSTRACT

To complement the epidemiological data and assist in the prophylaxis of Chagas disease in the State of São Paulo, we examined entomological lifting conducted in 40 municipalities of the Western region of the state from 2004 to 2008, highlighted the main vector species in this region, and reanalyzed the cytogenetic characteristics of *Rhodnius neglectus* from 3 different Brazilian states (Formoso/GO, Frutal/MG, Guaíra/SP, and Pitangueiras/SP). The municipalities of Castilho and Santo Antônio do Acaranguá registered the highest relative amounts of notifications. The main species notified in Western São Paulo were *Triatoma sordida* and *R. neglectus*. We collected a large number of *T. sordida* in 2005 and noted the absence of notification of infected insects in 2008. We observed no variation in chromosomal characteristics of *R. neglectus* of different states. These data are complementary to the survey presented from 1990 to 1999, as the vector species were the same (*T. sordida* and *R. neglectus*), with emphasis on *T. sordida*. We corroborate the future colonization domiciliary initially proposed for *T. sordida* in the region and underscore the importance of vector control programs in the prophylaxis of Chagas disease. Furthermore, we observed that the populations of *R. neglectus* in Brazil showed no intraspecific variation and we corroborated the chromosomal patterns originally described. These data are important for understanding the evolution of these hematophagous insects, which are vectors of Chagas disease.

INTRODUCTION

Triatomines belong to the Hemiptera order, Heteroptera suborder, Reduviidae family, and Triatominae subfamily (Lent and Wygodzinsky, 1979). The Triatominae subfamily is composed of 148 species distributed in 18 genera and 6 tribes (Tartarotti et al., 2006; Abad-Franch et al., 2013; Alevi et al., 2013a; Poinar, 2013). All triatomine species are hematophagous and potential vectors of Chagas disease. During the repast, these insects can become infected with the etiologic agent of Chagas disease *Trypanosoma cruzi*, a protozoan that can be transferred between hosts by these vectors (Noireau et al., 2009).

This disease is regarded as the second most endemic disease in Latin America. Approximately 8 million people are infected with *T. cruzi* worldwide, and approximately 2.5 million people are infected in Brazil (Neto and Pasternak, 2009; WHO, 2013). Because there is no specific treatment for Chagas disease, control of vector populations is the main method used for minimizing the incidence of this disease, which has recently been referred to as "the new acquired immunodeficiency syndrome (AIDS) of the Americas" (Hotez et al., 2012).

Currently, 65 triatomine species have been identified in Brazil (Gardim et al., 2014) and were found to be involved in the transmission of Chagas disease, highlighting *Triatoma infestans*, *Panstrongylus megistus*, *Triatoma brasiliensis*, *Triatoma sordida*, *Triatoma pseudomaculata*, and *Rhodnius neglectus* (Dias and Schofield, 1998; Silistino-Souza et al., 2013). After the International Certification of Elimination of Chagas Disease Transmission by *T. infestans*, conferred by the Pan American Health Organization, other species were found to be involved in the transmission of Chagas disease in the State of São Paulo; for *T. sordida*, *P. megistus*, and *R. neglectus*, recent entomological lifting showed a high incidence of these hemipterans inhabiting peri and intradomiciliary regions and, in some cases, were infected with *T. cruzi* (Wanderley et al., 2007; Silva et al., 2011; Silistino-Souza et al., 2013).

R. neglectus is important in the enzootic transmission of *T. cruzi* and *T. rangeli* (Gurgel-Gonçalves et al., 2004; Gurgel-Gonçalves and Cuba, 2009), and was recently found by different authors to inhabit palm trees in urban areas (Carvalho et al., 2014; Rodrigues et al., 2014). These insects have been observed in 13 Brazilian states, including Bahia, Distrito Federal, Goiás, Maranhão, Minas Gerais, Mato Grosso do Sul, Mato Grosso, Pernambuco, Piauí, Paraná, Rio de Janeiro, São Paulo, and Tocantins (Gurguel-Gonçalves et al., 2012). In Minas Gerais, São Paulo, and Goiás, evidence exists of colony formation in domiciliary regions (Barretto et al., 1968; Silva et al., 1999; de Oliveira and Silva, 2007; Silistino-Souza et al., 2013).

Cytogenetic analysis has shown to be an important cytotaxonomic tool for the differentiation of triatomines (Alevi et al., 2012a,b; 2013b, c, d, e; 2014). Barth (1956) described the karyotype of *R. neglectus* as 2n = 22 (20A + XY). The study also described some characteristics of the chromosomes of this vector, such as the size of the autosomes (3 pairs large + 6 medium + 1 small) and sex chromosomes (Y slightly bigger than X). Dujardin et al. (2002) described the pattern of constitutive heterochromatin of species and observed that *R. neglectus* only presented heterochromatin in the Y sex chromosome. However, Santos (2010) analyzed a population of Tocantins and reported that the species has heterochromatic blocks at the end of some autosomes.

In order to complement the epidemiological data and assist in the prophylaxis of Chagas disease in the State of São Paulo, we conducted an entomological lifting study in 40 municipalities of the Western region of the state from 2004 to 2008, highlighted the main vector species in this region, and reanalyzed the cytogenetic characteristics of *R. neglectus* in 3 different Brazilian states.

MATERIAL and METHODS

The analysis of the triatomines fauna of the Western region of the State of São Paulo was obtained from reports of triatomines by the population received from 2004 to 2008 by the Superintendência de Controle de Endemias (SUCEN) of Araçatuba. The SUCEN of Araçatuba is responsible for monitoring the 40 counties west of São Paulo (Alto Alegre, Andradina, Araçatuba, Auriflama, Avanhandava, Barbosa, Bento de Abreu, Bilac, Birigui, Braúna, Brejo Alegre, Buritama, Castilho, Clementina, Coroados, Gabriel Monteiro, Glicério, Guaraçaí, Guararapes, Guzolândia, Ilha Solteira, Itapura, Lavínia, Lourdes, Luiziânia, Mirandópolos, Murutinga do Sul, Nova Castilho, Nova Independência, Nova Luzitânia, Penápolis, Pereira Barreto, Placatú, Rubiácea, Santo Antônio do Aracanguá, Santopolos do Aguapeí, Sud Mennuci, Suzanópolis, Turiúba, and Valparaíso) (Figure 1). The insects found in these locations were collected and sent for laboratory exams to identify possible triatomines infected by the parasite *T. cruzi*. The feeding habits of insects collected were analyzed in the Laboratory of Chagas from SUCEN, located in Mogui Guaçu, São Paulo, Brazil. Based on the notifications registered by SUCEN from 2004 to 2008 in 40 counties, we calculated the mean, median, standard deviation, minimum, and maximum of the following variables: notification, triatomines captures, and examined species captured. For comparison between municipalities, we constructed 2 graphs using the data to the scale of the standardized measure by

transformation: variable frequency X divided by the population density of the municipality. The statistical software Minitab for Windows version 14.0 was used.

Based on information about the triatomines collected by SUCEN, we constructed 3 tables. Table 1 shows the relationship between collected specimens and infected specimens, based on the total number of insects collected in the 40 municipalities. Tables 2 and 3 show the relationship between vectors infected with the type of host based on blood found in the insect gut. After inspecting each notification site, the SUCEN performed nebulizations with insecticides.

For cytogenetics analysis, at least 10 specimens of adult male *R. neglectus* from each locality (Formoso/GO, Frutal/MG, Guaíra/SP, and Pitangueiras/SP) were analyzed cytogenetically using lacto-acetic orcein (De Vaio et al., 1985, with modifications according to Alevi et al., 2012a) and C-banding (Sumner, 1972).

RESULTS

Entomological lifting

Figure 1 shows the average, minimum, and maximum of notifications from each municipality. The municipalities of Castilho and Santo Antônio do Acaranguá registered the highest relative numbers of notifications. In addition, in the municipalities of Alto Alegre, Glicério, Guaraçaí, Itapura, Muritinga do Sul, and Nova Independencia, the number of notifications was higher than in other municipalities (Figure 2).

Figure 2 shows the frequency of notifications each year in the 40 municipalities. In 2005, the greatest frequency of notifications in most municipalities was reported, particularly in Castilho and Santo Antônio do Acaranguá. Furthermore, the record of notifications generally decreased, with the exception of Itapura and Murutinga do Sul, which registered an increase number of notifications in 2007 and 2008, respectively (Figure 3).

The main species notified by SUCEN in Western São Paulo were *T. sordida* and *R. neglectus*. Table 1 shows that the capture of *T. sordida* was much more frequent than that of *R. neglectus*. Based on the specimens collected, the SUCEN conducted tests of infection by *T. cruzi* (Table 1). A large number of *T. sordida* was collected in 2005 and, mainly, notification of infected insects was absent in 2008.

Based on the triatomines infected shown in Table 1, the SUCEN performed blood tests to detect the host on which the contaminated insect showed hematophagy. In *Triatoma sordida*

the presence of human blood, possum blood, rodent blood, and bird blood were notified (Table 2), and in *R. neglectus* was reported dog blood (Table 3).

Cytogenetic analysis

Cytogenetic analysis of the chromosome number and structure of the 4 populations was identical to that described by Barth (1956) (Figure 4). However, variations in the size of chromosomes as described by Barth (1956) were very small. The heterochromatic pattern was also identical to that described by Dujardin et al. (2002) (Figure 5). As all populations showed the same characteristics, we represent the heterochromatic pattern of populations of *R. neglectus* using one images of leptotene (Figure 5A) and one of diplotene (Figure 5B). The presence of heterochromatic blocks dispersed in the nucleus during leptotene and the presence of heterochromatic blocks during chromosome condensation (diplotene) would be easily detected if they were present in the chromosomes of *R. neglectus*.

DISCUSSION

In 1979, Forattini et al. (1979) reported the presence of *P. megistus*, *T. sordida*, and *R. neglectus* in the state of São Paulo. Of the total species collected, 2280 nymphs were *P. megistus*, 8009 were *T. sordida*, and 100 were *R. neglectus*, and 19, 9, 9.4, and 2% were observed in the same household regions.

Silva et al. (2010) conducted a survey in the municipality of Potirendaba from 2004 to 2006 and observed that of the 355 exemplary triatomines collected, 97.3% were *T. sordida* and 2.7% were *R. neglectus*. All insect specimens showed negative results for *T. cruzi*.

Silistino-Souza et al. (2013) conducted an epidemiological study of Chagas disease in the northwest of the State of São Paulo from 2004 to 2011. The authors also observed that the most common species were *T. sordida* and *R. neglectus*, mainly *T. sordida*. Of the 14,583 triatomines collected, only 6 exemplars of *T. sordida* were found to be infected with *T. cruzi*, representing approximately 0.04% of the total specimens collected.

Rodrigues et al. (2009) reported the presence of *R. neglectus* infestation in buildings in Araçatuba from 2004 to 2007. Twenty nymphs were collected in one of the rooms on the 10th floor of a building. Fortunately, all were negative for *T. cruzi*, as 3 nymphs were detected with human blood. In 2007, the authors studied the dispersion of *R. neglectus* in Araçatuba city and observed that of the 34 palm trees examined, 25 were infected with *R. neglectus*, allowing for

the collection of 357 species. It is thought that palm trees function as bridges to infestation of buildings by *R. neglectus*.

Silva et al. (2003) surveyed 21 municipalities in the region of Araçatuba from 1990 to 1999. In 20 municipalities analyzed, the predominant species was *T. sordida*. This species was detected mainly in the peridomicile regions, which may result in future colonization of domicile regions (Diotaiuti et al., 1994; Dias and Schofield, 1998). In the 2004-2008 survey, the main species in the Western region of the State of São Paulo also were *T. sordida* and *R. neglectus*. Of the 19,434 specimens collected, approximately 95% were also *T. sordida*. In the 40 municipalities analyzed, the vector in the domiciliary region was absent. According to Silistino-Souza et al. (2013), populations of *T. sordida* of Brazil may show from cryptic speciation, allowing for better adaptation of this vector to different environments.

Of the specimens of *T. sordida* and *R. neglectus* collected, approximately 0.17 and 0.4% were infected with *T. cruzi*. Although these percentages are low, and in 2008 no speci mens collected were found to be infected by the protozoan, it is important to continue the supervision of vector control programs as a prophylactic measure against Chagas disease, as the vectors are present in the State São Paulo.

In 32 exemplars of *T. sordida* contaminated by *T. cruzi* were detected mainly human, opossum and rodent, and bird blood. This test allows analyzing the organisms that are functioning as vertebrate hosts and possibly as reservoirs for *T. cruzi*. Birds are refractory and resistant to infection by *T. cruzi*, making it difficult for these vertebrates to operate as reservoirs of Chagas disease (Kierszenbaum et al., 1976).

The elimination of *T. infestans* has vacated new niches, and species of secondary importance in the transmission of Chagas disease, such as *R. neglectus*, were considered sylvatic and passed to domiciliary regions. The domiciliation process of triatomines can lead to chromosomal alterations, as observed by Panzera et al. (2004) to *T. infestans*. However, analysis of the 4 populations of *R. neglectus* revealed that these organisms had no intraspecific variation. Although we do not discard the possibility of polymorphism, the holocentric nature of chromosomes of the triatomines and small size of the chromosomes in the genus *Rhodnius* may have led to an incorrect interpretation of the heterochromatic pattern in the population of *R. neglectus* of Tocantins.

We surveyed the entoepidemiological data regarding Chagas disease in the Western region of the State of São Paulo from 2004 to 2008. These data are complementary to the survey conducted from 1990 to 1999, as the vector species were the same (*T. sordida* and *R.*

neglectus). We corroborate the possibility of future colonization of domiciliary regions and we underscore the importance of vector control programs for the prophylaxis of Chagas disease. Furthermore, the populations of *R. neglectus* in Brazil showed no intraspecific variation and we corroborate the chromosomal patterns originally described by Barth (1956) and Dujardin et al. (2002) for the species *R. neglectus*. These data are important for understanding the evolution of these hematophagous insects, which are vectors of Chagas disease.

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Figure 1. Forty counties of west of São Paulo monitored by Superintendency of Endemic Diseases Control of Araçatuba.

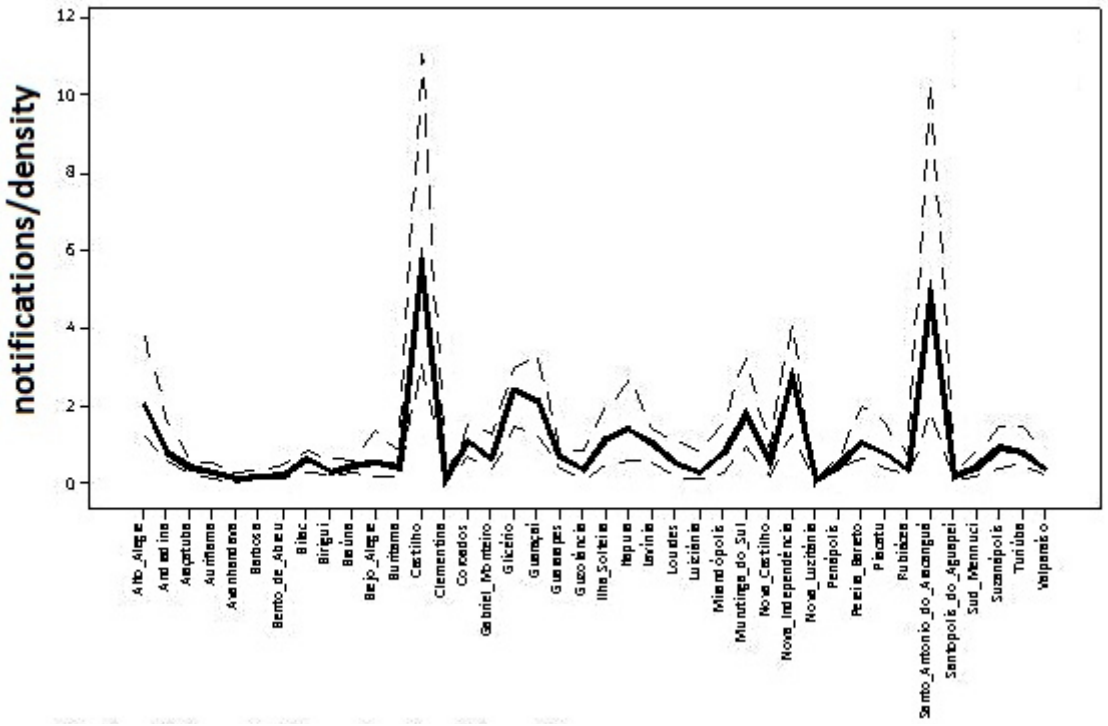

Figure 2. Average, minimum, and maximum of notifications in each municipality.

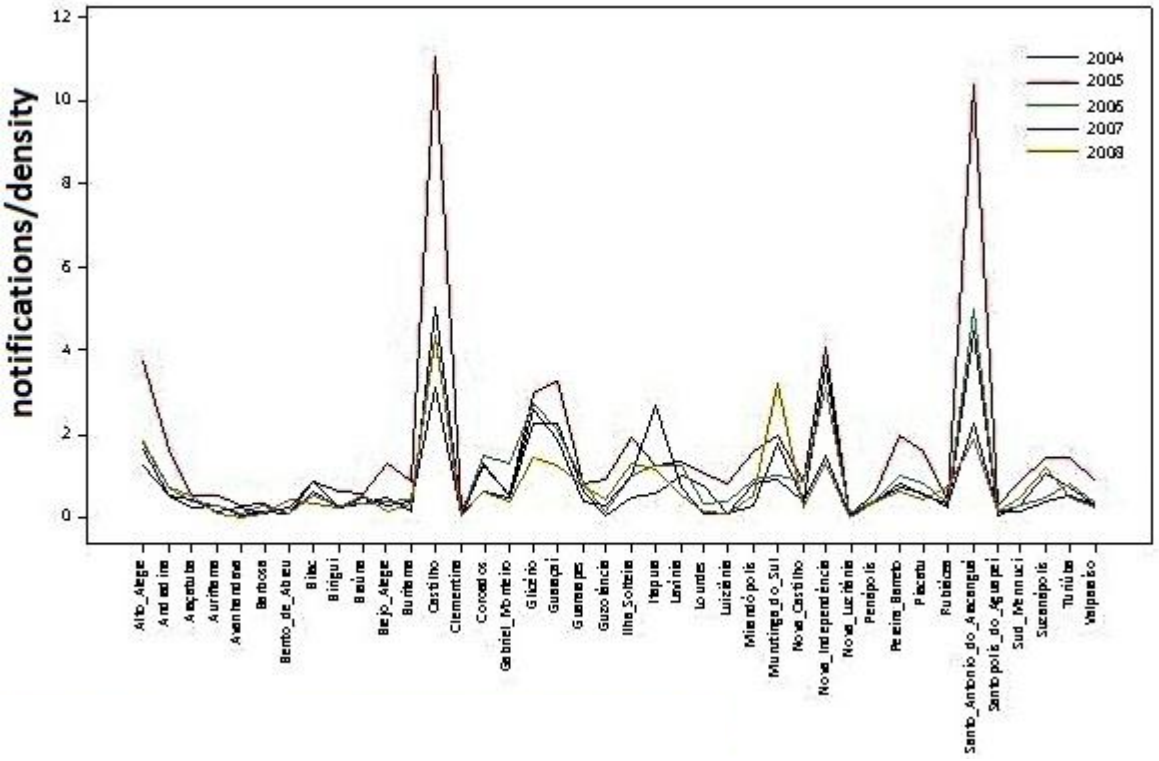

Figure 3. Notifications registered in the years 2004 to 2008 in 40 counties of West Paulista.

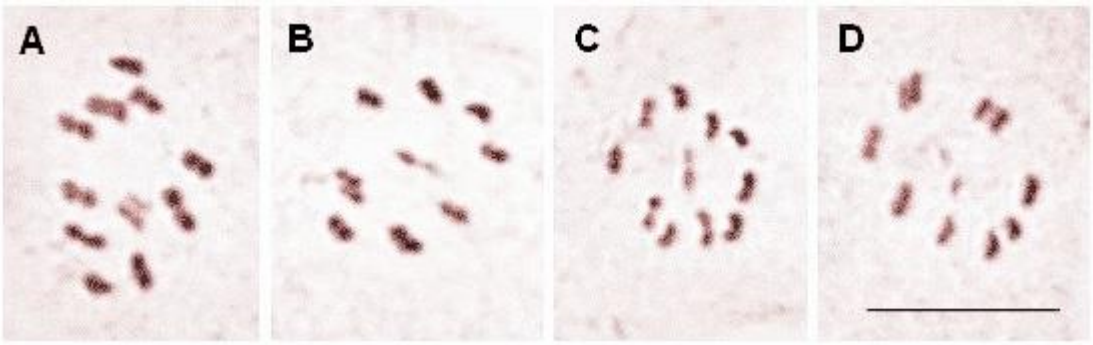

Figure 4. Meiotic metaphases of *Rhodnius neglectus*. (A) Metaphase I of Formoso/GO. (B) Metaphase II of Frutal/MG. (C) Metaphase II of Guaíra/SP. (D) Metaphase II of Pitangueiras/SP. Bar: 10 μm.

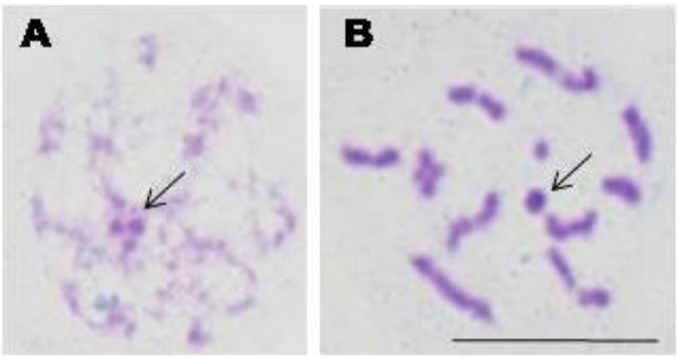

Figure 5. Stages of meiosis of *Rhodnius neglectus*. (A) Leptotene and (B) diplotene. Note the chromocenter (A) and sex chromosome Y (B) heterochromatics (arrows). Bar: 10 μm.

Table 1. List of *T. sordida* and *R. neglectus* samples collected and infected over 4 years in 40 municipalities of West Paulista.

Years	*Triatoma sordida*		*Rhodnius neglectus*	
	Collected	Infected	Collected	Infected
2004	3285	5	144	0
2005	7082	15	281	2
2006	3278	8	145	2
2007	2247	4	195	0
2008	2532	0	245	0
Total	18.424	32	1010	4

Table 2. List of *T. sordida* and type of host infected.

Years	Infected	Human	Opossum	Rodent	Bird	Dog	None
2004	5	2		2			1
2005	15	2	1	6	3*		3
2006	8	2			2		4
2007	4	1	2		1		
2008	0						

*Detected in bird blood and human blood.

Table 3. List of *R. neglectus* infected with the kind of host.

Years	Infected	Human	Opossum	Rodent	Bird	Dog	None
2004	0						
2005	2					1	1
2006	2					1	1
2007	0						
2008	0						

4.23 Anexo 23 (Artigo científico publicado na revista *Zookeys* FI 1,03)

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A new species of *Rhodnius* from Brazil (Hemiptera, Reduviidae, Triatominae)

Abstract

A colony was formed from eggs of a *Rhodnius* sp. female collected in Taquarussu, Mato Grosso do Sul, Brazil, and its specimens were used to describe *R. taquarussuensis* **sp. n.** This species is similar to *R. neglectus*, but distinct characters were observed on the head, thorax, abdomen, female external genitalia and male genitalia. Chromosomal differences between the two species were also established.

Introduction

In the subfamily Triatominae, the genera *Panstrongylus* (15 species), *Triatoma* (74 species) and *Rhodnius* (20 species) are of particular epidemiological importance, although the other 15 genera (containing 43 species) can also transmit *Trypanosoma cruzi*, which is the etiological agent of Chagas disease (Poinar 2013, Galvão 2014, Mendonça et al. 2016, Souza et al. 2016). Among the 152 species within the subfamily there are two fossils: *T. dominicana* Poinar, 2005 and *P. hispaniolae* Poinar, 2013.

The first two species identified as belonging to the genus *Rhodnius* were described by Stal (1859): *R. nasutus* and *R. prolixus*. From that year until 1979, a total of 12 species were identified (Lent & Wygodzinsky 1979). In 2003, Galvão et al. considered 16 valid species. The 17th, 18th, 19th and 20th species in that genus were respectively *R. zeledoni* Jurberg et al. 2009; *R. montenegrensis* Rosa et al. 2012; *R. barretti* Abad-Franch et al. 2013, and *R. marabaensis* Souza et al. 2016.

Most *Rhodnius* species live in palm trees, and several cases of transmission of Chagas disease have been associated with the consumption of açaí containing feces of triatomines infected by *T. cruzi* (Ferreira, Branquinho & Leite, 2014; Ministério da Saúde,

Brasil, 2017). Apart from such cases, which occur more frequently in the northern region of the country, it is worth noting that *R. neglectus* was found in palm trees (species of *Roystonea* , *Syagrus* and *Acrocomia*] in the city of Araçatuba, São Paulo, in 2009, as well as in palm trees (*Livistona australis*) located in the central square of the city of Monte Alto, São Paulo, in February 2012 (Rodrigues et al. 2014, Carvalho et al. 2013, respectively).

Based on morphological, morphometric and cytogenetic characters, this paper describes *R. taquarussuensis* **sp. n**., which is similar to *R. neglectus*. The first collected specimen of *R. taquarussuensis* was a female that invaded a domicile and laid eight eggs. The colony formed from those eggs resulted in the specimens used in this description.

Materials and methods

Morphological identification and description

On 10 November 2010 a female of *Rhodnius* sp. invaded a rural dwelling (22° 29′ 07.7″ S; 53° 21′ 08.9″ W) in the city of Taquarussu, Mato Grosso do Sul, Brazil, and was captured (Fig. 1). That specimen remained alive for a few days and laid eight eggs (Fig. 2). By means of macroscopic identification and subsequent optical microscopy (OM) and using the key of Lent & Wygodzinsky (1979), a clear similarity with *R. neglectus* was noticed. In view of that, all characters observed and documented for *Rhodnius* sp. were checked for *R. neglectus* CTA 229, which is a colony that has been kept since June 27, 2011 at the Triatominae Insectarium of the Faculty of Pharmaceutical Sciences, UNESP, Araraquara (ITPCF/UNESP). The temperature, humidity and light cycle conditions are not controlled due to the insect's biodiversity, but these parameters are measured daily, varying the temperature between 20-35ºC and humidity 50-80%. Insects kept in colonies are fed directly on ducks every 15 days and consists of specimens from the Brazilian National and International Triatominae Taxonomy Reference Laboratory at the Oswaldo Cruz Institute in Rio de Janeiro, Brazil (LNIRTT). The colony was kindly provided by Dr. José Jurberg and Dr. Cleber Galvão, and the specimens that originated it were collected in northern Formoso, Goiás, Brazil.

A colony was formed from the eight eggs laid by the *R. taquarussuensis* sp. n. female and identified as Araraquara Triatominae Colony (CTA) 277. The specimens of that colony were used to describe *R. taquarussuensis* sp. n.

Morphological study

The morphological study by OM and scanning electron microscopy (SEM) consisted of the observation of the head, thorax and abdomen of 30 adult females and 30 adult males, as well as 40 eggs of *R. taquarussuensis* **sp. n.** and the same number of specimens of *R. neglectus*, according to Barata (1981), Quintero (2003), Obara et al. (2007), Rosa et al. (2012), Rosa et al. (2014), Souza et al. (2016) (Figs 3 - 9).

Female external genitalia were observed from the dorsal, posterior, and ventral sides (Fig 6) by SEM, according to Rosa et al. (2010). The study of the male genitalia was carried out by OM (Figs 8, 9), following a technique developed by Jader de Oliveira based on Gallati (2016). The denominations used were those defined by Lent & Jurberg (1969).

The Leica MZ APO stereoscope from the Faculty of Pharmaceutical Sciences, UNESP, Araraquara, and the scanning electron microscope Topcon SM-300 located in the Department of Physical Chemistry at the Chemistry Institute, UNESP, Araraquara, were used for observation and capture of images.

Morphometric study

In the morphometric study by OM, 15 egg shells, 15 females and 15 males from the colony were measured, the same being done for *R. neglectus* CTA 229 (Table 1).The parameters measured were: total length, width of thorax and abdomen, length of the scutellum, three segments of the proboscis and four segments of the antenna, as well as five parameters of the head following Dujardin et al. (1999). Eggs had their length and the diameter of the opercular opening measured. The wings of *R. taquarussuensis* **sp. n.** and *R. neglectus* were studied by geometric morphometry using seven anatomical landmarks, according to parameters established by Gurgel-Gonçalves et al. (2008), as well as based on Rosa et al. (2012).

The observations and measurements were carried out on a Leica MZ APO stereoscope and the Motic Images Advanced System version 3.2.

Cytogenetic identification

In this study ten male specimens of *R. taquarussuensis* sp. n. were used for C and CMA_3/DAPI-banding analyses and ten male specimens of *R. neglectus* were used for CMA_3/DAPI-banding analyses. After being lacerated and placed on the slide, the seminiferous tubules underwent cytogenetic procedures following the C-banding (Sumner 1972) and CMA_3/DAPI-banding protocols [Schimid 1980, with modifications according to Severi-Aguiar et al. 2006]. C-banding was analyzed under a Jenaval (Zeiss) MO connected to a digital camera and the Axio Vision LE 4.8 image analyzer (Copyright ©2006-2009 Carl Zeiss Imaging Solutions Gmb H), whereas CMA_3/DAPI-banding was analyzed using Zeiss-Axioskop and Olympus BX-FLA fluorescence microscopes (FM).

Taxonomy

Family Reduviidae Latreille, 1807
Subfamily Triatominae Jeannel, 1919
Genus *Rhodnius* Stål, 1859
***Rhodnius taquarussuensis* sp. n.**

Holotype. BRAZIL: Mato Grosso do Sul: Taquarussu; Residence, 22° 29′ 07.7″ S; 53° 21′ 08.9″ W, 10 November 2010 H. E. G. Justino. UNESP (♀**).**

Paratypes. BRAZIL: Colony formed from eggs obtained from the holotype: Araraquara: Triatominae Insectarium of the Faculty of Pharmaceutical Sciences, Araraquara, January 3, 2017, J. A. da Rosa, UNESP (25 ♂ 25 ♀**).**

Additional Paratypes. CTIOC - Collection of Triatomines of the Oswaldo Cruz Institute, Rio de Janeiro - Brazil (2 ♂ 2 ♀). Entomological Reference Collection of the Faculty of Public Health - USP, São Paulo -Brazil (1 ♂ 1 ♀). Collection of the Institute of Entomology of the Metropolitan University of Education Sciences (IEUMCE), Santiago - Chile (2 ♂ 2 ♀).

Etymology. The name *Rhodnius taquarussuensis* sp. n. was chosen because this species was found in the city of Taquarussu, Mato Grosso do Sul, Brazil.

Diagnosis. *Rhodnius taquarussuensis* sp. n. is close to *R. neglectus*, their differences being the color and a variety of morphological, morphometric and cytogenetic characters (Tables 1, 2). The general color of *R. taquarussuensis* **sp. n.** is brown, whereas *R. neglectus* is dark brown, almost black. This difference is particularly noticeable on the hind wings. The stridulatory sulcus of *R. taquarussuensis* **sp. n.** is brown at the base and black on the sides, whereas on *R. neglectus* it is completely black.

On the head, differences were noticed on the vertex, genae, antennae and triangular furrow of the first segment of the rostrum. The vertex of the head of *R. taquarussuensis* **sp. n.** is quite visible, whereas on *R. neglectus* it is not (Fig 3 A, B, D, E). The genae of *R. taquarussuensis* **sp. n.** are longer than those of *R. neglectus* (Fig 3 A, D).On *R. taquarussuensis* **sp. n.** the 10^{th} part of the second segment of the antenna is brown; on *R. neglectus*, though, only the basis has that color. The triangular furrow of the first segment of the rostrum, towards the second segment, ends in a filamentous way on *R. taquarussuensis* **sp. n.** and in a rounded way on *R. neglectus* (Fig 3 C, F). On the thorax, differences can be found on the pronotum, wings, scutellum, prosternum, mesosternum and metasternum (Figs 4, 5). The membranous portion of the hind wings is brown on *R. taquarussuensis* **sp. n.** and dark brown on *R. neglectus*. The scutellum ends in a rounded apex on *R. taquarussuensis* **sp. n.** and in a filamentous apex on *R. neglectus* (Fig 4 A, B). On *R. taquarussuensis* **sp. n.** the apex of the scutellum covers the final portion of the urotergite I process, while on *R. neglectus* the apex of the process of the urotergite I is perfectly visible (Fig 4 A, B). The lines limiting the stridulatory sulcus are straight on *R. taquarussuensis* **sp. n**. and narrowed in the anterior third on *R.neglectus* (Fig 5 A, B). On *R. taquarussuensis* **sp. n**. the basis of the stridulatory sulcus is brown and the sides are black, whereas on *R. neglectus* the entire stridulatory sulcus is black. The central region of the limit between the mesosternum and the metasternum is regular and half-moon shaped on *R. taquarussuensis* **sp. n.**, while on *R. neglectus* it is pronounced and slightly irregular (Fig 5 C, D). The beginning of the metasternum is narrow on *R. taquarussuensis* **sp. n**. and wide on *R. neglectus* (Fig 5 C, D). The ventral abdomen of *R. taquarussuensis* **sp. n**. is light brown, and that of *R. neglectus* is dark brown (Fig 2). The terminal portion of the paramere of the male genitalia of *R. taquarussuensis* **sp. n.** is thinner than that of *R. neglectus* (Fig 9 A, C). The dorsal phallothecal sclerite has a trapezoidal shape

on *R. taquarussuensis* **sp. n.** and is rounded on *R. neglectus* (Fig 8 C, D). The external limit of the 10th segment of the dorsal side of the female external genitalia of *R. taquarussuensis* **sp. n.** presents a concavity in the middle portion, whereas on *R. neglectus* that limit is straight (Fig 6 A, B). From posterior view, the limits of the 9th segment with gonocoxite VIII are curve on *R. taquarussuensis* **sp. n.** and straight on *R. neglectus*, and the superior line limiting the 10th and 9th segments is straight on *R. taquarussuensis* **sp. n.** and curve on *R. neglectus* (Fig 6 C, D). In the ventral side of the female external genitalia of *R. taquarussuensis* **sp. n.** there is a concavity in the external limit with the 10th segment that is also noticed from dorsal view; on *R. neglectus* that limit is a straight line. From ventral view, the external limits of the 9th segment of the female external genitalia are curve on *R. taquarussuensis* **sp. n**. and straight on *R. neglectus* (Fig 6 E, F).

Among the 19 characters measured, 12 showed significant differences between *R. taquarussuensis* **sp. n.** and *R. neglectus* in both sexes and also the eggs of both species. Two characters showed differences only between males, and five characters did not show significant differences (Tables1, 2).

Description. A total of 15 adult females and 15 adult males of *R. taquarussuensis* **sp. n.** and *R. neglectus* were measured, as well as 15 egg shells of both species. Such measurements are detailed in Table 1.

The head of *R. taquarussuensis* **sp. n**. has a prominent brown vertex contrasting with the black sides. The clypeus is well defined. The genae are large, visible and dark brown, moving towards the anteclypeus (Figs 2 A, C, 3 A, B). The limits between the genae and the clypeus are brown.

The first segment of the antennae is black with mixes of brown. The articulation between the first and second segment of the antennae is brown. Roughly all the 10th part of the beginning of the second antennal segment is brown. The second segment is mostly black. In the articulation between the second and third antennal segment there is a black ring followed by a brown one. The beginning of the third segment (around 1/3) is black and the remaining portions (2/3) are brown. The articulation between the third and fourth antennal segment is brown. The beginning of the fourth segment is black and the remaining portions are brown with mixes of black (Fig 2 A, C).

The eyes are black and the ocelli are brown. The neck has a brown central dorsal strip flanked by two (1+1) black, narrower strips. The ventral portion of the neck between the ocelli is dark brown (Fig 2 A, B, C, D).

The pronotum of the thorax of *R. taquarussuensis* **sp. n**. has a trapezoidal shape and is limited by a brown carina. In the antero posterior direction the pronotum has other two brown carina in the middle portion and six black strips. The three carina and the three brown strips are interspersed with the six black strips, which are larger. The collar (first portion of the pronotum) in the central part is brown and is followed by two (1+1) black glabrous areas and the two (1+1) antero lateral angles. The anterior portion of the pronotum consists of three anterior lobes which are clearly distinct from the posterior portion (hindlobe). Those three anterior lobes are limited by the carina and on each of them there are two black glabrous areas with a lengthy and irregular outline (Fig 2 A, C).

The cuticle involving the veins of the hemelytron is light brown. The corium between the veins of the coriaceous region is dark brown, whereas that of the membrane is brown (Fig 2 A, C).

The prosternum contains the stridulatory sulcus, which moves along that segment in an antero-posterior direction, having a brown color in the background and black on the sides. Two elongated tubercles limit the anterior half of the stridulatory sulcus. In the superior portion and in diagonal direction from the tubercles there are two black glabrous areas surrounded by a set of brown sensilla (Fig 5 A).

The mesosternum is limited anteriorly by the prosternum and posteriorly by the metasternum, both limits being brown. The central line dividing two dark brown elevations is also brown. Those two elevations are limited by two (1+ 1) black side glabrous areas diagonally placed. The central region of the posterior limit of the mesosternum has a half-moon shape. The metasternum is brown and resembles an isosceles triangle. Its anterior portion, i.e., its limit with the mesosternum, corresponds to the vertex of the triangle and is narrow, whereas its posterior portion, i.e., its limit with the first abdominal segment, corresponds to the basis of the triangle (Fig 5 C).

The three pairs of coxae are brown, except for the black glabrous areas. The trochanters of the anterior pair of legs are brown, but mixed with black glabrous areas. The middle and posterior pairs of trochanters are brown and have no glabrous areas. The three pairs of femora are black and the same color prevails in the three pairs of tibiae, except in the articulations with the femur and the spongy fossula, which are brown. The spongy fossulae

are located in the first and second pairs of legs in the final portion of the tibia, alongside the articulations with the tarsi (Fig 2A, B, C, D).

The abdomen of *R. taquarussuensis* **sp. n**. presents a brown color in the longitudinal central portion. On the sides of each segment there are (3+3) black glabrous areas, which are mixed with brown and black areas. The connexivum of the dorsal portion lies between the second and seventh segment. For each of those segments the anterior half is black and the posterior one is brown. The dorsal connexivum, also lying between the second and seventh segment, has a black color in 2/3 of the anterior portion, but that black color ends in an irregular way over the remaining 1/3, which is brown. Therefore, the black portion of the connexivum presents two edges moving towards the brown portion: one in the internal limit of the connexivum and the other in the middle portion. However, the connexivum of the second dorsal segment is black in the anterior half and brown in the posterior one, the limit between the portions having a diagonal shape. The seventh segment, on the other hand, is practically all black, except for a small brown strip located in the external posterior half. Type 1 sensilla, which prevail on the head, thorax and abdomen, have a brown color (Fig 2 B, D).

Male genitalia have the typical aspect of the genus *Rhodnius*. The median process of the pygophore (PrP) is short and triangular, but the base is broad and the sides are elongated with a thin edge. Parameres are hairy with a thin edge. From ventral view, the phallosome (Ph) has a broad plate whose superior region has a trapezoidal shape and occupies the middle region of the aedeagus. The support of the phallosome plate (PrPh) is broad. Conjunctival process I (PrcjI) is present and II (PrcjII) is absent. Endosomal process (En) is well-developed when seen from dorsal and ventral view (Figs 8 A, C, 9 B).

The dorsal side of the female external genitalia presents a concavity in the middle portion of the 10^{th} segment. Seen from posterior view, the limits (1+1) of the 9^{th} segment with gonocoxite VIII are curve, whereas the superior line limiting the $10^{th}$and 9^{th}segments is straight. In the central portion of the 10^{th} segment of the ventral side of the female external genitalia there is another concavity that can be noticed from dorsal view. The external limits (1+1) of the 9^{th} segment of the female external genitalia are curve when seen from ventral view (Fig 6 A, C, E).

Egg shells of *R. taquarussuensis* **sp. n.** have a length of 1.72 mm and an opercular opening of 0.49 mm. They present lateral flattening, collar and exochorion cells, most with pentagonal or hexagonal shape (Fig 7 A,C).

Finally, although *R. taquarussuensis* **sp. n.** showed the same number of chromosomes as *R. neglectus* and all the tribe Rhodniini, i.e., 2n = 22 (Figure11 B), the constitutive heterochromatin pattern and the composition of the pairs of bases of DNA rich in AT and CG were completely different from *R. neglectus*, as the analysis of the nuclei of the initial prophases of *R. taquarussuensis* **sp. n.** has revealed a chromocenter consisting of sex chromosomes (arrow) and several heterochromatic blocks dispersed in the nucleus (Fig 11 A). The analysis of metaphase I of *R. taquarussuensis* **sp. n.** has demonstrated that this triatomine has heterochromatic blocks in both extremities of practically all the autosomes and in the Y sex chromosome(Fig11 B), unlike what has been recently stated for many populations of *R. neglectus* that do not present heterochromatin in autosomes (Alevi et al. 2015a). Furthermore, *R. taquarussuensis* **sp. n.** has the X sex chromosome rich in CG (Fig 12 C), the Y rich in AT (Fig 12D) and various blocks rich in CG dispersed in the prophase nucleus (Fig 12 C), while *R. neglectus* only has the X sex chromosome rich in CG (Fig 12 A) and the Y rich in AT (Fig 12 B), which proves the genetic differences between the two *Rhodnius* species.

Discussion

The subfamily Triatominae include 18 genera comprising 152 species, 20 of which belong to the genus *Rhodnius* (Galvão 2014, Mendonça et al. 2016, Souza et al. 2016).The difficulties involved in the specific identification of *Rhodnius* have already been noted by Neiva and Pinto (1923), as well as by Rosa et al. (2012) and Souza et al. (2016). However, even though it is difficult to specify the distinctions among the species of that genus, in the last eight years four species were described: *R. zeledoni*, *R. montenegrensis*, *R. barretti*, and *R. marabaensis*. Therefore, *R. taquarussuensis* **sp. n.** is the 21st species of the genus and the 5th described in the last eight years. Its similarity with *R. neglectus* was noticed after the capture of the first female specimen, as the most evident macroscopic characters, such as size, general aspect and connexivum, showed no differences. As a result, it was decided to base its description on the differences from *R. neglectus*.

In addition to the macroscopic characters, *R. taquarussuensis* **sp. n.** and *R. neglectus* were considered “close to” because the OM study indicated similar characters between them, including: placement of black and brown spots on the dorsal and ventral connexivum, length of the four segments of the antenna, pronotum, antero lateral angles, urotergite I process, geometric morphometry of the hind wings, median process of the pygophore and morphological characters of the eggs.

Rhodnius taquarussuensis **sp. n.** was considered distinct from *R. neglectus* on account of the observation of color, eleven morphological characters, twelve morphometric characters and cytogenetic features (Tables 1, 2). All those differences are consistent with Lent & Wygodzinsky (1979), whose descriptive key to 11 species of *Rhodnius* lists 13 morphological characters for specific distinction: color, tibia, legs, pronotum, head, posterior lobe of the pronotum, anterolateral angles, median process of the pygophore, connexivum, scutellum, eyes and antennae.

Regarding the color, the distinction between *R. taquarussuensis* **sp. n**. and *R. neglectus* was based on the general aspect, segments of the antenna, hind wings and stridulatory sulcus. The general color of *R. taquarussuensis* **sp. n**. is brown, whereas *R. neglectus* is dark brown, almost black, or "brown dark", as referred to by Lent & Wygodzinsky (1979).

Out of the eleven morphological characters that distinguish *R. taquarussuensis* **sp. n.** from *R. neglectus*, three are located on the head: dorsal vertex, genae and triangular furrow of the first segment of the proboscis. The difference related to the vertex was one of the characters used by Souza et al. (2016) to distinguish *R. marabaensis* from *R. prolixus* and *R. robustus*. The differences between the genae for specific characterization are being reported for the first time in this description. The ventral triangular furrow was mentioned by Rosa et al. (1999) in their study of *T. rubrovaria* and it was mapped by Lent & Wygodzinsky (1979), but it was not named and it is being used for the first time as a distinctive character.

In what refers to the thorax, differences on the scutellum, prothorax, mesothorax, and metathorax were noticed. The scutellum of *R. taquarussuensis* **sp. n** and *R. neglectus* differs in the shape of the apex and also the position on which that apex reaches urotergite I, since there is no significant difference in terms of length (Table 1). The taxonomic importance of the scutellum was tackled by Obara et al. (2007), who verified the differences of that character in eight species of *Triatoma*. Rosa et al. (2012) and Souza et al. (2016) also used it to describe *R. montenegrensis* and *R. marabaensis*, respectively.

The differentiation of seven genera of triatomines based on the shape of the prosternal stridulatory sulcus was carried out by Lent & Wygodzinsky (1979) and also by Souza et al. (2016) in the description of *R. marabaensis*. The description presented herein points out color and morphological differences between *R. taquarussuensis* **sp. n.** and *R. neglectus*. The mesothorax and metathorax, which have been used by Souza et al. (2016) to describe a new species, were found to be different in *R. taquarussuensis* **sp. n.** and *R. neglectus*.

Lent & Jurberg (1969) observed specific features in the characters of the male genitalia of 10 species of *Rhodnius* and since then that structure has been used to describe species of other genera of Triatominae, e.g., *Mepraia parapatrica* Frias, 2010, *P. mitarakaensis* Bérenger and Blanchet, 2007 and *T. jatai* Gonçalves et al., 2013. In the case of *R. taquarussuensis* **sp. n** and *R. neglectus*, the difference in the male genitalia is the shape of the phallosome. With respect to the female external genitalia, differences between *R. taquarussuensis* **sp. n.** and *R. neglectus* could be observed on the dorsal, posterior and ventral sides, which differ from other 13 *Rhodnius* species, according to Rosa et al. (2014).

The eggs of *R. taquarussuensis* **sp. n.** and *R. neglectus* showed differences in the measurement of their length and opercular opening, the same as *R. montenegrensis* and *R. marabaensis* on the occasion of their description. As for the morphological characters, no differences were recorded. On the other hand, it should be noted that morphological differences were found by Barata (1981) in eggs of 10 *Rhodnius* species, by Santos et al. (2009) in three species, by Rosa et al. (2012) in the description of *R. montenegrensis* and by Santos et al. (2016) when describing *R. marabaensis.*

Dujardin et al. (1999) established the geometric morphometry of the hind wings as a distinctive character among Triatominae while studying the sexual dimorphism of *R. domesticus* and *T. infestans*. The technique proved valid, for instance, to indicate the distinction between *Mepraia spinolai* and *M. gajardoi*; *T. bahiensis* and *T. lenti*; *R. colombiensis, R. ecuadoriensis* and *R. pallescens*; five populations of *T. patagonica* (Campos et al. 2011, Díaz et al. 2014, Nattero et al. 2016). However, even though that technique has contributed to distinguish even very close species, it showed no significant results to distinguish *R. taquarussuensis* **sp. n.** from *R. neglectus*.

According to Justi & Galvão (2016) the group *R. prolixus* comprise the following species: *R. baretti, R. dalessandroi, R. domesticus, R. marabaensis, R.milesi, R.montenegrensis, R.nasutus, R. neglectus, R. neivai, R. prolixus* and *R. robustus.* Since *R.taquarussuensis* sp. n. is close to *R. neglectus* we suggest the inclusion of *R. taquarussuensis* sp. n. in the *R. prolixus* group and we present the main differences between the twelve species (Table 3).

Cytogenetic analyses of *R.taquarussuensis* **sp. n.** made it possible to describe the karyotype (2n = 22) and observe the constitutive heterochromatin pattern in the chromosomes (extremities of most autosomes), which are rich in CG. All the species in the tribe Rhodniini have 22 chromosomes (Alevi et al. 2013, 2015b). On the other hand, out of the 14 species of

the genus *Rhodnius* whose chromosomes have been studied in the literature, only four show heterochromatic blocks in the autosomes, namely *R. colombiensis*, *R. nasutus*, *R. pallescens* and *R. pictipes* (Dujardin et al. 2002). *R. neglectus*, which is a similar species for *R. taquarussuensis* **sp. n.,** does not show heterochromatic blocks in the autosomes (Dujardin et al. 2002; Panzera et al., 2012; Alevi et al. 2015a).

Although the evolutionary process in triatomine is disruptive (Dujardin et al. 2009) and intraspecific chromosome variation has been described for *R. ecuadoriensis* (Pita et al., 2013), *R. pallescens* (Gómez-Palacio et al., 2008), *P. geniculatus* (Crossa-Pérez et al., 2002), *T. dimidiata* (Panzera et al., 2006) *T. infestans* (Panzera et al., 2004, 2012) and *T. sordida* (Panzera et al., 1997), generally the distribution of species is associated with different countries [for exemple, *R. ecuadoriensis* from Peru and Ecuador (Pita et al., 2013) and *T. sordida* from Brazil and Argentina (Panzera et al., 1997)] or different regions [for example, *R. pallescens* from North and West regions from Colombia (Gómez-Palacio et al., 2008) and *T. infestans* from Andean group and Non-Andean group (Panzera et al., 2004, 2012)]. However, a population study was previously performed with *R. neglectus* (endemic species of Brazil) coming from different Brazilian states (Alevi et al., 2015a) and the authors pointed out that there is no intraspecific chromosome variation for this species. This fact and the morphological data described sustain the specific status of *R. taquarussuensis* sp. n., since the gain and loss of heterochromatin in the autosomes of *Rhodnius* are adaptive processes that can be linked to speciation processes, as recently noted for the group *pallescens* (Alevi et al.2015c).

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Table 1. Mean of measurement (mm) of 15 females and 15 males of *R. taquarussuensis* **sp. n.** and *R. neglectus*.

	Female		**Male**	
	R. taquarussu	*R.neglectus*	*R. taquarussu*	*R. neglectus*
TL	17,25	17,25	**15,24**	**15,96**
MLA	9,86	10,03	**8,41**	**9,05**
MLT	**5,00**	**4,11**	**4,54**	**3,79**
R1	**0,75**	**0,92**	**0,71**	**0,92**
R2	**3,38**	**3,58**	**3,16**	**3,59**
R3	**0,85**	**0,95**	**0,77**	**0,96**
HL	**4.44**	**5,81**	**4.20**	**5,24**
EO	**1.53**	**1,98**	**1.42**	**1,80**
IE	**0.61**	**0,77**	**0.54**	**0,66**
PO	**1.00**	**3,67**	**1.04**	**3,37**
AO	**2.62**	**0,90**	**2.44**	**0,91**
AT	**1.93**	**2,30**	**1.82**	**2,30**
SC	1,96	2,03	1,69	1,86
A1	0,45	0,55	0,29	0,59
A2	3,56	4,13	2,24	4,35
A3	2,03	2,43	1,28	2,50
A4	1,36	1,82	0,87	1,92
Eggs	*R. taquarussuensis*		*R. neglectus*	
TE	**1,72**		**1,62**	
OO	**0,49**		**0,52**	

*30 eggshells were used for each species.

TL, Total length of the triatomine; **MLA**, maximum length of the abdomen; **MLT**, maximum length of the thorax; **R1**, **R2** and **R3**, lengths of first, second, and third rostral segments, respectively; **HL**, head length; **EO**, external distance between ocelli; **IE**, inner distance between eyes; **PO**, postocular distance (excluding neck); **AO**, anteocular distance; **AT**, antenniferous tubercle; **SC**, Scutellum; **A1**, **A2**, **A3** and **A4**, 1st, 2nd, 3rd, and 4th left antennal segments, respectively; **TE**, Total egg length; **OO,** egg opercular opening. The values in bold were significant at $\alpha = 0.05$, using unpaired t-test.

Table 2 – Main distinguishing characters between *R.taquarussuensis* **sp. n.** and *R. neglectus.*

Distinguishing characters		Species	
		R.taquarussuensis	*R. neglectus*
Overall color		Brown	Dark brown
Genae		Lengthier longer	Longer
Vertex		Quite visible	Not visible
Ventral triangular furrow		Filamentous way	Rounded way
Scutellum		Covers the final portion of the urotergite I process	The apex of the process of the urotergite I is perfectly visible
Stridulatory sulcus		Straight	Waisted
Mesothorax		Half-moon shaped and regular	Pronounced and slightly irregular
Female external genitalia	Dorsal side	10^{th} segment presents a concavity in the middle portion	10^{th} segment is straight
	Posterior side	The limits of the 9^{th} segment with gonocoxite VIII are curve	The limits of the 9^{th} segment with gonocoxite VIII are straight
	Ventral side	There is a concavity in the external limit with the 10^{th} segment	There is a straight line in the external limit with the 10^{th} segment
Male genitalia	Phallothecal sclerite	Trapezoidal shape	Rounded shape
	Tip of parameres	Thinner	Thin
Heterochromatin in the autosomes		Present	Absent
CMA^{+} in autosomes		Present	Absent

Table 3 – Distinguishing characters among twelve species of the group *Rhodnius prolixus.*

Species	Distinctive characters	References
R. barretti	The third antennal segment appears to be relatively shorter. The scutellar process is narrowly pointed.	Abad-Franch et al 2013
R. dalessandroi	Antenniferous tubercle slightly pilose and with triangular glabrous depression in the upper region. Semicircular spot on the posterior end of the neck.	Carcavallo & Barreto 1976
R. domesticus	Head comparatively long, distinctly longer that pronotum. Process of pygophore rectangular.	Lent & Wygodzinsky 1979
R. marabaensis	The second antennal segment is 10.3 times larger than the first. The scutellum is larger and includes two prominent internal lateral carinea.	Souza et al 2016
R. milesi	The male genitalia presents a second process of the phallosoma. Divergent antennal tubercle with an apical denticle.	Valente et al 2001
R. montenegrensis	Anterior wings with well-demarcated veins, notable the Sc by a yellow tonality. Abdomen presents yellow spots interposed with dark ones over the ventral abdomen lengthwise.	Rosa et al 2012
R. nasutus	Overall color light reddish brown, trochantera not contrasting conspicuously with femora. Median process of pygophore wide at base.	Lent & Wygodzinsky 1979
R. neglectus	Overall color dark brown, trochantera very light colored. Median process of pygophore narrow at base.	Lent & Wygodzinsky 1979
R. neivai	Pronotum entirely dark brown or black, including the carine. Connexivum blackish, with very small reddish spots.	Lent & Wygodzinsky 1979
R. prolixus	Anteocular region slightly over three times as long as postocular. Distance between eyes dorsally larger than width of eyes in dorsal view.	Lent & Wygodzinsky 1979
R.robustus	Anteocular region about four times as long as postocular. Specimens distance between eyes dorsally smaller than, or equal to, width of eye in dorsal view.	Lent & Wygodzinsky 1979
R. taquarussuensis **sp.n.**	Head with a prominent brown vertex contrasting with the black sides. The phallosome (Ph) has a broad plate whose superior region has a trapezoidal shape and occupies the middle region of the aedeagus.	This work

*group *R. prolixus* according to Justi & Galvão 2016.

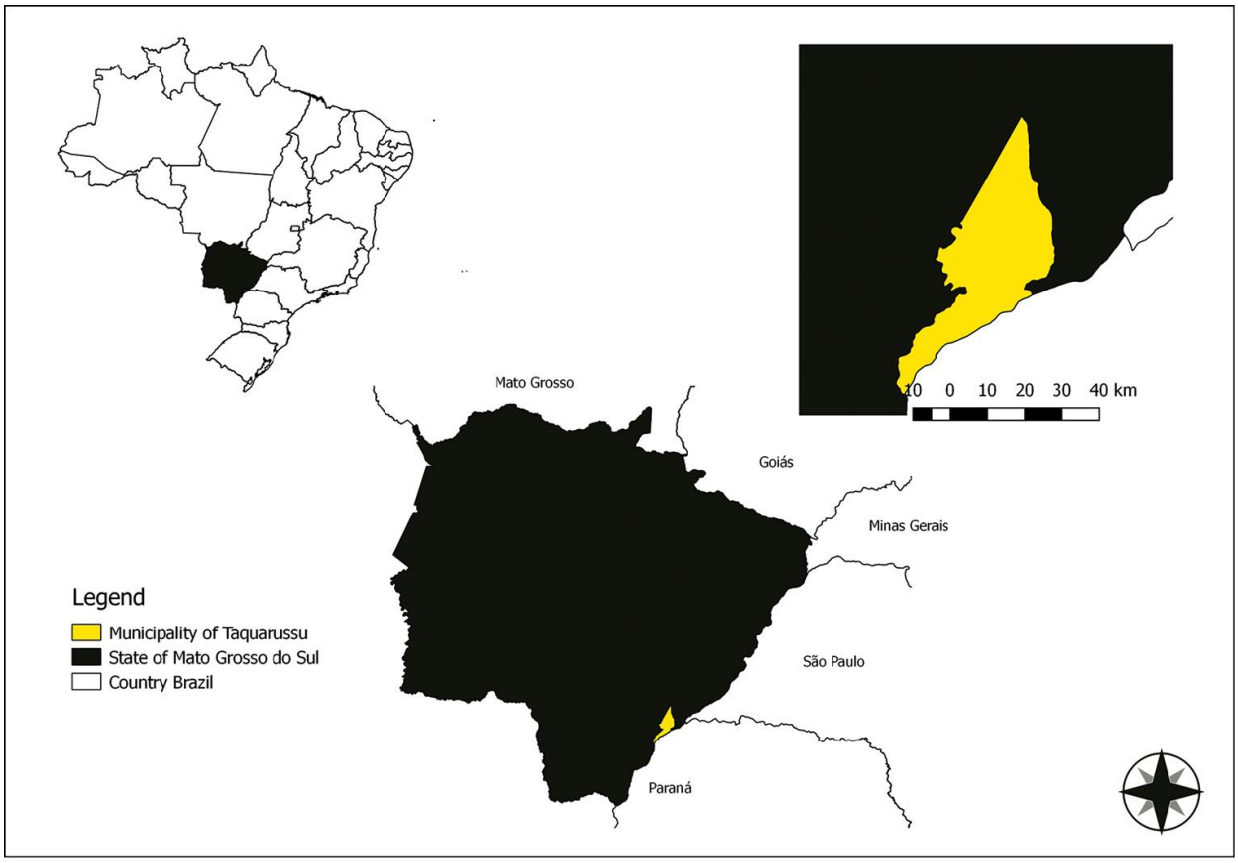

Figure 1. Localization of Taquarussu - MS where female of *R. taquarussu* sp. n. is collected (22° 29' 233" S, 053° 21' 107" W).

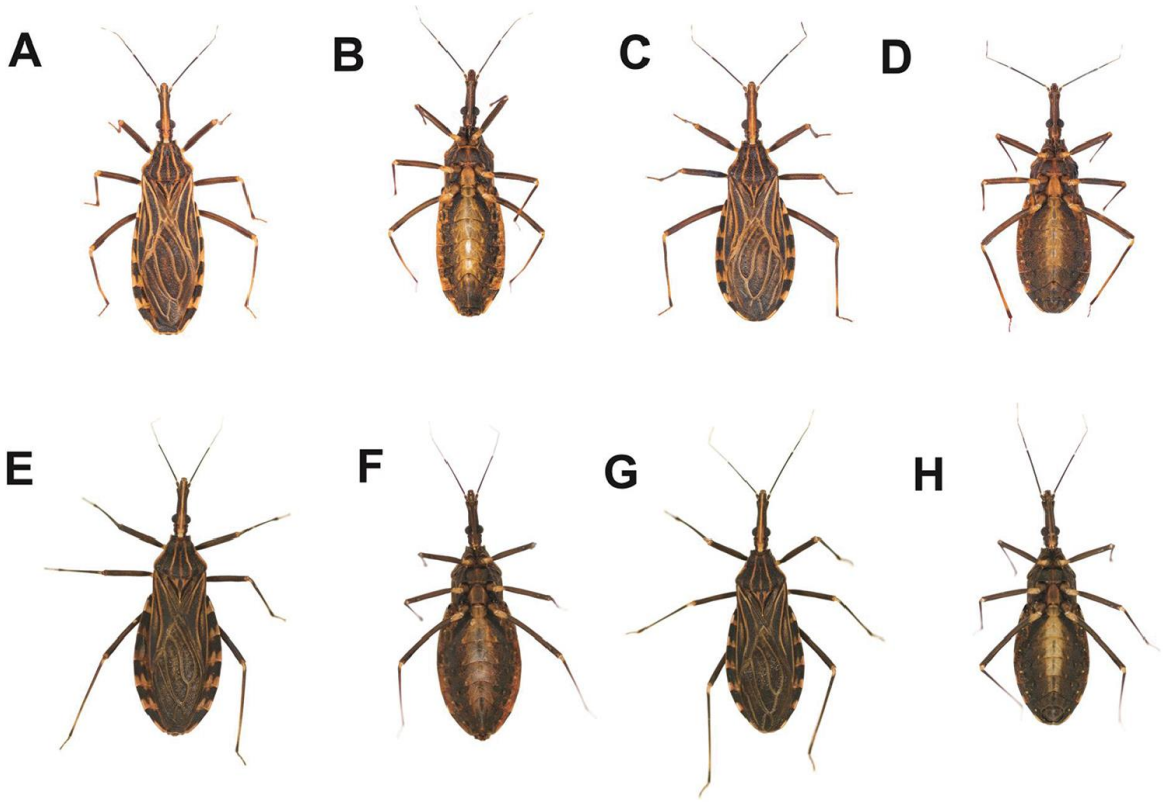

Figure 2. *R. taquarussuensis* sp. n. female **(A)** dorsal side, **(B)** ventral side, *R. taquarussuensis* sp. n. male **(C)** dorsal side, **(D)** ventral side, *R. neglectus* female **(E)** dorsal side, **(F)** ventral side), *R. neglectus* male **(G)** dorsal side, **(H)** ventral side.

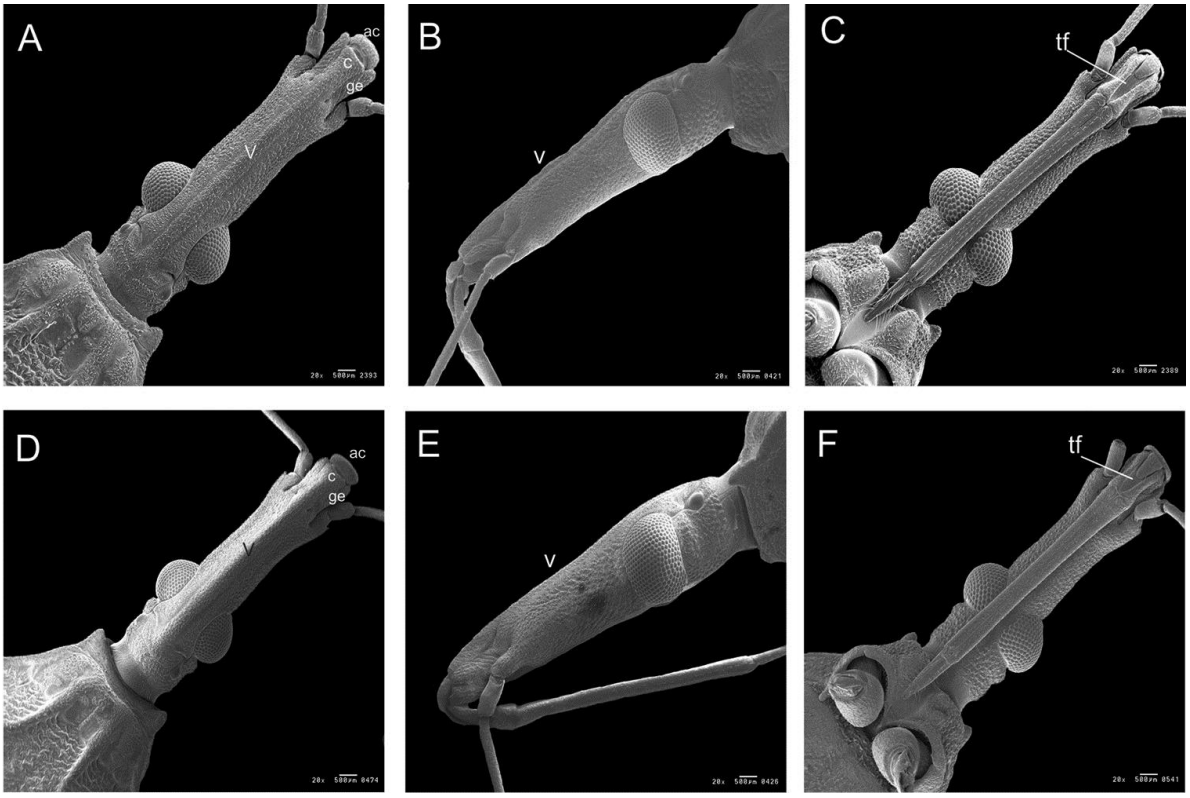

Figure 3. Head by SEM of *R. taquarussuensis* sp. n. **(A)** dorsal view, **(B)** lateral view, **(C)** ventral view, *R. neglectus* **(D)** dorsal view, **(E)** lateral view, **(F)** ventral view. v: vertice, ge: gena, c: clypeus, ac: anteclypeus, tf: triangular furrow.

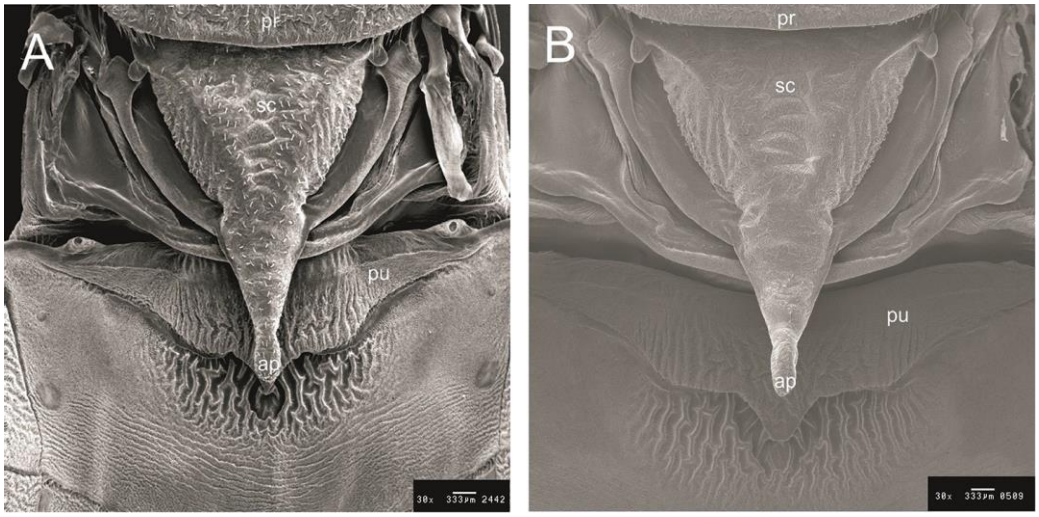

Figure 4. Escutellum and process of I urotergit by SEM. **(A)** *R. taquarussuensis* sp. n., **(B)** *R. neglectus*. pr: pronotum, sc: escutelum, pu: process of I urotergit, ap: apex of escutelum.

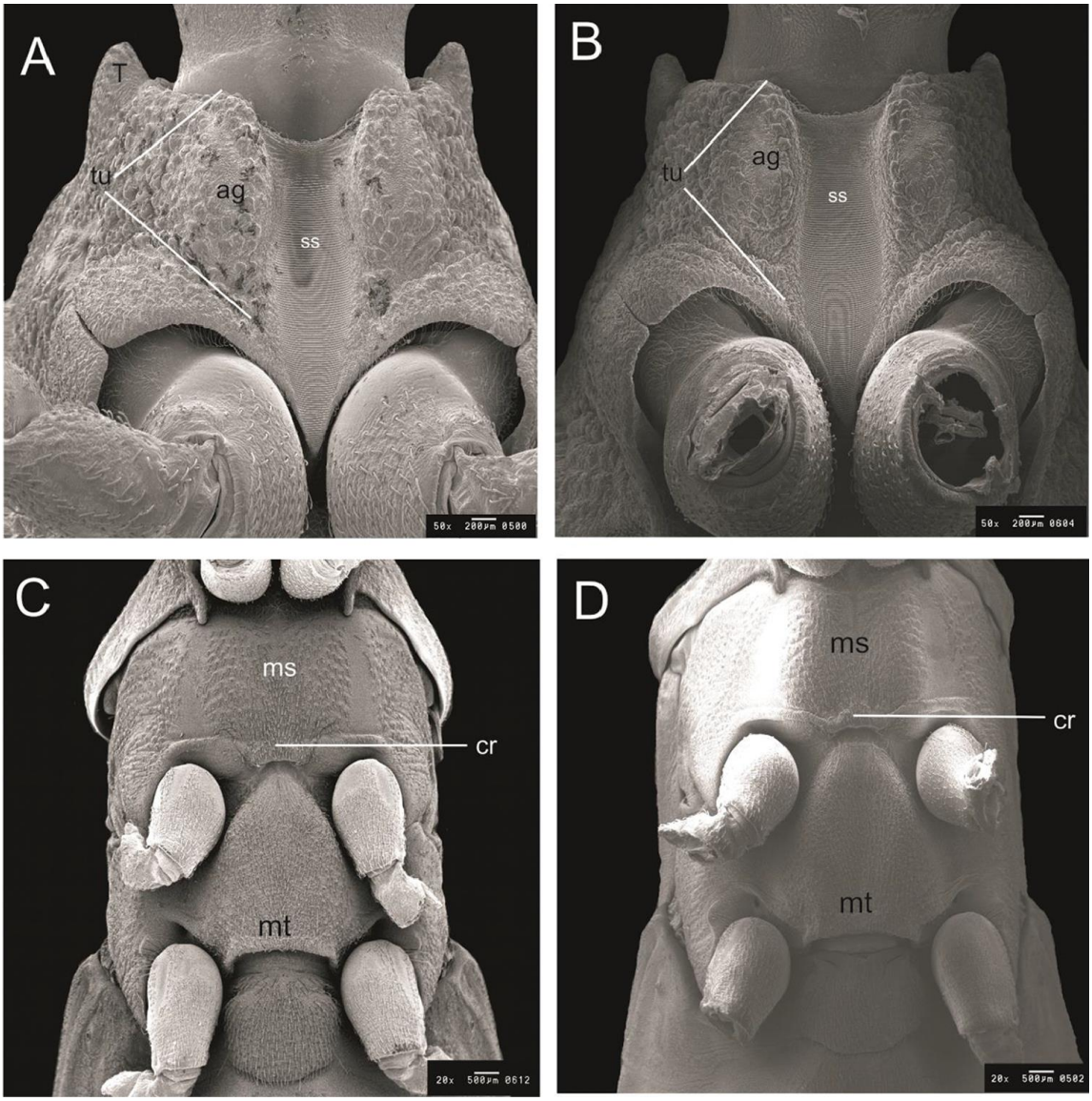

Figure 5. Thorax ventral by SEM. **(A, C)** *R. taquarussuensis* sp. n., **(B, D)** *R. neglectus*.

ss: stridulatory sulcus, ms: mesosternum, mt: metasternum, tu: tubercle, ga: glabrous area, cr: central region.

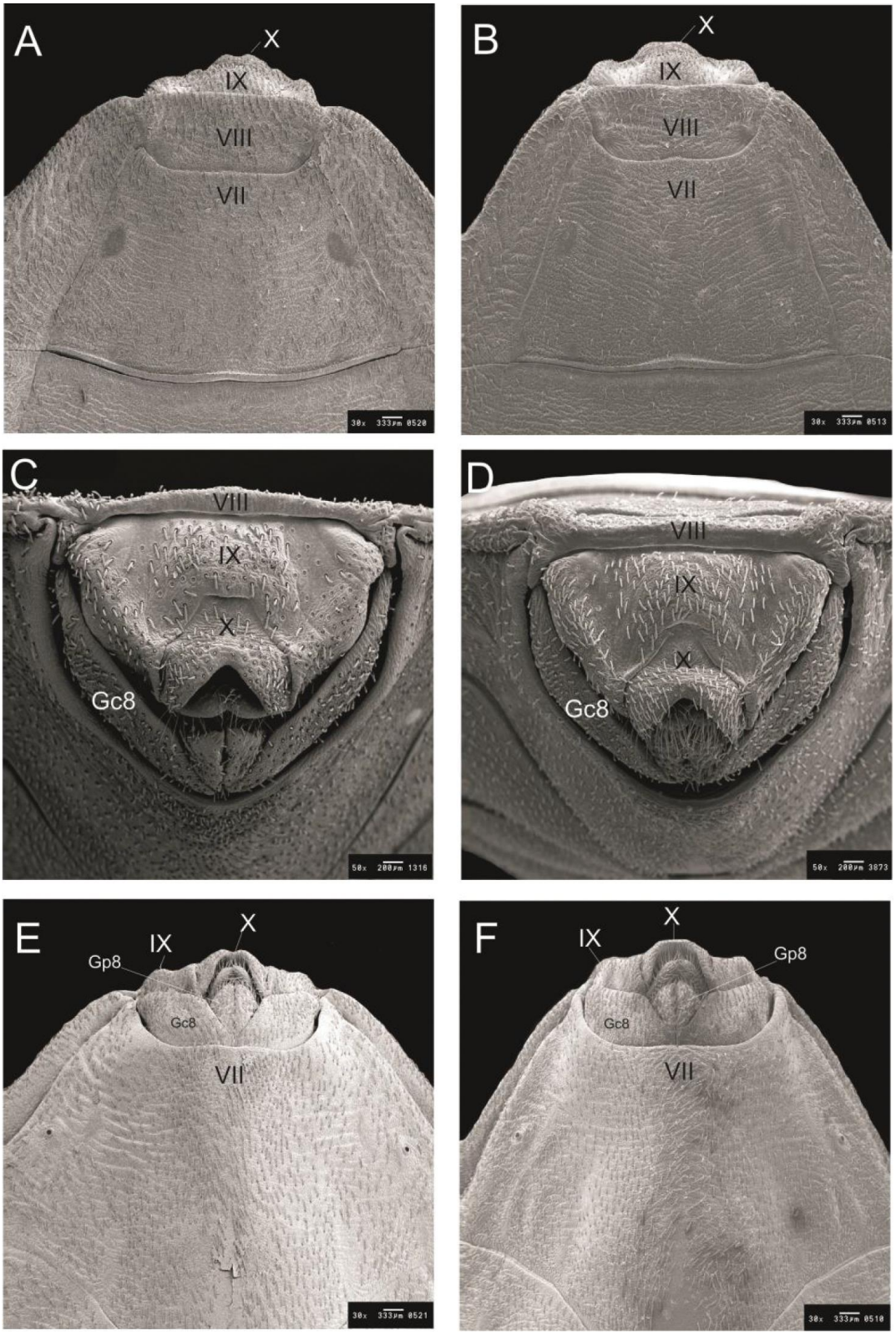

Figure 6. Female external genitalia by SEM *R. taquarussuensis* sp. n. **(A)** dorsal view, **(C)** posterior view, **(E)** ventral view, *R. neglectus* **(B)** dorsal view, **(D)** posterior view, **(F)** ventral view. Gc 8: gonocoxite VIII; Gc 9: gonapophyse IX; Gp 8: gonapophyse VIII; VII, IX: tergites; X: segment.

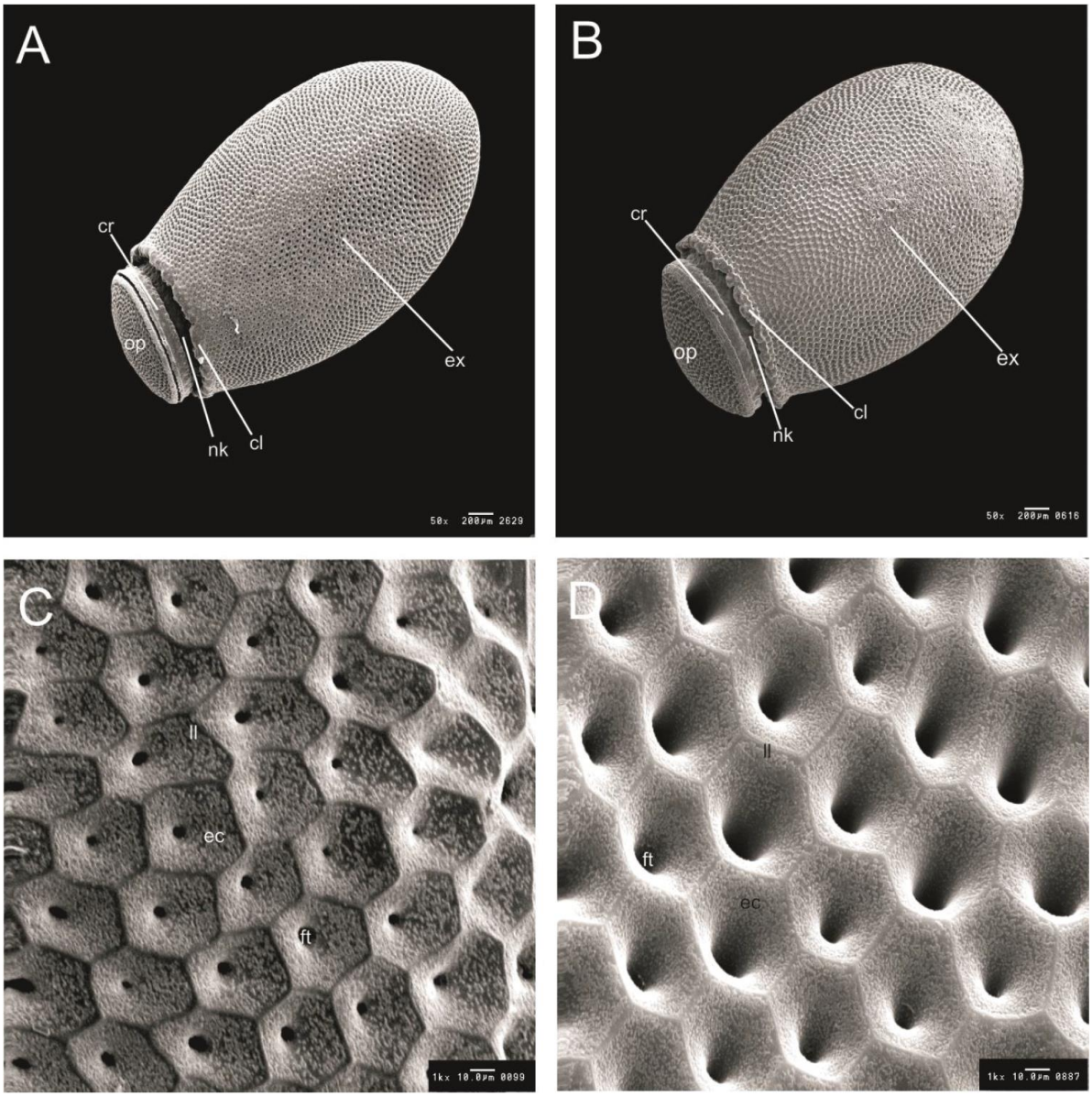

Figure 7. Eggs general vision and egg exochorium detail of *R. taquarussuensis* sp. n. **(A, C)**, *R. neglectus* **(B, D)**. cl: colar, cr: chorial rim, ex: exochorium, nk: neck, op: operculum, ec: exochorium cell, ft: follicular tubes, ll: limiting line.

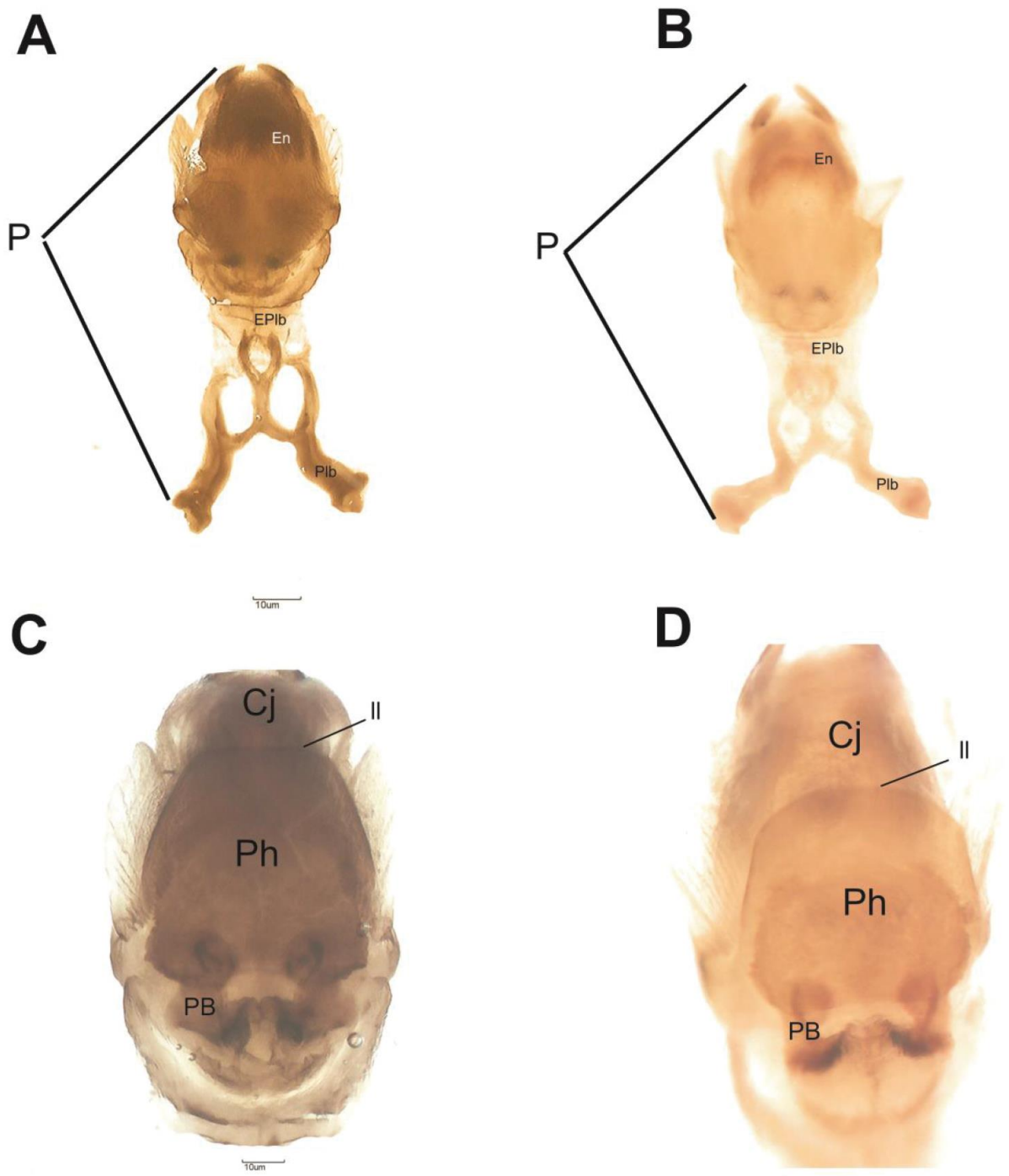

Figure 8. Phallus of *R. taquarussuensis* sp. n. **(A)** dorsal view, **(C)** ventral view, *R. neglectus* **(B)** dorsal view, **(D)** ventral view). Cj: conjunctive, En: endosome, EPlb: median extencion of basal plate, P: phallus, Plb: basal plate, PrG: gonopore process, PrPh: phallossoma process, Ph: phallosoma, PrCj: conjunctive process, ll: line limit.

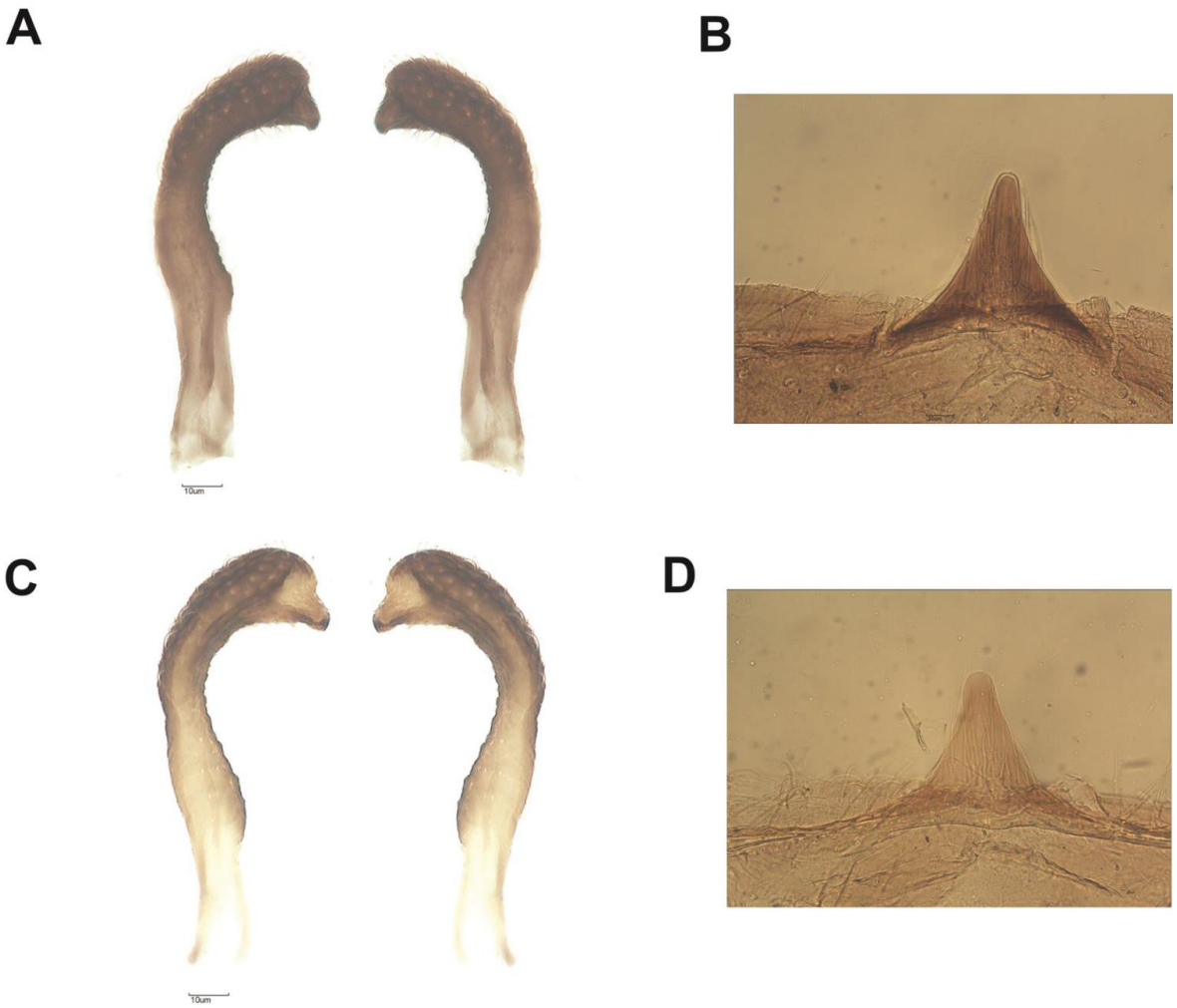

Figure- 9. Parameres dorsal view of *R. taquarussuensis* sp. n**. (A)**, Median process of the pygophore of *R. taquarussuensis* sp. n.**(B)**, Parameres dorsal view of *R. neglectus* (**C),** Median process of the pygophore of *R. neglectus* **(D)**.

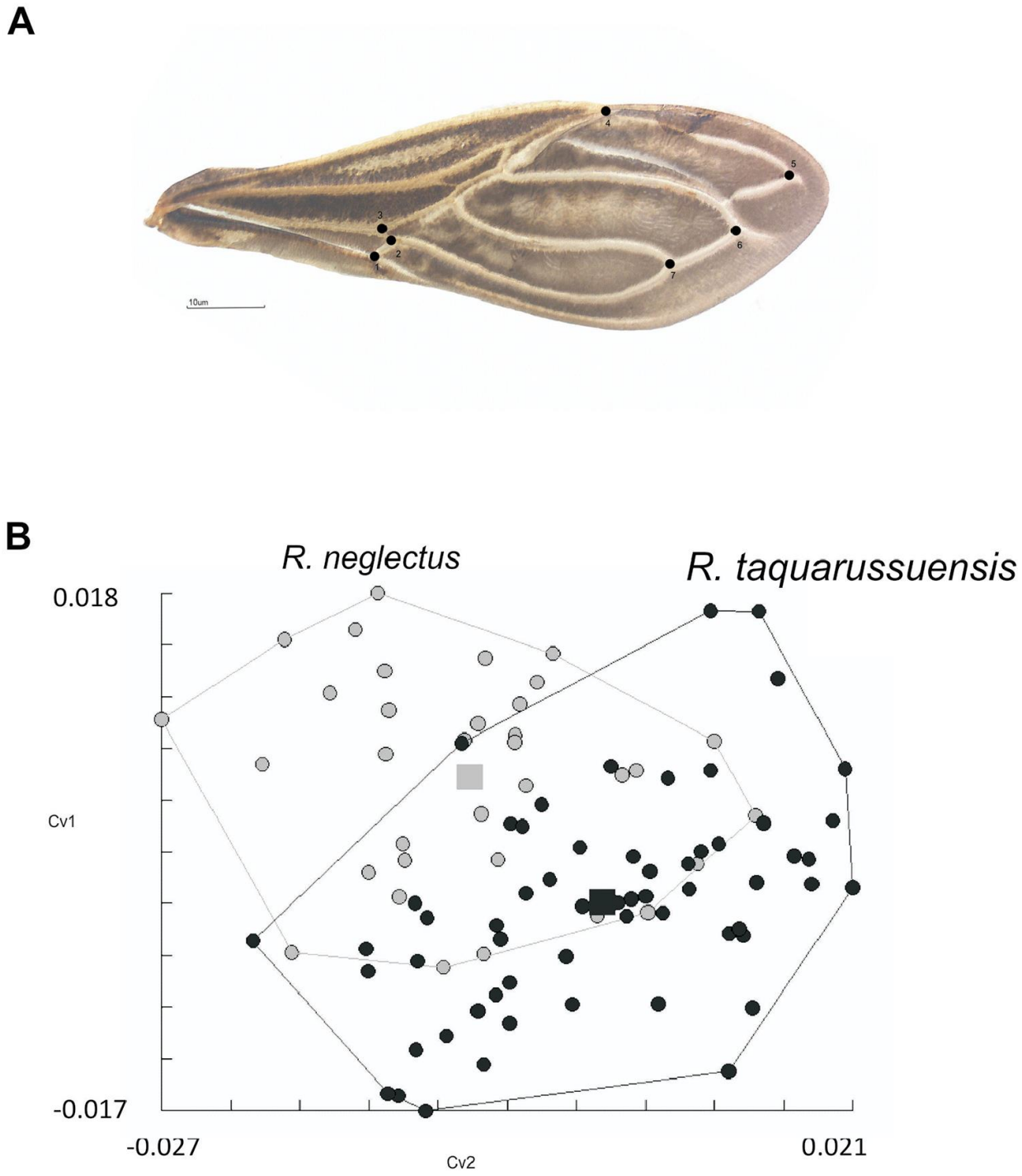

Figure 10. A - Right wing of *R. taquarussuensis* sp. n. with the seven landmarks used in morphometric analysis. Following Bookstein (1990), all points correspond to type I landmarks (venation intersections), **(B)** Factorial maps in the plane of the two discriminant factors of wing shape variation (canonical variables 1 and 2, or CV1 and CV2) presenting the distribution of specimens of *R. taquarussuensis* sp. n. (Rta, black cicle) and *R. neglectus* (Rne, silver cicle).

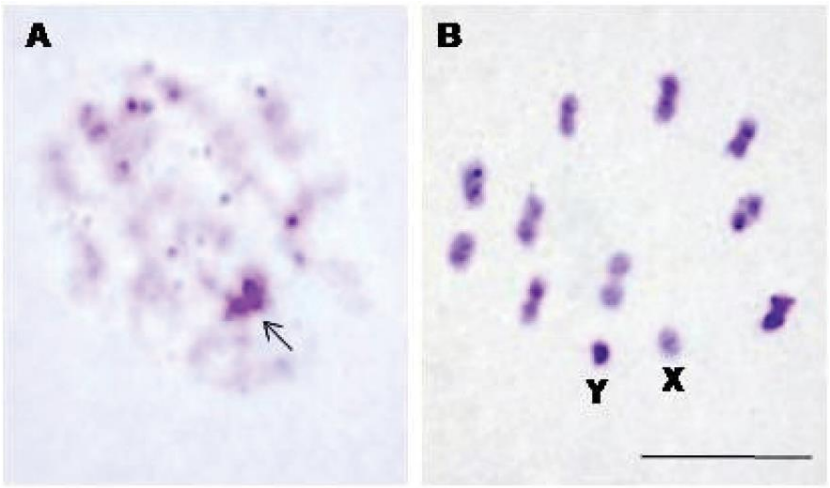

Figure 11. Constitutive heterochromatin pattern in *R. taquarussuensis*. **(A)** Initial prophases with a chromocenter heterochromatic consisting of sex chromosomes (arrow) and several heterochromatic blocks dispersed in the nucleus. **(B)** Metaphase I with heterochromatic blocks in both extremities of practically all the autosomes and in the Y sex chromosome. X: X sex chromosome, Y: Y sex chromosome. Bar: 10 μm.

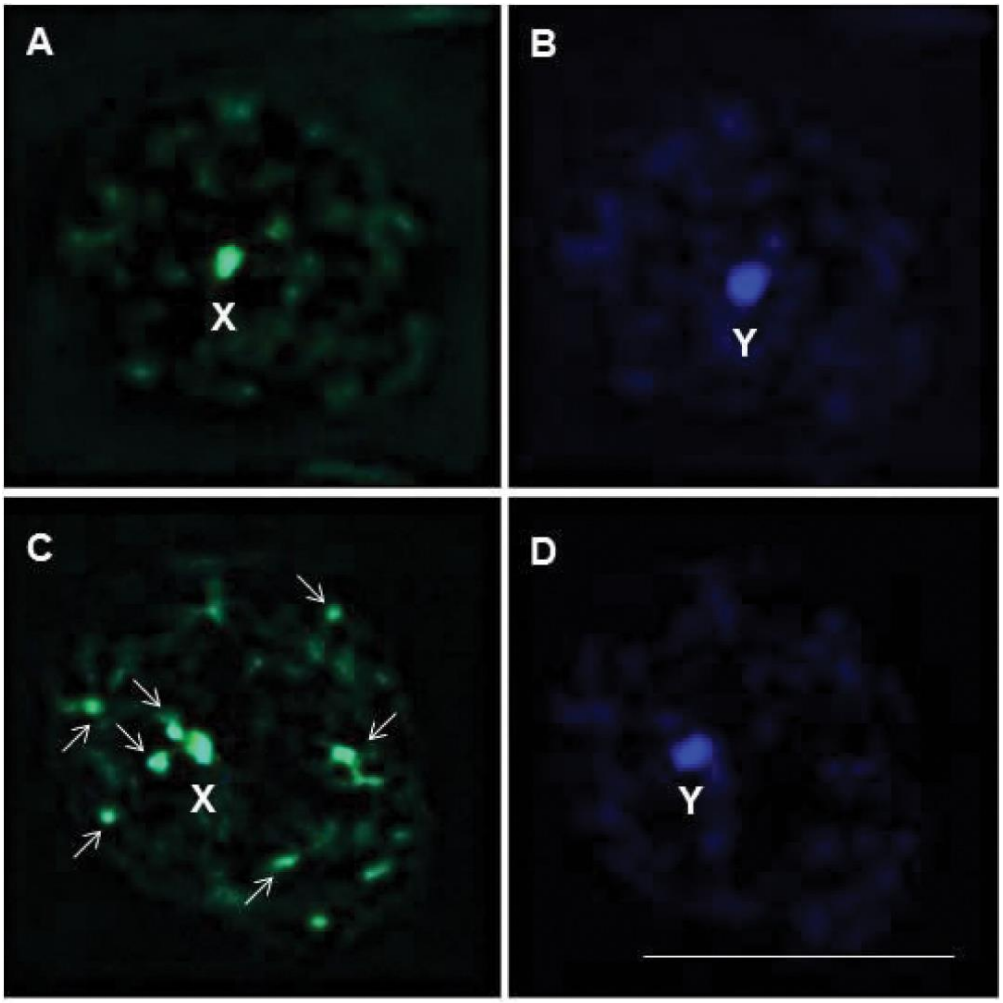

Figure 12. Composition of the pairs of bases of DNA rich in AT and CG in *R. neglectus* **(A, B)** and *R. taquarussuensis* **(C, D)**. **(A)** X sex chromosome rich in CG. **(B)** Y sex chromosome rich in AT. **(C)** X sex chromosome and various blocks dispersed in the prophase nucleus (arrows) rich in CG. **(D)** Y sex chromosome rich in AT. X: X sex chromosome, Y: Y sex chromosome. Bar: 10μm.

4.24 Anexo 24 (Artigo científico submetido para publicação na revista *The American Journal of Tropical Medicine & Higyene* FI 2,54)

New evidence on the genetic structure of Brazilian populations of *Triatoma sordida* (Stål, 1859) (Hemiptera, Triatominae), by means of chromosomal markers

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Abstract

Triatoma sordida is among the main Brazilian species considered as vectors of Chagas disease. The genetic studies are directed mainly to phylogenetic questions, because this species possibly has suffered cryptic speciation. Furthermore, there are punctual studies that analyzed the structure and genetic variability of specimens from Minas Gerais, Brazil that showed low genetic diversity and strong genetic structuring of the population samples. Therefore, due to great epidemiological importance of *T. sordida* in Brazil and mainly the restriction of genetic characterization of this vector only for populations of Minas Gerais state, this paper presents new evidence on the structure of Brazilian populations of *T. sordida* from seven different states representing different biomes, by means of cytogenetic markers. All analyzed specimens presents the same cytogenetic characteristics: early meiotic prophase with several heterochromatic bodies dispersed in the nucleus (CG-rich), being one of them formed by the associated sex chromosomes surrounded by some autosomal heterochromatic regions, meiotic metaphase with most autosomal pairs exhibits a C-heterochromatic block in one chromosomal end (CG-rich), Y sex chromosome fully heterochromatin (AT-rich) and X chromosome may present a small C-block (CG-rich). These results are important because the chromosomal markers suggest low genetic diversity for all Brazilian states occupied by *T. sordida*, also suggest that all Brazilian populations were originated from a small ancestral population and dispersed by founder effect and demonstrate that Brazilian *T. sordida* are not suffering cryptic speciation and are classified as *T. sordida sensu stricto*.

Introduction

The Chagas disease is a neglected disease considered as a potentially life-threatening illness caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909), distributed mainly in endemic areas of 21 Latin American countries, where it is mostly vector-borne transmitted to humans by contact with feces of triatomines. It is estimated that about 6 million to 7 million people are infected worldwide, mostly in Latin America where Chagas disease is endemic.[1]

Presently, there are 152 species of triatomines, distributed in 18 genera and five tribes, being all species considered as potential vector of Chagas disease.[2] In Brazil, there are 68 species distributed throughout the country's 27 states, and 64% of them are endemic species.[3-6] *Triatoma infestans* (Klug, 1834), *T. brasiliensis* Neiva, 1911, *T. pseudomaculata* Corrêa and Espínola, 1964, *T. sordida* (Stål, 1859) and *Panstrongylus megistus* (Burmeister, 1835) are the main species of triatomines related to the domestic transmission of Chagas disease.[3,7]

Triatoma sordida is distributed in 13 Brazilian states (Bahia, Goiás, Mato Grosso, Mato Grosso do Sul, Maranhão, Minas Gerais, Paraná, Pernambuco, Piauí, Rio Grande do Sul, Santa Catarina, São Paulo e Tocantins)[3,8], being considered the species with the highest frequency of domiciliary captures in Brazil.[3] Although *T. sordida* exhibits endemism centered in the Cerrado, this species is also prevalent in Bahia and the states of the Southeast and Midwest regions of Brazil, including transition areas to the Amazon[3], occupying thus other biomes besides the Cerrado: Pantanal, Atlantic forest and Caatinga.[8]

The epidemiological importance of *T. sordida* is increasing due to its tendency to invade houses, particularly in areas where *T. infestans* has been controlled[9,10], being thus considered as "semi-domicile".[11] The anthropic activities have influence in the richness of this species, because the areas of higher occurrence of *T. sordida* are the ones related to the agricultural activities in the past, what could explain its presence in areas that suffered ecologic impact due to significant loss of vegetation (Forattini, 1980). Furthermore, *T. sordida* also is associated with the reinfestation of dwellings treated with insecticides.[12]

Although *T. sordida* exhibit wide geographic distribution in Brazil[3,8] genetic studies are directed mainly to phylogenetic questions[13-17], because this species is also found in Argentina, Bolivia, Paraguay and Uruguay[3], and possibly has suffered cryptic speciation.[14-18] Furthermore, there are exact studies that analyzed the genetic structure and genetic variability of specimens from Minas Gerais, Brazil that showed low genetic diversity and strong genetic structuring of the population samples.[19,20]

Recently, Pessoa et al.[20] related to low genetic diversity and strong genetic structuring of the population samples of *T. sordida* with five possible factors: possible geographical isolation due to an obstacle to Triatominae flow between neighboring localities, focal distribution of insects in small colonies usually comprised of a few individuals, thereby limiting gene flow between them; the low dispersal capacity of *T. sordida*, the long Triatominae life cycle, which ensures that contributions from young adults able to reproduce (and consequently exchange genetic material) occurs over long intervals that differ among triatomine species and continuous pressure from insecticides used since the 1950s to triatomines control.

As Chagas disease has no cure and treatment with benznidazole and nifurtimox is effective only in the acute phase of the disease (which is often asymptomatic), vector control is the most effective method of preventing this neglected disease[1] and all knowledge about these hematophagous insects is important and can generate subsidies to assist the vector control programs. Thus, due to great epidemiological importance of *T. sordida* in Brazil and mainly the restriction of genetic characterization of this vector only for populations of Minas Gerais state, this paper performs a microevolutionary analysis of the *T. sordida* from seven different Brazilian states representing different biomes, by means of cytogenetic markers.

Material and Methods

Material

100 adult males coming from the municipalities described in Table 1 (at a minimum three copies of each locality) were used in the study (males were utilized because spermatogenesis is continued in adulthood, allowing the visualization of chromatin and chromosomes). The triatomines were provided by the insectariums of FCFAR/UNESP, Araraquara, SP, Brazil, of IOC/FRIOCRUZ, Rio de Janeiro RJ, Brazil and of CPqRR/FIOCRUZ, Belo Horizonte, MG, Brazil. The characterization of biomes was performed according to the Brazilian Institute of Geography and Statistics (IBGE).[21]

Chromosome preparation

For cytological preparations, testes were removed from adult insects alive, fixed in an ethanol–acetic acid (3:1) and stored at -20°C. Squashes were made in a 50% acetic acid drop,

coverslips were removed after freezing in liquid nitrogen and the slides were air dried and then stored at 4°C.

C and CMA_3/DAPI banding

C-banding was performed according to Sumner et al.[22] for the characterization of constitutive heterochromatin and CMA_3/DAPI banding was performed according to Schimid[23], with modifications according to Severi-Aguiar et al.[24], for differentiating the regions of heterochromatin rich in AT ($DAPI^+$) and CG (CMA_3^+).

Analysis of biological material

At least 50 cells in prophase (for analysis of the chromatin) and 50 in metaphase (for analysis of the chromosomes) of *T. sordida* from each location were analyzed. The analysis of C-banding preparations was made 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) and of CMA_3/DAPI banding preparation was analyzed using in fluorescence microscopy Zeiss-Axioskop and Olympus BX-FLA.

Results

All analyzed specimens, regardless the locality (Table 1) presents the same cytogenetic characteristic: C-Banding - during early meiotic prophase, several heterocromatic bodies are observed, being one of them formed by the associated sex chromosomes surrounded by some autosomal heterocromatic regions (Fig 1A, *); Furthermore, during the meiotic metaphase the most autosomal pairs present a C-heterochromatic block in one chromosomal end and X chromosome may present a small C-block (Fig 1B) (Y sex chromosome is heterochromatin in all species of triatomine); CMA_3/DAPI Banding: several bodies CG-rich dispersed in the nucleus of early meiotic prophase, being one of them formed by the association of the X sex chromosome surrounded by some autosomal (Fig 1C, *) and Y sex chromosome AT-rich DNA (Fig 1D).

Discussion

Cytogenetic markers have been used because initially Panzera et al.[14] differentiated *T. sordida* from Brazil and Argentina by the pattern of heterochromatin and recently Panzera et al.[16] recognized five chromosomal taxa for *T. sordida* subcomplex and three chromosomal taxa for *T. sordida* (*T. sordida sensu stricto*, T. sordida Argentina and *T. sordida* La Paz). The authors analyzed Brazilian representatives from Minas Gerais, Piauí and Mato Grosso with C-banding and observed the same results that we observed for all analyzed specimens. Beyond that, we characterized the composition of DNA rich in AT and CG in chromatin of these vectors and all the specimens of *T sordida* analyzed showed the same disposition in chromatin and chromosomes observed by Bardella et al.[17] for a population of Minas Gerais, Brazil.

These results are important because the chromosomal markers enable confirm and expand the results described by Monteiro et al.[19] and Pessoa et al.[20] (for molecular markers) and Vendrami et al.[25] for all Brazilian states occupied by *T. sordida*. In addition, the low genetic diversity can also be confirmed by the low genetic distance observed between *T. sordida* from different Brazilian states, for example, 0.011 among populations of Mato Grosso (Pocotó) and Mato Grosso do Sul (Pantanal) with the cytochrome oxidase I gene; 0.007 among populations of Mato Grosso (Rondonópolis) and Mato Grosso do Sul (Pantanal) with cytochrome oxidase II gene; and 0.006 among populations from municipalities in the state of Mato Grosso (Rondonópolis and Pocotó), and 0.004 and 0.002 distance observed between Rondonópolis and Pantanal and Pocotó and Pantanal, respectively, both with the 16S r DNA gene.[26]

Some authors suggest that *T. sordida* probably have originated in the Brazilian Cerrado[13,27,28] and as a consequence of deforestation and *T. infestans* control strategies, it has dispersed towards the south.[29,30] Our results, together with the results of Justi et al.[26] and Vendrami et al.[25] also suggest that all Brazilian populations originated from a small ancestral population (possibly in the Cerrado, as suggested above) and dispersed to other biomes by founder effect (possibly by active dispersal by flight[31] or passive dispersion nymphs associated with bird feathers[32]). However, since there are evidences that genetic diversity of *T. infestans* is reduced in areas treated with insecticides due to bottleneck events[33,34], we cannot also dismiss this hypothesis for *T. sordida*.

Noireau et al.[15] reported for the first time the presence of cryptic speciation to *T. sordida* specimens from Bolivia. Monteiro et al.[19] analyzed this phenomenon for four

populations from Minas Gerais, Brazil and found no cryptic speciation. Our results demonstrate that *T. sordida* Brazilians are not suffering cryptic speciation, because if this evolutionary event was taking place, the microevolutionary results with chromosomal markers would demonstrate differences, as well as were recently observed for specimens of Bolivia.[16] Wing geometry also reinforce low genetic variability among *T. sordida* populations from Brazil[25], confirms that all triatomine collected in Brazil and maintained in Brazilian insectariums, initially classified as *T. sordida* by morphology, exhibit the same chromosome pattern characterized by Panzera et al.[16] as *T. sordida sensu stricto*, assisting directly in the taxonomy of these vectors.

Thus, by means of the microevolutionary study of Brazilian *T. sordida* was observed that this vector species although widely distributed in Brazil has extremely low genetic diversity. Moreover, we confirm the classification of all Brazilian examples as *T. sordida sensu stricto*. Our results are important to direct further studies (such as biogeography) and, especially, to assist in developing tools for the control of this vector species by vector control programs.

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Table 1. Geographical origin of the specimens used in the study microevolutionary and their corresponding biomes.

Triatoma sordida	**Collection sites**	**Brazilian biomes**
Brazilian states	**municipalities**	
Mato Grosso	Rondonópolis	Cerrado
Goiás	Posse	Cerrado
	São Luiz dos Montes Belos	Cerrado
	Guarani do Goiás	Cerrado
Minas Gerais	Matias Cardoso and Monte Azul	Cerrado and Caatinga
	Itinga	Atlantic forest
	Lontra	Cerrado
Bahia	Seabra, Macaúbas and Boquira	Caatinga
Rio Grande do Sul	São Francisco de Assis	Pampa and Atlantic forest
São Paulo	Mirassol and Guaíra	Atlantic forest and Cerrado
	São Paulo and Castilho	Atlantic forest
Mato Grosso do Sul	Rio Verde de Mato Grosso	Cerrado and Pantanal
	Corumbá	Pantanal
	Brasilândia	Atlantic forest and Cerrado

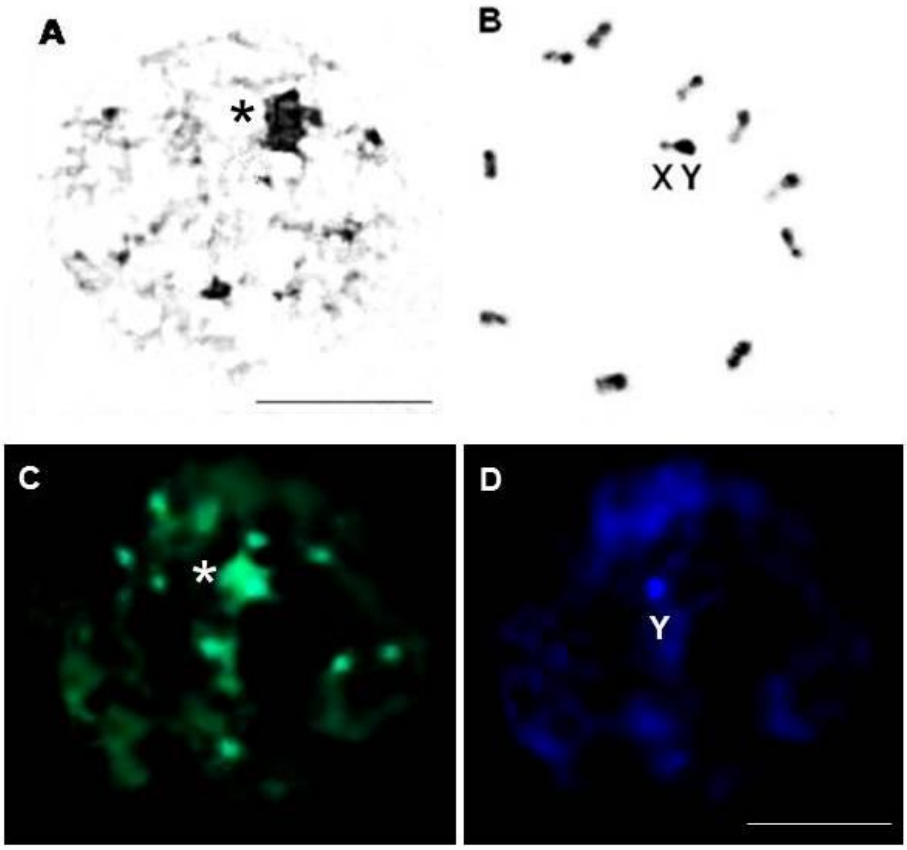

Figure 1. Constitutive heterochromatin pattern and composition of AT-rich and CG-rich DNA observed in *T. sordida* Brazilians. (A) Early meiotic prophase prepared with C-banding. Note the several heterochromatic bodies dispersed in the nucleus, being one of them formed by the associated sex chromosomes surrounded by some autosomal heterochromatic regions (*). (B) Meiotic metaphase prepared with C-banding. Note the most autosomal pairs presents a C-heterochromatic block in one chromosomal end, Y sex chromosome is fully heterochromatin and X chromosome may present a small C-block. (C and D) Early meiotic prophase prepared with CMA_3/DAPI banding. (C) Note the several bodies CG-rich dispersed in the nucleus, being one of them formed by the associated X sex chromosome surrounded by some autosomal (*). (D) Note that only the Y sex chromosome is AT-rich. X: X sex chromosome Y: Y sex chromosome. Bar = 10 μm.

4.25 Anexo 25 (Artigo científico aceito para publicação na revista *The American Journal of Tropical Medicine & Higyene* FI 2,54)

Karyotype evolution of Chagas disease vectors (Hemiptera, Triatominae)

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Abstract

The Triatominae subfamily is composed of 153 hematophagous species that are potential vector of *Trypanosoma cruzi*, the etiological agent of Chagas disease. Karyotypic studies in triatomines were initiated in 1909. There are 92 karyotypes described, all grouped into the Rhodniini and Triatomini tribes. Recently, a phylogenetic study of the triatomines that combines molecular data with geological changes was performed. We now discuss how the karyotype evolved with the diversification of the triatomines.

Short Report

The Triatominae subfamily is composed by 153 hematophagous species (151 live species and two fossil) considered as potential vector of *Trypanosoma cruzi* (Chagas, 1909), etiological agent of Chagas disease.[1,2,3] Chagas disease is a neglected disease distributed in 21 Latin American countries, being actually estimated that approximately 6 million to 7 million people are infected worldwide.[4]

Actually the Triatominae is divided into 18 genera and five tribes (Alberproseniini, Bolboderini, Cavernicolini, Rhodniini and tribes Triatomini) [1], which are distributed from the southern USA to Patagonia, with a few species of Triatomini known from India and Australia. According to Justi et al.[5] the first three tribes (Alberproseniini, Bolboderini and Cavernicolini) comprise only 15 out of the 153 known species. Rhodniini and Triatomini are the most diverse and epidemiologically relevant tribes and are, therefore, the best studied.[1]

Karyotypic studies in Triatominae were initiated in 1909 with the description of the karyotype of *Triatoma sanguisuga* (Leconte, 1855).[6] In 1950, the karyology were resumed and new karyotypes were described.[7] In 1966, Ueshima[8] described 20 new karyotype and proposed cytogenetic studies as a tool in the taxonomy of these vectors (cytotaxonomy). Actually, there are 92 karyotypes described,[3,9,10,11] being all species grouped into Rhodniini and Triatomini tribes.

Recently, was proposed a phylogenetic study of the triatomines that combines molecular data with geological changes.[5] The authors observed that most of the diversification events that occurred in Rhodniini and Triatomini tribes are linked mainly to the climatic and geological changes caused by the Andean uplift in South America. Based on phylogenetic relationships presented by Justi et al.[5], we will discuss for the first time as the karyotype evolved according to the diversification of the triatomines.

Triatominae subfamily was recovered as monophyletic by Justi et al.[5]. For a long time, was discussed if Triatominae subfamily is monophyletic, paraphyletic or polyphyletic.[12] Nokkala and Nokkala[13] suggest that primitive karyotype of the order Hemiptera is XY. Thus, taking as a basis the latest theory where triatomine arose from a common ancestor (monophyletic group)[5] and that the main events of variation in the number of chromosomes of triatomines are involved with fission (agmatoploidy) and fusion (simploidy) of the X sex chromosome[9], we can confirm that all triatomine evolved of an ancestral karyotype 2n = 22 (20A + XY) (as initially suggested by Ueshima[8]).

The species of the Alberproseniini, Bolboderini and tribes Cavernicolini does not have the described karyotype. However, as Cavernicolini was presented as sister tribe to Rhodniini and all *Rhodnius* (15 of the 20 species) and *Psammolestes* (2 of the 3 species) species analyzed have 2n = 22 (20A + XY)[3,9], we suggest that all species of Rhodniini and Cavernicolini tribes have 22 chromosomes. A recent study cytogenetic of *Cavernicola pilosa* Barber, 1937 suggests that this species has sex determination system of the type XY[14], supporting our hypothesis.

The most diverse genera within the Rhodniini (*Rhodnius*) and Triatomini (*Triatoma*) have classically been divided into subgroups (groups, complexes and subcomplexes) primarily based on morphology and geographical distribution.[15] Between the different groups of *Rhodnius* (*pallescens*, *pictipes* and *prolixus* group) were not observed events that resulted in changes in the number of chromosomes of these vectors when compared to the ancestral karyotype 2n = 22.[3,9] On the other hand, there are various events associated with the chromosomal evolution of Triatomini that are discussed next.

Justi et al.[5] observed that the uplift of the Western Cordillera acted as a vicariant event separating the *venosa* clade from the remaining Triatomini. The *venosa* clade, represented by the species of the *dispar* complex (*T. boliviana* Martinez et al., 2007, *T. carrioni* Larrousse, 1926, *T. dispar* Lent, 1950, *T. nigromaculata* (Stål, 1872) and *T. venosa* (Stål, 1872)[15] was the first to diverge from Triatomini tribe. Recently it was described the karyotype 2n = 22

(20A + XY) to *T. boliviana* and *T. carrioni*.[10] Thus, we suggest that all species of this complex have 2n = 22 chromosomes and haven't suffered events of the agmatoploidy and simploidy during the divergence of clade from the karyotype ancestor.

Subsequently, Justi et al.[5] observed that the Northern Andean uplift separated *T. maculata* (restricted to the Amazon) from the other members of the *infestans* group (evolutionary event that has not led to change in the number of chromosomes). Furthermore, the authors report that *T. melanocephala* Neiva and Pinto, 1923 and *T. vitticeps* (Stål, 1859) though present in South America are considered as an exception, because which appear to have reached the Atlantic coast by dispersal and diversified prior to that event. Different from the species of the *infestans* group that present 2n = 22 chromosomes[9,11], *T. melanocephala* and *T. vitticeps* have 2n = 24 (20A + $X_1X_2X_3Y$).[6,16] The evolutionary event that led to karyotypic diversification of species (*vitticeps* subcomplex) is known as agmatoploidy and resulted in a karyotype which enables distinguish these species from all other South America.[17]

The *infestans* group is formed by *brasiliensis*, *infestans*, *maculata*, *matogrossensis*, *rubrovaria* and *sordida* subcomplexes.[15,18] Many karyotypes of these subcomplexes have been described and all species analyzed has 2n = 22 (20A + XY).[9,11] Thus, same as the different subcomplexes emerged from different selective pressures (*brasiliensis* subcomplex in the Caatinga Province, *rubrovaria* subcomplex in the Pampean Province, *infestans* subcomplex in the Chacoan Province and *sordida*, *matogrossensis* and part of species of the *maculata* subcomplex in the Cerrado Provinces)[5,18], we suggest that all species of *infestans* group has 2n = 22 chromosomes. Although the *spinolai* complex was long considered as sister group of South American triatomine (being part of the *infestans* group)[15], the recent study presented by Just et al.[5] demonstrated that the species of this complex are more closely related to the *geniculatus* and *rubrofasciata* clades.

The *spinolai* complex is composed by the species *T. breyeri* Del Ponte, 1929, *T. eratyrusiformis* Del Ponte 1929, *Mepraia spinolai* (Porter, 1934), *M. gajardoi* Frias, Henry & Gonzalez, 1998 and *M. parapatrica* Frías-Lasserre, 2010.[15] *T. eratyrusiformis* (and possibly *T. breyeri* by proximity phylogenetic)[5] presents 2n = 24 (20A + $X_1X_2X_3Y$)[8] and *Mepraia* spp. presents 2n = 23 (20A + X_1X_2Y).[19] From the ancestral karyotype (2n = 22), the species of this complex that diverged in *Mepraia* have suffered one agmatoploidy event for the X sex chromosome (X_1X_2) and the species that diverged in *Triatoma* have suffered two events ($X_1X_2X_3$). These results corroborate the phylogenetic relationship presented by Just et al.[5]

because the majority of species of the *geniculatus* and *rubrofasciata* clades have 23 (sex-determination system X_1X_2Y), 24 (sex-determination system $X_1X_2X_3Y$) or exceptionally 25 chromosomes (sex-determination system X_1X_2Y).[8,17,18,19]

Gardim et al.[20] evidenced the need for a generic revision in the Triatomini, because *Panstrongylus* cannot be clustered isolated of *Triatoma*. The *geniculatus* clade, for example, is composed of *Panstrongylus* spp. + *flavida* complex + *T. tibiamaculata*.[5] All studied species of this clade (except *P. megistus* (Burmeister, 1835) and *P. lutzi* (Neiva & Pinto, 1923)[21,22] presented 2n = 23 (20A + X_1X_2Y)[9,21], confirming the evolutionary relationship proposed. Based on the ancestral karyotype (2n = 22), we suggest that during the divergence of the common ancestor of the *geniculatus* clade one event of agmatoploidy in the X sex chromosome has happened, which resulted in karyotype 2n = 23 (karyotype shared by *Panstrongylus* spp., *Nesotriatoma* spp. and *T. tibiamaculata*). However, during the karyotypic evolution of *Panstrongylus*, two events occurred: simploidy in a pair of autosomes for *P. megistus* 2n = 21 (18A + X_1X_2Y)[21] (event less common, possibly related to the divergence by vicariant between *P. megistus* and *T. tibiamaculata* (Pinto, 1926) from the separation of the common ancestor by disappeared of the connection between the Amazonian Forest and the Atlantic Forest because climatic changes caused by Andean uplift)[4] and agmatoploidy in the X sex chromosome for *P. lutzi* 2n = 24 (20A + $X_1X_2X_3Y$).[22]

Justi et al.[5] presented the *phyllosoma* group composed by *T. mexicana* (Herrich-Schaeffer,1848), *T. dimidiata* (Latreille,1811), *T. recurva* (Stål, 1868), *T. gerstaeckeri* (Stål,1858), *Meccus pallidipennis* (Stål, 1872), *M. longipennis* (Usinger, 1939), *M. mazzottii* (Usinger, 1941) and *M. picturatus* (Usinger, 1939) and suggest that *T. sanguisuga* was separated from the other members of the *phyllosoma* group by vicariant event (high sea level that inundated Florida and the Gulf Coast). This group is basically a combination of species of the *lecticularia* (*T. gerstaeckeri, T. indictiva* Neiva, 1912, *T. lecticularia* (Stål, 1859), *T. recurva, T. rubida* (Uhler, 1894) and *T. sanguisuga*) and *phyllosoma* complex (*M. bassolsae* Aguillar et al., 1999, *T. bolivari* Carcavallo, Martinez & Pelaez, 1987, *M. longipennis, M. mazzottii, T. mexicana, M. pallidipennis, M. phyllosoma, M. picturata* and *T. ryckmani* Zeledón & Ponce, 1972).[15] With the exception of *T. lecticularia* (which was recovered as the sister of *Paratriatoma hirsuta* Barber, 1939 and both have karyotype 2n = 22), all the species of *lecticularia* and *phyllosoma* complex studied cytogenetically have karyotype 2n = 23 (20A + X_1X_2Y).[9] We suggest that this is the karyotype of all species of *phyllosoma* group.

Considering that *T. ryckmani* and *T. rubida* present 2n = 23 and *T. lecticularia* and *P. hirsuta* 2n = 22, we can suggest two hypotheses: i) the ancestor of these triatomine had 22 chromosomes and during the divergence of *T. ryckmani* and *T. rubida* occurred one agmatoploidy event of X sex chromosome or ii) the common ancestor had 2n = 23 and during the divergence of *T. lecticularia* and *P. hirsuta* had a simploidy event of X sex chromosome. However, as the agmatoploidy event is much more common in subfamily Triatominae[21] and simploidy event is known only to autosomes[9], we suggest that the first hypothesis is correct.

Lastly, Just et al.[5] showed that the separation of the Old World clade (composed by *T. rubrofasciata* (De Geer, 1773) and *Linshcosteus* spp., dates to a time as late as the Mid-Oligocene. Furthermore, the authors note that *T. rubrofasciata* and the species of genus *Linshcosteus* (*L. carnifex* Distant, 1904, *L. chota* Lent & Wygodzyski, 1979, *L. confumus* Ghauri, 1976, *L. costalis* Ghauri, 1976, *L. kali* Lent & Wygodzyski, 1979 and *L. karupus* Galvão et al., 2002) form a monophyletic group. Based on the peculiar karyotype 2n = 25 (22A + X_1X_2Y) of *T. rubrofasciata* (which presents one event at agmatoploidy in the autosomes, which allows for differentiate it from all triatomine species)[23] and the phylogenetic relationship presented between *T. rubrofasciata* and the species of genus *Linshcosteus*,[5] we suggest that agmatoploidy event has occurred in the common ancestor of Old World clade, that is, we believe that all species of the genus *Linshcosteus* also have 25 chromosomes.

Thus, based primarily in the evolutionary data presented by Justi et al.[5], we highlight new and important information (being some hypotheses) on the karyotype evolution of the triatomines that will drive new studies on these vectors of Chagas disease.

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4.26 Capítulo 26 (Artigo científico publicado na *Cell Biology International*)

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Nucleolar persistence in spermatogenesis of the genus *Rhodnius* (Hemiptera, Triatominae)

Abstract

The Triatominae subfamily comprises of 18 genera and six tribes. The tribe Rhodniini comprises of two genus (*Rhodnius* and *Psammolestes*). The nucleolar persistence is defined by the presence of the nucleolus or nucleolar corpuscles during meiotic metaphase. This phenomenon, to date, has been described for 13 species of triatomine between *Triatoma*, *Rhodnius* and *Panstrongylus* genera. Thus, taking into consideration that the phenomenon of nucleolar persistence has only been described in two species of the genus *Rhodnius*, this paper aims to analyze the nucleolar behavior during spermatogenesis of eight species of the genus *Rhodnius* (*R. colombiensis*, *R. montenegrensis*, *R. nasutus*, *R. neglectus*, *R. neivai*, *R. pictipes*, *R. prolixus*, *R. robustus*), focusing on nucleolar persistence. By means of cytogenetic analysis with silver ions, we describe the nucleolar behavior during spermatogenesis of the eight species of *Rhodnius* analyzed. Thus, as in all species the nucleolar behavior was similar and mainly the phenomenon of nucleolar persistence was observed. Therefore, based on our work, we confirm the nucleolar persistence as a peculiarity of the genus *Rhodnius*. However, we emphasize that new cytogenetic analysis should be performed in Triatominae subfamily, more specifically between 15 genera that do not exhibit the nucleolar behavior described, to assess whether this phenomenon is really a synapomorphy of these hematophagous insects.

1. Introduction

Triatomines are insects that are taxonomically included in the Hemiptera order and Heteroptera suborder, within the Reduviidae family, and Triatominae subfamily (Lent and Wygodzinsky, 1979). The Triatominae subfamily consists of 145 species (Alevi et al., 2013a).

All triatomine species are susceptible to infection by the protozoan *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae) and therefore potential vectors of Chagas disease. Infection occurs through food with infected blood, and all instars subject to ingest the parasite, since hematophagous is mandatory in all life stages of the insects (Noireau et al., 2009).

The Triatominae subfamily comprises of six tribes, out more, Alberproseniini, Bolboderini, Cavernicolini, Linshcosteini, Rhodniini and Triatomini (Galvão et al., 2003; Tartarotti et al., 2006; Alevi et al., 2013a). The tribe Rhodniini comprises of two genus (*Rhodnius* and *Psammolestes*) and 21 nominal species (18 of *Rhodnius* and 3 of *Psammolestes*) (Alevi et al., 2013a).

In cytogenetics, triatomines are important biological models because they have holocentric chromosomes, which have diffuse kinetochores and these insects also perform an unusual meiosis in which the segregation of sex chromosomes is post-reductional (Barth, 1956; Ueshima, 1966; Alevi et al., 2012). In addition, these insects have a peculiar behavior of the nucleolus during spermatogenesis termed nucleolar persistence (Tartarotti and Azeredo-Oliveira, 1999).

The phenomenon of nucleolar persistence, to date, has been described for 13 species of triatomine among *Triatoma* (Severi-Aguiar et al., 2005; Severi-Aguiar et al., 2006; Morielle and Azeredo-Oliveira, 2007; Bardella et al., 2008; Costa et al., 2008; Alevi et al., 2013b), *Rhodnius* (Morielle and Azeredo-Oliveira, 2004; Morielle and Azeredo-Oliveira, 2007) and *Panstrongylus* genera (Tartarotti and Azeredo-Oliveira, 1999).

Thus, taking into consideration that the phenomenon of nucleolar persistence has only been described in two species of the genus *Rhodnius*, this paper aims to analyze the nucleolar behavior during spermatogenesis of eight species of the genus *Rhodnius* focusing on nucleolar persistence.

2. Material and methods

Spermatogenesis of at least five adult males of each *Rhodnius* was analyzed: *R. colombiensis* (Mejía et al., 1999), *R. montenegrensis* (Rosa et al., 2012), *R. nasutus* (Stal, 1859), *R. neglectus* (Lent, 1954), *R. neivai* (Lent, 1953), *R. pictipes* (Stal, 1872), *R. prolixus* (Stal, 1859) and *R. robustus* (Larrousse, 1927). 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. The seminiferous tubules of

adult males were torn and fixed to a cover slip. They then underwent the cytogenetic technique of impregnation by silver ions (Howell and Black, 1980). 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.

3. Results and discussion

The nucleolar persistence is defined as the nucleolus or nucleolar fragments persistent during metaphase, as in eukaryotes, according to Gonzalez-Garcia and Rufas (1995) during cell division, the cells cease transcription of ribosomal RNA (rRNA) in prophase and nucleolar fragmentation occurs. Only in anaphase the rRNA transcription is reactivated followed by gradual reorganization of the nucleolus.

The nucleolar proteins responsible for silver ion impregnation are nucleolin (C23) and the numatrin (B23) (Howell and Black, 1980). By means of cytogenetic analysis with silver ions, we describe the nucleolar behavior during spermatogenesis of the eight species of *Rhodnius*. Spermatogenesis consists of three different phases: spermatocitogenesis, which is a phase of proliferation; meiosis, which is the multiplication phase; and spermiogenesis, which is the differentiation phase (Johnson et al., 1997). Thus, as in all species the nucleolar behavior was similar and mainly the phenomenon the nucleolar persistence was observed (Table 1). We represent the nucleolar behavior by means of the meiosis in *R. montenegrensis* (Figure 1A-E). Moreover, we represent the persistent nucleolar in *R. nasustus* (Figure 2A), *R. robustus* (Figure 2B) and *R. pictipes* (Figure 2C).

During prophase did not occur nucleolar fragmentation (Figure 1A-C). In meiotic metaphase (Figure 1D, arrow), it was possible to note the presence of the nucleolus, which characterized the nucleolar persistence. This nucleolus was only maintained during anaphase (Figure 1D, arrow) and, unlike other eukaryotes, nucleolar reorganization did not occur.

Severi-Aguiar et al. (2005) reported that the nucleolus during spermiogenesis of triatomines is inactivated. Alevi et al. (2013b) believed that persistent nucleolar observed in the meiosis of the triatomines is a very important event, because this material together with the nucleolar organizer regions (NORs) present on chromosomes are major transcriptional factors that allow spermiogenesis to occur. However, further studies should be performed to evaluate the transcriptional activity of the nucleolus in haploid cells, such as the direct

relationship between nucleolar persistence and the process of cell differentiation during spermiogenesis.

Therefore, based on our work, we confirm the nucleolar persistence as a peculiarity of the genus *Rhodnius*. However, we emphasize that new cytogenetic analysis with silver ions focusing on nucleolar behavior should be performed in Triatominae subfamily, more specifically between 15 genera that do not exhibit the nucleolar behavior described, to assess whether this phenomenon is really a synapomorphy of these hematophagous insects.

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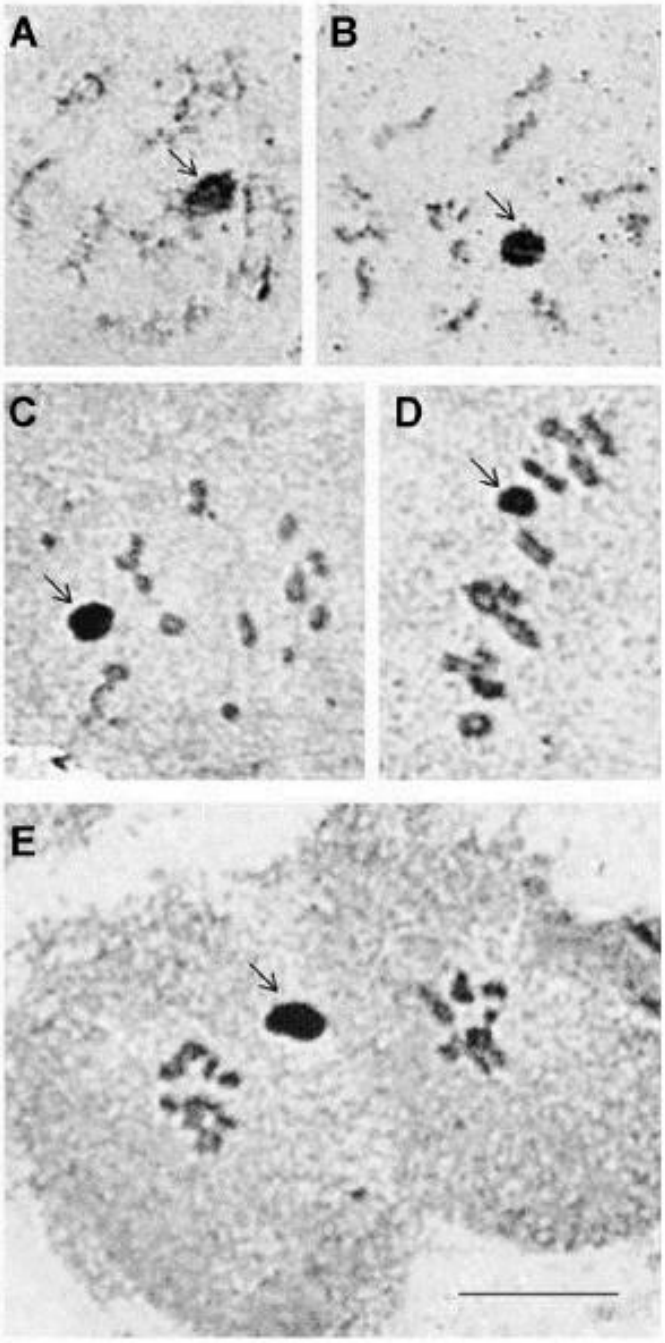

Figure 1: Cytogenetic analysis of spermatogenesis of *Rhodnius montenegrensis*. (A-C) Note the nucleolus during prophase (arrows). (D) Note the phenomenon of nucleolar persistence in metaphase (arrow) and the presence of this nuclear structure in anaphase (Figure 1E, arrow). Bar: 10 mm.

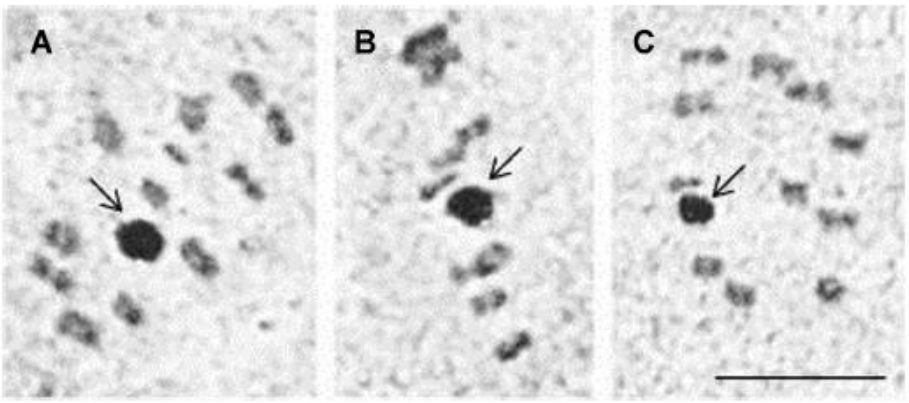

Figure 2: The phenomenon of nucleolar persistence (arrow) in *R. nasustus* (A), *R. robustus* (B) and *R. pictipes* (C). Bar: 10 mm.

Table 1: Species of the genus *Rhodnius* to the phenomenon of nucleolar persistence described.

Species	**Autors**
Rhodnius colombiensis	First description
Rhodnius domesticus	Morielle and Azeredo-Oliveira, 2004
Rhodnius montenegrensis	First description
Rhodnius nasutus	First description
Rhodnius neglectus	First description
Rhodnius neivai	First description
Rhodnius palescens	Morielle-Souza and Azeredo-Oliveira, 2007
Rhodnius pictipes	First description
Rhodnius prolixus	First description
Rhodnius robustus	First description

4.27 Anexo 27 (Artigo científico publicado na revista *The American Journal of Tropical Medicine & Hygiene*)

MADEIRA, F. F.; BORSATTO, K. C.; LIMA, A. C. C.; RAVAZI, A.; OLIVEIRA, J.; ROSA, J. A.; AZEREDO-OLIVEIRA, M. T. V.; ALEVI, K. C. C. Nucleolar persistence: peculiar characteristic of spermatogenesis of the vectors of Chagas disease (Hemiptera, Triatomine). **The American Journal of Tropical Medicine & Hygiene**, v. 95, p. 1118-1120, 2016.

Nucleolar persistence: peculiar characteristic of spermatogenesis of the vectors of Chagas disease (Hemiptera, Triatomine)

Abstract

All the species of triatomines are considered potential vectors of the Chagas disease and the reproductive biology of these bugs has been studied by different approaches. In 1999, nucleolar persistence during meiosis was observed in the subfamily for the first time. Recently, it has been observed that all species within the genus *Rhodnius* exhibit the same phenomenon, suggesting that it may be a synapomorphy of the triatomines. Thus, this paper aims to analyze the nucleolar behavior during spermatogenesis of 59 triatomine species. All analyzed species exhibited nucleolar persistence during meiosis. Recently, it has been suggested that nucleolar persistence may be fundamental for the spermatogenesis of these vectors, since it is related to the formation of the chromatoid body (CB). Therefore, we emphasize that this phenomenon is a peculiarity of the Triatominae subfamily and that further studies are required in order to analyze if the nucleolar material that persists is active.

Short Report

The Triatominae subfamily is composed of 150 species (148 living species and two fossils), grouped into 18 genera and five or six tribes. All triatomine species are considered potential vectors of the *Trypanosoma cruzi*, etiologic agent of the Chagas disease. [1]

The Chagas disease is a neglected disease that has no cure. The main way to minimize the incidence of this disease in human populations is through vector control[1]. It is estimated that more than five million people are infected by *T. cruzi*, the parasite that causes the Chagas disease, and that 70 million still live at risk, which places this illness among the most serious

parasitic disease in the world[1]. Thus, improving the knowledge on several fields on triatominae vector potentiality (such as ecology[2] and biology[3]), may provide important information for control measures.

The reproductive biology of these bugs has been studied by different approaches, such as cytogenetic[4], structural[5], and ultrastructural[6] analysis. Furthermore, the spermatogenesis of the triatomines is characterized as cystic[5,6] and it has been suggested that, during imaginal molt (transition from the fifth instar nymph to adult), the cell division is disrupted, aiming to reduce energy costs, and the differentiation into sperm is stimulated to ensure the paternity of the adult male.[7]

In 1999, Tartarotti and Azeredo-Oliveira[8], while studying the spermatogenesis of *Panstrongylus megistus* and *P. herreri* (= *P. lignarius*), noted that these triatomines exhibited a different nucleolar behavior than the one described for other eukaryotes: the nucleolus persisted during all stages of meiosis. The authors characterized this phenomenon as nucleolar persistence.

Recently, Alevi and colleagues[9] have observed that all species within the genus *Rhodnius* feature nucleolar persistence during meiosis as well, and they suggested that analyses of the nucleolar behavior should be carried out in a large range of species of triatomines to examine if that characteristic is a synapomorphy of the Triatominae subfamily.

Thus, this paper aims to analyze the nucleolar behavior during spermatogenesis of 59 triatomine species in order to determine if nucleolar persistence occurs in all bugs of the Triatominae subfamily.

Were analyzed at least two adult male specimens of each species (*Cavernicola pilosa*, *Psammolestes tertius*, *Rhodnius brethesi*, *R. colombiensis*, *R. domesticus*, *R. ecuadoriensis*, *R. milesi*, *R. montenegrensis*, *R. nasutus*, *R. neglectus*, *R. neivai*, *R. pallescens*, *R. pictipes*, *R. prolixus*, *R. robustus*, *R. stali*, *Dipetalogaster maxima*, *Eratyrus cuspidatus*, *Meccus pallidipennis*, *M. longipennis*, *Mepraia spinolai*, *Nesotriatoma bruneri* (= *N. flavida*), *Panstrongylus lignarius*, *P. megistus*, *Triatoma arthurneivai*, *T. baratai*, *T. brasiliensis*, *T. b. macromelasoma*, *T. carcavalloi*, *T. circummaculata*, *T. costalimai*, *T. delpontei*, *T. dimidiata*, *T. garciabesi*, *T. guasayana*, *T. guazu*, *T. infestans*, *T. juazeirensis*, *T. jurbergi*, *T. klugi*, *T. lectularia*, *T. lenti*, *T. maculata*, *T. matogrossensis*, *T. melanica*, *T. melanocephala*, *T. petrochiae*, *T. platensis*, *T. protracta*, *T. pseudomaculata*, *T. pintodiasi*, *T. rubrovaria*, *T. sherlocki*, *T. sordida*, *T. tibiamaculata*, *T. vandae*, *T. vitticeps*, *T. williami*, *T. wygodzinskyi*) that were provided by the "Insetário de Triatominae", from FCFAR/UNESP, Araraquara, São

Paulo, Brazil, and by the "Insetário do Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos", from FIOCRUZ, Rio de Janeiro, Brazil. The bugs were dissected, the testicles were removed, and slides were mounted through cell squashing and stained through silver impregnation[10].

The analysis of the nucleolar behavior during the meiosis of these bugs has highlighted that all species feature the nucleolar persistence phenomenon. Therefore, as the observed results were the same for all triatomines, we represent the nucleologenesis (Figure 1A-D) and the nucleolar persistence phenomenon (Figure 1C, D, arrow) with *Dipetalogaster maxima*. Our results are in agreement with the initial works carried out for the *Triatoma* [11, 12, 13, 14, 15, 16, 17], *Meccus*[18], *Rhodnius*[19, 9] and *Panstrongylus*[9] genera, which likewise have exhibited this phenomenon during spermatogenesis of these vectors.

Recently, it has been suggested that the nucleolar persistence can be essential for the spermiogenesis of these vectors, since during this differentiation stage, the nucleolus – although present – is inactive by epigenetic factors, and the chromatoid body (CB) is an essential organelle to supply all the transcriptional activity required for spermiogenesis[20]. Thus, it has been suggested that the nucleolus that persists during meiosis can be a structure responsible for forming the CB, because if it is active, all the transcribed RNA is directed to the formation of the CB[17].

Therefore, we describe the nucleolar persistence as a synapomorphy of the triatomines and suggest that further analysis should be conducted in order to assess whether the persistent nucleolus is really active. Thus clarifying the importance of the persistence of the nucleolus for spermatogenesis of these vectors of the Chagas disease.

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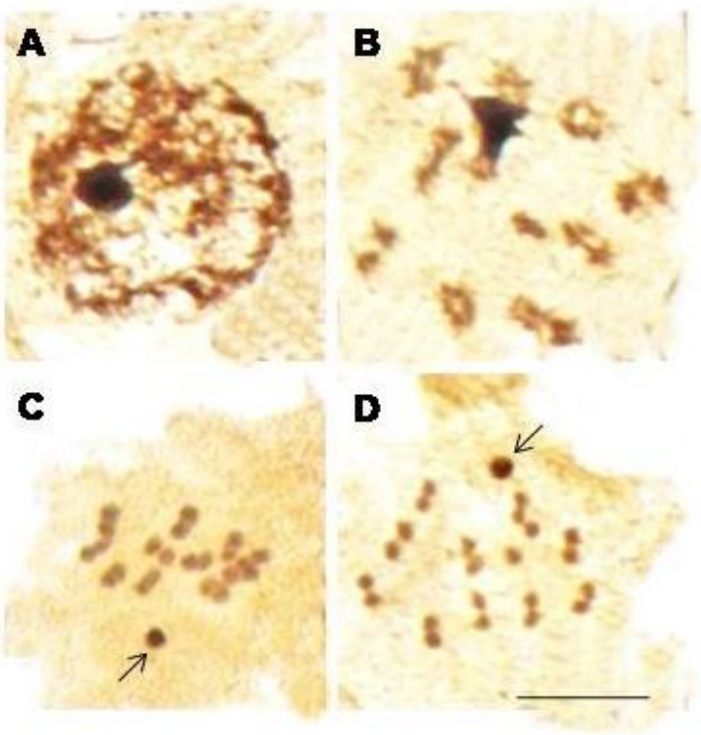

Figure 1: Nucleologenesis of *Dipetalogaster maxima*. Note the nucleolar persistence phenomenon (arrow). Bar: 10 μm.

4.28 Anexo 28 (Artigo científico publicado na revista *Biota Neotropica* FI 0,73)

Alevi, K.C.C.; Oliveira, J.; Rosa, J.A.; Azeredo-Oliveira, M.T.V.. Coloration of the testicular peritoneal sheath as a synapomorphy of triatomines (Hemiptera, Reduviidae). **Biota Neotropica**, v. 14, p. 1-3, 2014.

Coloration of the testicular peritoneal sheath as a synapomorphy of triatomines (Hemiptera, Reduviidae)

Abstract

Recently, were described some morphological characteristics of 18 species of terrestrial hemipteran grouped in the Alydidae, Coreidae, Corimelaenidae, Lygaeidae, Rhopalidae, Scutelleridae, Tingidae and Reduviidae family that presented found variation in coloration of testicular peritoneal sheath (Reddish, Orange, Yellowish or Transparent). Thus, this study aims to analyzed the coloration of the peritoneal sheath in 44 species of triatomines grouped in nine different genera, with the aim of analyze whether the insects of the Triatominae subfamily also show variations in coloration. By means of analysis of the sheath was possible to observe that members of this subfamily have no interspecific differences, because all species have a transparent sheath. Thus, this paper describes the coloring of the peritoneal sheath in 44 species of the subfamily Triatominae and mainly suggests that the transparent color is one synapomorphy of this important group of insect vectors.

Introduction

Triatomines are hematophagous insects of medico-sanitary importance because they are considered as the main vector of Chagas disease in the human population. These vectors are included in the Hemiptera order, Heteroptera suborder, Reduviidae family and Triatominae subfamily (Lent & Wygodzinsky 1979).

The knowledge of the biology of these vectors is of great importance to public health, because the main way to minimize the incidence of Chagas disease is by controlling populations (Dias & Schofield 1998, Alevi et al. 2012a). The reproductive biology of hemipterans was extensively studied by many aspects, such as anatomical (Barth 1956, Freitas et al. 2008), morphological (Gomes et al. 2013, Rosa et al. 2014), structural (Freitas et al. 2010, Silistino-Souza et al. 2012), ultrastructural (Morielle-Souza et al. 2010, Freitas et al.

2010, Silistino-Souza et al. 2012) and cytogenetic (Alevi et al. 2012a, b, 2013a, b, c, d, 2014a, b).

Recently, Gomes et al. (2013) described some morphological traits of 18 species of terrestrial hemipteran grouped in the Alydidae, Coreidae, Corimelaenidae, Lygaeidae, Rhopalidae, Scutelleridae, Tingidae and Reduviidae family. Among the characteristics, the color of the peritoneal sheath covering the seminiferous tubules was analyzed. The authors found variation in coloration of sheath (Reddish, Orange, Yellowish or Transparent). Thus, this study aims to analyze the peritoneal sheath in 44 species of triatomines grouped in nine different genera (Table 1), with the aim of analyze whether the insects of the Triatominae subfamily also show variations in coloration.

Material and Methods

Exemplars males of 44 species (Table 1) were provided by "Triatominae Insectarium" within the Department of Biological Sciences, in the College of Pharmaceutical Sciences, at Sao Paulo State University's Araraquara campus, Brazil (FCFAR/UNESP). The testicles were removed and photographed according to the methodology of Gomes et al. (2013).

Results and Discussion

The analysis of the peritoneal sheath of 44 species possible to observe that members of Triatominae subfamily have no interspecific differences, as described by Gomes et al (2013) for phytophagous hemipterans, because all species have a transparent sheath. Like all species of nine genera analyzed show the same coloring, we represent with image the testicle of one species of each genus (Figures 1 and 2).

The insects of the subfamily Triatominae share synapomorphies related to reproductive biology, such as the presence of chromosomes holocentric (Panzera et al. 1996), inverted meiosis for sex chromosomes (Gómez-Palacio et al. 2008), the phenomenon of nucleolar persistence during meiosis (Tartarotti & Azeredo-Oliveira 1999, Alevi et al. 2014c), nucleolar inactivation during spermiogenesis (Alevi et al. 2014b), presence seven testicular follicles (Schreiber et al. 1968) and testicular peritoneal sheath transparent (this paper), demonstrating that during the evolution and speciation of the triatomines, the reproductive aspects undergone few modifications.

Thus, this paper describes the coloring of the peritoneal sheath in 43 species of the subfamily Triatominae and mainly suggests that the transparent color is one synapomorphy of this important group of insect vectors.

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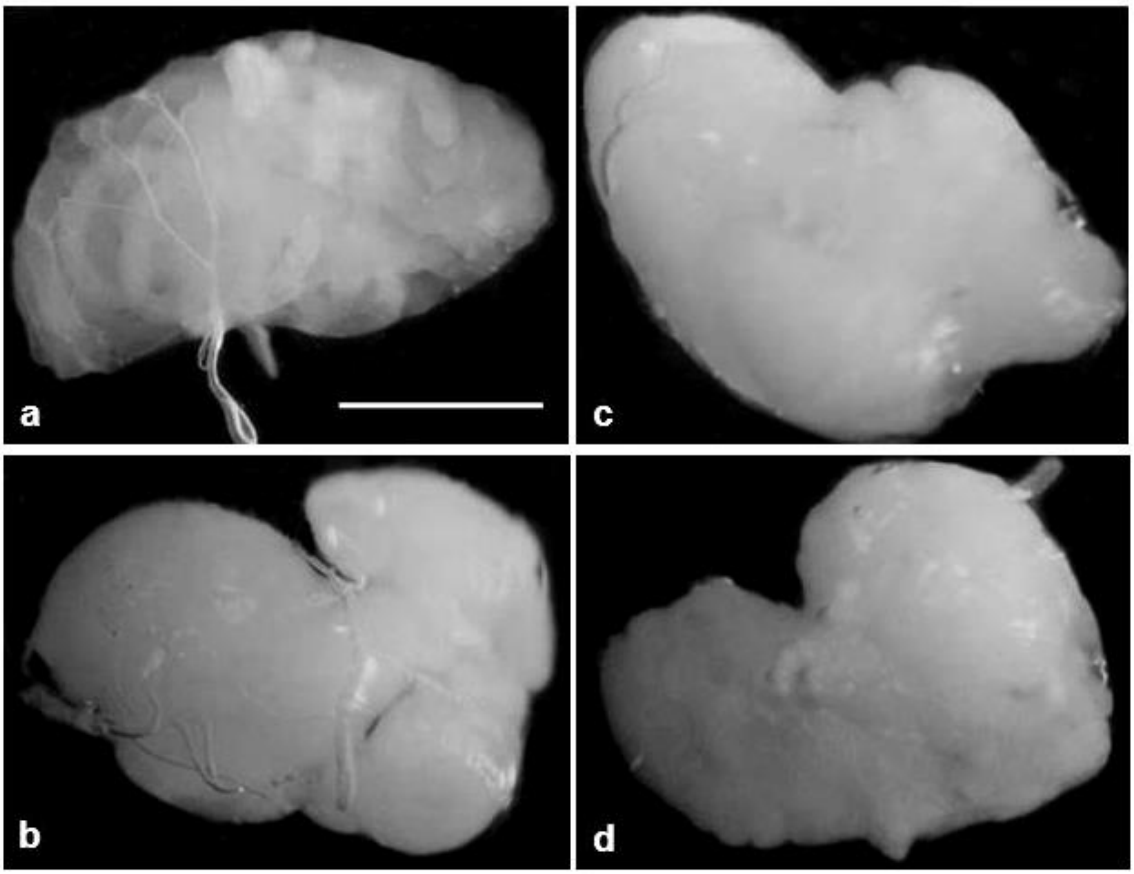

Figure 1. Peritoneal sheath of different genus of the subfamily Triatominae analyzed. Note that in all genus the sheath is transparent. (a) *T. infestans*. (b) *M. pallidipennis*. (c) *P. megistus*. (d) *D. maximus*. Bar: 10 mm.

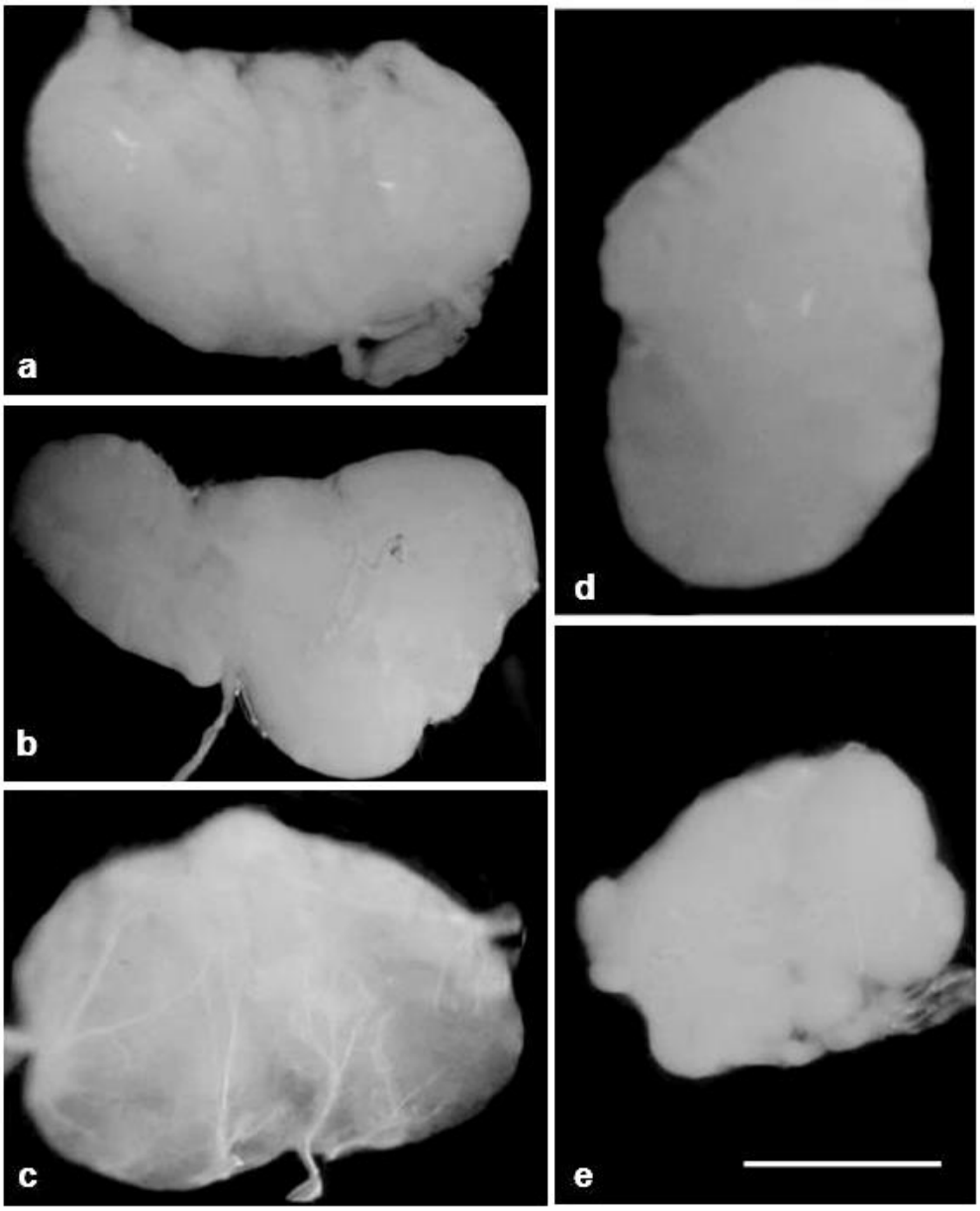

Figure 2. Peritoneal sheath of different genus of the subfamily Triatominae analyzed. Note that in all genus the sheath is transparent. (a) *M. spinolai*. (b) *E. cuspidatus*. (c) *P. tertius*. (d) *R. montenegrensis*. (e) *C. pilosa*. Bar: 10 mm.

Table 1. Genus and species of Triatominae who had the peritoneal sheath analyzed.

Genus	Species
Cavernicola	*C. pilosa*
Psammolestes	*P. tertius*
Rhodnius	*R. brethesi*, *R. colombiensis*, *R. domesticus*, *R. montenegrensis*, *R. nasutus*, *R. neglectus*, *R. neivai*, *R. palescens*, *R. pictipes*, *R. prolixus*, *R. robustus*
Dipetalogaster	*D. maximus*
Eratyrus	*E. cuspidatus*
Meccus	*M. pallidipennis*, *M. longipennis*
Mepraia	*M. spinolai*
Panstrongylus	*P. lignarius* (= *P. herreri*), *P. megistus*
Triatoma	*T. baratai*, *T. brasiliensis*, *T. b. macromelasoma*, *T. carcavalloi*, *T. costalimai*, *T. infestans*, *T. guazu*, *T. juazeirensis*, *T. klugi*, *T. lectularia*, *T. lenti*, *T. maculata*, *T. melanica*, *T. melanocephala*, *T. platensis*, *T. protracta*, *T. pseudomaculata*, *T. rubrovaria*, *T. rubrofasciata*, *T. sordida*, *T. tibiamaculata*, *T. vandae*, *T. vitticeps*, *T. williami*

4.29 Anexo 29 (Artigo científico publicado na revista *The American Journal of Tropical Medicine & Hygiene* FI 2,54)

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Study of the salivary glands in Triatominae (Hemiptera, Reduviidae, Triatominae): their color and application to the Chagas disease vector evolution

Abstract

Chagas disease is caused by *Trypanosoma cruzi* and transmitted by feces of a triatomine that has the habit of defecating during blood feeding. The salivary glands of triatomines are important to hematophagy because their saliva is rich in anticoagulant and hemolytic proteins. The salivary glands of some *Rhodnius* species analyzed are reddish due to the presence of nitrophorins (antihemostatic activity). The present study aimed to analyze the color pattern of the salivary glands of 67 triatomine species in order to evaluate whether the presence of nitrophorins is a synapomorphy of *Rhodnius* or the tribe Rhodniini, or if it is shared with triatomines of the tribes Triatomini and Cavernicolini. Since only the species of the tribe Rhoniini present red glands, it is admitted that the presence of nitrophorin proteins is a synapomorphy of the tribe Rhodniini and that this tribe has derived more recently when compared with Triatomini and Cavernicolini.

Short Report

Chagas disease is a vector-borne and potentially life-threatening illness caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909). It occurs mainly in endemic areas in 21 Latin American countries, where it is transmitted to humans mostly by contact with feces of triatomines, known as 'kissing bugs'. It is estimated that about 6 to 7 million people are infected worldwide, mostly in Latin America.[1]

Currently, there are 151 species of triatomines distributed in 18 genera and five tribes, all species being considered potential vectors of Chagas disease.[2,3] As Chagas disease has no

cure and treatment with benznidazole and nifurtimox is effective only in the acute phase of the disease (which is often asymptomatic), vector control is the most effective method of preventing this neglected disease.[1] Thus, all knowledge about these hematophagous insects is important and can help and improve vector control programs.

Although the transmission of *T. cruzi* to the host occurs mostly through the feces of triatomines, hematophagous behavior is fundamental to the contamination with the protozoan, as such insects have the habit of defecating during blood feeding.[2] It is believed that hematophagy was derived from ancestral generalist predators that initiated this behavior as opportunistic hematophagy, then it became facultative and finally evolved to mandatory hematophagy.[4]

The salivary glands of triatomines perform a fundamental role during hematophagy because their saliva is rich in proteins and anticoagulant and hemolytic enzymes.[5,6] These structures have been studied anatomically[7], histologically[7], biochemically[5], molecularly[8] and cytogenetically[9] based on a few triatomines of the genera *Triatoma*, *Rhodnius* and *Panstrongylus*.

Based on the analysis of *R. prolixus*, it has been suggested that the salivary glands (principal glands) of the species of the genus *Rhodnius* are reddish.[10] This characteristic results from the presence of nitrophorins[10], which are proteins with antihemostatic activity[11] whose molecules contain a heme group responsible for that color. The relationship between red salivary glands and the presence of nitrophorins has recently been confirmed by Pacheco[12] which inoculated nitrophorin inhibitors into 80 Rhodnius eggs and the adult salivary glands were transparent.

Considering that the presence of nitrophorins is suggested for all species of the genus *Rhodnius*,[13] and only *R. prolixus*,[13] *R. robustus*,[13] and *R. domesticus*[14] have been studied, the present study aimed to analyze the color pattern of the salivary glands of 67 triatomine species, distributed in ten different genera and grouped into three tribes, in order to evaluate whether the presence of nitrophorins is a synapomorphy of *Rhodnius* or the tribe Rhodniini, or if it is shared with triatomines of the tribes Triatomini and Cavernicolini.

At least two adult specimens of each species (males and/or females) were analyzed (tribe Cavernicolini: *Cavernicola pilosa*; tribe Rhodniini: *Psammolestes tertius*, *P. coreodes*, *P. arthuri*, *R. brethesi*, *R. colombiensis*, *R. domesticus*, *R. ecuadoriensis*, *R. marabaensis*, *R. milesi*, *R. montenegrensis*, *R. nasutus*, *R. neglectus*, *R. neivai*, *R. pallescens*, *R. pictipes*, *R. prolixus*, *R. robustus*, *R. stali*, tribe Triatomini: *Dipetalogaster maxima*, *Eratyrus cuspidatus*,

Meccus pallidipennis, *M. longipennis*, *M. picturata*, *M. phylossoma*, *Mepraia spinolai*, *Nesotriatoma bruneri* sn *N. flavida*, *P. herreri* sn *P. lignarius*, *P. lignarius*, *P. megistus*, *P. lutzi*, *T. arthurneivai*, *T. bahiensis*, *T. baratai*, *T. brasiliensis*, *T. b. macromelasoma*, *T. carcavalloi*, *T. circummaculata*, *T. costalimai*, *T. delpontei*, *T. dimidiata*, *T. garciabesi*, *T. guasayana*, *T. guazu*, *T. infestans*, *T. juazeirensis*, *T. jurbergi*, *T. klugi*, *T. lectularia*, *T. lenti*, *T. maculata*, *T. matogrossensis*, *T. melanica*, *T. melanocephala*, *T. petrocchiae*, *T. platensis*, *T. protracta*, *T. pseudomaculata*, *T. pintodiasi*, *T. rubrovaria*, *T. sherlocki*, *T. sordida*, *T. tibiamaculata*, *T. vandae*, *T. vitticeps*, *T. williami*, *T. wygodzinskyi*). The specimens were provided by the Triatominae Insectarium of FCFAr/Unesp, Araraquara, São Paulo, Brazil, and the Insectarium of the National and International Triatominae Taxonomy Reference Laboratory at Fiocruz, Rio de Janeiro, Brazil. The bugs were dissected and the salivary glands were then removed and examined by stereoscope microscope.

Through the analysis of the salivary glands, it was observed that the species of the tribe Rhodniini present red glands [represented by *P. tertius* (Figure 1A) and *R. montenegrensis* (Figure 1B)]. On the other hand, all the other species analyzed exhibited transparent glands [represented by *T. infestans* (Figure 1C)].

It is estimated that the tribes Triatomini and Rhodniini diverged at 48.9-64.4 mya, when South America was already separated from Africa.[15] Recently, it was suggested that the uplift of the Andes in South America and the variations in sea levels in North America are the events involved in the diversification of these tribes.[16] Ntrophorin heme proteins could have appeared (and later they were positively selected) after the divergence of the tribes, more specifically in the common ancestor of the tribe Rhodniini.

Although there is no dating of the divergence of the tribe Cavernicolini, recently this tribe was presented as a brother group of Rhodniini.[16] However, the absence of nitrophorins in the salivary glands of *C. pilosa* suggest that this tribe derived before Rhodniini. A possible conclusion is that the tribe Triatomini derived first, followed by Cavernicolini and finally came the tribe Rhodniini. This highlights the need for studies using molecular clocks in Triatominae with representatives of all the tribes.

One of the antihemostatic activities of the nitrophorins is the storage and transport of nitric acid ligated into the center of ferric heme[6], which promotes vasodilation and inhibition of platelet aggregation when it is released in the microcirculation.[17] This study demonstrates that the species of the tribes Triatomini and Cavernicolini do not have this heme protein in the composition of their salivary glands. In the few studies that characterize the salivary glands of

other genera of the tribe Triatomini, the substances isolated were: triabin and pallidipin in *M. pallidipennis*[18], triafestins, triplatin and trialysin in *T. infestans*[19,20], procalin in *T. protracta*[20], dipetalodipin in *D. maxima*[20], and lipocalin in *T. lectularia*[8] and *P. herreri*.[8]

Therefore, this study highlights the presence of nitrophorin proteins as a synapomorphy of the tribe Rhodniini and suggests that this tribe has derived more recently when compared with the tribes Triatomini and Cavernicolini, which contributes to understanding the evolutionary history of this important vector group.

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Figure 1. Salivary glands of *P. tertius* (A), *R. montenegrensis* (B) and *T. infestans* (C). Note the red glands in *P. tertius* (A) and *R. montenegrensis* (B) (tribe Rhodniini).

Discussão

5. DISCUSSÃO GERAL

5.1 Subcomplexo Brasiliensis

O subcomplexo Brasiliensis [= complexo *T. brasiliensis*, segundo OLIVEIRA et al., 2017)], composto, atualmente, pelas espécies *T. brasiliensis*, *T. b. macromelasoma*, *T. melanica*, *T. juazeirensis*, *T. sherlocki*, *T. lenti* e *T. petrocchiae* (MENDONÇA et al., 2014; OLIVEIRA et al., 2017), representa o principal grupo de espécies vetoras da região nordeste (COSTA et al., 2003a, 2014), sendo *T. brasiliensis* uma das principais espécies vetoras do Brasil (GALVÃO et al., 2014; ALMEIDA et al., 2016; LILOSO et al., 2017).

A citotaxonomia desse subcomplexo foi iniciada, em 2000, com análises cromossômicas de diferentes populações de *T. brasiliensis* (PANZERA et al., 2000). Todas as espécies do subcomplexo Brasiliensis apresentam características citogenéticas peculiares que podem ser utilizadas como diagnósticas para o agrupamento/exclusão de espécies nesse subcomplexo, pois permitem diferenciá-las de todas as outras espécies de triatomíneos, a saber, presença de um cromocentro profásico formado pelos cromossomos sexuais mais um par de autossomos, presença de blocos heterocromáticos/heteropicnóticos dispersos na cromatina dos núcleos profásicas, assim como presença de heterocromatina constitutiva em uma ou ambas as extremidades dos autossomos (PANZERA et al., 2000; ALEVI et al., 2013b; MENDONÇA et al., 2016).

As análises citogenéticas de *T. melanica*, por exemplo, possibilitaram confirmar o posicionamento da espécie no subcomplexo Brasiliensis (ALEVI; ROSA; AZEREDO-OLIVEIRA, 2014d), uma vez que essa espécie é considerada como uma das formas evolutivas mais diferenciada dentro do subcomplexo, pois apresenta incompatibilidade genética com os outros membros do subcomplexo (COSTA et al., 2003; COSTA; ARGOLO; FELIX, 2006). Além disso, sugerimos que, possivelmente, o tamanho relativamente grande dos gametas masculinos de *T. melanica* possa ser uma das barreiras de isolamento reprodutivo (isolamento gamético) (ALEVI; ROSA; AZEREDO-OLIVEIRA, 2014d) observadas para cruzamentos experimentais entre *T. melanica* e os outros membros do subcomplexo Brasiliensis (COSTA et al., 2003).

Lucena (1970) e Schofield; Galvão (2009), com base em dados morfológicos e na distribuição geográfica, agruparam *T. lenti* no subcomplexo Brasiliensis. Essa espécie de triatomíneo apresenta características morfológicas que a aproxima de *T. sherlocki* (PAPA et al., 2002). Além das características morfológicas, ambas as espécies apresentam

características citogenéticas idênticas (ALEVI et al., 2012b, 2013b; ALEVI; ROSA; AZEREDO-OLIVEIRA, 2014b) e produzem híbridos férteis em primeira geração (F1) (MENDONÇA et al., 2014). Contudo, análises citogenéticas durante a espermatogênese dos híbridos em segunda geração (F2) permitiram observar erros no pareamento entre os cromossomos homeólogos (que resultaram em monovalentes), o que leva a formação de gametas inviáveis (fenômeno conhecido como desmoronamento do híbrido) (MENDONÇA et al., 2014). Esse fenômeno de isolamento pós-zigótico é, possivelmente, devido à desregulação gênica que ocorreu durante a recombinação dos cromossomos homeólogos nos híbridos e caracteriza diminuição da viabilidade/inviabilidade híbrida a partir da F2, como observado para os híbridos do cruzamento entre *T. lenti* e *T sherlocki*.

O desmoronamento do híbrido, caracterizado pela primeira vez em Triatominae, ressalta o *status* específico de *T. lenti* e *T. sherlocki* (MENDONÇA et al., 2014). No entanto, deve-se ressaltar que a compatibilidade genética inicial observada pelo grau de pareamento em F1 só é possível porque essas espécies são evolutivamente muito próximas. Esses dados, em conjunto com as análises citogenéticas clássicas (ALEVI et al., 2012b, 2013b; ALEVI; ROSA; AZEREDO-OLIVEIRA, 2014b), sugerem que *T. lenti* deve ser considerado como o sexto membro do subcomplexo Brasiliensis. Mendonça et al. (2016) corrobora a inclusão da espécie no subcomplexo Brasiliensis por meio de reconstrução filogenética com gene mitocondrial (*cyt b*) (MENDONÇA et al., 2016).

Triatomíneos semelhantes à *T. lenti* foram capturados na Bahia. Análises morfológicas iniciais levaram a caracterização desses insetos como *T. bahiensis*. No entanto, desde 1979 essa espécie havia sido sinonimizada com *T. lenti* (LENT; WYGOFZINSKY, 1979). Com base em análises morfológicas, morfométricas e de cruzamento experimental foi possível revalidar *T. bahiensis* (MENDONÇA et al., 2016; ALEVI et al., 2017a). Além disso, análises moleculares e citogenéticas permitiram destacar que essa espécie deve ser considerada como o sétimo membro do subcomplexo Brasiliensis (MENDONÇA et al., 2016).

Lucena (1970) e Schofield; Galvão (2009) também agruparam *T. petrocchiae* como membro do subcomplexo Brasiliensis. Por muito tempo, essa espécie foi considerada como sinônima de *T. brasiliensis* (LUCENA, 1970). O *status* específico de *T. petrocchiae* foi confirmado por meio de cruzamentos experimentais que não resultaram em híbridos F1 (isolamento reprodutivo pré-zigótico) (ESPÍNOLA, 1971). Recentes estudos morfométricos (OLIVEIRA et al., 2017), filogenéticos (OLIVEIRA et al., 2017) e citogenômicos confirmaram a posição de *T. petrocchiae* como oitavo membro desse subcomplexo.

Além disso, com o intuito de auxiliar no conhecimento da biologia reprodutiva de *T. brasiliensis*, que é a principal espécie vetora da região Nordeste (COSTA et al., 2003a), a espermatogênese desse vetor foi analisada durante a muda imaginal (passagem de ninfa de quinto instar para adulto) e pôde-se observar que esse vetor interrompe a divisão celular meiótica (possivelmente para reduzir os gastos energéticos) e induz a diferenciação celular (possivelmente para chegar à fase adulta com muitos espermatozóides e totalmente apto à reprodução) durante essa fase da metamorfose.

5.2 Subcomplexos Matogrossensis e Rubrovaria

Todas as espécies do subcomplexo Matogrossensis (ALEVI et al., 2015e) e Rubrovaria (ALEVI et al., 2015a) apresentam o mesmo número de cromossomos, a saber, 2n = 22 (20A + XY). Além disso, todas as espécies também apresentam o mesmo padrão heterocromático: presença de heterocromatina constitutiva restrita apenas ao cromossomo sexual Y (ALEVI et al., 2015b). Embora essas características não permitam diferenciar as espécies agrupadas no subcomplexo Matogrossensis do subcomplexo Rubrovaria, são importantes para diferenciar esses subcomplexos dos outros subcomplexos de triatomíneos presentes na América do Sul (Brasiliensis, Maculata, Sordida e Infestans) (ALEVI et al., 2015b), destacando-as como importante ferramenta citotaxonômica.

T. pintodiasi foi descrita como espécie críptica de *T. circummaculata* e morfologicamente relacionada com *T. carcavalloi* (JURBERG et al., 2013). Embora as espécies apresentem o mesmo número de cromossomos e o mesmo padrão de heterocromatina [assim como todas as espécies do subcomplexo Rubrovaria (ALEVI et al., 2015b)], esses insetos puderam ser diferenciados pelo comportamento meiótico, pois em *T. pintodiasi* é possível observar um cromocentro formado pelos dois cromossomos sexuais individualizados nas prófases iniciais e em *T. circummaculata* e *T. carcavalloi*, esse cromocentro forma um único corpúsculo heteropicnótico (ALEVI et al., 2015a), assim como pela alta distância genética observada entre *T. pintodiasi* e as espécies do subcomplexo Rubrovaria para o gene mitocondrial 16S (ALEVI et al., 2016c), ressaltando, dessa forma, o *status* específico de *T. pintodiasi*.

Embora a citotaxonomia clássica não permitiu diferenciar os membros dos subcomplexos Matogrossensis e Rubrovaria, a citotaxonomia molecular, com base na disposição do DNAr 45S, sugere que o subcomplexo Matogrossensis seja eliminado e as espécies inicialmente agrupadas nesse subcomplexo sejam reagrupadas nos subcomplexos

Sordida (RONs localizada em um ou ambos os cromossomos sexuais) e Pseudomaculata (RONs localizadas em um par de autossomos) (novo subcomplexo sugerido) (PITA et al., 2016). Além disso, os autores sugerem que *T. guasayana* e *T. patagonica*, inicialmente agrupadas no subcomplexo Sordida (SCHOFIELD; GALVÃO, 2009), façam parte do subcomplexo Rubrovaria (RONs localizadas em um par de autossomos).

5.3 Subcomplexo Maculata

O subcomplexo Maculata é composto pelas espécies *T. maculata*, *T. pseudomaculata*, *T. arthurneivai* e *T. wygodzinskyi* (SCHOFIELD; GALVÃO, 2009). *T. maculata*, *T. pseudomaculata* e *T. arthurneivai* foram analisadas citogeneticamente e, embora apresentassem o mesmo número de cromossomos, puderam ser diferenciadas pela análise das células profásicas e pela disposição do padrão de heterocromatina constitutiva (SANTOS et al., 2007). No entanto, Carbajal-de-La-Fuente et al. (2010), por meio de análises morfométricas, sugerem que os exemplares de *T. arthurneivai* analisados por Santos et al. (2007), eram *T. wygodzinskyi*.

Por meio de análises citogenéticas de prófases iniciais de *T. arthurneivai* e *T. wygodzinskyi*, foi possível caracterizar as duas espécies de triatomíneos e diferenciá-las de *T. maculata* e *T. pseudomaculata*. Além disso, foi possível confirmar o sugerido por Carbajal-de-La-Fuente et al. (2010), uma vez que o padrão descrito por Santos et al. (2007) (presença de apenas um corpúsculo heteropicnótico nas células profásicas) está presente em *T. wygodzinskyi* e não em *T. arthurneivai*.

Durante a evolução cromossômica desses vetores, ocorreram eventos de ganho e/ou perda de heterocromatina, o que permite caracterizar citotaxonomicamente as quatro espécies do subcomplexo Maculata: *T. maculata* apresenta um cromocentro heterocromatico formado pelos cromossomos sexuais e pequenos blocos de heterocromatina dispersos no núcleo das células profásicas [equivalentes a pequenos blocos heterocromáticos nas extremidades de todos os autossomos, assim como inicialmente descrito por Santos et al. (2007)], *T. pseudomaculata* apesenta um cromocentro heterocromático formado pelos cromossomos sexuais mais um par de autossomos e alguns blocos de heterocromatina dispersos no núcleo (equivalentes aos cinco cromossomos heterocromáticos descritos para a espécie por Imperador et al. (2016)], *T. wygodzinskyi* apresenta apenas um cromocentro heterocromático formado pelos cromossomos sexuais (equivalente a autossomos eucromáticos) e *T. arthurneivai* apresenta o cromocentro heterocromático formado pelos cromossomos sexuais e

grandes blocos de heterocromatina dispersos no núcleo (demonstrando que essa espécie apresenta muita heterocromatina nos autossomos) (IMPERADOR et al., 2017).

Pita et al. (2016), com base na disposição do DNAr 45S, sugerem que o subcomplexo Maculata deva ser formado apenas por *T. maculata* (que apresenta marcação nos cromossomos sexuais X e Y), sendo as três outras espécies inicialmente agrupadas nesse subcomplexo transferidas para o subcomplexo Pseudomaculata, pois apresentam marcação em um par de autossomos.

5.4 Subcomplexo Sordida

O subcomplexo Sordida, proposto por meio de dados morfológicos e disposição geográfica, agrupou, inicialmente, as espécies *T. garciabesi, T. guasayana, T. patagonica* e *T. sordida* (SCHOFIELD; GALVÃO, 2009). No entanto, um extenso estudo de Panzera et al. (2015) demonstra que as espécies desse subcomplexo são crípticas e reclassifica, com base em dados cromossômicos e moleculares, *T. garciabesi* e *T. sordida* em *T. garciabesi, T. sordida sensu stricto, T. sordida Argentina* e *T. sordida La Paz*. Mais recentemente, Pita et al. (2016), com base em análises citogenômicas envolvendo a disposição do DNAr 45S, propõem que o subcomplexo Sordida deve ser formado pelas espécies *T. garciabesi*, *T. sordida sensu strictu, T. sordida* Argentina, *T. jurbergi, T. matogrossensis* e *T. vandae*.

Noireau et al. (1998) propuseram que *T. sordida* da Bolívia estão sofrendo especiação críptica. Dados cromossômicos diferenciam *T. sordida* da Bolívia em dois citótipos: *T. sordida sensu stricto* e *T. sordida La Paz* (PANZERA et al., 2015). Análises citogenéticas e citogenômicas em populações brasileiras, representando diferentes biomas, demonstraram que todos os vetores caracterizados inicialmente como *T. sordida* são *T. sordida sensu stricto* e não estão sofrendo especiação críptica. Além disso, pela ausência de variabilidade cromossômica (que representa baixa variabilidade genética) foi possível corroborar que os *T. sordida* brasileiros tiveram origem de uma pequena população (possivelmente no Cerrado brasileiro) e sugerir que, possivelmente, se dispersaram por efeito fundador, assim como recentemente demonstrado para *T. infestans* por dados cromossômicos (PEREIRA et al., 2016).

5.5 Subcomplexo Rubrofasciata

O subcomplexo Rubrofasciata é composto pelas espécies *T. amicitiae, T. bouvieri, T. cavernicola, T. leopoldi, T. migrans, T. pugasi, T. rubrofasciata, T. sinica* e está presente na

América do Norte e no Novo Mundo (SCHOFIELD; GALVÃO, 2009), exceto *T. rubrofasciata* que ocupa 45 países distribuídos no continente Americano, Africano, Asiático e na Oceania (DIOTAIUT; PEREIRA; ESPÍNOLA, 2000; GALVÃO et al. 2003). Alevi et al. (2016a) sugerem que essa grande capacidade de habitar diferentes países possa estar relacionada com a alta atividade metabólica das células císticas de *T. rubrofasciata*.

A única espécie do subcomplexo Rubrovaria que apresenta o número de cromossomos descrito é *T. rubrofasciata*. Essa espécie pode ser caracterizada pelo cariótipo, uma vez que das 88 espécies com o cariótipo descrito, *T. rubrofasciata* é a única que apresenta 2n = 25 (22A + X_1X_2Y) (ALEVI et al., 2015f), destacando, dessa forma, as análises citogenéticas de extrema importância para a taxonomia dessa espécie. Além das análises cariotípicas, *T. rubrofasciata* também pode ser caracterizado pela análise das prófases iniciais, o que garante a correta identificação dessa espécie de importância global (ALEVI et al., 2016b).

Acredita-se que as espécies do gênero *Linshcosteus*, presente na Índia (GALVÃO et al. 2003), foram originadas a partir de *T. rubrofasciata* (HYPSA et al. 2002). Destacamos que as análises citogenéticas nas seis espécies de *Linshcosteus* poderiam elucidar a relação evolutiva entre essas espécies, uma vez que *T. rubrofasciata* apresenta um número de cromossomos bastante peculiar em Triatominae.

5.6 Tribo Rhodniini

Com a recente descrição de *R. taquarussuensis*, a tribo Rhodniini passou a ser composta por 24 espécies, sendo 21 do gênero *Rhodnius* e três do gênero *Psammolestes* (ALEVI et al., 2015a; SOUZA et al., 2016; ROSA et al., 2017) e a subfamília Triatominae por 152 espécies (OLIVEIRA; ALEVI, 2017). Todas as espécies dessa tribo apresentam 22 cromossomos (20A + XY) (ALEVI et al., 2015g; ROSA et al., 2017) (demonstrando que não sofreram eventos de agmatoploidia (fissão) e/ou simploidia (fusão) durante a evolução cromossômica da tribo) e DNAr 45S restrito aos cromossomos sexuais (X, Y ou X e Y) (PITA et al., 2013). Além disso, as espécies da tribo Rhodniini apresentam espermatogênese cística (ALEVI et al., 2015h) e persistência nucleolar durante a meiose (ALEVI et al., 2014a).

As espécies do gênero *Rhodnius* são muito semelhantes do ponto de vista morfológico (MONTEIRO et al., 2000). Recentemente, o *status* específico de várias espécies foi questionado (ABAD-FRANCH et al., 2013). Diante disso e, principalmente, da contribuição da espermiotaxonomia no estudo do gênero *Triatoma* (ALEVI et al., 2013c, d), espermátides de espécies do gênero *Rhodnius* foram analisadas. Muito embora não tenha sido possível

diferenciar as espécies do gênero *Rhodnius*, a espermiotaxonomia permitiu corroborar a monofilia da tribo Rhodniini, uma vez que *Psammolestes* também apresentou as mesmas características nas espermátides que as espécies do gênero *Rhodnius*.

R. montenegrensis foi uma das espécies que teve seu *status* específico questionado (ABAD-FRANCH et al., 2013). Os autores sugerem que *R. montenegrensis* provavelmente não seja uma espécie nova, assim como descrita por Rosa et al. (2012), mas sim apenas represente uma das linhagens de *R. robustus* já descrita por Monteiro et al. (2003). No entanto, por meio da análise da localização das RONs nos cromossomos desses vetores foi possível diferenciar ambas as espécies, pois *R. montenegrensis* apresentou marcação nos cromossomos sexuais X e Y e *R. robustus* apenas no X, corroborando, dessa forma, o *status* específico de *R. montenegrensis*. Recentes estudos transcriptômicos também observaram grandes divergências entre *R. montenegrensis* e *R. robustus* (CARVALHO et al., 2017). Os resultados de transcriptoma e a hipótese de que a transferência do DNAr 45S entre os cromossomos sexuais dessa espécie tenha ocorrido por meio de elementos de transposição suportam o *status* específico de *R. montenegrensis* e sugerem que essas espécies divergiram há muito tempo.

Devido à relação morfológica das espécies do gênero *Rhodnius*, esses vetores foram agrupados em grupos ou complexos monofiléticos (MONTEIRO et al., 2003). O grupo pallescens é formado pelas espécies *R. pallescens*, *R. colombiensis* e *R. ecuadoriensis*. Acredita-se que a divergência entre *R. colombiensis* e *R. pallescens* esteja associada com o Istmo do Panamá (DÍAZ et al., 2014) e entre *R. ecuadoriensis* e *R. pallescens* esteja associada com a cordilheira dos Andes (ABAD-FRANCH e MONTEIRO, 2007). As análises citogenéticas desses vetores possibilitaram observar que a divergência dessas espécies está associada com perda de heterocromatina constitutiva, uma vez que *R. pallescens* apresenta heterocromatina nas extremidades de todos os autossomos, *R. colombiensis* apresenta heterocromatina nas extremidades de cinco pares os autossomos e *R. ecuadoriensis* não apresenta heterocromatina nos autossomos (ALEVI et al., 2015i). De acordo com Panzera et al. (2004), a perda de heterocromatina constitutiva é uma alteração genômica adaptativa que pode contribuir para a capacidade de adaptação, reprodução e dispersão das espécies aos novos ambientes durante a especiação.

Variação cromossômica intraespecífica foi descrita para *R. pallescens* e *R. ecuadoriensis* (GÓMEZ-PALACIO et al., 2008; PITA et al., 2013). Análises citogenéticas de diferentes populações de *R. neglectus*, uma das principais espécies vetoras da região noroeste

(SILISTINO-SOUZA et al., 2013) e oeste paulista (ALEVI et al., 2015c), demonstrou que essa espécie não apresenta polimorfismo cromossômico. Contudo, análises citogenéticas em exemplares inicialmente classificados como *R. neglectus* monstraram um padrão totalmente diferente dessa espécie e, com base em estudos morfológicos, morfométricos e citogenéticos, suportaram a descrição de *Rhodnius taquarussussuensis* sp. n. A nova espécie de triatomíneo é afim de *R. neglectus*, mas pode ser facilmente diferenciada pelas análises citogenéticas, pois enquanto *R. neglectus* apresenta heterocromatina restrita apenas ao cromossomo sexual Y (rica em AT), *R. taquarussuensis* apresenta heterocromatina em uma ou ambas as extremidades de todos os autossomos (rica em CG), o que suporta a descrição da nova espécie, pois essas reorganizações genômicas com ganho e/ou perda de heterocromatina é um processo adaptativo que pode ser associado com especiação no gênero *Rhodnius* [como acontece no grupo pallescens (ALEVI et al., 2015i)].

5.7 Subfamília Triatominae

Recentemente, dados geológicos e moleculares foram associados para auxiliar no entendimento evolutivo da diversificação das espécies de triatomíneos (JUSTI; GALVÃO; SCHRAGO, 2016). Com base na história evolutiva dos triatomíneos, foi possível entender como a evolução cariotípica ocorreu nesses insetos hematófagos (ALEVI et al., 2017b). Em geral, foi possível corroborar a hipótese de Ueshima (1966) que sugere que o ancestral dos triatomíneos apresenta o cariótipo 2n = 22 (20A + XY), suportar a relação entre as tribos Rhodniini e Cavernicolini (sistema de determinação do sexo do tipo XY), sugerir que todas as espécies do clado venosa apresentam 22 cromossomos, confirmar a divergência entre *T. melanocephala* e *T. vitticeps* [que apresentam 2n = 24 (20A + $X_1X_2X_3Y$) cromossomos], em relação aos outros triatomíneos da América do Sul, que apresentam 2n = 22 cromossomos, sugerindo a criação de um novo subcomplexo denominado *T. vitticeps* (ALEVI et al., 2017c), corroborar a relação do complexo *spinolai* com os clados *geniculatus* e *rubrofasciata* e desvinculá-lo dos triatomíneos do grupo *infestans*, corroborar a relação entre a espécie *T. tibiamaculata* e o complexo *flavida* com o clado geniculatus, corroborar o agrupamento das espécies dos complexos *lecticularia* e *phyllosoma* no grupo *phyllosoma* e sugerir que *Linshcosteus* spp. apresentam o mesmo cariótipo peculiar de *T. rubrofasciata* (2n = 25).

As análises da nucleologênese das espécies da sufamília Triatominae permitiram corroborar que a persistência nucleolar, caracterizada pela presença do nucléolo ou de corpúsculos nucleolares durante a metáfase, é uma sinapomorfia desses insetos vetores

(ALEVI et al., 2014a; MADEIRA et al., 2016). Recentemente, observou-se que esse material nucleolar persistente apresenta atividade transcricional e relacionou-se essa peculiariedade com a formação do corpo cromatoide que é uma organela citoplasmática responsável por toda a diferenciação celular durante a espermiogênese (MADEIRA et al., 2017).

Conclusões

6. CONCLUSÕES

- ✓ Análises citogenéticas e moleculares agruparam *T. lenti* como novo membro do subcomplexo Brasiliensis (= complexo *T. brasiliensis*);
- ✓ Análises morfológicas, morfométricas, citogenéticas, moleculares e de cruzamentos experimentas revalidaram *T. bahiensis* e a agruparam no subcomplexo Brasiliensis (= complexo *T. brasiliensis*);
- ✓ Análises citogenéticas e citogenômicas confirmaram o agrupamento de *T. petrocchiae* como novo membro do subcomplexo Brasiliensis (= complexo *T. brasiliensis*);
- ✓ Cruzamentos experimentais corroboraram o *status* específico de *T. lenti*, *T. sherlocki* e *T. bahiensis*, por meio do desmoronamento do híbrido;
- ✓ Análises da gametogênese durante a muda imaginal de *T. brasiliensis* sugeriram que os triatomíneos cessam a meiose (para diminuir os gastos energéticos) e estimulam a espermiogênese (para garantir a chance de paternidade) nessa fase da metamorfose;
- ✓ Análises citogenéticas clássicas não permitiram diferenciar as espécies dos subcomplexos Matogrossensis e Rubrovaria, mas possibilitaram diferenciar esses triatomíneos dos outros subcomplexos presentes na América do Sul;
- ✓ Análises citogenéticas moleculares sugeriram que o subcomplexo Rubrovaria é composto pelas espécies *T. carcavalloi*, *T. circummaculata*, *T. klugi*, *T. limai*, *T. oliveirai*, *T. rubrovaria*, *T. pintodiasi*, *T. guasayana* e *T. patagonica*;
- ✓ Análises citogenéticas moleculares sugeriram que o subcomplexo Matogrossensis seja eliminado;
- ✓ Análises citogenéticas moleculares sugeriram que as espécies *T. baratai*, *T. costalimai*, *T. deaneorum*, *T. guazu*, *T. williami* e *T. jatai* (subcomplexo Matogrossensis) sejam reagrupadas com *T. arthurneivai*, *T. pseudomaculata* e *T. wygodzinskyi* em um novo subcomplexo intitulado, a saber, Pseudomaculata;
- ✓ Análises citogenéticas moleculares sugeriram que as espécies *T. jurbergi*, *T. matogrossensis* e *T. vandae* (subcomplexo Matogrossensis) sejam reagrupadas no subcomplexo Sordida;
- ✓ Análises citotaxonômicas clássicas possibilitaram distinguir as quatro espécies do complexo Maculata, com ênfase para *T. wygodzinskyi* que, por muito tempo, foi classificada errada como *T. arthurneivai*;

- ✓ Análises citogenômicas e cariossistemáticos, combinadas com estudos fenotípicos e genotípicos, permitiram a criação de um novo subcomplexo denominado *T. vitticeps*, composto pelas espécies *T. vitticeps* e *T. melanocephala*;
- ✓ Análises citotaxonômicas e cariossistemáticas permitiram diferenciar *T. rubrufasciata* de todas as outras espécies de triatomíneos;
- ✓ Análises citogenômicas permitiram diferenciar *R. montenegrensis* (DNAr presente nos cromossomos sexuais X e Y) e *R. robustus* (DNAr presente no cromossomo sexual X), corroborando o *status* específico de *R. montenegrensis*;
- ✓ Análises citogenéticas permitiram diferenciar *R. pallescens*, *R. colombiensis* e *R. ecuadoriensis* e, principalmente, relacionar a perda de heterocromatina constitutiva dos autossomos com a ocupação de novos ambientes;
- ✓ Análises citogenéticas dos gametas masculinos de *Rhodnius* e *Psammolestes* permitiram corroborar a monofilia da Tribo Rhodniini;
- ✓ Análises citogenéticas demonstraram que *R. neglectus* não apresenta variação cromossômica intraespecífica;
- ✓ Análises citogenéticas clássicas e moleculares, combinadas com dados morfológicos e morfométricos, possibilitaram descrever uma nova espécie do gênero *Rhodnius* a fim de *R. neglectus*, a saber, *R. taquarussuensis*;
- ✓ Análises citogenéticas do conjunto cromossômico diploide permitiram discorrer sobre a evolução cariotípica nas tribos Rhodniini, Triatomini e Cavernicolini;
- ✓ Análises da nucleologênese permitiram corroborar que o fenômeno de persistência nucleolar é uma sinapomorfia de Triatominae;
- ✓ Análises citogenéticas clássicas e moleculares de *T. sordida* demonstraram que os espécimes brasileiros não estão sofrendo especiação críptica (ausência de variação cromossômica intraespecífica) e permitiram classificar esses triatomíneos como *T. sordida sensu stricto*.

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