Universidade Estadual Paulista
"JÚLIO DE MESQUITA FILHO"
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Jader de Oliveira

Estudo citogenético, molecular, morfométrico e morfológico de espécies do gênero
Psammolestes **Bergroth, 1911 (Hemiptera, Reduviidae, Triatominae).**

Araraquara - SP
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Tese apresentada ao Programa de Pós-Graduação em Biociências e Biotecnologia Aplicadas à Farmácia, área de concentração: Parasitologia, da Faculdade de Ciências Farmacêuticas, UNESP, como parte dos requisitos para obtenção do Título de Doutor em Biociências e Biotecnologia aplicadas à Farmácia.

Orientador: Prof. Dr. João Aristeu da Rosa
Co-orientador: Dr. Carlos Eduardo Almeida

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UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Araraquara

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Estudo citogenético, molecular, morfométrico e morfológico de espécies do gênero *Psammolestes* Bergroth, 1911 (Hemiptera, Reduviidae, Triatominae).

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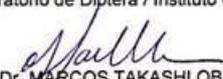
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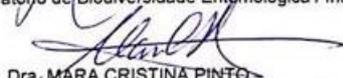
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Araraquara, 16 de abril de 2019

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Dedicatória

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Jader de Oliveira

Errare Humanum Est

Tem uns dias
Que eu acordo
Pensando e querendo saber
De onde vem
O nosso impulso
De sondar o espaço
A começar pelas sombras sobre as estrelas-las-las
E depensar que eram os deuses astronautas
E que se pode voar sozinho até as estrelas-las-las
Ou antes dos tempos conhecidos
Conhecidos
Vieram os deuses de outras galáxias-xias-xias
Ou de um planeta de possibilidades impossíveis
E de pensar que não somos os primeiros seres terrestres
Pois nós herdamos uma herança cósmica
Errare humanum est
Errare humanum est
Nem deuses
Nem astronautas
Jorge Ben Jor – A tábua de esmeraldas (1974)

Resumo

A subfamília Triatominae inclui com as tribos: Alberproseniini, Bolboderini, Cavernicolini, RhodniiniRhodniini e Triatomini, dentre as quais 19 gêneros e 154 espécies. As tribos Rhodniinini e Triatomini contém o maior número de espécies e epidemiologicamente as mais relevantes. O gênero *Psammolestes* que pertence à tribo Rhodniinini e conta com as espécies *P. arthuri*, *P. coreodes e P. tertius*. Foram realizados estudos citogenéticos, morfológicos, morfométricos, morfometricos geométricos e inferências evolutivas do gênero *Psammolestes*. Os estudos citogenéticos dos testículos foram feitos por meio de técnicas citogenéticas convencionais e indicaram a evolução cromossômica das três espécies de *Psammolestes*; Os aspectos morfológicos de ovos, adultos machos e fêmeas foram estudados por meio de microscopia eletrônica de varredura; a morfometria clássica por meio de mensuração de ovos e avaliada por analise estatística; o estudo da morfometria geométrica de cabeças foi conduzido por análises multivariadas e gráficos gerados no programa CLIC *"CollectingLandmarks for IdentificationandCharacterization"*. Foram propostas hipóteses filogenéticas baseando-se na matriz morfométrica, padrão da coloração da glândula salivar e evolução cromossômica.

Palavras chaves: Triatominae, Rhodniini, Sistemática, Evolução e Cruzamentos.

Abstract

The subfamily Triatominae counts on the tribes: Alberproseniini, Bolboderini, Cavernicolini, Rhodniinini and Triatomini, among which 19 genera and 154 species. The Rhodniinini and Triatomini tribes contain the largest number of species and epidemiologically the most relevant. The genus Psammolestes belongs to the Rhodniinini tribe and has the species *P. arthuri, P. coreodes* and P. *tertius.* Cytogenetic, morphological, morphometric, morphometric and evolutionary inferences of the genus *Psammolestes* were performed. Cytogenetic studies of the testis were made using conventional cytogenetic techniques and showed the chromosomal evolution of the three species of *Psammolestes*; The morphological aspects of eggs, adult males and females were studied by means of scanning electron microscopy; The classical morphometry by means of egg measurement and evaluated by statistical analysis; the study of the geometric morphometry of heads was conducted by multivariate analyzes and graphs generated in the CLIC program "CollectingLandmarks for Identification and Characterization". Phylogenetic hypotheses were proposed based on the morphometric matrix, salivary gland coloration pattern and chromosome evolution.

Keysword: Triatominae, Rhodniini, Systematics, Evolution and Crossovers.

Sumário

Capitulo I

Introdução

A subfamília Triatominae descrita por (JEANNEL, 1919) a subfamília Triatominae é reconhecida pela hematofagia obrigatória das espécies pertencentes à mesmas. Pode ser diferenciada das demais subfamílias de Reduviidae por apresentarem uma membrana entre o segundo e o terceiro segmento do aparelho bucal picador-sugador. Atualmente, Triatominae é composta por 154 espécies e 19 gêneros, a subfamília Triatominae e está dividida em cinco tribos: Alberproseniini, Bolboderini, Cavernicolini, Rhodniinini e Triatomini, das quais as duas últimas são as que apresentam o maior número de espécies e epidemiologicamente mais relevantes (DORN et al., 2018; GALVÃO, 2014; GALVÃO et al., 2003; LENT; WYGODZINSKY, 1979; LIMA-CORDÓN et al., 2019; POINAR, 2019).

Os triatomíneos possuem desenvolvimento hemimetabólico com cinco estádios ninfais anteriores à fase adulta. As ninfas podem ser diferenciadas dos adultos por serem ápteras, pela ausência de ocelos e de fossetas esponjosas. O 5º estádio apresenta diferenciação sexual externa, enquanto os adultos apresentam evidente dimorfismo sexual (ESPÍNOLA, 1966; LENT; WYGODZINSKY, 1979; ROSA et al., 1992).

Os adultos possuem cabeça geralmente alongada e subcilíndrica, dividida em regiões ante e pós-ocular; olhos bem desenvolvidos e presença de ocelos; um par de antenas com quatro artículos com funções olfatória e auditiva. Na base de cada antena pode ser encontrada uma estrutura chamada de tubérculo antenífero, situada lateralmente. O aparelho bucal do tipo picador-sugador é curto e retilíneo, formado por três segmentos. O tórax com o pronoto bem desenvolvido e escutelo proeminente exibe características com

grande importância taxonômica. O abdome achatado dorso-ventralmente, apresenta conexivos laterais(LENT; WYGODZINSKY, 1979).

A maioria das espécies de triatomíneos podem ser encontradas no ambiente silvestre, vivem em ninhos de aves, tocas de animais, sob cascas ou ocos de árvores, em bromélias, palmeiras, abaixo de pedras e outros ecótopos, associados a mamíferos, aves, anfíbios, répteis e invertebrados dos quais se alimentam. Algumas espécies, no decurso da evolução, adquiriram a capacidade de colonizar as estruturas artificiais construídas pelo homem nas proximidades de suas casas, como galinheiros, pocilgas e estábulos e são denominadas peridomiciliares. Outras são capazes de colonizar o interior das habitações humanas, principalmente daquelas construídas de maneira a propiciar condições de albergar os triatomíneos, construções feitas de barro e bambu com folhas de palmeira. Essas são as espécies domiciliares, as mais importantes tratando se de transmissão vetorial, uma vez que, são as principais responsáveis pela transmissão da doença de Chagas (CARCAVALLO et al., 1998a, 1998b).

Psammolestes Bergroth, 1911

Bergroth (1911) descreveu gênero *Psammolestes* e a espécies *P. coreodes.* (BERGROTH, 1911). A seguir, em 1926, Cesar Pinto descreveu *Eutriatoma arthuri*, que posteriormente foi transferida para o gênero *Psammolestes* por Del Ponte em 1930. Em 1965 Lent & Jurberg descreveram *P. tertius,* a terceira e última espécie reconhecida até o momento no gênero *Psammolestes* (BERGROTH, 1911; DEL PONTE, 1930; LENT; JURBERG, 1965; PINTO, 1926).

A estreita associação de *Psammolestes* com aves sugere que essas constituem a única fonte de alimentação para esses triatomíneos na natureza. São varias as espécies de aves encontradas em associação com as três espécies de *Psammolestes* (LENT;

WYGODZINSKY, 1979). Espécies do gênero de *Psammolestes* já foram encontradas em ninhos das seguintes famílias de aves: Dendrocolaptidae (arapaçus), Troglodytidae (curruíra, garrinchão), Furnariidae (João graveteiro e João de barro), Icteridae (corrupião, japu, João pinto amarelo e xexéu)(BARRETTO; ALBUQUERQUE, 1969; CRUZ-GUZMÁN et al., 2014; GONÇALVES DIOTAIUTI et al., 2018; PIFANO, 1938; PINTO; LENT, 1935; SHERLOCK; GUITTON, 1973).

Psammolestes arthuri (Pinto, 1926)

O comprimento total dos machos:11,0 e 12,0 mm, das fêmeas entre 12,0 e 14,0 mm. Coloração geral amarelo palha com áreas irregularmente manchadas de castanho. Tegumento rugoso com cerdas curtas e douradas, cabeça muito curta com 3 mm de comprimento, antenas inseridas próximas aos olhos; primeiro segmento antenal curto quase atingindo a extremidade apical da cabeça, segundo, terceiro e quarto segmentos antenais quase do mesmo tamanho e todos com coloração castanha; cabeça castanha possuindo uma estria longitudinal mais clara e disposta centralmente, olhos negros e ocelos claros (LENT; WYGODZINSKY, 1979; PINTO, 1926).

O lábio é reto com três segmentos, o segundo segmento é o mais longo de todos; o terceiro é igual ao primeiro em extensão. A soma do 1° e 3° é menor do que o comprimento do 2° segmento. Escutelo triangular colorido escuro e castanho no ápice; conexivo estreito, escuro quase negro com cinco pequenas manchas transversais castanhas, com coloração mais intensa no ápice. Hemiélitros com divisão normal entre cório e membrana: Corio castanho, membrana um pouco mais escura; face ventral de colorido castanho e irregularmente salpicada de negro. As pernas são de colorido castanho, e as tíbias são mais escuras (LENT; WYGODZINSKY, 1979; PINTO, 1926).

Psammolestes arthuri já foi encontrado na Colombia (Meta) e na Venezuela (Aragua, Cojedes, Miranda, Guárico, Portuguesa, Yaracuy, Anzoátegui, Apure, Lara, Táchira, Barinas,Monagas)(GALVÃO et al., 2003).

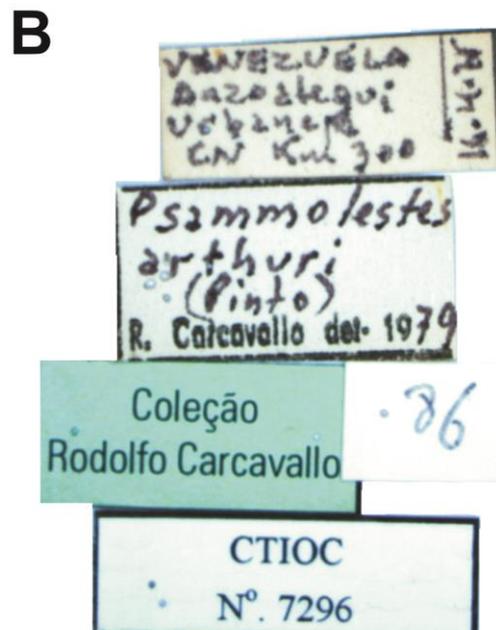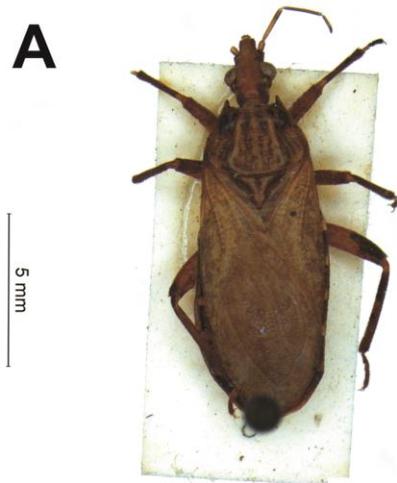Material tipo: Instituto Oswaldo Cruz – Rio de Janeiro.

Figura 1 – A – Espécime macho de *P. arthuri*; B- etiquetas com informação sobre o espécime.   (Imagens cedidas pelo Dr. Cleber Galvão do Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomineos – LNIRTT).

Psammolestes coreodes (Bergroth, 1911).

O comprimento total dos machos varia entre 12 e 14 mm, das fêmeas entre 13,5 e 15 mm. Coloração geral marrom amarelada com áreas irregularmente manchadas de marrom escuro ou negro. Tegumento rugoso com cerdas curtas e douradas. Cabeça tão longa ou ligeiramente mais longa que larga,distintamente mais curta que o pronoto; de coloração amarelada e irregularmente manchada de marrom escuro (BERGROTH, 1911; GALVÃO, 2014; LENT; WYGODZINSKY, 1979).

Genas visíveis, fortemente divergentes, ultrapassando consideravelmente o clípeo. Olhos grandes, em vista lateral podem alcançar, mas não ultrapassar a face ventral da cabeça. Ocelos inseridos em nítidas elevações. Antenas amareladas, manchadas com negro; segundo artículo negro no ápice, terceiro escurecido submedianamente. Segundo e terceiro segmentos labiais com longas cerdas. Ângulos ântero-laterais do pronoto curtos, não atingindo o nível dos ocelos, aplainados e acuminados com pequenas áreas negras. Lobo posterior do pronoto irregularmente rugoso. Escutelo rugoso transversalmente, processo apical se estreitando apicalmente. Hemiélitro da mesma coloração do corpo, células da membrana distintamente escurecidas. Hemiélitros dos machos atingindo o ápice do abdômen, enquanto o das fêmeas apenas se aproxima. (BERGROTH, 1911; GALVÃO, 2014; LENT; WYGODZINSKY, 1979).

Pernas de cor marrom claro, com manchas marrons escuras irregularmente espalhadas. Abdômen ventral marrom amarelado, irregularmente reticulado com manchas negras e com filas de manchas escuras longitudinais. Segmentos conexivais amarelados,

geralmente com duas distintas marcas marrom escuro ao longo da margem lateral.(BERGROTH, 1911; GALVÃO, 2014; LENT; WYGODZINSKY, 1979).

Psammolestes coreodes encontra-se distribuido na Argentina (Catamarca, Córdoba, Corrientes, Chaco, Entre Rios, Formosa, Santa Fé, Santiago Del Estero, Salta, Jujuy, Tucumán), Bolivia (Santa Cruz), Brasil (Mato Grosso), Paraguay (Central) (GALVÃO et al., 2003).

Material tipo: *Universitetets Zoologiska Museum, Helsinki, Finland.*

Figura 2 – A – Espécime de *P. coreodes*; B- etiquetas com informação sobre o espécime.   (Imagens cedidas pelo Dr. Cleber Galvão do Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomineos – LNIRTT).

Psammoles tertius Lent e Jurberg, 1965

Comprimento total dos machos de 11,5 a 12,5 mm, das fêmeas de 12,5 a 13,5 mm. Coloração geral marrom amarelada com áreas irregularmente manchadas de marrom escuro ou negro. Tegumento rugoso com sensilas curtas e douradas. Cabeça ligeiramente mais longa que larga e mais curta que o pronoto; coloração marrom amarelada dorsalmente com manchas escuras distribuídas irregularmente. Clípeo ligeiramente alargado anteriormente. Genas visíveis, fortemente divergentes, ultrapassando consideravelmente o clípeo. Olhos grandes, em vista lateral podem alcançar o nível da superfície ventral da cabeça e se aproximando do nível da superfície dorsal. Ocelos inseridos em nítidas elevações (um pouco menos proeminentes que em *P. coreodes*). Segundo e terceiro segmentos do lábio com longas cerdas. Pronoto com numerosas manchas escuras no lobo anterior. Escutelo rugoso transversalmente, processo apical afilando para o ápice. Hemiélitro atingindo o ápice do abdômen em ambos os sexos, de cor marrom geral, com cerdas da membrana distintamente escurecidas. Pernas da cor do corpo, com manchas marrons escuras irregularmente espalhadas. Abdômen e conexivo como em *P. coreodes*(GALVÃO, 2014; LENT; JURBERG, 1965; LENT; WYGODZINSKY, 1979)

P. tertius encontra-se distribuído no Brasil (Bahia, Ceará, Goiás, Mato Grosso, Maranhão, Minas Gerais, Pará, Paraíba, Pernambuco, São Paulo e Rio Grande do Norte) e no Peru (San Martin)(CABRERA, 2006; GALVÃO et al., 2003; SILVA et al., 2018).

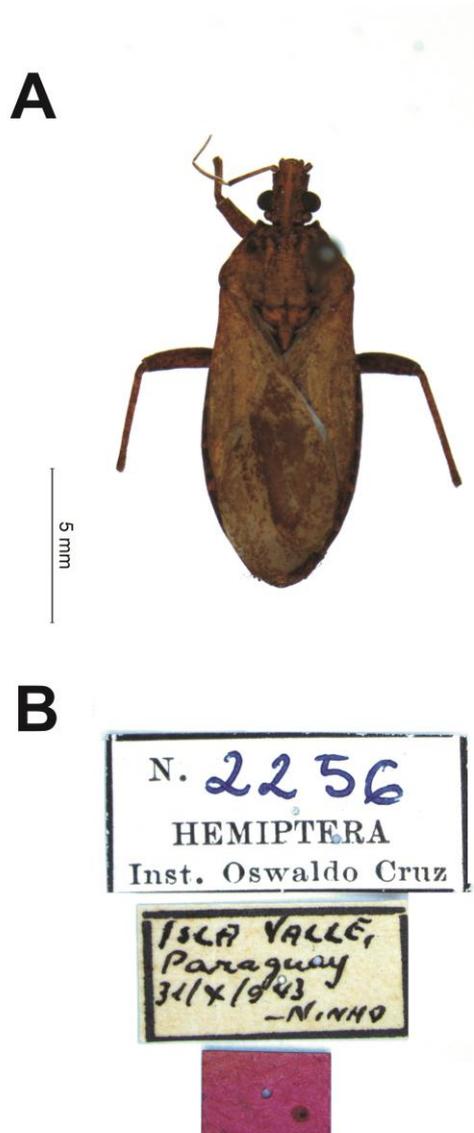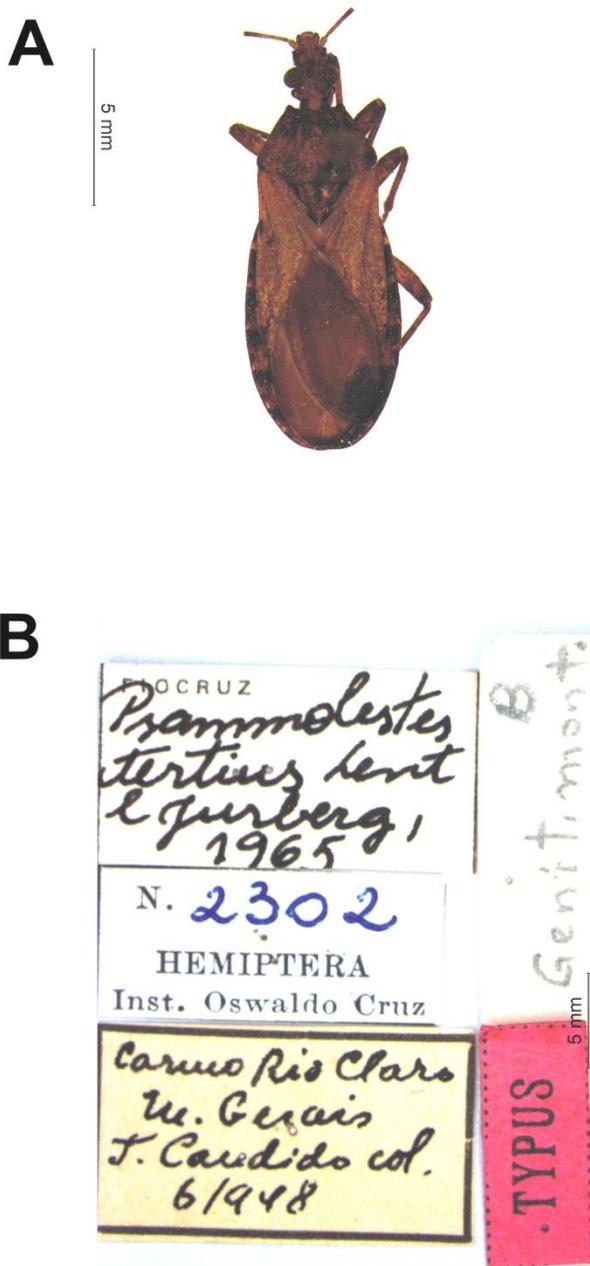Material tipo: Instituto Oswaldo Cruz – Rio de Janeiro.

Figura 03 – A – Espécime tipo de *P. tertius*; B- etiquetas com informação sobre o espécime. (Imagens cedidas pelo Dr. Cleber Galvão do Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomineos – LNIRTT).

Tribo Rhodniini

A tribo Rhodniini é composta por 23 espécies distribuídas em dois gêneros, a saber, *Rhodnius* e *Psammolestes*. As espécies do gênero *Rhodnius* são difíceis de distinguir apenas por dados morfológicos, pois apresentam plasticidade fenotípica e eventos de especiação críptica (ABAD-FRANCH et al., 2013), o que ressalta a importância dos dados moleculares para a correta identificação dos diferentes táxons. Dos dados, quando aplicados em estudos filogenéticos, sugiram que a tribo Rhodniini é um grupo monofilético e o gênero *Rhodnius* é parafilético (MONTEIRO et al., 2000, 2018), o que ressalta a necessidade de novas análises para avaliar as questões taxonômicas e a hipótese do parafiletismo de *Rhodnius*(DE CARVALHO et al., 2017; ROSA et al., 2017).

Eventos de hibridização natural foram relatados para a tribo Rhodniini (DIAS; JARAMILLO-O; DIOTAIUTI, 2014). Além disso, cruzamentos experimentais entre *R. prolixus* x *R. neglectus*, *R. prolixus* x *R. robustus*, *R. prolixus* x *R. pictipes* e *R. pallescens* x *R. colombiensis* (CARVALHEIRO JR, 1976; DÍAZ et al., 2014; GALÍNDEZ et al., 1994) produziram híbridos em pelo menos uma das direções, ressaltando ausência de isolamento reprodutivo entre as espécies dessa tribo, o que também pode acarretar em erros taxonômicos caso esses organismos sejam encontrados na natureza. O cruzamento experimental entre *R. taquarussuensis* x *R. neglectus*, associado com dados de sistemática filogenética, demonstrou que *R. taquarussuensis* é uma entidade taxonômica sinônima de *R. neglectus* (RAVAZI et al., 2019; ROSA et al., 2017), destacando, assim, a importância de estudos filogenéticos associados à análise de híbridos experimentais para a taxonomia, sistemática e evolução da tribo Rhodniini.

Estudos mais aprofundados e dinâmicos devem ser desenvolvidos para solucionar as derivações evolutivas que permanecem desconhecidas sobre a tribo Rhodniini em

especial, espécies do gênero *Psammolestes,* pois as suas características biológicas e ecológicas são distintas dos demais Triatominae .

Em vista do referido anteriormente, este trabalho tem o intuito de contribuir para aprofundar os estudos das três espécies do gênero *Psammolestes* por meio de métodos citogeneticos, morfométricos e morfológicos.

Objetivos

Geral

Estudar a evolução cromossômica, os caracteres morfológicos e morfométricos, bem como propor uma organização sistemática para as três espécies do gênero *Psammolestes.*

Específicos

Analisar a morfologia de ovos, glândulas salivares, genitálias masculina, feminina, cabeça e tórax das espécies: *P. arthuri, P.coreodes, P.tertius* capturados em diferentes áreas do Brasil e Venezuela.

Avaliar os aspectos morfometricos de cascas de ovos e da cabeça de adultos fêmeas das espécies do gênero *Psammolestes.*

Analisar a evolução cromossômica das três espécies do gênero *Psammolestes*.

Realizar cruzamentos experimentais entre espécies do gênero *Psammolestes* e *Rhodnius,* a fim de verificar a compatibilidade genômica entre a tribo Rhodniini.

Referências

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Capitulo II - (Artigo científico publicado na revista *Genetics and Molecular Research*)

New record and cytogenetic analysis of *Psammolestes tertius* Lent & Jurberg, 1965 (Hemiptera, Reduviidae, Triatominae) from Bahia State, Brazil

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Abstract

This short communication reports on the first occurrence of *Psammolestes tertius* in the Chapada Diamantina region, located in the city of Seabra, Bahia State, in northeastern Brazil. On an active search, 24 *P. tertius* specimens were collected from *Phacellodomus rufifrons* (rufous-fronted thornbird) nests. The *P. tertius* specimens were identified based on the criteria previously described by Lent and Wygodzinsky in 1979. The insects did not present infection by *Trypanosoma cruzi*. *P. tertius* males were cytogenetically analyzed, and the results were compared to the data on other specimens from the Brazilian state of Ceará. Triatomines from both locations presented the same cytogenetic characteristics: 22 chromosomes, little variation in the size of the autosomes, Y chromosomes that were larger than the X chromosomes, a chromocenter formed only by the sex chromosomes during prophase, and autosomes lacking constitutive heterochromatin. However, it is important to note that this species presents intraspecific chromosomal variation. In light of the results obtained, it is recommended that more studies be performed to characterize *P. tertius*. These studies will be particularly helpful in understanding this species in ecological, biological, biogeographical, and phylogenetic terms.

Key Words: Triatominae subfamily, Chapada Diamantina, chromosomal variation

INTRODUCTION

The Triatominae subfamily is composed of 150 species grouped into 18 genera [1]. All triatomine species are bloodsucking and are potential vectors of the protozoan *Trypanosoma cruzi* Chagas, 1909, an etiological agent of Chagas disease.

In Brazil, 65 triatomine species have been reported. They are distributed into ten genera: *Alberprosenia* Martínez and Carcavallo, 1977, *Belminus* Stål, 1859, *Cavernicola* Barber, 1937, *Eratyrus* Stål, 1859, *Microtriatoma* (Lent, 1951), *Panstrongylus* Berg, 1879, *Parabelminus* Lent, 1943, *Psammolestes* Bergroth, 1911, *Rhodnius* Stål, 1859, and *Triatoma* Laporte, 1832. With 23 species, the Brazilian state of Bahia has presented the greatest species diversity thus far. It is believed that one of the reasons for this richness in registered species is the variety of biomes and ecoregions in the state, which include the Cerrado, the Caatinga scrubland, and the Atlantic Forest [2].

Chapada Diamantina is located in the center of Bahia State. This region has been the focus of many studies [3,4] because it is considered a refuge for many species [5]. Sherlock and Serafim [6] reported the presence of nice triatomine species in the Chapada Diamantina: *Panstrongylus diasi, P. geniculatus, P. lutzi, P. megistus, Triatoma bahiensis, T. maculata, T. melanocephala, T. sordida*, and *T. tibiamaculata*. Forty years later, Souza et al. [7] reported the occurrence of ten triatomine species in the Chapada Diamantina: *P. diasi, P. geniculatus, P. lutzi, P. megistus, T. brasiliensis, T. infestans, T. melanocephala, T. lenti, T. pseudomaculata*, and *T. sordida*. In other words, in the last forty years, only *T. brasiliensis* was newly reported in the Chapada Diamantina. It is important to note that Sherlock and Serafim [6] misidentified *T. pseudomaculata* as *T. maculata*, since *T. maculata* is a species restricted to the state of Roraima within Brazil [8]. In addition, *T. bahiensis* was found to be synonymous with *T. lenti* [9].

Some triatomine species are associated with bird nests [9]. *Psammolestes tertius* is one of the species with which triatomines have been associated. In most cases, these insects are found living in furnariid nests, particularly in those of the species *Phacellodomus rufifrons* Wied, 1821, *P. ruber* Vieillot, 1817 [10], and *Anumbius annumbi* Vieillot, 1817 [11]. Thus, in order to determine whether this species is present in the Chapada Diamantina of Brazil, active searches of *P. rufifrons* nests were performed in the city of Seabra, Bahia State, Brazil (12°32.449'S and 41°32.896'W), and *P. tertius* specimens were captured.

The triatomines were collected with help from the Central Laboratory for Public Health (LACEN-BA), and the Endemic Disease Control Center in the city of Seabra, Bahia. On an active search in *P. rufifrons* nests made of thorny twigs and hung 1064 meters high (Figure 1), 24 *P. tertius* specimens were collected, five of which were adult females, two of which were adult males, and 17 of which were nymphs. Beetles from the families Cerambycidae, Chrysomelidae, and Curculionidae were also identified around the nests, as were phytophagous hemipterans from the family Lygaeidae.

The *P. tertius* specimens were identified in the Parasitology Laboratory within the Department of Biological Science, part of the School of Pharmaceutical Sciences at São Paulo State University, Araraquara (UNESP/FCFAR) in São Paulo, Brazil. Identification was based on the criteria described previously by Lent and Wygodzinsky [9]. Feces from the specimens were examined. For this examination, the feces samples were diluted in a saline solution and observed under a Leica MZ APO stereomicroscope. No Trypanosomatid forms were found. After the identification, these insects were also

cytogenetically analyzed using lacto-aceto orcein techniques [12], with modifications, according to Alevi et al. [13], and also using C-banding [14]. The results were compared to those obtained by Panzera et al. [15] in a study on *P. tertius* from the Brazilian state of Ceará in order to determine whether *P. tertius* exhibits any intraspecific chromosomal variation.

The genus *Psammolestes* Bergroth, 1911 is composed of three species grouped into the Rhodniini tribe: *P. arthuri* (Pinto, 1926), *P. coreodes* Bergroth 1911, and *P. tertius* Lent and Jurberg 1965. *P. arthuri* distribution was found to be restricted to Colombia and Venezuela, while *P. coreodes* was reported in Argentina, Bolivia, Brazil, and Paraguay. *P. tertius* distribution is restricted to Brazil [2].

P. tertius is an exclusively sylvatic species that has not been associated with the transmission of Chagas disease, particularly because of its feeding habits, which are restricted to Chagas-resistant birds. Though the results obtained were negative for infection by *T. cruzi*, this species was found to be infected by the protozoan in nature [9]. The authors report that these flagellates were likely acquired from opossums that were found in abandoned bird nests that were still inhabited by triatomines.

The cytogenetic analyses performed on *P. tertius* revealed that this species does not present intraspecific chromosomal variation, since the specimens from Bahia presented the same characteristics described for the samples from Ceará [15]: 22 chromosomes (20A + XY) (Figure 2A); little variation in autosome size (Figure 2A); Y chromosomes that were larger than X chromosomes (Figure 2A), a chromocenter formed only by X and Y chromosomes during initial prophase (Figure 2B, arrow); and autosomes lacking constitutive heterochromatin, which was restricted to the Y chromosomes (Figure 2C, arrow).

Intraspecific chromosomal variation in the Rhodniini tribe was observed in the case of *R. ecuadoriensis* [16] and *R. pallescens* [17]. *As was found with P. tertius*, Rhodnius neglectus presented chromosomal homogeneity among specimens from different Brazilian states [18]. Soares et al. [19] analyzed two *P. tertius* populations through the use of morphometry, isoenzymes, and genetic analyses; they were able to distinguish between specimens from Ceará and specimens from the Brazilian state of Minas Gerais. The authors suggest that these populations have not performed genetic exchanges, largely because they are geographically isolated. Though the results herein did not reflect intraspecific variation, it is important to note that genetic evolution and chromosomal evolution are each guided by very different factors. The events that may lead to variations in DNA sequences are more common than those that lead to chromosomal changes [20].

Thus, the presence of *P. tertius* in the Chapada Diamantina region of Brazil is being described for the first time, keeping in mind that this species presents intraspecific chromosomal homogeneity. Thus, more studies are necessary to characterize this species. These studies will be particularly helpful in understanding this species in ecological, biological, biogeographical, and phylogenetic terms. More studies are also necessary to determine triatomine fauna and their distribution around Brazil, particularly in the case of species from the genus *Psammolestes*.

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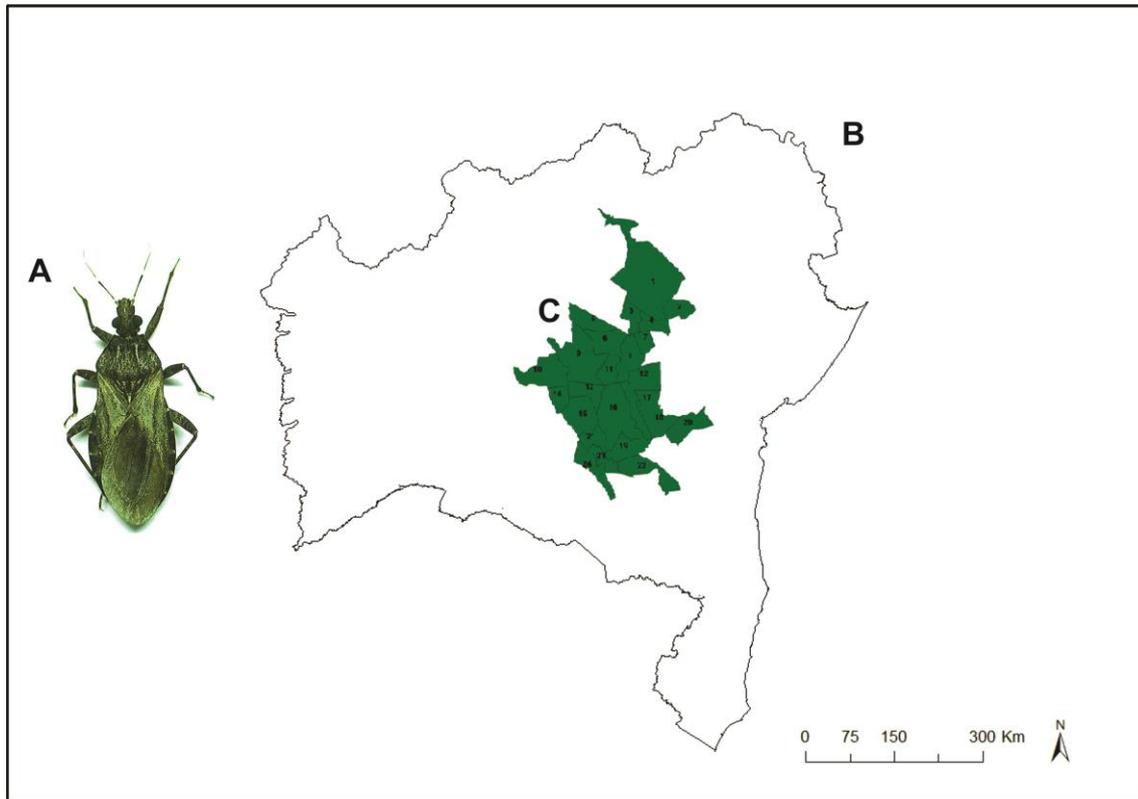
Figure 1: *P. tertius* males (A), Map of the Brazilian state of Bahia (B), cities within the Chapada Diamantina region: 1- Morro do Chapéu, 2- Tapiramuta, 3- Bonito, 4- Utinga, 5- Souto Soares, 6- Iraquara, 7- Wagner, 8- Lençóis, **9- Seabra**, 10- Ibitiara, 11- Palmeira, 12- Andarai, 13- Boninal, 14- Novo Horizonte, 15- Piatã, 16- Mucugê, 17- Nova Redenção, 18- Itaetê, 19- Ibicoara, 20- Marcíonilio Souza, 21- Abaira, 22- Barra da Estiva, 23- Jussiape, and 24- Rio de Contas (C).
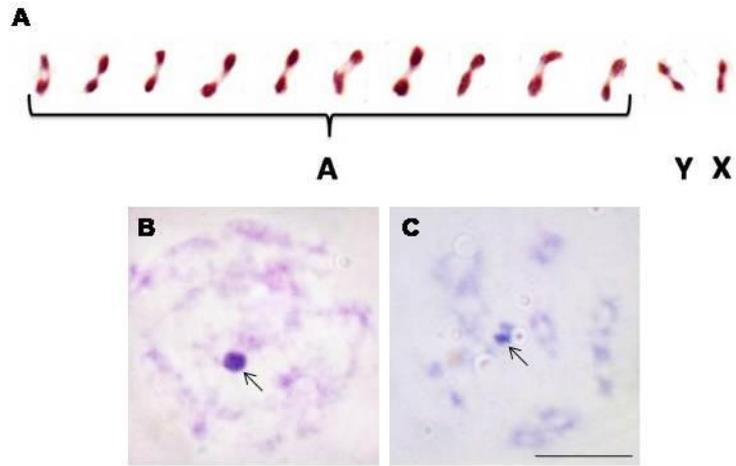
Figure 2. Karyotype (A) and constitutive heterochromatin arrangement (B, C) in *P. tertius*. A: Metaphase I: Note that the species presents 22 chromosomes, little variation in autosome size, and a Y chromosome that is larger than the X chromosome. B: Initial prophase. Note the heterochromatic chromocenter formed by the sex chromosomes (arrow). C: Final prophase. Note that only the Y chromosome is heterochromatic (arrow). Bar scale used: 10 μm.

Capitulo III (Artigo científico publicado na revista *The American Journal of Tropical Medicine & Higyene*).

SHORT REPORT

Running title: **Study of the salivary glands in Triatominae**

Study of the salivary glands in Triatominae (Hemiptera, Reduviidae, Triatominae): coloration and its applications to the Chagas disease vector evolution

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Abstract

Chagas disease is caused by *Trypanosoma cruzi* and transmitted by feces of triatomine that has the habit of defecating during the blood feeding. The salivary glands of triatomines are important to hematophagy, because the saliva is abounds in proteins anticoagulant and hemolytic. The salivary glands of some *Rhodnius* species analyzed are reddish by presence of nitrophorins (anti-hemostatic activity). Thus the present study aimed to analyze the pattern of coloration of the salivary glands of 67 triatomines species in order to evaluate whether the presence of nitrophorins is a synapomorphy of *Rhodnius*, of the Rhodniini tribe or it is shared with triatomines from the Triatomini and Cavernicolini tribes. Only the species of the tribe Rhoniini present red glands. Thus, we highlight the presence of the nitrophorins proteins as a synapomorphy the Rhodniini tribe and suggest that this tribe has derived more recently when compared with Triatomini and Cavernicolini tribes.

Key Words: taxonomy; tribe Rhodniini; tribe Triatomini; tribe Cavernicolini; nitrophorins

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 triatomines, 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]

Currently, there are 151 species of triatomines, distributed in 18 genera and five tribes, being all species considered as potential vector of Chagas disease.[2,3,4,5] 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.

Though the transmission of *T. cruzi* to the host occurs through the feces of triatomines, the behavior of hematophagy is fundamental to contamination with the protozoan, once the triatomines present the habit of defecate during blood feeding.[2] It is believed that hematophagy was derived from generalist predators ancestral who initiated the behavior as opportunistic hematophagy, passed for a facultative hematophagy and evolved for mandatory hematophagy.[6]

The salivary glands of triatomines perform a fundamental role during hematophagy, because the saliva is abounds in proteins and anticoagulant and hemolytic enzymes.[7,8] These structures have been studied anatomically[9], histologically[9], biochemically[7], molecularly[10] and cytogenetically[11] based on few triatomines of the genus *Triatoma*, *Rhodnius* and *Panstrongylus*.

Based on the analysis of *R. prolixus* has been suggested that salivary glands (principal glands) of the species of the genus *Rhodnius* is reddish.[12] This characteristic is coming from the presence of nitrophorins.[12] These proteins present a heme group in its molecule (which confer the reddish coloration) are responsible for many anti-hemostatic activity.[13]

Considering that the presence nitrophorins is suggested for all species of the genus *Rhodnius*,[14] and only *R. prolixus*,[14] *R. robustus*,[14] and *R. domesticus*[15] have been studied, the present study aimed to analyze the pattern of coloration 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*, of the tribe Rhodniini or it is shared with triatomines from the Triatomini and Cavernicolini tribes.

Were analyzed at least two adult male specimens of each species (tribe Cavernicolini: *Cavernicola pilosa*; tribe Rhodniini: *Psammolestes tertius*, *P. coreodes*, *P. arthuri*, *Rhodnius 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*, *Panstrongylus herreri* sn *P. lignarius*, *P. lignarius*, *P. megistus*, *P. lutzi*, *Triatoma 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, 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 salivary glands were removed, and examined by stereoscope microscope.

Through the analysis of the salivary glands, it was observed that the species of the tribe Rhoniini present red glands [represented by *P. tertius* (Figure 1A) and *R. montenegrensis* (Figure 1B)]. On the other hand, all other species analyzed exhibited transparent glands [represented by *T. infestans* (Figure 1C)].

It is estimated that the Triatomini and Rhodniini tribes diverged at 48.9-64.4 mya, when South America was already separated from Africa.[16] 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.[17] During the diversification of these tribes from the common ancestor, we suggest that nitrophorins hemeproteins appeared (and was selected positively) after the divergence of the tribes, more specifically in the common ancestor of Rhodniini tribe.

Although there is no dating to the divergence of Cavernicolini tribe, recently this tribe was presented as a brother group of Rhodniini.[17] However, the absence of nitrophorins in the salivary glands of *C. pilosa* also allows us to suggest that this tribe derived before Rhodniini tribe. Thus, we suggest that Triatomini tribe derived first, followed by Cavernicolini tribe and lastly the Rhodniini tribe, highlighting the need for studies using molecular clocks in Triatominae with representatives of all the tribes.

Between the anti-hemostatic activities from the nitrophorins, can highlight storage and transport of nitric acid ligated into the center of ferric heme[8] which promotes vasodilation and inhibition of platelet aggregation when it is released in the microcirculation.[18] Our study demonstrates that the species of Triatomini and Cavernicolini tribe not show this hemeprotein in the composition of salivary glands. Among the few studies that characterize the salivary glands of other genera the Triatomini tribe, the anti-hemostatic substances isolated were: triabin and pallidipin in *M. pallidipennis*,[19] triafestins in *T. infestans*,[20] and lipocalin in *T. lectularia*[10] and *P. herreri*.[10]

Thus, we highlight the presence of the nitrophorins proteins as a synapomorphy of the Rhodniini tribe and suggest that this tribe has derived more recently when compared with Triatomini and Cavernicolini tribes, contributing to the knowledge of the evolutionary history of this important vector group.

Acknowledgments

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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). Bar = 10 μm.

Capitulo IV (**Artigo científico publicado na revista** *The American Journal of Tropical Medicine & Higyene*)**.**

New evidence of the monophyletic relationship of the genus *Psammolestes* Bergroth, 1911 (Hemiptera, Reduviidae, Triatominae)

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Abstract

The genus *Psammolestes* within the subfamily Triatominae and tribe Rhodniini comprises the species *P. arthuri*, *P. coreodes* and *P. tertius*, all potential vectors of Chagas disease. A feature of *Psammolestes* is their close association with birds, which makes them an interesting model for evolutionary studies. We analyzed cytogenetically *Psammolestes* spp., with the aim of contributing to the genetic and evolutionary knowledge of these vectors. All species of the *Psammolestes* showed the same chromosomal characteristics: chromocenter formed only by sex chromosomes X and Y, karyotype 2n = 22 and constitutive heterochromatin and AT base pairs restricted to the sex chromosome Y. These results corroborate the monophyly of the genus and leads to the hypothesis that during the derivation of *P. tertius, P. coreodes* and *P. arthuri* from their common ancestor there was no reorganization in the number or structure of chromosomes.

Keywords: cytogenetic, evolution, Rhodniini, wild species.

Short report

Chagas disease is caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909) and transmitted mainly by triatomines[1]. Presently, the subfamily Triatominae (Hemiptera, Reduviidae) consists of 152 species (150 living species and two fossil ones) distributed in 18 genera and five tribes (Alberproseniini, Bolboderini, Cavernicolini, Rhodniini and tribes Triatomini)[2], all the species being potential vectors of *T. cruzi*.

The tribe Rhodniini is a monophyletic group[3] consisting of two genera with different phenotypes: one with long thin legs and a long head, living mainly in palm trees (genus *Rhodnius* Stål, 1859), and the other having a short head, strong legs, wide femora, a very wide rostrum (the widest in all the subfamily) and living in nests of birds of the

family Furnariidae (genus *Psammolestes* Bergroth, 1911)[4]. The genera include 21 species of *Rhodnius* [divided into the groups *pallescens*, *pictipes* and *prolixus*] and three species of *Psammolestes*.[2,5]

Psammolestes coreodes Bergroth, 1911 is distributed in Argentina (Catamarca, Corrientes, Chaco, Entre Rios, Formosa, Santa Fe, Santiago Del Estero, Salta, Jujuy and Tucumán), Bolivia (Santa Cruz), Brazil (Mato Grosso), Paraguay (Central); *P. tertius* Lent & Jurberg, 1965 is distributed in Brazil (Bahia, Ceará, Goiás, Mato Grosso, Maranhão, Minas Gerais, Para, Paraíba, Pernambuco and São Paulo) and Peru (San Martin) and *P. arthuri* (Pinto, 1926) is distributed in Colombia (Meta) and Venezuela (Aragua, Cojedes, Miranda, Guárico, Portuguesa, Yaracuy, Anzoátegui, Apure, Lara, Táchira, Barinas and Monagas).[6,7]

Phylogenetic analyses of *P. tertius* and *P. coreodes* suggest that this genus is monophyletic[3] (there are no phylogenetic studies of *P. arthuri* in the literature) and presents a phylogenetic relationship with the species of the *prolixus* group[3], which led to suggest the inclusion of the genus *Psammolestes* in the genus *Rhodnius*.[8] Monteiro et al.[3] suggest that perhaps *Psammolestes* should be regarded as a specialized lineage from the *prolixus* group of *Rhodnius*, because the genus *Psammolestes* and species of the *prolixus* group share a common ancestral, which highlights the paraphyly of the genus *Rhodnius*[5].

Based on mitochondrial DNA data presented by Monteiro et al.[3], Soares et al.[9] suggest that *Psammolestes* has derived from a form similar to *R. robustus*. In addition, the authors suggest that these triatomines spread from the Amazon region northward into the llanos of Venezuela, where *P. arthuri* is now abundant in furnariidae nests, and southeastward into the caatinga-cerrado path of Central Brazil. Furthermore, as predicted by Schofield and Dujardin[10] the authors suggest subsequent differentiation of *P. tertius* along a north–south cline, from the larger specimens of the northeastern caatinga region to

the smaller individuals of the central cerrado. According to them, the third species of the genus, *P. coreodes*, from the Chaco region of Argentina and Paraguay, may represent the southernmost differentiation of these descending populations.

Cytogenetic studies on the genus *Psammolestes* started in 1950 with the description of the karyotype of *P. coreodes*[11]. After 48 years the karyotype of *P. tertius* was described[12] and in 2012 the constitutive heterochromatin pattern of the species was characterized by Panzera et al.[13] (Table 1). In addition, a more recent cytogenetic study comparing *P. tertius* of different Brazilian states (Bahia and Ceará) was performed and showed absence of intraspecific chromosome variation[14]. The present work seeks to characterize the karyotype evolution and the chromatin composition of the species of the genus *Psammolestes*, with the aim of contributing to the genetic and evolutionary knowledge of these potential vectors.

Five adult males of each species were used for cytogenetic analysis. The wild species considered herein were *P. tertius* [Castro Alves, Bahia, Brazil], *P. coreodes* [Corumbá, Mato Grosso do Sul, Brazil] and *P. arthuri* [Maracay, Aragua, Venezuela], all of them being from the field. The seminiferous tubules were torn apart, crushed and fixed on slides in liquid nitrogen. The cytogenetic technique of Lacto-Acetic Orcein[15,16] and C-banding[17] were applied for description of karyotype, characterization of meiosis and description of heterochromatin pattern, respectively. Then the cytogenomic technique of CMA_3/DAPI banding was applied Schimid[18] with the modifications provided by Severi-Aguiar et al.[19] to differentiate the heterochromatin regions rich in AT and CG. The biological material was analyzed using a Jenaval light microscope (Zeiss) and Olympus BX-FLA fluorescence microscope.

All species of the genus *Psammolestes* presented the same chromosomal characteristics, namely chromocenter formed only by sex chromosomes X and Y during

the prophase (Fig. 1a-c), euchromatic autosomes and sex chromosomes X and heterochromatic sex chromosome Y (Fig. 1d-f), sex chromosome X rich in CG (Fig. 1g-i) and Y rich in CG (Fig. 1j-l), and karyotype 2n = 22 (Fig. 1m-o). These characteristics confirm the data already described in the literature (Table 1) and corroborate the monophyly of this genus.

The karyotype 2n = 22 is present in all species of the tribe Rhodniini[20,21]. This karyotype is the same number of chromosomes as the ancestor of Triatominae[17] which indicates that the genomic reorganization events that occurred during the evolution of the tribe Rhodniini did not lead to numerical alterations in the chromosomes, unlike what happened to the tribe Triatomini, which presents karyotypes ranging from 2n = 21 to 25.[22,23]

Chromocenter formed only by sex chromosomes X and Y is also shared with the species of *Rhodnius*[13,24], which demonstrates that this meiotic behavior is present in all species of the tribe Rhodniini. In the tribe Triatomini there are species with the pattern described for *Psammolestes* and species that present union of autosomes with sexual chromosomes in the formation of the chromocenter[25]. This meiotic behavior can be used as a taxonomic tool to group related species. For example, all species of the *T. brasiliensis* complex present chromocenter formed by the sex chromosomes plus a pair of autosomes [characteristics that make it possible to differentiate seven species in this complex from all other triatomine complexes[26].

With the exception of *R. colombiensis* Mejia, Galvão & Jurberg, 1999, *R. nasutus* Stål, 1859, *R. pallescens* Barber, 1932, *R. pictipes* and *R. taquarussuensis* Rosa et al. 2017[21,27] all *Rhodnius* species also have constitutive heterochromatin restricted to the sex chromosome Y, as well as observed in *Psammolestes* spp. The distribution pattern of constitutive heterochromatin is extremely important for the taxonomy of Triatominae,

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being one of the main tools used in the description of the last species of the tribe Rhodniini, namely *R. taquarussuensis* [21]. In addition, the three species of the genus *Psammolestes* showed the same DNA composition rich in AT and CG.

Heterochromatin loss/reorganization in AT and CG composition could have occurred during the speciation of *Psammolestes* spp, as observed for the species of the group *pallescens*[28]. However, considering that the ancestor of *P. tertius*, *P. coreodes* and *P. arthuri* was similar to *R. robustus* Larrousse, 1927 (which does not present heterochromatin in the autosomes either, only in the sexual chromosome Y[27]) and especially the degree of specialization of these species during their evolution (they inhabit only bird nests), it can be stated that the species maintained the genetic material without chromosomal changes.

The cytogenetic characteristics analyzed indicate chromosomal homogeneity in the genus *Psammolestes*, which corroborates the monophyletic feature of the genus and suggests that during the derivation of *P. tertius, P. coreodes* and *P. arthuri* from the common ancestor there was no reorganization in the number or structure of chromosomes.

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Table 1 - Cytogenetic characteristics of species of the genus *Psammolestes.*

Species	Karyotype	Meiosis	C-Banding			CMA₃/DAPI		
		Chromocenter	A	X	Y	A	X	Y
P. arthuri	2n = 22 (20A + XY)	XY	-	-	+	-	CMA[+]	DAPI[-]
P. coreodes	2n = 22 (20A + XY)[1]	XY	-	-	+	-	CMA[+]	DAPI[-]
P. tertius	2n = 22 (20A + XY)[2,4]	XY[3]	-	-	+[3,4]	-	CMA[+]	DAPI[-]

[9] Schreiber and Pellegrino (1950); [10] Panzera et al. (1998); [11] Panzera et al. (2012); [12] Oliveira et al. (2016)
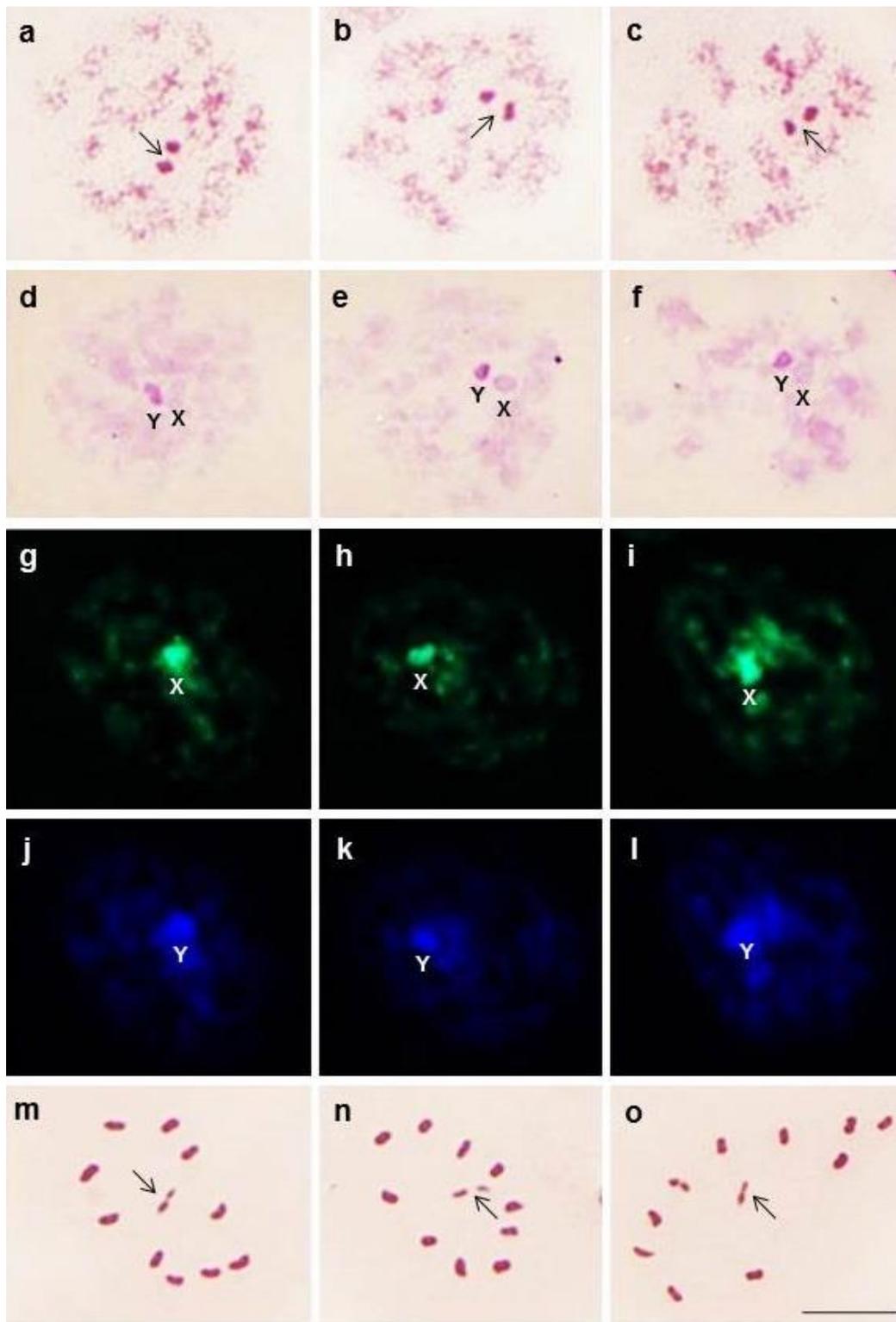
Fig. 1 Cytogenetics analyses of *Psammolestes* spp. Note the X and Y sex chromosomes forming the chromocenter of *P. tertius* (**a, arrow**), *P. coreodes* (**b, arrow**), and *P. arthuri* (**c, arrow**). Note that the autosomes and X chromosome are euchromatic and Y is heterochromatic in *P. tertius* (**d**), *P. coreodes* (**e**), and *P. arthuri* (**f**). Note that the X chromosome is rich in CG (**g-i**) and the Y chromosome is rich in AT (**j-l**) and the karyotype is 2n = 22 (20A + XY) (**m-o**) for *P. tertius* (**g, j, m**), *P. coreodes* (**h, k, n**), and *P. arthuri* (**i, l, o**). X: X sex chromosome, Y: Y sex chromosome. *Scale-bar*: 10 μm.

Capitulo V (Artigo científico que será submetido na revista *Journal Medical Entomology*).

Review of the genus *Psammolestes* (Bergroth, 1911): A morphological and morphometric approach.

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Abstract

Psammolestes Bergroth 1911 is revised and the eggs is redescribed. First morphometric study relating Rhodniini tribe species. Notes on taxonomical history based in SEM of *Psammolestes* are provided. An updated key to the genera of *Psammoleste* is presented.

Keywords: Heteroptera, Triatominae**,** multidisciplinary study, biodiversity and Rhodniini.

Introduction

Composed of 154 species and 19 genera, the subfamily Triatominae is divided into five tribes: Alberproseniini, Bolboderini, Cavernicolini, RhodniiniRhodniini and Triatomini, of which the last two are the ones with the highest number of species and epidemiologically relevant(Lent and Wygodzinsky 1979, Dorn et al. 2018, Oliveira et al. 2018, Lima-Cordón et al. 2019, Nascimento et al. 2019, Poinar 2019).

Most Triatominae species can be found in the wild, live in bird nests, burrow animal, under bark or hollow of trees, in bromeliads, palm trees, under rocks and other ecotypes, associated with mammals, birds, amphibians, reptiles and invertebrates from which they feed. Some species, in the course of evolution, have acquired the ability to colonize artificial structures built by man in the vicinity of their houses, such as chicken coops, pens and stables and are called peridomiciliary. Others are able to colonize the interior of the human habitations, especially those built to provide conditions to house the triatomines, buildings made of mud and bamboo with palm leaves. These are the domiciliary species, the most important, since, they are the main responsible for the transmission of the disease to the man (Carcavallo et al. 1998a, 1998b).

The genus *Psammolestes* was described by Bergroth, 1911. In this generic description, Begroth describes *P. coreodes*. After Cesar Pinto describes *Eutriatoma arthuri* in 1926, which was later transferred to the genus *Psammolestes* by Del Ponte in 1930. In 1965 Lent and Jurberg described *P. tertius*, the third and last species until the time of the genus *Psammolestes* (Bergroth 1911, Pinto 1926, Del Ponte 1930, Lent and Jurberg 1965)

The close association of *Psammolestes* with birds suggests that these are the only source of food for these triatomines in nature. There are several species of birds found in association with the three species of *Psammolestes* (Lent and Wygodzinsky, 1979). Species of the genus *Psammolestes* have already been found in nests of the following families of birds: Dendrocolaptidae (Arapaçus), Troglodytidae (Curruíra, Garrinchão), Furnariidae (João Gravteiro, Casaca de couro and João de barro), Icteridae (Corundum)(Pinto and Lent 1935, Pifano 1938, Barretto and Albuquerque 1969, Sherlock and Guitton 1973, Cruz-Guzmán et al. 2014, Gonçalves Diotaiuti et al. 2018).

Psammolestes arthuri has already been found in Colombia and Venezuela (Galvão et al. 2003). Type material is deposited in the Oswaldo Cruz Institute - Rio de Janeiro - Brazil. *Psammolestes coreodes* is distributed in Argentina, Bolivia, Brazil, Paraguay (Galvão et al. 2003, Santos et al. 2015) and the type material is deposited in Universitetets Zoologiska Museum, Helsinki, Finland. The last species of the genus: *P. tertius* is distributed in Brazil and Peru (Galvão et al. 2003, Cabrera 2006, Silva et al. 2018).

More detailed and dynamic studies should be developed to solve the evolutionary derivations that remain unknown about the Rhodniini tribe in particular, species of the genus *Psammolestes*, because their biological and ecological characteristics are distinct from the other Triatominae. This work intends to contribute through morphometric and morphological methods to deepen and better characterize the three species of the genus *Psammolestes*.

Material and Methods

The fieldwork occurred during the day and at points where he was spotted if nests of the species *Phacellodomus rufifrons* (João Graveteiro) due to the record of encounter of the species of *Psammolestes* in their nests. The fieldwork were conducted in wild and rural environments. The nests were removed from the trees with the aid of a rope and carefully placed in a white cloth to assist in the visualization of the insects. Later, a triage was used to separate the triatomines from the branches. The triatomines were organized in plastic

bottles with filter paper and labeled with the data referring to the collected point. The information of the populations used accounts in table 1.

Table 1 - Collection data of specimens of the genus *Psammolestes*.

Espécie	País	Estado	Município	Localidade
Psammolestes tertius	Brasil	Bahia	Seabra	Agreste
Psammolestes tertius	Brasil	Bahia	Castro Alves	Melancia II
Psammoleste scoreodes	Brasil	Mato Grosso do Sul	Corumbá	Estrada de acesso ao Que Qué
Psammolestes coreodes	Brasil	Mato Grosso do Sul	Corumbá	Estrada de acesso ao Rio Paraguai
Psammolestes tertius	Brasil	Mato Grosso do Sul	Corumbá	Estrada de acesso a fazenda Alegria (Partindo Que Que)
Psammoleste sarthuri	Venezuela	Aragua	Maracay	Campus Universidade Central da Venezuela

Observations were made using a stereoscope microscope (Leica 205A). Measurements were made using the MoticAdvanced 3.2 plus image analysis system. The total length of the head was measured excluding the neck, for better uniformity of this measurement. Were utilized 50 eggshells of the species of the genus *Psammolestes*. During the maintenance of the colonies, hatched eggs were collected from which 50 shells were randomly taken to measure them, and the mean, standard deviation, maximum and minimum for the width, length, area and diameter of the opercular opening were determined. All measurements are in millimeters (mm).

Dissections of the male genitalia were made by first removing the pygophore from the abdomen with a pair of forceps and then clearing it in 20% NaOH solution for 24 hours. The dissected structures were studied and photographed in glycerol similarly as described by (Lent and Jurberg 1965, Gil-Santana and Oliveira 2016).

Scanning electron microscopy images were obtained by the first author and the last author (JO and JAR). A female and male of *Psammolestes* species and shelleggs were cleaned in an ultrasound machine. Subsequently, the samples were dehydrated in alcohol, dried in an incubator at 45 ℃ for 20 min, and fixed in small aluminum cylinders with transparent glaze. Sputtering metallization was then performed on the samples for 2 minutes at 10 mA in an Edwards sputter coater. After this process, the samples were studied and photographed using a high resolution field emission scanning electron microscope (JEOL, JSM-7500F), similarly as described by (Rosa et al. 2010, 2014).

To explore patterns of morphological variation of the head. We used geometric morphometry. For geometric morphometric analyses were used in *P.arthuri Maracay*, Aragua-Venezuela (15), *P. coreodes* Corumbá, Mato Grosso do Sul, Brasil (15), *P.tertius* Castro Alves, Bahia-Brazil (15), *Rhodnius neglectus* Formoso, Goiás-Brazil (15) and *R. paraensis* Guiana Francesa (1). The number of specimens used is summarized in Table 1. The selections of landmarks coordinates were selection according to (Bookstein 1991) and (Oliveira et al. 2017). Heads of females species were used to evaluate the shape variables. Nine anatomical landmanrks (Figure) were colleted at intersecctions between venations and processed by researcher using modules available at the software XYOM-CLIC (Dujardin et al. 2010). Centroid size am isometric size estimator derived from coordinate data (Bookstein 1991)**,** was used to analyze wing size variation. Shape variables were obtained using the generalized procrustes analyses superimposition algorithm (Rohlf 1996). The phenogram was constructed with the Mahalanobis distances matrix by the PAST software.

General morphological terminology mainly follows (Lent and Wygodzinsky 1979). The (visible) segments of labium are numbered as II to IV, given that the first segment is lost or fused to the head capsule in Reduviidae (Weirauch 2008). The terminology of the male genitalia structures follows (Lent and Wygodzinsky 1979)and female genitalia follows (Rosa et al. 2010).

The specimens used here will be deposited in the Dr Jose Maria Soares Barata Triatominae Collection (CTJMSB) of the São Paulo State University Julio de Mesquita Filho (Unesp), School of Pharmaceutical Sciences (FCFAR), Araraquara, São Paulo, Brazil.

Results

In November of 2015, 172 triatomines of the *P. tertius* species were collected in the municipality of Castro Alves and 26 in Seabra, State of Bahia, Brazil. In Corumbá, Mato Grosso do Sul State, Brazil, triatomine collections were collected in December 2015 and 137 specimens of *P. coreodes* were obtained. On the campus of the Central University of Venezuela in the municipality of Maracay, Aragua, Venezuela, 40 specimens of *P.arthuri* were collected in March 2017. All detailed information was organized in table 1.

Morphological aspects

Redescription of the male genitalia.

P. arthuri (Pinto, 1926)

The phallosoma (Ph) had a quadrangular shape, the anterior borders are rounded and the (PrPh) has a small rounded curvature, the extension of the basal plate (Eplb) is medium in size but broad in shape (Figure 2A). A cylindrical and hollow gonopore (PrG) process with internal edges joined at the base and at the apex, in which each extension consists of two parts; inserted into the basal bridge by a narrow rod (1 + 1) (Figure 2A). The phalossoma (Ph) plate is convex in shape (Figure 2J). The parameres (Pa) are of medium length, with cylindrical conformation and through the side view are little arcuate. In the general profile they are sinuous with pointed apex projected coming out of a side flap; the outer face has many sensilla; the inner face has sensilla and the protruding edges; the end has a chitinized tip of different coloration from the body and that coloring keeps the insertion thereof (Figure 2D). Median pygophore process (PrP) is pointed with a blunt apex, and the end of the plate where it is inserted is poorly chitinized, located inside the outer edge of the pygophore (Fig. 2G).

P. coreodes Bergroth 1911

The morphology of the phallosoma (Ph) had an ovoid shape, the anterior borders are acuminate and the (PrPh) has a small curvature, the extension of the basal plate (Eplb) is short but broad (Figure 2B). A cylindrical and hollow gonopore (PrG) process with internal edges joined at the base and at the apex where each extension consists of two parts; inserted into the basal bridge by a short rod (1 + 1) (Figure 2BA). The phallosoma plate shows an equilateral triangle shape with the rounded apex (Figure 2K). The parameres (Pa) are short, with cylindrical conformation through the lateral view, besides being slightly arcuate. In the general profile they are sinuous with pointed apex projected coming out of a side flap; the outer face has many sensillae, the inner face few and the tip has a different colored tip of the body (Figure 2E). The median process of the pygophoro (PrP) is pointed with a very sharp apex, and the end of the plate where it is inserted has two strongly chitinized striae, located inside the outer border of the pygophoro (Fig. 2H).

P. tertius Lent e Jurberg 1965

The phallosoma (Ph) has a rounded shape, the anterior edges are rounded and the (PrPh) has a small irregular curvature, the one has a long size (Figure 2C). (PrG) with inner edges joined at the base and at the apex where each extension consists of two parts which are inserted in the basal bridge by a narrow rod (1 + 1) (Figure 2C). A has rounded trapezoidal shape (Figure 2L).   The parameres (Pa) are of medium length, with cylindrical conformation, by side view are few arched. In the general profile they are sinuous with pointed apex and projected coming out of a side flap; the outer face has many bristles, the inner face has fewer sensillae and the edges protrude to the point of having a chitinized tip of different coloring from the body (Figure 2F). The median process of the pygophoro (PrP) is pointed with a sharp apex, and the end of the plate where it is inserted is little chitinizada, situated inside the outer edge of the pygophoro (Fig. 2I).

Egg morphometry of *Psammolestes.*

The mean, maximum, and minimum deviation of the measurements of total length and egg shell length of populations of *P. arthuri*, *P. coreodes* and *P. tertius* are presented below in the form of tables (Table 2).

Table 2 - Results of the measurements and respective statistical analyzes of the opercular opening and the total length of 50 eggshells of populations of three species of *Psammolestes*.

Population	Opercular opening					Total length			
	Minimum	Maximum	Average	Standard Deviation		Minimum	Maximum	Average	Standard Deviation
P. tertius POP1	0,3	0,37	0,34	0,017		1,27	1,47	1,36	0,052
P. tertius POP2	0,3	0,38	0,35	0,02		1,32	1,5	1,4	0,046
P. coreodes POP3	0,29	0,35	0,32	0,016		1,25	1,43	1,34	0,05
P. coreodes POP4	0,29	0,37	0,32	0,018		1,26	1,43	1,34	0,046
P. coreodes POP5	0,26	0,36	0,31	0,022		1,2	1,46	1,33	0,051
P. coreodes POP6	0,28	0,34	0,31	0,015		1,25	1,44	1,36	0,051
P. arthuri POP7	0,39	0,47	0,43	0,02		1,34	1,71	1,54	0,085

Subtitle:POP1 : Castro Alves - BA;POP2: Santa Therezinha – BA; POP3: (Que Que) Corumbá – MS; POP4: (Acesso Faz. Alegria) Corumbá – MS; POP5: (Acesso ao Rio Paraguai) Corumbá – MS; POP6: (Acesso ao morro do Urucum) Corumbá-MS and POP7: Maracay , Aragua –Venezuela

When performing the intraspecific comparison, regarding significance, it was observed that they did not present any significance in any of the parameters used in the study. When performing the interspecific comparison, the species present a significant difference in the diameter of the opercular opening, but the total length is very significant (Table 03).

TABLE 03 - Statistical analysis using unpaired t-test with Welch's correction for measurements of the opercular opening and total length of 50 shells of the genus *Psammolestes* populations eggs.

| Populations | Opercular opening | | | Total length | |
	P value	Significance		P. value	Significance
POP1 vs POP2	P>0,05	NS		P>0,05	NS
POP1 vs POP3	P>0,05	NS		P>0,05	NS
POP1 vs POP4	P>0,05	NS		P>0,05	NS
POP1vs POP5	P>0,05	NS		P>0,05	NS
POP1 vs POP6	P>0,05	NS		P>0,05	NS
POP2 vs POP3	P>0,05	NS		P<0,05	*
POP2 vs POP4	P>0,05	NS		P<0,05	*
POP2 vs POP5	P>0,05	NS		P<0,05	*
POP2 vs POP6	P>0,05	NS		P>0,05	NS
POP3 vs POP4	P>0,05	NS		P>0,05	NS
POP3 vs POP5	P>0,05	NS		P>0,05	NS
POP3 vs POP6	P>0,05	NS		P>0,05	NS
POP7 vs POP1	P>0,05	NS		P<0,01	**
POP7 vs	P>0,05	NS		P<0,01	**

POP2				
POP7 vs POP3	P<0,05	*	P<0,01	**
POP7 vs POP4	P<0,05	*	P<0,01	**
POP7 vs POP5	P<0,05	*	P<0,01	**
POP7 vs POP6	P<0,05	*	P<0,01	**

Subtitle: POP1 *P.tertius*: Castro Alves - BA;POP2:*P.tertius*: Santa Therezinha – BA; POP3:*P.coreodes* (Que Que) Corumbá – MS; POP4:*P.coreodes* (Acesso Faz. Alegria) Corumbá – MS; POP5:*P.coreodes* (Acesso ao Rio Paraguai) Corumbá – MS; POP6: *P.coreodes*(Acesso ao morro do Urucum) Corumbá-MS and POP7:*P.arthuri*:Maracay , Aragaua –Venezuela. NS = not significant (P>0,05);* significant (P<0,05);** very significant (P<0,01);*** extremely significant (P<0,001).

Head

Ocelli: In *P. coreodes* and *P. tertius* (Fig. 3B, 3C) the ocellus form a well symmetrical cylindrical shape and are larger in *P.tertius* when compared to *P.coreodes* e *P. arthuri*. In *P. arthuri* (Fig. 3A), the ocelli are separated and arranged in a straight line. Clypeus: In *P.arthuri* it is broad in the anterior portion and narrow in the posterior portion with lateral lines transversely arranged (Fig. 3A). In *P. coreodes* is rectangular in shape with straight lateral lines (Fig. 3B) and *P.tertius* with a small narrowing in the anterior portion (Fig. 3C). Anteclypeus: In *P.arthuri* the anteclypeus is concave and narrow in the anterior and straight and wide portion in the posterior portion (Fig. 3A), *P. coreodes* the anteclypeus is straight and broad in the anterior and posterior portion (Figs 3B) and *P. tertius* the anteclypeus is concave and broad in the anterior and straight and wide portion in the posterior portion (Fig. 3C).

Tórax

Anterolateral angle: In *P.arthuri* the insertion is done near the border of the ocelli, are pronounced and the posterior extremities are triangular in shape (Figure 3D). In *P.coreodes* the insertion is below the dividing line between the neck and the protorax, they are short, triangular, and not very pronounced (Fig. 3E). In *P. tertius* the insertion is made below the dividing line between the neck and the protorax, are short and the ends are rounded (Fig. 3F). Submedian carina: In *P. arthuri* the carinas are elevated from the anterior to the posterior lobe (Fig. 3D). In *P.coreodes* e *P.tertius* are present but distributed with little prominence in the posterior lobe (Figs 3E, 3F). Discal tubercle: In *P. arthuri, P. coreodes*e *P. tertius* the discal tubercle are not present (Figures 3D, 3E, 3F). Glabrous areas: all three species are distributed regularly in the central region of the pronotum and irregularly on the sides of the anterior lobe; in *P. arthuri* you can view six glabrous areas; *P. coreodes* presents another layout pattern with eight glabrous areas and *P. terius* has a pattern with twelve glabrous areas. The

posterior lobe of *P. arthuri* is rough (Fig. 19A); in *P. coreodes* and *P. tertius* is striated (Figure 19 B and C).

Apical process: In *P.arthuri, P.coreodes* and *P.tertius* the apical processes are extremely short; however, the tips of the apical processes are distinct due to the differences of the triangular conformation (Figure 4 A,B and C). Central Depression: In *P.arthuri* is clearly delimited by two areas a small fluted and a larger flat area (Figure 4A). In *P.coreodes* and *P.tertius* the delimitations are not accentuated and have irregular striations distributed throughout the region (Figure 4 B and C).

In *P.arthuri, P.coreodes* and *P.tertius* the limiting line of the estridulatory sulcus is slightly curved in the anterior portion (Figure 4 G,H and I). In *P. arthuri* the groove is short and broad throughout the groove body and shows a narrowing in the posterior portion (Figure 4G). In *P. coreodes* the groove is short and broad in the anterior portion and presents two straight limiting lines in the medial and posterior "V" (Fig. 4H). In *P.tertius* the border is wide initially and suffers a narrowing in the median region and the posterior portion is re-sharpened at the end of the posterior V-shaped portion (Fig. 4I).

Abdômen

Urotergite I process: In *P.arthuri* the urotergite has a striated general appearance, but with vertical striae and ribs distributed throughout the upper and lower areas. Its apex is subtly rounded and does not extend beyond the central area. (Figure 4D); *P. coreodes* presented a urotergite with striated aspect vertically on the sides and lower region, but with discrete ridges and ribs in the upper region and marked in the lower region. Its apex is pointed and has a rounded marking in the central region (Figure 4E). *P.tertius* presents a urotergite with striations and discrete veins and its apex is pointed with a central depression. (Figure 4F).

The line dividing the seventh and eighth segments: In *P.arthuri* (Fig.5A) the line is concave, in *P.coreodes* (Fig. 5B) it is straight transverse; in *P.tertius* (Fig. 5C) the line is convex in the central portion. The line dividing the eighth of the ninth segment: In *P. arthuri* (Fig. 5A) the line is convex and in *P. coreodes* and *P.tertius* (Fig. 5B and C) the line is straight. The third segment of *P.arthuri* (Fig. 5A) laterally presents (1+ 1) symmetric curves differing from *P.coreodes* (Fig. 5B) and *P. tertius* (Fig. 5C) which are straight and diagonally arranged.

In *P. arthuri*, *P.coreodes* and *P.tertius* (Fig. 5D, E and F) the ninth segment is flattened frontally, being sharper in *P.tertius* (Fig. 5F). Tenth segment: in *P.arthuri, P.coreodes* and *P.tertius* (Figure 5D, E and F) is in the form of a "U" with more closed or more open contours. Gonocoxito8: It is visible in *P.arthuri* and not visible in *P.tertius* (Fig. 5D and F) have peniform format, which vary in size, markedly higher in *P.arthuri* (Fig. 5D). Gonopófise8: It is visible in *P.arthuri* is subtly in *P. tertius* (Figure 5D and F) and is not visible in *P. coreodes* (Fig. 5E).

The dividing line of segment VII with gonocoxites8: This line is concave and similar in *P.arthuri* and *P.coreodes* (Figure 5G and H) and differs in the central part in *P. tertius* (Figure 5I). Segment IX: It is irregular and has an undulating shape throughout its length in all three species (Fig. 5G, H and I). Gonocoxito8: In *P.arthuri* are rounded (Fig. 5G), *P.coreodes* are convex in the lateral and border with gonapophysis 8 and rounded in all others (Fig. 5H), *P. tertius* have a trapezoidal shape, gonapophysis 8 (Fig. 5I). Gonapófise8: In *P.arthuri* (Figure 5G) is short and curved; in *P.coreodes* and *P. tertius* are triangular and similar (Figure 5 H and I).

Morphological description of eggs

Eggs of the three known species of the genus *Psammolestes* were studied, eggs of this genus are strongly attached to the substrate and are of medium and small size.

Psammolestes arthuri

Coloration ranging from off-white to brown coming to light brown when about to hatch. Egg with continuous coloring having a small stripe on the chorion border lighter than the body of the egg. They do not have a "collar," they have a long, narrow "collar", often presenting a "lateral flattening" of coloration equal to the body of the egg. The operculum is light in color, in the same shade of the corial border in tone lighter than the body of the egg, with translucent and whitish edges. Its shape is circular with elevated, irregular filamentous projections and the opercular borders are narrow and of a light color, in some cases, a slight inclination of the operculum in relation to the "lateral flattening". Eggs, significantly cylindrical, average 1.54 mm in length per table (Table 2). The general aspect of the exocorial is not uniform, but the body is not organized by people who are not regular and the patients do not have access to the exocoriais cells. The pattern of the exocoriais cells is irregular and is not standardized of smooth appearance. In optical microscopy (OM), by transparency, only the small central holes at the entrance of the tubes are evident. In scanning electron microscopy (SEM) the "boundary lines" (LL) are high and well visible in the presence of granulations (Figure 6 A – E).

Psammolestes coreodes

Coloration that varies from whitish to brown reaching light brown when about to hatch, with a pronounced brown pigmentation pattern, with specular appearance. Egg with double coloration being one the color just below the "colo" and corial board always clear. They do not have a "collar," they have a long, narrow "collar" and often have a "lateral flattening" of coloration equal to the body of the egg. The operculum is light in color, in the same shade of the corial border in tone lighter than the body of the egg, with translucent and whitish edges. Its shape is circular with a subtle central projection and has no obvious opercular edges, more evident pattern of pores in the central region. The eggs, markedly oval, have a mean diameter of 1.34 mm (Table 2). The general appearance of the exocory is uniform, in both the egg body and the operculum, the polygonal areas vary from pentagonal to hexagonal, most of which are hexagonal. In optical microscopy (OM), by transparency, only the small central holes at the entrance of the tubes are evident. In scanning electron microscopy (SEM) the "limiting lines" (LL), due to the presence of granulations, have a slightly rough appearance. By transparency, they are refringent and, consequently, not very evident. The granulations that cover the tegument are irregular in size, agglutinated, distributed throughout the area of the exocorial cell (Figure 7 A- F).

Psammolestes tertius

The egg color has four patterns: one whitish with small spots of light brown color (17D), one second with a gray color with larger brown stains (17A), one full brown (17B) and one light brown with the white collar and corial lip (17C); marginal pigmentation pattern determined spotted appearance. They are "collared", have a long, narrow "collar", often showing a "lateral flattening" of coloration equal to the body of the egg. The operculum is light in color, in the same shade of the corial border in tone

lighter than the body of the egg, with translucent and whitish edges. Its shape is circular with a subtle central projection and has no obvious opercular edges, more evident pattern of pores in the central region. The eggs, markedly oval, have an average diameter of 1.40 mm (Table 2). The general appearance of the exocoria is uniform, both in the egg body and in the operculum, the non-polygonal areas. In optical microscopy (OM), by transparency, only the small central holes at the entrance of the tubes are evident. In scanning electron microscopy (SEM) the "boundary lines" (LL) are not present, cells have a slightly rough appearance. The granulations that cover the integument are of irregular size, agglutinated, distributed throughout the area of the exocoriaal cell (Figure 8 A-G).

Dichotomous key for species of the genus *Psammolestes*, based on eggs:

1 - Long cylindrical shaped eggs with lateral flattening ……........................*P.arthuri* (Figure 6)

Oval and circular shaped eggs…..2

2 – Eggs of oval shape without the presence of the collar............................*P.coreodes* (Figure 7)

Eggs in circular format with the presence of the collar....................................*P. tertius* (Figure 8)

Morphometric geometric of head

Results of Principal Component Analysis (PCA), to compare the female species of *R.neglectus, R. paraensis, P.arthuri, P.coreodes* and *P.tertius*, showed that Principal Component 1 represented 90.04% and the Principal Componet 2 represented 7.67%, together they corresponded to 97.71% of the total variability of shape and size (Figure X). The factorial map generated from main component 1 (CP1) and main component 2 (CP2) both influence the two variables (size and shape) and serve as a tool to show the distribution of each species within the map and its similarities, represented by the polygons (Figure 9 A). Because CP1 is more influenced by shape than size, the results of female heads indicate that the distances between the groups were more influenced by the shape. Principal component 1 was responsible for most of the differentiation between species.

By means of comparisons of centroid size, *P.tertius* differed significantly from all other populations (Figure 9B) The groupings are supported by Mahalanobis distances among groups and presented as phenogram (Figure 10).

Advances in triatomine studies are necessary in order to understand biology, biogeography, reproduction and their morphological characters. Although *Psammolestes* is not directly involved in the transmission cycle of *Trypanosoma cruzi*, the results contribute to the evolutionary and taxonomic studies of the subfamily Triatominae.

The phenotypic identification of the three species was based on the following descriptions (Bergroth 1911), (Pinto 1926) and (Lent and Jurberg 1965), the marked

character for the separation of the species was the median process of pygophore characterized and described by (Lent and Jurberg 1965) and (Lent and Wygodzinsky 1979) describe a dichotomous key for the genus *Psammolestes*, it was used and enriched with new characters to improve the separation of the species collected in the field.

In 1965 Lent and Jurberg conducted a review of the genus *Psammolestes*, in which they describe the species *P. tertius*. In this work a brief redescription of the three species was made, describing the following characteristics: Median process of pygophore, paramere, median extension of the basal plaque and phallosoma, the redescription of male internal genitalia was performed, the following details: phallosoma, gonopore process, phallosoma plate, parameres and median process of the pygophoro

In the triatomines the eggs present useful differential characteristics for the taxonomic study (Barata 1981, Obara et al. 2007). The results of the morphological and morphometric studies of the eggs allowed to differentiate the three species of the genus *Psammolestes*. In 1975 Carcavallo and collaborators published the first characteristics of *P. arthuri* eggs; in 1997 Barata when publishing a study on eggs of Triatominae succinctly describes eggs of the genus *Psammolestes*. Our results confirmed both evidence and described diagnostic characters for the three species in dichotomous key identification form (Carcavallo et al. 1975, Barata 1998).

The morphological study conducted by scanning electron microscopy on characters of the head, thorax and abdomen. In the head were observed the differences between the three species in the arrangement and size of the ocelli, the shape of the clypeus and the anteclypeus. (Hypša et al. 2002) suggested an inclusion of the genus in comparison with the morphological patterns of the heads of the genus. The results showed that the morphology of the head by SEM of *Psammolestes* does not corroborate the insertion in *Rhodnius* genus.

In the dorsal portion of the thorax, differences were noted between the three species in the pronotum, anterior lateral angles, the sub median carinas, glabrous areas and the scutellum. In the ventral portion of the thorax, differences in the shape of the stridulatory sulcus of the three species were observed. (Lent and Wygodzinsky 1979) described the first dichotomous key for the genus *Psammolestes*, but is not considered the thorax as the diagnosis of the character; in this work we describe the glabrous areas as a diagnosis of character.

In the abdomen the female external genitalia of the three species were examined and differences were observed when examined by the dorsal, posterior and ventral view. Thus, this study confirms the observations of (Rosa et al. 2010, 2012, 2014, Rivas et al. 2017, Rodrigues et al. 2018, Lasserre et al. 2019) who verified that the female external genitalia has taxonomic validity. In this way, contributions were made for the phenotypic distinction of three species of the genus *Psammolestes*, that is, *P. arthuri* and *P.coreodes*, *P.tertius*.

Geometrical morphometric studies are bringing valuable contributions to the subfamily Triatominae ((Dujardin et al. 2008, Nattero et al. 2016, Vendrami et al. 2016, Oliveira et al. 2017) the results obtained through geometric morphometry showed that *R. paraensis* may be a species link in the Rhodniini tribe based on the morphology of the head. With the redescription of the male genitalia, eggs and morphological study by SEM, it was possible to better characterize the three species of the genus *Psammolestes*, as well as to increase the number of distinguishing characters among the three.
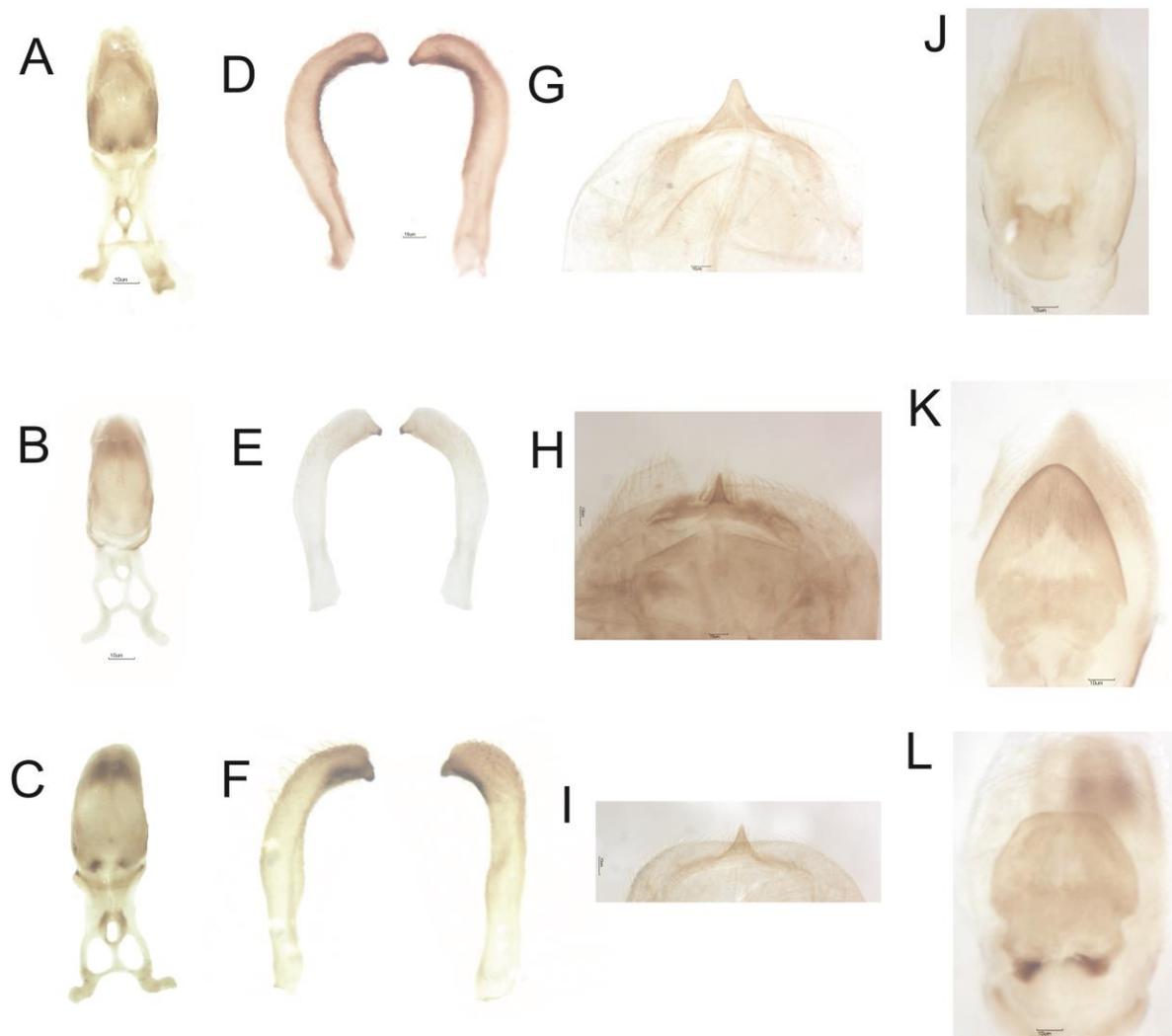
Figure 2 – Structures of diaphanized male genitalia: Phallus: A – *P. arthuri:* B - *P. coreodes;* C – *P.tertius;*   Parâmeres: D – *P.arthuri;* E- *P.coreodes*; F- *P.tertius;* Medium process of pygofphore : G- *P.arthuri;* H- *P.coreodes;* I- *P.tertius;* Phallotheca plate: J- *P.arthuri;* K- *P.coreodes* and L- *P.tertius.*
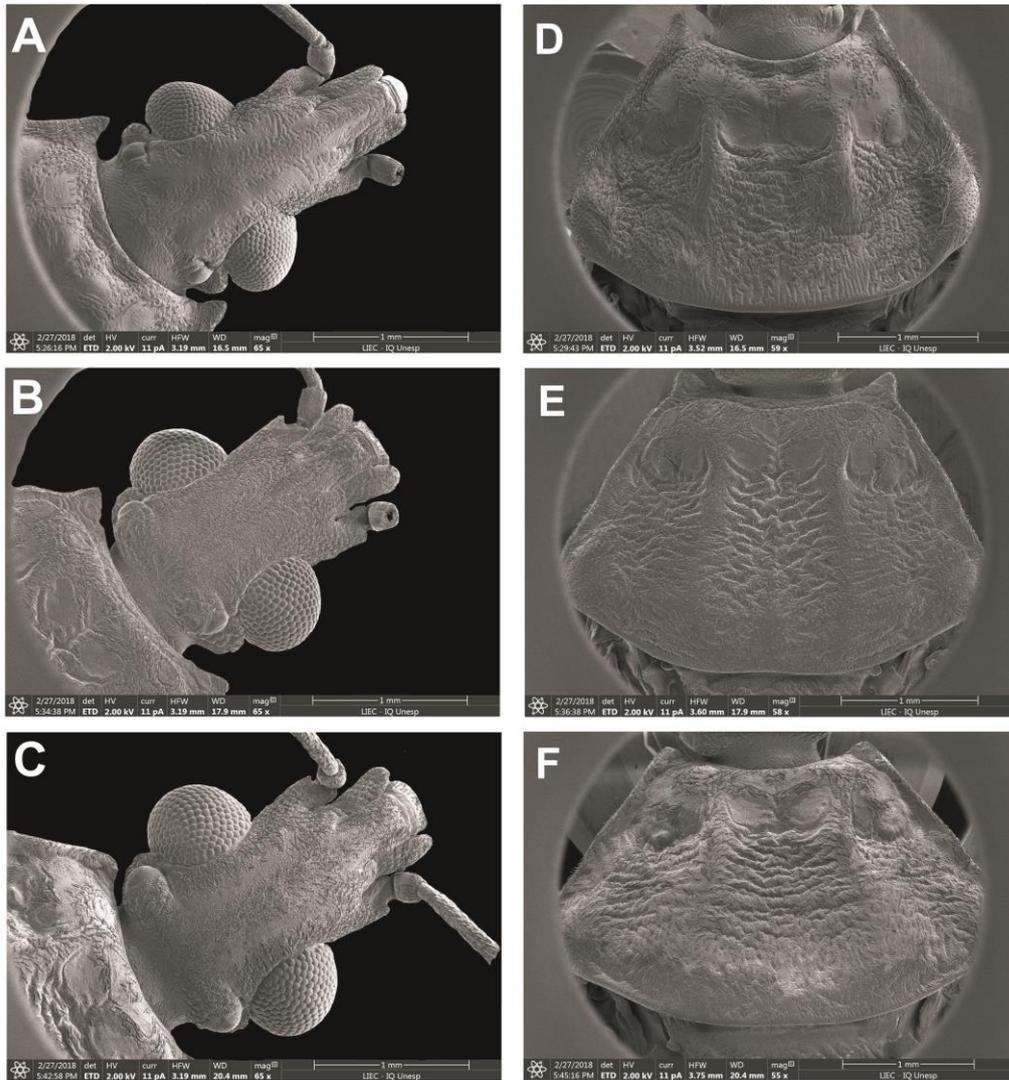
Figure 03- Dorsal view of head: A – *P. arthuri;* B – *P. coreodes*e C – *P.tertius;* Pronotum de D- *P.arthuri*; E- *P.coreodes* and F- *P. tertius.*
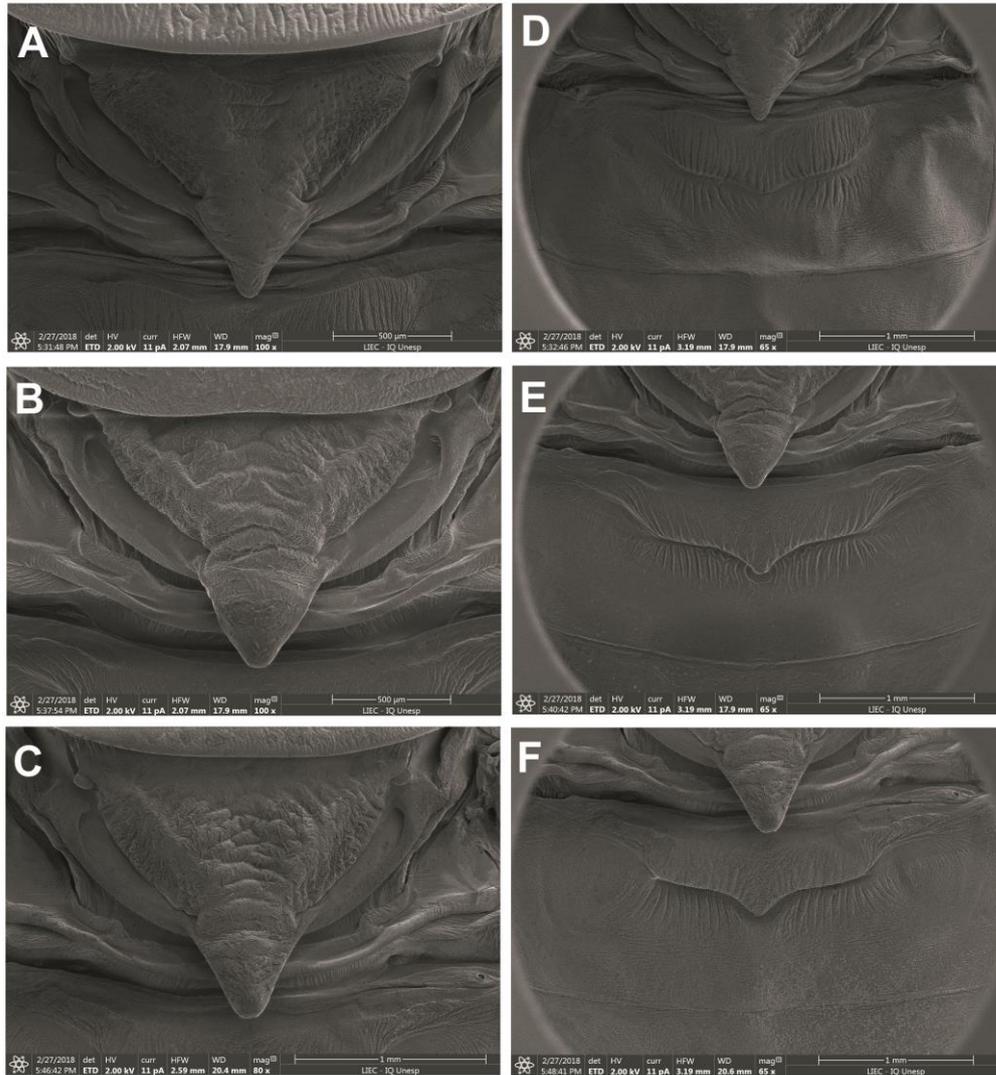
Figure 04 –Scutellum – A – *P.arthuri,* B – *P. coreodes*e C – *P.tertius;* Median Process I of urotergite -D- *P.arthuri,* E - *P. coreodes,* F-   *P.tertius*; Groove sulcus: G- *P. arthuri,* H - *P.coreodes* and I- *P. tertius.*
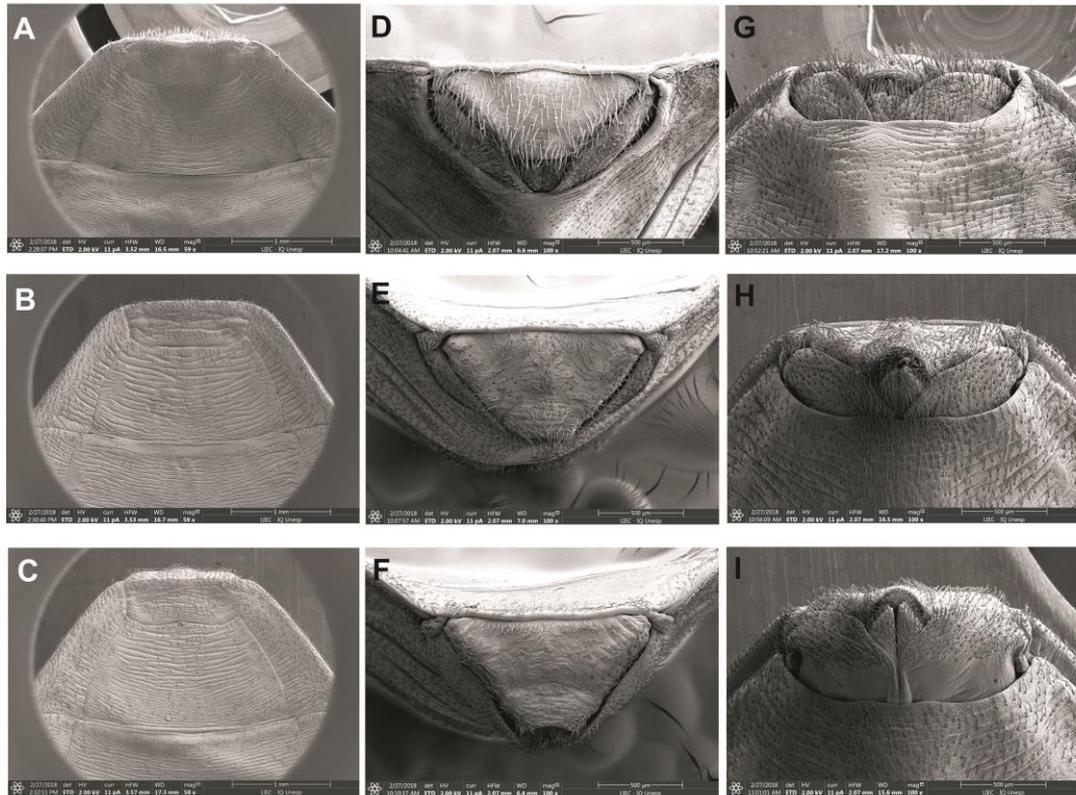
Figure 05 - Female external genitalia by SEM: Dorsal view A- *P.arthuri,* B- *P.coreodes,* C- *P.tertius.* Posterior view: D- *P.arthuri,* E- *P.coreodes*, F - *P.tertius.* Ventral view: G – *P.arthuri,* H- *P.coreodes* e I - *P. tertius.*
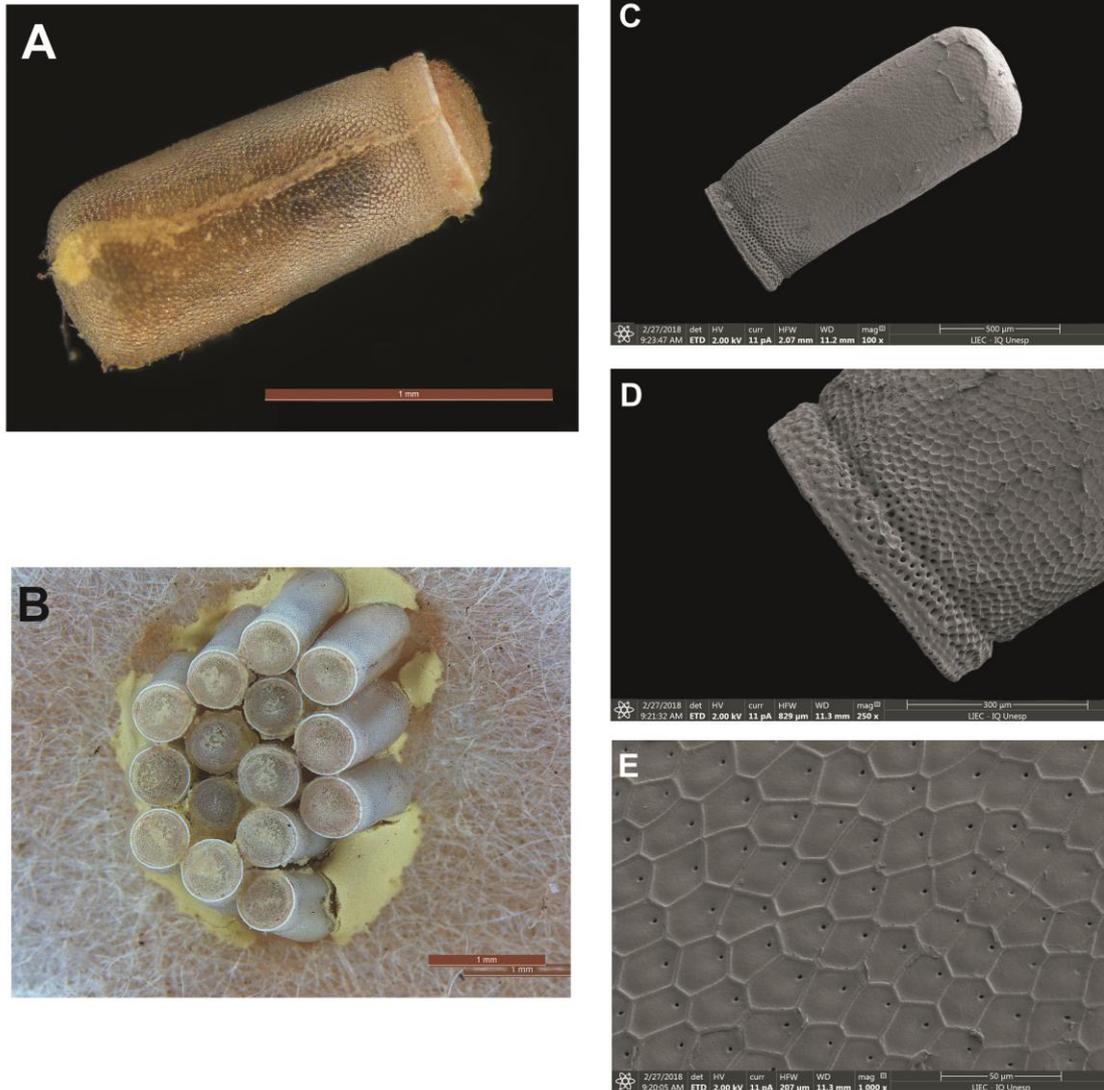
Figure 06 - Eggs of *P. arthuri*- A - Egg by means of optical microscopy; B - Eggs adhered together in a substrate; C- Egg shell by means of scanning electron microscopy; D - Detail of the chorion border by means of MEV and E - Pattern of exocorial cell by MEV.

Figure 07 - Eggs of *P. coreodes* - A - C - Chromatic variations of eggs by means of light microscopy; D - Eggshell by means of scanning electron microscopy; E - Detail of the central border of the SEM and F - Pattern of exocorial cells by SEM.
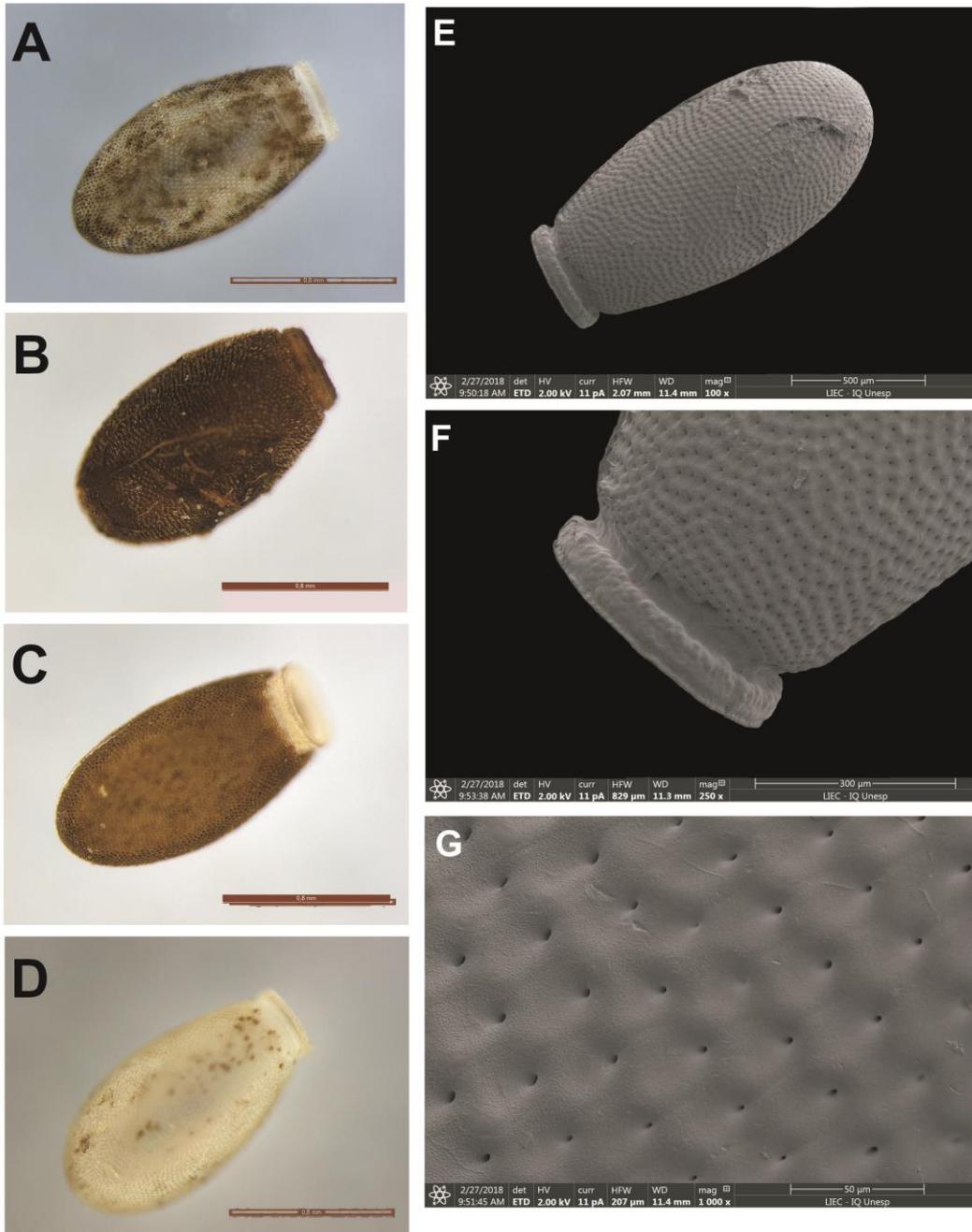
Figure 08: - Eggs of *P. tertius*- A - D - Chromatic patterns of eggs by means of optical microscopy; E- Shell of eggs by means of scanning electron microscopy; F - Detail of the chorion border by means of SEM and G - Pattern of exocorial cell by MEV.
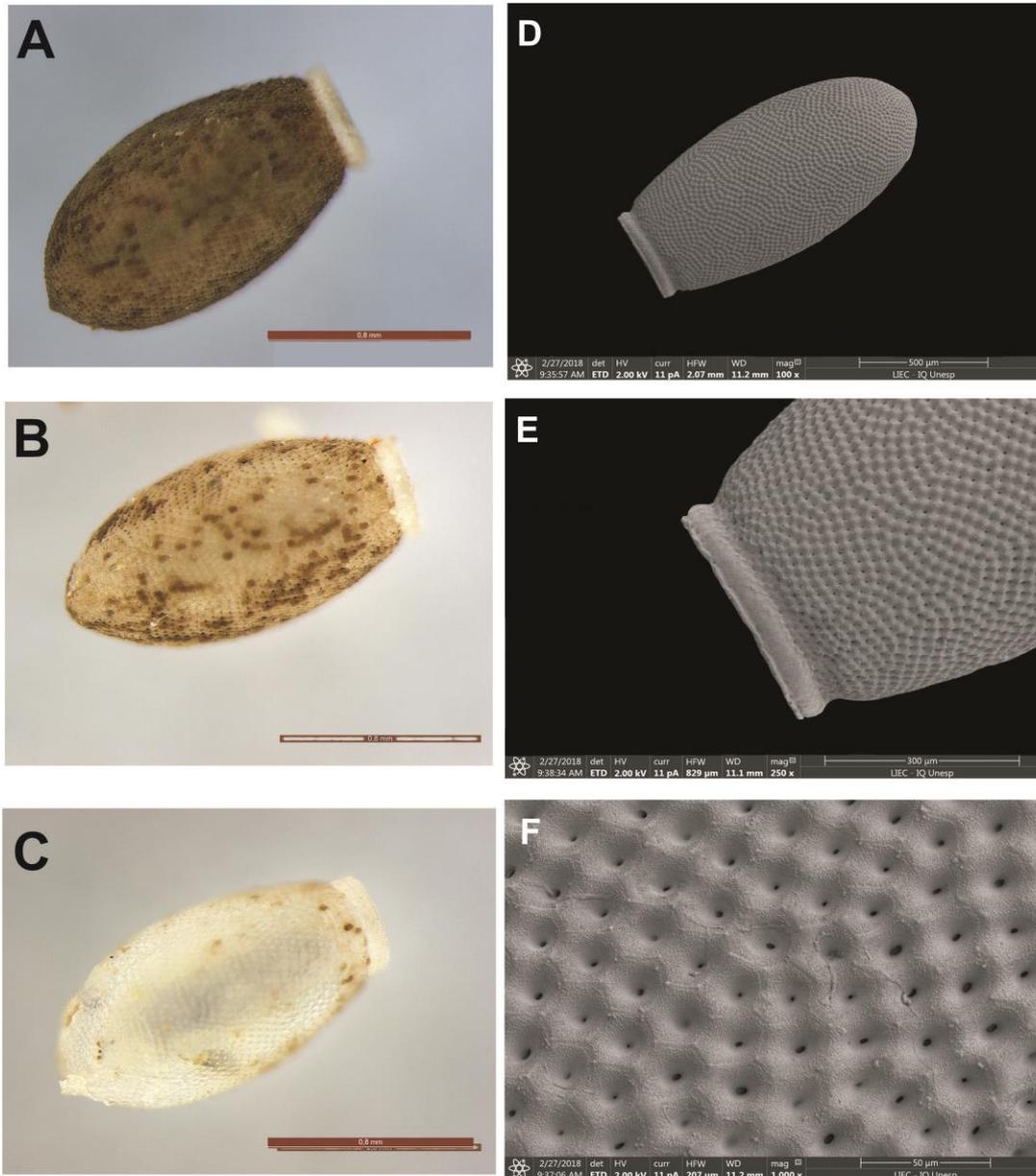
Figure 9 – A - Factorial maps in the plane of the two discriminant factors of head shape variation (canonical variables 1 and 2, or CV1 and CV2) presenting the distribution of specimens females of Triatominae species. *P. arthuri* (Yellow), *P.coreodes* (lilac), *P.tertius* (purple), *R. neglectus* (emerald) and *R. paraensis* (Green). B - Centroid size variation of the head.

Figure 10 - The Phenogram with the clusters is supported by the Mahalanobis distance of the species studied.

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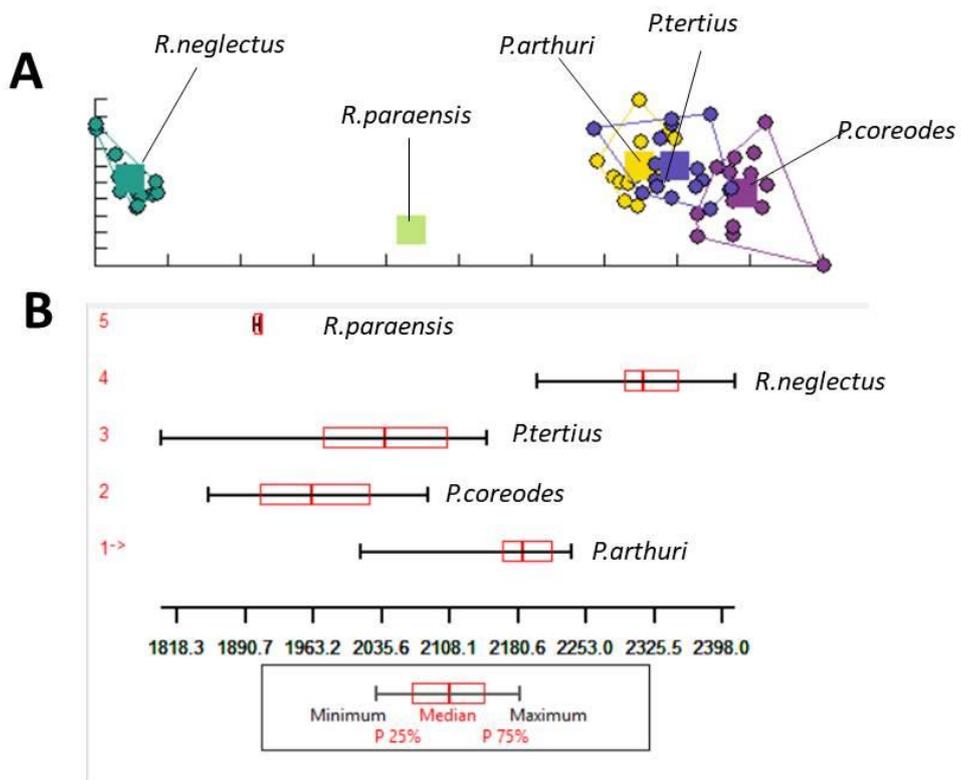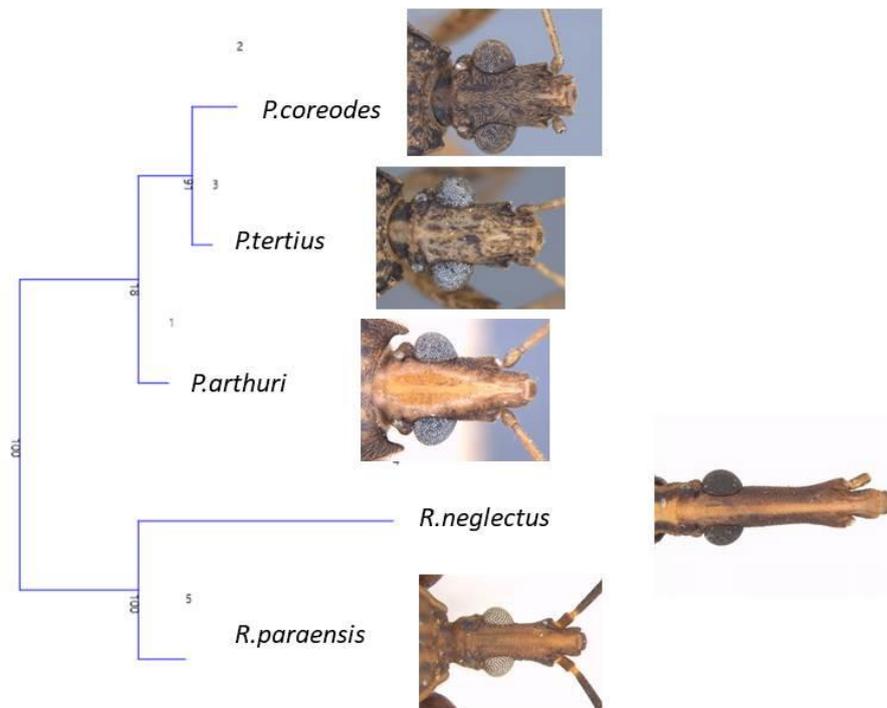**Barata, J. M. 1981**. Aspectos morfologicos de ovos de Triatominae II- Caracteristicas macroscopicas e exocoriais de dez espécies do gênero *Rhodnius* Stal, 1859 (Hemiptera-Reduviidae). Rev. Saude Publica. 15: 490–542.

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Capitulo VI (Artigo científico que será submetido para a revista *Infection, genetics and Evolution*).

Trends in Evolution of the Rhodniini tribe (Hemiptera, Triatominae): intergeneric genomic incompatibility between *Psammolestes* Bergroth, 1911 and *Rhodnius* Stål, 1859

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Short Communication

Chagas disease is a neglected disease caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae) which affects about 8 million people worldwide (CDC, 2017; WHO, 2018). The main form of transmission of the protozoan is through hematophagous insects known as triatomines (CDC, 2017; WHO, 2018). Currently 154 species of triatomines are known, divided in 18 genera and five tribes (Galvão, 2014; Oliveira and Alevi, 2017; Oliveira et al., 2018; Dorn et al., 2018; Poinar, 2019) and all are considered as potential Chagas disease vectors.

The tribe Rhodniini Pinto, 1926 is a monophyletic group composed of 24 species grouped in two genera: one with long thin legs and a long head, living mainly in palm trees (genus *Rhodnius* Stål, 1859), and the other having a short head, strong legs, wide femora, a very wide rostrum (the widest in all the subfamily) and living in nests of birds of the family Furnariidae (genus *Psammolestes* Bergroth, 1911) (Lent and Wygodzinsk,

1979), although there are reports of *P. tertius* Lent & Jurberg, 1965 living in sympathy with *R. neglectus* Lent, 1954 in nests of *Phacellodomus ruber* (Vieillot, 1817) (Passeriformes, Furnariidae) present in the crown of *Mauritia flexuosa* Linnaeus 1782 (Arecaceae) (Gurgel-Gonçalves et al., 2004). Based on mitochondrial DNA data, Soares et al., 2001 suggest that *Psammolestes* has derived from an ancestral form similar to *R. robustus* Larrousse, 1927.

Rhodnius is a paraphyletic genus formed by 21 species divided into three groups: trans-Andean *Rhodnius* clade (*pallescens* group) and cis-Andean *Rhodnius* clade (*pictipes* + *prolixus* groups) (Justi and Galvão, 2017). The event of paraphilia is supported by the greater evolutionary proximity of the species of the *prolixus* groups with the genus *Psammolestes* than with the other groups of *Rhodnius* (Justi et al., 2016). Based on this phenomenon, Hypsa et al., 2012 proposed the alteration of the genus of the species of *Psammolestes* to *Rhodnius*: *P. arthuri* (Pinto, 1926) for *R. arthuri* (Pinto, 1926), *P. coreodes* Bergroth, 1911 for *R. coreodes* (Bergroth, 1911) and *P. tertius* Lent & Jurberg, 1965 for *R. tertius* (Lent & Jurberg, 1965).

Thus, considering that studies of hybridization can help to understand the taxonomy and systematics of species, can be used to analyze the isolating mechanisms that limit gene flow between species, as well as experimental hybridization can be employed to establish the role of natural hybridization in generating new genetic variants (that may lead to adaptive evolution and/or in founding new evolutionary lineages) (Arnold, 1997; Monteiro et al., 2018), we performed experimental crosses between *Psammolestes* and *Rhodnius* to assess the degree of intergeneric genomic compatibility between the species of the Rhodniini tribe.

In order to evaluate the genetic and reproductive compatibility (Mayr, 1963) between the genus of the Rhodniini tribe, reciprocal crossing experiments were conducted between *Rhodnius* and *Psammolestes* (Table 1). The crossing experiments were conducted in the Triatominae insectary of the School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, São Paulo, Brazil, in according to the experiments of Costa et al., 2003 and Mendonça et al., 2014: the insects were sexed as 5th instar nymphs (Rosa et al., 1992), and males and females were kept separately until they reached the adult stage in order to cross adult virgins (Martinez-Ibarra et al., 2011). For the crosses, 3 couples from each set were placed in plastic jars (5 cm in diameter

×10 cm in height) and kept at room temperature. Furthermore, intragenerics crosses were also performed for control (Table 1). The eggs were collected weekly throughout the female's oviposition periods and the egg fertility rate was calculated.

The chromosome evolution of the Rhodniini tribe was quite homogenous and resulted in several identical characteristics for most taxa (karyotype, sex determination system, heterochromatin pattern, AT and CG base pair composition and arrangement of the 45S rDNA probes (Pita et al., 2013; Oliveira et al., 2018), which, at first, could allow the formation of hybrids. However, our results demonstrate that there is a reproductive barrier in the intergeneric hybridization between *Rhodnius* and *Psammolestes* (Table 1).

We suggest that after the divergence of the genera from the common ancestor, genotypic modifications (at the genomic level, which did not lead to changes in the numerical and structural composition of the chromosomes) and phenotypic [excluding mechanical isolation, since intergeneric couplings were observed during the experiment] resulted in reproductive isolation (even if the generes lived in sympathy, as already reported [Cruz-Gusman et al., 2014]). These observations are of great taxonomic and evolutionary importance, since it supports the particularities observed between the genera and, above all, it corroborates the generic status of *Psammolestes* questioned by Hypsa et al., 2012.

Based on this, we have demonstrated that *Rhodnius* and *Psammolestes* have intergeneric genomic incompatibility, contributing to the evolutionary and taxonomic knowledge of the Rhodniini tribe, an important group of vectors of Chagas' disease.

Table 1. Experimental crosses performed between the genus *Rhodnius* and *Psammolestes*

	Number of eggs				Egg Fertility
Intergeneric	**C1**	**C2**	**C3**	**Total**	
P. coreodes ♀ x *R. neglectus* ♂	55	74	52	181	0%
R. neglectus ♀ x *P. coreodes* ♂	60	121	156	337	0%
P. tertius ♀ x *R. neglectus* ♂	1	26	18	45	0%
R. neglectus ♀ x *P. tertius* ♂	114	130	97	341	0%
Control experiments	**C1**	**C2**	**C3**	**Total**	
R. neglectus ♀ x *R. neglectus* ♂	337	409	174	900	88%
P. coreodes ♀ x *P. coreodes* ♂	61	10	90	161	68%
P. tertius ♀ x *P. tertius* ♂	52	48	54	154	77%

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Capitulo VII (Artigo científico que será submetido para a revista *PLOS ONE*).

Trends in evolution of the Rhodniini tribe (Hemiptera, Triatominae): experimental crosses between *Psammolestes tertius* Lent & Jurberg, 1965 and *P. coreodes* Bergroth, 1911 and analysis of the reproductive isolating mechanisms

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Introduction

Chagas disease is a neglected disease which affects about 8 million people and puts at risk of infection approximately 25 million people in the world, being the treatment with the anti-trypanosomatids (Benznidazole and Nifurtimox) effective only in the acute phase of the disease [1, 2]. This disease is caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae) and the main form of transmission is through hematophagous insects known as triatomines [2]. Currently are known 154 species of triatomines, divided in 18 genera and five tribes [3-7] and all are considered as potential Chagas disease vectors.

The tribe Rhodniini Pinto, 1926 is a monophyletic group composed of 24 species grouped in two genera morphologically and ecologically distinct: one with long thin legs and a long head, living mainly in palm trees (genus *Rhodnius* Stål, 1859), and the other having a short head, strong legs, wide femora, a very wide rostrum (the widest in

all the subfamily) and living in nests of birds of the family Furnariidae (genus *Psammolestes* Bergroth, 1911) [8]. Soares et al. [9] suggest that *Psammolestes* has derived from an ancestral form similar to *R. robustus* Larrousse, 1927.

Rhodnius is a paraphyletic genus formed by 21 species divided into trans-Andean *Rhodnius* clade (*pallescens* group) and cis-Andean *Rhodnius* clade (*pictipes* + *prolixus* groups) [10]. The event of paraphilia is supported by the greater evolutionary proximity of the species of the *prolixus* groups with the genus *Psammolestes* which groups these species into a single clade [11]. Based on this phenomenon, Hypsa et al. [12] proposed the alteration of the genus of the species of *Psammolestes* to *Rhodnius*: *P. arthuri* (Pinto, 1926) for *R. arthuri* (Pinto, 1926), *P. coreodes* Bergroth, 1911 for *R. coreodes* (Bergroth, 1911) and *P. tertius* Lent & Jurberg, 1965 for *R. tertius* (Lent & Jurberg, 1965).

The species of the genus *Psammolestes* present a distribution restricted to Latin America, namely, *P. coreodes* was notified in Argentina, Bolivia, Brazil and Paraguay, *P. tertius* in Brazil and Peru and *P. arthuri* in Colombia and Venezuela [13, 14]. Phylogenetic and cytogenetics analyses suggest that this genus is monophyletic [15, 16]. Monteiro et al. [15] suggest that perhaps *Psammolestes* should be regarded as a specialized lineage from the *prolixus* group of *Rhodnius*, because the genus *Psammolestes* and species of the *prolixus* group share a common ancestral, which highlights the paraphyly of the genus *Rhodnius*.

Soares et al. [9] suggest that *Psammolestes* spp. spread from the Amazon region northward into the llanos of Venezuela (where *P. arthuri* is abundant in furnariidae nests) and southeastward into the caatinga-cerrado path of Central Brazil (with subsequent differentiation of *P. tertius* along a north–south cline and *P. coreodes* in the Chaco region of Argentina and Paraguay) [15, 17].

Events of natural hybridization were reported for the Rhodniini tribe, more specifically for the genus *Rhodnius* [18]. Considering that studies of hybridization can help to understand the taxonomy and systematics of species, can be used to analyze the isolating mechanisms that limit gene flow between species, as well as experimental crosses can be employed to establish the role of natural hybridization in generating new genetic variants (that may lead to adaptive evolution and/or in founding new evolutionary lineages) [19, 20], we performed experimental crosses between *P. tertius*

and *P. coreodes* and we analyzed the reproductive and morphological aspects of the hybrids in order to characterize the possible barriers reproductive and the segregation of the phenotypic characters, respectively.

Materials and Methods

Experimental crosses

In order to evaluate the pre and post-zygotic barriers between the species of the Rhodniini tribe, crossing experiments were conducted among between *P. tertius* and *P. coreodes* (Table 1).

The crossing experiments were conducted in the Triatominae insectary of the School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, São Paulo, Brazil, in according to the experiments of Costa et al. [21] and Mendonça et al. [22]: the insects were sexed as 5th instar nymphs [23], and males and females were kept separately until they reached the adult stage in order to cross adult virgins [24].

For the crosses, three couples from each set were placed in plastic jars (5 cm in diameter ×10 cm in height) and kept at room temperature. Furthermore, intraspecific crosses were also performed for control (Table 1). The eggs were collected weekly throughout the females' oviposition periods and the egg fertility rate and mortality rate of the hybrids was calculated (Table 1). After the hybrids from the first generation (F1) reached the fifth instar (N5), a hybrid pair F1 was formed for each direction (Table 1) and the same parameters described above being used in the evaluation of these crosses.

Cytogenetics analysis

After the experimental crosses, the F1 males were dissected, the testes were removed and stored in methanol: acetic acid solution (3: 1). Slices were prepared by the cell-crushing technique (as described by Alevi et al. [25]) and the cytogenetic analyzes were applied with the aim of characterizing spermatogenesis, with emphasis on the degree of pairing between the homeopathic chromosomes (Pérez et al. 20] and Mendonça et al. [22]), using the Lacto-Acética Orceína technique (De Vaio et al. [26], with modifications according to Alevi et al. [25].) The slides will be examined in light microscopy Jenaval (Zeiss), coupled to the digital camera and the Axio Vision LE 4.8 image analyzer system, with a 1000-fold increase.

Morphology of the gonads

The morphology of the male gonads was analyzed and photodocumented under a Leica MZ APO stereomicroscope microscope with Motic Advanced 3.2 plus image analysis system to evaluate the presence of gonadal dysgenesis (which may be uni or bilateral) [27]. For this, the F1 hybrids of the experimental crosses were used, which were dissected and had their gonads analyzed.

Segregation of phenotypic characteristics

The F1 male hybrids used in the experimental crosses had the head measured in a Leica MZ APO stereomicroscope and Motic Advanced 3.2 plus image analysis system to analyze the segregation of phenotypic characteristics, based on the main parameter used in the taxonomic key of Lent and Wygodzinsky [8] to characterize *P. tertius* (anteocular region with two to two and a half times the length of the ocular post) and *P. coreodes* (anteocular region no more than twice the length of the post-ocular).

Results and Discussion

It is estimated that the ancestors of the Rhodniini and Triatomini tribes diverged at 48.9-64.4 million of years ago, at about the time when South America was beginning to separate from Antarctica and Australia during the lower tertiary period [28]. It is believed that the radiation of the genus *Rhodnius* occurred from the Amazon region and resulted in three main evolutionary lineages present in the south (Brazilian Cerrado), in the north (Venezuela) and in the northwest (passing through the Andean Cordillera into the Magdalena valley in Colombia) [29]. In addition, the *Psammolestes* adapted to exploit bird nest microhabitats and currently occur over the open ecoregions north and south of the moist Amazon forests – *P. arthuri* in the Orinoco and Venezuelan coastal basins, *P. tertius* primarily in the Cerrado-Caatinga, and *P. coreodes* primarily in the Chaco [15, 30].

Interspecific experimental crosses between *P. tertius* and *P. coreodes* (Figure 1), demonstrated genomic compatibility intrageneric, since hybrids were produced in both directions for all crosses (Table 1) (Figure 2). Likewise, interspecific crosses between *Rhodnius* (*R. prolixus* x *R. negletcus*, *R. prolixus* x *R. robustus*, *R. prolixus* x *R. pictipes*

and *R. pallescens* x *R. colombiensis*) also resulted in the production of hair hybrids at least one of the directions [31, 32]. Considering the biological concept of species proposed by Mayr et al. [33], that is, "groups of natural populations that actually or potentially intersect and are reproductively isolated from other groups," this feature is extremely important from an evolutionary point of view because it demonstrates that evolutionary events that result in pre- total zygotic isolation between species have not yet been installed in the Rhodniini tribe. .

Table 1. Experimental crosses performed between *P. coreodes* and *P. tertius*.

Crossing experiments	Number of eggs				Egg Fertility	Mortality F1	Mortality F2
	C1	C2	C3	Total			
P. coreodes ♀ x *P. tertius* ♂	3	24	27	54	43%	91,3%	-
P. tertius ♀ x *P. coreodes* ♂	40	23	54	117	26%	93,3%	-
Hybrid ♀ x Hybrid ♂[1]	0	-	-	-	-	-	-
Hybrid ♀ x Hybrid ♂[2]	14	-	-	-	57%	-	100%
Control experiments	**C1**	**C2**	**C3**	**Total**			
P. coreodes ♀ x *P. coreodes* ♂	61	10	90	161	68%	-	-
P. tertius ♀ x *P. tertius* ♂	52	48	54	154	77%	-	-

[1] Hybrids of the cross between *P. tertius* ♀ x *P. coreodes* ♂; 2 Hybrids of the cross between *P. coreodes* ♀ x *P. tertius* ♂.

Figure 1. Interspecific experimental crosses between *P. coreodes* ♀ and *P.tertius* ♂ (A) *P. tertius* ♀ and *P. coreodes* ♂ (B), demonstrating the occurrence of copula between species.

Figure 2. Adult hybrids from the experimental crossing between *P. coreodes* ♀ and *P.tertius* ♂ (A) and *P. tertius* ♀ and *P. coreodes* ♂.

The main mechanisms of pre-zygotic reproductive isolation observed in the subfamily Triatominae are: ecological isolation between *T. infestans* and *T. platensis* Neiva, 1913 which are phylogenetically related [10], but present different habits (*T. infestans* is associated with domiciliary and is fed on mammalian blood [3] and *T. platensis* is associated with bird nests and feeds preferentially from the blood of these

vertebrates [34]) and mechanical isolation that is associated with genitalia incompatibility and happens with a certain frequency in only one direction of the crosses, as occurs, for example, at the crossing of *T. platensis* female with males of *T. delpontei* Romaña & Abalos, 1947 [35].

Although intrageneric genomic homology to *P. tertius* and *P. coreodes* was confirmed by the hatching of first-generation hybrids (Table 1), different fertility rates were observed for each specific crossover (Table 1). For crosses that have a very low fertility rate, it is possible to use this tool to corroborate the specific status of the parents (as used by Mendonça et al. [36] to revalidate *Triatoma bahiensis*). n addition, the high mortality rate of F1 hybrids (Table 1) makes it possible to characterize the evolutionary phenomena of the non-viability of hybrids, which consists of extremely low (low adaptive value) viability of F1 hybrids, not reaching the adult stage for the hybrids resulting from the crossing between *T. mexicana* (Herrich-Schaeffer, 1848) and the six species of *Meccus spp.* [37])

The first specimens of F1 that reached adulthood had a dominant phenotypic segregation pattern for *P. tertius* in both directions (anterior region measured at least twice the size of the post-ocular region) (Figure 3). These triatomines were crossed again, with 100% of the F2 offspring infeasible (Table 1), characterizing the hybrid breakage. Although these insects did not present gonadal dysgenesis (Figure 4), cytogenetic analyzes of male gonads demonstrated both metaphases with 100% matching of homeopathic chromosomes (Figure 5A) (which resulted in viable gametes) and metaphases with pairing errors in one (Figure 5B, arrows) (which resulted in non-viable gametes), which justifies the hatching of hybrids in F2 (Table 1).

These results are important from a systematic and taxonomic point of view, since according to Dewey [38], the evaluation of the behavior of the chromosomes in metaphase I in interspecific hybrids, allows the establishment of phylogenetic structures, as well as can be used in the definition of placements taxonomic and evolutionary. In addition, according to Riley [39], two species possess distinct genomes when their chromosomes are different in structure and genetic content, so that there is no pairing between one or more pairs of homeopaths during hybrid meiosis. This behavior leads to sterility and, consequently, to genetic isolation between species.
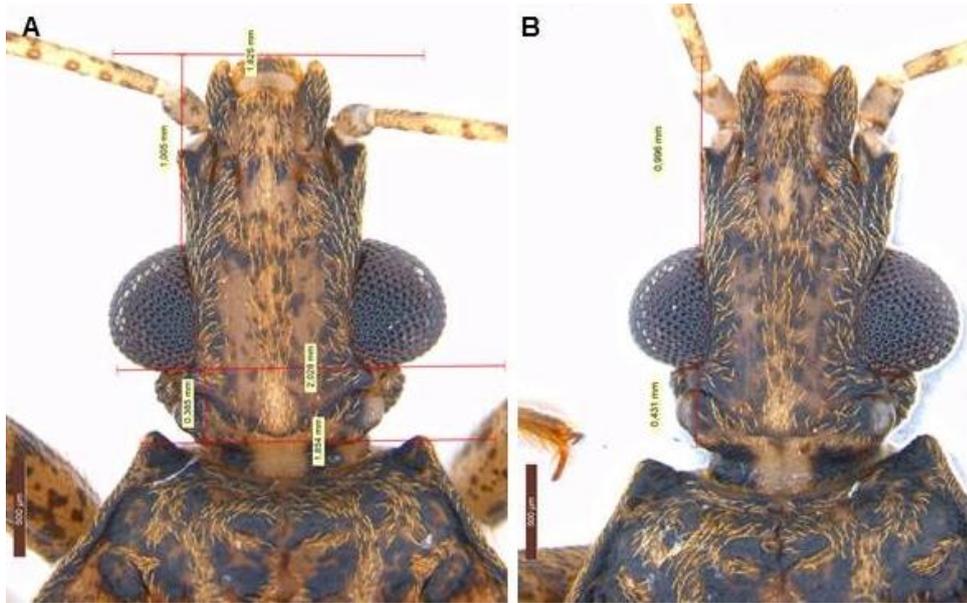
Figure 3. Adult male hybrids of the experimental cross between *P. coreodes* and *P. tertius* submitted to morphometric analysis of the head. A = *P. coreodes* ♀ x *P. tertius* ♂ (anteocular region = 1.005mm; post-ocular region = 0.385 mm. Anteocular region 2.6 times greater than the post-ocular region). B = *P. tertius* ♀ x *P. coreodes* ♂ (anteocular region = 0.996 mm; post-ocular region = 0.431. Anteocular region 2.3 times greater than the post-ocular region).
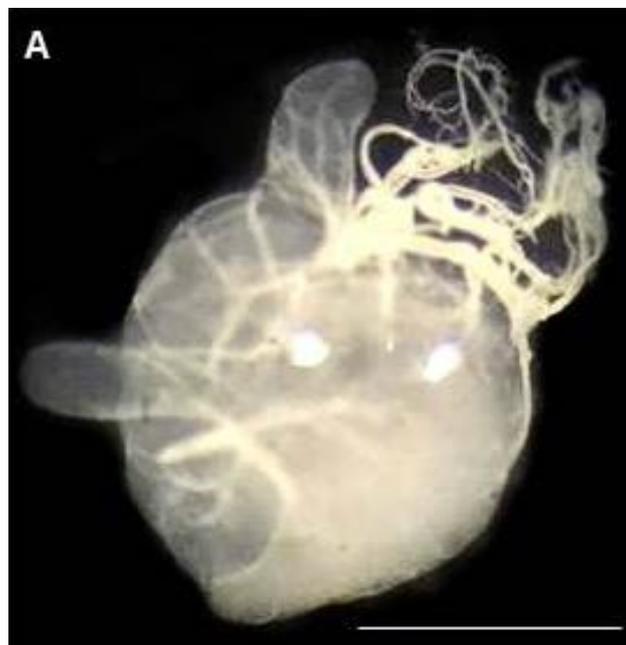

Figure 4. Not atrophied testicle of adult crossbreeding between *P. coreodes* ♀ x *P. tertius* ♂, demonstrating the absence of gonadal dysgenesis at this junction, in both directions.
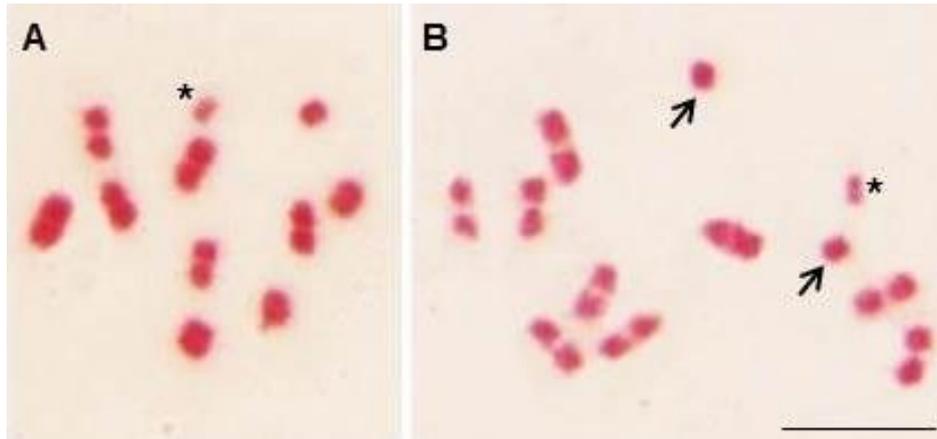
Figure 5. Metaphases meiotics from the experimental crossing between *P. coreodes* and *P. tertius.* A = Metaphases with correct pairing of the homeologous chromosomes of *P. coreodes* ♀ x *P. tertius* ♂. B = Metaphases with chromosome pairing error of *P. tertius* ♀ x *P. coreodes* ♂ as indicated by the arrow. (* = sex chromosomes X and Y).

Based on this, we have demonstrated that *Psammolestes* spp. have compatibility genomic intrageneric, contributing to the evolutionary and taxonomic knowledge of the Rhodniini tribe, an important group of vectors of Chagas' disease.

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Anexo

Licença para realização das coletas e transporte em território nacional.

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 50299-1	Data da Emissão: 21/08/2015 11:17	Data para Revalidação*: 19/09/2016

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Jader de Oliveira	CPF: 382.454.058-41

Título do Projeto: Estudo citogenético, molecular, morfométrico e morfológico de espécies do gênero Psammolestes Bergroth, 1911 (Hemiptera, Reduviidae, Triatominae).

Nome da Instituição : Faculdade de Ciências Farmacêuticas	CNPJ: 48.031.918/0025-00

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta de Triatomíneos	10/2015	01/2019

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas à autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio nº 03/2014 ou na Instrução Normativa ICMBio nº 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para Importação ou exportação de flora e fauna - CITES e não CITES).
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6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

1	Solicita-se que o pesquisador, de acordo com o Plano de Manejo do Parque Nacional da Chapada Diamantina (PNCD), encaminhe à Sede Administrativa desta Unidade um texto, escrito de forma didática e acessível ao grande público, com informações que considerar mais pertinentes (fisiologia, biologia, ecologia, etc.) referentes aos organismos objeto de sua pesquisa, com ilustrações e/ou fotos das mesmas, objetivando a divulgação e sensibilização da sociedade acerca da biodiversidade existente no PNCD. O pesquisador deverá contactar a Sede do PNCD (pamadiamantina@yahoo.com.br; telefone (75) 3332-2310) antes do início das atividades de campo, para informar seu cronograma e itinerário. Finalmente, as publicações geradas deverão ser encaminhadas, preferencialmente por meio eletrônico, ao PNCD.
2	1) Fazer contato com a administração do Parque Nacional do Pantanal Matogrossense (pamapantanal@icmbio.gov.br) com pelo menos 30 dias antes das expedições de campo. 2) A UC oferece alojamento e, quando possível, um servidor para apresentar a área da UC aos pesquisadores.

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	CUIABA	MT	Cuiaba	Fora de UC Federal

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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