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Análise das barreiras reprodutivas no complexo *Triatoma brasiliensis* (Hemiptera, Triatominae): *T. petrocchiae* Pinto e Barreto, 1925 x *T. brasiliensis* Neiva, 1911 e *T. lenti* Sherlock e Serafim (1967)

Botucatu
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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Zoologia), junto ao Programa de Pós-Graduação em Ciências Biológicas (Zoologia) do Instituto de Biociências de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu.

Orientador: Prof. Dr. Kaio CesarChaboli Alevi

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E, por fim, aos membros da banca examinadora que aceitaram o convite de avaliar a dissertação de mestrado.

RESUMO

Os triatomíneos são insetos hematófagos de grande importância epidemiológica, pois atuam como vetores do protozoário *Trypanosoma cruzi*, agente etiológico da doença de Chagas. As espécies de *Triatoma* foram agrupadas em complexos e subcomplexos, com base, principalmente, em dados morfológicos e distribuição geográfica. O complexo *T. brasiliensis* é um grupo monofilético formado pelas espécies *T. juazeirensis*, *T. melanica*, *T. petrocchiae*, *T. lenti*, *T. bahiensis*, *T. sherlocki* e pelas subespécies *T. b. brasiliensis* e *T. b. macromelasoma*. Embora a relação entre *T. petrocchiae* e as espécies do complexo *T. brasiliensis* tenha sido sugerida desde 1970, apenas recentemente estudos morfométricos e filogenéticos confirmaram a inclusão da espécie no complexo. Cruzamentos experimentais entre *T. petrocchiae* e *T. b. brasiliensis* detectaram barreira pré-zigótica interespecífica. No entanto, todas as outras espécies do complexo *T. brasiliensis* são capazes de produzir híbridos, sendo barreiras pós-zigóticas responsáveis pela quebra do híbrido. Com base nisso, o presente trabalho teve como objetivo realizar cruzamentos experimentais entre *T. petrocchiae* e algumas espécies do complexo *T. brasiliensis* (*T. b. brasiliensis* e *T. lenti*) para avaliar/reavaliar as barreiras reprodutivas interespecíficas instaladas entre essa espécie e as espécies/subespécies do complexo *T. brasiliensis*. Híbridos não foram produzidos em nenhuma das direções dos cruzamentos, evidenciando a presença de isolamento pré-zigótico entre *T. petrocchiae* e as outras espécies do complexo *T. brasiliensis*. Dessa forma, confirmamos o *status* específico de *T. petrocchiae*, demonstramos a presença de isolamento reprodutivo em relação à *T. b. brasiliensis* e *T. lenti*, e sugerimos que *T. petrocchiae* é a espécie mais derivada do complexo *T. brasiliensis*.

Palavras-chave: Triatomíneos. Taxonomia. Cruzamentos experimentais. Doença de Chagas.

ABSTRACT

Triatomines are hematophagous insects of great epidemiological importance, as they act as vectors of the protozoan *Trypanosoma cruzi*, the etiologic agent of Chagas disease. The species of *Triatoma* were grouped into complexes and subcomplexes, based mainly on morphological data and geographic distribution. The *T. brasiliensis* complex is a monophyletic group composed of the species *T. juazeirensis*, *T. melanica*, *T. petrocchiae*, *T. lenti*, *T. bahiensis*, *T. sherlocki* and the subspecies *T. b. brasiliensis* and *T. b. macromelasoma*. Although the association between *T. petrocchiae* and the species of the *T. brasiliensis* complex has been suggested since 1970, only recently morphometric and phylogenetic studies have confirmed the inclusion of the species in the complex. Experimental crosses between *T. petrocchiae* and *T. b. brasiliensis* detected an interspecific pre-zygotic barrier. However, all other species of the *T. brasiliensis* complex are capable of producing hybrids, being post-zygotic barriers responsible for the breakage of the hybrid. Based on this, the present work aimed to carry out experimental crosses between *T. petrocchiae* and some species of the *T. brasiliensis* complex (*T. b. brasiliensis* and *T. lenti*) to evaluate/re-evaluate interspecific reproductive barriers installed between this species and the species/subspecies of the *T. brasiliensis* complex. Hybrids were not produced in either direction of the crosses, evidencing the presence of pre-zygotic isolation between *T. petrocchiae* and the other species of the *T. brasiliensis* complex. In this way, we confirm the specific status of *T. petrocchiae*, demonstrate the presence of reproductive isolation in relation to *T. b. brasiliensis* and *T. lenti*, and we suggest that *T. petrocchiae* is the species most derived from the *T. brasiliensis* complex.

Keywords: Triatomines. Taxonomy. Experimental crosses. Chagas disease.

SUMÁRIO

1 INTRODUÇÃO

Os triatomíneos (Hemiptera, Triatominae) são insetos hematófagos de grande importância epidemiológica, pois atuam como vetores do protozoário *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae), agente etiológico da doença de Chagas (WHO, 2020) – enfermidade negligenciada que atinge cerca de oito milhões de pessoas e coloca em risco de infecção, aproximadamente, outras 25 milhões (WHO, 2020). A principal forma de minimizar a incidência de novos casos se fundamenta no controle das populações de vetores (WHO, 2020), sendo os estudos relacionados com esses insetos de extrema importância para a saúde pública, uma vez que podem gerar subsídios para auxiliar os programas de controle de vetores na profilaxia da doença de Chagas.

Atualmente, existem 157 espécies descritas na subfamília Triatominae (sendo 154 espécies vivas e três espécies fósseis), agrupadas em 18 gêneros e cinco tribos (ALEVI et al., 2020, 2021; GALVÃO, 2020; DALLE et al., 2021; ZHAO et al., 2021). Os gêneros *Triatoma* Laporte, 1832, *Rhodnius* Stål, 1859 e *Panstrongylus* Berg, 1879 são os mais importantes do ponto de vista epidemiológico para a doença de Chagas (JUSTI et al., 2014). As espécies de *Triatoma* foram agrupadas em complexos esubcomplexos, com base, principalmente, em dados morfológicos e distribuição geográfica (SCHOFIELD e GALVÃO, 2009). Estudos genéticos complementares agrupam *Triatoma* spp. em oito complexos e nove subcomplexos (Tabela 1) (PITA etal. 2016; ALEVI et al. 2017).

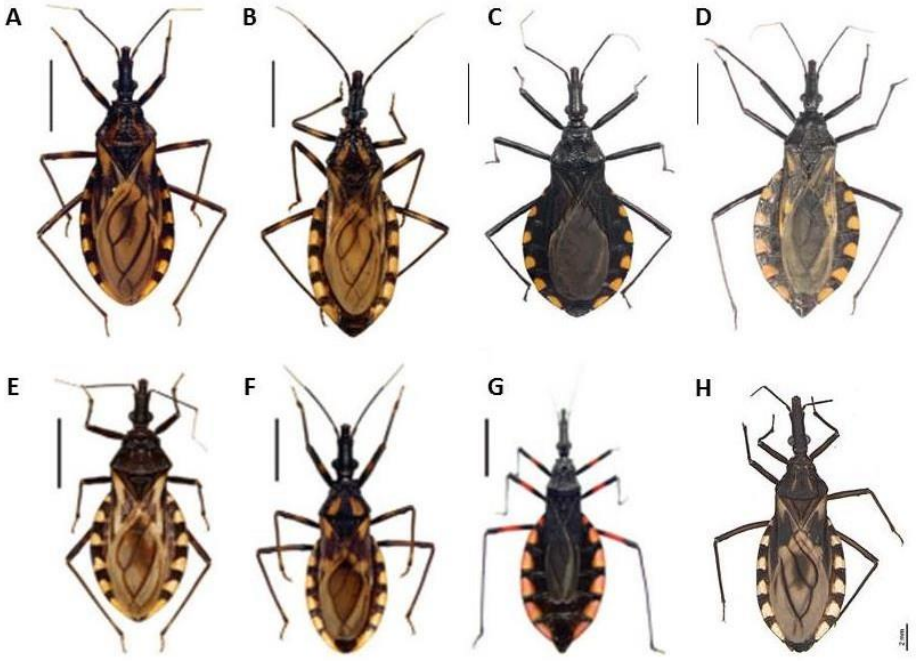
Tabela 1 — Complexos e subcomplexos de *Triatoma* spp.

Complexos	**Subcomplexos**
Phyllosoma	Dimidiata
	Phyllosoma
Flavida	
Rubrofasciata	
Protracta	
Lecticularia	
Dispar	
Infestans	Brasiliensis
	Infestans
	Maculata
	Pseudomaculata
	Rubrovaria
	Sordida
	Vitticeps
Spinolai	

Fonte: Elaborado pela autora.

Embora os complexos e subcomplexos não sejam reconhecidos pelo Código Internacional de Nomenclatura Zoológica (ICZN, 1999), Justi et al. (2014) sugerem que esses agrupamentos de espécies devem formar grupos naturais, ou seja, grupos monofiléticos. O complexo *T. brasiliensis* foi inicialmente proposto por Lucena (1970) como um arranjo sistemático composto, principalmente, por táxons encontrados na região Nordeste do Brasil, a saber: *T. b. brasiliensis* Neiva, 1911, *T. petrocchiae* Pinto & Barreto, 1925, *T. lenti* Sherlock & Serafim, 1967, *T. pessoai* Sherlock e Serafim, 1967 [atualmente sinônimo de *T. lenti* (LENT e WYGODZINSKY, 1979)], *T. bahiensis* Sherlock & Serafim, 1967, *T. b. melanica* Neiva & Lent, 1941 [que teve o *status* específico elevado para *T. melanica* (COSTA et al., 2006)] e *T. b. macromelasoma* Galvão, 1965. Recentemente, estudos filogenéticos demonstram que o complexo *T. brasiliensis* é um grupo monofilético formado pelas espécies *T. melanica*, *T. petrocchiae*, *T. lenti*, *T. bahiensis*, *T. sherlocki* Papa et al., 2002, *T. juazeirensis* Costa & Félix, 2007, e pelas subespécies *T. b. brasiliensis* e *T. b. macromelasoma* (Figura 1) (MONTEIRO et al, 2004; MENDONÇA et al., 2009, 2014; GARDIM et al., 2014; OLIVEIRA et al., 2017).

Figura 1 — Espécies que compõem o complexo *T. brasiliensis*.



Legenda: A. *T. b. brasiliensis*, **B.** *T. b. macromelasoma*, **C.** *T. lenti*, **D.** *T. bahiensis*, **E.** *T. juazeirensis*, **F.** *T. melanica*, **G.** *T. sherlocki*, **H.** *T. petrocchiae*. **Fonte:** Adaptado de Costa et al. (2013), Mendonça et al. (2016) e Oliveira et al. (2022).

Além dessas espécies, Schofield e Galvão (2009), com base em caracteres morfológicos e na distribuição geográfica, agruparam *T. melanocephala* Neiva & Pinto, 1923, *T. tibiamaculata* (Pinto, 1926) e *T. vitticeps* (Stål, 1859) no complexo *T. brasiliensis*. No entanto, Alevi et al. (2012), por meio de dados cariotípicos, propuserama exclusão dessas espécies do complexo *T. brasiliensis*, uma vez que apresentam cariótipos totalmente diferentes dos observados para os outros membros do complexo (sendo *T. tibiamaculata* com 2n = 23 e *T melanocephala* e *T. vitticeps* com 2n = 24 cromossomos, enquanto as espécies do complexo *T. brasiliensis* apresentam 2n = 22). Esses resultados foram corroborados por análises filogenéticas (GARDIM et al., 2014; OLIVEIRA et al., 2017) e cruzamentos experimentais (NEVES et al., 2020).

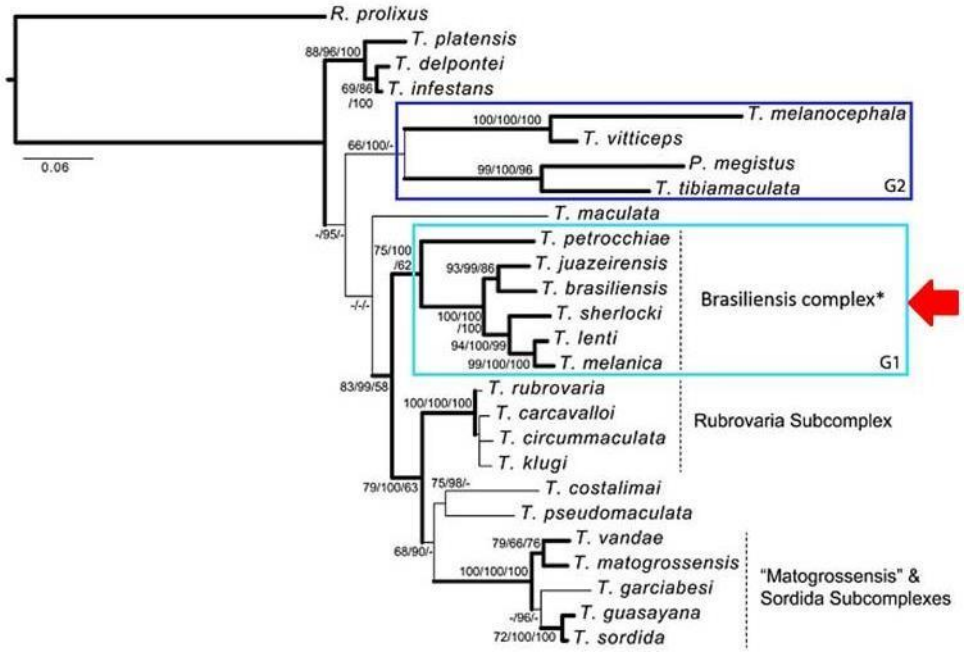
Embora a relação entre *T. petrocchiae* e as espécies do complexo *T. brasiliensis* tenha sido sugerida por Lucena (1970), apenas recentemente estudos morfométricos e filogenéticos confirmaram a inclusão da espécie no complexo (Figura 2) (OLIVEIRA et al., 2017). Essa espécie foi descrita por Pinto e Barreto (1925), baseada em apenas um exemplar proveniente

do Rio Grande do Norte. Os autores consideraram *T. petrocchiae* idêntica a *T. b. brasiliensis*, sendo diferenciadas apenas por *T. petrocchiae* não ter manchas claras nos fêmures. Lent e Wygodzinsky (1979) confirmaram a similaridade existente entre as duas espécies, contudo ressaltaram que *T. petrocchiae* apresentava algumas diferenças morfológicas, tais como ausência de fosseta esponjosa nas tíbias dosmachos, rostro praticamente glabro e primeiro segmento antenal mais curto. Além disso,Monteiro et al. (1998), por meio de parâmetros genéticos, e Espínola (1971), por meio de cruzamento experimental, confirmaram o *status* específico de *T. petrocchiae*.

A realização de cruzamentos experimentais e a análise dos híbridos – com ênfase na taxa de eclosão e mortalidade, bem como na viabilidade reprodutiva e proporção entre machos e fêmeas [uma vez que a regra de Haldane prediz que caso haja eclosão de híbridos, o sexo heterogamético é o primeiro afetado pelos eventos evolutivos que inviabilizam ou levam a esterilidade desse organismo (TURELLI; ORR, 1995)] – é importante para a taxonomia dos triatomíneos, principalmente porque, de acordo com o conceito biológico de espécie apresentado por Mayr et al. (1963) e Dobzhansky (1970), "espécies são grupos de populações naturais que se intercruzam real ou potencialmente e que são isoladas reprodutivamente de outros grupos (produzindo qualquer incompatibilidade reprodutiva entre os parentais, esterilidade dos híbridos ou ambos)".

Espínola et al. (1971) realizaram cruzamentos experimentais entre *T. petrocchiae* e *T. b. brasiliensis* (provenientes de Paulo Afonso, Bahia, Brasil) e detectaram barreira pré-zigótica interespecífica. No entanto, todas as outras espécies do complexo *T. brasiliensis*, quando cruzadas, são capazes de produzir híbridos (COSTA etal., 2003; CORREIA et al., 2013; MENDONÇA et al., 2014, 2016; PINOTTI et al., 2021), sendo barreiras pós-zigóticas responsáveis pela quebra do híbrido (MENDONÇAet al., 2014, 2016; ALEVI et al. 2018). Com base nisso, realizar cruzamentos experimentais entre *T. petrocchiae* e alguns táxons do complexo *T. brasiliensis* (por exemplo, *T. b. brasiliensis* e *T. lenti*) mostra-se importante para avaliar/reavaliar as barreiras reprodutivas interespecíficas instaladas entre essa espécie e as espécies/subespécies do complexo *T. brasiliensis*.

Figura 2 — Filogenia desenvolvida com genes mitocondriais (12S, 16S, COI e Cytb), confirmando a inclusão de *T. petrocchiae* no complexo *T. brasiliensis* (seta).



Fonte: Adaptado de Oliveira et al. (2017).

2 OBJETIVOS

2.1 Objetivo geral

Realizar cruzamentos experimentais interespecíficos entre *T. petrocchiae* e *T. b. brasiliensis* e entre *T. petrocchiae* e *T. lenti*, com o intuito de avaliar/reavaliar as barreiras reprodutivas interespecíficas instaladas entre essa espécie e as espécies/subespécies do complexo *T. brasiliensis*.

2.2 Objetivos específicos

a) Avaliar a dinâmica evolutiva dos cruzamentos experimentais, por meio da análise da cópula interespecífica, da oviposição, da taxa de eclosão dos ovos e da viabilidade dos híbridos;

b) Realizar um levantamento sobre os triatomíneos já notificados no município de Paulo Afonso, Bahia, com intuito de confirmar se o membro do complexo *T.*

brasiliensis que Espínola (1971) cruzou com *T. petrocchiae* foi realmente *T. b. brasiliensis*;

c) Avaliar exemplares de *T. b. brasiliensis* e *T. petrocchiae* coletados em Paulo Afonso, Bahia e depositados em coleções entomológicas, a fim de garantir que os triatomíneos foram identificados de forma correta.

3 RESULTADOS (apresentados na forma de artigo científico)

3.1 Artigo científico publicado na revista internacional *Insects*

DELGADO L.M.G, et al. Revisiting the Hybridization Processes in the *Triatoma brasiliensis* Complex (Hemiptera, Triatominae): Reproductive Isolation between *Triatoma petrocchiae* and *T. b. brasiliensis* and *T. lenti*. Insects, v. 12, p. 1015, 2021. Doi: https://doi.org/10.3390/insects12111015.

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Communication

Revisiting the Hybridization Processes in the *Triatoma brasiliensis* Complex (Hemiptera, Triatominae): Reproductive Isolation between *Triatoma petrocchiae* and *T. b. brasiliensis* and *T. lenti*

Luiza Maria Grzyb Delgado [1,†], Jader de Oliveira [2,3,†], Amanda Ravazi [1], Fernanda Fernandez Madeira [4], Yago Visinho dos Reis [1], Heloisa Pinotti [3], Ana Beatriz Bortolozo de Oliveira [4], Isabella da Silva Masarin [1], Maurício Lilioso [5], Elaine Folly-Ramos [6], Cleber Galvão [7,*], Maria Tercília Vilela de Azeredo-Oliveira [4], João Aristeu da Rosa [3] and Kaio Cesar Chaboli Alevi [1,2,3]

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Simple Summary: Although all triatomines are potential vectors of Chagas disease, there are species with greater or lesser vectorial importance. Therefore, the correct identification of triatomine species is essential for vector control programs. In general, triatomines are identified by external morphological characters. However, some species are very similar or even morphologically identical, making the use of complementary analyses for the correct identification of species important. For this reason, this study focused on the use of experimental crosses to assess the specific status of species of the *Triatoma brasiliensis* species complex. The crosses did not result in hybrids, demonstrating that there are pre-zygotic reproductive barriers installed between *T. petrocchiae* and the other species of the *T. brasiliensis* complex (which confirms the specific status of the species according to the biological species concept). On the basis of the results above, we demonstrated that *T. petrocchiae* is reproductively isolated from *T. b. brasiliensis* and *T. lenti*. Furthermore, we suggest that *T. petrocchiae* is the species most derived from the *T. brasiliensis* complex.

Abstract: *Triatoma petrocchiae* is a species morphologically similar to *T. b. brasiliensis* (which resulted in a synonymization event); despite this similarity, genetic, morphological, and experimental crossbreeding studies confirmed the specific status of *T. petrocchiae*. Considering that both species have been reported living in sympatry and that, for a long time, most species of the *T. brasiliensis* complex were considered only chromatic variants of *T. b. brasiliensis*, we carried out experimental crosses between *T. b. brasiliensis* and *T. petrocchiae* (to confirm whether these species are reproductively isolated) and

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between *T. lenti* and *T. petrocchiae* (to assess whether *T. petrocchiae* also presents prezygotic isolation with the other species of the *T. brasiliensis* complex). Reciprocal experimental crosses were conducted, and weekly, the eggs were collected, counted, and separated in new containers to assess the hatch rate. Neither cross resulted in hybrids, demonstrating that there are pre-zygotic reproductive barriers installed between *T. petrocchiae* and the other species of the *T. brasiliensis* complex. On the basis of the results above, we demonstrated that *T. petrocchiae* is reproductively isolated from *T. b. brasiliensis* and *T. lenti*. Furthermore, we suggest that *T. petrocchiae* is the species most derived from the *T. brasiliensis* complex.

Keywords: triatomines; reproductive barriers; hybridization; speciation; Chagas disease vectors

1. Introduction

Triatomines (Hemiptera, Triatominae) are hematophagous insects of great importance to public health, as they are considered the main form of transmission of the protozoan *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae), the etiological agent of Chagas disease [1] (a neglected disease that affects about 8 million people and results in 10,000 deaths per year [1]). There are currently 157 species grouped into 18 genera and five tribes [2–5]. The Triatomini and Rhodniini tribes have the largest number of species (115 and 24, respectively) and are the most important from an epidemiological point of view (since *Panstrongylus megistus* (Burmeister, 1835), *Triatoma infestans* Klug, 1834, *T. brasiliensis brasiliensis* Neiva, 1911, *T. dimidiata* (Latreille, 1811), and *Rhodnius prolixus* Stål, 1859 are of worldwide importance in the transmission of the disease [6]).

The genus *Triatoma* Laporte, 1832 is the most representative (81 species) and the most morphologically diversified [6,7]. This genus is paraphyletic [8,9], and species are grouped into complexes and subcomplexes [9–12]. The *T. brasiliensis* complex is a grouping of endemic species from Brazil [13] composed of six species and two subspecies that share a common ancestry: *T. b. brasiliensis* Neiva, 1911, *T. b. macromelasoma* Galvão, 1965, *T. juazeirensis* Costa and Félix, 2007, *T. sherlocki* Papa et al., 2002, *T. petrocchiae* Pinto and Barreto, 1925, *T. lenti* Sherlock and Serafim, 1967, *T. bahiensis* Sherlock and Serafim, 1967, and *T. melanica* Neiva and Lent, 1941 [14–18]. The last taxon grouped in this complex was *T. petrocchiae* [18], a species reported in the states of Bahia, Ceará, Pernambuco, Paraíba, and Rio Grande do Norte [13,19]; however, the potential distribution map published by Caranha et al. [20] suggests that this species could also be found in the states of Piauí, Alagoas, and Sergipe, where the species has not been recorded to date.

The species *T. brasiliensis sensu stricto* is currently divided into two subspecies (*T. b. brasiliensis* and *T. b. macromelasoma*), which can be differentiated by morphological characters: *T. b. brasiliensis* presents a pronotum with 1 + 1 brownish-yellow areas extending from the posterior portion of the anterior lobe to the posterior lobe, femora with broad brownish-yellow rings, and membrane of hemelytra with a lumen of cells that are not darkened; *T. b. macromelasoma* presents a pronotum with 1 + 1 narrow brownish-yellow stripes on the submedian carinae, not attaining its apex, legs with an incomplete brownish-yellow ring on the apical half of the femora, and hemelytra with membrane cells that are darkened on the central portion [21]. In addition to the phenotypic divergences, these species have a different geographic distribution: while *T. b. brasiliensis* has been noted in the states of Ceará, Maranhão, Paraíba, Piauí, and Rio Grande do Norte, *T. b. macromelasoma* is endemic to Pernambuco [13,19].

Triatoma petrocchiae is a species morphologically similar to *T. b. brasiliensis*, which led Lucena [22] to propose the synonymization of species, considering *T. petrocchiae* only as a chromatic variant of *T. b. brasiliensis*. However, Espínola [23] carried out experimental crosses between *T. b. brasiliensis* and *T. petrocchiae* from Paulo Afonso, Bahia, Brazil, and observed that these species did not produce viable hybrids. On the basis of this, Lent and

Wygodzinsky [24] revalidated the specific status of *T. petrocchiae* from morphological data (the status was corroborated with genetic analyses using allozyme electrophoresis [25]).

The interspecific crosses performed by Espínola [23] were proposed because chromatic variations were observed in the populations of *T. b. brasiliensis* from Paulo Afonso, Bahia. The authors indicated that there are similarities in the coloration between *T. b. brasiliensis* and *T. petrocchiae* and, above all, these species share the same ecological niche. Considering that these species have been reported living in sympatry [26,27] and that, in 1971, most species of the *T. brasiliensis* complex were still considered only chromatic variants of *T. b. brasiliensis*, there is a need to confirm whether *T. b. brasiliensis* and *T. petrocchiae* are really reproductively isolated (mainly because all other species in this complex are capable of producing hybrids [15,28–32]). On the basis of the assumptions above, we carried out experimental crosses between *T. b. brasiliensis* and *T. petrocchiae* (to corroborate the results of Espínola [23]) and between *T. lenti* and *T. petrocchiae* (to assess whether *T. petrocchiae* also presents prezygotic isolation with the other species of the *T. brasiliensis* complex).

2. Materials and Methods

Reciprocal experimental crosses were conducted between *T. b. brasiliensis* (from Currais Novos (Pedra do Sino), Rio Grande do Norte, Brazil, collected in wild ecotopes (geographic coordinates: 6°17′06.8″ S 36°29′51.9″ W)) and *T. petrocchiae* (from Caicó, Rio Grande do Norte, Brazil, collected in wild ecotopes (geographic coordinates: 6°27′47.8″ S 37°09′11.3″ W)) and between *T. lenti* (from Macaúbas, Bahia, Brazil, collected in peridomiciliary ecotopes (geographic coordinates: 13°11′25.7″ S 42°31′56.3″ W)) and *T. petrocchiae* (Figure 1). The insects used in the experiment came from colonies kept in the Triatominae insectary of the School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, São Paulo, Brazil. The experimental crosses were conducted in the Triatominae insectary, according to the experiments of Mendonça et al. [30], Neves et al. [33], and Pinotti et al. [32]: the insects were sexed as 5th instar nymphs [34], and males and females were kept separately until they reached the adult stage to guarantee the virginity of the insects used in the crosses. For the experimental crosses, three couples from each set were placed in plastic jars (diameter 5 cm × height 10 cm) (each couple in a jar) and kept at room temperature (average of 24 °C [35]) and an average relative humidity of 63% [35]). Weekly, the couples were fed on duck blood, and the eggs were collected, counted, and separated into new containers to assess the hatch rate.

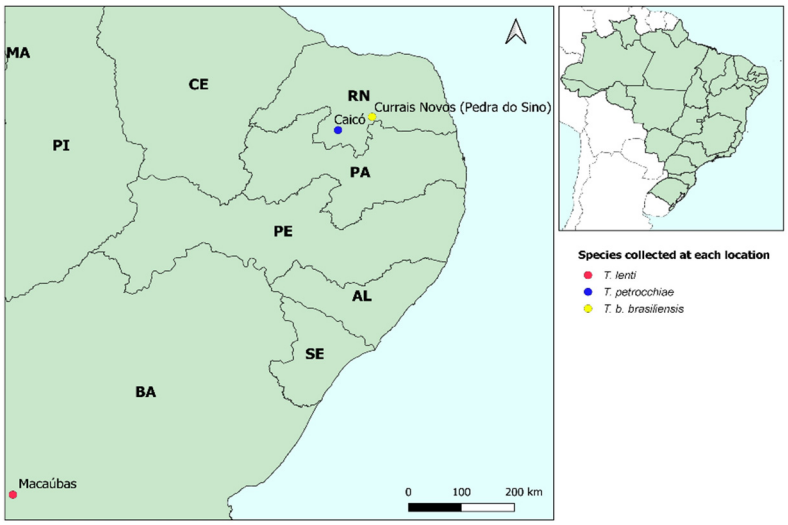

Figure 1. Distribution map of species used in experimental crosses.

3. Results and Discussion

The experimental crosses between *T. petrocchiae* and *T. b. brasiliensis* did not result in hybrids (Table 1), demonstrating that there are pre-zygotic reproductive barriers installed

between these species (confirming the specific status of *T. petrocchiae* according to the biological species concept [36,37]). These results obtained for the cross between *T. b. brasiliensis* and *T. petrocchiae* from Rio Grande do Sul (the state where the species were also collected in the same rock outcrop spot [38]) agree with those obtained by Espínola [23] when they crossed specimens from Paulo Afonso, Bahia.

Table 1. Experimental crosses performed between *T. petrocchiae* x *T. b. brasiliensis* and *T. lenti*.

Crossing Experiments					Number of Eggs				Egg Fertility
					C1	C2	C2	Total	
♀	*T. b. brasiliensis*	x	*T. petrocchiae*	♂	48	40	56	144	0%
♀	*T. petrocchiae*	x	*T. b. brasiliensis*	♂	38	45	37	120	0%
♀	*T. lenti*	x	*T. petrocchiae*	♂	42	33	27	102	0%
♀	*T. petrocchiae*	x	*T. lenti*	♂	36	28	22	86	0%
Parental Crossings									
♀	*T. b. brasiliensis*	x	*T. b. brasiliensis*	♂	-	-	-	414	95,4%
♀	*T. petrocchiae*	x	*T. petrocchiae*	♂	-	-	-	58	86,2%
♀	*T. lenti*	x	*T. lenti*	♂	-	-	-	179	57,5%

Whereas in 1971, the current species *T. melanica*, *T. sherlocki*, and *T. juazeirensis* were considered only phenotypic variants and/or subspecies of *T. b. brasiliensis* [14,39–42], we conducted a survey of the literature on the triatomine already noted in Paulo Afonso, Bahia to confirm which species Espínola [22] had crossed with *T. petrocchiae*, and we observed that only *T. b. brasiliensis* and *T. petrocchiae* were the species of the *T. brasiliensis* complex notified for the municipality [43]. Furthermore, to ensure that the *T. brasiliensis* complex triatomines collected in Paulo Afonso, Bahia, were correctly identified as *T. b. brasiliensis* and *T. petrocchiae*, we evaluated some specimens collected in this municipality that were deposited in the entomologic collections of the Faculty of Public Health of the University of Sao Paulo, Brazil (Figure 2). On the basis of this information, we confirmed that the specimens used in the experiments of Espínola [23] were *T. b. brasiliensis*.

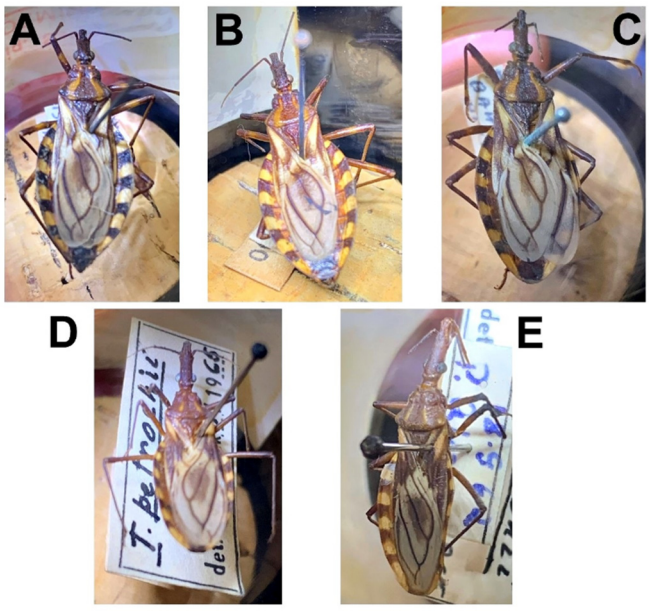

Figure 2. Triatomines deposited in the entomologic collections of the Faculty of Public Health of the University of Sao Paulo, Brazil. (**A**,**B**) *T. b. brasiliensis* ♀(Brazil, Paulo Afonso, BA. Ident. Sherlock/68); (**C**) *T. b. brasiliensis* ♂(Brazil, Paulo Afonso, BA. Ident. Sherlock/68); (**D**,**E**) *T. petrocchiae* ♂(Brazil, Paulo Afonso, BA. Ident. Sherlock/62).

Although Espínola [23] suggested that *T. petrocchiae* and *T. b. brasiliensis* share the same ecological niche, Liloso et al. [27] recently demonstrated that while *T. b. brasiliensis* is mainly associated with rodents, the food sources of *T. petrocchiae* were strongly associated with reptiles of the *Tropidurus* and *Hemidactylus* genera; this suggests that *T. petrocchiae* is the single member within this complex that is associated with reptiles, indicating a distinct niche occupation related to the trophic resources. These results point to the possible presence of a prezygotic reproductive barrier due to ecological isolation between *T. petrocchiae* and members of the *T. brasiliensis* complex. However, other possible prezygotic barriers cannot be ruled out, such as mechanical isolation, as the morphological analysis of the external female genitalia evidenced some unique characteristics for *T. petrocchiae* [44].

Experimental crosses between *T. petrocchiae* and *T. lenti* also did not result in hybrids (Table 1). Unlike *T. b. brasiliensis*, which cohabits rock outcrops with *T. petrocchiae* [27,38], there are no reports of *T. petrocchiae* and *T. lenti* living in sympatry (on the contrary, they inhabit municipalities in the state of Bahia that are at least 800 km away [43]). This result is in accordance with what was proposed by Oliveira et al. [18], which suggests that *T. petrocchiae* is the most distant species from the *T. brasiliensis* complex. The genomic incompatibility resulting in the inability to produce hybrids with *T. lenti* points to the hypothesis that *T. petrocchiae* was possibly the first species to be derived from the common ancestor of the *T. brasiliensis* complex (since all other species in the complex produce hybrids [15,28–32]).

The prezygotic isolation observed between *T. petrocchiae* and species of the *T. brasiliensis* complex was only observed when members of this complex (*T. b. brasiliensis*) were crossed with other subcomplexes, such as *T. sordida* [45], *T. infestans* [45], and *T. vitticeps* subcomplexes [33] (which are species phylogenetically distant from *T. b. brasiliensis* [8,9]). The reproductive barrier that possibly prevents hybrids between other species of the *T. brasiliensis* complex is based on post-zygotic reproductive isolation due to hybrid collapse (as noted by Mendonça et al. [30] and Alevi et al. [31]). It was believed that *T. melanica* was the most differentiated form of the complex [37]; however, according to the results of experimental crosses and the high genetic distance observed between *T. petrocchiae* and members of the *T. brasiliensis* complex [46], the most differentiated species from a genetic point of view is *T. petrocchiae*.

Before concluding, it is worth mentioning that the low number of eggs produced by crosses can be a limiting factor for the research. Furthermore, it is important that new crosses between *T. petrocchiae* and all members of the *T. brasiliensis* complex be carried out to confirm that this species is indeed reproductively isolated from all other species in the complex or whether prezygotic isolation is restricted to *T. b. brasiliensis* and *T. lenti* (as noted by Espínola et al. [23] and in the present manuscript).

4. Conclusions

On the basis of the results above, we demonstrated that *T. petrocchiae* is reproductively isolated from *T. b. brasiliensis* and *T. lenti* (confirming the specific status of *T. petrocchiae*). Furthermore, we demonstrated that these species have prezygotic reproductive isolation and suggest that *T. petrocchiae* is the species most derived from the *T. brasiliensis* complex.

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4 CONCLUSÃO

Com base nos cruzamentos experimentais, confirmamos o status específico de *T. petrocchiae*, demonstramos a presença de isolamento reprodutivo em relação à *T. b. brasiliensis* e *T. lenti*, e sugerimos que *T. petrocchiae* é a espécie mais derivada do complexo *T. brasiliensis*.

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