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Especiação em Triatominae (Hemiptera, Reduviidae): seria o número de cromossomos uma barreira reprodutiva pré-zigótica para os vetores da doença de Chagas?

Botucatu
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Tese apresentada como parte dos requisitos para obtenção do título de Doutor 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 Cesar Chaboli Alevi

Coorientadora: Prof. Dra. Mara Cristina Pinto

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"Nada em biologia faz sentido exceto à luz da evolução"

(DOBZHANSKY, 1973, p. 125-129)

RESUMO

Os triatomineos são insetos hematófagos de grande importância epidemiológica, pois podem transmitir o protozoário *Trypanosoma cruzi*, agente etiológico da doença de Chagas. Diversos estudos citogenéticos já foram realizados na subfamília Triatominae, contribuindo para a elucidação de problemáticas relacionadas com a taxonomia e sistemática desses vetores. Os cariótipos de varias espécies já foram descritos, sendo as alterações no número de cromossomos (que variam de 2n = 21 a 25 cromossomos no sexo heterogamético) relacionadas a eventos de fusão e fissão cromossômica a partir do cariótipo ancestral 2n = 22 (proposto com base no número modal dos cariótipos descritos em Triatominae). Essas alterações já foram relacionadas a eventos de isolamento reprodutivo e especiação em outros grupos de insetos e plantas. Além disso, recentemente foi levantada uma hipótese de que diferentes cariótipos, possivelmente, podem inviabilizar a formação de híbridos de triatomíneos. Dessa forma, caracterizamos quais barreiras de isolamento reprodutivo estão presentes entre espécies de triatomíneos com número de cromossomos diferentes e avaliamos as implicações de possíveis eventos de anagênese e cladogênese relacionados a mudanças no número de cromossomos ao longo do processo evolutivo dos vetores da doença de Chagas. Nossos resultados demonstram que: (1) o cariótipo ancestral de Triatominae é 2n = 22; (2) durante o processo evolutivo, pelo menos nove eventos cladogenéticos associados a alterações no número de cromossomos podem ter ocorrido nessa subfamília; e que (3) essas alterações podem atuar como barreira pré-zigótica em Triatominae (isolamento cariotípico), sendo importantes eventos evolutivos para a diversificação das espécies.

Palavras-chaves: triatomineos; cruzamentos experimentais; barreira cariotípica; ChromoSSE.

ABSTRACT

Triatomines are hematophagous insects of great epidemiological importance, as they can transmit the protozoan *Trypanosoma cruzi*, the etiological agent of Chagas disease. Several cytogenetic studies have already been carried out in Triatominae subfamily, contributing to the elucidation of problems related to the taxonomy and systematics of these vectors. The karyotypes of many species have already been described, with changes in the number of chromosomes (ranging from 2n = 21 to 25 chromosomes in the heterogametic sex) related to chromosomal fusion and fission events from the ancestral karyotype 2n = 22 (proposed based on the modal number of the karyotypes described in Triatominae). These changes have already been related to reproductive isolation and speciation events in other groups of insects and plants. In addition, a hypothesis was recently raised that different karyotypes may possibly make the formation of triatomine hybrids unfeasible. Thus, we characterized which reproductive isolation barriers are present between triatomine species with different chromosome numbers and evaluated the implications of possible anagenesis and cladogenesis events related to changes in chromosome number throughout the evolutionary process of Chagas disease vectors. Our results demonstrate that: (1) the ancestral karyotype of Triatominae is 2n = 22; (2) during the evolutionary process, at least nine cladogenetic events associated with alterations in the number of chromosomes may have occurred in this subfamily; and that (3) these alterations can act as a pre-zygotic barrier in Triatominae (karyotypic isolation), being important evolutionary events for species diversification.

Keywords: triatomines; experimental crosses; karyotypic barrier; ChromoSSE.

SUMÁRIO

1. INTRODUÇÃO

A doença de Chagas, causada pelo protozoário *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae), é uma enfermidade negligenciada transmitida, principalmente, de forma vetorial, por meio de insetos da subfamília Triatominae (Hemiptera, Reduviidae) (WHO, 2022). Esses insetos são hematófagos obrigatórios em todos os estádios do desenvolvimento (ninfais e adultos de ambos os sexos), e estão suscetíveis a ingerirem o parasito durante o repasto sanguíneo (GALVÃO, 2014). A transmissão, por outro lado, ocorre por meio das fezes/urina desses insetos, uma vez que eles possuem o hábito de defecar/urinar durante ou logo após a hematofagia (DIAS; NETO; LUNA, 2011; GALVÃO, 2014).

Estima-se que cerca de sete milhões de pessoas estejam infectadas ao redor do mundo, além de haver mais de 100 milhões de pessoas em risco de infecção por habitarem áreas endêmicas (RASSI et al., 2010). São conhecidas, atualmente, 154 espécies viventes de triatomíneos (e mais três espécies fósseis), distribuídas em 18 gêneros e cinco tribos (ALEVI et al., 2021; CORREIA et al., 2022; GIL-SANTANA et al., 2022). Apesar de todas as espécies serem potenciais vetoras do *T. cruzi*, existem aquelas de importância primária, secundária e silvestres (GALVÃO, 2014).

As espécies de importância primária apresentam alta antropofilia, colonizando regiões domiciliares de maneira permanente; já as de importância secudária apresentam diferentes graus de antropofilia e se adaptam bem aos ecótopos artificias (geralmente associados ao peridomicílio), podendo formar pequenas colônias intradomiciliares transitórias (principalmente na ausência dos vetores primários) (FIOCRUZ, 2017). Por fim, os triatomíneos silvestres são responsáveis pela manutenção do *T. cruzi* no ambiente silvestre, podendo eventualmente participar do ciclo peridoméstico e doméstico (principalmente quando os vetores são atraídos pela luz) (FIOCRUZ, 2017).

Os relatos de hibridização experimental são relativamente comuns na subfamília Triatominae, já tendo sido obtidos híbridos interespecíficos em diversos gêneros, como *Mepraia*

Mazza, Gajardo & Jörg, 1940 (CAMPOS-SOTO et al., 2016), *Panstrongylus* Berg, 1879 (VILLACÍS et al., 2020), *Psammolestes* Bergroth, 1911 (RAVAZI et al., 2021), *Rhodnius* Stål, 1859 (DÍAZ et al., 2014) e, principalmente, *Triatoma* Laporte, 1832 (COSTA et al., 2003; PÉREZ et al., 2005; BELISÁRIO et al., 2007, CORREIA et al., 2013; MENDONÇA et al., 2014, 2016; ALEVI et al., 2018; CESARETTO et al., 2021; PINOTTI et al., 2021). Conhecer o potencial de hibridização entre esses vetores é muito importante do ponto de vista epidemiológico, uma vez que os híbridos podem apresentar maior capacidade vetorial em relação aos parentais (MARTINEZ-IBARRA et al., 2016, 2017, 2021; MERAZ-MEDINA et al., 2019).

Além dos aspectos epidemiológicos, quando experimentos de hibridização demonstram isolamento reprodutivo entre os táxons avaliados, é possível corroborar o *status* específico dos parentais, baseando-se no conceito biológico de espécie (MAYR, 1963, 2001). Dessa forma, os cruzamentos experimentais interespecíficos são grandes aliados para auxiliar na taxonomia de espécies relacionadas. Em Triatominae, por exemplo, o *status* específico de espécies do complexo *brasiliensis* (COSTA et al., 2003; CORREIA et al., 2013; MENDONÇA et al., 2014, 2016, DELGADO et al., 2021; PINOTTI et al., 2021), do complexo *phyllosoma* (MARTÍNEZ-IBARRA et al., 2008, 2011, 2016) e do complexo *dimidiata* (GARCÍA et al., 2013) já foram validados por meio de cruzamentos experimentais.

Sabe-se que o isolamento reprodutivo geralmente evolui de forma lenta, ao longo de diversas gerações, sendo muitas vezes necessário um conjunto de barreiras para, de fato, as espécies estarem totalmente isoladas (KULMUNI et al, 2020). Por outro lado, as alterações cromossômicas (estruturais e numéricas), por exemplo, podem estar associados a um forte isolamento reprodutivo (ESCUDERO et al., 2016; DE VOS et al., 2020; KULMUNI et al, 2020). Para os triatomineos, foram observadas, recentemente, barreiras pré-zigóticas entre algumas espécies com cariótipo diferente, as quais foram relacionadas às diferenças no número de cromossomos (NEVES et al., 2020; REIS et al., 2022). Assim, a realização de novos cruzamentos experimentais abrangendo um maior número de espécies com números de

cromossomos diferentes pode contribuir para a elucidação de como essas alterações cariotípicas atuam no isolamento reprodutivo dos triatomineos.

Atualmente, os cariótipos de 102 táxons são conhecidos, os quais variam de 2n = 21 a 25 cromossomos (nos sexos heterogaméticos) (PANZERA et al., 1996, 2010; REIS; ALEVI, 2021). Os cromossomos desses vetores (assim como de todos os outros hemípteros) são de natureza holocêntrica, ou seja, apresentam cinetócoro difuso (diferente dos monocêntrico em que o cinetócoro é restrito à região centromérica) (UESHIMA, 1966). Em teoria, eventos de reorganização do genoma nesse tipo de cromossomo podem acarretar em menos problemas de segregação durante a divisão celular, uma vez que as fibras do fuso meiótico/mitótico podem se ligar em qualquer região cromossômica (LUCEK; AUGUSTIJNEN; ESCUDERO, 2022).

A origem desses cariótipos tem sido relacionada a eventos de fusão e fissão cromossômica a partir do cariótipo ancestral [o qual é proposto como 2n = 22 cromossomos, baseado no número modal (UESHIMA, 1966)] (ALEVI et al., 2018; PANZERA; PITA; LORITE, 2021). Considerando a hipótese de que as alterações no cariótipo podem atuar no isolamento reprodutivo e, consequentemente, na diversificação das espécies dessa subfamília, os estudos de evolução cariotípica envolvendo modelos, como o ChromoSSE (FREYMAN; HOHNA, 2018), podem contribuir para o conhecimento evolutivo desses vetores.

Dessa forma, com base na importância dos estudos cariotípicos, bem como dos estudos de hibridização experimental para o conhecimento evolutivo e epidemiológico dos triatomineos, realizamos uma revisão acerca dos cariótipos já conhecidos nessa subfamília (Capítulo 1) e avaliamos o papel das alterações cariotípicas no isolamento reprodutivo dos triatomineos, discutindo como essas mudanças no número de cromossmos ocorreram ao longo do processo evolutivo desses vetores (Capítulo 2).

2. OBJETIVOS

2.1 Objetivo geral

Caracterizar quais barreiras de isolamento reprodutivo estão presentes entre espécies de triatomíneos com número de cromossomos diferentes e avaliar as implicações de possíveis eventos de anagênese e cladogênese relacionados a mudanças no número de cromossomos ao longo do processo evolutivo dos vetores da doença de Chagas.

2.2 Objetivos específicos

a) Avaliar a dinâmica dos cruzamentos experimentais entre espécies com diferentes cariótipos, por meio da análise da cópula interespecífica, da oviposição, da taxa de eclosão dos ovos e da viabilidade dos híbridos;

b) Testar a hipótese de que o número de cromossomos pode atuar como uma barreira reprodutiva pré-zigótica para Triatominae;

c) Realizar estudos de evolução cariotípica em Triatominae, utilizando modelagem de evolução cromossômica (como o ChromoSSE).

3. RESULTADOS e DISCUSSÃO

Os resultados e a discussão serão apresentados na forma de capítulo de livro e artigo científico.

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REVISÃO CARIOTÍPICA DOS VETORES DA DOENÇA DE CHAGAS

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RESUMO

Os triatomíneos (Hemiptera, Triatominae) são insetos de grande importância para a saúde pública por serem considerados como a principal forma de transmissão da doença de Chagas (DC). São conhecidas 157 espécies, distribuídas em 18 gêneros e cinco tribos, sendo todas elas potenciais vetores da DC. A citogenética tem sido amplamente utilizada como ferramenta taxonômica para esses insetos. Algumas características cromossômicas, como o cariótipo, por exemplo, podem ser utilizadas como diagnósticas para algumas espécies, bem como para discutir questões sistemáticas e, até mesmo, para a criação de chaves dicotômicas de identificação. Atualmente, 102 cariótipos são conhecidos, o que representa 65% das espécies válidas. Com base no exposto, agrupamos as informações cariotípicas disponíveis na literatura e ressaltamos a importância dessas análises nas demais espécies da subfamília Triatominae, uma vez que essas informações são de grande importância para o conhecimento evolutivo, taxonômico e, até mesmo, entomoepidemiológico dos vetores da DC.

Palavras-chave: Triatomíneos, Cariótipo e Conjunto cromossômico diploide.

ABSTRACT

Triatomines (Hemiptera, Triatominae) are insects of great importance for public health because they are considered as the main form of transmission of Chagas disease (CD). There are 157 species, distributed in 18 genera and five tribes, all of which are potential vectors of CD. Cytogenetics has been widely used as a taxonomic tool for these insects. Some chromosomal characteristics, such as karyotype, for example, can be used as diagnoses for some species, as well as to discuss systematic issues and even for the creation of dichotomous identification keys. Currently, 102 karyotypes are known, representing 65% of valid species. Based on the above, we grouped the karyotype information available in the literature and we emphasize the importance of these analyses in other species of the Triatominae subfamily, since this information is of great importance for the evolutionary, taxonomic and even entomoepidemiological knowledge of CD vectors.

Keywords: Triatomines, Karyotype and Diploid chromosome set.

1. INTRODUÇÃO

A doença de Chagas (DC), causada pelo protozoário *Trypanosoma cruzi* (Chagas, 1909) (Kinetoplastida, Trypanosomatidae), afeta cerca de sete milhões de pessoas ao redor do mundo (WHO, 2021). A transmissão do parasito ocorre, principalmente, por meio das fezes/urina de triatomíneos (Hemiptera, Triatominae) infectados, os quais têm o hábito de defecar durante o repasto sanguíneo (GALVÃO, 2014; WHO, 2021). Apesar de vários surtos de infecção oral terem sido notificados nas últimas décadas (DIAS et al., 2008; COURA, 2015), vale destacar que o inseto também está envolvido nessa forma de transmissão (MONSALVE-LARA et al., 2021), ressaltando, assim, a importância do controle populacional dos vetores para a prevenção da doença.

São conhecidas 157 espécies de triatomíneos (sendo três fósseis), as quais são divididas em 18 gêneros e cinco tribos (Tabela 1) (ALEVI et al., 2020; DALE; JUSTI; GALVÃO, 2021; ZHAO; GALVÃO; CAI, 2021). Todas elas são consideradas como potenciais vetoras do *T. cruzi*, mas variam no grau de importância epidemiológica (sendo classificadas como espécies de importância primária, secundária e silvestres) (GALVÃO, 2014). Dessa forma, o conhecimento dos aspectos biológicos, ecológicos, genéticos, comportamentais, taxonômicos e epidemiológicos desses insetos auxilia no direcionamento dos órgãos responsáveis pelo controle vetorial (GALVÃO, 2014; OLIVEIRA et al., 2020).

Tabela 1. Tribos, gêneros e espécies agrupados na subfamília Triatominae.

Tribos	**Gêneros**	**Espécies**
Alberproseniini	*Alberprosenia*	2
Bolboderini	*Belminus*	9
	Bolbodera	1
	Microtriatoma	2
	Parabelminus	2
Cavernicolini	*Cavernicola*	2
Rhodniini	*Psammolestes*	3
	Rhodnius	21
Triatomini	*Dipetalogaster*	1
	Eratyrus	2
	Hermanlentia	1
	Linshcosteus	6
	Mepraia	3
	Nesotriatoma	3
	Panstrongylus	15
	Paratriatoma	2
	Triatoma	81
	Paleotriatoma	1
Total		**157**

Os triatomíneos também são modelos biológicos clássicos para estudos celulares (CARVALHO; RECCO-PIMENTEL, 2013). Esses insetos apresentam algumas peculiaridades relacionadas à morfologia (cromossomos holocêntricos) e o comportamento dos cromossomos (meiose invertida para os cromossomos sexuais) (PANZERA et al., 1996). Além disso, informações cromossômicas têm contribuído para a taxonomia e sistemática dos triatomíneos (ALEVI et al., 2012; ALEVI et al., 2020; PANZERA; PITA; LORITE, 2021), o que torna os estudos citogenéticos recorrentes na subfamília Triatominae.

Entre as diversas análises cromossômicas que podem ser aplicadas nos estudos taxonômicos e sistemáticos, a caracterização do cariótipo é a mais antiga, sendo o primeiro conjunto cromossômico diploide descrito em 1909 (PAYNE, 1909). Atualmente, 102 cariótipos são conhecidos (PANZERA et al., 1996, ALEVI; ROSA; AZEREDO-OLIVEIRA, 2013; ALEVI et al., 2016; PANZERA et al., 2021). Considerando a importância desses estudos para a classificação dos vetores da DC (BORSATTO et al., 2019, BORSATTO; AZEREDO-OLIVEIRA; ALEVI, 2019), agrupamos as informações cariotípicas disponíveis na literatura.

2. REVISÃO DA LITERATURA

Como já mencionado acima, o cariótipo de *Triatoma sanguisuga* (LeConte, 1856) foi o primeiro descrito na literatura (PAYNE, 1909). Após 41 anos, novos cariótipos foram descritos (SCHREIBER; PELLEGRINO, 1950) e, em 1966, Ueshima (1966), além de descrever o cariótipo de 20 espécies de triatomíneos, propôs, pela primeira vez, a aplicação da citogenética como ferramenta taxonômica (citotaxonomia). Até o momento, 102 cariótipos foram caracterizados (Tabela 2) (PANZERA et al., 1996; ALEVI; ROSA; AZEREDO-OLIVEIRA, 2013; ALEVI et al., 2016; PANZERA; PITA, LORITE, 2021), representando 65% das espécies conhecidas da subfamília Triatominae.

A utilização do número de cromossomos na taxonomia das espécies de triatomíneos foi iniciado em 2012, quando Alevi et al. (2012) propuseram a exclusão das espécies *T. melanocephala* Neiva & Pinto, 1923, *T. vitticeps* (Stål, 1859) e *T. tibiamaculata* (Pinto, 1926) do subcomplexo *T. brasiliensis*. Atualmente os triatomíneos estão agrupados em oito complexos e nove subcomplexos (Tabela 3) (SCHOFIELD; GALVÃO, 2009; PITA et al., 2016; ALEVI et al., 2017), sendo a maioria dos agrupamentos de espécies com um número padrão de cromossomos, com exceção dos complexos protracta, lecticularia e spinolai (Tabela 3).

Tabela 2. Número de cromossomos de 102 táxons da subfamília Triatominae.

Cariótipo (2n)	**Gêneros:** Espécies
21 = 18A + X_1X_2Y	***Panstrongylus****: megistus* ***Triatoma****: nitida*
22 = 20A + XY	***Psammolestes****: arthuri, coreodes, tertius* ***Rhodnius****: brethesi, colombiensis, domesticus, ecuadoriensis, marabaensis, milesi, montenegrensis, nasustus, neglectus, neivai, pallescens, pictipes, prolixus, robustus, stali* ***Dipetalogaster****: maximus* ***Paratriatoma****: hirsuta, lecticularia* ***Triatoma****: arthurneivai, bahiensis, baratai, boliviana, brasiliensis (b. brasiliensis, b. macromelasoma), carcavalloi, carrioni, circummaculata, costalimai, delpontei, dispar, garciabesi, guasayana, guazu, infestans, jatai, juazeirensis, jurbergi, klugi, lenti, maculata, matogrossensis, melanica, patagonica, petrocchiae, pintodiasi, platensis, pseudomaculata, rosai, rubrovaria, sherlocki, sordida, vandae, venosa, williami, wygodzinskyi*
23 = 20A + X_1X_2Y	***Belminus****: herreri, corredori* ***Eratyrus****: cuspidatus, mucronatus* ***Mepraia****: gajardoi, parapatrica, spinolai* ***Nesotriatoma****: confusa, flavida* ***Panstrongylus****: chinai, geniculatus, howardi, lignarius, rufotuberculatus, tupynambai* ***Triatoma****: barberi, bassolsae, dimidiata, gerstaeckeri, hegneri, huehuetenanguensis, longipennis, mazzotti, mexicana, mopan, pallidipennis, peninsularis, phyllosoma, picturata, protracta, recurva, rubida, ryckmani, sanguisuga, sinaloensis, tibiamaculata*
24 = 20A + $X_1X_2X_3Y$	***Panstrongylus****: lutzi* ***Triatoma****: eratyrusiformis, melanocephala, vitticeps, breyeri*
25 = 22A + X_1X_2Y	***Triatoma****: rubrofasciata*

Tabela 3. Número de cromossomos presentes nos complexos e subcomplexos de vetores da doença de Chagas.

Complexos	Subcomplexos	Cariótipo
Phyllosoma	Dimidiata	2n = 23
	Phyllosoma	2n = 23
Flavida		2n = 23
Rubrofasciata		2n = 25
Protracta		2n = 21, 23
Lecticularia		2n = 22, 23
Dispar		2n = 22
Infestans	Brasiliensis	2n = 22
	Infestans	2n = 22
	Maculata	2n = 22
	Pseudomaculata	2n = 22
	Rubrovaria	2n = 22
	Sordida	2n = 22
	Vitticeps	2n = 24
Spinolai		2n = 24, 23

Embora a maioria das espécies de triatomíneos apresente 22 ou 23 cromossomos (Figura 1), o cariótipo foi proposto como característica diagnóstica para algumas espécies: *T. rubrofasciata* (De Geer, 1773), por exemplo, é a única espécie de Triatominae com 25 cromossomos, o que permite diferenciá-la de todas as espécies dessa subfamília (ALEVI et al., 2015); *T. nitida* Usinger, 1939, por sua vez, apresenta 21 cromossomos e também pode ser diferenciada de todas as outras do gênero *Triatoma* Laporte, 1832 (SCHREIBER; PELLEGRINO, 1950); além disso, *Panstrongylus lutzi* (Neiva & Pinto, 1923) (2n = 24) (ALEVI et al., 2017) e *P. megistus* (Burmeister, 1835) (2n = 21) (SCHREIBER; PELLEGRINO, 1950) podem ser diferenciadas de todas as outras espécies de *Panstrongylus* Berg, 1879 que apresentam 23 cromossomos (Tabela 2).

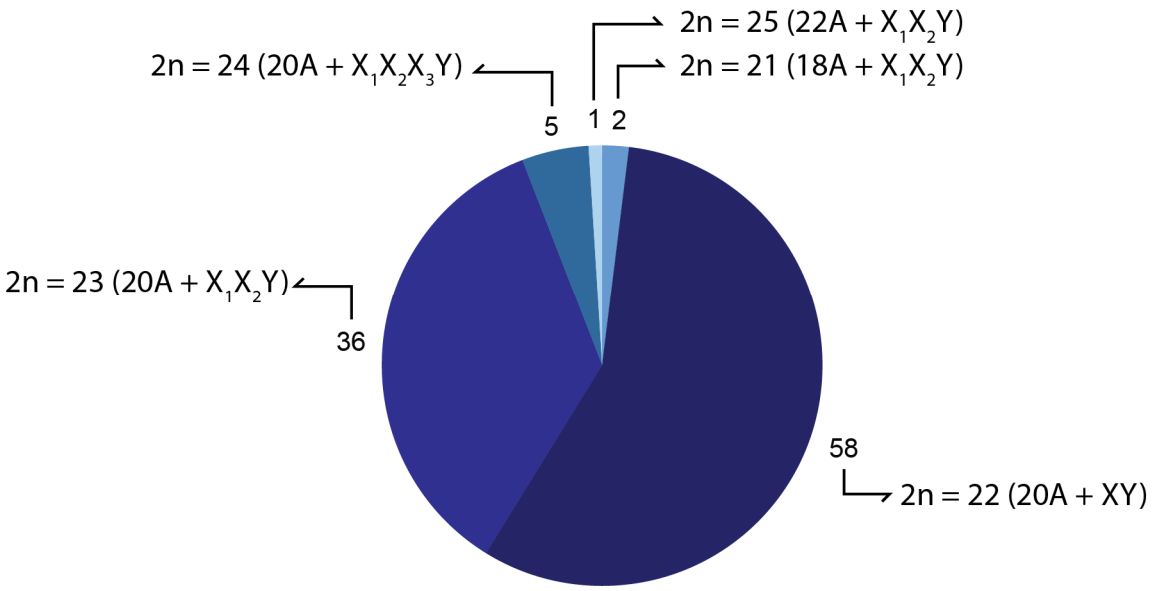

Figura 1. Número de táxons com cada cariótipo conhecido em Triatominae.

Panzera, Pita e Lorite (2021) apresentaram, pela primeira vez, uma possível espécie nova de *Panstrongylus* com 2n = 22 cromossomos, no entanto, ressaltamos que na descrição formal dessa espécie é necessário que análises moleculares sejam feitas para confirmar se realmente é um *Panstrongylus* ou uma nova espécie de outro gênero com convergência evolutiva para as características morfológicas de *Panstrongylus*. Esse mesmo evento, por exemplo, foi sugerido para *T. tibiamaculata* (JUSTI et al., 2014). Essa espécie, embora ainda seja considerada um *Triatoma*, apresenta relação filogenética com *Panstrongylus* (JUSTI et al., 2014, JUSTI; GALVÃO; SCHRAGO, 2016). Do ponto de vista cariotípico, *T. tibiamaculata* diverge de todas as espécies de *Triatoma* da América do Sul (2n = 22 ou 24) e se assemelha a *Panstrongylus* spp. (2n = 23) (PANZERA et al., 1998; ALEVI et al., 2018).

Alevi et al. (2018) realizaram um amplo estudo relacionado a evolução cariotípica em Triatominae. Os autores sugeriram que a divergência de cariótipo entre as espécies do complexo lecticularia [*Paratriatoma hirsuta* Barber, 1938 e *P. lecticularia* (Stål, 1859), com 2n = 22 cromossomos; e *T. rubida* (Uhler, 1894) e *T. ryckmani* Zeledón & Ponce, 1972, com 2n = 23 cromossomos], tenha sido decorrente de uma fissão no cromossomo sexual X do ancestral comum desses dois grupos [uma vez que essas espécies formam um clado monofilético (JUSTI; GALVÃO; SCHRAGO, 2016)], que provavelmente possuía 2n = 22 cromossomos (ALEVI et al., 2018). Da mesma forma, no complexo spinolai, a variação no número de cromossomos também é atribuída a um evento de fissão no cromossomo X, sendo observado em *T. eratyrusiformis* Del Ponte, 1929 e *T. breyeri* Del Ponte, 1929 2n = 24

cromossomos, enquanto que nas demais espécies, 2n = 23 cromossomos (ALEVI et al., 2018).

Como observado acima, a maioria dos eventos relacionados à evolução cariotípica dos triatomíneos estão associados ao cromossomo sexual X, tendo ocorrido diversas vezes, de forma independente, durante a diversificação das espécies (PANZERA; PITA, LORITE, 2021). Os eventos relacionados a alterações nos autossomos são bastante pontuais (PANZERA et al., 2021). No complexo protracta, por exemplo, todas as espécies apresentam 2n = 23 cromossomos, com exceção de *T. nitida*, a única espécie de *Triatoma* com 21 cromossomos (SCHREIBER; PELLEGRINO, 1950; PANZERA et al., 1996). Essa alteração numérica é decorrente de divergência no número de autossomos, sendo 20 presentes na maioria das espécies do complexo protracta e 18 presentes em *T. nitida* (PANZERA et al., 1996, ALEVI; ROSA; AZEREDO-OLIVEIRA, 2013). Possivelmente, essa diferença é decorrente de um evento de fusão, assim como sugerido para *P. megistus* (ALEVI et al., 2018), embora eventos de perda cromossômica não possam ser descartados. Além dessa espécie, divergência no número de autossomos também pode ser observada em *P. megistus* (18 autossomos) e *T. rubrofasciata* (22 autossomos) (SCHREIBER; PELLEGRINO, 1950; PANZERA et al., 1996, ALEVI et al., 2015).

Recentemente, o cariótipo tem sido agregado com outras informações citogenéticas dos triatomíneos e chaves dicotômicas foram desenvolvidas: Borsatto et al. (2019), Borsatto, Azeredo-Oliveira e Alevi (2019) e Oliveira, Rosa e Alevi (2021) apresentaram chaves de classificação para diferentes estados brasileiros (Alagoas, Amapá, Ceará, Roraima, Santa Catarina, São Paulo e Espírito Santo) e, sobretudo, Gonzalez-Britz et al. (2021) apresentaram uma chave para o Paraguai.

3. CONSIDERAÇÕES FINAIS

Com base nas informações cariotípicas, fica evidente a importância de caracterizar o número de cromossomos dos vetores da DC. Assim, destacamos que novos estudos devem ser realizados, principalmente entre as 52 espécies que ainda não tiveram o cariótipo descrito, uma vez que esse conhecimento pode auxiliar no entendimento evolutivo, taxonômico e, até mesmo, entomoepidemiológico dos triatomíneos.

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3.2 Artigo científico aceito para publicação na revista internacional *International Journal of Molecular Science* (FI 6,2)

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Article

Karyotype Evolution in Triatominae (Hemiptera, Reduviidae): the Role of Chromosomal Rearrangements in the Diversification of Chagas Disease Vectors

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Abstract: Several cytogenetic studies have already been performed in Triatominae, such that different karyotypes could be characterized (ranging from 2n = 21 to 25 chromosomes), being the changes in the number of chromosomes related mainly to fusion and fission events. These changes have been associated with reproductive isolation and speciation events in other insect groups. Thus, we evaluated whether different karyotypes could act in the reproductive isolation of triatomines and we analyzed how the events of karyotypic evolution occurred along the diversification of these vectors. For this, experimental crosses were carried out between triatomine species with different karyotypes. Furthermore, based on a phylogeny with 88 triatomine taxa (developed with different molecular markers), a reconstruction of ancestral karyotypes and of anagenetic and cladogenetic events related to karyotypic alterations was performed through the ChromoSSE chromosomal evolution model. All crosses performed did not result in hybrids (prezygotic isolation in both directions). Our modeling results suggest that during Triatominae diversification, at least nine cladogenetic events may be associated with karyotype change. Thus, we emphasize that these alterations in the number of chromosomes can act as a prezygotic barrier in Triatominae (karyotypic isolation), being important evolutionary events during the diversification of the species of Chagas disease vectors.

Keywords: ChromoSSE; experimental crosses; phylogenetic analysis; karyotypic isolation

1. Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi* (Chagas, 1909) (Kineto-

plastida, Trypanosomatidae), has no cure in the chronic phase and affects about seven million people worldwide [1,2]. The main form of transmission of this protozoan is vectorial, through the feces/urine of infected triatomines (Hemiptera, Triatominae) [3,4], since these insects are obligatory hematophagous and have the habit of defecating/urinating during the blood meal [3,4]. Currently, 157 species of triatomines are known (three are fossils), which are distributed in 18 genera and five tribes [5–7].

The generation of hybrids is relatively common between the species of this subfamily, with the main evolutionary events responsible for the decline of the hybrid lineage (postzygotic barriers) being inviability (offspring mortality before reaching adulthood) [8], sterility (partially or completely infertile offspring) [9,10] and the collapse of the hybrid (high offspring mortality from the second generation) [11,12]. Several studies involving experimental crosses have already been carried out among the triatomines, focusing mainly on taxonomic [8,11,13–23] and epidemiological aspects [24–27]. On the other hand, although prezygotic barriers are less frequent and, generally, are present in only one direction of the crossings, as observed between *Rhodnius colombiensis* Mejia, Galvão and Jurberg, 1999 and *R. pallescens* Barber, 1932 [28], *Triatoma pseudomaculata* Corrêa and Espínola, 1964 and *T. infestans* (Klug, 1834) [29], *T. delpontei* Romaña and Abalos, 1947 and *T. platensis* Neiva, 1913 [30] and *T. longipennis* Usinger, 1939 and *T. mopan* Dorn et al., 2018 [17], they may be present between species that are very distant from the phylogenetic point of view [29,31] and between species of different genera (*Triatoma* Laporte, 1832 × *Panstrongylus* Berg, 1879, *Triatoma* × *Rhodnius* Stål, 1859 and *Rhodnius* × *Psammolestes* Bergroth, 1911 [18,23,31]).

Recently, experimental crosses between triatomine species with different chromosome numbers were performed, and it was demonstrated that there is prezygotic isolation [18,32]. Based on these observations, the authors suggested karyotypic variation may be an important factor in the reproductive isolation of these insects [18,32]. In Lepidoptera, for example, it has been suggested that in some genera, karyotypic changes may be related to speciation events [33]. Thus, it is possible that these numerical changes also played a relevant role in the diversification of Triatominae species.

Ueshima [34], based on the modal number, proposed that the ancestral karyotype of triatomines would be 2n = 22 chromosomes (20A + XY, in males; 20A + XX, in females), so that the karyotypic variation present in the current species (2n = 21 to 25 chromosomes) would have arisen after chromosomal fission and fusion events (although aneuploidy events related to chromosomal loss cannot be ruled out) [34–39]. Several studies involving the evolution of the karyotype in Triatominae were carried out from inferences made based on the phylogenetic relationships of the species [36–38]. However, there are specific models for chromosomal evolution studies, such as ChromEvol [40,41] and ChromoSSE [42], which can help to understand how these changes occurred throughout the evolutionary process of these vectors.

In general, the application of the ChromEvol model allows the changes in the number of chromosomes along the branches of a phylogenetic tree (anagenesis) to be evaluated, also allowing to infer the ancestral karyotype at each phylogeny node [41]. The ChromoSSE model allows the evaluation, under a Bayesian approach, not only of anagenetic processes, but also of cladogenetic events related to karyotypic changes [42].

Based on the above, we performed several interspecific crosses to evaluate the role of the karyotype in the reproductive isolation of triatomine species. In addition, we evaluated the implications of possible anagenesis and cladogenesis events related to changes in the number of chromosomes throughout the evolutionary process of Chagas disease vectors.

2. Results and Discussion

All interspecific crosses performed between species with different chromosome numbers did not result in hybrids (Table 1). On the other hand, intraspecific crosses (control) showed hatching rates ranging from 51 to 86% (Table 1). Furthermore, the

Bayesian phylogeny obtained (Figure S1) and used in studies related to karyotypic evolution (Figure 1) showed a topology similar to that of the main phylogenetic reconstructions for Triatominae available in the literature [43,44], being most clusters with good support (posterior probability > 0.8).

Table 1. Number of eggs and hatching rate resulting from experimental crosses between species with different chromosome numbers. CN: diploid chromosome number.

Experimental Crosses			Number of Eggs	Hatching Rate (%)	Reference
Female (CN)	**x**	**Male (CN)**			
Interspecific cross					
T. longipennis (23)	x	*T. vitticeps* (24)	26	0	This paper
T. vitticeps (24)	x	*T. longipennis* (23)	72	0	This paper
T. longipennis (23)	x	*T. infestans* (22)	38	0	This paper
T. infestans (22)	x	*T. longipennis* (23)	45	0	This paper
T. infestans (22)	x	*T. protracta* (23)	146	0	This paper
T. protracta (23)	x	*T. infestans* (22)	93	0	This paper
T. protracta (23)	x	*P. lecticularia* (22)	160	0	This paper
P. lecticularia (22)	x	*T. protracta* (23)	82	0	This paper
P. tibiamaculatus (23)	x	*T. brasiliensis* (22)	60	0	This paper
T. brasiliensis (22)	x	*P. tibiamaculatus* (23)	193	0	This paper
T. pseudomaculata (22)	x	*P. tibiamaculatus* (23)	150	0	This paper
P. tibiamaculatus (23)	x	*T. pseudomaculata* (22)	102	0	This paper
T. melanocephala (24)	x	*P. tibiamaculatus* (23)	102	0	This paper
P. tibiamaculatus (23)	x	*T. melanocephala* (24)	237	0	This paper
T. rubrovaria (22)	x	*P. tibiamaculatus* (23)	21	0	This paper
P. tibiamaculatus (23)	x	*T. rubrovaria* (22)	53	0	This paper
T. infestans (22)	x	*P. tibiamaculatus* (23)	174	0	This paper
P. tibiamaculatus (23)	x	*T. infestans* (22)	93	0	This paper
T. brasiliensis (22)	x	*T. vitticeps* (24)	90	0	[18]
T. vitticeps (24)	x	*T. brasiliensis* (22)	147	0	[18]
T. melanocephala (24)	x	*T. brasiliensis* (22)	78	0	[18]
T. brasiliensis (22)	x	*T. melanocephala* (24)	63	0	[18]
P. megistus (21)	x	*P. tibiamaculatus* (23)	107	0	[32]
P. tibiamaculatus (23)	x	*P. megistus* (21)	265	0	[32]
P. megistus (21)	x	*P. lignarius* (23)	157	0	[32]
P. lignarius (23)	x	*P. megistus* (21)	523	0	[32]
Intraspecific cross					
T. infestans	x	*T. infestans*	439	62	This paper
T. protracta	x	*T. protracta*	278	86	This paper
P. lignarius	x	*P. lignarius*	700	51	[32]
P. megistus	x	*P. megistus*	372	68	[32]
P. tibiamaculatus	x	*P. tibiamaculatus*	190	65	[18]
T. brasiliensis	x	*T. brasiliensis*	271	59	[18]
T. melanocephala	x	*T. melanocephala*	302	63	[18]
T. vitticeps	x	*T. vitticeps*	353	70	[18]

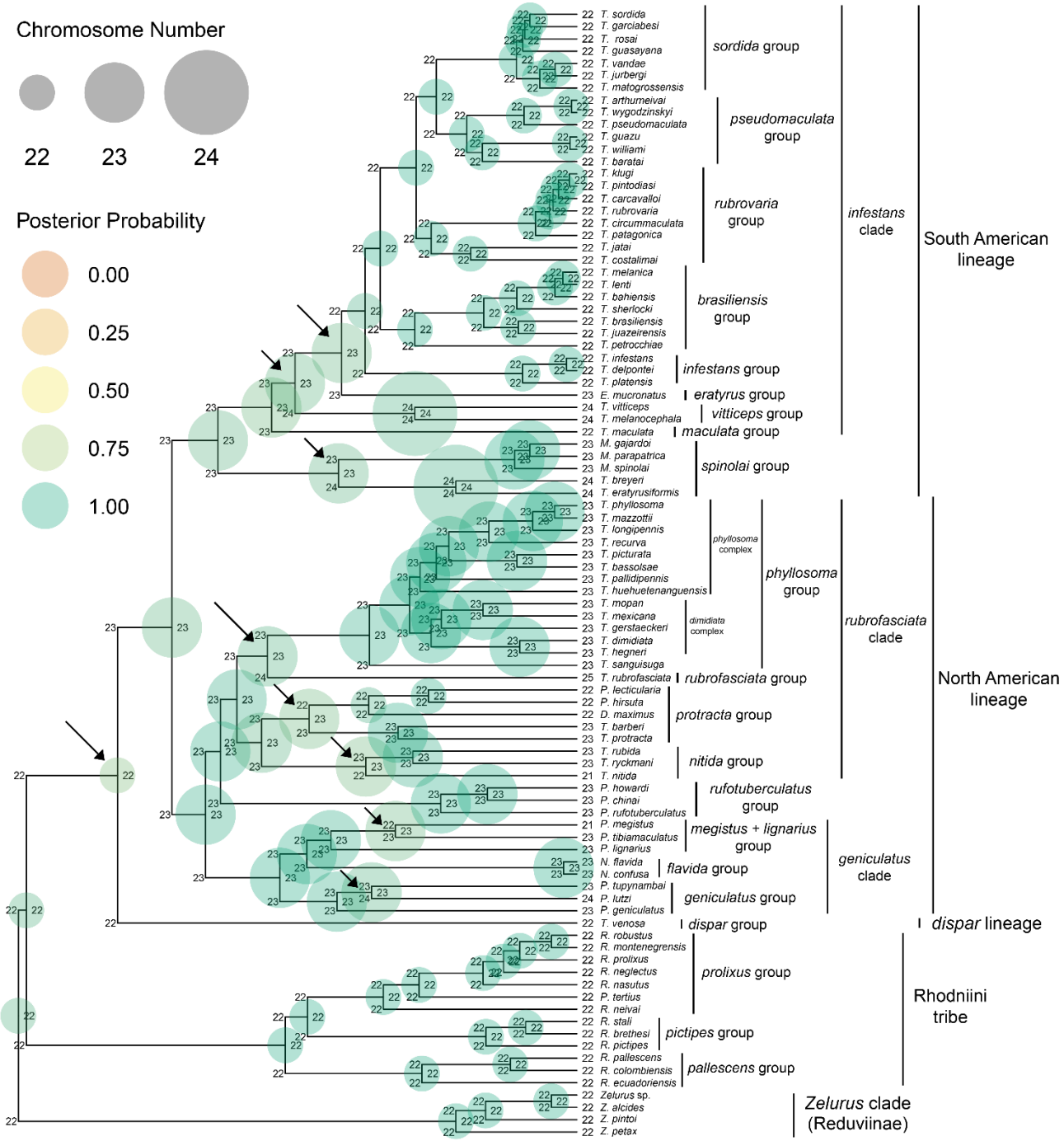

Figure 1. Maximum a posteriori (MAP) estimate of the chromosome number in triatomine ancestors (nodes). The number observed on the "shoulders" shows the karyotype of each lineage just after cladogenesis. Arrows indicate where karyotype alterations may have played a role in cladogenesis.

In general, the subfamily Triatominae and the Rhodniini and Triatomini tribes were recovered as monophyletic groups (Figure 1). In addition, all species groups were recovered as monophyletic, satisfying the proposal by Justi et al. [43] who consider that the clusters (groups, complexes and subcomplexes) should represent natural groups. Curiously, *T. guasayana* Wygodzinsky and Abalos, 1949 was recovered together with the *sordida* group and not with the *rubrovaria* group (Figure 1) (different from what was recently proposed by Belintani et al. [45]) and the *rufotuberculatus* group did not group with the rest of the genus *Panstrongylus* (Figure 1) (different from what was recently observed by

Bittinelli et al. [46]). In addition, our results demonstrated that the species of the *spinolai* complex are more closely related to triatomines from South America (Figure 1) (as noted by Justi et al. [43] and Pita et al. [47]).

As previously mentioned, the number of chromosomes in the Triatominae subfamily ranges from 21 to 25 (in males): 2n = 21 (18A + X_1X_2Y), 2n = 22 (20A + XY), 2n = 23 (20A + X_1X_2Y), 2n = 24 (20A + $X_1X_2X_3Y$) and 2n = 25 (22A + X_1X_2Y) [37–39]. The phylogenetic reconstruction recovered the ancestral karyotype of triatomines as 2n = 22 (PP = 1.0) (Figure 1), corroborating the proposal made by Ueshima [34] based on the modal number. However, the analysis also suggested that the current species of the Triatomini tribe with 22 chromosomes (with the exception of the *dispar* group) may have arisen from ancestors who already had X chromosome fragmentation (Figure 1). In addition, nine cladogenetic events related to changes in chromosome number may have occurred in Triatominae (Figure 1, arrows).

Chromosomal changes associated with cladogenetic events occurred in both autosomes and sex chromosomes. It is important to emphasize that, in species in which there was an increase or a decrease in a pair of autosomes [*T. nitida* Usinger, 1939, *T. rubrofasciata* (De Geer, 1773) and *P. megistus* (Burmeister, 1835)], the model considered this change as two independent events for each homologous chromosome: for *T. nitida* and *P. megistus*, from 23 to 22 (as a cladogenetic event) and later from 22 to 21 (anagenetic); for *T. rubrofasciata*, from 23 to 24 (cladogenetic) and from 24 to 25 (anagenetic) (this was because the model considers only one modification at a time). However, as the chromosomes are organized in pairs in 2n (bivalent) cells, we considered this as just a cladogenetic event.

Our results indicated that the Rhodniini tribe is the most basal group and presents the same karyotype as the ancestor 2n = 22 (Figure 1). However, we emphasize that if species from the Cavernicolini, Bolboderini and Alberprosini tribes (which have few sequences deposited and/or have never been studied cytogenetically) were included, a different topology could have been recovered. Thus, we will discuss the cladogenetic events related to the karyotypic alterations of the Triatomini tribe (Figure 1, arrows) for the three groupings: the dispar lineage, the South American lineage and the North American lineage.

2.1. Dispar Lineage

The vicariance event related to the separation of the *dispar* group [represented by *T. venosa* (Stål, 1872)] from the other species of the Triatomini tribe is related to the uplift of the Western Cordillera of North America [44]. Our results suggest that the first cladogenetic event related to the fission of the X sex chromosome may have occurred during this separation (Figure 1). However, as *Belminus* Stål, 1859 species (Bolboderini tribe) were recently recovered as a sister group to Triatomini [48], we cannot rule out the possibility that the ancestor of the Triatomini tribe had X chromosome fragmentation, since species of the genus *Belminus* have 2n = 23 [36,39]. We highlight that *Belminus* spp. were not included due to the low availability of related sequences in GenBank. Thus, when considering the Bolboderini tribe (and, consequently, the ancestral karyotype 2n = 23), it is possible that the diversification of the ancestors of the *dispar* group in relation to the other species of the Triatomini tribe may be due to a fusion event (or loss) of one X chromosome.

2.2. North American Lineage

North American triatomines are divided into two clades and eight groups (the *phyllosoma* group is composed of the *phyllosoma* and *dimidiata* complexes) (Figure 1). Alevi et al. [38] suggested that the karyotype differences observed between the species of the *geniculatus* clade may be due to the fusion (or loss) of a pair of autosomes (in the ancestors of *P. megistus*) and the fission of the X chromosome [in the ancestors of *P. lutzi* (Neiva and Pinto, 1923)]. Our modeling results suggest that these chromosomal changes may have

played a role in cladogenesis between *P. lutzi* and *P. tupynambai* Lent, 1942 (Figure 1, arrow) and between *P. megistus* and *P. tibiamaculatus* (Pinto, 1926) (Figure 1, arrow). The separation of the ancestors of *P. megistus* and *P. tibiamaculatus* has already been related to the formation of a dry corridor between the Atlantic Forest and the Amazon rainforest after the uplift of the Andes, which acted as a vicariant event [44]. Considering that allopatric species may not develop mechanisms that make hybridization between them unfeasible [49], the prezygotic isolation observed in experimental crosses between *P. megistus* and *P. tibiamaculatus* [also observed between *P. megistus* and *P. lignarius* (Walker, 1873)] (Table 1) may be related to the difference in the number of chromosomes [32]. Thus, the alteration of the karyotype in the ancestors of *P. megistus* may have contributed to the isolation between these species.

The position of the *rufotuberculatus* group in the phylogeny is quite intriguing, since it is placed outside the *geniculatus* clade and is closer to the *rubrofasciata* clade (composed of species of the genus *Triatoma*) (Figure 1). The position of *P. rufotuberculatus* (Champion, 1899) has previously been questioned [48,50,51], a greater phylogenetic proximity of this species to the *rubrofasciata* clade being observed [48,50]. Thus, further studies should be performed in order to elucidate these evolutionary relationships.

Panstrongylus noireaui Gil-Santana et al., 2022 was recently described from specimens initially classified as *P. rufotuberculatus* from Bolivia [7]. Phylogenetic studies have recovered *P. noireaui* as a sister species of *P. rufotuberculatus* [52]. However, in contrast to *P. rufotuberculatus* and all other *Panstrongylus* species, this species has 22 chromosomes [52]. The authors suggest that the size of the X chromosome of this species is equivalent to that of the sum of the X chromosomes (X_1 and X_2) of *P. rufotuberculatus*, which indicates the possible occurrence of chromosomal fusion [52]. Thus, despite *P. noireaui* was not included in our analysis, we emphasize the possibility that this chromosomal fusion had a cladogenetic role between these two species. Although the authors suggested that hybridization may occur between them [52], we believe that the difference in the number of chromosomes acts as a prezygotic barrier between *P. noireaui* and *P. rufotuberculatus*.

Cladogenetic events resulting from karyotypic changes may also have occurred in the *nitida* and *protracta* groups (Figure 1). The origin of the *T. nitida* karyotype is related to the fusion (or loss) of a pair of autosomes (as in *P. megistus*) [38]. Thus, it is possible that this change in the number of chromosomes have promoted a reproductive isolation between the ancestor of *T. nitida* and the ancestor of the lineage *T. rubida* and *T. ryckmani* (Figure 1). In the *protracta* group, the fusion (or loss) of an X chromosome in the ancestors of the *Paratriatoma* Barber, 1938 and *Dipetalogaster* Usinger, 1939 clades may have occurred in a cladogenetic manner (Figure 1), resulting in the isolation of the ancestors of these species from the ancestors that gave rise to *T. protracta* (Uhler, 1894) and *T. barberi* Usinger, 1939. Experimental crosses have already been carried out between *T. protracta* and *T. barberi*, revealing reproductive compatibility, obtaining hybrids up to the second generation [53]. Already in crosses between *T. barberi* and *T. rubida* (Uhler, 1894), hybrids were produced, but they were not viable [53]. However, in the crosses carried out between *T. protracta* and *P. lecticularia* (Stål, 1859) (previously included in the genus *Triatoma* [54]) we found the presence of a prezygotic barrier (Table 1). This reinforces the importance of karyotypic alterations in the reproductive isolation of these vectors, since even between species of these groups that are phylogenetically more distant but that present the same karyotype (*T. barberi* and *T. rubida*), there are no prezygotic barriers [53].

Regarding the *rubrofasciata* clade, the gain of a pair of autosomes in *T. rubrofasciata* [38] may also have acted as a cladogenetic event, resulting in the isolation of the ancestors of this species (Figure 1). However, several phylogenetic analyses have grouped *T. rubrofasciata* with species of the genus *Linshcosteus* Distant, 1904 and other species of Old World *Triatoma* [43,44,48], for which there are no cytogenetic data (thus, they were not included in phylogenetic studies). Justi et al. [44] suggested a Neotropical origin for the clade involving these species (25–10 Ma), with later separation of the ancestors that would originate the Old World species (from those that would give rise to *T. rubrofas-*

ciata). Therefore, the dispersion of *T. rubrofasciata* to the locations where it is found today (more than 40 countries [55]) would have occurred only recently by ships [44,51]. On the other hand, Kieran et al. [48] observed that *T. rubrofasciata* clusters with *T. bouvieri* Larrousse, 1924 and *T. migrans* Breddin, 1903 (present in the Old World), suggesting that the origin of this species is in the Old World. Considering the possibility of hybridization after secondary contact between allopatric species [56], if Old World species have the same number of chromosomes as *T. rubrofasciata*, it is possible that natural crosses followed by introgression events may have occurred between these species, as observed in *Rhodnius* [57]. Thus, the grouping of these species observed by Kieran et al. [48] may be related to these events, and the origin of *T. rubrofasciata* is in fact neotropical, emphasizing the need for cytogenetic studies, as well as tests of experimental crosses between these species to elucidate the origin and diversification of these triatomines.

2.3. South American Lineage

The South American triatomines are divided into nine groups (Figure 1). The first cladogenetic event related to the variation in the number of chromosomes that may have occurred in this lineage may be associated with the diversification of the species of the *spinolai* group (Figure 1). This group is formed by *T. breyeri* Del Ponte, 1929, *T. eratyrusiformis* Del Ponte 1929 and by *Mepraia* spp. [51]. The phylogenetic position of this group within the Triatomini tribe is still controversial, as in some phylogenetic reconstructions, they are grouped in the South American lineage [as well as in our analysis (Figure 1)] [43], while in other studies, in the North American one [44,48]. Based on karyotypic studies, it was suggested that the karyotype origin of *T. breyeri* and *T. eratyrusiformis*, both with 2n = 24 chromosomes, was due to one X chromosome fission event in the ancestors of the *spinolai* group [36,38]. Our results support the origin of the 2n = 24 karyotype from the 2n = 23 ancestor and suggest that this modification in the number of chromosomes may have acted in the reproductive isolation between these lineages (Figure 1).

The next two possible cladogenetic events related to karyotypic changes occurred within the *infestans* clade (Figure 1). Our results suggest that the fission of one of the X chromosomes [event attributed to the karyotype origin of *T. vitticeps* (Stål, 1859) and *T. melanocephala* Neiva and Pinto, 1923 (both with 2n = 24 chromosomes) from the ancestral karyotype 2n = 23] [36,38] may have promoted reproductive isolation between the ancestors of the *vitticeps* group and those of other groups of the *infestans* clade (Figure 1). After that, strains with 23 chromosomes would continue to diverge and would undergo a new cladogenetic event, this time, by the fusion of the X_1 and X_2 chromosomes or the loss of one of the X sex chromosomes (becoming 2n = 20A + XY) (Figure 1). The lineage with 23 chromosomes would originate the species of the genus *Eratyrus* Stål, 1859, while the lineage with 22 chromosomes would originate the other species of the other groups. Experimental crosses have already been carried out between species of the *vitticeps* group and other groups from South America with 22 chromosomes (Table 1), and in all combinations, there was no hatching of eggs, confirming the role of the karyotype in the reproductive isolation and, consequently, in the diversification of some groups of South American triatomines.

In our analyses, only the chromosomal alteration that occurred in the ancestors of *T. maculata* (Erichson, 1848) was not related to a cladogenetic event (Figure 1). Based on our phylogeny and the phylogenetic reconstruction of Justi et al. [44], this species is positioned at the base of the *infestans* clade. Considering the ancestral karyotype 2n = 23 recovered in our analysis (PP > 0.75), we suggest that fusion (or loss) of an X chromosome may have occurred during the diversification of *T. maculata*. On the other hand, analyses with ultraconserved elements recovered this species between the *infestans* and the *brasiliensis* groups (both with 2n = 22 chromosomes) [48], so that the karyotype of *T. maculata* would not have changed in relation to the ancestor. Thus, further studies with this species may contribute to the understanding of these phylogenetic relationships.

2.4. Chromosomal Changes as a Reproductive Barrier

Some chromosomal changes have already been suggested as a mechanism of isolation between the species of Triatominae, such as, for example, the difference in heterochromatin pattern [58]. However, the hybridization capacity observed between species that have different heterochromatin patterns, such as those of the *infestans* group (*T. infestans*, *T. delpontei* and *T. platensis*), does not support this hypothesis [30]. Furthermore, it has recently been suggested that 45S rDNA translocations between sex chromosomes and autosomes may also contribute to the reproductive isolation of triatomines [37]. The authors suggested that, possibly, hybrids between species with different patterns of 45S rDNA present on autosomes/sex chromosomes may have lower fertility due to unbalanced gamete production [37].

The role of chromosomal changes in reproductive isolation and, consequently, in the speciation process has been extensively discussed over the years, with emphasis on chromosomal inversions [59–65]. Initially, it was believed that the crossing between polymorphic individuals for chromosomal changes would produce heterozygous hybrids, which would be sterile [65,66]. However, this has been questioned, since if these chromosomal changes promoted strong isolation, it would be difficult for this characteristic to be fixed in the population and, on the other hand, if the isolation was weak, it would be difficult for it to lead to speciation [65,66]. Subsequently, it was proposed that reproductive isolation would result from the suppression of recombination in areas where these inversions occurred, so that genes in these regions could differentiate, leading to the accumulation of divergence and, consequently, speciation [65]. Despite this, there is still no consensus on how and if, in fact, chromosomal speciation can occur.

On the other hand, most chromosomal speciation studies have been carried out with organisms that have monocentric chromosomes [61,65]. Fissions in this type of chromosome are more difficult to occur and to fixate in the population, due to the possibility that one of the fragments does not have centromeric regions and does not segregate correctly in meiosis [67]. Fusions can happen, but mainly between two acrocentric chromosomes (Robertsonian fusion) [67,68]. On the other hand, in holocentric chromosomes (which present the kinetochore diffuse along the chromosome), there is a greater facility for the occurrence of chromosomal fusion and fission [36,67].

In Triatominae, all changes related to a variation in the chromosome number involve fusion or fission events [36]. However, as mentioned several times in the manuscript, we do not have enough information to confirm whether fusion or just loss of one or more chromosomes has occurred. Recently, Pita et al. [52] suggested, based on the size of the sex chromosome X, that the karyotype 2n = 22 of *P. noireaui* is the result of a fusion event of the X chromosomes, from the ancestral karyotype 2n = 23. Furthermore, Alevi et al. [38] also suggested that the 2n = 21 karyotype of *P. megistus* is the result of a fusion event of a pair of autosomes, from the ancestral karyotype 2n = 23. We emphasize the importance of using appropriate techniques (such as chromosomal microdissection and the development of species-specific probes) to confirm whether the evolutionary event related to the chromosomal diversification of these *Panstrongylus* species was really associated with fusion or chromosomal loss.

Changes in chromosome number have already been related to reproductive isolation in organisms with holocentric chromosomes, such as plants [69] and, more recently, Lepidoptera [36]. The authors verified that for some groups of this order, these alterations may have played a role in cladogenesis among some species [36]. For triatomines, we could observe that nine cladogenetic events may be related to changes in the number of chromosomes (Figure 1, arrows). Adding these evolutionary events to the fact that in none of the experimental crosses between species with different karyotypes the eggs hatched (Table 1), we suggest that these alterations may play a role in the reproductive isolation of triatomines (we will call it karyotypic isolation) and can promote speciation (if fixed).

Considering the problems of chromosomal speciation (which also apply to karyotypic speciation), one of the suggested requirements for it to occur would be the population size [66], since in small populations, these changes would have more facility to become fixed by genetic drift [66]. The cladogenesis among some triatomine species (such as the *venosa* clade/remainder of Triatomini tribe, the *T. maculata/infestans* clade and *P. megistus/P. tibiamaculatus*) has been linked to vicariant events [44]. Thus, it is possible that in ancestral populations, with the emergence of a geographic barrier, this requirement was met, allowing the different karyotypes to be fixed and leading to cladogenesis (Figure 1).

3. Materials and Methods

3.1. Chromosomal Evolution Modeling

For the modeling study, a phylogenetic analysis with sequences of seven molecular markers obtained from GenBank for 88 triatomine species (Table S1) was initially performed (Figure S1). Molecular markers of four species of *Zelurus* spp. (Hemiptera, Reduviinae) were included as an outgroup, since this genus has been recovered close to Triatominae [44]. Although *Opisthacidius* spp. are closer to Triatominae [44], the species of this genus have never been studied cytogenetically. Thus, the choice of the taxa was mainly due to the availability of molecular markers and karyotype data for these species (Table S1).

The sequences were aligned in the Mega11 program [70], using the Muscle method [71] and concatenated in Seaview4 [72]. The phylogenetic tree of Bayesian inference was reconstructed in the program BEAST 1.8.4 [73], under the substitution model GTR +I +G and Yule Process prior [74,75], in a total of 100 million generations. The burn-in was adjusted to 25% of the samples, and convergence (ESS > 200) was evaluated in Tracer 1.8 [76].

The resulting tree (Figure S1) was used as a basis for the analysis in the RevBayes v. 1.1.1. program [77], using the ChromoSSE model [42], to infer the ancestral karyotype at each node, as well as the karyotype changes that occurred anagenetically and cladogenetically along the phylogeny. The triatomine karyotypes used in the modeling study were obtained from Panzera et al. [36] and Reis and Alevi [39] (Table S1). In addition, the number of chromosomes considered for the *Zelurus* clade (2n = 22) was proposed based on the karyotypes of *Z. ochripennis* (Stål, 1854) and *Z. femoralis longispinis* Lent and Wygodzinsky, 1954 [78,79] (for which molecular data are not available), because unfortunately there are no cytogenetic data for the *Zelurus* species used in the phylogenetic analysis. The analyses were carried out with a total of 20,000 generations, with burn-in adjusted in 25% of the samples, and convergence (ESS > 200) was evaluated in Tracer 1.8 [76]. The resulting tree was then plotted in R 4.2 [80], using the ggtree [81–85] and Revgadgets [86] packages. Vertical bars and grouping names were inserted using Adobe Illustrator CS6.

3.2. Experimental Crosses

To assess the reproductive compatibility between species with different chromosome numbers, three crosses were performed for each couple, as shown in Table 1. For each species used, intraspecific crosses were also performed (control). However, due to the low availability of live insects, intraspecific crosses between *P. lecticularia*, *T. longipennis* and *T. rubrovaria* (Blanchard, 1843) were not performed.

The species used were provided by the Triatominae Insectarium of the School of Pharmaceutical Sciences (FCFAR/UNESP), Araraquara, São Paulo, Brazil, where the crossings were also carried out. We emphasize that the choice of the species was based on the availability of live insects with different karyotypes kept in the FCFAR/UNESP insectarium.

To ensure the virginity of the tested insects, fifth instar nymphs were separated and sexed. After reaching the adult stage, the crosses were initiated and lasted 4 months. Insect feeding and oviposition counting were performed weekly during this period. The insects were kept at room temperature (average of 24 °C) and relative humidity of 63% [87]. After the crossing period, the eggs were kept for another two months to check the hatching rate.

4. Conclusions

Based on the above, we can conclude that: i. the ancestral karyotype of Triatominae is 2n = 22 chromosomes; ii. during the evolutionary process, at least nine cladogenetic events associated with alterations in the number of chromosomes may have occurred in triatomines; iii. these alterations could act as a prezygotic barrier in Triatominae (karyotypic isolation) and, consequently, promote species diversification; and iv. the description of new karyotypes (for example, species of the genus *Linshcosteus*, the Old World *Triatoma*, species of the Alberprosini, Cavernicolini and Bolboderini tribes and reduvids, phylogenetically close to Triatominae), the use of new molecular markers, the development of species-specific probes by chromosomal microdissection, and carrying out studies of experimental crosses can contribute to the elucidation of the evolutionary history of this group of vectors.

Finally, we emphasize that some phylogenetic relationships need to be better elucidated, namely, i. the position of *T. maculata* in relation to the *brasiliensis* group; ii. the position of *T. guasayana* in relation to the *sordida* group; iii. the position of the *spinolai* group in relation to the North and South American lineages; iv. the position of the *Eratyrus* group in relation to the *infestans* clade; and v. the position of the *rufotuberculatus* group in relation to the *geniculatus* clade, since the species of this group were recovered closer to the *rubrofasciata* clade (genus *Triatoma*).

Supplementary Materials: The following supporting information can be downloaded at www.mdpi.com/xxx/s1, Table S1: GenBank accession code and karyotype for each species. Number of chromosomes obtained from Panzera et al. [36] and Reis and Alevi [39]; Figure S1: Bayesian phylogeny of the Triatominae subfamily based on seven molecular markers. The posterior probability is shown in the nodes.

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Table S1: GenBank accession code and karyotype for each species. Number of chromosomes obtained from Panzera et al. [36] and Reis; Alevi [39].

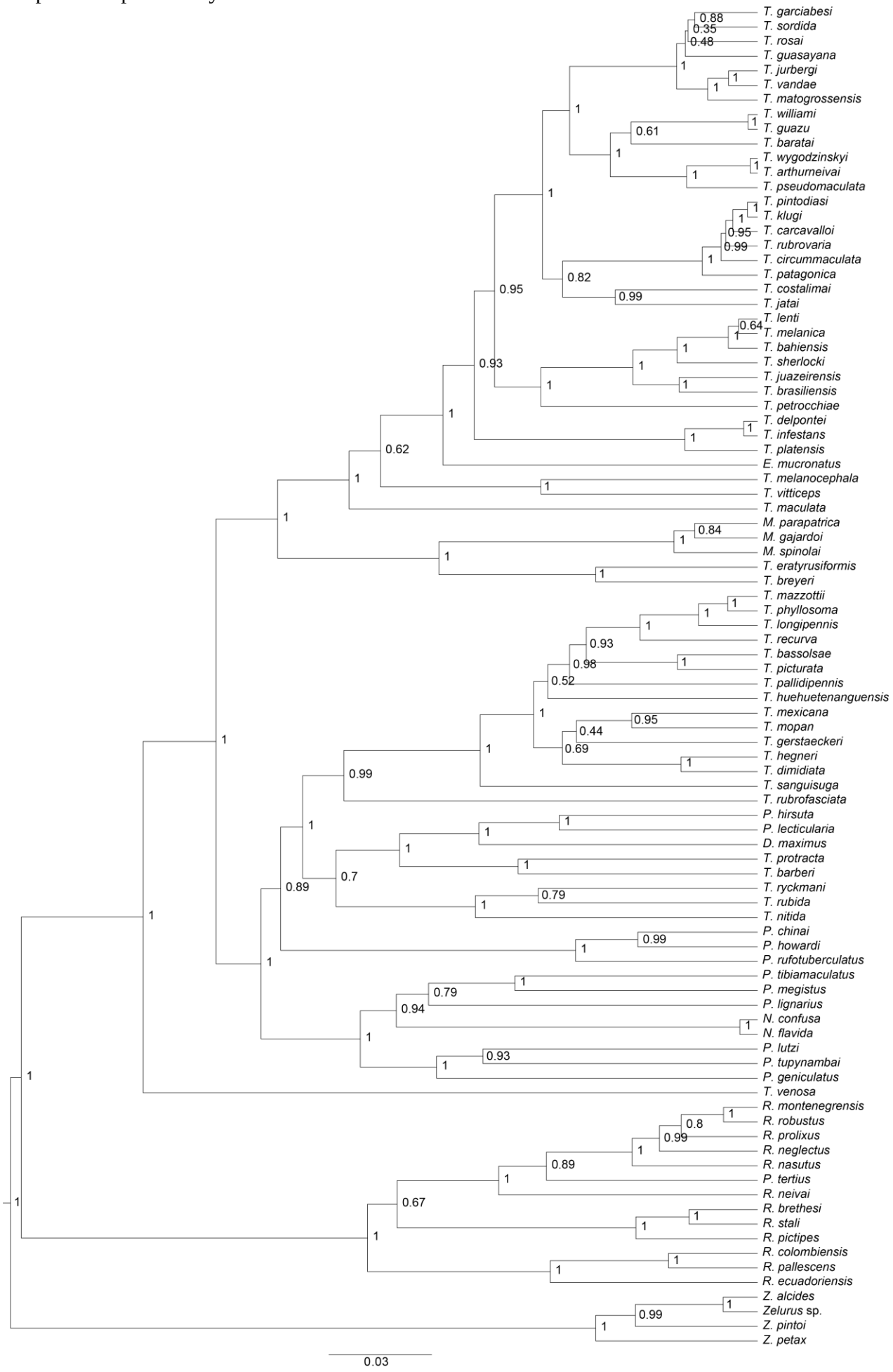
Species	Marker							Chromosome number
	Cytb	16S	18S	28S	COI	COII	ITS2	
D. maximus	KC249226	KC248968	KC249092	KC249134	KC249306	KC249399	AJ286887	22
E. mucronatus		JQ897794	JQ897555	JQ897635	AF449140		EF550126	23
M. gajardoi	JN102359				GQ336896			23
M. parapatrica	MN117884				MN117870			23
M. spinolai	JN102358	AF324518	AJ421961		MN117874			23
N. confusa		KC248989		KC249146		KC249418		23
N. flavida	JX848648	AY035451	AJ421959				AM286732	23
P. chinai	JX400960				MN504941	MN504970	AJ306547	23
P. geniculatus	KX109903	AF394593	JQ897583	KX109907	MW983060			23
P. hirsuta		FJ230443	FJ230521	FJ230604				22
P. howardi	JX400969					MN504980	MN505070	23
P. lecticularia	MT702379	KC249029	KC249111	KC249176	NC_050326	NC_050326	AY860407	22
P. lignarius	MK829849	AY185833	JQ897584	KX109906	AF449141		AJ306549	23
P. lutzi	KC249227	AY035449		KC249135	KC249307	KC249401		24
P. megistus	KC249228	KC248970	AJ243336	KC249137	KC249308	KC249402	AJ306542	21
P. rufotuberculatus	JX400989	KY748239	AJ421955		MF614953	MF614953	AJ306546	23
P. tertius		AY035439	Y18751				AJ286891	22
P. tibiamaculatus	KC249296	KC249081	KC249127	KC249214	KC249389	KC249485		23
P. tupynambai	KC249233	KC248978		KC249142	MZ643677	KC249404		23
R. brethesi	KC249235	KC248980			KC249313	KC249405		22
R. colombiensis	FJ229360	AY035438		KC543516			KT351069	22
R. ecuadoriensis	AF045715	AF028746		KC543518		GQ869665	KT267950	22
R. montenegrensis	KR072682							22

Species	Marker							Chromosome number
	Cytb	16S	18S	28S	COI	COII	ITS2	
R. nasutus	JX273155	AF028749		AF435856			KT317022	22
R. neglectus	KT317068	JQ897839	JQ897601	JQ897670			KT317033	22
R. neivai		AY035441			AF449137			22
R. pallescens	EF071584			KC543527			KT351070	22
R. pictipes	KC249236	JQ897840	KC249093		KC249314	KC249407	FJ887796	22
R. prolixus	EF043579	AF028747	AJ421962	AF435862	AF449138	NC_050328	AJ286888	22
R. robustus	AF421343	MF966360		MF966331			MK411278	22
R. stali	KC249237	KC248984	KC249095	KY111699	KC249317	KC249410	FJ887794	22
T. arthurneivai		AY035460					AM286736	22
T. bahiensis	KT347298							22
T. baratai	KC249238	KC571991		KC249143		KC249411		22
T. barberi	MT556655	JX872242	AJ421958		MT556655	MT556655	AJ293590	23
T. bassolsae	AY859410						MK248256	23
T. brasiliensis	KC249239	KC248985	AJ421957	KC249145	KC249318	KC249413	AJ293591	22
T. breyeri	KC249242	KC248988			KC249321	KC249417		24
T. carcavalloi	KC249244	KC248990	KC249097		KC249322	KC249419		22
T. circummaculata	KC249245	KC248992	KC249099	KC249148	KC249325	KC249422		22
T. costalimai	KC249246	KC248997	KC249101	KC249149	KC249327	KC249425		22
T. delpontei	KC249248	KC249000		KC249150	KC249330	KC249427	AJ576060	22
T. dimidiata	FJ197155	KC249004	JQ897609	KC249152		KC249431	AM286693	23
T. eratyrusiformis	JN102360	AY035466			GQ336898		FN396537	24
T. garciabesi	KC249249	KC249006	KC249102	KC249158	KC249338	KC249434		22
T. gerstaeckeri	JQ282723			KF188642	MT587783		AM286734	23

Species	Marker							Chromosome number
	Cytb	16S	18S	28S	COI	COII	ITS2	
T. guasayana	KC249252	KC249012	KC249103	KC249162	KC249343	KC249438		22
T. guazu	KC608976	KC249013	KC249105	KC249164	KC608984	KC249440		22
T. hegneri	JN585830						AM286727	23
T. huehuetenanguensis	MG951755				NC_050325	NC_050325	MG947606	23
T. infestans	KC249256	KC249016	KC249109	KC249168	KC249349	KC249442	AJ289876	22
T. jatai		KT601153			KT601164			22
T. juazeirensis	KC249263	KC249026		KC249173	KF826892			22
T. jurbergi	KC249264	KC249027	KC249110	KC249174		KC249448		22
T. klugi	KC249265	KC249028			KC249356	KC249449		22
T. lenti	KY576789	KY576788			KY576792			22
T. longipennis	KC249267	KC249031	AJ243331	KC249177	KC249357	MT556658	AJ286883	23
T. maculata	KC249268	KC249035		KC249178	AF449139	KC249455	AJ582027	22
T. matogrossensis	KC249269	KC249036	KC249114	KC249179	KC249359	KC249456		22
T. mazzottii	DQ198816	AY035446	AJ243333		NC_050327	NC_050327	AY860393	23
T. melanica		KC249041		KC249183	KJ580495	KC249461		22
T. melanocephala	KF826898	KF769450			KF826895			24
T. mexicana	DQ118976	JX872251			NC_050324	NC_050324	AM286728	23
T. mopan	JN585883						MG954252	23
T. nitida	AF045723	JX872239			MT556667	MT556667	JX872260	21
T. pallidipennis	EU790632	KC249044	KC249115	KC249184	MT556659	MT556659	AM286730	23
T. patagonica	MG241451	AY035464			KR139999			22
T. petrocchiae	KY654075	KY654073			KY654074			22
T. phyllosoma	DQ198818		AJ243329		MT556660	MT556660	AJ286881	23

Species	Marker							Chromosome number
	Cytb	16S	18S	28S	COI	COII	ITS2	
T. picturata	DQ198817	AY185840	AJ243332		MT556661	MT556661	AJ286884	23
T. pintodiasi		MG264738			MZ345607			22
T. platensis	KC249274	KC249047		KC249186	KC249363	KC249462	AJ576062	22
T. protracta	MT239325	KC249048	KT231850	KC249187	MT556662	MT556662	JX872263	23
T. pseudomaculata	KC249275	KC249051		KC249189	KC249366	KC249464		22
T. recurva	DQ198813	FJ230417	FJ230496	FJ230577	MT556663	MT556663	MK248251	23
T. rosai	KC249295	KC249078		KC249213	MH029697			22
T. rubida	DQ198809	AY035445		GQ853391	MT556664	MT556664	AM286735	23
T. rubrofasciata	MH368021	AY127046	AJ421960	KR632546	MH934953	MH934953		25
T. rubrovaria	KC249281	KC249067	KC249117	KC249198	KC249370	KC249471	AJ557261	22
T. ryckmani		JX872248					AM286731	23
T. sanguisuga	HQ141317	JX890269		GQ853392	NC_050329	NC_050329	KF142511	23
T. sherlocki	KC249288	KC249068		KC249205	KC249377	KC249478		22
T. sordida	MH054940			KC249210	MH029692			22
T. vandae	KC249298	KC249083	KC249128	KC249216	KC249391	KC249487		22
T. venosa		JQ897850	JQ897611	JQ897681			AJ582026	22
T. vitticeps	KC249303	KC249087	KC249132	KC249220	KC249396			24
T. williami	KC608981	KC249089			KC608990	KC249493		22
T. wygodzinskyi		KC249090	KC249133	KC249222	KC249398	KC249494		22
Z. alcides		JQ897855	JQ897615	JQ897686				22
Z. petax		FJ230416	FJ230495	FJ230576	JQ888708			22
Z. pintoi		JQ897856	JQ897616	JQ897687				22
Zelurus sp.	GQ869679	JQ897857	JQ897618		JQ888708	GQ869673		22

Figure S1: Bayesian phylogeny of the Triatominae subfamily based on seven molecular markers. The posterior probability is shown in the nodes.



4. CONCLUSÕES GERAIS

Nossos resultados demonstram que: (1) o cariótipo ancestral de Triatominae é 2n = 22 cromossomos; (2) durante o processo evolutivo, pelo menos nove eventos cladogenéticos associados á alterações no número de cromossomos podem ter ocorrido em triatomíneos; (3) essas alterações podem atuar como barreira pré-zigótica em Triatominae (isolamento cariotípico), sendo importantes eventos evolutivos para a diversificação das espécies; e (4) a descrição de novos cariótipos (por exemplo, espécies do gênero *Linshcosteus*, bem como de *Triatoma* do Velho Mundo, das tribos Alberprosini, Cavernicolini e Bolboderini e de reduvídeos filogeneticamente próximos de Triatominae), o uso de novos marcadores moleculares, o desenvolvimento de sondas para os cromossomos sexuais (por microdissecção cromossômica) e a realização de estudos de cruzamentos experimentais entre espécies com número de cromossomos diferentes podem contribuir para a elucidação da história evolutiva desse grupo de vetores.

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