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Evolución cariotípica en Triatominae (Hemiptera: Reduviidae), análisis de secuencias de ADN repetido

Mag. Sebastián Pita Mimbacas

FACULTAD DE CIENCIAS
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UNIVERSIDAD DE JAÉN

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Evolución cariotípica en Triatominae (Hemiptera: Reduviidae), análisis de secuencias de ADN repetido.

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Orientadores:

Dr. Francisco Panzera Arballo, Sección Genética Evolutiva, Facultad de Ciencias, UdelaR

Dr. Pedro Lorite Martínez, Departamento de Biología Experimental, Universidad de Jaén.

Tribunal:

Dr. Gustavo Folle, Instituto de investigaciones Biológicas Clemente Estable, MEC (presidente)

Dra. Cristina Mazzella, Facultad de Agronomía, Universidad de la República (vocal)

Dra. Magdalena Vaio, Facultad de Agronomía, Universidad de la República (vocal)

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A mis abuelos,

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Resumen

Los insectos vectores de la enfermedad de Chagas involucran 152 especies agrupadas en la subfamilia Triatominae, familia Reduviidae del orden Hemiptera. La relevancia del análisis de este grupo radica fundamentalmente por actuar como vectores del parásito causante de la enfermedad de Chagas. Además el estudio de este grupo de insectos resulta interesante debido a que posee una clase especial de cromosomas denominados holocéntricos, caracterizados por presentar un centrómero no localizado o difuso (sin constricción primaria) que segregan diferencialmente en meiosis y mitosis. La falta de una constricción primaria, el pequeño tamaño cromosómico, y la escasez de diferenciación longitudinal dificultan enormemente el análisis de la variabilidad cariotípica de estos insectos. Sin embargo, mediante la observación de la heterocromatina constitutiva y la localización de los genes ribosomales se reveló que los genomas de los triatominos presentan una extensa variación cromosómica. El uso de estos marcadores han permitido la determinación de especies crípticas con diferente importancia vectorial así como la detección de variaciones poblacionales en distintas especies pertenecientes a los tres géneros principales: *Triatoma, Rhodnius* y *Panstrongylus*. Es por ello que además de ampliar el análisis de los genes ribosomales a nuevas especies y poblaciones, hemos abordado otras metodologías capaces de revelar otras secuencias de ADN repetido. Con el objetivo de ampliar el conocimiento sobre la evolución cariotípica en esta subfamilia enfocada al análisis de sus secuencias repetidas, en esta tesis se planteó obtener nuevos marcadores genómicos mediante la aplicación de las técnicas de microdisección cromosómica e hibridación *in situ* genómica (GISH), hasta ahora no empleadas en triatominos. Además se abordó un análisis más detallado del genoma de *T. infestans*, determinando la totalidad de las secuencias repetidas de su genoma (repeatoma).

Los resultados obtenidos nos han permitido ampliar sustancialmente nuestro conocimiento acerca de la evolución cariotípica en los triatominos. El análisis de la localización cromosómica de los genes ribosomales nos han permitido comprobar su importancia como marcador cromosómico capaz de diferenciar especies crípticas, y al mismo tiempo ser utilizado para sugerir cambios evolutivos, gracias a las distintas tasas de cambio observadas en los diferentes grupos. El uso de sondas de ADN repetido determinó que estas secuencias tienen dos tipos de localización en el genoma: dispersa en todo el cromosoma y/o clusterizada en regiones específicas. Mediante microdisección, hemos comprobado que las secuencias repetidas de los cromosomas Xs se encuentran también en la eucromatina de los autosomas de forma dispersa. Estos cromosomas Xs, a su vez, presentan un alto grado de diferenciación con el cromosoma Y. El cromosoma Y está formado por secuencias repetidas, altamente conservadas y compartidas por todos los miembros analizados de la tribu Triatomini. Sin embargo, en *R. prolixus*, perteneciente a la tribu Rhodniini, el cromosoma Y no se encuentra conformado por secuencias repetidas. Por otro lado utilizando sondas genómicas hemos propuesto una hipótesis sobre la evolución de las secuencias que conforman la heterocromatina de los cromosomas autosómicos. Nuestros análisis de GISH junto con el análisis del repeatoma de *T. infestans* sobre una docena de especies sugieren que especies cercanas comparten sus repetidos, pero especies evolutivamente alejadas poseen repetidos diferentes.

El análisis del repeatoma de *T. infestans* mostró que las secuencias de ADN repetido más abundantes son las de ADN satélite, que están presentes tanto dispersas en la eucromatina, como clusterizadas en las regiones heterocromáticas. Estos bloques heterocromáticos están conformados por sólo unas pocas familias de ADN satélite, pero son los responsables principales de la diferencia observada en el ADN genómico de los dos linajes de *T. infestans*. Nosotros sugerimos que a nivel evolutivo, especies próximas comparten secuencias de ADN satélite en su heterocromatina, mientras que en especies más alejadas la heterocromatina está formada por familias de ADN satélite diferentes y propias de cada grupo.

Por último, gracias al análisis de los repetidos en el genoma de *T. infestans*, hemos logrado identificar las secuencias teloméricas, formadas por el pentanucleótido (TTAGG)n, motivo repetido ancestral para los artrópodos. Además hemos comprobado la presencia de este repetido en otras especies de triatominos de los géneros *Triatoma*, *Dipetalogaster* y *Rhodnius*. De esta forma describimos por primera vez las secuencias teloméricas en heterópteros del suborden Cimicomorpha, oponiéndonos a la hipótesis imperante sobre la pérdida del repetido ancestral en la mayoría de los heterópteros.

Summary

Chagas disease vectors encompass 152 insect species, grouped within the Triatominae subfamily, Reduviidae family, Heteroptera order. The relevance of this group lies on the fact that they are vectors of the parasitic CHagas disease. Moreover, there is another interesting issue on the biology of this insect group which is the presence of holocentric chromosomes in their cells. These chromosomes are characterized by a non-localized or diffuse centromere (without primary constriction) that segregate differentially in meiosis and mitosis. The lack of a primary constriction, small chromosome size, and the scarcity of longitudinal differentiation make it extremely difficult to analyze the karyotype variability of these insects. However, by observing constitutive heterochromatin and localization of ribosomal genes it was revealed that the genomes of triatomines exhibit extensive chromosomal variation. The use of these markers allowed the determination of cryptic species with different vectorial importance as well as the detection of population variations in different species belonging to the three main genera: *Triatoma, Rhodnius* and *Panstrongylus*. This is why, in addition to expanding the analysis of ribosomal genes to new species and populations, we have addressed other methodologies capable of revealing other repeated DNA sequences. The aim of this thesis was to obtain new genomic markers by means of the application of chromosomal microdissection and genomic *in situ* hybridization (GISH) techniques, in order to increase the knowledge about karyotype evolution in this subfamily, focused on the analysis of its repeated sequences, not used in triatomines hitherto. In addition, a more detailed analysis of the genome of *T. infestans* was carried out, determining the totality of the repeated sequences of its genome (repeatome).

The obtained results have allowed us to substantially expand our knowledge about karyotype evolution in triatomines. The ribosomal genes chromosomal location analysis has allowed us to verify its importance as a chromosomal marker capable of differentiating cryptic species, and at the same time be used to suggest evolutionary changes, thanks to the different rates of change observed in the different groups. The use of repetitive DNA probes determined that these sequences have two types of localization in the genome: dispersed throughout the chromosome and / or clustered in specific regions. Through microdissection, we have verified that the repeated sequences of the Xs chromosomes are also found in the euchromatin of the autosomes in a dispersed form. These Xs chromosomes, in turn, have a high degree of differentiation with the Y chromosome. The Y chromosome is formed by highly conserved repetitive sequences, shared by all analyzed members of the Triatomini tribe. However, in *R. prolixus*, belonging to the Rhodniini tribe, the Y chromosome is not formed by repeatitive sequences. On the other hand using genomic probes we have proposed a hypothesis about the evolution of the sequences that make up the heterochromatin of the autosomal chromosomes. Our GISH analyzes -together with the *T. infestans* repeatome analysis- on a dozen species suggest that close species share their repeats, but evolutionarily distant species ones have different repetitive DNA.

Repeatome analysis of *T. infestans* showed that the most abundant repetitive DNA sequences are those of satellite DNA, which are present both dispersed in euchromatin and clustered in

the heterochromatic regions. These heterochromatic blocks are made up of only a few satellite DNA families, but are the main responsible for the difference observed in the genomic DNA amount of the two *T. infestans* lineages. We suggest that at an evolutionary level, close species share satellite DNA sequences in their heterochromatin, whereas in more distant species the heterochromatin is formed by different satellite DNA families, which belongs to each group.

Finally, thanks to the analysis of the repetitive DNA in the *T. infestans* genome, we have succeeded in identifying the telomeric sequences, formed by the pentanucleotide $(TTAGG)_n$, ancestral repetitive motif for the arthropods. In addition we have verified the presence of this repeat in other triatomine species of the *Triatoma*, *Dipetalogaster* and *Rhodnius* genus. In this way we first describe the telomeric sequences in heteroptera of the suborder Cimicomorpha, opposing to the prevailing hypothesis about the loss of the repeated ancestor in most of the heteroptera.

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INTRODUCCIÓN

Los insectos vectores de la enfermedad de Chagas

Los insectos vectores de la enfermedad de Chagas involucran 152 especies agrupadas en la subfamilia Triatominae, pertenecientes a la familia Reduviidae, suborden Heteroptera del orden Hemiptera (Justi & Galvão 2017; da Rosa et al. 2017). Vulgarmente se las denomina de distintas formas en Latinoamérica, tales como vinchucas, chinches hociconas, chipo, pic, barbeiro (en Brasil) o chinches besuconas (*kissing bugs*) en Estados Unidos. Estos insectos triatominos habitan principalmente en el continente americano, desde el sur de los Estados Unidos hasta el sur de Chile y Argentina, aunque también existen algunas pocas especies en Asia (Schofield & Galvão 2009; Gorla et al. 1997). Estas especies han sido agrupadas debido a su hábito hematófago, es decir, se alimentan de sangre de vertebrados (Lent & Wygodzinsky 1979). Excepcionalmente algunas especies también tienen comportamientos predatorios sobre otros invertebrados (Schofield & Galvão 2009; Hwang & Weirauch 2012).

La relevancia de estos insectos es fundamentalmente debida a su capacidad de actuar como trasmisores del protozoo *Trypanosoma cruzi* (Euglenozoa, Kinetoplastida), agente causante de la enfermedad de Chagas en el hombre. Esta enfermedad parasitaria es endémica del continente americano y afecta entre 6 y 8 millones de personas (WHO 2017). Se desarrolla fundamentalmente en un contexto de gran pobreza, siendo catalogada por la Organización Mundial de la Salud (OMS) como *neglected disease* (enfermedad olvidada). La casi totalidad de las especies de triatominos descritas han sido encontradas infectadas con *T. cruzi* (Lent & Wygodzinsky 1979), aunque solo una veintena de ellas representan una amenaza al hombre, debido, fundamentalmente, a su adaptación al ambiente humano. Cada una de estas 20 especies con importancia epidemiológica tiene diferentes características a tener en cuenta a la hora de combatirlas, como su efectividad en la trasmisión del parásito al hombre, su capacidad de dispersión y domiciliación, la duración de su ciclo de vida o la existencia de poblaciones silvestres (Schofield & Galvão 2009; Gourbière et al. 2012). Las especies más destacadas como vectores más fecuentes de la enfermedad de Chagas son *Rhodnius prolixus, Triatoma infestans* y *T. dimidiata*.

Debido a que no existe vacuna ni tratamiento farmacológico eficaz contra el parásito en fase crónica, el combate contra la enfermedad de Chagas se ha centrado principalmente en la erradicación del vector del domicilio mediante el rociado de las casas con insecticidas piretroides, y en interrumpir la transmisión transfusional mediante el control sanguíneo en los bancos de sangre (Guhl et al. 2009; Dias 2009). Para el establecimiento de estrategias adecuadas de control vectorial, o para optimizar las existentes, es imprescindible la correcta identificación taxonómica de las especies o poblaciones que actúan como vectores, el análisis de su capacidad de dispersión, determinar la habilidad de poblaciones genéticamente diferenciadas para invadir y colonizar ambientes domésticos, así como lograr

establecer el origen de individuos que reaparecen después del rociado de las casas con insecticidas (Dias & Schofield 2010; Justi & Galvão 2017).

El análisis genético de los insectos vectores se ha abordado con distintos marcadores, tales como isoenzimáticos (Noireau et al. 2002), cromosómicos (Panzera et al. 2010) y de ADN, tanto de secuencias nucleares como mitocondriales (Justi & Galvão 2017). Estos estudios han contribuido de forma sustancial a la disminución drástica de la transmisión vectorial en diversas regiones de Latinoamérica. En numerosos casos las relaciones filogenéticas obtenidas con secuencias de ADN nucleares y mitocondriales presentan resultados contradictorios con las clasificaciones basadas en caracteres morfológicos y ecológicos, así como en la identificación de poblaciones de especies con gran importancia vectorial como *T. infestans* o *T. dimidiata* (Schofield & Galvão 2009). Por tanto es imprescindible la búsqueda de nuevos marcadores que nos permitan establecer cambios evolutivamente muy rápidos ocurridos en los genomas de los triatominos.

Hábitats y hábitos

A excepción de algunas especies pertenecientes al género *Belminus*, todos los miembros de Triatominae son hematófagos obligatorios (necesitan una ingesta de sangre para progresar al siguiente estado ninfal) que viven en asociación con sus hospedadores vertebrados. Se las encuentra tanto en ambientes naturales (rocas, nidos, cuevas, huecos de árboles y palmeras) como domésticos y peridomésticos (gallineros, corrales, galpones), donde anidan en techos, grietas de muros y colchones, siempre cercanos a sus posibles fuentes de alimento (hombre y animales domésticos). Algunas especies ocupan diversos ecotopos y se alimentan de diferentes hospedadores, pero otras especies son más especializadas. Por ejemplo las especies del género *Psammolestes* están en nidos de aves de la familia Furnariidae, *T. delpontei* que está asociada a la especie de loros *Myiopsitta monachus*, mientras que *T. rubrofasciata* y *Cavernicola pilosa* se alimentan preferentemente de roedores y murciélagos respectivamente (Gaunt & Miles 2000; Galvão & Justi 2015; Otálora-Luna et al. 2015).

Tanto en ambientes domésticos como silvestres, la regulación del tamaño poblacional de los triatominos depende de la densidad de sus huéspedes. A mayor número de individuos en la población e igual número de huéspedes la competencia por el alimento aumenta. Un factor clave es la duración de la ingesta, la cual es interrumpida en casos de gran competencia por el alimento. Normalmente una ingesta completa dura entre 10 a 20 minutos cada cuatro a nueve días, dependiendo de la especie. La reducción de la nutrición tiene tres grandes consecuencias: 1) la velocidad del desarrollo de la ninfa disminuye, reduciendo la velocidad de muda a los estadios sucesivos, de manera que las hembras emergen más lentamente, 2) las hembras depositan menos huevos, 3) tiende a aumentar la probabilidad de que inicien un vuelo dispersivo, lo cual permite la invasión de sitios alternativos, como por ejemplo las viviendas humanas (Schofield 1994).

En general los triatominos se dispersan activamente, preferentemente caminando o mediante el vuelo atraídos por fuentes de luz o estimulados por la hambruna (Wisnivesky-Colli et al. 1993; Almeida et al. 2012; Pacheco-Tucuch et al. 2012). En las

especies domésticas un factor muy importante es la dispersión pasiva realizada por el hombre (Cortez et al. 2010).

El hábito hematófago marcó la historia natural de los triatominos. Cómo pasaron a la hematofagia dependió del repertorio genético que estos organismos heredaron de sus ancestros no hematófagos (seguramente predadores) y de las restricciones impuestas por su entorno físico. Los ancestros predadores redúvidos ya habían desarrollado piezas bucales perforadoras, enzimas digestivas para proteínas, comportamientos agresivos y de acecho ante sus presas, así como asociaciones con vertebrados nidícolas (Otálora-Luna et al. 2015).

Ciclo de vida

Los triatominos son insectos hemimetábolos, con un ciclo de vida marcado por tres etapas: huevo, ninfa y adulto. En total, la duración del ciclo abarca un año, pero este período depende de la especie y del ambiente. En el caso de los triatominos el estado ninfal atraviesa por cinco estadios. El cambio entre los estadios ninfales ocurre por muda del exoesqueleto, lo que permite el crecimiento del insecto y es dependiente del estadio de desarrollo, temperatura, humedad y alimento disponible (o densidad poblacional). La última muda provoca la metamorfosis de la ninfa a adulto y está marcado por la salida de las alas. En promedio el adulto vive entre 8 a 16 meses. Las hembras pueden ser fecundadas poco después de la última muda y depositan unos 80-100 huevos por postura, comenzando a los 8-10 días post cópula hasta incluso un año después; dependiendo de la especie y del alimento ingerido. Los huevos eclosionan entre los 10 a 40 días posteriores a su puesta (Buxton 1930; Wigglesworth 1972).

Clasificación

Los insectos vectores de la enfermedad de Chagas se agrupan dentro de la subfamilia Triatominae, pertenecientes a la familia Reduviidae (Hemiptera, Heteroptera). Se encuentra integrado por 150 especies (más dos registros fósiles), agrupadas en cinco tribus y entre 15 y 17 géneros de acuerdo a los distintos autores (Tabla 1) (Justi & Galvão 2017; da Rosa et al. 2017).

Las cinco tribus son: Alberproseniini, Bolboderini, Cavernicolini, Rhodniini y Triatomini. Las tres primeras son tribus con pocas especies y se conoce poco de ellas. La tribu Rhodniini agrupa 23 especies en dos géneros, *Psammolestes* y *Rhodnius* (Tabla 2). La tribu Triatomini es la más numerosa, incluyendo siete u ocho géneros, dependiendo de la clasificación usada ya que el *Mepraia* es considerado un género válido por algunos autores (Galvão et al. 2003) o es incluido dentro de *Triatoma* por otros autores (Lent & Wygodzinsky 1979). Los géneros incluidos en la tribu Triatomini son *Dipetalogaster*, *Eratyrus, Hermanlentia, Linshcosteus, Mepraia, Panstrongylus, Paratriatoma* y *Triatoma*. Este último es el género más numeroso, donde se reconocen tres grupos distintos, los cuales se subdividen en complejos y subcomplejos (Tabla 3). Estos agrupamientos taxonómicos se basan principalmente en caracteres morfológicos, ecológicos ó biogeográficos, y sólo en algunos de ellos existen estudios genéticos que los sustenten. La aplicación de distintos marcadores genéticos están modificando sustancialmente los agrupamientos de las especies tradicionalmente establecidos por morfología (Justi et al. 2014).

Tabla 1. Conformación taxonómica de la subfamilia Triatominae en tribus, géneros y número de especies, de acuerdo a Schofield & Galvão (2009) con modificaciones.

Tribu	Géneros	Número de especies
Alberproseniini	*Alberprosenia*	2
Bolboderini	*Belminus*	8
	Bolbodera	1
	Microtriatoma	2
	Parabelminus	2
Cavernicolini	*Cavernicola*	2
Rhodniini	*Psammolestes*	3
	Rhodnius	21
Triatomini	*Dipetalogaster*	1
	Eratyrus	2
	Hermanlentia	1
	Linshcosteus	6
	Mepraia	5
	Panstrongylus	14
	Paratriatoma	1
	Triatoma	79
Total		**150**

Tabla 2. Especies reconocidas en los linajes evolutivos de la tribu Rhodniini, integrada por los géneros *Rhodnius y Psammolestes* de acuerdo a Monteiro et al. (2000) con modificaciones.

Linaje	Especies
pictipes	*R. amazonicus, R. brethesi, R. paraensis, R. pictipes, R. stali, R. zeledoni*
pallescens	*R. colombiensis, R. ecuadoriensis, R. pallescens*
prolixus	*R. barreti R. dalessandroi, R. domesticus, R. milesi, R. marabaensis, R. montenegrensis, R. nasutus, R. neglectus, R. neivai, R. prolixus, R. robustus, R. taquarussuensis, Ps. arthuri, Ps. coreodes, Ps. tertius*

Tabla 3: Especies integrantes del género *Triatoma*, distribuidas en grupos, complejos y subcomplejos, de acuerdo a Schofield & Galvão (2009) con modificaciones.

Grupo	Complejo	Subcomplejo	Especies
Rubrofasciata	Phyllosoma	Dimidiata	*T. dimidiata, T. hegneri, T. brailovskyi, T. gomeznunezi*
		Phyllosoma (=Meccus)	*T. bassolsae, T. bolivari, T. longipennis, T. mazzotti, T. mexicana, T. Pallidipennis, T. phyllosoma, T. picturata, T. ryckmani*
	Flavida		*T. flavida, T. bruneri, T. obscura*
	Rubrofasciata		*T. amacitiae, T. bouvieri, T. cavernicola, T. leopoldi, T. migrans, T. pugasi, T. rubrofasciata, T. sinica*
	Protracta		*T. barberi, T. incrassata, T. neotomae, T. nitida, T. peninsularis, T. protracta, T. sinaloensis*
	Lecticularia (=Nesotriatoma)		*T. gerstaeckeri, T. indictiva, T. lecticularia, T. recurva, T. rubida, T. sanguisuga*
Dispar	Dispar		*T. boliviana, T. carrioni, T. dispar, T. nigromaculata, T. venosa*
Infestans	Infestans	Brasiliensis	*T. bahiensis, T. b. brasiliensis,*

		T. b. melanosoma, *T. juazeirensis*, *T. melanica*, *T. petrochiae*, *T. lenti*, *T. sherlocki*
	Infestans	*T. delpontei*, *T. infestans*, *T. platensis*
	Maculata	*T. arthurneivai*, *T. maculata*, *T. pseudomaculata*, *T. wygodzinskyi*
	Matogrossensis	*T. baratai*, *T. costalimai*, *T. deaneorum*, *T. guazu*, *T. jatai*, *T. jurbergi*, *T. matogrossensis*, *T. vandae*, *T. williami*
	Rubrovaria	*T. carcavalloi*, *T. circunmaculata*, *T. klugi*, *T. limai*, *T. oliverai*, *T. pintodiasi*, *T. rubrovaria*
	Sordida	*T. garciabesi*, *T. guasayana*, *T. patagonica*, *T. sordida*
Spinolai (=Mepraia)		*T. breyeri*, *T. eratyrusiformis*, *T. (M.) spinolai*, *T. (M.) gajardoi*, *T. (M.) parapatrica.*
Sin asignar grupo		*T. melanocephala*, *T. tibiamaculata*, *T. vitticeps*

Origen Evolutivo de la subfamilia Triatominae

Está ampliamente aceptado que los triatominos derivan de redúvidos que tenían hábitos predadores. Sin embargo, existe un gran debate sobre si la subfamilia Triatominae es monofilética o polifilética. La respuesta a esta pregunta está ligada a conocer cuántas veces surgió en la evolución la hematofagia. Es decir, si el surgimiento de la hematofagia en cierto grupo de predadores es seguido por la adaptación a diferentes hábitats (teoría monofilética), o si por lo contrario, distintas especies de predadores se adaptaron a diferentes hábitats y luego desarrollaron la hematofagia independientemente (teoría polifilética) (Schofield & Galvão 2009).

La subfamilia Triatominae fue considerada tradicionalmente como un grupo monofilético en base a su hematofagia obligatoria y a que el tercer segmento rostral ascendente es flexible cuando el rostro está en posición de alimentación, características no compartidas con ningún otro grupo de redúvidos (Lent & Wygodzinsky 1979).

Debido a las grandes diferencias morfológicas y fisiológicas observadas entre las tribus Rhodniini y Triatomini, se

desarrolló la teoría polifilética que propuso que la hematofagia es una convergencia evolutiva que surge en diversas ocasiones y en distintos linajes con hábitos predatorios de la familia Reduviidae (Schofield 1988). Estudios usando secuencias de ADN apoyaron el polifiletismo sugiriendo que las dos tribus se originaron de grupos distintos dentro de Reduviidae (De Paula et al. 2005; Schofield & Galvão 2009). El argumento fundamental de la teoría polifilética radica en que la hematofagia surge de un proceso paso a paso. El estado ancestral sería un redúvido predador libre. Un primer paso hacia la hematofagia es su especialización a predar en nidos o viviendas de sus presas. Luego se convertiría en un hematófago facultativo, para por último desarrollar la hematofagia obligatoria. Este proceso con tantas etapas sugiere que la hematofagia puede haberse desarrollado en diferentes oportunidades en distintos linajes, y por ende conformando un grupo polifilético (Schofield & Galvão 2009).

Sin embargo, otros análisis filogenéticos y cladísticos sugirieron nuevamente la monofilia de la subfamilia (Hypša et al. 2002; Weirauch & Munro 2009; Patterson & Gaunt 2010). La ventaja de estos nuevos estudios fue el aumento del número de especies analizadas, tanto de triatominos como de otros redúvidos emparentados. Por ejemplo se incluyó por primera vez una especie de la tribu Bolboderini (*Microtriatoma trinidadensis*) (Patterson & Gaunt 2010). Las primeras evidencias fijaron el origen monofilético del grupo hace 95 millones de años (MA), coincidiendo con la formación del continente sudamericano, lo cual a su vez explicaría la distribución exclusivamente neotropical de los triatominos (Gaunt & Miles 2002). Sin embargo, más recientemente, análisis realizados con un reloj molecular más relajado, no fijo, e incluyendo nuevos registros fósiles de triatominos y otros redúvidos, han acotado este tiempo a 35 MA (Justi et al. 2016; Justi & Galvão 2017). El origen de la subfamilia Triatominae estaría representado por el nodo de la separación entre las tribus Triatomini y Rhodniini. Las otras tribus, Bolboderini y Cavernicolini estarían más emparentadas con Rhodniini (Weirauch & Munro 2009; Patterson & Gaunt 2010; Hwang & Weirauch 2012; Justi et al. 2016).

Recientemente se planteó una tercera visión acerca del origen de los triatominos, la cual sostiene un origen parafilético. La subfamilia Triatominae sería parafilética con respecto al género *Opisthacidius,* integrante de la subfamilia Reduviinae (Hwang & Weirauch 2012). Es decir que la hematofagia pudo haber evolucionado una o dos veces entre los redúvidos predadores. En el análisis presentado por estos autores se observa a la subfamilia Triatominae subdividida en dos clados; por un lado la tribu Triatomini y por otro la tribu Rhodniini junto con el género *Opisthacidius*. Otro aporte del mismo trabajo fue el desarrollo de un nuevo reloj molecular que dató el origen de la subfamilia Triatominae (+ *Opisthacidius*) en 24 a 32 MA (Oligoceno). Por lo tanto, se establece que el surgimiento de los triatominos es posterior a la formación de Sudamérica, y que coincide con una radiación de mamíferos y aves neotropicales (Hwang & Weirauch 2012).

En resumen, los últimos datos se perfilan más hacia una teoría mono o parafilética, dejando la polifilia de lado. El último modelo de reloj molecular establece que los Triatominae se habrían originado hace 30 a 40 MA en el norte de Sudamérica desde donde se dispersarían al resto del continente y luego también a Asia a través

del estrecho de Bering (Justi et al. 2016; Justi & Galvão 2017).

Citogenética

Además de la importancia sanitaria, este grupo de insectos resulta interesante debido a que posee una clase especial de cromosomas denominados holocéntricos u holocinéticos, los cuales exhiben un comportamiento muy particular durante las divisiones celulares. Los cromosomas holocéntricos presentan la particularidad de tener un centrómero no localizado o difuso, y como consecuencia no se observa una constricción primaria (Hughes-Schrader & Schrader 1961).

Los cromosomas holocéntricos no sólo están presentes en los hemípteros como los triatominos, sino que también están descriptos para casi todos los taxones eucariotas, incluyendo protistas, animales y plantas. El gusano *Caenorabditis elegans* es la especie más utilizada para el estudio de estos cromosomas (Mandrioli & Manicardi 2012). Recientemente se ha sugerido que es un carácter derivado, surgido en la evolución animal unas trece veces (Escudero et al. 2016), de ellas cinco han sido en insectos, estando presentes en Odonata, Zoraptera, Dermaptera, Paraneoptera (Hemiptera + Thysanoptera + Psocoptera) y Amphiesmenoptera (Lepidoptera + Trichoptera). Su presencia en taxones evolutivamente muy distantes sería el resultado de convergencias evolutivas (Melters et al. 2012). Además, los cromosomas holocéntricos en los diferentes organismos donde están presentes tienen diferencias en la conformación de la placa cinetocórica (policéntricos u holocéntricos propiamente dicho) y de comportamiento durante la segregación cromosómica tanto en mitosis como en meiosis. Toda esta diversidad refuerza un origen independiente durante la evolución de los distintos taxones (Melters et al. 2012).

Existen varias hipótesis por las cuales cromosomas monocéntricos pasarían a ser holocéntricos. Éstas implican una extensión del centrómero debido a una expansión de elementos transponibles (ETs), o debido a la que la formación del cinetocoro gira en 90° (revisado en Escudero et al. 2016). Otra hipótesis muy interesante involucra a la formación de los telómeros y los centrómeros cromosómicos. Ésta propone que en la transición de un genoma circular a uno linear, lo primero que se formaría serían las estructuras teloméricas, para darle estabilidad a la molécula de ADN lineal. La acumulación de retroelementos sería la responsable de tales estructuras. Posteriormente, la formación del centrómero se debería a la migración de las secuencias repetitivas teloméricas a una región subterminal, donde adquirirán la actividad centromérica. A su vez, el cambio de una segregación mediada por filamentos de actina a microtúbulos, también reforzaría la creación de los centrómeros mediante este proceso. Una migración continua de estos retroelementos al resto del cromosoma daría lugar a cromosomas holocéntricos (Villasante et al. 2007). Por último, la formación de cromosomas holocéntricos se ha asociado al proceso de *centromere drive*, proceso por el cual determinados centrómeros son seleccionados en la meiosis femenina. Esta hipótesis propone que a mayor cantidad de secuencias repetitivas centroméricas, el centrómero tiene mayor capacidad de ser atraído hacia el polo que va a dar lugar al gameto (y no al corpúsculo polar). Sin embargo este proceso acarrearía consecuencias negativas a nivel poblacional como aneuploidías, baja en la fertilidad

masculina o modificaciones en la tasa machos/ hembras. Por ello se propuso que la supresión de este proceso derivaría en cromosomas holocéntricos (Malik & Henikoff 2009). Recientemente se ha detectado el proceso selectivo de *centromere drive* en el género *Caenorabditis* (Zedek & Bureš 2012).

Otro hecho que refuerza la hipótesis de que los cromosomas holocéntricos son derivados de los monocéntricos es la constitución de sus histonas. En cromosomas monocéntricos de animales, la variante histónica CEN-H3 es la responsable del ensamblaje del cinetocoro. Sin embargo, en insectos con cromosomas holocéntricos se ha documentado la pérdida de este gen. Una reconstrucción filogenética demostró que en insectos el gen CEN-H3 se ha perdido varias veces en la evolución de este grupo de forma independiente. Estas evidencias refuerzan la hipótesis de que los cromosomas holocéntricos han surgido en distintos grupos con cromosomas monocéntricos. La pérdida del gen CEN-H3 no permitiría una reversión hacia el monocentrismo (Drinnenberg et al. 2014).

Sin embargo, hasta el día de hoy no se ha podido descartar la hipótesis que sugiere que los cromosomas holocéntricos son ancestrales. Según esta última hipótesis los cromosomas holocéntricos tienen la ventaja de que ante roturas de doble cadena los fragmentos continúan segregando correctamente al poseer actividad cinética, evitándose así la pérdida de material genético. Pero la mayor prevalencia de cromosomas monocéntricos en la escala eucariota sugiere que esta ventaja de los cromosomas holocéntricos es contrarrestada por otras desventajas, probablemente relacionadas con la segregación de los cromosomas recombinantes durante la meiosis (Mandrioli & Manicardi 2012).

El comportamiento mitótico de los cromosomas holocéntricos es diferente al observado en cromosomas monocéntricos. En los cromosomas holocéntricos de los heterópteros, las cromátidas hermanas en anafase migran paralelas al eje de división (Figura 1). En meiosis el comportamiento es aún más complejo, ya que la resolución del quiasma implica un problema geométrico. Esto es debido a la dificultad que presenta la resolución del bivalente para migrar a polos opuestos. La estructura cruciforme del bivalente hace imposible que exista actividad cinética a lo largo de todo el cromosoma. Los cromosomas holocéntricos pueden teóricamente unirse al huso de la meiosis I en muchas posiciones a lo largo de su longitud. Por lo tanto, si un bivalente holocéntrico no tiene ninguna modificación en su estructura cromosómica o posición de cinetocoro, sus superficies de captura de microtúbulos se enfrentarán en todas las direcciones. Los distintos organismos con cromosomas holocéntricos han desarrollado diferentes mecanismos para asegurar una meiosis exitosa. En el caso de los heterópteros existe un proceso llamado restricción de la actividad cinética (Melters et al. 2012) (Figura 2) que consiste en que la actividad cinética se localiza sólo en una pequeña región del cromosoma cercana a uno de los dos telómeros (Pérez et al. 1997). Generalmente, en Heteroptera, los bivalentes muestran un solo quiasma, que puede estar colocado en cualquier parte del cromosoma, y no está terminalizado (movimiento del quiasma, desde donde ha ocurrido hacia un extremo cromosómico) (Panzera et al. 2010).

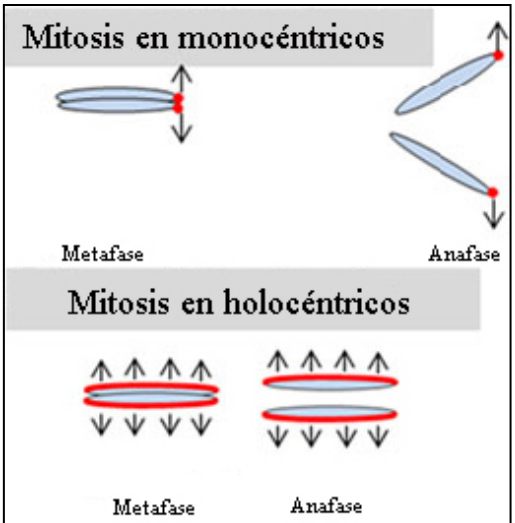

Figura 1. Diferencias entre la mitosis en cromosomas holocéntricos y monocéntricos. En la parte superior se esquematiza la actividad centromérica en un cromosoma monocéntrico, mientras en la parte inferior se

En los cromosomas holocéntricos de Heteroptera también existen diferencias en el comportamiento meiótico entre los cromosomas sexuales y los autosomas. La primera división meiótica (meiosis I) es reduccional para los autosomas y ecuacional para los cromosomas sexuales. Además, los cromosomas sexuales son aquiasmáticos y se comportan como univalentes en la meiosis I (Pérez et al. 1997). Por otro lado, ya que cualquiera de los dos extremos puede presentar actividad cinética, los autosomas tienen diferentes orientaciones en metafase I y II respectivamente, dependiendo además de la localización del quiasma (Figura 2). Los cromosomas sexuales también adquieren diferentes orientaciones en metafase I y II según el extremo cinéticamente activo (Pérez et al. 1997; Pérez et al. 2000). Se ha demostrado que existe una actividad cinética inversa, es decir, el extremo activo en meiosis I es inactivo en meiosis II. Pese a estas diferencias, la meiosis I de los cromosomas sexuales y la meiosis II de los autosomas son similares en comportamiento, lo cual sugiere un mecanismo común determinado por la cantidad de cromátidas en los cromosomas a ser segregados (Pérez et al. 2000).

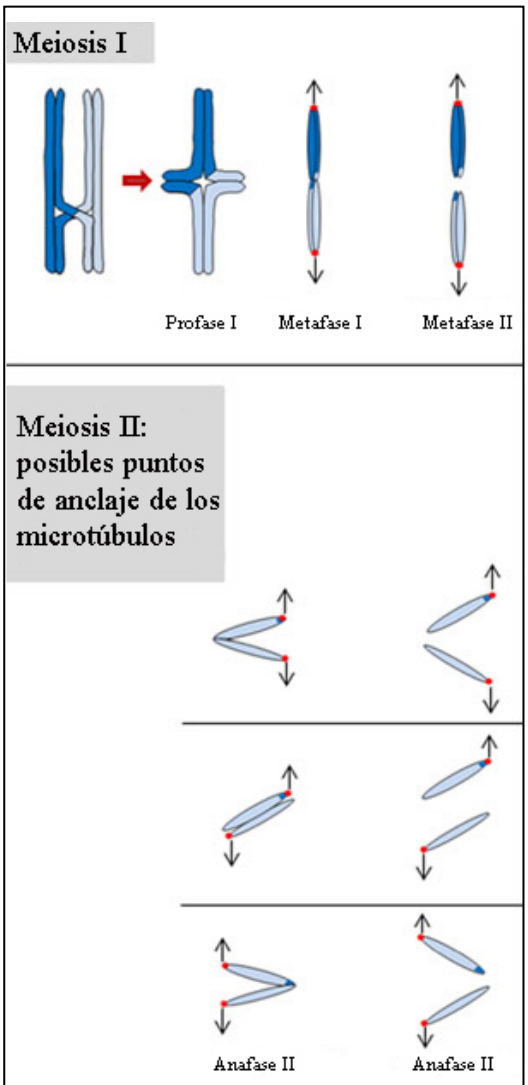

Figura 2. Mecanismo de migración de los cromosomas holocéntricos en la meiosis de los Heteroptera: restricción de la actividad cinetocórica. Parte superior esquema de un bivalente en meiosis I. Parte inferior esquema de un cromosoma en meiosis II. Modificado de Melters et al. (2012).

Se han descrito los cariotipos de más de 80 especies de triatominos (Panzera et al. 2010; Panzera et al. 2012). Todos ellos presentan cromosomas de pequeño y en

general de similar tamaño, con una escasa diferenciación longitudinal, lo cual dificulta enormemente su análisis citogenético. A esto se le suma la homogeneidad de los triatominos en su número cromosómico, el cual varía entre 21 y 25 cromosomas en los machos. La presencia de 20 autosomas es lo común en los triatominos analizados, variando solo en tres especies; *T. nitida* y *P. megistus* con 18 autosomas y *T. rubrofasciata* con 22 autosomas. La variación en el número cromosómico diploide es principalmente causada por la existencia de tres sistemas de cromosomas sexuales: XY, X_1X_2Y, y $X_1X_2X_3Y$ (en machos) (Panzera et al. 2010). Por ello se ha propuesto que 20 autosomas es el carácter ancestral (Ueshima 1966) y que los mecanismos principales para su variación son las fusiones y fisiones, como es lo más común en todos los Heteroptera (Ueshima 1966; Papeschi & Bressa 2006; Poggio et al. 2012). Sin embargo la homogeneidad del número diploide revela una baja ocurrencia de estos reordenamientos cromosómicos, contrariamente a lo pensado para los cromosomas holocéntricos. Probablemente la baja ocurrencia de estos reordenamientos en triatominos sea debida a sus efectos negativos. Generalmente, en meiosis, los univalentes y los fragmentos cromosómicos se asocian a los cromosomas sexuales, afectando su correcta segregación y produciendo gametos genéticamente no balanceados, con variaciones en el número de autosomas y de sexuales. Por ello, se sugiere que la constancia en el número cromosómico no es un reflejo de una estabilidad genómica, sino el resultado de una fuerte presión selectiva (Pérez et al. 2004).

Con respecto a los cromosomas sexuales, la hipótesis más aceptada es que el sistema XX/XY es el ancestral en la subfamilia Triatominae. El origen de los sistemas sexuales múltiples se debería a la fragmentación del cromosoma X ancestral (Ueshima 1966). Sin embargo, la homeología de los cromosomas X no ha sido comprobada entre las distintas especies de triatominos. El análisis mediante bandeo C ha mostrado una gran variabilidad en la composición de los cromosomas X, lo cual sugiere que podrían existir otros reordenamientos cromosómicos involucrados en la formación de los sistemas múltiples. Por ejemplo, la ocurrencia de reordenamientos autosómicos pueden generar fragmentos cromosómicos que posteriormente se podrían comportar como cromosomas sexuales adicionales (Pérez et al. 2004).

Debido a todo lo mencionado anteriormente acerca de las características de estos cromosomas, el análisis citogenético en especies con cromosomas holocéntricos se centra fundamentalmente en encontrar marcadores cromosómicos capaces de revelar variabilidad y, como consecuencia, analizar su evolución entre distintas especies. Así, nuestro grupo de investigación ha logrado optimizar el uso de diferentes marcadores cromosómicos en la subfamilia Triatominae. La aplicación del bandeo C permitió detectar una gran variabilidad, tanto inter como intraespecífica, en la cantidad, tamaño, localización y comportamiento de la heterocromatina constitutiva durante las divisiones meióticas. La cantidad de heterocromatina varía entre 0 y 45% del total del complemento autosómico. También el tamaño de las bandas heterocromáticas presenta una gran variación, pudiendo estar ausentes o ser

diminutas en especies del género *Rhodnius*, u ocupando el 80% del cromosoma en el caso de *T. nitida*. A su vez los bloques heterocromáticos pueden encontrarse en uno o ambos extremos cromosómicos, tanto en uno como en todos los pares autosómicos (Panzera et al. 2010). Este marcador ha sido muy útil para la detección de especies crípticas (Panzera et al. 1997; Panzera et al. 2006), y en la detección de variaciones poblacionales en los tres géneros principales: *Triatoma, Rhodnius* y *Panstrongylus* (Panzera et al. 2004; Crossa et al. 2002; Gómez-Palacio et al. 2008). En relación a los cromosomas sexuales, todas las especies de triatominos poseen un cromosoma Y heterocromático, mientras que el cromosoma X es heterocromático solo en algunas especies (Panzera et al. 2010). Hay que destacar que el comportamiento de la heterocromatina constitutiva durante la profase I temprana también es una importante variable entre especies, observándose distintas configuraciones. Por último, otro carácter significativo de diferenciación cromosómica en meiosis es la presencia o ausencia de asociaciones heterólogas mediadas por regiones heterocromáticas, lo cual se ve influenciado por el número y tamaño de los bloques heterocromáticos (Panzera et al. 2010).

La variabilidad encontrada en la heterocromatina pudo ser relacionada directamente con el tamaño de los genomas de los triatominos. La aplicación de la citometría de flujo láser permitió determinar que los genomas de los triatominos difieren en su contenido haploide de ADN en cuatro veces, entre 0.7 pg y 2.8 pg. Sin embargo, la media hallada es muy similar a la propuesta para todos los heterópteros. Los resultados mostraron que el género *Triatoma* es el más variable en su tamaño genómico, mientras el género *Rhodnius* se presentó como el más constante (Panzera et al. 2007). Considerando que los triatominos poseen complementos cromosómicos similares (entre 21 y 25 cromosomas), la gran variación encontrada en la cantidad de ADN genómico debería resultar de diferencias en el número y tipo de secuencias que contienen. Entre especies filogenéticamente cercanas hay una correlación positiva entre el tamaño genómico y la cantidad de heterocromatina. Mientras que en especies evolutivamente más distantes, las diferencias en la cantidad de ADN involucran además diferencias en la cantidad de eucromatina (Panzera et al. 2007).

La búsqueda de otros marcadores cromosómicos llevó a la implementación de la técnica de hibridación *in situ* fluorescente (FISH) para el mapeo cromosómico de las regiones del organizador nucleolar (NOR) que contienen las unidades transcripcionales para el ADNr 45S. Estas unidades incluyen una región intergénica o espaciador no transcripto (IGS o NTS) y tres regiones que codifican para los ADNs ribosómicos 18S, 5,8S y 28S separados entre sí por dos espaciadores transcriptos internos (ITS- 1 e ITS- 2). El análisis del ADNr 45S en unas pocas especies de triatominos mostró una llamativa variabilidad entre especies (Severi-Aguiar & de Azeredo-Oliveira 2005; Morielle-Souza & Azeredo-Oliveira 2007; Bardella et al. 2010). Sin embargo, estos autores utilizaron una sonda heteróloga sintetizada a partir del genoma de *Drosophila melanogaster*, por lo que su aplicación en triatominos exhibió en muchos casos señales de hibridación no específicas. Por ello, nuestro laboratorio

generó una sonda del gen 18S (incluido en el cluster ribosomal 45S) a partir del genoma de *T. infestans*. Resultados previos de nuestro grupo (los cuales incluyen mi tesis de Maestría) utilizando esta sonda mostraron una gran variación en los triatominos, incluyendo variabilidad poblacional en dos especies (*T. infestans* y *R. ecuadoriensis*), validando la hipótesis de partida (Panzera et al. 2012; Pita et al. 2013; Panzera et al. 2014). En total, las 71 especies analizadas durante mi tesis de Maestría mostraron uno o dos loci con cuatro patrones para la localización de los NORs: en un par autosómico; en el cromosoma X; en dos cromosomas sexuales o en un par autosómico y el cromosoma X. Se sugirió que el cariotipo ancestral de la familia constaría de 20 pares autosómicos y un sistema sexual simple XX/XY con un NOR localizado en un par autosómico. Además los resultados obtenidos permitieron concluir que la localización del NOR no está relacionada con variaciones en el sistema sexual, o con el número de autosomas de las especies ni tampoco con la presencia de heterocromatina constitutiva. Probablemente las fuerzas que gobiernan la evolución de la heterocromatina constitutiva y los tándem de ADN ribosomal 45S son distintas. Al igual que la variación excepcional en los patrones de bandeo C, las distintas localizaciones de los NORs demuestran una gran dinámica en los genomas de las distintas especies de triatominos. La variabilidad observada podría ser explicable mediante un mecanismo de asociación entre los genes del ARN ribosomal 45S y elementos transponibles. Otro posible mecanismo de cambio en la localización de los genes ribosomales es la recombinación ectópica, o sea recombinación de secuencias homólogas entre cromosomas no homólogos (Panzera et al. 2012; Pita et al. 2013).

Sin embargo, aún restan muchas preguntas por responder sobre la evolución cariotípica en los triatominos, entre otras ¿Qué tipos de secuencias son las responsables de los cambios en el tamaño de los genomas? ¿Qué secuencias están presentes en las regiones heterocromáticas? ¿Estas secuencias son compartidas o diferentes entre las especies? ¿Comparten secuencias los cromosomas autosómicos y los sexuales? ¿Existe algún rastro filogenético o evolutivo en la variabilidad en el número y localización de los NORs? ¿Esta variabilidad es una herramienta adecuada para abordar problemáticas poblacionales en otras especies como fue en *T. infestans*?

Es por ello que además de ampliar el análisis de las regiones NOR por FISH, hemos buscado nuevas herramientas capaces de analizar otras secuencias de ADN repetido, las cuales creemos que nos ayudarían a responder a las preguntas antes formuladas. Durante esta tesis, se planteó adquirir nuevos marcadores genómicos mediante la aplicación de técnicas de microdisección cromosómica e hibridación *in situ* genómica (GISH), hasta ahora no aplicadas en triatominos.

La microdisección cromosómica es una técnica por la cual cromosomas enteros o segmentos cromosómicos son extraídos bajo un microscopio invertido. Este ADN aislado es amplificado por PCR y marcado para ser utilizado como sonda específica sobre preparaciones cromosómicas. Esta técnica fue originalmente desarrollada en los cromosomas politénicos de *Drosophila* para obtener marcadores de ADN de regiones cromosómicas específicas, siendo rápidamente empleada en genomas de

mamíferos. Estas técnicas han sido desde entonces aplicadas en una gran diversidad de organismos, especialmente en cromosomas de mamíferos y plantas, con múltiples objetivos y variaciones metodológicas. En insectos, exceptuando *Drosophila*, esta técnica ha sido aplicada en muy pocas especies. Son de destacar los trabajos de microdisección de cromosomas X y de cromosomas B (o supernumerarios) en especies de Orthoptera (Teruel et al. 2009). En organismos con cromosomas holocéntricos está técnica fue aplicada en Lepidoptera (Vítková et al. 2007) y en una especie de Heteroptera (Bressa et al. 2009). No existen antecedentes de la aplicación de la microdisección cromosómica en triatominos.

La técnica de *Genomic in situ hibridization* (GISH) es una modificación de la técnica de FISH que se ha convertido en una de las más importantes y versátiles herramientas de investigación en citogenética que se han desarrollado en los últimos años para comparar secuencias repetidas entre especies. El ADN genómico de una especie se hibrida sobre los cromosomas de otra especie, permitiendo identificar cromosomas y genomas en híbridos, localizar y detectar cromatina de introgresión así como otros aspectos de la evolución cromosómica. La técnica de GISH es ampliamente aplicada en híbridos inter-genéricos e inter-específicos, principalmente en plantas de interés económico para, entre otras cosas, comprobar la transferencia de cromosomas, segmentos e incluso genes insertados artificialmente (Raina & Rani 2001). Además la técnica de GISH puede generar patrones específicos de bandeo cromosómico, permitiendo entre otras cosas determinar los reordenamientos cromosómicos involucrados en la evolución de las especies. Esta técnica ofrece información única sobre las similitudes de ADN entre especies relacionadas, determinado la distribución física de secuencias repetidas, las cuales pueden ser comunes o distintas entre las especies analizadas. El uso de esta técnica se ha incrementado significativamente en los últimos años pues permite no sólo establecer relaciones evolutivas entre las especies sino que también los resultados obtenidos se pueden comparar y analizar conjuntamente con árboles filogenéticos basados en secuencias de ADN (Markova & Vyskot 2009). El uso de esta técnica en cromosomas holocéntricos es excepcional habiendo sido hasta ahora solamente aplicada en Lepidoptera (Yoshido et al. 2005; Yoshido et al. 2006).

Secuencias de ADN repetido

El ADN repetido, constituido por secuencias que se repiten cientos o miles de veces en el genoma, constituye generalmente la mayor parte del ADN nuclear en eucariotas (Palomeque & Lorite 2008). Por este motivo su estudio es considerado fundamental para la comprensión de la evolución de los genomas y de su diferenciación cariotípica. Su gran diversidad y el rápido ritmo evolutivo son de gran utilidad para realizar estudios filogenéticos y poblacionales (Charlesworth et al. 1994). Además la naturaleza repetida de estas secuencias facilita enormemente su mapeo físico sobre los cromosomas mediante distintas técnicas de hibridización *in situ*, por lo que son considerados marcadores idóneos para analizar la organización y estructura del genoma, particularmente en insectos, dado que revelan la ocurrencia de distintos tipos de reordenamientos cromosómicos (Cabral-de-Mello et al. 2011).

Al conjunto de todas las secuencias de ADN repetidas presentes en el genoma nuclear de una especie se denomina "repeatoma" (Maumus & Quesneville 2014). Se reconocen diferentes clases de secuencias repetidas según su longitud, grado de repetición, estructura interna y distribución en el genoma. Según este último criterio, se distinguen dos tipos de ADN repetidos: secuencias en tándem o dispersas. Dentro de las secuencias repetidas en tándem se reconocen el ADN satélite (ADNsat) y algunas familias multigénicas como las histonas y los genes ribosomales (45S y 5S) (Charlesworth et al. 1994; Palomeque & Lorite 2008). Comúnmente se clasifica al ADNsat en micro, mini y macrosatélites según la extensión del monómero repetido y la cantidad de veces que éste se repite. Los microsatélites se definen por tener 1-6 pares de bases (pb) de longitud de repetición, con 5-100 repeticiones por locus. Los minisatélites de 7 a 100 pb de longitud, con dos a varios cientos de repeticiones por locus. Los macrosatélites presentan de dos a varios miles de bases de longitud, con alrededor de 100 repeticiones (Tautz et al. 1993).

Las secuencias repetidas en forma dispersa incluyen principalmente los elementos genéticos móviles o también llamados elementos transponibles (ETs). Los ETs son secuencias de ADN capaces de insertar copias de sí mismas en diferentes lugares del genoma. En eucariotas, los ETs están divididos en dos clases según su estructura y mecanismo de transposición (Wicker et al. 2007). Los ETs de clase I, llamados retrotransposones, necesitan de un intermediario de ARN, el cual es retrotranscripto para lograr su transposición en otro lugar del genoma. Como resultado de este mecanismo de acción, los ETs clase I aumentan su número en el genoma cuando se transponen y representan, en general pero no siempre, los ETs más abundantes del ADN genómico (Charlesworth et al. 1994). Los retroelementos se dividen en dos órdenes que difieren en su estructura, los LTR (*Long Terminal Repeats*) y los no LTR (NLTR). A su vez, dentro de cada orden existe una gran diversidad de superfamilias que son clasificadas según el grado de conservación de sus secuencias de ADN. Así dentro del orden LTR se encuentran por ejemplo los elementos *Pao/BEL*, *copia, gypsy* y ERV (Havecker et al. 2004). O en el orden NLTR las superfamilias R2, L1, RTE, I, Jockey entre otras (Kapitonov et al. 2009). La clasificación no termina aquí ya que cada superfamilia puede ser subdividida por criterios filogenéticos en subfamilias y a medida que el conocimiento avanza, nuevas superfamilias y familias son descritas (Wicker et al. 2007). Por otro lado, los ETs de clase II o transposones de ADN necesitan un intermediario de ADN de simple o doble cadena para su transposición. Se reconocen 2 grupos o subclases que se distinguen por su mecanismo de transposición (Wicker et al. 2007; Piégu et al. 2015). La primera subclase incluye los clásicos transposones "corte y pegue" ("*cut-and-paste*") que se escinden e insertan como ADN de doble cadena. Se caracterizan por codificar una transposasa y por la presencia de secuencias terminales específicas llamadas ITRs (*Inverted Terminal Repeats*), las cuales son reconocidas por la propia transposasa (Wicker et al. 2007). En los ETs de clase II, subclase transposones, también se reconocen diferentes superfamilias que se distinguen según la secuencia del gen de la transposasa, por ejemplo Tc1-*Mariner*, hAT o *PiggyBac* (Wicker et al. 2007). La segunda subclase de los ETs clase II son los llamados Helitrons, caracterizados por un proceso de

transposición llamado círculo rodante (*rolling-circle*), que implica la replicación con un corte de cadena simple (Kapitonov & Jurka 2001). Las tercera subclase de ETs clase II son los llamados Mavericks también conocidos como Polintons. Son transposones muy extensos con largas secuencias ITRs que codifican para múltiples proteínas, incluyendo una ADN polimerasa que actúa en su propia transposición (Feschotte & Pritham 2007). Aunque Helitrons y Mavericks se trasponen por mecanismos distintos, ambos involucran la formación de un ADN monocatenario intermedio mediante procesos de "copiado y pegado" ("copy-and-paste"). Su inserción puede provocar distintos tipos de mutaciones que incluso pueden afectar al fenotipo del individuo. De esta forma los ETs juegan un rol fundamental en la evolución de los organismos, ya que por su capacidad de movilidad participan activamente en la reestructuración de los genomas generando diversidad genética. La movilidad de los ETs podrían causar importantes reordenamientos cromosómicos tales como translocaciones, inversiones, duplicaciones y deleciones, lo cual fue comprobado en distintos organismos (Weil 2009; Zhang et al. 2009).

El significado funcional del ADN repetitivo ha sido muy controvertido. Durante mucho tiempo se pensaba que carecía de función, por lo que había sido denominado ADN basura o parásito (Palazzo & Gregory 2014). Sin embargo, numerosas evidencias contradicen esta afirmación, habiéndose comprobado su importante papel en varias funciones del genoma. Por ejemplo, el ADNsat es el principal constituyente de la heterocromatina, la cual posee un papel esencial en la estructura del centrómero, formación del cinetocoro y en el apareamiento y segregación de los cromosomas. Además, se ha relacionado al ADNsat con el aislamiento reproductivo y por lo tanto con la aparición de nuevas especies (Biscotti et al. 2015a). En los últimos años numerosos trabajos evidencian la existencia de ARNs transcritos a partir de ADNsat de diferentes especies de vertebrados, invertebrados y plantas. La mayor parte de estos transcritos son ARNs no codificantes (ncRNAs) y están implicados en la regulación de la expresión génica bajo determinadas circunstancias como estrés celular y ciertas enfermedades como por ejemplo el cáncer (Rošić et al. 2014; Biscotti et al. 2015b). En insectos, el papel de la transcripción del ADNsat ha sido especialmente estudiado en *Drosophila melanogaster*. Donde, se han encontrado transcritos del ADNsat 1.688 en embriones y en adultos procedentes de los cromosomas 2 y 3 daban lugar a ARN de interferencia (siRNAs) que participaban en la formación de heterocromatina en estos dos cromosomas (Usakin et al. 2007). Mientras que transcritos del cromosoma X son necesarios para una correcta segregación cromosómica (Rošić et al. 2014). También se han relacionado estos transcritos con la remodelación de la cromatina asociada a la compensación de dosis génica, posiblemente interaccionando con el complejo MSL (Menon et al. 2014). En otra especie de insecto, el escarabajo *Palorus subdepressus* se ha observado la expresión de una familia de ADNsat en diferentes estadios de desarrollo, habiéndose asociado la transcripción al estrés. En esta especie se observó un aumento de la transcripción como respuesta al choque térmico, considerándose que los transcritos podrían estar implicados en la remodelación de la cromatina y participando así en la

activación de la expresión génica en condiciones de estrés (Pezer & Ugarković 2009).

En resumen, diversos autores han destacado la importancia del ADNsat en la integridad del genoma y en la evolución cariotípica (revisión en (Biscotti et al. 2015a).

Por otro lado los ETs han recibido desde hace mucho tiempo una gran atención respecto a su potencial papel dentro del genoma, más allá de un mero ADN egoísta. Además del efecto mutagénico de la propia inserción, algunos ETs tienen la capacidad de capturar y mover otras secuencias genómicas adyacentes a ellos por un proceso denominado transducción. En el maíz por ejemplo, determinados ETs se mueven junto con fragmentos de genes adyacentes que capturan durante su mecanismo de replicación (Yang & Bennetzen 2009). En varias especies de animales y plantas, la extensa variabilidad intraespecífica en la localización cromosómica de los genes repetidos ribosomales 45S y 5S es debida a la movilidad de ETs adyacentes o intercalados entre ellos (Raskina et al. 2008). También, su naturaleza repetitiva facilita la recombinación entre fragmentos de ADN no-homólogo. En relación con la especiación, en los últimos años se ha demostrado, tanto en vertebrados como invertebrados, que los ETs son los responsables de extensos cambios cromosómicos que pueden conducir a la formación de nuevas especies (Lonnig & Saedler 2002; Feschotte & Pritham 2007; Weil 2009; Zhang et al. 2009), e incluso invadir nuevos genomas mediante el fenómeno de transferencia horizontal (Kidwell & Lisch 2001). Otro aspecto interesante del aporte que han significado los ETs en la remodelación de los genomas, es que estos son una materia prima para la generación de nuevos genes o estructuras cromosómicas. La domesticación de elementos transponibles, proceso por el cual un ET se convierte en un gen funcional para el organismo, está ampliamente documentada (Feschotte & Pritham 2007; Mateo & Gonzalez 2014). A su vez, la hipótesis más aceptada es que los primeros telómeros y centrómeros derivan de la acumulación de secuencias de ETs (Villasante et al. 2007).

Pese a que con el tiempo el análisis de las secuencias repetitivas ha adquirido mayor relevancia para la comprensión del funcionamiento del genoma, su estudio ha recibido menor atención que el de las secuencias codificantes. El análisis de los genomas completos suele abarcar y profundizar en mayor medida en las regiones codificantes del genoma debido a la dificultad que representa la secuenciación y ensamblaje de las regiones repetidas.

Hoy en día, el análisis del ADN repetido se ha beneficiado de novedosas estrategias de análisis genómicos (*genome wide*) basadas en nuevas tecnologías de secuenciación denominadas tecnologías de secuenciación masiva o de próxima generación (*Next generation sequencing*, NGS). Acompañando estas tecnologías se han desarrollado herramientas bioinformáticas que permiten analizar la gran cantidad de datos que resultan de la secuenciación. Una de estas herramientas, denominada RepeatExplorer (Novák et al. 2013) permite analizar la totalidad del repeatoma, ya que identifica de una manera gráfica los diferentes tipos de secuencias repetidas, además de permitir establecer su abundancia. Este programa aplica un enfoque muy diferente para el análisis global del ADN repetido que facilita la

identificación *de novo* de repeticiones. Se basa en la búsqueda y la cuantificación de las similitudes (90% de similitud en 55% del largo de la secuencia) entre las lecturas de secuencias individuales obtenidas por los nuevos sistemas de secuenciación, que utilizan segmentos de ADN genómico cortados al azar. Este método y otros similares se han aplicado con éxito por ejemplo en el estudio del ADNsat humano. Los *gaps* más largos dentro de la secuencia del genoma humano se corresponden con regiones heterocromáticas de un tamaño muy grande (multi-mega bases) compuestas principalmente de dos familias relacionadas de repeticiones en tándem, en concreto los satélites humanos 2 y 3 (HSat2, 3). La abundancia de ADN repetido en estas regiones ha constituido un auténtico reto, aún sin solución, para las técnicas de mapeo y secuenciación por ensamblaje mediante algoritmos estándares, por lo que la composición de las secuencias repetidas y sus posibles funciones biológicas siguen siendo, en gran parte, desconocidas (Altemose et al. 2014; Chu et al. 2016).

Muy recientemente, estas nuevas herramientas informáticas se han comenzado a aplicar con éxito en insectos. Por ejemplo estos análisis bioinformáticos junto con la localización cromosómica por FISH de las secuencias repetidas han aportado novedosos datos sobre la evolución del ADNsat en especies de Orthoptera (Camacho et al. 2015; Ruiz-Ruano, López-León, et al. 2016; Ruiz-Ruano, Cabrero, et al. 2016; Palacios-Gimenez et al. 2017) y *Drosophila* (de Lima et al. 2017). Sin embargo en insectos con cromosomas holocéntricos, no existen antecedentes.

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Hipótesis de trabajo

Tal como se ha ido poniendo de manifiesto en la introducción de este trabajo, las secuencias repetitivas tienen un papel fundamental en la organización y evolución de los genomas eucariotas. El estudio de la composición y localización de las secuencias repetidas ha sido una herramienta útil para ampliar el conocimiento sobre los procesos evolutivos que han acompañado la evolución y diferenciación de las especies. Consideramos que la realización de análisis similares en Triatominos puede resultar igualmente útiles para analizar los cambios genómicos ocurridos durante la evolución de este importante grupo de insectos.

Objetivos

Objetivo general

Ampliar el conocimiento sobre los procesos evolutivos a nivel citogenético ocurridos en la subfamilia Triatominae (Hemiptera, Reduviidae) mediante la caracterización y análisis del ADN repetitivo.

Objetivos específicos

1. Extender el análisis sobre la localización de las regiones del organizador nucleolar a nuevas especies y poblaciones no analizadas durante la tesis de maestría.

2. Determinar el grado de diferenciación de los cromosomas sexuales X e Y entre sí y con los autosomas mediante sondas cromosómicas obtenidas por microdisección cromosómica.

3. Análisis comparativo de secuencias de ADN repetido, entre distintas especies utilizando la técnica de *Genomic in situ hybridization* (GISH).

4. Búsqueda, identificación y mapeo cromosómico de secuencias de ADN repetido (elementos transponibles y familias génicas) mediante *Fluorescence in situ hybridization* (FISH).

Capítulo I:

Genes ribosomales y otras familias multigénicas

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RESEARCH Open Access

Cryptic speciation in the *Triatoma sordida* subcomplex (Hemiptera, Reduviidae) revealed by chromosomal markers

Francisco Panzera[1*], Sebastián Pita[1*], Julieta Nattero[2,3], Yanina Panzera[1], Cleber Galvão[4], Tamara Chavez[5], Antonieta Rojas De Arias[6], Lourdes Cardozo Téllez[7] and François Noireau[8^]

Abstract

Background: Chagas disease vectors (Hemiptera-Reduviidae) comprise more than 140 blood-sucking insect species of the Triatominae subfamily. The largest genus is *Triatoma*, subdivided in several complexes and subcomplexes according to morphology, ecology and genetic features. One of them is the sordida subcomplex, involving four species: *Triatoma sordida, T. guasayana*, *T. garciabesi* and *T. patagonica*. Given the great morphological similarity of these species, their taxonomic identification, evolutionary relationships and population differentiation have been controversial for many years and even today remain under discussion.

Methods: We simultaneously analyzed two chromosomal markers, C-heterochromatin distribution and 45S ribosomal genes chromosomal position, of 139 specimens from several sordida subcomplex populations from Argentina, Bolivia, Brazil and Paraguay, collected both in nature and from several established insectaries. Our results were compared with COI sequences deposited in GenBank.

Results: We recognized five chromosomal taxa with putative hybrids, which each differ in at least one chromosome marker. Most of them present significant differences in their mtDNA sequences.

Conclusion: The chromosomal taxa here show a significant chromosome differentiation involving changes in the C-heterochromatin content and in the ribosomal clusters position. This paper identifies several erroneously classified populations by morphological methods, delimits the geographical distribution of each taxon and proposes the existence of a new cryptic species, widely distributed in Argentina. We also suggest that sordida sibling species involve closely related as well as evolutionary distant species. Taxonomic status of each chromosomal taxon is discussed considering phenotypic and genetic results previously published.

Keywords: Sordida subcomplex species, Triatominae, Chagas disease vectors, Cryptic species, Holocentric chromosomes, FISH

Background

The subfamily Triatominae (Hemiptera, Reduviidae) comprises more than 140 blood-sucking insect species – most of them vectors of the protozoan *Trypanosoma cruzi*, causative agent of Chagas disease or American trypanosomiasis. Of these, the most conspicuous genus is *Triatoma* with 80 species, grouped in 8 complexes and 8 subcomplexes according to morphology, habitat, ecology and genetic analyses [1]. One of these groups is the sordida subcomplex, which traditionally included four species: *Triatoma sordida* (Stål 1859), *T. patagonica* Del Ponte 1929, *T. guasayana* Wygodzinsky & Abalos 1949 and *T. garciabesi* Carcavallo, Cichero, Martínez, Prosen & Ronderos 1967. *Triatoma sordida* was the first described species in 1859 and it presents the most extensive distribution in parts of central Argentina, Bolivia, Brazil, Paraguay, and Uruguay. *T. guasayana* is found in Argentina, Bolivia and Paraguay, while *T. garciabesi* and *T. patagonica* are recorded only from Argentina [2, 3].

* Correspondence: fcopanzera@gmail.com; spita@fcien.edu.uy
^Deceased
[1]Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Calle: Iguá 4225, 11400 Montevideo, Uruguay
Full list of author information is available at the end of the article

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The taxonomic validity of sordida subcomplex species, their evolutionary relationships, and especially population differentiation within *T. sordida*, have been controversial for many years and even today remain under discussion.

The first report regarding morphological variation among *T. sordida* populations appeared in 1951 [4]. These authors compared smaller and darker sylvatic individuals from Santiago del Estero (northeastern Argentina) with domestic and peridomestic specimens from the rest of Argentina. In 1965, electrophoretic profiles of hemolymph proteins showed significant differentiation among Argentinean sylvatic samples with domestic specimens from Brazil [5]. These sylvatic specimens were then described as a new species called *T. garciabesi* based on morphological differences from *T. sordida* and *T. guasayana* [6], but this new species was then synonymized with *T. sordida* [2]. Several years later, isoenzymatic and chromosomal studies of several *T. sordida* populations from Argentina and Brazil suggested the existence of at least two distinct forms, one from Brazil and other from Argentina [7]. Based on these results, *T. garciabesi* was revalidated as a species, according to differences in their morphology (overall size, color, head and genitalia), isoenzymes (10 diagnostic loci) and chromosomal characteristics (C-heterochromatin distribution) [8]. In Bolivia, isoenzymes studies suggested the existence of two distinct forms within the *T. sordida* populations, named group 1 and group 2, differentiated by their Idh-2 and Mdh-2 loci [9]. All domestic *T. sordida* populations in Bolivia were of group 1, although this form could also be found in peridomestic and sylvatic environments [10]. *T. sordida* group 2 is predominantly sylvatic and seems to be restricted to the Chaco region. In the Bolivian Chaco, both *T. sordida* groups and *T. guasayana* occurred in sympatry, including putative hybrids [9–11]. Morphometric (wings and heads) and cuticular hydrocarbon analyses confirmed a distinction between *T. garciabesi* and *T. sordida*, and also show significant differences between *T. sordida* populations from Argentina and Brazil [12, 13]. More recently, population studies on *T. sordida* from Paraguay revealed ecological, genetic and morphometric differences between specimens from Western (Chaco region) and Eastern Paraguay [14].

Karyotypic information for more than 80 species of Triatominae is currently available, showing a highly conserved diploid chromosome number ranging from 21 to 25 chromosomes in males [15]. In spite of this homogeneity, cytogenetic analyses have shown that the subfamily is one of the most variable and chromosomally diverse of the Heteroptera. Distribution, size and amount of C-heterochromatin and the chromosomal location of 45S ribosomal genes presented a striking differentiation among triatomine species. Although there are exceptionally polymorphic species, both traits are species-specific characters, and therefore very useful for species discrimination [15–18].

The four sordida subcomplex species exhibit the same diploid number: 22 chromosomes composed of 20 autosomes plus XY/XX (male/female) sex chromosomes [7, 8]. The original *T. sordida* karyotypic information was described for Brazilian specimens [19]. With C-banding, *T. guasayana* and *T. garciabesi* did not present autosomal C-heterochromatin while *T. sordida* (Brazilian populations) and *T. patagonica* presented C-blocks comprising around the 30 % of their autosomes [7]. Currently, Fluorescence in situ hybridization (FISH) analyses are restricted to one *T. sordida* population from Brazil and one *T. garciabesi* population. In both species 45S ribosomal DNA clusters are located on X chromosome [17].

In the current work, we analyzed both chromosomal markers simultaneously on specimens from Argentina, Bolivia, Brazil and Paraguay. This study does not include *T. patagonica* because it does not present morphological identification problems with the other species of the subcomplex [20]. The application of two cytogenetic techniques offers the possibility of differentiate populations that show similar characteristics with a single chromosomal marker, and the analysis with both markers allows us to detect the existence of hybrids, either between species or populations chromosomally differentiated, as recently reported in *T. infestans* [16]. Both markers are mainly inherited in a Mendelian fashion and evolved independently [16, 17, 21], so they are suitable for analyzing different evolutionary pathways.

Methods

Insects and sampling sites

We carried out chromosomal analyses of 139 insects of the *T. sordida* subcomplex from Argentina, Bolivia, Brazil and Paraguay, including field-caught and insectary specimens. The geographic origin of each population, habitat, and *a priori* species determination are given in Tables 1, 2, 3, 4 and 5. Some individuals examined cytogenetically in this paper have been previously studied using various morphological and genetic analyses (Tables 1–5). When possible, we determined the chromosomal characteristics of each species based on topotype specimens (i.e. individuals from the same geographical origin as the holotype). For *T. sordida,* named here *T. sordida sensu stricto,* we considered as topotypes the specimens from Minas Gerais, Brazil, because the holotype locality is not specified. For *T. garciabesi* we analyzed the same individuals used in their redescription [8] and chromosomal characteristics were also determined from specimens from the holotype locality (Santiago del Estero, Argentina) and from the same specimens previously identified by

Table 1 Populations identified as *T. guasayana* by C-banding and rDNA FISH studies

Country, Province, Department, habitat	*N*	Original species assignment	Previous morphological and genetic analyses
Argentina, Santiago del Estero, P. CRV. 4thgener.	3	*T. guasayana*	Cuticular hydrocarbons, iso-enzymes, C-banding [7, 13, 39].
Argentina, Córdoba, Cruz del Eje, S. CRV. 2thgener.	5	*T. guasayana*	Isoenzymes, C-banding [7].
Argentina, Córdoba, Sobremonte, S.	3	*T. guasayana*	NS
Argentina, La Rioja, P. CRV. Colony 0297, 4thgener.	4	*T. guasayana*	Isoenzymes, C-banding [7].
Bolivia, Santa Cruz, Boyuibe, S.	2	*T. guasayana*	Isoenzymes [9].
Bolivia, Santa Cruz, Izozog, S.	4	*T. guasayana*	Isoenzymes [9, 10].
Bolivia, Cochabamba, Mataral, S.	1	*T. guasayana*	NS
Bolivia, Cochabamba, Chujillas. S. LEN. 2thgener.	2	*T. guasayana*	NS

Geographical origin, number of analyzed individuals and previous reports
N number of analyzed individuals in this paper, *CRV* Centro de Referencia de Vectores, Servicio Nacional de Chagas (Córdoba, Argentina), *LNIRTT* Laboratório Nacional e Internacional de Referencia em Taxonomia de Triatomíneos, Instituto Oswaldo Cruz (Fiocruz) (Rio de Janeiro, Brazil), *LEN* Laboratorio de Entomología Médica, Instituto Nacional de Laboratorios de Salud (INLASA) (La Paz, Bolivia), *D* domestic, *P* peridomestic, *S* sylvatic, *gener* generation, *RAPD* Random Amplified Polymorphic DNA, *NS* No Studied

isoenzymes [7, 9]. A new chromosomal taxon, here referred to as *T. sordida Argentina*, was differentiated for specimens from San Luis del Palmar (Corrientes, Argentina), and a further chromosomal taxon was differentiated from domestic populations from La Paz (Bolivia) provisionally named *T. sordida La Paz.*

Chromosome preparations and banding procedures

For chromosome preparations, testes were removed from freshly killed adults, and fixed in an ethanol–glacial acetic acid mixture (3:1,v:v). Subsequently, we performed C-banding to establish the diploid chromosome number (2n) and the C-heterochromatin distribution [21]. We also applied FISH analyses to determine the location of 45S ribosomal clusters [17]. For each specimen, at least 20 cells were analyzed. Chromosome preparations were examined under a Nikon Eclipse 80i microscope and the images were obtained with a DS-5Mc-U2 digital camera using Nikon Nis Element s3.1 Advanced Research software and processed with Adobe Photoshop® software.

Molecular datasets, sequence alignments and phylogenetic analyses

Fourteen *T. sordida, T. garciabesi* and *T. guasayana* partial COI gene sequences deposited in GenBank were employed for phylogenetic analyses, plus two *T. rubrovaria* sequences used as outgroup. Other sequences of this subcomplex available in GenBank were excluded from this study since their geographical origin was unknown. All sequence accession numbers are specified in Fig. 3. Maximum likelihood (ML) phylogenetic analyses were implemented in MEGA 6 [22] with statistical support for the nodes evaluated with 1000 bootstrap replicates. The best fitted substitution model was also determined using MEGA 6 software [22]. The alignment included 522 bp including 113 variable sites (21.6 %), 101 of which were informative for parsimony (19.3 %).

Results

Male specimens from all populations showed the same diploid number (2n = 22) constituted by 20 autosomes

Table 2 Populations identified as *T. garciabesi* by C-banding and rDNA FISH studies

Country, Province, Department, habitat	*N*	Original species assignment	Previous morphological and genetic analyses
Argentina, Salta, Rivadavia, S. LNIRTT.	6	*T. garciabesi*	Morphology of head and genitalia, isoenzymes, C-banding, FISH [8, 17].
Argentina, La Rioja, P. CRV. Colony 0303, 3thgener.	2	*T. sordida/T. garciabesi*	Geometric morphology of wings and heads, isoenzymes, C-banding [7, 12].
Argentina, Santiago del Estero, Rio Hondo, S.CRV. 4th gener.	2	*T. sordida/T. garciabesi*	Cuticular hydrocarbons, isoenzymes, C-banding [7, 13].
Argentina, Santiago del Estero, Aguirre, P.	3	*T. garciabesi*	NS
Argentina, Chaco, Guemes, P. CRV. Colony 352, 1thgener.	5	*T. sordida*	NS
Argentina, Formosa, Patiño, P. CRV. Colony 335, 1thgener.	3	*T. sordida*	NS
Paraguay, Presidente Hayes & Boquerón. Several localities. P.	9	*T. sordida*	Geometric morphology of wings and heads, RAPD [14].
Bolivia, Santa Cruz, Izozog. S.	9	*T. sordida Group 2*	Isoenzymes [9, 10].

Geographical origin, number of analyzed individuals and previous reports. Abbreviations described in Table 1

Table 3 Populations identified as *T. sordida sensu stricto* by C-banding and rDNA FISH studies

Country, Province, Department, habitat	*N*	Original species assignment	Previous morphological and genetic analyses
Brazil, Minas Gerais, Montes Claros, P. LNIRTT. 1th-2thgener.	8	*T. sordida*	Morphology of head and genitalia, isoenzymes, C-banding [7, 8].
Brazil, Minas Gerais, Uberaba. P.	4	*T. sordida*	Isoenzymes, C-banding [7].
Brazil, Piaui, P. LNIRTT.	7	*T. sordida*	Isoenzymes, C-banding [7].
Brazil, Matto Grosso, São José do Povo, P.	2	*T. sordida*	C-banding, FISH [17].
Bolivia, Santa Cruz, Izozog. P.	2	*T. sordida Group 1*	Isoenzymes [9, 10].
Bolivia, Santa Cruz, Cotoca. D.	2	*T. sordida Group 1*	Isoenzymes [9, 10].
Paraguay, San Pedro, P.	2	*T. sordida*	Geometric morphology of wings and heads, RAPD [14].
Paraguay, Concepción, P.	7	*T. sordida*	NS

Geographical origin, number of analyzed individuals and previous reports. Abbreviations described in Table 1

plus two sex chromosomes (XY). All individuals, including putative hybrids, presented normal meiosis without irregularities in the chromosome behavior. With C-banding, all specimens had a C-heterochromatic Y chromosome. Different C-banding patterns are observed according to the number of autosomes with C-regions. The ribosomal cluster has one or two chromosome loci per haploid genome, showing 4 location patterns: one autosomal pair, X chromosome, both sex chromosomes or on an autosomal pair and one sex chromosome simultaneously.

Analysis of each individual with both chromosomal markers allowed us to identify five different chromosomal taxa, which are described below. Tables 1–5 show the populations identified within each taxon. Table 6 summarizes chromosomal characteristics of the five chromosomal taxa identified.

T. guasayana (Table 1 and Fig. 1a, b): The 10 autosomal pairs do not show striking size differences, being two or three pairs slightly larger. The X chromosome and all bivalents have no C-bands (Fig. 1a). Ribosomal clusters are located in one autosomal pair (arrow Fig. 1b). This species included samples from Argentina and Bolivia. In several individuals our chromosomal identification matches the species assignment by isoenzymes (Table 1).

T. garciabesi (Table 2 and Fig. 1c, d): The 10 bivalents exhibit similar size, but two or three of them slightly larger. Autosomes and X chromosome are euchromatic (without C-bands) (Fig. 1c). The 45S rDNA clusters are localized on the X chromosome (arrow Fig. 1d). This species included specimens from Argentina, Western Paraguay and Bolivian Chaco.

T. sordida sensu stricto (Table 3 and Fig. 1e, f): Two or three autosomal pairs are slightly larger than the rest. Most autosomal pairs present a C-heterochromatic block in one chromosomal end, while the others are euchromatic (Fig. 1e). The X chromosome may present a small C-block or not. The 45S rDNA clusters are localized on the X chromosome (arrow Fig. 1f). This species included samples from Brazil, Central and Eastern Paraguay, and Bolivian Chaco.

T. sordida Argentina (Table 4 and Fig. 1g, h): The 10 autosomal pairs and the X chromosome do not have C-heterochromatin (Fig. 1g). Ribosomal clusters are located on both sex chromosomes (X and Y) (arrows Fig. 1h). This putative chromosomal species included samples from Argentina, Eastern Paraguay, and Bolivian high valleys (Table 4).

T. sordida La Paz (Table 5 and Fig. 1i, j): Seven to eight autosomal pairs present a C-heterochromatic region

Table 4 Populations identified as *T. sordida Argentina* by C-banding and rDNA FISH studies

Country, Province, Department, habitat	*N*	Original species assignment	Previous morphological and genetic analyses
Argentina, Corrientes, San Luis del Palmar. P.	4	*T. sordida*	Geometric morphology of wings and heads [12].
Argentina, Santiago del Estero. S. LNIRTT.	2	*T. sordida*	NS
Argentina, Chaco, Guemes, El Colchón, P.	6	*T. sordida*	NS
Argentina, Formosa, Pirane, P. CRV. 1th gener.	2	*T. sordida*	NS
Argentina, Tucumán, San Miguel, D.	3	*T. sordida*	NS
Bolivia, Cochabamba, Quilacollo, Cotapachi, S.	3	*T. sordida*	NS
Paraguay, Paraguari, Carepaguá, P.	6	*T. sordida*	Geometric morphology of wings and heads, RAPD [14].
Paraguay, San Pedro, Itacurubí& Villa Rosario, P.	3	*T. sordida*	Geometric morphology of wings and heads, RAPD [14].

Geographical origin, number of analyzed individuals and previous reports. Abbreviations described in Table 1

Table 5 Populations identified as *T. sordida La Paz* by C-banding and rDNA FISH studies

Country, Province, Department, habitat	*N*	Original species assignment	Previous morphological and genetic analyses
Bolivia, La Paz, Inquisivi, D	4	*T. sordida*	NS
Bolivia, La Paz, Apolo, D	5	*T. sordida*	NS

Geographical origin, number of analyzed individuals and previous reports. Abbreviations described in Table 1

in only one chromosomal end, similar to that observed in *T. sordida sensu stricto* (Fig. 1i). However, the ribosomal clusters are located on one autosomal pair (arrow Fig. 1j). This putative chromosomal species included exclusively domestic individuals from Bolivian highlands of La Paz (Table 5).

Chromosomal hybrids (Fig. 2a, c): We detected 4 individuals from Bolivia (two from Apolo-La Paz and two from Izozog-Santa Cruz) with chromosomal patterns that could represent hybrids between some of the above mentioned taxa. With C-banding, each individual presented three types of bivalents: heterochromatic with C-region in one chromosomal end, euchromatic and heterozygote bivalents (one homologous heterochromatic and the other euchromatic) (arrowheads Fig. 2a). With FISH technique, they exhibit two different ribosomal cluster locations: either in one heterozygote autosomal pair, or in an autosomal pair plus X-chromosome (arrows Fig. 2b, c, respectively).

Phylogenetic analyses

The maximum likelihood tree was obtained using Tamura 3-parameters plus Gamma distribution (T92 + G). The resulting ML tree topology (Fig. 3) showed four well supported groups or clades. Clade 1 or *T. sordida*: include 6 *T. sordida* samples (4 from Brazil and 2 from Bolivia) and 1 specimen identified as *T. guasayana* (Bolivia); Clade 2 or *T. sordida Argentina*: include 2 samples identified as *T. sordida* from Corrientes (Argentina); Clade 3 or *T. garciabesi*: Include 1 *T. garciabesi* individual from Argentina (Salta) and 2 specimens identified as *T. sordida* from Bolivian Chaco (Romerillo); Clade 4 or *T. guasayana:* involve 2 *T. guasayana* individuals from Argentina (Santiago del Estero).

The mean pairwise nucleotide distances (Tamura-3 parameters) between the four clades reflect significant differences between them (from 5.3 to 14.9 %) (Table 7). Considering the robustness of the four clades supported by their genetic distance, we can suggest errors in the original taxonomic determination of some individuals (*T. guasayana*/KC249342; *T. sordida* from Romerillo/KC249379 and KC249381), since they grouped within a clade formed by individuals from another species (Fig. 3). These misidentifications were corroborated by our cytogenetic results (see discussion) using individuals from the same or similar geographic locations.

Discussion

Cryptic species, also called sibling or isomorphic species, are identical in their external appearance, or differ in apparently minor and not easily visible traits. In Triatominae, the use of different phenotypic and genetic markers has shown that cryptic speciation is a widespread phenomenon in this subfamily. The existence of sibling species has been described in different *Triatoma* groups such as the brasiliensis, dimidiata, phyllosoma and sordida subcomplexes, as well as in several species of *Rhodnius* and *Panstrongylus* (for review see [1]).

Sordida subcomplex species except *T. patagonica* are morphologically very similar, with overlapping geographical distribution and even sympatric in many regions with putative hybrids, which significantly increase the taxonomic confusion. Although there are phenotypic and genetic markers able to differentiate the species, its recognition requires high expertise which greatly hinders their application by vector control staff– leading to mistaken determination even within insectary material (see Tables 1–4). Below, we discuss our results for each chromosomal taxon here identified, in comparison with previously published determinations.

T. guasayana: The morphological differentiation of *T. guasayana* with *T. sordida* is very difficult, especially in

Table 6 Comparative summary of the 5 chromosomal taxa and putative hybrids identified by two chromosomal markers

Chromosomal taxon (*n*)	Autosomal C-heterochromatin by C-banding	Location of 45S ribosomal clusters by FISH
T. guasayana (24)	NO	One autosomal pair
T. garciabesi (39)	NO	X chromosome
T. sordida sensu stricto (34)	YES	X chromosome
T. sordida Argentina (29)	YES	X and Y chromosomes
T. sordida La Paz (9)	YES	One autosomal pair
Hybrids from Apolo-La Paz (2) and Izozog-Santa Cruz (2)	YES	Polymorphic: One autosomal pair plus X chromosome or on a heterozygote autosomal pair.

We included the total number of analyzed individuals between brackets

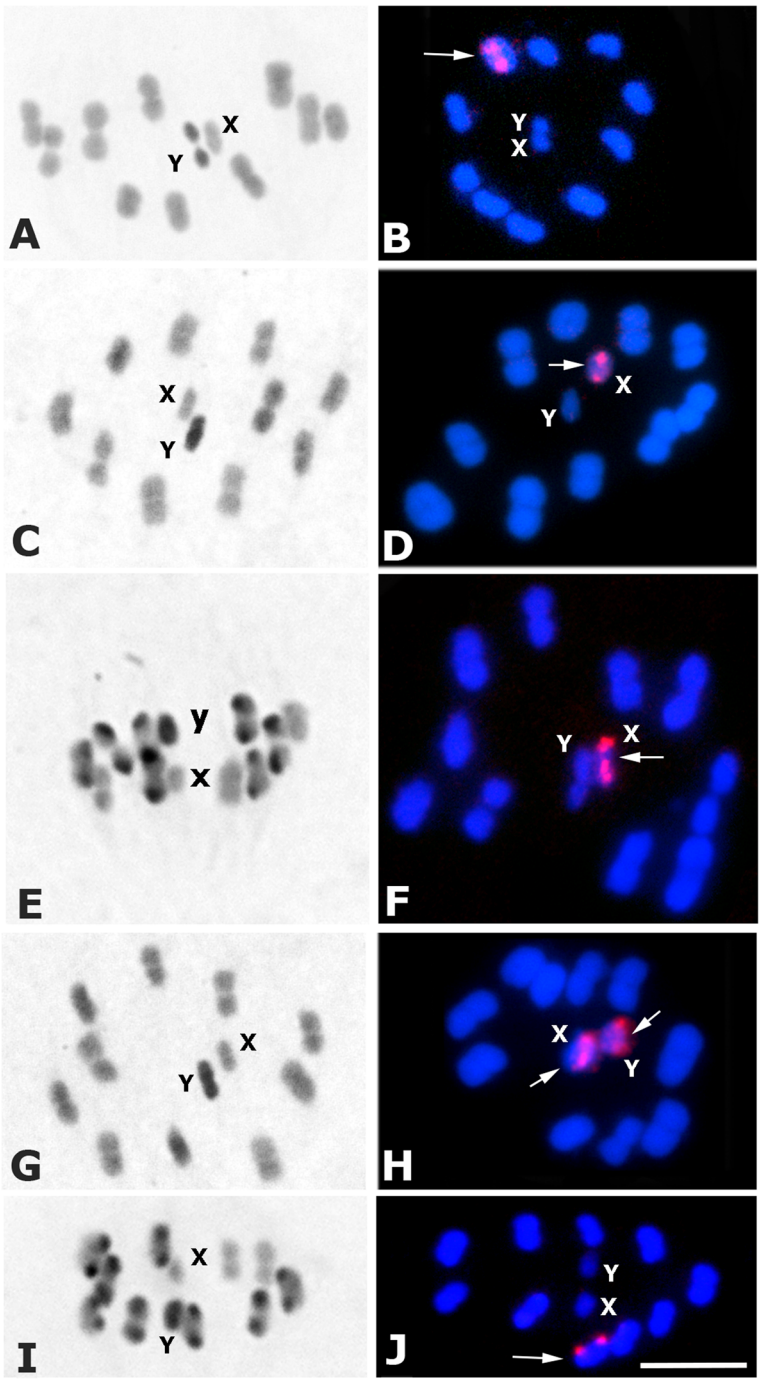

Fig. 1 Male meiosis in different *Triatoma sordida* subcomplex species (2n = 20A + XY). (**a**-**c**-**e**-**g**-**i**): C-banding. (**b**-**d**-**f**-**h**-**j**): Fluorescent in situ hybridization with 45S ribosomal DNA probe. **a**-**b**: *T. guasayana* **a**: Metaphase I (MI). All autosomal bivalents and the X chromosome are euchromatic while that the Y appears C-heterochromatic. **b**: Metaphase II (MII). rDNA signals are located in one autosomal bivalent (*arrow*). **c**-**d**: *T. garciabesi* **c**: MI. C-heterochromatin distribution similar as observed in *T. guasayana* (**d**): M I. Ribosomal signals on the X chromosome (*arrow*). **e**-**f**: *T. sordida sensu stricto*. **e**: MII. Seven autosomal pairs exhibit C-blocks while 3 pairs are euchromatic (**f**): MI. rDNA signals on the X chromosome (*arrow*). **g**-**h**: *T. sordida Argentina*. **g**: MI. All chromosome complement is euchromatic, except for the heterochromatic Y chromosome. **h**: MI. Ribosomal signals on both sex chromosomes (*arrows*). **i**-**j**: *T. sordida La Paz*. **i**: MII. Eight autosomal pairs present C-blocks, while 2 pairs are euchromatic. **j**: MII. rDNA clusters on one autosomal pair (*arrow*). Bar = 10 μm

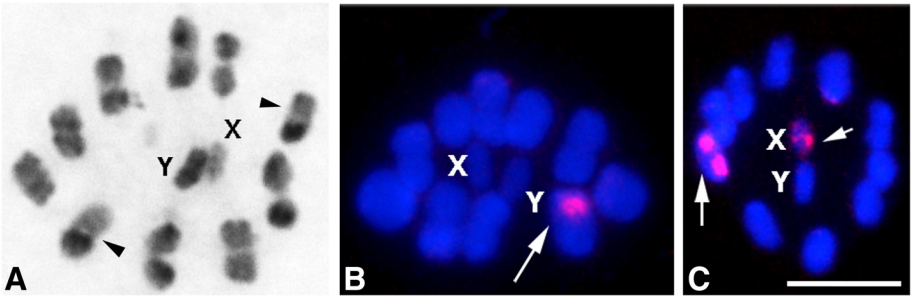

Fig. 2 Male meiosis in putative hybrids of *Triatoma sordida* subcomplex (2n = 20A + XY). **a**: Metaphase I with C-banding. Three types of bivalents are observed: heterochromatic (C-region in one chromosomal end), euchromatic (without C-region) and heterozygote bivalents with one homologous heterochromatic and the other euchromatic (*arrowheads*). **b**-**c**: Metaphase I and metaphase II, respectively. Fluorescent *in situ* hybridization with 45S ribosomal DNA probe. Ribosomal signals (*arrows*) can be located in one heterozygote autosomal pair (**b**) or in an autosomal pair and X chromosome simultaneously (**c**). Bar = 10 μm

the nymphal stages. The geographic distribution of both species overlaps in northern Argentina and in the Chaco region from Bolivia and Paraguay. *T. guasayana* is mainly sylvatic, occupying a great variety of habitats including bromeliads, similar to *T. sordida*. Peridomiciliary colonies are frequent, side by side with *T. infestans* and *T. sordida*, especially in chicken houses [2]. Isoenzymes studies in Bolivia show the absence of hybrid forms confirming the reproductive isolation in nature of both species [10].

Genetic markers (isoenzymes, chromosomal and mitochondrial sequences) clearly differentiated *T. guasayana* from the other species of sordida subcomplex [7, 9, 10, 23–25]. However, probably due to their morphological similarity, COI sequences deposited in GenBank reveal at least one mistaken identification: a specimen from Santa Cruz, Bolivia (Chaco, Tita) (KC249342) originally identified as *T. guasayana*, should be recognized as *T. sordida* (Fig. 3). At phylogenetic level, genetic distances between *T. guasayana* and the other sordida taxa are the most extreme, between 14 and 14.9 % (Table 7). In fact *T. guasayana* seems more related to *T. rubrovaria* (10.3 %) (Table 7), as suggested by other authors using different mitochondrial genes [23–25]. Chromosomal similarities between these two species (lack of autosomal heterochromatin and ribosomal clusters on one autosomal pair) would also support the inclusion of *T. guasayana* in the rubrovaria subcomplex.

T. garciabesi: Until now, this species can be only described in Central and Northern Argentina [8, 12, 13]. Our

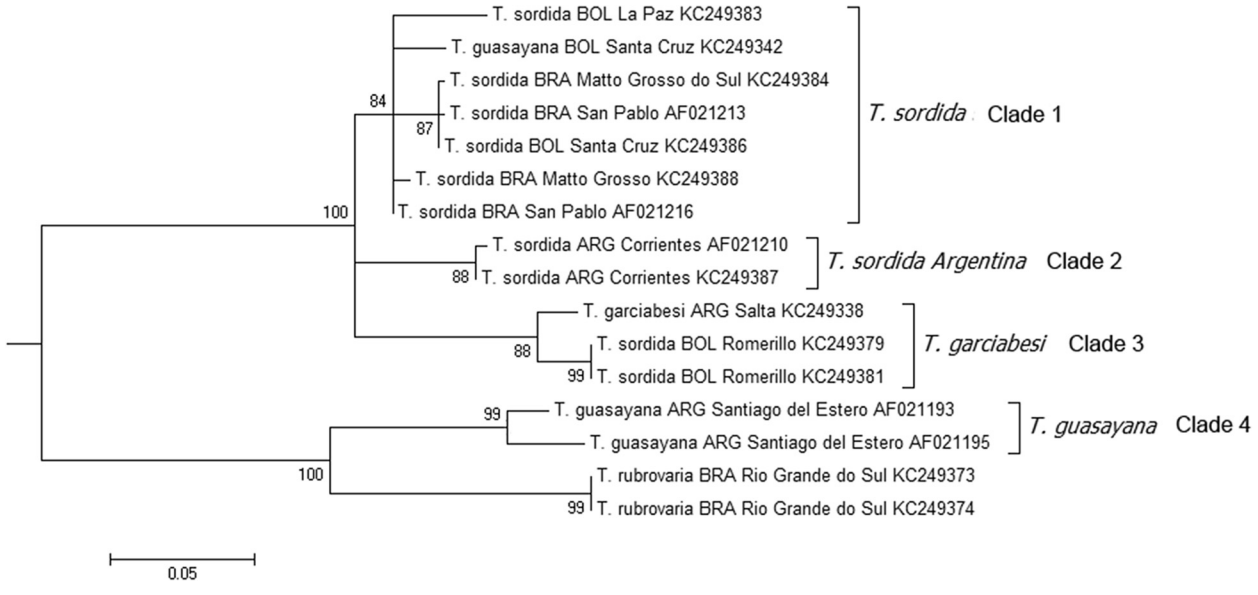

Fig. 3 Maximum likelihood phylogenetic tree obtained with COI gene partial sequences deposited in GenBank. This tree clearly shows four well supported clades and mistakes in the primary species identification of some individuals (KC249342, KC249379 and KC249381). Numbers on nodes represent statistical support obtained through 1000 bootstrap replications. ARG = Argentina; BOL = Bolivia; BRA = Brazil

Table 7 Mean Tamura-3 parameters pairwise genetic distances between the four clades for the COI gene fragments

	CLADE 1	CLADE 2	CLADE 3	CLADE 4	*OUTGROUP*
	T. sordida	*T. sordida Argentina*	*T. garciabesi*	*T. guasayana*	*T. rubrovaria*
CLADE 1	**1.4**				
CLADE 2	5.3	**0.4**			
CLADE 3	7.1	7.0	**2.0**		
CLADE 4	14.9	14.6	14.0	**3.6**	
OUTGROUP	15.7	13.5	14.6	10.3	**0.0**

Intergroup distances are in the lower left section; mean intragroup distances are in bold

results extend the *T. garciabesi* geographical distribution to other Chaco regions previously not described: Bolivian Chaco (Santa Cruz), western Paraguay (Departments of Boquerón and Presidente Hayes) and the Argentine provinces of Tucumán and Santiago del Estero (Table 2). This geographical distribution closely matches the predicted distribution for *T. garciabesi* based on ecological niche modeling [12]. Most of them are sylvatic, but occasionally occupy peridomestic environments.

Different approaches support the taxonomic validity of this species: head and genitalia morphology [8], head and wing morphometrics [12], cuticular hydrocarbons [13], isoenzymatic and cytogenetic traits [7, 17], and molecular analyses [26] (Fig. 3). This paper establishes the chromosomal identity between *T. garciabesi* and the *T. sordida* group 2 from Bolivia defined by isoenzymes [9, 10].

Despite the genetic and phenotypic differentiation, taxonomic problems still persist in terms of distinguishing *T. garciabesi* from other species of the subcomplex. Individuals from established laboratory colonies (Salta, La Rioja, Santiago del Estero, Formosa and Chaco) which were originally identified as *T. sordida,* presented the chromosomal characteristics of *T. garciabesi* (Table 2). Hence, incorrect identification of this species is also seen in the sequences deposited in GenBank. The two individuals from the Bolivian Chaco (Romerillo) probably belong to *T. garciabesi* rather than *T. sordida* (Fig. 3). Recently, comparative analyses of *T. sordida* populations from Western (Chaco) and Eastern regions of Paraguay reveal striking differences in the feeding patterns, random amplified polymorphic DNA profiles (RAPD), and head and wing morphometrics [14]. These authors suggested that this differentiation is associated to eco-geographical isolation by distance. However, our chromosomal studies suggest that *T. sordida* populations in Paraguay involve at least three taxa. In the Chaco region (Western Paraguay) we only found *T. garciabesi*, while in the Eastern region it appears that *T. sordida Argentina* and *T. sordida sensu stricto* coexist in sympatry. The ecological differentiation and distinct feeding patterns described by [14] support our results. We suspect that besides *T. garciabesi* and *T. guasayana* [27], also other cryptic sordida species may be present in Paraguay.

T. sordida sensu stricto: This species is the most geographically widespread species of the sordida subcomplex, found in large parts of Argentina, Brazil, Bolivia (Santa Cruz) and Paraguay (Central and Eastern) [2]. Surprisingly according to our results, this species was not detected in Argentina despite the large number of populations and individuals analyzed (Tables 2–4). Individuals recognized as *T. sordida* group 1 in Bolivia by isoenzymes [9–11] belong to this chromosomal group (Table 3). All these populations occupy domestic and peridomestic habitats, although they are also found in sylvatic habitats such as birdnests, tree holes and under dry tree bark [9–11]. Historically, *T. sordida* is forming abundant colonies in peridomestic habitats (particularly chicken coops), with the ability to invade and colonize human habitations in Brazil [28, 29], Bolivia [9–11, 30] and Paraguay [14]. Considering the sibling taxa here analyzed, *T. sordida sensu stricto* would be the most significant in terms of Chagas disease transmission [31], being the most common synanthropic species captured in Brazil [28].

T. sordida Argentina: For this chromosomal taxon, we considered as topotypes the individuals from San Luis del Palmar (Corrientes, Argentina). Chromosomal characteristics (without autosomal C-heterochromatin and ribosomal clusters in both sex chromosomes) were determined in most individuals from Argentina and some from Bolivia and Paraguay, all of them initially identified as *T. sordida* (Table 4). Ribosomal genes located on both sex chromosomes (Fig. 1g, h) is an uncommon feature in the genus *Triatoma*, only previously observed in four unrelated species of the 27 so far analyzed [17]. The mtDNA sequence divergence between *T. sordida Argentina* with sympatric *T. garciabesi* is 7.0 % (Table 7), similar to that used to support specific denominations in the brasiliensis subcomplex [32] or *Mepraia* species [33]. Sequence divergence between *T. sordida Argentina* and *T. sordida sensu stricto* is 5.3 %, similar to that observed between *T. sanguisuga* subspecies [34]. However, isoenzymatic analyses involving 19 loci revealed a striking differentiation between *T. sordida Argentina* and *T. sordida sensu stricto* from Brazil, showing 3 different fixed alleles and 4 polymorphic loci [7]. Considering this isoenzymatic diversity and the extreme chromosomal distinction (C-heterochromatin amount and

ribosomal clusters location) between these taxa we suggest there may be complete genetic isolation between them. We therefore propose that *T. sordida Argentina* presents characteristics consistent with its designation as a new species. At the epidemiological level, *T. sordida Argentina* were found in various sylvatic ecotopes and peridomestic habitats, but in very low frequency in domestic environments compared to, say, *T. sordida* from Brazil [35, 36].

T. sordida La Paz: This chromosomal taxon was identified in domestic specimens collected from La Paz (Bolivia) (Table 5). These individuals exhibit heterochromatic autosomes similar to what is observed in *T. sordida sensu stricto*, but they differ in the position of the ribosomal clusters (Fig. 1). *T. sordida La Paz* showed rDNA clusters in one autosomal pair, while *T. sordida sensu stricto* has them on the X chromosome. Considering almost 100 heteropteran species analyzed to date, including 40 triatomine species, the chromosomal position of ribosomal genes appears to be a species-specific character, although variation in ribosomal gene location was reported in *T. infestans* [16, 17]. For this reason we cannot rule out that *T. sordida sensu stricto* and *T. sordida La Paz* are conspecific populations with different ribosomal gene locations. Furthermore, some hybrid individuals detected in Bolivia (Fig. 2) could plausibly have originated from crosses between these two chromosomal taxa, thereby strengthening the idea of an intraspecific variation in ribosomal genes. Unfortunately we do not have information about COI sequences of individuals from this chromosomal taxon.

Putative hybrids by chromosomal markers

Experimental crosses between *T. sordida* populations and with *T. guasayana* have been made by many researchers, showing either fertility or F1 sterility [8, 20, 37–39]. These results, apparently contradictory, can now be explained by the fact that the crossings involved distinct chromosomal taxa, as suggested in this paper. Currently, there is only one report that demonstrates the existence of natural hybrids in sordida subcomplex species. By isoenzymes, putative hybrids in low frequency (3 %) were recorded in two localities from Santa Cruz in Bolivia (Izozog and Tita) [9]. According to these authors, in these regions at least three sordida subcomplex species (*T. guasayana* and *T. sordida* group 1 and group 2) coexist in sympatry, together with the putative hybrids. Here, in Izozog we also identified three species (*T. guasayana*, *T. sordida sensu stricto* and *T. garciabesi*, respectively) and putative hybrids, similar to isoenzymes [9]. Since both heterochromatin and the ribosomal genes are inherited in Mendelian fashion [17, 21], the occurrence of heterozygous chromosomes for C-heterochromatin and the FISH patterns observed in the ribosomal cluster location (Fig. 2) suggests that these individuals are hybrids resulting from crosses among different taxa.

Conclusions

By chromosomal markers, we recognize five chromosomal taxa and putative hybrids within the sordida subcomplex species in Argentina, Bolivia, Brazil and Paraguay. These morphologically similar taxa exhibit a striking karyological differentiation involving changes in the heterochromatin content and genome reorganization in the ribosomal clusters chromosomal position. In several regions, some of these taxa are sympatric and putative hybrids are detected. This paper identifies several erroneously classified populations, delimits the geographical distribution of each taxon and proposes the existence of a new cryptic species, widely distributed in Argentina. Most extensive population analyses (particularly from La Paz) and the application of other genetic techniques could resolve the taxonomic status of each chromosomal taxon. Considering the different epidemiological importance of these species, a morphological recognition key should be implemented for the selection of appropriate strategies for vector control.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: FP, SP, FN. Collected the bugs: FP, JN, CG, TC, ARA, LC, FN. Performed the experiments: FP, SP, JN, YP, TC, LC. Analyzed the data: FP, SP, JN, YP, TC, LC. Contributed reagents/materials/analysis tools: FP, YP, CG, ARA, FN. Wrote the manuscript: FP, SP, JN, YP, ARA. All authors read and approved the final version of the manuscript.

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Author details

[1]Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Calle: Iguá 4225, 11400 Montevideo, Uruguay. [2]Cátedra Introducción a la Biología, Facultad de Ciencias Exactas Físicas y Naturales, Instituto de Investigaciones Biológicas y Tecnológicas (IIByT) CONICET, Universidad Nacional de Córdoba, Córdoba, Argentina. [3]Present address: Departamento de Ecología, Genética y Evolución, Laboratorio de Eco-Epidemiología, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina. [4]Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos (LNIRTT), Instituto Oswaldo, Cruz, Rio de Janeiro, Brazil. [5]Instituto Nacional de Laboratorios de Salud (INLASA), Laboratorio de Entomología Médica, La Paz, Bolivia. [6]Centro para el Desarrollo de la Investigación Científica (CEDIC)/Díaz Gill Medicina Laboratorial/Fundación Moisés Bertoni, Asunción, Paraguay. [7]Laboratorio de Biotecnología, Centro de Investigación Hernando

Bertoni, Instituto Paraguayo de Tecnología Agraria, Asunción, Paraguay.
[8]Interactions hôtes-vecteurs-parasites dans les infections par trypanosomatidae (INTERTRYP), Institut de Recherche pour le Développement (IRD), Montpellier, France.

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Research paper

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

Sebastián Pita [a], Pedro Lorite [b], Julieta Nattero [c,1], Cleber Galvão [d], Kaio C.C. Alevi [e], Simone C. Teves [f,g], Maria T.V. Azeredo-Oliveira [e], Francisco Panzera [a,*]

[a] Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
[b] Departamento de Biología Experimental, Área de Genética, Universidad de Jaén, Jaén, Spain
[c] Cátedra de Introducción a la Biología, Facultad de Ciencias Exactas Físicas y Naturales, Instituto de Investigaciones Biológicas y Tecnológicas (IIByT), Universidad Nacional de Córdoba (UNC-CONICET), Córdoba, Argentina
[d] Laboratório Nacional e Internacional de Referência em Taxonomia de Triatomíneos, Instituto Oswaldo Cruz – FIOCRUZ, Rio de Janeiro, Brazil
[e] Laboratorio de Biologia Celular, Departamento de Biologia, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista "Júlio de Mesquita Filho" (IBILCE-UNESP), São José do Rio Preto, São Paulo, Brazil
[f] Laboratório Interdisciplinar de Vigilância Entomológica em Diptera e Hemiptera (LIVEDIH), Instituto Oswaldo Cruz/Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
[g] Programa de Pós-Graduação em Biologia Animal, Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Brazil

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ABSTRACT

The hemipteran subfamily Triatominae includes 150 blood-sucking species, vectors of Chagas disease. By far the most specious genus is *Triatoma*, assembled in groups, complexes and subcomplexes based on morphological similarities, geographic distribution and genetic data. However, many molecular studies questioned the species integration of several subcomplexes as monophyletic units. In triatomines, chromosomal position of major ribosomal DNA (rDNA) loci is extremely variable but seems to be species-specific and an evolutionary conserved genetic trait, so that closely related species tend to have ribosomal clusters in the same chromosomal location. Considering that the autosomal position as the ancestral character for all heteropteran species, including triatomines, we suggest that the movement of rDNA loci from autosomes to sex chromosomes rapidly established reproductive barriers between divergent lineages. We proposed that the rDNA translocation from the autosomes to the sex chromosomes restrict reproductive compatibility and eventually promote speciation processes. We analyzed the chromosomal position of 45S rDNA clusters in almost all species of the matogrossensis, rubrovaria, maculata and sordida subcomplexes. The fluorescent in situ hybridization results are discussed considering the available genetic data and we proposed new arrangements in the species that constitute each one of these subcomplexes.

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1. Introduction

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

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

* Corresponding author at: Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay.
E-mail address: fcopanzera@gmail.com (F. Panzera).
[1] Current address: Laboratorio de Eco-Epidemiología, Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (IEGEBA-UBA-CONICET), Buenos Aires, Argentina.

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

Triatominae subfamily species, similar to other heteropterans have holocentric chromosomes, i.e. chromosomes with diffuse or non-localized centromeres (Hughes-Schrader and Schrader, 1961). The absence of a primary constriction and their homogeneity in their autosomal number limited comparative and evolutionary chromosomal studies (Panzera et al., 2010). As a taxonomic tool, banding techniques application has been a valuable for karyotypic differentiation and to detect cryptic species, particularly in sordida subcomplex (Panzera et al., 1997, 2015). Recent application of fluorescent in situ hybridization (FISH) to determine the chromosomal position of major ribosomal clusters shows that this trait is species-specific and also with a striking inter-specific variability, revealing an extraordinary dynamics of change in the genomes during the evolution in this insect group (Panzera et al., 2012). Ribosomal ribonucleic acid (rRNA) is the main structural and catalytic component of the ribosome, being essential for protein synthesis in all living organisms. It is indispensable for cell viability and is one of only a few gene products present in all cells. In eukaryotes, the genes encoding ribosomal RNA (rDNA) are present in multiple copies, arranged as clusters and located in one or more chromosomes, named nucleolar chromosomes. Typically, each repeat unit of the major ribosomal cluster (45S rDNA) contains three regions encoding the 18S, 5.8S and 28S rRNAs.

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

In Heteroptera order, FISH analyses of a hundred species from 38 genera showed that the rDNA clusters are restricted to one or two loci per haploid genome. The chromosomal position is extremely variable; on a pair of autosomes (exceptionally 2), on m-chromosomes, on one or two sex chromosomes or simultaneously on a pair of autosomes and the X chromosome. The most predominant location is on one autosomal pair, recorded in species with different chromosome numbers and sex chromosome systems, involving eight of ten studied families including the most ancient groups (Grozeva et al., 2015), being considered as an ancestral character of the Heteroptera order. In spite of that ribosomal loci are regularly inherited by Mendelian fashion, changes in the position of rDNA loci are often originated by chromosomal rearrangements such as fusions, fissions or translocations. In some ants and heteropteran species, these changes may imply modifications in the chromosome number (Hirai et al., 1996; Bressa et al., 2009). However, in Triatominae, given that the number of autosomes remains almost unaffected (almost all species have 20 autosomes), we can rule out that these chromosomal rearrangements are responsible for variation of rDNA position. Inter-chromosomal mobility of rDNA by other mechanisms such as ectopic recombination and transposition is frequently reported in several insect groups (Cabrero and Camacho, 2008; Nguyen et al., 2010; Cabral de Mello et al., 2011) that could be also the origin of the rDNA location changes observed in Heteroptera (Panzera et al., 2012). In triatomines, chromosomal position of rDNA loci seems to be an evolutionary conserved genetic trait, so that closely related species tend to have the ribosomal genes in the same chromosomal location (Panzera et al., 2012, 2015).

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

2. Materials and methods

In this paper we compared the 45S rDNA clusters chromosomal location in 21 of the 25 recognized species of matogrossensis, rubrovaria, maculata and sordida subcomplexes, ten of them described here for the first time (Table 1). About previously reported species, we improve our analysis including new populations. The four misplaced species (*Triatoma deanorum*, *Triatoma limai*, *Triatoma oliveirai*, and *Triatoma arthurneivai*) were not analyzed because of the great difficulty to collect and keep them alive in insectariums (Noireau et al., 2002). Geographical origin, number of individuals analyzed and results about the chromosome location of ribosomal clusters are detailed on Table 1, including previous FISH data.

FISH was carried out using squashed gonad preparations. The gonads were extracted from living adult insects and fixed in ethanol-acetic acid (3:1). FISH procedure was applied using as probe an 18S rDNA fragment of 807-bp isolated from *T. infestans* from Uruguay as described by Panzera et al. (2012). Chromosome slides were examined under a Nikon Eclipse 80i microscope and the images were obtained with a DS-5Mc-U2 digital camera. For each specimen, at least 20 cells in meiotic (metaphase I, II or diplotene) or mitotic divisions were analyzed to determine 45S rDNA clusters chromosomal location. Images were processed with the Adobe Photoshop® software.

3. Results

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

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

Considering the subcomplexes until now recognized (Table 1), all rubrovaria subcomplex species are homogenous in their ribosomal clusters chromosomal location, presenting the rDNA signals on an autosomal pair (Fig. 1A). On the contrary, matogrossensis, maculata and sordida subcomplexes include species with rDNA clusters in different chromosomes. Matogrossensis and maculata subcomplex species present two ribosomal patterns: some species with 45S rDNA on an

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

Species	Geographical origin	45S rDNA location
Rubrovaria subcomplex		
T. carcavalloi	São Jerônimo, RS, Brazil. LNIRTT. [2]	One autosomal pair[b]
T. circummaculata	Cerro Largo, Uruguay. S. [2]	One autosomal pair[d]
	Vila São Jerónimo, RS, Brazil. LNIRTT. [2]	One autosomal pair[d]
T. klugi	Nova Petrópolis, RS, Brazil. LNIRTT. [2]	One autosomal pair[d]
T. rubrovaria	Artigas, Uruguay. S. [2]	One autosomal pair[b]
	ND. Araraquara (SP) & LNIRTT insectaries. [10]	One autosomal pair[a]
T. pintodiasi	Caçapava do Sul, RS, Brazil. LNIRTT. [2]	One autosomal pair[d]
Matogrossensis subcomplex		
T. baratai	Corumbá, MS, Brazil. LNIRTT. [2]	One autosomal pair[d]
T. costalimai	Posse, GO, Brazil. LNIRTT. [2]	One autosomal pair[d]
	Aurora de Tocantins, TO, Brazil. LIVEDIH. [2]	One autosomal pair[d]
T. guazu	Barra do Garças, MT, Brazil. LNIRTT. [3]	One autosomal pair[d]
T. jatai	Paranã, TO, Brazil. LIVEDIH. [3]	One autosomal pair[d]
T. williami	Fazenda Nova, GO, Brazil. LNIRTT. [2]	One autosomal pair[d]
	Barra do Garças, MT, Brazil. LNIRTT. [2]	One autosomal pair[d]
T. jurbergi	Alto Garças, MT, Brazil. LNIRTT. [3]	X & Y chromosomes[d]
T. matogrossensis	Serra das Arenas, MT, Brazil. LNIRTT. [3]	X & Y chromosomes[b]
	ND. Araraquara (SP) & LNIRTT insectaries. [11]	X & Y chromosomes[a]
T. vandae	Rondonópolis, MT, Brazil. LNIRTT. [3]	X & Y chromosomes[b]
Maculata subcomplex		
T. maculata	Boa Vista, RO, Brazil. P. [2]	X & Y chromosomes[b]
	Bolivar, RO, Venezuela. LNIRTT. [2]	X & Y chromosomes[d]
T. pseudomaculata	Sobral, CE, Brazil. LNIRTT. [2]	One autosomal pair[b]
	Alem Paraiba, MG, Brazil. S. [2]	One autosomal pair[d]
T. wygodzinsky	São João da Boa Vista, SP, Brazil. S. [2]	One autosomal pair[b]
	Espírito Santo do Pinhal, SP, Brazil. LNIRTT. [2]	One autosomal pair[d]
Sordida subcomplex		
T. sordida Argentina	Populations from Argentina, Bolivia & Paraguay. D, P, S. [29]	X & Y chromosomes[c]
T. sordida sensu stricto	Populations from Brazil, Bolivia & Paraguay. D, P. [34]	X chromosome[b,c]
T. garciabesi	Populations from Argentina, Bolivia & Paraguay. P, S. [39]	X chromosome[b,c]
T. patagonica	9 de julio, Santa Fe, Argentina. P. [4]	One autosomal pair[d]
	Mitre, Santiago del Estero, Argentina. P. [4]	One autosomal pair[d]
	San Martin, San Luis, Argentina. P. [1]	One autosomal pair[d]
	Utracan, La Pampa, Argentina, P. [3]	One autosomal pair[d]
	Avellaneda, Rio Negro, Argentina, P. [3]	One autosomal pair[d]
T. guasayana	Populations from Argentina & Bolivia. S. [24]	One autosomal pair[c]

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

autosomal pair (Fig. 1B), while other carry them on both sex chromosomes (X and Y) (Fig. 1D). Sordida subcomplex species have three ribosomal patterns: on an autosomal pair (Fig. 1C), on both sex chromosomes (Fig. 1E) or on one sex chromosome (X chromosome) (Fig. 1F).

4. Discussion

4.1. Chromosomal location of rDNA clusters as taxonomic marker

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

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

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

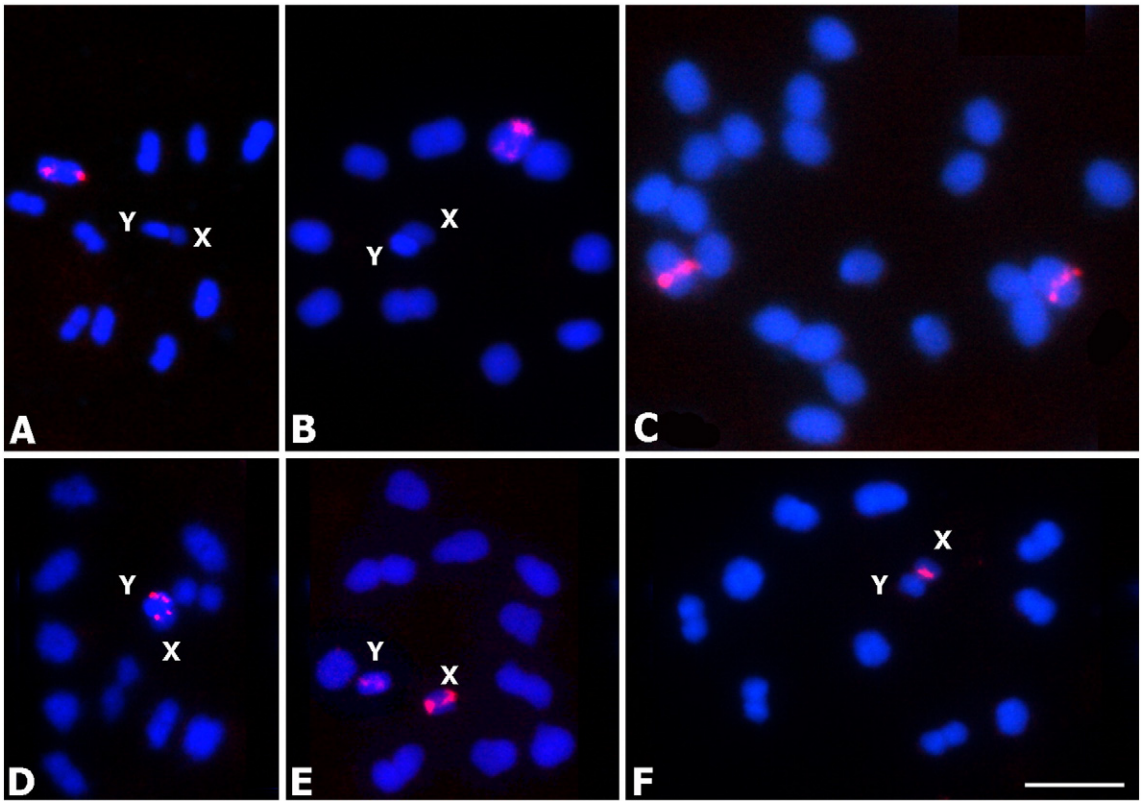

Fig. 1. Localization of 45S ribosomal DNA by fluorescent in situ hybridization (FISH) in male meiosis and spermatogonial prometaphase in different *Triatoma* species from South America, using 18S rDNA as probe. All species showed a diploid chromosome number of 22 chromosomes (2n = 20 autosomes plus XY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

Another important consequence of the rDNA change from autosomes to sex chromosomes is the generation of new linkage groups in the X chromosomes. According to the available information in Diptera, controlling sex genes and reproduction-related traits genes are abundant on the X chromosomes, and many of them are involved in barriers to gene flow between diverging lineages (Noor and Feder, 2006). These genes, so-called "speciation" or "barrier" are related with reproductive isolation, including both pre-zygotic (such as pheromones) and post-zygotic isolations (hybrid sterility and hybrid inviability) (for review see Qvarnström and Bailey, 2009). As a result of the insertion of the ribosomal genes it is likely that the formation of new linkage groups in the X chromosome establishes reproductive isolation among populations with different locations of the ribosomal genes. The translocation of ribosomal genes to sex chromosomes results in changes of the evolutionary dynamics and also has an effect on speciation. A hybrid resulting from a cross between two individuals with different localization of the rDNA loci (autosomes and sex chromosomes) produces unbalanced gametes in the number of rDNA loci resulting in reproductive disadvantage. Depending on the particular genotypes participating in a breeding, the combination of certain gametes could lead to a significant proportion of unviable zygotes (e.g., without rDNA clusters), selecting against heterozygotes and maintenance of polymorphisms in a population. These negative effects can be overcome if the two populations have fixed rDNA loci in both chromosomal positions. The simultaneous presence of rDNA loci in at least one sex chromosome and autosomes in reported in few species of Diptera (Roy et al., 2005), Coleoptera (Cabral de Mello et al., 2011) and Orthoptera (Cabrero and Camacho, 2008). In holocentric chromosomes, this double location is exceptional, having been reported only in two triatomine species: *T. delpontei* and *T. infestans* (Panzera et al., 2012, 2014). The last one exhibits an extensive rDNA polymorphism involving more than one autosomal pair together with the X chromosome in their putative center of origin, but shows a strong tendency to fix the ribosomal clusters on the X chromosome during its dispersion process (Panzera et al., 2014). In conclusion, we propose that the rDNA translocation from the autosomes to the sex chromosomes limits reproductive compatibility and eventually promote speciation, similar as reported for other chromosomal rearrangements (Butlin, 2005). Perhaps the reverse movement of rDNA loci, i.e. from the sex chromosomes to autosomes is highly unlikely, given the new gene linkage relationships in the X chromosome and the establishment of isolation barriers to gene flow. Probably this mechanism may be acting on other insect groups, including with monocentric chromosomes such as Coleoptera, provided that the number of ribosomal clusters is present on one or two chromosomes per haploid complement.

4.3. Phylogenetic relationships in Triatominae subcomplexes

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

mainly based on morphologically similarities (Schofield and Galvão, 2009), the morphological plasticity confused both species identification and the establishment of evolutionarily related groups.

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

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

In this paper, considering the ribosomal clusters chromosomal location by FISH and in the light of the available molecular data, we have evaluated the species integration of maculata, matogrossensis, rubrovaria and sordida subcomplexes, and propose new arrangements that reflect their evolutionary relationships more accurately.

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

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

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

4.3.1. Rubrovaria subcomplex

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

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

Our FISH results show that all rubrovaria species analyzed hitherto present the ribosomal clusters on an autosomal pair (Tables 1 and 2), confirming their close phylogenetic relationships. Furthermore, two other species belonging to sordida subcomplex show the same chromosome location: *T. guasayana* and *T. patagonica* (Table 1). Analyses of several nuclear and mitochondrial fragments also cluster these two species in the same clade within rubrovaria subcomplex (García et al., 2001; Hypša et al., 2002; Sainz et al., 2004; Paula et al., 2005; Almeida et al., 2009; Gardim et al., 2014). The close association among *T. sordida* and *T. guasayana* observed in phylogenetic trees with COI and Cyt b fragments reported by Gardim et al. (2013) and Justi et al. (2014) probably are due to an incorrect identification of the analyzed specimens since to their great morphological similarity, as has been suggested by Panzera et al. (2015).

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

4.3.2. Maculata subcomplex

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

According to Schofield (1988), *T. maculata* and *T. pseudomaculata* resulted from the evolution of two geographic populations derived from a common ancestor. Our FISH results clearly splits the maculata subcomplex in two clades: species with the ribosomal genes in an autosomal pair (*T. pseudomaculata* and *T. wygodzinskyi*) and a species having the ribosomal genes in both sex chromosomes (*T. maculata*) (Table 1, Fig. 1). This clear division (*T. maculata* vs *T. pseudomaculata*/*T. wygodzinskyi*) and the close evolutionary relationship between *T. pseudomaculata* and *T. wygodzinskyi* were also been reported by isoenzymes (Dos Santos et al., 2007), nuclear (Bargues et al., 2008; Justi et al., 2014) and mitochondrial sequences (Hypša et al., 2002; Paula et

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

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

4.3.3. Matogrossensis subcomplex

Includes nine species which share morphological characteristics, all terrestrial and distributed throughout the Pantanal ecosystem in Central-Western Brazil and Paraguay: *T. baratai*, *T. costalimai*, *T. deaneorum*, *T. guazu*, *T. jurbergi*, *T. matogrossensis*, *T. vandae* and *T. williami* (Schofield and Galvão, 2009). Recently, *T. jatai* was described and incorporated in the matogrossensis subcomplex due to its close morphological, morphometric and genetic similarities with *T. costalimai* (Gonçalves et al., 2013; Teves et al., 2016). Molecular data of *T. deaneorum* are not available.

None DNA sequences analysis succeeded to recover a clade formed by matogrossensis subcomplex species, reflecting a conflict between ecologic and genetic data (Hypša et al., 2002; Sainz et al., 2004; Paula et al., 2005; Gardim et al., 2013; Justi et al., 2014; Teves et al., 2016). Our FISH results clearly splits the matogrossensis subcomplex in two clusters: species with the ribosomal genes on an autosomal pair (*T. baratai*, *T. costalimai*, *T. guazu*, *T. jatai* and *T. williami*), and species bearing the ribosomal clusters on both sex chromosomes (*T. jurbergi*, *T. matogrossensis* and *T. vandae*) (Table 1, Fig. 1). A same dichotomy was also reported by morphometry (eight measurements of head and thorax) and isoenzyme (18 loci) analyses (Noireau et al., 2002) on the former called "*T. oliveirai complex*" (Carcavallo et al., 2001) currently matogrossensis subcomplex. Several analyses with mitochondrial genes (mainly 12S and 16S rDNA) have shown that *T. jurbergi*, *T. matogrossensis* and *T. vandae* are closely related among them and with sordida species (*T. sordida* and *T. garciabesi*) (García et al., 2001; Hypša et al., 2002; Sainz et al., 2004; Paula et al., 2005; Gardim et al., 2013; Justi et al., 2014; Teves et al., 2016). All these species showed the rDNA clusters on one or two sex chromosomes (Table 1). In conclusion, considering all available genetic data and the similar ribosomal clusters localization (all bearing ribosomal genes on sex chromosomes, either both or just the X chromosome), we proposed that the sordida subcomplex must be constituted by the following species: *T. garciabesi*, *T. sordida*, *T. sordida* Argentina (putative new species suggested by Panzera et al., 2015) plus *T. jurbergi*, *T. matogrossensis* and *T. vandae* (from matogrossensis subcomplex). From this subcomplex we excluded *T. guasayana* and *T. patagonica* which has been proved to be related with the rubrovaria subcomplex (Table 2).

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

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

Our FISH results in *T. baratai*, *T. costalimai*, *T. guazu*, *T. jatai* and *T. williami* (from matogrossensis subcomplex) as well as in *T. pseudomaculata* and *T. wygodzinskyi* (from maculata subcomplex) showed that the ribosomal clusters are localized on one autosomal pair. Evolutionary relationships between these two groups of species are not constant, and vary according to the specimens used and the molecular markers analyzed (Gardim et al., 2013). These inconsistencies clearly reveal improper sequencing or misidentification of species, such as above mentioned for *T. costalimai*. In spite of this, several phylogenetic trees, mainly with 12S and 16S fragments, show a close association among the two species groups afore mentioned (Hypša et al., 2002; Paula et al., 2005; Justi et al., 2014).

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

5. Conclusions

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

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Capítulo II:

Diferenciación de los cromosomas sexuales

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Chromosome Painting in Triatomine Insects Reveals Shared Sequences Between X Chromosomes and Autosomes

Sebastián Pita,[1] Francisco Panzera,[1,2] Antonio Sánchez,[3] Teresa Palomeque,[3] and Pedro Lorite[3]

[1]Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay (spita@fcien.edu.uy; fcopanzera@gmail.com), [2]Corresponding author, e-mail: fcopanzera@gmail.com, and [3]Departamento de Biología Experimental, Área de Genética, Universidad de Jaén, Av. Lagunillas s/n., 23071 Jaén, Spain (abaca@ujaen.es; tpalome@ujaen.es; plorite@ujaen.es)

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Abstract

In order to provide a broad picture on the origin and evolution of holocentric X chromosomes in heteropteran species, we prepared a sex chromosome painting probe by microdissection of the X_1 and X_2 chromosomes from a kissing bug *Mepraia spinolai* (Hemiptera: Reduviidae: Triatominae). Fluorescence in situ hybridization on four species of the Triatomini having different amounts of autosomal heterochromatin and sex chromosome systems show that the Xs probe hybridizes on the euchromatin, located both on autosomes and X chromosomes. The heterochromatic Y chromosome and autosomal heterochromatic regions always appear free of hybridization signals. The hybridization results of the Xs probe on *Rhodnius prolixus* (Rhodniini) is completely different to that observed in Triatomini species. The hybridization signals are small and scattered on all euchromatin, without specific regions including the X chromosome. These results are in accordance with previous data obtained by genomic in situ hybridization and fluorescent banding, suggesting a clear differentiation in the repeat sequence composition of both sex chromosomes between Triatomini and Rhodniini tribes. These results also support that each sex chromosome in Triatomini has evolved independently from different autosomal pairs of a common ancestor, as described in other insect orders.

Key words: fluorescence in situ hybridization, holocentric chromosome, kissing bug, microdissection X chromosome, sex chromosome evolution

Chromosomal painting analysis using whole chromosome-specific probes has contributed to the knowledge on the origin and evolution of sex chromosomes in a great diversity of organisms such as mammals (Cortez et al. 2014), fish (Cioffi et al. 2011a,b), reptilia (Matsubara et al. 2014), platyhelminthes (Hirai et al. 2012), and plants (Kejnovsky and Vyskot 2010). However, the application of chromosome painting for comparative chromosome studies in insects, excepting Diptera, is very limited due to the difficulties in obtaining probes. In fact, just a few studies with chromosome painting probes have already been conducted in this animal group, including studies focused on both autosomes and sex chromosomes (Traut et al. 1999; Rutten et al. 2004; Bressa et al. 2009; Teruel et al. 2009a,b; Martins et al. 2013; Yoshido et al. 2013; Menezes-de-Carvalho et al. 2015).

Insects of the Heteroptera order (true bugs) represent an attractive group to study sex chromosome evolution due to holocentric nature of their chromosomes and their particular segregation during cell divisions. Holocentric chromosomes, also called chromosomes with diffuse or nonlocalized centromeres, are characterized by lack of a primary constriction (Hughes-Schrader and Schrader, 1961). In Triatominae subfamily, three male sex systems are recorded: XY, X_1X_2Y, and $X_1X_2X_3Y$, the first one considered to be ancestral (Ueshima 1966). Sex chromosomes are very well differentiated from autosomes by their behavior during meiotic divisions. As a rule, heteropteran sex chromosomes are considered asynaptic, achiasmatic, and behave as univalents during the first meiotic division (Solari 1979). From first meiotic prophase until diakinesis, the sex chromosomes are grouped together forming a positive heteropycnotic body. At first metaphase, the sex chromosomes appear clearly separated but lying side by side, without any visible physical connection between them. During anaphase I, the two sister chromatids of each sex chromosome segregate to opposite poles resulting in their equal division. In metaphase II, the sex chromosomes appear associated end-to-end to form a pseudobivalent (pseudotri or tetravalent according to the sex system), which is located in the center of the ring formed by the autosomes. In anaphase II, these chromatids segregate to opposite poles resulting in two gametes with different sex

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chromosomes. So, in this group, sex chromosomes exhibit an equational division in first meiotic division and reductional division in meiosis II. This reversion in the order of segregation of sex chromosomes is called postreductional segregation or inverted meiosis (Hughes-Schrader and Schrader 1961; Ueshima 1979). The meiotic association between sex chromosomes does not involve recombination or association by homologous regions.

In different insect groups such as Diptera, Lepidoptera, and Orthoptera distinct types of DNAs have been identified on sex chromosomes, such as different repetitive DNA sequences and transposable elements (Kaiser and Bachtrog 2010). However, in Heteroptera, knowledge about the molecular composition and evolution of sex chromosomes is very limited. Recently, the sequencing of the *R. prolixus* genome has allowed the identification of nine Y-linked genes (Mesquita et al. 2015). Of the nearly 90 Triatominae species cytogenetically studied so far, the Y chromosome is entirely C-heterochromatic (Panzera et al. 2010). However, at the molecular level, the Y chromosome shows striking differences between the two main tribes of the subfamily, Triatomini and Rhodniini, which comprise almost 90% of the 150 recognized species (Galvão and Paula 2014). In Triatomini, the Y chromosome is mainly composed of A + T rich repeated DNA sequences (Bardella et al. 2014, 2016). Genomic in situ hybridization (GISH) studies reveal that these repetitive sequences are highly conserved and probably represent an ancestral character of this tribe (Pita et al. 2014). In Rhodniini, the heterochromatic Y chromosome is constituted by other types of DNA sequences that are not revealed by GISH or fluorescence analyses (Pita et al. 2014, Bardella et al. 2016; respectively).

Unlike the Y chromosome, the X chromosomes of Triatominae presented an extensive variability with C and fluorescence bandings. In most species, they are euchromatic, but in others, the X chromosomes revealed heterochromatic regions (Panzera et al. 2010). Several of these heterochromatic regions are CMA positive (G + C rich) and are associated with ribosomal clusters (Bardella et al. 2016). Currently, there are no data on sequence composition of the X chromosomes in this group, nor in the Heteroptera order.

In order to provide a preliminary study on the origin and evolution of X chromosomes, chromosome painting analyses were performed in several triatomine species. We obtained a sex chromosome painting probe by microdissection of the X_1 and X_2 chromosomes from *Mepraia spinolai* meiotic nuclei, followed by polymerase chain reaction (PCR) amplification and labeling. We have tested the probe by fluorescence in situ hybridization (FISH) on meiotic preparations of the same species. For comparative chromosome studies, we applied this probe on male preparations of other triatomine species with different sex mechanisms and amounts of C-heterochromatin (Panzera et al. 1995, 2010, 2014; Calleros et al. 2010).

Materials and Methods

Biological Material

Table 1 summarizes the geographic origin and cytogenetic data of the material here analyzed. Microdissection was performed on two individuals of *M. spinolai* (Chile, Til Til). Hybridizations were performed on at least two individuals of each species, whose geographical origin is specified in Table 1.

Chromosome Preparations, C-Banding, and Microdissection of X Sex Chromosomes

Cytological preparations (squashes) from microdissection, hybridizations, and C-banding were obtaining from testes removed from alive adult insects and fixed in an ethanol–glacial acetic acid mixture (3:1) and finally stored at −20 °C. Microdissection of sex chromosomes was performed from *M. spinolai* individuals (2n = 20 autosomes plus X_1X_2Y sex chromosomes) without pooling chromosomes. C-banding was performed as described by Panzera et al. (1995).

Microdissection was performed using an inverted microscope (Zeiss Axiovert 200, Göttingen, Germany) with a sterile glass needle attached to a micromanipulator (Eppendorf Transfer Man NK2, Hamburg, Germany). Forty chromatids of each X chromosome (total = 80 chromatids) obtained from metaphase I or II cells were microdissected. Before degenerate oligonucleotide-primed PCR (DOP-PCR) amplification, the microdissected chromosomes were pretreated by using a precycling incubation of 15 cycles at 30°C/1 min and 50°C/1 min. For the DOP-PCR amplification, 30 µl of a PCR reagent mix was added to give a final concentration of 200 µM dNTPs, 2.5 mM $MgCl_2$, 100 pg/µl BSA, 2 U Biotaq DNA polymerase, and 1 µM DOP primer (50-CCG ACT GCA GNN NNN NAT GTG G).

Degenerate oligonucleotide-primed PCR was carried out with an initial denaturation at 94°C for 5 min, followed by eight cycles at 94°C/1 min, 45°C/1 min, and 72°C/3 min, and finally 28 cycles at 94°C/1 min, 56°C/1 min, and 72°C/3 min, with a final extension

Table 1. Geographic origin and chromosomal traits of the species here analyzed.

Species	Male diploid number (2n)	Autosomal C-banding	Y chromos.	X chromos.	Geographic origin
Mepraia spinolai	20A + X_1X_2Y	10 II with blocks in 2 ends	C+, DAPI+, CMA-	X_1 & X_2 with dots in both ends (C+ & CMA+)	Chile, Metropolitan Region, Til Til, S.
Triatoma infestans Non-Andean Group	20A + XY	3–4 II with blocks in 1 or 2 ends; 6–7 II euchromatic	C+, DAPI+, CMA-	Euchromatic with DAPI dot in 1 end	Argentina, Chaco, Tres Estacas, P.
T. infestans Andean Group	20A + XY	8–10 II with blocks in 1 or 2 ends; 0–2 II euchromatic	C+, DAPI+, CMA-	C+ dot & DAPI+ in both ends	Bolivia, Potosí, Palquiza, S.
T. delpontei	20A + XY	10 II with block in only 1 end	C+, DAPI+, CMA-	C+ dot & DAPI+	Bolivia, Santa Cruz, Tita, S.
T. dimidiata	20A + X_1X_2Y	10 II with dots in 2 ends	C+, DAPI+, CMA-	Euchromatic	Guatemala, Jutiapa, Carrizal, D.
Rhodnius prolixus	20A + XY	No, all II euchromatic	C+, DAPI-, CMA-	Euchromatic	Guatemala, Quezaltenango, D. Insectary CDC (USA)

Chromosome data from Calleros et al. (2010), Panzera et al. (1995, 2010, 2014), and Bardella et al. (2016).

A, autosomes; II, bivalents; P, peridomiciliary; D, domiciliary; S, sylvatic; DAPI, 4',6-diamino-2-fenilindol; CMA, chromomycin A_3.

at 72°C for 7 min. A second labeling DOP-PCR amplification was made with 5 µl of the first PCR product as template in a reaction mixture (30 ml volume) containing of 200 µM dNTPs, 2.5 mM $MgCl_2$, 4 µM DOP primer, 2 U Taq DNA polymerase (Roche), and 20 µM Biotin-16-dUTP (Roche). The amplification was carried out with an initial denaturation at 95°C for 5 min, followed by five cycles at 94°C/30 s, 30°C/30 s, and 72°C/1.5 min, and finally 35 cycles at 94°C/30 s, 62°C/30 s, and 72°C/1.5 min, with a final extension at 72°C for 7 min.

Squashed preparations for FISH were dehydrated in an increasing series of ice-cold ethanol and then air-dried. Chromosomes were denaturized in 70% deionized formamide and 2×SSC at 70°C for 3.5 min, then incubated in 2×SSC at room temperature for 1 min and dehydrated in an ethanol series. Approximately 200 ng of probe (chromosome paint) was coprecipitated with 5 µg of denatured salmon sperm DNA and was finally dissolved in 10 µl of hybridization solution containing 50% deionized formamide, 10% dextran sulfate, and 2×SSC. After denaturation (6 min at 73°C), the probe was dropped onto each slide and spread over the hybridization area with a 22- by 22-mm glass cover slip. Hybridization was conducted at 37°C overnight in a moist chamber. Fluorescence immunological detection was performed using the avidin-Fluorescein/anti-avidin-biotin system with two amplification rounds. The slides were counterstained with DAPI and mounted in an antifade solution (Vectashield from Vector laboratories). Finally, images were captured using a fluorescence microscope (Olympus BX51) equipped with a CCD camera (Olympus DP70). Hybridization pattern for each species was determined by the chromosomal analyses of at least two individuals.

Results

Control Hybridization of the Xs Probe (Self-Hybridization)

The sex chromosome-painting probe derived from microdissected X_1 and X_2 chromosomes of *M. spinolai* was first hybridized on meiotic cells from male individuals of *M. spinolai* with the same geographical origin of the microdissected X chromosomes in order to verify the probe specificity (Fig. 1a-b). The Xs probe displayed strong hybridization signals on both X chromosomes; however, it did not paint the Y chromosome evenly. Also most of the chromatin in all autosomes (10 pairs) presents strong hybridization signals, excepting their terminal regions (arrowheads Fig. 1a-b). This hybridization pattern is exactly the opposite of that observed with C-banding: the Y chromosome is entirely C-heterochromatic, while the 10 autosomal pairs exhibit terminal C-blocks (Table 1, Fig. 1c). In conclusion, in our control species the Xs probe hybridizes with the euchromatin of both X chromosomes and on the euchromatic regions of all 10 autosomal pairs, but not with the heterochromatic regions of autosomes and Y chromosome.

Interspecific X Probe Hybridization

In the analyzed species from the genus *Triatoma*, the Xs probe hybridized with the euchromatic regions. The Xs probe on *T. infestans* (non-Andean group) male chromosomes displays strong labeling on the X chromosome and in the seven minor autosomal bivalents while the heterochromatic Y chromosome is free of labeling (Fig. 1d). The three largest autosomal bivalents show a nonuniform hybridization with regions without hybridization signals (arrowheads Fig. 1d), which correspond with the large C-heterochromatic regions presented in these chromosomes (Table 1, Fig. 1e). The pattern of hybridization in *T. infestans* (Andean group) is similar to that observed in the non-Andean group, but the level of hybridization is less uniform and less strong (Fig. 1f). As in the non-Andean group, the heterochromatic regions of the autosomal bivalents lack hybridization signals, especially visible in the largest bivalents (arrowheads Fig. 1f).

In *T. delpontei* the Y chromosome appears without hybridization. Unlike the results in the other X chromosomes, in this species this chromosome shows scattered signals (Fig. 1g), probably due to possessing heterochromatic regions (Table 1). The 10 autosomal bivalents have strong signals located in the middle regions that correspond to one of the chromosomal ends (arrowheads Fig. 1g). The other chromosome ends are heterochromatic (Fig. 1h) and lack hybridization signals (Fig. 1g).

In *T. dimidiata*, the 10 autosomal bivalents and both X chromosomes (X_1 and X_2) show strong hybridizations signals (Fig. 1i). Only the heterochromatic Y chromosome does not display labeling. Finally, in *R. prolixus* (Rhodniini), all autosomal bivalents as well as the heterochromatic Y chromosome and the euchromatic X chromosome show scattered hybridization signals (Fig. 1j).

Discussion

Knowledge of the sequences that compose the X chromosomes in organisms with holocentric chromosomes is very limited. In parthenogenetic aphids with X0/XX system, the X chromosome presents levels of genetic diversity, allelic richness, and recombination rates similar as autosomes (Jaquiéry et al. 2012). In species with heteromorphic sex chromosomes, several studies support the accumulation of different sequences between both sex chromosomes. In lepidopteran species with a ZZ/ZW system, the overall sequence composition of Z chromosome (equivalent of X chromosome) is much like that of autosomes than the W chromosome, showing extensive conserved synteny in their gene content (Sahara et al. 2012). In Heteroptera, only one report using an X probe has been published, involving *Dysdercus* species (Pentatomomorpha) with X0 and neo-X neo-Y sex systems (Bressa et al. 2009). These authors showed the existence of conserved regions among X chromosomes that are not present in the neo-Y chromosome.

The results obtained here provide new information about the origin of the sex chromosomes in triatomines. The DOP-PCR of microdissected chromosomes preferentially amplifies anonymous repeat DNA sequences (Houben et al. 2001). The hybridization of *M. spinolai* X_1-X_2 probe on the same species chromosomes (self-hybridization) and other three Triatomini species shows the existence of shared repeat DNA sequences between the X chromosomes and the autosomes. Since hybridization signals are located in euchromatic regions it is very likely that they recognize interspersed repeat sequences, common between autosomes and X chromosomes. This hybridization pattern is completely reverse to that observed with C-banding that point that these sequences are not present in the Y chromosome heterochromatin neither in the autosomal heterochromatic ends, which appear free of labeling (Fig. 1a-i). Our results are similar to the obtained in two grasshopper species *Locusta migratoria* and *Eyprepocnemis plorans* (Teruel et al. 2009a, 2009b). In both species, with localized centromere, chromosome painting using an X chromosome probe results in the presence of dot-like painting pattern over euchromatic autosomal regions. The authors suggested that this hybridization pattern is due to the presence of some repetitive elements interspersed in euchromatin that are shared between the X chromosome and the autosomes.

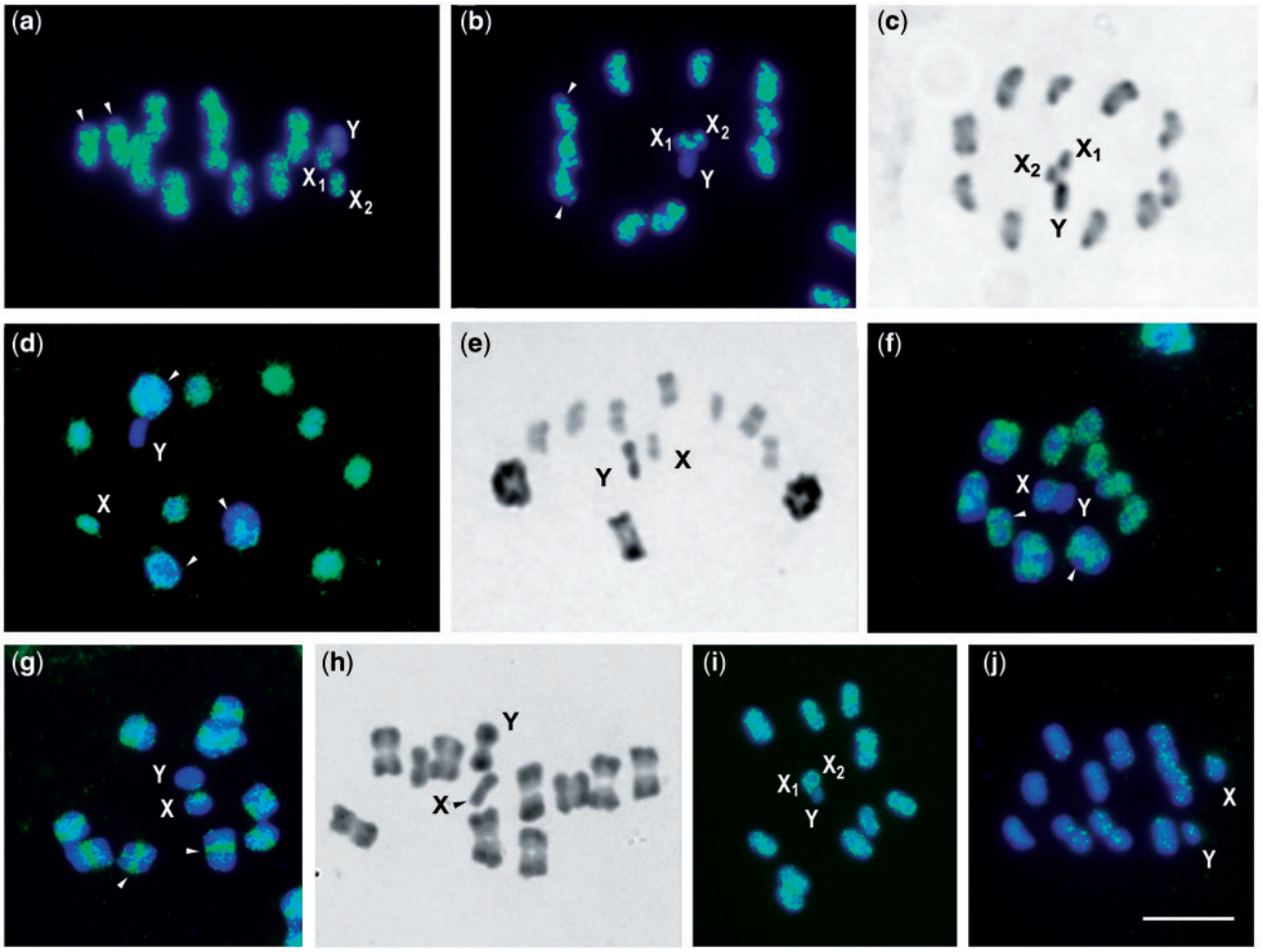

Fig. 1. Hybridization results using an Xs probe obtained by the microdissection of the X_1 and X_2 chromosomes of *M. spinolai* on male meiotic chromosomes from different triatomine species. (**a**) Metaphase I (MI) and (**b**) Metaphase II (MII) of *M. spinolai*. Both X chromosomes and all autosomes (10 pairs) show strong hybridization signals (in green), except on their terminal regions, which do not have labeling (arrowheads). The Y chromosome is completely unlabeled. (**c**) C-banding in MII of *M. spinolai* showing the heterochromatic Y chromosome and the presence of small and terminal heterochromatic regions in all autosomes. (**d**) *Triatoma infestans* (non-Andean group). MI. Hybridization signals involve almost completely the seven smaller bivalents and the X chromosome. The three largest autosomal bivalents have regions lacking hybridization signals (arrowheads) while the Y chromosome is completely unlabeled. (**e**) C-banding in *T. infestans* (non-Andean group). MI showing the heterochromatic nature of the Y chromosome and the euchromatic X chromosome. Only the three biggest bivalents show prominent heterochromatic regions, the remaining autosomes being entirely euchromatic. (**f**) *T. infestans* (Andean group). MI. The 10 bivalents and the X chromosome present hybridization signals. The heterochromatic Y chromosome is free of labeling as well as the heterochromatic regions of the autosomes (arrowheads). (**g**) *T. delpontei*. MI. Hybridization signals are located in the middle regions of 10 bivalents that correspond to one of the chromosomal ends (arrowheads). The X chromosome shows label in a small region while the Y chromosome does not show hybridization signals. (**h**) C-banding in *T. delpontei*. MI shows the presence of large heterochromatic blocks in the terminal regions of all autosomes as well the heterochromatic Y chromosome. (**i**) *T. dimidiata*. MII. All autosomes and both X chromosomes appear almost entirely labeled while the Y chromosome is completely unlabeled. (**j**) *Rhodnius prolixus*. MI. Autosomes and sex chromosomes show small and scattered hybridization signals. Scale bar = 10 μm.

Previous GISH and fluorescent banding analyses (Pita et al. 2014, Bardella et al. 2016) and the results presented here support that the X and Y chromosomes in Triatomini species presented substantial differences in their chromatin constitution. Bardella et al. (2016) showed that C-heterochromatic Y chromosome is $DAPI^+$, preferentially formed by A + T rich repeated sequences, being extremely conserved among all Triatomini species (Pita et al. 2014). By contrast the X chromosome would consist of dispersed repeated sequences located in euchromatin, and similar to those observed in euchromatic autosomal regions (this paper; Fig. 1a, b, d, f). Occasionally in some species, the X chromosomes may have heterochromatic regions which may be similar to those observed in the autosomes, as in *T. delpontei* (Fig. 1g).

In several insects orders such as Coleoptera, Diptera, and Lepidoptera, different authors suggest that each sex chromosome (X and Y) have evolved independently from different pairs of autosomes from a common ancestor (Carvalho 2002, Kaiser and Bachtrog 2010, Pease and Hahn 2012, Yoshido et al. 2013). For example in *Drosophila melanogaster*, the Y chromosome does not share any genes with the X chromosome, except for Ste-Su (Ste) and rDNA gene clusters (Carvalho 2002). In Triatominae, the XY sex chromosomes contain different types of repeat sequences, as revealed by GISH studies (Pita et al. 2014), fluorescent banding (Bardella et al. 2016), and this paper. All these results strongly support that heteromorphic XY sex chromosomes in Triatomini species derived from different autosomes of a common ancestor, as has been described in other insect groups.

The results of the Xs probe on *R. prolixus* (Rhodniini) are completely different to that observed in Triatomini species (Fig. 1j). The hybridizations signals are small and scattered on all euchromatin,

without specific regions including the X and Y chromosomes. The Xs probe results joint with fluorescent banding data indicate that the X and Y chromosomes in Triatomini and Rhodniini are very different in each tribe. In Rhodniini, the C-heterochromatic Y chromosome has not fluorescence signals and probably it is constituted by other types of repeated sequences (Bardella et al. 2016). The results obtained with chromosome painting show that a similar phenomenon occurs with the X chromosomes. The *M. spinolai* Xs probe resulted in a strong hybridization on the euchromatin of the four Triatomini species here analyzed (Fig. 1a–f), suggesting that the X chromosome sequences are widely conserved among them. However, this Xs probe, obtained from a Triatomini species, does not show hybridization on the *R. prolixus* X chromosome (Fig. 1j).

The high chromatin differentiation between X and Y chromosomes in Triatomini and Rhodniini could help to elucidate the controversial evolutionary origin of the Triatominae subfamily. Phylogenetic trees based on nuclear and mitochondrial sequences support Triatominae as monophyletic (Hypša et al. 2002, Justi et al. 2016), polyphyletic (Schofield and Galvão 2009), or paraphyletic group (Hwang and Weirauch 2012). If Triatominae is monophyletic, the sex chromosomes could have diverged after separation between both tribes about 40 million years ago (Justi et al. 2016). Conversely, if Triatominae is polyphyletic, they might have been derived from different ancestors with dissimilar sex chromosomes. The molecular characterization of specific sequences on sex chromosomes and their comparison with putative ancestors or sister species could clarify the origin of the Triatominae subfamily.

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Capítulo III:

Evolución cariotípica de las secuencias de ADN repetido

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RESEARCH ARTICLE

Distribution and Evolution of Repeated Sequences in Genomes of Triatominae (Hemiptera-Reduviidae) Inferred from Genomic In Situ Hybridization

Sebastian Pita[1], Francisco Panzera[1]*, Antonio Sánchez[2], Yanina Panzera[1], Teresa Palomeque[2], Pedro Lorite[2]*

1. Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay, **2.** Departamento de Biología Experimental, Área de Genética, Universidad de Jaén, Jaén, Spain

*plorite@ujaen.es (PL); fcopanzera@gmail.com (FP)

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Abstract

The subfamily Triatominae, vectors of Chagas disease, comprises 140 species characterized by a highly homogeneous chromosome number. We analyzed the chromosomal distribution and evolution of repeated sequences in Triatominae genomes by Genomic in situ Hybridization using *Triatoma delpontei* and *Triatoma infestans* genomic DNAs as probes. Hybridizations were performed on their own chromosomes and on nine species included in six genera from the two main tribes: Triatomini and Rhodniini. Genomic probes clearly generate two different hybridization patterns, dispersed or accumulated in specific regions or chromosomes. The three used probes generate the same hybridization pattern in each species. However, these patterns are species-specific. In closely related species, the probes strongly hybridized in the autosomal heterochromatic regions, resembling C-banding and DAPI patterns. However, in more distant species these co-localizations are not observed. The heterochromatic Y chromosome is constituted by highly repeated sequences, which is conserved among 10 species of Triatomini tribe suggesting be an ancestral character for this group. However, the Y chromosome in Rhodniini tribe is markedly different, supporting the early evolutionary dichotomy between both tribes. In some species, sex chromosomes and autosomes shared repeated sequences, suggesting meiotic chromatin exchanges among these heterologous chromosomes. Our GISH analyses enabled us to acquire not only reliable information about autosomal repeated sequences distribution but also an insight into sex chromosome evolution in Triatominae. Furthermore, the differentiation obtained by GISH might be a valuable marker to

establish phylogenetic relationships and to test the controversial origin of the Triatominae subfamily.

Introduction

The repetitive DNAs distribution along chromosomes is one of the essential elements in evolutionary genetics for understanding the organization and the evolution of genomes [1]. Analyses of these sequences are even more important in organisms with holocentric chromosomes, such as hemipteran insects, where the lack of primary constriction, small chromosome size and the limited banding procedures makes chromosomal studies harder to achieve.

The subfamily Triatominae include 140 species called kissing bugs, vectors of Chagas disease or American trypanosomiasis, recognized as the most serious human parasitic disease of Latin America with around 7–8 million people infected [2]. Karyotypic information is currently available for more than 80 species, showing a highly conserved diploid chromosome number, ranging from 21 to 25 chromosomes in males [3]. The number of autosomes is remarkably constant; all species except 3 present 20 autosomes. They have three sex systems in males (XY, X_1X_2Y and $X_1X_2X_3Y$), being the sex chromosomes achiasmatic and showing a particular segregation called inverted meiosis or post-reduccional segregation [4].

In triatomines, the heterochromatin (revealed by C-banding) and the 45S ribosomal genes chromosomal mapping (revealed by fluorescent in situ hybridization) are the principal repetitive DNAs used for studying karyotypic diversification and make evolutionary inferences [3, 5, 6]. Heterochromatin variation includes remarkable changes in the quantity, size, composition, chromosome location and behavior of autosomal C-blocks during cell divisions. Autosomal C-heterochromatin differences are positively correlated with an extensive variation in the total DNA content measured by laser flow cytometry. The haploid genome size varies 4-fold, from 0.72 pg in *Rhodnius* species to 2.90 pg in *Triatoma delpontei* [3, 7]. FISH technique application in 46 triatomine species has also shown a high variability in the 45S ribosomal DNA chromosomal location, never reported so far in holocentric chromosomes, demonstrating that these repeated DNA sequences are an important marker to disclose chromosomal differentiation [5, 6].

The most striking example of intraspecific variation of repeated sequences is *Triatoma infestans*, which involved polymorphism in the number of heterochromatic chromosomes, different molecular composition within the C-blocks and variation on the rDNA genes localization [5, 8–11]. This species comprises two main evolutionary lineages, known as the Andean and non-Andean groups, defined by substantial differences (from 30% to 50%) in nuclear DNA amount, due to dissimilar quantities of highly repeated DNA revealed as C-heterochromatin [8]. In Andean group, the number of autosomes with heterochromatin

fluctuates from 14 to 20, while in the non-Andean group varies from 4 to 7 [8]. Fluorochromes staining shown that the C-blocks in both chromosomal groups are conformed by different DNA repeats [10, 11]. In the three largest autosomal pairs, each C-block is subdivided into two different repeats regions: a telomeric DAPI-positive region (AT-rich) and a subtelomeric chromomycin A3-positive region (GC-rich). In the other autosomes, the C-blocks are exclusively formed by a DAPI positive telomeric region. The Y chromosome is almost totally C-heterochromatic and DAPI positive, while that the X chromosome is polymorphic. In non-Andean group this chromosome is euchromatic with a DAPI-positive signal in one chromosomal end [10, 12] while that in Andean group, the X chromosome is almost entirely C-heterochromatic and displayed two DAPI bands on each chromosomal end [13].

Inter-species distribution of repeated sequences by Genomic in situ hybridization (GISH) was applied in different organisms with dissimilar objectives such as genome analysis, determination of phylogenetic relationships, detection of chromosomal aberrations and alien chromatin [14]. In holocentric systems, GISH approaches have been applied primarily to the study of the evolution of sex chromosomes, particularly in Lepidoptera [15]. In Heteroptera, only one report has been published to explore the evolution of neo-sex chromosomes in the genus *Dysdercus* (Pyrrhocoridae) [16].

The aim of this paper is to analyze the similarities and differences of repeated DNA sequences among several Triatominae species by means of GISH. For this purpose, we made three genomic probes derived from the total DNA of two triatomine species with the highest DNA content of the subfamily: *T. delpontei* and *T. infestans* (Andean Group) [7]. These DNA probes were hybridized both on their own chromosomes (self-GISH) and on other species included in six different genera, belonging to the two main Triatominae tribes. With this strategy, we can establish a preliminary but broad overview on the repeated sequences evolution both in autosomes and sex chromosomes in the subfamily Triatominae.

Materials and Methods

Material

Table 1 summarizes the geographic origin, relevant cytogenetic traits and GISH results of the material here analyzed. No specific permissions were required for insect collections performed in this work, and did not involve endangered or protected species.

Chromosome preparations for GISH analyses were obtained from males of 11 triatomine species, included in the two principal tribes of the subfamily: Rhodniini and Triatomini (Table 1), which involve almost 90% of the 140 recognized species [17]. For Rhodniini tribe, which included 19 species in 2 genera, we analyzed one species: *Rhodnius prolixus*. For Triatomini tribe, which involved 104 species in eight genera, we studied species from the following five genera: *Dipetalogaster*, *Eratyrus*, *Mepraia*, *Panstrongylus* and *Triatoma*. For the

Table 1. Geographic origin, relevant cytogenetic traits and GISH results of the eleven Triatominae species analyzed by three genomic probes.

SPECIES	Male Diploid Number (2n)	Amount (%) autosomal C-heterochromatin	Chromosome location of Autosomal C-bands	X chromosome	GISH results. Hybridization signals	Geographic Origin. Country: Department, Locality, habitat
TRIBE TRIATOMINI						
Triatoma delpontei	20A + XY	Polymorphic, 45–50%	9–10 autosomal pairs with large C-blocks in only one chromosomal end (Figure 1g)	Almost entirely C-heterochromatic	All chromatin. 9–10 bivalents and X chromosome with strongest hybridization signals in only one chromosomal end. Y chromosome almost totally labeled (Figure 1c and 1f)	Bolivia: Santa Cruz, Tita, S. 18˚ 34′ 31″ S, 62˚ 40′ 05″ W.
Triatoma infestans Andean Group	20A + XY	Polymorphic, 40–50%	7–9 autosomal pairs with C-blocks of different size in one or two chromosomal ends (Figure 1i)	Almost entirely C-heterochromatic	All chromatin. 6 bivalents with strongest hybridization signals (different size and chromosome location). Y chromosome intensively and totally labeled while that X chromosome has a small signal (Figure 1h)	Bolivia: Potosí, Palquiza, S. 21˚ 31′ 41″ S, 65˚ 45′ 04″ W, and Potosí, Thago Thago, S. 18˚ 00′ 44″ S, 65˚ 48′ 31″ W.
Triatoma infestans Non-Andean Group	20A + XY	Polymorphic, 24–30%	3 autosomal pairs with C-blocks in one or two chromosomal ends (Figures 2c and 2e)	Euchromatic	All chromatin. Hybridization signals strongest in 3 larger autosomal pairs and the whole Y chromosome. The X no have strong labeled (Figures 2b and 2d)	Argentina: Chaco. Tres Estacas, P. 26˚ 54′ 30″ S, 51˚ 40′ 23″ W.
Triatoma platensis	20A + XY	Polymorphic, 10–12%	2–4 autosomal pairs with small C-blocks in one or two chromosomal ends	Almost entirely C-heterochromatic	All chromatin. 3 largest bivalents with strong and small signals. Y and X chromosomes intensively and totally labeled (Figure 2f)	Uruguay: Paysandú, S. 32˚ 18′ 28″, 58˚ 02′ 59″ W.
Mepraia spinolai	20A + X_1X_2Y	Polymorphic, 15–25%	All autosomes with C-dots in one or two chromosomal ends (Figure 2l)	Small C-dots in both Xs chromosomes	All chromatin (Figures 2j and 2k). All bivalents with strong hybridization signals in chromosomal ends. Y chromosome intensively and totally labeled (Figure 2i)	Chile: Metropolitan Region of Santiago, Colina, S. 33˚ 11′ 53″ S, 70˚ 39′ 42″ W.
Triatoma dimidiata	20A + X_1X_2Y	Polymorphic, 5–10%	All autosomes with C-dots in one or both ends	Euchromatic	All chromatin. Only Y chromosome intensively and totally labeled (Figure 3b)	Guatemala: Jutiapa, Carrizal, D. 14˚ 25′ 48″ N, 89˚ 57′ 28″W
Triatoma carrioni	20A + XY	5%	2 autosomal pairs with C-dots in one chromosomal end	Euchromatic	All chromatin. Only Y chromosome intensively and totally labeled (Figure 3c)	Peru: Piura, Ayabaca, S. 4˚ 35′ 00″ S, 79˚ 43′ 00″ W.
Triatoma protracta	20A + X_1X_2Y	Polymorphic, 35–45%	All autosomes with C-blocks in one or two chromosomal ends (Figure 3e)	X_1 with C-dots in both ends. X_2 euchromatic	All chromatin. Only Y chromosome intensively and totally labeled (Figure 3d)	Insectary Justin Schmidt (USA). Origin colony: USA, Arizona, S.
Dipetalogaster maxima	20A + XY	0%	Without autosomal C-heterochromatin	Euchromatic	All chromatin. Only Y chromosome intensively and totally labeled (Figure 3f)	Mexico: Baja California Sur, La Paz, S. 24˚ 09′ N, 110˚ 17′ W.
Eratyrus mucronatus	20A + X_1X_2Y	Polymorphic, 0–5%	0–1 autosomal pair with C-blocks	Euchromatic	All chromatin. Only Y chromosome intensively and totally labeled (Figure 3g)	Insectary E. Chagas. Origin: Brazil, Para, S.

Table 1. Cont.

SPECIES	Male Diploid Number (2n)	Amount (%) autosomal C-heterochromatin	Chromosome location of Autosomal C-bands	X chromosome	GISH results. Hybridization signals	Geographic Origin. Country: Department, Locality, habitat
TRIBE TRIATOMINI						
Panstrongylus geniculatus	20A + X_1X_2Y	0%	Without autosomal C-heterochromatin	Both X chromosomes with C-dots	All chromatin. Only Y chromosome intensively and totally labeled (Figure 3h)	Colombia: Antioquia, Amalfi, S. 6° 55′ 58″ N, 75° 05′ 30″ S.
TRIBE RHODNIINI						
Rhodnius prolixus	20A + XY	0%	Without autosomal C-heterochromatin	Euchromatic	Hybridization signals scattered throughout all chromosomes. No chromosomal region was observed with specific labeling, including the heterochromatic Y chromosome (Figure 3i)	Insectary CDC (USA). Origin: Colombia. S.

These genomic probes produce the same hybridization pattern in each species but the chromosomal location of the most intense signals allows recognizing a species-specific hybridization patterns. P= peridomiciliary; D= domiciliary; S= sylvatic.

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Triatoma genus, we studied the three main clades or groups: a) the Rubrofasciata Group (from Central and North America and Old World species): *T. protracta* and *T. dimidiata*, b) the Dispar Group (west of the Amazon region): *Triatoma carrioni* and c) the Infestans Group (from south and east of the Amazon region). For this group we included three closely related species of the infestans subcomplex (*T. delpontei*, *T. infestans* and *T. platensis*). In *T. infestans* we analyzed the two chromosomal groups: Andean and non-Andean with striking differences in the amount and chromosome distribution of the C-heterochromatin, both in autosomes and sex chromosomes (see Table 1).

Chromosome preparations and C-banding

For cytological preparations, testes were removed from adult insects alive, fixed in an ethanol–glacial acetic acid mixture (3:1) and stored at -20℃. Squashes were made in a 50% acetic acid drop, coverslips were removed after freezing in liquid nitrogen and the slides were air dried and then stored at 4℃. C-banding was performed according to Panzera *et al.* [8]. The analysis of C-banded preparations was made using a Nikon Eclipse 80i microscope with a DS-5Mc-U2 digital camera.

Genomic DNA Isolation and probe labeling for GISH techniques

Three genomic DNA probes were used from two species. Each one isolated from one adult individual: one from a male of *T. infestans* (Andean group) collected in Bolivia (Potosí, Palquiza, sylvatic) and the others two probes were obtained from one male and one female of *T. delpontei* collected from Bolivia (Santa Cruz, Tita,

sylvatic). These two species have the highest DNA content in triatomines: *T. delpontei* (2.90 pg) and *T. infestans* -Andean group- (1.98 pg) [3]. In both species, C-heterochromatin amount represent approximately 45% of the autosomal complement [18], but with different chromosome localization (see Table 1).

Genomic DNA was purified from adult legs following the NucleoSpin Tissue kit (MACHEREY-NAGEL). For the probes, total genomic DNAs were labeled with biotin-16-dUTP (Roche) using a Nick Translation Kit (Roche), following manufacturer's instructions.

In situ hybridization was carried out as described previously [19]. Hybridization solutions were prepared to a final concentration of 0.5–2 ng probe/mL in 50% formamide. Hybridization was conducted at 37℃ overnight. Fluorescence immunological detection was performed using the avidin-FICT/anti-avidin-biotin system with two amplification rounds. Slides were mounted with Vectashield (Vector). DAPI in the antifade solution was used to counterstain chromosomes. The hybridized chromosomes were observed and photographed using a BX51 Olympus fluorescence microscope equipped with a CCD camera (Olympus DP70) and merged using the DPManager software. Hybridization pattern for each species was determined by the chromosomal analyses of at least two individuals.

Results

Our GISH results reveal the occurrence of two chromosomal hybridization configurations: a) very intense hybridization signals concentrated on specific chromosomal regions or particular chromosomes and b) lower intensity hybridization signals dispersed along all chromosomes (Figures 1, 2 and 3). In some cases the lower intense hybridization signals dispersed along the chromosomes were masked by the DAPI signal in the merged figures. The three genomic probes produce the same hybridization pattern in each species. The chromosomal location of the most intense signals allows recognize a species-specific hybridization patterns.

Self-GISH or Auto-GISH. Genomic DNA from *T. delpontei* (male or female) on *T. delpontei* male meiotic chromosomes

All chromatin present scattered hybridization signals. In addition 9 of the 10 bivalents and the X chromosome exhibit strongest hybridization signals in only one chromosomal end, while the Y chromosome appears almost totally hybridized (Figures 1a to 1c). This *T. delpontei* individual does not have C-block in one autosomal bivalent (data not shown), consequently this chromosome do no have hybridization signals on the chromosomal ends (Figures 1b and 1c). Male meiotic prophase clearly shows the two hybridization configurations: a large hetero-pycnotic chromocenter with a strong hybridization signal and the rest of the chromatin with weaker signals (Figures 1d and 1e). This meiotic chromocenter is

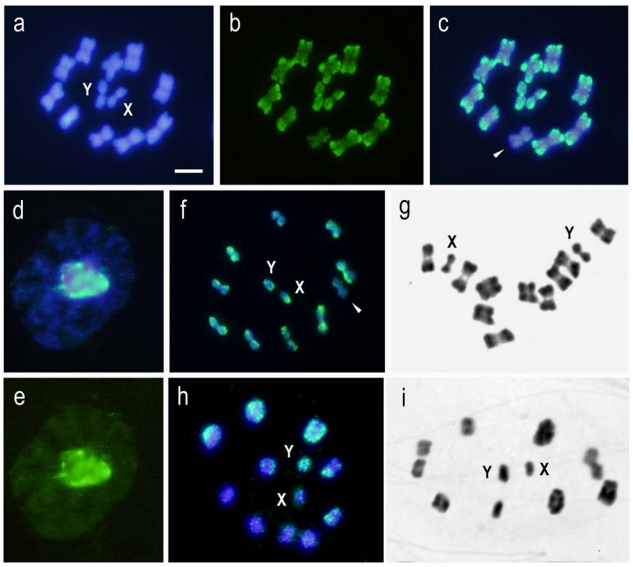

Figure 1. Self-GISH using three genomic DNA (gDNA) probes: *Triatoma delpontei* (TD) (male or female) and *Triatoma infestans* (TI) (Andean group) on own chromosomes. Bar =5 μm. (a-b-c) TD gDNA on TD chromosomes. Metaphase I (2n=20A + XY). **(a)** DAPI staining. **(b)** FITC. **(c)** Merged. Hybridization signals appear scattered on all chromatin. Furthermore, strongest signals are preferably located at one chromosomal end on nine of the ten autosomal bivalents. Both sex chromosomes (X and Y) appear almost entirely labeled. The bivalent without signal is pointed out by arrowhead. **(d–e)** TD gDNA on TD. Early male meiotic prophase. **(d)** DAPI staining. **(e)** FITC. A large heteropycnotic chromocenter appear with a strong hybridization signal and the rest of the chromatin with weaker signals. **(f)** TD gDNA (female) on TD chromosomes. Metaphase II (2n=20A + XY). Terminal regions of 9 autosomal pairs and both sex chromosomes, including the Y chromosome, appear strongly labeled. The autosomal pair without strong hybridization signals is pointed out by arrowhead. **(g)** TD. C-banding. Metaphase I. All autosomal bivalents (10) show C-blocks in only one chromosomal end. Both sex chromosomes (XY) appear almost entirely. C-heterochromatin distribution similar as observed with GISH in (c). **(h)** TI (Andean group) gDNA on TI (Andean group) chromosomes. Metaphase I (2n=20A + XY). Six autosomal bivalents show hybridization signals with different intensity and size. The Y chromosome appears almost entirely labeled while that X chromosome shows a small hybridization region. **(i)** TI (Andean group). C-banding. Metaphase I. Seven to nine autosomal bivalents appear heterochromatic with C-blocks of different size in one or both chromosomal ends, while X and Y sex chromosomes are almost entirely C-heterochromatic.

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formed by the heterologous association of the C-heteropycnotic regions of all autosomal bivalents and both sex chromosomes [18]. In metaphase II, each autosomal pair has a strong signal in only one chromosomal end and both sex chromosomes (X and Y) appear labeled (Figure 1f). This GISH pattern is very similar compared with those observed with C-banding: figure 1g shows a C-banded metaphase I with a C-block in only one chromosomal end of all bivalents while both sex chromosomes are C-heterochromatic (compare Figure 1c and 1g).

Self-GISH or Auto-GISH. Genomic DNA from *T. infestans* Andean Group (male) on *T. infestans* Andean Group male meiotic chromosomes

All chromosomes show hybridization signals but with differences in size and intensity. The strongest signals are observed in the terminal regions of 6 bivalents,

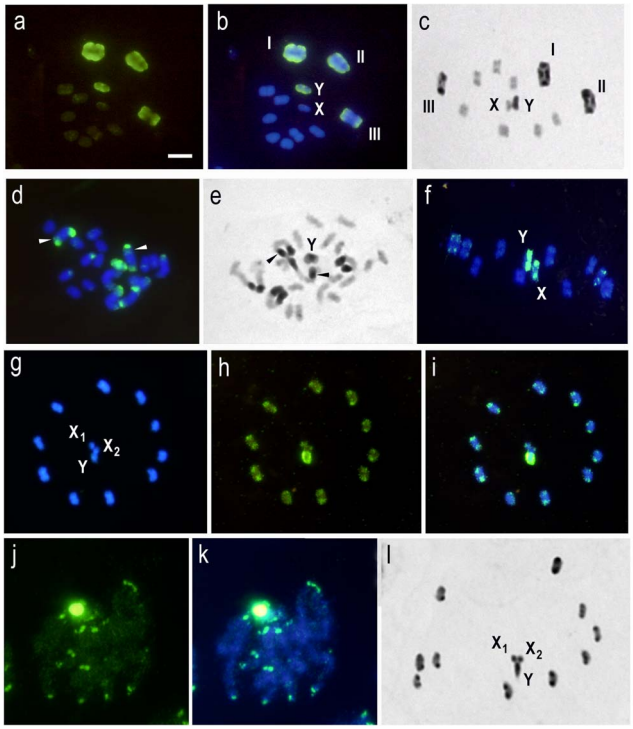

Figure 2. Inter-specific GISH of triatomine species using three genomic DNA (gDNA) probes: *Triatoma delpontei* (TD) (male or female) and *Triatoma infestans* (TI) (Andean group). Bar =5 μm. (a–b) TI (Andean group) gDNA on TI (non-Andean group) chromosomes. Metaphase I (2n=20A + XY). **(a)** FITC. **(b)** Merged. All chromatin appear labeled. Strong hybridization signals are restricted to the three largest autosomal pairs and the Y chromosome: in two largest bivalents (I and II) the signals are localized in both chromosomal ends while that in the third bivalent (III) the label in restricted to only one chromosomal end. The remaining seven autosomal bivalents and X chromosome did not display strong labeling. **(c)** TI (non-Andean group). C-banding. Metaphase I. Three bivalents present C-blocks: two of them in both chromosomal ends (I and II) and the third in only one end (III). The Y chromosome is C-positive, and seven autosomal bivalents and X chromosome are euchromatic. C-heterochromatin distribution similar as observed with GISH in (b). **(d)** TD gDNA on TI (non-Andean group) chromosomes. Spermatogonial mitotic prometaphase (2n=22 chromosomes). Six autosomes appear with strong and telomeric hybridization signals: 4 of them in both chromosomal ends and the other 2 in only one telomeric region. The Y chromosome is entirely hybridized. Arrowheads pointed out subtelomeric regions (DAPI negative) without GISH label localized on the largest autosomes. **(e)** C-banding. TI (non-Andean group). Spermatogonial mitotic prometaphase (2n=22). Six autosomes with C-blocks: 4 of them in both chromosomal ends, and the other 2 in only one end. The Y chromosome appears almost totally C-heterochromatic. Each autosomal C-block is subdivided into 2 regions: a darker subtelomeric region (arrowheads) and a clearer telomeric region. **(f)** TI (Andean group) gDNA on *T. platensis* chromosomes. Metaphase I (2n=20A + XY). Hybridization signals are restricted to small regions of 3 autosomal bivalents. Both sex chromosomes (X and Y) appear almost entirely labeled. **(g-h-i)** TI (Andean group) gDNA on *M. spinolai* chromosomes. Metaphase II (2n=20A + X_1X_2Y). **(g)** DAPI staining. **(h)** FITC. **(i)** Merged. All chromatin appear labeled. Autosomal telomeric regions present small and intense hybridization signals. The Y chromosome appears strongly and totally labeled. **(j–k)** TI (Andean group) gDNA on *Mepraia spinolai* chromosomes. Early male meiotic prophase. **(j)** FITC **(k)** Merged. All chromatin appear labeled but strong signals are restricted to telomeric regions and on the meiotic chromocenter constituted by the association of sex chromosomes with some autosomes. **(l)** *M. spinolai*. C-banding. Metaphase II (2n=20A + X_1X_2Y). C-heterochromatin distribution similar as observed with GISH in **(c)**.

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in the whole Y chromosome and more scarcely on the X chromosome (Figure 1h). Also in this case the hybridization pattern is very similar to the C-banding pattern: *T. infestans* (Andean group) exhibit 7–9 autosomal bivalents with C-blocks of

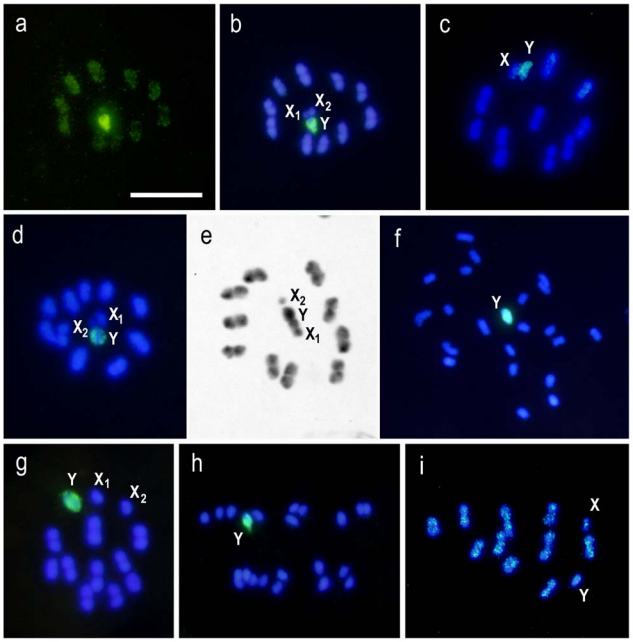

Figure 3. Inter-specific GISH of triatomine species using three genomic DNA (gDNA) probes: *Triatoma delpontei* (TD) (male or female) and *Triatoma infestans* (TI) (Andean group). Bar =5 μm. (a–b) TD gDNA on *Triatoma dimidiata* chromosomes. Metaphase II (2n=20A + X_1X_2Y). **(a)** FITC **(b)** Merged. All chromatin present dispersal hybridization signals but only the Y chromosome exhibits a very strong labeled. (c) TI (Andean group) gDNA on *T. carrioni* chromosomes. Metaphase I (2n=20A + XY). Strong hybridization signals are restricted to the heterochromatic Y chromosome. **(d)** TD gDNA on *T. protracta* chromosomes. Metaphase II (2n=20A + X_1X_2Y). Only the Y chromosome presents strong hybridization signal in spite of the autosomal C-bands in all autosomal pairs and in one X chromosome. **(e)** *T. protracta*. C-banding. Metaphase II. All autosomal pairs, the Y chromosome and one X chromosome present C-blocks in one or both chromosomal ends. **(f)** TD gDNA on *Dipetalogaster maxima* chromosomes. Spermatogonial mitotic prometaphase with 22 chromosomes (2n=20A + XY). Only the Y chromosome presents intense hybridization signal. **(g)** TI (Andean group) gDNA on *Eratyrus mucronatus* chromosomes. Metaphase I (2n=20A + X_1X_2Y). Only the Y chromosome presents hybridization signal. **(h)** TI (Andean group) gDNA on *Panstrongylus geniculatus* chromosomes. Anaphase II (2n=20A + X_1X_2Y) showing a post-reductional sex chromosomes segregation. Only the Y chromosome has strong hybridization signals. **(i)** TI (Andean group) gDNA on *Rhodnius prolixus* chromosomes. Metaphase I (2n=20A + XY). The hybridization signals are scattered throughout all chromosomes. No chromosomal region was observed with intense labeled signals, including the heterochromatic Y chromosome.

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different size in one or both chromosomal ends. Both sex chromosomes (X and Y) appear C-heterochromatic (Figure 1i).

Inter-specific GISH

Genomic probes produce a specific pattern on each one of the 11 species analyzed. However, since each probe recognizes the same repeated sequences, the chromosome hybridizations results on each species are the same with the three applied probes. For this reason, to illustrate the patterns of the 11 analyzed species we combine different genomic probes in figures 1, 2 and 3. Genomic DNA probe of *T. infestans* (Andean group) on *T. delpontei* chromosomes and the reverse

hybridization, i.e., *T. delpontei* gDNA probe on *T. infestans* (Andean group) chromosomes shows the same patterns described in the corresponding self-GISH.

Genomic DNA probe of *T. infestans* (Andean group) on *T. infestans* (non-Andean group) chromosomes shown strong hybridization signals on the Y chromosome (almost entirely) and on the three largest autosomal bivalents (Figures 2a and 2b). These signals are localized in both chromosomal ends of the two largest bivalents (I and II, Figure 2b) and in one end of the third largest autosomal pair (III, Figure 2b). The remaining seven autosomal bivalents and X chromosome not display strong labeling. Similar pattern is observed with C-banding (Figure 2c). On the mitotic chromosomes, more detail about the chromosomal localization of the hybridization signals can be seen (Figure 2d). The Y chromosome appears almost completely labeled. Terminal or telomeric positions of the autosomal hybridization signals are clearly observed. Furthermore a subterminal region (negative for GISH and DAPI) is observed between the telomeric hybridization signal and the euchromatin (Figure 2d, arrowheads). This pattern is similar to C-banding, in which the Y chromosome and six autosomes (three pairs) appear with C-blocks in one or both chromosomal ends (Figure 2e). The size of the C-heterochromatic regions is larger than those obtained with GISH, and they are formed by a darker subtelomeric region (arrowheads) and a clearer telomeric region with Giemsa staining. These results indicated that telomeric C-bands are strongly hybridized with the GISH probes while the subtelomeric regions are not.

In *T. platensis*, the three largest autosomal bivalents present small but intense signals on only one chromosomal end and both sex chromosomes (X and Y) are almost entirely hybridized (Figure 2f). This GISH pattern is very similar to the described by C-banding (Table 1). In *M. spinolai*, all chromatin appear labeled but the ten autosomal pairs exhibit strong hybridization dots on chromosomal ends, clearly observed during early meiotic prophase and metaphase II (Figures 2g to 2k). The Y chromosome appears strongly and totally labeled (Figures 2h to 2k). This hybridization pattern is similar to C-banding pattern (Figure 2l). In the other seven Triatomine tribe species, including *Triatoma*, *Dipetalogaster*, *Eratyrus*, and *Panstrongylus* genera, strong hybridization signal is only observed on the C-heterochromatic Y chromosome (Figures 3a to 3d, and 3f to 3h). However, some of these species exhibit autosomal C-heterochromatin (Table 1), which clearly is not labeled with our GISH probes, as observed in autosomes and one X chromosome of *T. protracta* (compare Figures 3d with 3e).

Finally in *R. prolixus* from the Rhodniini tribe, a species with C-band only on the Y chromosome, the hybridization signals are scattered throughout all chromosomes (Figure 3i). No chromosomal region was observed with specific labeling, including the heterochromatic Y chromosome.

Discussion

Heteropteran have holocentric or holokinetic chromosomes, i.e. chromosomes with diffuse or non-localized centromeres [4]. The absence of a primary constriction, the similar and small chromosome size and the minor number of chromosomal landmarks limited comparative and evolutionary chromosomal studies in holocentric systems. In many different heteropteran groups, including triatomines, the main source of karyological differentiation has been the identification of highly repeated DNA regions included in the heterochromatin, revealed by the classical C-banding technique [3, 20]. Fluorescent banding and FISH with ribosomal DNA probes (18S and 28S rDNA) have shown a high chromosomal diversity in triatomines [5, 6, 11]. Unlike other insects such as Orthoptera, Diptera and Coleoptera, FISH analyses with DNA probes of other repeated sequences, such as the 5S rDNA cluster and histone genes [21], failed to achieve satisfactory results in Heteroptera. To better understand the chromatin organization and composition of the holocentric chromosomes it is essential to find methodological approaches that allow the detection of other repeated sequences.

Our GISH results reveal the occurrence of two hybridization configurations: scattered hybridization signals along the entire chromosome length and more intense hybridization signals concentrated in specific chromosomal regions or the whole chromosomes (Figures 1, 2 and 3). Most likely both hybridization types are reflecting two main classes of repeated DNA elements within eukaryotic genomes: dispersed and accumulated repeats. Regions with accumulated repeats, usually C-band positive, comprise mainly satellite DNAs (included in the heterochromatin) and multigene families such ribosomal RNA (rRNA) and the histone gene families [22], although dispersed repeats could be also present in euchromatic regions [23, 24]. The dispersed repeats mainly include transposable genetic elements. In several insect species, such as *Drosophila* and *Anopheles*, transposable elements constitute about 15% of its genome [25]. The scatter hybridization configuration here obtained is a typical pattern observed in other insects with probes of different mobile genetic elements [26].

All analyzed species in this paper exhibit scattered hybridizations signals on their chromatin, suggesting that transposable elements constituted a very important component in the triatomine genomes. Furthermore, all species except *R. prolixus*, present a specific intense hybridization pattern on regions of autosomes and/or sex chromosomes that vary in number, size, and chromosomal localization. *R. prolixus* present all chromatin, including Y chromosome, with dispersed hybridization without strong hybridization regions, suggesting that their repetitive DNA is mainly formed by dispersed repeats without cluster repeats, at least recognized by our genomic probes. Hence, the heterochromatic Y chromosome in *R. prolixus* is constituted by different repetitive sequences than the observed in Triatomini species (Figure 3i). Previous studies suggest the existence of different families of transposable elements in this species [27, 28].

In summary, these results reveal that the genomes of the 10 tribe Triatomini studied species share dispersed repetitive DNA sequences. In addition *T. infestans*, *T. delpontei, T. platensis* and *Mepraia spinolai* share also the accumulated repetitive sequences located in the C-band positive regions, while in the remaining species these sequences are only present on the heterochromatic Y chromosome. Among Triatomini species and *Rhodnius prolixus* (Tribe Rhodniini) only dispersed repetitive DNA sequences are shared.

Correlation between GISH and DAPI positive C-band

Genome-specific repeats have frequently a non-random distribution, forming clusters within heterochromatin blocks. In most organisms, including many plant species, the GISH hybridization signals often coincide with C-bands and also with DAPI positive regions, suggesting the presence of AT-rich DNA sequences at these regions [14]. Our GISH results in triatomines are partially consistent with that observed in plants, when evolutionarily related species are compared. However, the correspondence between C and DAPI patterns with GISH hybridizations are not observed when more evolutionarily distant species are analyzed.

In the three closely related infestans subcomplex species (*T. infestans, T. delpontei*, and *T. platensis*) and *Mepraia spinolai*, the strong hybridization signals mainly co-localized with their C-banding patterns (Figures 1, 2 and 3). However, more detailed analysis in *T. infestans* demonstrated that GISH signals coincide exactly with autosomal DAPI positive regions described for this species (Figure 2d). Hence, our hybridization signals are not able to label the subterminal CMA_3 positive C-heterochromatic regions (GC-rich) observed in the largest autosomal pairs of *T. infestans* (arrowheads Figures 2d and 2e). This concordance between GISH patterns and DAPI regions in the infestans subcomplex and *M. spinolai* species is not observed when we analyze more evolutionarily distant species, even within the genus *Triatoma*. For example, the DAPI positive C-heterochromatin regions in all autosomes and X chromosome of *T. protracta* do not show strong hybridization signals (compare Figures 3d and 3e). Similar results were obtained in other species with autosomal C-heterochromatin such as *T. dimidiata* and *T. carrioni* (Figures 3b and 3c). Our GISH results are in agreement with the Triatominae molecular phylogeny, which shown that *M. spinolai* is closed relater with South American than North American *Triatoma* species [29]. Hence, repetitive sequences shared between *M. spinolai* and infestans subcomplex species is showing this evolutionary relationship.

Recent analyses of the AT-rich satDNA portion of *T. infestans* using reassociation kinetics (C_0t) found two repetitive arrays located on the terminal regions of autosomal C-heterochromatin but not on the sex chromosomes heterochromatin [13]. Due to the similar chromosome localization, our GISH probes probably identify these repetitive arrays (Figure 2d) and others A–T rich sequences present on the C-heterochromatin of the X and Y sex chromosomes of *T. infestans*, not identified by C_0t studies (Figures 1h, 2b and 2d).

These results clearly show that: (a) infestans subcomplex closely related species share the majority of their repetitive sequences, in spite of their different size and chromosome distribution, (b) our genomic probes identify different repetitive DNA families, which are A-T rich, (c) autosomal and X heterochromatin regions from other evolutionary distant species are integrated by other divergent repetitive DNA, without homology to our genomics probes, hence not identified by GISH. Isolation and characterization of repeated sequences, either by GISH or others methodological approaches, seems to be appropriate genetic markers to infer evolutionary relationships between different species groups.

Evolution of sex chromosomes inferred by GISH

In all triatomine species, sex chromosomes are very well differentiated from autosomes by their particular behavior during meiosis. They are considered asynaptic and achiasmatic during male meiotic division [30, 31] showing an inverted meiosis: in first meiotic division the sister chromatids of each sex chromosome separate equationally and in the second division the sex chromosomes segregate to opposite poles [4].

In insect groups such as Diptera, Orthoptera and Lepidoptera distinct DNAs classes have been mapped on sex chromosomes [32–35]. However in Heteroptera there is a lack of knowledge about the molecular composition and evolution of sex chromosomes. In all of the 80 cytogenetically Triatominae studied species, the Y chromosome is almost entirely C-heterochromatic [3]. Molecular studies on the Y chromosome sequences are limited to fluorescence banding, showing that it is constituted by DAPI positive AT-rich sequences [10, 12, 13]. Our GISH studies reveal that in the ten Triatomini species, the Y chromosome is mainly constituted by accumulated repetitive sequences. It is well known that most Y chromosomes (from mammals, fish, insects and plants) are enriched with repeats in a process of heterochromatin accumulation. However, a striking result is that 10 species of triatomines, including 5 different genera, present and share the same types of repeated DNA sequences. In Diptera and Lepidoptera, unlike triatomines, Y chromosome (or their equivalent W chromosome) sequence composition appears to have a rather high turnover evolution rate because even in related species, the Y chromosome exhibit different repeated sequences or gene content [34, 36]. For this reason the Y chromosome repeated sequences conservation in Triatomini tribe species here analyzed is a very uncommon phenomenon and probably these repeated sequences represent an ancestral character of this tribe (Figures 1, 2 and 3).

On the other hand, the *R. prolixus* Y chromosome, species which belongs to the Rhodniini tribe, does not shared the same conserved repetitive DNA sequences observed in the analyzed Triatomini species (Figure 3i). This result suggests an early evolutionary differentiation or dichotomy within Triatominae subfamily in two clearly distinct clades: the Rhodniini and Triatomini tribes. Our results are in agreement with phylogenetic studies with nuclear and mitochondrial DNA sequences which suggested a Rhodniini–Triatomini node as the oldest known split

within the triatomine bugs [39, 40]. Another explanation could be a different origin of the Triatomini and Rhodniini Y chromosomes, either deriving from different autosomal pairs or B chromosomes as suggested in homopteran and *Drosophila* species [41].

In triatomines, sex chromosomes are achiasmatic, so surely completely differentiated between each other, which implicate a scenario with an old Y chromosome. As has been described in *Drosophila* and mammals, old Y chromosome evolution is mostly driven by genetic hitchhiking, where evidence of positive selection evidence was found [36, 37]. In addition, the Y chromosome is a very important determinant of male fitness [38], so conservation in triatomines could be caused by a selective pressure on important male fitness genes. Maybe genome project data on Y-linked genes would bring light on this question.

One of the most striking results of this paper is that, in infestans subcomplex and *M. spinolai* species, the sex chromosomes (Xs and Y) with each other and with some autosomes share highly repeated sequences (Figures 1 and 2). A distinctive cytogenetic feature of these species is that during meiotic prophase chromosomal associations among autosomes with both sex chromosomes occur [18, 42]. These associations would facilitate the sequences exchange among these non-homologous chromosomes [9]. If we consider that the highly repeated sequences localized in the Y chromosome are the plesiomorphic state, a possible scenario could be a sequences transfer from Y chromosome to the autosomes and X chromosomes. Consequently, the sequence homology between them could be considered a secondary character (apomorphic) restricted to closely related species groups. Comparative analyses on the 45S rDNA clusters chromosome position in several triatomine species also suggested the existence of chromatin exchange between sex chromosomes [6], in contrast to the widely accepted idea that the achiasmatic sex chromosomes of Heteroptera do not interchange sequences [31]. Furthermore, the simultaneous presence of 45S ribosomal clusters in autosomes and the X chromosome observed in *T. infestans* and *T. delpontei* also suggests the occurrence of sequences exchanges among autosomes and sex chromosomes [5, 9]. It has been suggested that transposable elements could play an important role in the mobilization of repeat sequences among chromosomes [23]. Nevertheless, in order to advance in the knowledge of sex chromosomes evolution, a molecular characterization of their sequences will be necessary.

Phylogenetic origin of the subfamily Triatominae

The phylogenetic origin of blood-feeding Triatominae has received considerable attention due to the epidemiological significance as vectors of Chagas disease. Conflicting hypotheses support Triatominae as monophyletic [29, 39, 43], polyphyletic [17, 44] or paraphyletic group [40]. The answer to this question goes beyond the academic interest, since it could represent a very important issue to know if hematophagy in Triatominae arose from a single evolutionary event or as multiple independent evolutionary processes. According to the polyphyletic and paraphyletic proposals, the Triatomini and Rhodniini tribes are derived from

quite different reduviid subfamilies. The sister group of the Triatomini seems most likely to be the Reduviinae, while the sister group for the Rhodniini may possibly be the Stenopodainae or Salyavatinae [17, 43]. Considering the extreme Y chromosome molecular differentiation between the two tribes described here, the application of GISH methodology on the suspected sister species of Triatominae could help elucidate the monophyletic or polyphyletic origin of this subfamily.

Author Contributions

Conceived and designed the experiments: PL FP. Performed the experiments: SP FP AS PL. Analyzed the data: SP FP AS YP TP PL. Contributed reagents/materials/analysis tools: SP FP AS YP TP PL. Wrote the paper: SP FP AS YP TP PL.

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RESEARCH Open Access

Holocentric chromosome evolution in kissing bugs (Hemiptera: Reduviidae: Triatominae): diversification of repeated sequences

Sebastián Pita[1], Pedro Lorite[2], Jesús Vela[2], Pablo Mora[2], Teresa Palomeque[2], Khoa Pham Thi[3] and Francisco Panzera[1*]

Abstract

Background: The analysis of the chromosomal and genome evolution in organisms with holocentric chromosomes is restricted by the lack of primary constriction or centromere. An interesting group is the hemipteran subfamily Triatominae, vectors of Chagas disease, which affects around 6 to 7 million people worldwide. This group exhibits extensive variability in the number and chromosomal location of repeated sequences such as heterochromatin and ribosomal genes. This paper tries to reveal the significant differences of the repeated sequences among *Triatoma* species through the use of genomic DNA probes.

Methods: We analysed the chromosomal distribution and evolution of repeated sequences in *Triatoma* species by genomic in situ hybridization (GISH) using genomic DNA probes from two North American *Triatoma* species. These genomic probes were hybridized both on their own chromosomes and on other *Triatoma* species from North and South America, with different amounts and chromosome location of C-heterochromatin. The results were compared with those previously described using South American *Triatoma* genomic probes.

Results: We observed two chromosomal hybridization patterns: (i) very intense hybridization signals concentrated on specific chromosomal regions or particular chromosomes; and (ii) lower intensity hybridization signals dispersed along all chromosomes. Self-GISH on *T. rubrofasciata* and *T. dimidiata* chromosomes presented strong hybridization signals on all C-heterochromatin regions. However, when we perform genomic cross-hybridizations, only strong signals are detected on the Y chromosome, leaving the C-heterochromatic autosomal regions unmarked.

Conclusions: We confirm that repeated DNA of the Y chromosome is shared among *Triatoma* species and probably represents an ancestral character of the Triatomini tribe. On the contrary, autosomal heterochromatic regions are constituted by species-specific DNA repeats, most probably satDNA families, suggesting that *Triatoma* speciation involved the amplification of diverse types of autosomal repeats. Molecular characterization of principal repetitive DNAs seems to be an appropriate approach to infer evolutionary relationships in triatomines.

Keywords: Chagas disease vectors, Genomic in situ hybridization, Holocentric chromosomes, Triatominae

* Correspondence: fcopanzera@gmail.com
[1]Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Calle Iguá 4225, 11400 Montevideo, Uruguay
Full list of author information is available at the end of the article

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Background

Repetitive DNA sequences consist of a large portion of eukaryotic genomes including tandem and dispersed repeats [1, 2]. Tandem repeats are organized in arrays in which the monomers (or repeat units) are repeated in a head-to-tail fashion, including multigene families (ribosomal DNAs, histone genes and small nuclear DNA) and satellite DNA (satDNA). Dispersed repeats are mainly represented by transposable elements, which are DNA segments able to change from one locus to another within the genome of their host [3].

Repetitive DNA fractions are usually larger than the coding sequence component of a genome and are essential for different genomic functions such as replication, transcription and expression [4]. For example in *Drosophila,* the important role that heterochromatin plays in the chromosome pairing, gene silencing via position-effect variegation and maintaining genome stability is well documented [5]. The chromosomal distribution of repeated DNA sequences, mainly located in the heterochromatic regions, confers a specific nuclear architecture, which establishes distinct transmission and expression characteristics even with the same coding sequences [4]. Hence, alterations in the location and composition of repeated DNA would have a diversification role in the evolution of the species [6], such as it was observed in the reproductive isolation of *Drosophila* sister species [7]. Evolutionary analyses of these sequences are even more significant in organisms with holocentric chromosomes, where karyotypes generally lack appropriate chromosomal markers for this type of study. In insects, different orders that include species with significant economic (agricultural pests) and medical importance (vectors of human diseases) exhibit this type of chromosomes. These orders are Dermaptera (earwigs), Hemiptera (true bugs), Lepidoptera (butterflies and moths), Odonata (dragonflies and damselflies), Phthiraptera (lice), Psocoptera (book lice), Zoraptera (angel insects) and Trichoptera (cloth moths) [8]. In the Hemiptera, the reduviid subfamily Triatominae comprises vectors for Chagas disease, an anthropozoonotic illness caused by the protozoan parasite *Trypanosoma cruzi*, which affects six to seven million people worldwide, mostly in Latin America but is increasingly detected in USA, Canada, and many European countries [9]. In this hemipteran group, the lack of primary constriction and their small chromosome size greatly hamper chromosome studies. Although their holokinetic structure is expected to facilitate karyotype evolution through fusions and chromosome fissions, the number of autosomes in triatomines appears to be quite stable: almost all species have a diploid autosomal number of 20 [10]. In spite of this extensive uniformity in their autosomal number, the subfamily Triatominae exhibits a great variability of the genome size [11], in the ribosomal clusters chromosome location [12–14] and in the amount, distribution and composition of the repetitive sequences included in the constitutive heterochromatin [10, 15]. Nevertheless, unlike other insects or even other hemipteran families such as Coreidae or Pentatomidae, FISH analyses using other multigene families' probes such as 5S rDNA, U2 snDNA or histone genes, failed to achieve satisfactory results in this insect group. In this way, different methodological approaches were applied to analyse repetitive sequences of triatomines such as genomic in situ hybridization (GISH) [16] and chromosomal microdissection [17]. In insects with holocentric chromosomes, GISH approaches are scarce and have been applied to the study of sex chromosomes evolution in the Lepidoptera (reviewed by [18]) and Hemiptera (Pyrrhocoridae) [19]. A previous GISH study using genomic probes of *Triatoma* species from South America revealed that closely related species share their repetitive sequences. Furthermore, all Triatomini tribe species, including *Triatoma* and other genera, always have a Y chromosome with similar highly repeated sequences [16]. To further analyse the evolution of the repeated sequences both in autosomes and sex chromosomes in the genus *Triatoma*, in this paper we applied genomic probes from two North American *Triatoma* species. These DNA probes were hybridized both on their own chromosomes (self-GISH) and on other *Triatoma* species, with different amount and chromosomal location of the C-heterochromatin.

Methods

The subfamily Triatominae includes 151 species in 15 genera, being the genus *Triatoma* (Triatomini tribe) the most numerous and diverse (84 species) and the only one found in the New and Old World [20]. We studied species included in two of the three main groups of this genus: (i) the rubrofasciata group (from Central and North America and Old World species): *T. barberi, T. dimidiata, T. lecticularia, T. nitida* and *T. rubrofasciata*; and (ii) the infestans group (from South America): *T. infestans* and *T.* (*Mepraia*) *spinolai*. All these species are important vectors of Chagas disease due to their presence in domestic and peridomestic environments, excepting *T. lecticularia* which is strictly sylvatic [21].

Geographical origin and cytogenetic traits of the *Triatoma* species analysed are detailed in Table 1. These species were selected by their different number of autosomes (18, 20 and 22), male sex systems (XY and X_1X_2Y), and differences in the amount and chromosomal location of autosomal heterochromatin [15, 22–25].

Genomic DNA (gDNA) probes were made from two *Triatoma* species of the rubrofasciata group: *T. rubrofasciata* collected in Vietnam (Hanoi city, Tu Liem

Table 1 Geographical origin and chromosomal traits of seven *Triatoma* species analysed in the present studyk

Species and male diploid chromosome number (2n)	Geographical origin	%, chromosome location and size of autosomal C-heterochromatin
T. rubrofasciata (2n = 22A + X_1X_2Y)	Vietnam, Hanoi, Tu Liem district. P. 21°2′48″N, 105°44′54″E	40%; 11 II with C-blocks in both chromosomal ends [22]
T. dimidiata (2n = 20A + X_1X_2Y)	Guatemala, Jutiapa, Carrizal. D. 14°25′48″N, 89°57′28″W	10%; 10 II with C-dots in both ends [23]
T. barberi (2n = 20A + X_1X_2Y)	Mexico, Queretaro, La Cueva. P. 20°29′4″N, 100°26′20″W	35%; 10 II with C-blocks in both ends [15]
T. nitida (2n = 18A + X_1X_2Y)	Guatemala, Quiché, Zacualpa, D. 15°1′34″N, 90°52′42″W	25%; 2 II almost entirely C-heterochromatic [15]
T. lecticularia (2n = 20A + XY)	Insectary CDC (Atlanta). Origin: USA, Oklahoma, Walkiria.	30%; 10 II with C-blocks in both ends [15]
T. infestans (non-Andean lineage) (2n = 20A + XY)	Argentina, Chaco, Tres Estacas. P. 26°54′30″S, 51°40′23″W	24–30%; 2–4 II with C-blocks in one or both ends [24]
T. (*Mepraia*) *spinolai* (2n = 20A + X_1X_2Y)	Chile, Metropolitan Region of Santiago, Colina. S. 33°11′53″S, 70°39′42″W	15%; 10 II with C-dots in both ends [25]

Abbreviations: *A* autosomes, *D* domestic, *P* peridomestic, *S* sylvatic, *II* bivalents

district, Dai Mo commune, Ngoc Truc village) and *T. dimidiata* from Guatemala (Jutiapa, Carrizal). Genomic DNA isolation for probes generation was made from legs of one adult male individual using the NucleoSpin Tissue kit (Macherey-Nagel Co., Düren, Germany). For probe labelling, total genomic DNA was labelled with biotin-16-dUTP (Roche, Mannheim, Germany) using a Nick Translation Kit (Roche, Shanghai, China), following manufacturer's instructions. To compare the results obtained with these two probes, we applied a third genomic probe (*T. infestans*) that was already used in a previous paper [16] but applied here for the first time on *T. rubrofasciata* chromosomes.

Chromosome preparations for C-banding and GISH analyses were obtained from males. Gonads were removed from live adult insects, fixed in ethanol: glacial acetic acid mixture (3:1) and stored at -20 °C. Squashes were made in a 50% acetic acid drop, coverslips were removed after being frozen in liquid nitrogen and the slides were air dried and then stored at 4 °C. C-banding was performed according to Panzera et al. [24].

In situ hybridizations were carried out as described by Palomeque et al. [26]. Hybridization solutions were prepared to a final concentration of 0.5–2.0 ng probe/ml in 50% formamide. Hybridization was conducted at 37 °C overnight. Fluorescence immunological detection was performed using the avidin-FITC/ anti-avidin-biotin system with two amplification rounds. Slides were mounted with Vectashield (Vector, Burlingame, USA). DAPI in the antifade solution was used to counterstain chromosomes. The hybridized chromosomes were observed and photographed using a BX51 Olympus fluorescence microscope equipped with a CCD camera (Olympus DP70) and merged using the DP Manager software. Hybridization pattern for each species was determined by the chromosomal analyses of at least two individuals.

Results

We observed two chromosomal hybridization patterns: (i) very intense hybridization signals concentrated on specific chromosomal regions or particular chromosomes; and (ii) lower intensity hybridization signals dispersed along all chromosomes (Figs. 1, 2 and 3). In some cases, the lower intensity hybridization signals were masked by the DAPI signal in the merged figures. Table 2 summarises the GISH results described below.

Triatoma rubrofasciata gDNA probe

Self-GISH results on *T. rubrofasciata* showed that almost all chromatin presented scattered hybridization signals. Strong hybridization signals were observed in C-heterochromatin regions: chromosomal ends of the 11 half-bivalents plus the Y chromosome, but neither X chromosome presented strong hybridizations signals (Fig. 1a). A similar pattern was observed with C-banding (Fig. 1b). Hybridization of all North American *Triatoma* species showed strong hybridization signals only on the Y chromosome (Fig. 1c-g), without labelling on other heterochromatic regions (compare Fig. 1e with f, arrows). In *T. barberi*, in addition to the Y chromosome, one of the two X chromosomes is also labelled (X_1) (Fig. 1g).

Triatoma rubrofasciata gDNA probe on chromosomes of South American *Triatoma* showed differences between the two species analysed. In *T.* (*Mepraia*) *spinolai,* only the Y chromosome exhibited hybridization signals (Fig. 1h). In *T. infestans*, in addition to the Y chromosome, 2 to 3 autosomal pairs showed terminal hybridization signals (Fig. 1i).

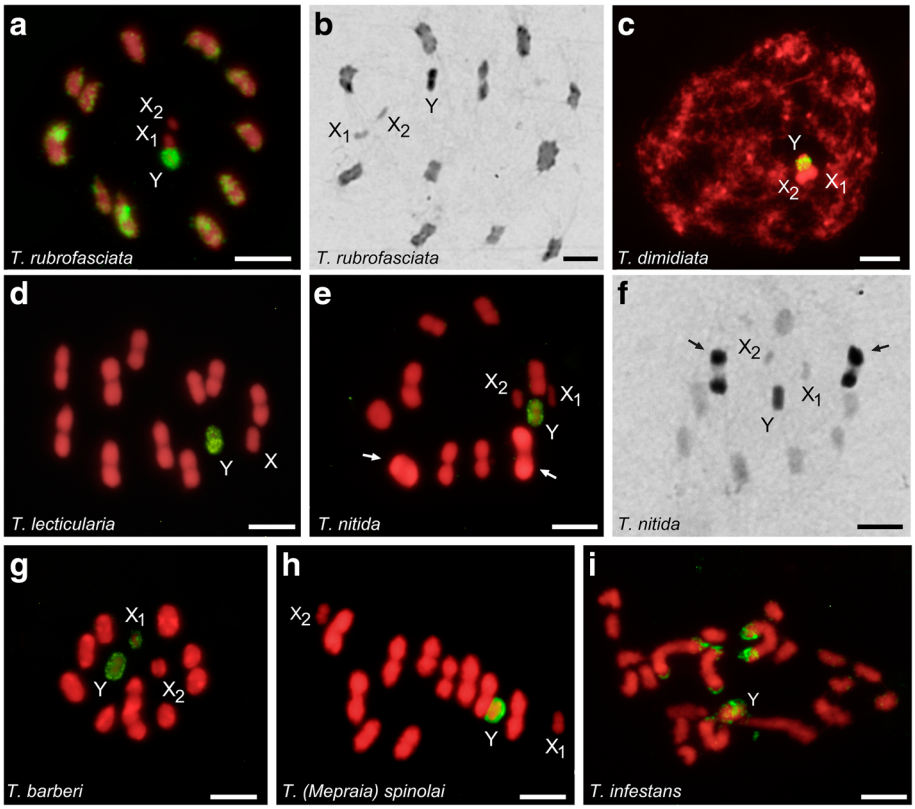

Fig. 1 GISH results using *Triatoma rubrofasciata* genomic DNA (gDNA) probe (labelled in yellow-green) on male chromosomes of different *Triatoma* species (labelled in red). **a** Self-GISH on own chromosomes of *T. rubrofasciata* (2n = 22A + X_1X_2Y): Second meiotic metaphase. Hybridization signals appear scattered on all chromatin, but the strongest signals are preferably located at the autosomal chromosome ends plus the Y chromosome. Both X chromosomes did not present hybridization signals. **b** *T. rubrofasciata*. First meiotic metaphase (MI) with C-banding. Heterochromatic regions with the same distribution pattern as observed with self-GISH. **c** *T. dimidiata* (2n = 20A + X_1X_2Y). Early meiotic prophase. **d** *T. lecticularia* (2n = 20A + XY): MI. **e** *T. nitida* (2n = 18A + X_1X_2Y): MI. In (**c**) to (**e**), strong hybridization signals are restricted to the heterochromatic Y chromosome. **f** *T. nitida*: MI with C-banding. The two C-heterochromatic bivalents did not exhibit hybridization signals (compare chromosomes pointed out with arrows in **e** and **f**). **g** *T. barberi* (2n = 20A + X_1X_2Y): MI. Y chromosome and one of the X chromosomes (X_1) appear with strong signals. **h** *T.* (*Mepraia*) *spinolai* (2n = 20A + X_1X_2Y): MI. Only the Y chromosome shows hybridization signals. In (**c**) to (**h**), autosomal C-heterochromatic regions appear labelling free. **i** *T. infestans* (2n = 20A + XY): Spermatogonial prometaphase. Strong hybridization signals are observed on the Y chromosome and five autosomes. *Scale-bars*: 5 μm. *Abbreviations*: A, autosomes; MI, metaphase I

Triatoma dimidiata gDNA probe

Self-GISH results on *T. dimidiata* showed strong hybridization signals on the Y chromosome as well as scattered hybridization signals on all chromatin, including both X chromosomes (Fig. 2a). However, on less condensed chromosomes (early meiotic prophase), strong hybridization signals were observed as spots on the chromosomal ends of all autosomes (Fig. 2b). These hybridization signals correspond to the heterochromatic dot regions located on the chromosomal ends observed with C-banding (Fig. 2c). When *T. dimidiata* gDNA probe was employed on chromosomes of *T. rubrofasciata* and other North American *Triatoma* species, only the Y chromosome presented positive hybridization signals (Fig. 2d, e), with the exception of *T. barberi*, which exhibited the same pattern observed with *T. rubrofasciata* probe (one X chromosome also labelled, data not shown). In South American *Triatoma* species, hybridization patterns were the same as the obtained with *T. rubrofasciata* genomic probe, i.e. only on the Y chromosome in *T. spinolai* (Fig. 2f) and 2–3 bivalents plus the Y chromosome in *T. infestans* (Fig. 2e).

Triatoma infestans (non-Andean lineage) gDNA probe

GISH results on *T. dimidiata* (Fig. 3a) and *T. rubrofasciata* (Fig. 3b) chromosomes showed strong hybridization signals only on the Y chromosome, while the autosomal heterochromatic regions remain unmarked.

Discussion

GISH technique and DNA repeat sequences

This paper and the previous one [16] show that triatomine GISH probes reveal two hybridization patterns: scattered hybridization signals along almost all chromatin and

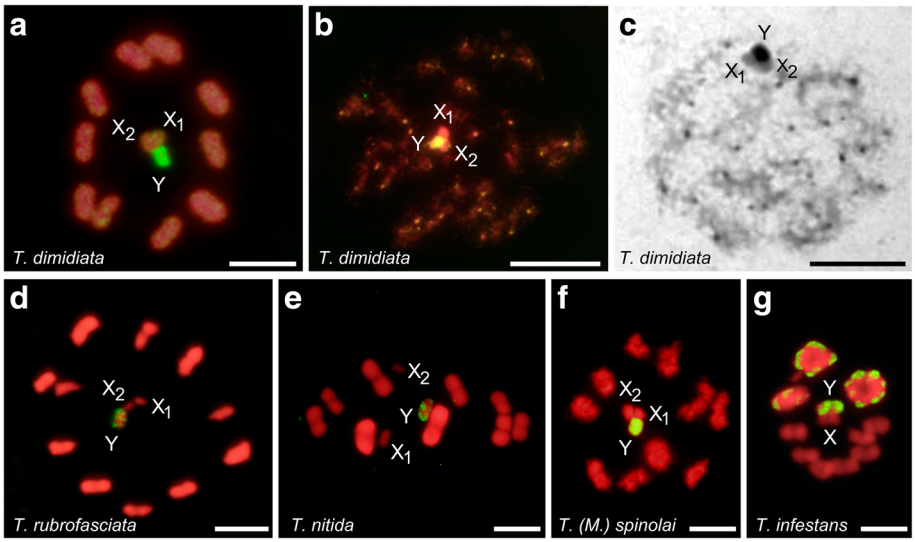

Fig. 2 GISH results using *Triatoma dimidiata* genomic DNA (gDNA) probe (labelled in yellow-green) on chromosomes of different *Triatoma* species (labelled in red). **a** Self-GISH on own chromosomes of *T. dimidiata* (2n = 20A + X_1X_2Y): Second meiotic metaphase (MII). All chromatin presented scattered hybridization signals, but strong signals were observed only on the Y chromosome. **b** *T. dimidiata*: Early meiotic prophase showing dot hybridization signals at the chromosome ends of all autosomes and on the Y chromosome. **c** *T. dimidiata*: Early meiotic prophase with C-banding. C-Heterochromatin regions with the same distribution pattern as observed with the genomic probe in **b**. **d** *T. rubrofasciata* (2n = 22A + X_1X_2Y): MII. **e** *T. nitida* (2n = 18A + X_1X_2Y): Metaphase I (MI). **f** *T.* (*Mepraia*) *spinolai* (2n = 20A + X_1X_2Y): Diakinesis. In **c**-**e**, the Y chromosome exhibited strong hybridization signals. **g** *T. infestans* (2n = 20A + XY): MI. Strong hybridizations signals were observed on the Y chromosome and chromosomal ends of three bivalents. *Scale-bars*: 5 μm. *Abbreviations*: A, autosomes; MI, metaphase I; MII, metaphase II

stronger hybridization signals concentrated on particular chromosomal regions or on the entire chromosomes (Figs. 1, 2 and 3). The stronger signals could be caused by satellite DNA families clustered in long tandem arrays as was seen using specific satDNA probes [27]. Meanwhile, the scattered signals might be produced by small amounts of satellite DNA families [27, 28], or by transposable genetic elements, as has been observed in other insects [29, 30].

From GISH results obtained here (Table 2, Figs. 1, 2 and 3) we can draw the following assumptions. First, in the two Self-GISH or Auto-GISH analyses, i.e. *T. rubrofasciata* genomic probe on *T. rubrofasciata* chromosomes and *T. dimidiata* gDNA probe on *T. dimidiata* chromosomes, the strong hybridization signals coincide exactly with the C-heterochromatic regions described for each species (compare Fig. 1a with b, and Fig. 2b with c, respectively). However, when we perform genomic cross-hybridizations, i.e. *T. rubrofasciata* gDNA on *T. dimidiata* chromosomes (Fig. 1c) and vice versa (Fig. 2d), only strong signals are detected on the Y chromosome, leaving the C-heterochromatic autosomal regions unmarked. This reveals that the heterochromatic regions of *T. rubrofasciata* and *T. dimidiata* are constituted by different classes of satellite DNA families, which appear undifferentiated with

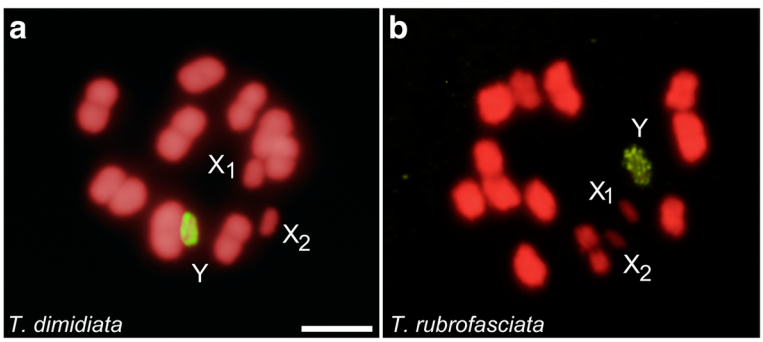

Fig. 3 GISH results using *Triatoma infestans* genomic DNA (gDNA) probe (labelled in yellow-green) on metaphase I chromosomes (labelled in red) of *T. dimidiata* (**a**) and *T. rubrofasciata* (**b**). In both species, only the heterochromatic Y chromosome appears with strong hybridization signals, while autosomal heterochromatic regions appear label free. *Scale-bars*: 5 μm

Table 2 Summary of GISH results using two genomic DNA probes of North American *Triatoma* on the chromosomes of seven *Triatoma* species

Species	*T. rubrofasciata* genomic DNA probe	*T. dimidiata* genomic DNA probe
T. rubrofasciata	Self-GISH. All autosomal pairs (11 half-bivalents) with strong hybridization signals in one or both chromosomal ends. Y chromosome intensively and totally labelled (Fig. 1a)	Only Y chromosome (Fig. 2d)
T. dimidiata	Only Y chromosome (Fig. 1c)	Self-GISH. All bivalents (10) with strong hybridization signals in both chromosomal ends. Y chromosome intensively and totally labelled (Fig. 2a, b)
T. lecticularia	Only Y chromosome (Fig. 1d)	Only Y chromosome.
T. nitida	Only Y chromosome (Fig. 1e)	Only Y chromosome (Fig. 2e)
T. barberi	Y chromosome plus one X chromosome (Fig. 1g)	Y chromosome plus one X chromosome
T. (*Mepraia*) *spinolai*	Only Y chromosome (Fig. 1h)	Only Y chromosome (Fig. 2f)
T. infestans (non-Andean lineage)	2–3 autosomal pairs with strong hybridization signals in 1 or 2 chromosomal ends plus the Y chromosome (Fig. 1i)	2–3 bivalents with strong hybridization signals in 1 or 2 chromosomal ends plus the Y chromosome (Fig. 2g)

C-banding and fluorescence staining [15]. This same conclusion can be extended to the other North American *Triatoma* (*T. lecticularia, T. nitida* and *T. barberi*) (Fig. 1d-g; Fig. 2e), which have a different amount and chromosomal localization of heterochromatin (Table 1). This evidence is conclusive in showing that, at least in the species here analysed, chromosome diversification in North American *Triatoma* involved a differential amplification of diverse types of autosomal repeated DNA sequences. This is very different from what has been described for the infestans subcomplex species (*T. infestans*, *T. platensis* and *T. delpontei*), where these closely related species shared their repeated DNA sequences [16].

Secondly, the hybridization patterns of both genomic probes on *T. infestans* chromosomes showed strong signals on autosomal heterochromatic regions (Figs. 1i and 2g). These results confirm those obtained with South American *Triatoma* genomic probes, revealing that in the infestans subcomplex the repeated sequences of the Y chromosome are also present on the autosomal heterochromatic regions [16]. Genomic hybridizations using *T. infestans* gDNA probe on *T. dimidiata* [16] and *T. rubrofasciata* chromosomes (Fig. 3a, b, respectively) showed strong hybridization signals only on the Y chromosome. These results reveal that autosomal heterochromatic regions of *T. dimidiata* and *T. rubrofasciata* are constituted by different DNA repeats, most probably satDNA families, which are also different from what was observed for the Y chromosome.

Evolution of sex chromosomes

In triatomines, sex chromosomes are very well differentiated from autosomes by their distinct behaviour during meiosis. During male meiotic division, X and Y chromosomes are asynaptic and achiasmatic, showing an inverted meiosis [31, 32]. Three male sex systems have been reported in Triatominae subfamily: XY, X_1X_2Y, and $X_1X_2X_3Y$. The first system is considered ancestral so that the multiple Xs are due to fission processes of the original X [33]. Multiple sex chromosomes have been reported in several triatomine genera including *Triatoma* species from North and South America [10]. Given that these multiple sex systems appear in triatomine species that are not closely related, it is probable that the fission processes of the X chromosome have occurred several times during the evolution of this group.

Knowledge about the DNA sequences that composed sex chromosomes in heteropteran species is very limited. Recently, Gallo et al. [34] have analysed the repetitive DNA composition of the Y and X chromosomes in several species of the giant water bugs (Heteroptera: Belostomatidae). These authors showed that repetitive DNA was absent on the Y chromosome while X chromosomes and autosomes shared repetitive sequences. Our previous results obtained by GISH [16], fluorescent banding [15], X chromosome microdissection [17] and the results displayed here support the idea that the X and Y chromosomes in triatomines presented substantial differences in their DNA composition. All of these studies pointed out that the Y chromosome is extremely conserved among all Triatomini species. It is C-heterochromatic and DAPI positive [10, 15], very different to that observed in Belostomatidae. This study confirms that the Y chromosome in North and South American *Triatoma* share repeated DNA sequences (Figs. 1, 2 and 3). Very recently we proved that the Y chromosome in *T. infestans* is constituted by at least two satDNA families [35]. The sequence conservation of the Y chromosome in a speciose and diverse insect group is very uncommon, and probably represents an ancestral character of Triatomini tribe, such as suggested by Pita et al. [16].

By contrast, the X chromosome is euchromatic, without fluorescence staining [15] but with dispersed repeated sequences similar to those observed in euchromatic autosomal regions [17, 35]. Occasionally in some species, like *T. barberi* (Fig. 1g) and the infestans subcomplex species

(*T. infestans* Andean lineage, *T. platensis* and *T. delpontei*) [16], the X chromosomes have heterochromatic regions similar to those observed in the Y chromosome. Probably this sequence homology between the X and Y chromosomes are due to repeated sequences transferred from Y chromosome to the X chromosome. Since *T. barberi* and the infestans subcomplex species are evolutionarily distant species, this transference appears to have occurred several times in the evolutionary history of *Triatoma*, perhaps as a product of secondary differentiation processes. The occurrence of shared sequences between X and Y chromosomes have also been seen with the major ribosomal genes in several *Triatoma* and *Rhodnius* species [12–14, 36]. The existence of shared repeated sequences between the X and the Y chromosomes is not consistent with the accepted notion that the sex chromosomes of Heteroptera do not exchange sequences [32]. Mobilization of repeated sequences between sex chromosomes without recombination or chromosomal pairing could be due to transposition events, as has been suggested in ants [37] and triatomines [13].

Another alternative hypothesis to explain the sequence homology between the Y chromosome and one of the X chromosomes in *T. barberi* is that this heterochromatic X_1 chromosome was originated by fission from the Y chromosome, rather than fissions of an ancestral X as is currently accepted. Considering that the X_1X_2Y sex system is the only one present in 22 North American *Triatoma* species currently analysed (except *T. lecticularia* with XY) and that the X chromosome is almost always euchromatic [10], this hypothesis seems improbable but cannot be ruled out.

Conclusions

Our GISH results allow us to draw the following conclusions: (i) Y chromosome repeated sequences seem to be the only ones shared among all *Triatoma* species, and even in the whole Triatomini tribe (which also involves six other genera); (ii) the five North American *TriatoXma* species here analysed did not share their autosomal repeated DNA sequences, in spite of their close evolutionary relationships; and (iii) this divergence in the molecular composition of autosomal repetitive DNA is also observed between North and South American *Triatoma*. These results suggest that autosomal heterochromatin in triatomines consists of different classes of repeated sequences, probably satDNA, so that during the speciation of this genus amplifications of different repeated DNA families have occurred. Molecular characterization of such repetitive DNA families seems to be an appropriate approach to identify the diversification mechanisms of the repeated sequences and to infer evolutionary relationships in triatomines.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

SP, PL, JV, PM, TP, KPT and FP conceived and designed the experiments. KPT and FP collected the bugs. SP, PL, JV, PM and FP performed the experiments. SP, PL, JV, PM, TP and FP analyzed the data. PL, TP, KPT and FP contributed reagents/materials/analysis tools. SP, PL, TP and FP wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author details

[1]Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Calle Iguá 4225, 11400 Montevideo, Uruguay. [2]Departamento de Biología Experimental, Área de Genética, Universidad de Jaén, Paraje Lagunillas s/n, 23071 Jaén, Spain. [3]Center for Molecular Biology, IRD, Duytan University, Danang, Vietnam.

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Capítulo IV:

Determinación del repeatoma de *T. infestans*

PLOS ONE

RESEARCH ARTICLE

Comparative repeatome analysis on *Triatoma infestans* Andean and Non-Andean lineages, main vector of Chagas disease

Sebastián Pita[1]*, Francisco Panzera[1], Pablo Mora[2], Jesús Vela[2], Ángeles Cuadrado[3], Antonio Sánchez[2], Teresa Palomeque[2], Pedro Lorite[2]

1 Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay,
2 Departamento de Biología Experimental, Área de Genética, Universidad de Jaén, Jaén, Spain,
3 Department of Cell Biology and Genetics, University of Alcalá de Henares, Alcalá de Henares, Madrid, Spain

* spita@fcien.edu.uy

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Abstract

Triatoma infestans is the most important Chagas disease vector in South America. Two main evolutionary lineages, named Andean and non-Andean, have been recognized by geographical distribution, phenetic and genetic characteristics. One of the main differences is the genomic size, varying over 30% in their haploid DNA content. Here we realize a genome wide analysis to compare the repetitive genome fraction (repeatome) between both lineages in order to identify the main repetitive DNA changes occurred during *T. infestans* differentiation process. RepeatExplorer analysis using Illumina reads showed that both lineages exhibit the same amount of non-repeat sequences, and that satellite DNA is by far the major component of repetitive DNA and the main responsible for the genome size differentiation between both lineages. We characterize 42 satellite DNA families, which are virtually all present in both lineages but with different amount in each lineage. Furthermore, chromosomal location of satellite DNA by fluorescence *in situ* hybridization showed that genomic variations in *T. infestans* are mainly due to satellite DNA families located on the heterochromatic regions. The results also show that many satDNA families are located on the euchromatic regions of the chromosomes.

Introduction

Triatoma infestans, hemipteran insect of the subfamily Triatominae, is the most important vector of the protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease. Until 1990s, *T. infestans* had a wide geographical distribution occupying more than 6 million km^2 in seven South American countries, and was responsible for well over half of the 18 million people affected by Chagas disease [1]. This species included two main evolutionary lineages, named Andean and non-Andean, recognized by genetic [2,3] and phenetic characteristics [4,5]. These lineages present a clearly differentiated geographic distribution. Andean lineage is dispersed in high altitude valleys of Bolivia and Peru (above 1700 meters above sea level, masl) and also in

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lower altitudes in Southern Peru. Non-Andean lineage was found in lowland regions (0 to 1400 masl) in Argentina, Brazil, Chile, Paraguay, Uruguay and Bolivia (Chaco region) and also at higher altitudes in some localities of Argentina. Since the 1990s until now, control interventions by multinational initiatives coordinated by the Pan American Health Organization/ Word Health Organization (PAHO/WHO), mainly based in houses spraying with pyrethroid insecticides have reduced more than 80% of the original *T*. *infestans* distribution occupying less than 1 million of km^2 [1]. Non-Andean lineage was virtually eliminated from Brazil, Chile, Uruguay, and in substantial areas of Argentina, Bolivia and Paraguay, persisting only in the Gran Chaco region (southern Bolivia, north-western Paraguay and northern Argentina). On the contrary, the Andean lineage still persists in many localities of Bolivia, probably due to the frequent occurrence of pyrethroid resistant populations [6]. The drastic reduction of the non-Andean group in most of its distribution suggests that *T*. *infestans* lineages have different genetic backgrounds in relation to their susceptibility to pyrethroid insecticides.

The main genetic difference between both lineages is their nuclear DNA content, varying from 1.487 Gbp (non-Andean) to 1.936 Gbp (Andean) per haploid genome, without modifications in their chromosome number (2n = 22). This variation in the genome size has been related with differences in the C-heterochromatin amount [7,8]. Non-Andean group bear C-heterochromatin on 2 to 4 autosomal pairs plus on the Y chromosome, while Andean group presents heterochromatin on 6 to 9 pairs and on both sex chromosomes (XY) [9,10]. Both lineages also differ in the number and chromosomal location of other class of repetitive DNA: the major ribosomal clusters. Non-Andean lineage always exhibits one rDNA locus in the X chromosome, while Andean lineage is polymorphic in number (1–4 loci) and position (on 1, 2 or 3 autosomal pairs and the X chromosome) [10].

Repetitive DNA component is not only often the largest component of the genomes, but it is also essential for different genomic functions. The repetitive DNA distribution, mainly the heterochromatin, establishes a particular nuclear architecture, which can determine distinct transmission and expression properties even with the same coding sequences [11]. So changes in the composition and location of repeated DNA would have a very important role in the evolutionary diversification of the species [12]. In disease vectors, including *T*. *infestans*, it has been suggested that heterochromatin organization, histone modifications, and nuclear architecture can play important roles to determine phenotypes with different vectorial capacity [13]. Probably in *T*. *infestans* lineages, the striking differences in the genome size, C-heterochromatin amount and ribosomal genes location are involved together with other genomic changes, affecting differentially the susceptibility to pyrethroid insecticides as observed in both lineages.

Next-generation sequencing (NGS) techniques are powerful tools that allow carry out a global analysis of the repetitive sequences that form part of a genome [14–16]. The repetitive DNA fraction of a genome, named "repeatome" by Maumus and Quesneville [17], has been frequently omitted or superficially analyzed in most genome-wide analyses [18], probably by their non-coding nature or by the difficulty of the repetitive DNAs assembly. However, there are numerous studies showing the fundamental role of the repetitive sequences in the genome conservation and evolution [19].

Despite *T*. *infestans* medical and social relevance, the research on the nature of its repetitive sequences is very scarce. Using reassociation kinetics (C_0t) followed by cloning and sequencing, three satellite DNA (satDNA) families have been isolated and determined their chromosome location only in Andean *T*. *infestans* [20]. In this paper NGS techniques are applied in both *T*. *infestans* lineages for first time, in order to characterize and compare the repeat sequences composition of both genomes. The objective was to identify the main repetitive DNA changes occurred during *T*. *infestans* differentiation process. Since results showed that

satDNA is the main responsible for the genome size differences between lineages, a thorough analysis of this kind of repetitive DNA was carried out, including the chromosomal location determination for the eleven most abundant satDNA families by fluorescence *in situ* hybridization (FISH).

Material and methods

Samples

Triatoma infestans samples for DNA sequencing from Tacuarembó (Uruguay) and Tres Estacas (Argentina) were selected for the non-Andean lineage (1 male individual each) and a Cochabamba (Bolivia) sample was used for the Andean lineage (1 male individual). The membership of the selected individuals to each lineage was previously confirmed by C-banding and FISH localization of ribosomal clusters following [10]. Chromosome preparations for FISH analyses were obtained from several samples from Argentina and Uruguay (non-Andean lineage), and Bolivia (several localities from Cochabamba and La Paz) (Andean lineage).

No specific permissions were required for insect collections performed in this work, and did not involve endangered or protected species.

DNA sequencing and data analyses

For each sample, approximately 3 μg of genomic DNA were employed in Illumina® Hiseq™ 2000 paired-end sequencing–with 100 bp reads. 2.4 Gbp of sequences were obtained from Uruguay sample (coverage 1.35x), 1.9 Gbp from Argentina sample (1.7x) and 4.4 Gbp from the Bolivian sample (2.3x). Graph-based clustering analyses on the three samples were first carried on separately using RepeatExplorer, implemented within the Galaxy environment (http://repeatexplorer.org/) [14,21]. In addition, we performed a combined data set analysis, since facilitates finding shared repeat families of unequal abundance among species, while the individual genome screening facilitates the detection of species specific repeat families [14]. Sequences corresponding to mitochondrial DNA were eliminated for the repeat analyses. The genome proportion for each cluster was calculated as the reads percentage. Additionally, repeat type identification was corroborated by sequence-similarity searches of the assembled contigs against GenBank using BlastN and BlastX (http://www.ncbi.nlm.nih.gov/) and Repbase using CENSOR (http://www.girinst.org/). To identify potential satellite repeats, contigs were analyzed using Dotmatcher (available on http://emboss.bioinformatics.nl/cgi-bin/emboss/dotmatcher/). Estimates of evolutionary divergence between sequences were conducted with MEGA 6 using p-distance.

Cytogenetic mapping

Meiotic chromosome preparations for FISH analyses were obtained from male gonads. Testes were removed from living adult insects, fixed in an ethanol–glacial acetic acid mixture (3:1) and stored at -20˚C. Squashes were made in a 50% acetic acid drop, coverslips were removed after freezing in liquid nitrogen and the slides were air dried and then stored at 4˚C.

The consensus sequence of each satDNA family (S1 Table) was used to designed one or two oligonucleotides (S2 Table). SatDNA families with monomer longer than 85 bp were amplified by PCR in 25 μl reaction mixtures containing 50 ng of genomic DNA, 0.5 mM dNTPs, 50 pmol of the primers and 1 U of Taq polymerase (Bioline). The PCR program used was 2 min at 92˚C and 35 cycles: 20 sec at 92˚C, 60 sec at 51˚C, 2 min at 72˚C, with a final extension of 5 min at 72˚C. PCR products were analyzed by electrophoresis in agarose gels and amplified bands were eluted from the gel and inserted into the pGEMT-Easy vector (Promega). Recombinant plasmids

were sequenced. FISH using plasmids probes were performed as described by Palomeque *et al.* [22]. Probe labeling was carried on with biotin-16-dUTP (Roche®) using a Nick Translation Kit (Roche®), following manufacturer's instructions. Hybridization solutions were prepared to a final concentration of 5 ng probe/ml in 50% formamide. Hybridization was conducted at 37˚C overnight. For satDNA families with monomer shorter than 85 bp (and for TinfSat06-181), one or two oligonucleotides based on the most conserved regions were directly labeled with biotin-16-dUTP using terminal transferase (Roche®) and following the instructions of the supplier. Hybridization solutions were prepared to a final concentration of 200 pmol probe/ml in 50% formamide. Hybridization was also conducted at 37˚C overnight. Fluorescence immunological detection was performed using the avidin-FITC/ anti-avidin-biotin system with two rounds of amplification. Slides were mounted with Vectashield (Vector®). DAPI in the antifade solution was used to counterstain the chromosomes.

Images capture and analysis were made using a BX51 Olympus® fluorescence microscope equipped with a CCD camera (Olympus® DP70) and processed with Adobe® Photoshop® software.

Results

Triatoma infestans repeatome composition

Graph-based clustering was used to characterize, quantify and compare highly repetitive DNA sequences in Andean and non-Andean *T. infestans* genomes. In the Andean sample (Bolivia) the clustering of 199,646 reads produced 164 clusters. While the two non-Andean samples (Argentina and Uruguay individuals) retrieved clustering of 270,604 and 274,852 reads which produced 131 and 136 clusters respectively, excluding clusters matching to mitochondrial DNA. Since each repetitive DNA category (see later) percentages were practically the same in both non-Andean individuals; data about non-Andean lineage is an average between both analyzed individuals. All repetitive clusters (repeatome) corresponded to 44% and 34% of the total genome in Andean and non-Andean lineages respectively. Clusters could be classified into 6 categories or groups: Long Terminal Repeats (LTR), non Long Terminal Repeats (non-LTR), class II elements or DNA transposons (DNA TEs), satellite DNA (satDNA), ribosomal DNA (rDNA) and undetermined repeats (unclassified) (Fig 1). This last group includes clusters that

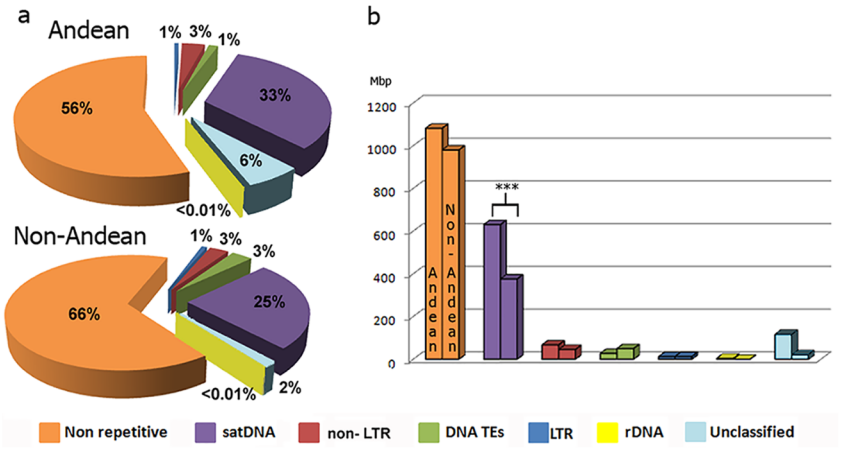

Fig 1. *T. infestans* from Andean and non-Andean lineages repeatomes. (a) Pie charts showing total percentages of each category in the genome, including repetitive and non repetitive DNA. (b) Comparative charts showing the amount of each category expressed in mega base pairs (Mbp) per haploid genome. Chi-square test significant differences are depicted with asterisks ($p < 0.001$).

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could not be assigned to any category. Considering the haploid genome, frequency and amount in Mega base pairs (Mbp) of each category are shown in Fig 1B. Non-repetitive sequences represent 56% and 66% in each genome, equivalents to 1081.92 and 980.31 Mbp per haploid genome, Andean and non-Andean lineages respectively (Fig 1A and 1B). When we developed the combined data analysis on RepeatExplorer among the three samples, the same results were retrieved for the Andean lineage. However, it was successful to identify several satDNA families also present in the non-Andean lineage. These families are depicted on the S1 Table as <0.1% abundance on the genome.

SatDNA is by far the main component of the repetitive DNA in both lineages, being the 33% in the Andean and the 25% in the non-Andean genomes (Fig 1A). The second most common fraction is composed of the non-LTR sequences with a 3% in both genomes (Fig 1A). No substantial variations were observed in richness and kinds of non-LTR between both lineages. Most non-LTR clusters belong to the LOA clade, but other clades detected were: I, CRE, R1, R2, jockey, L2 and Penelope, which are not strictly non-LTR elements but here we considered together to simplify. DNA TEs represents the 1% and 3% in Andean and non-Andean genomes respectively. Within them, *mariners*-like elements constituted the half of them, while helitrons represented about 10% of DNA TEs. Other families found in low percentages were hAT, mutator and PIF-Harbinger. Also a small fraction of DNA TEs were not classified into any family. Lastly LTR elements were scare, representing the 1% of both genomes. Most of them are represented by Gypsy elements, followed by BEL/Pao and copia elements.

We compared by Chi-square test the amount of all genome fractions between both lineages, including non-repetitive DNA. Only satDNA have statistically significant differences ($p<0.001$). Hence, the genome content difference between both lineages is mainly due to the quantity of satDNA fraction (Fig 1B).

Triatoma infestans satellitome composition

RepeatExplorer analysis using the Illumina reads from the Andean and non-Andean samples resulted in the characterization of 42 different satDNA families (S1 Table, Acc. Numbers: KY242402-KY242443). The nomenclature used for each satDNA family was the proposed by Ruiz-Ruano *et al*. [16], which include: species name abbreviation, a number in decreasing abundance order and the repeat unit size. Since there are differences in the amount of each satDNA family between both lineages, the Andean lineage was chosen to designate satDNA family names. So the most abundant family in the Andean lineage was called as TinfSat01-33, the second one as TinfSat02-79 and so on.

Thirty-four of the 42 satDNA families have been found in both lineages. The sequence of each satDNA is conserved between both lineages, with the same consensus sequence. One family was only detected in the Andean lineage and seven only in the non-Andean lineage (S1 Table). The five most frequent satDNA families represent the 94% and the 83% of the total satDNA in Andean and non-Andean lineages respectively. However, the amount of each satDNA family is highly variable between them. For instance, TinfSat01-33 is the main family in the Andean sample (11.77% of the genome), but not in the non-Andean sample (3.55%). In the non-Andean genome the main family is TinfSat02-79, representing a 10.03% of the genome (S1 Table).

There is a great variation in relation with the repeat unit length (4 to 1,000 bp) (S1 Table). However, most of the satDNAs (34 of the 42 families) have a repeat unit below 120 bp. The A+T content in the satDNA families ranging between 55.0 and 81.3%, with the exception of the TinfSat37-314 family (44.6%).

Most of the different satDNA families seem to be non-related. The existence of satDNAs with similar monomer length could indicate that they are related, such as TinfSat37-314 and

TinfSat38-315. Consensus sequence alignment of both satDNAs shows that they have a similarity of 58.5% that could suggest a common origin. Regions with similarity have been also found between satDNA families with different monomer length. So, TinfSat22-64 sequence contains a 23 bp region with a 91% of similarity with the TinfSat09-113 monomer. A more complex relationship could exist between other families, as TinfSat04-1000 and TinfSat42-112, which despite the size differences of the repeat units, share a 104 bp region with an 84% of similarity.

The existence of higher order repeats (HOR) has been observed for other satDNA families, as TinfSat09-113. The analysis of the sequence of this satDNA shows that contains four internal subrepeats (S1 Fig). This satDNA would therefore has originated from a 25 bp satDNA that has diverged during evolution by duplications and probably by short insertions. The existence of HOR has been also observed for other satDNA families, specifically for TinfSat03-4 and TinfSat05-4. Both satDNAs have a 4 bp repeat unit (GATA and CATA respectively) and the majority of the analyzed contigs are formed by a tandem repeat of these simple sequences. However, for TinfSat03-4 it is possible to detect other contigs with new repeat units probably generated from these simple repetitions. So, in Bolivia and Argentina individuals there are tandem repeats with the $(GATAGTTA)_n$ sequence and in Uruguay with $(GATAGATTA)_n$ or (GATAGGTA). For the TinfSat05-4 family a new repeat unit has been detected in Andean as well in non-Andean individuals, $(CAATACATACATACATA)_n$.

An interesting satDNA family is TinfSat04-1000. This satellite DNA contains an internal microsatellite $(CA)_{6\text{-}20}$, so the length of the monomeric repeat is variable in function of the microsatellite repeats number. A similar organization has been recently described for a satDNA family in *Locusta migratoria*, with different size variants due to the number of repeats of a GA microsatellite [16].

Main satDNA families' chromosome location

We have located by FISH eleven satDNA families that were shared between both lineages and that represent more than 0.03% in each genome. Their richness in the genome and chromosome location of each one in both lineages is showed in Table 1, Fig 2 and Fig 3. We have found that the different satDNA families could be located on the heterochromatic regions as well as on euchromatic regions. The hybridization patterns between Andean and non-Andean

Table 1. *Triatoma infestans* satDNA families' quantification in Andean and non-Andean lineages. : All data are expressed in relation to the haploid genome. Abbreviations: A = Autosomes, E = euchromatin, H = heterochromatin, X = euchromatic X chromosome, X* = heterochromatic X chromosome in Andean lineage, Y = Y chromosome. Nucleotide motifs of the 11 satDNA families are included in S1 Table.

SatDNA family	Andean (Mbp)	Non-Andean (Mbp)	Chromosome Localization
TinfSat01-33	228.78	37.76	H: A+X*+Y
TinfSat02-79	174.83	149.63	H: A
TinfSat03-4	88.87	66.29	H: A+X*+Y
TinfSat04-1000	82.86	40.52	E: A+X
TinfSat05-4	27.56	14.15	H: A
TinfSat06-181	4.66	1.42	E: A+X
TinfSat07-10	2.53	0.15	E: A+X
TinfSat08-239	1.55	1.05	E: A+X
TinfSat09-113	1.36	3.35	E: A+X
TinfSat10-53	0.97	0.45	E: A+X
TinfSat11-85	0.58	0.45	E: A+X
TOTAL Mbp	614.53	315.19	

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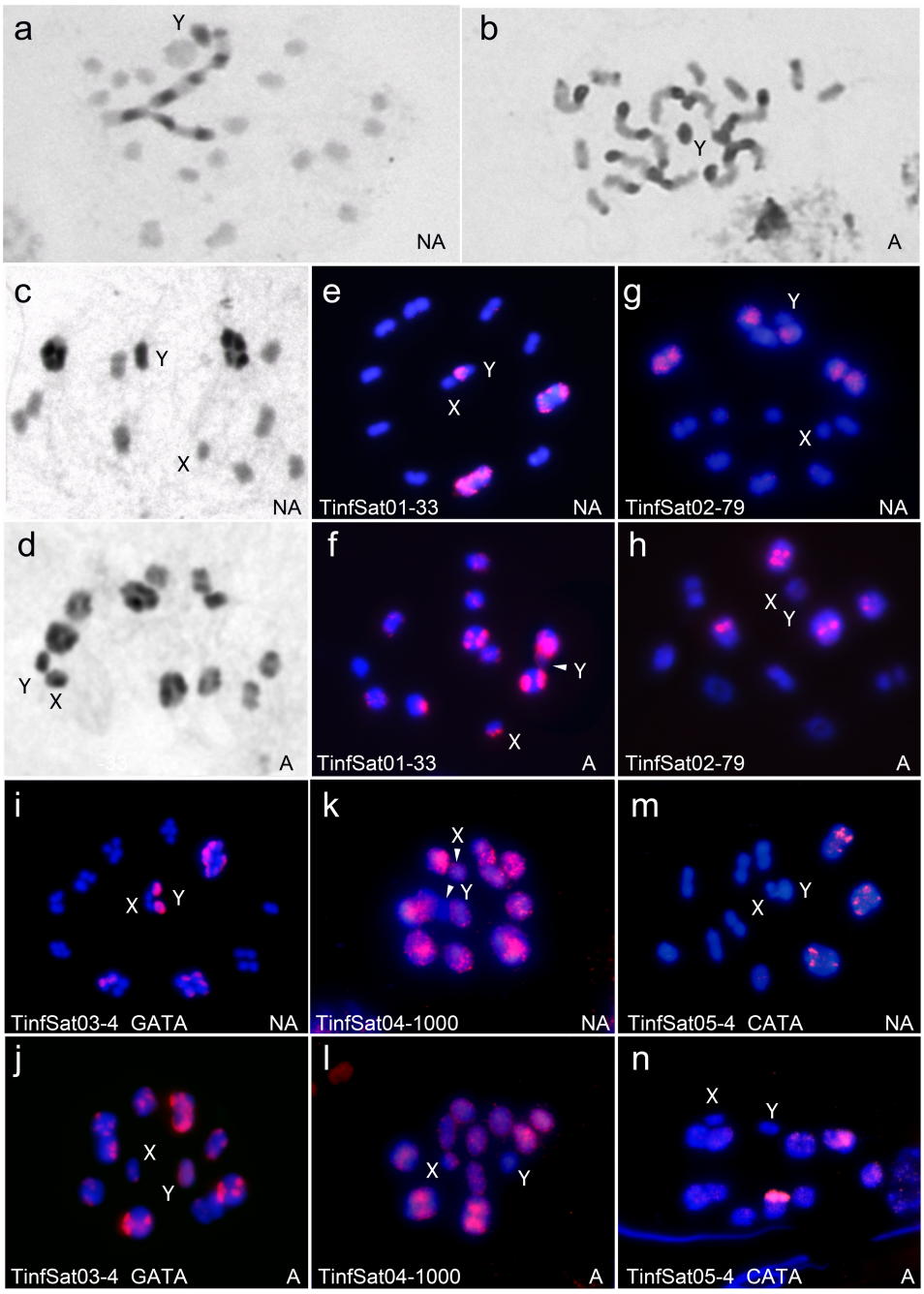

Fig 2. C-banding patterns and satDNA families' hybridization observed in *Triatoma infestans* in non-Andean (NA) and Andean (A) lineages (2n = 20 autosomes plus XY in males). Abbreviations: first meiotic metaphase (MI), anaphase I (AI) and second meiotic metaphase (MII). (a) C-banding. NA lineage (spermatogonial mitotic metaphase): four autosomes and the Y chromosome present C-heterochromatic regions. (b) C-banding. A lineage (spermatogonial metaphase): almost all chromosomes present C-bands in addition to the Y chromosome. (c) C-banding. NA lineage (MI): only two bivalents and the Y chromosome present heterochromatic regions. The X chromosome is euchromatic. (d) C-banding. A lineage (MI): heterochromatic Y and almost all bivalents present C-bands. The X chromosome also shows a heterochromatic block. (e) TinfSat01-33. NA lineage (MI): hybridization signals on heterochromatic regions of two bivalents and the Y chromosome almost entirely. (f) TinfSat01-33. A lineage (MI): hybridization signals on nine bivalents, the Y chromosome and a region of the X chromosome. (g) TinfSat02-79. NA lineage (early AI): Hybridization signals are restricted to heterochromatic regions of three autosomal bivalents. The heterochromatic Y chromosome appears labeled free. (h) TinfSat02-79. A lineage (MI): Hybridization signals on four bivalents. X and Y chromosomes lack

hybridization signals. (i) TinfSat03-4 $(GATA)_n$ repeats. NA lineage (early AI): hybridization signals on heterochromatic regions of three bivalents and the Y chromosome. (j) TinfSat03-4 $(GATA)_n$ repeats. A lineage (MI): hybridization signals on heterochromatic regions of nine bivalents and both sex chromosomes (X and Y). (k) TinfSat04-1000. NA lineage (MI): hybridization signals on euchromatic regions of all bivalents (10) and the X chromosome. The heterochromatic Y chromosome did not display labeling. (l) TinfSat04-1000. A lineage (MI): hybridization signals on euchromatic regions of all bivalents (10) and the euchromatic region of the X chromosome. The heterochromatic region of the autosomes, the X and the Y chromosome did not display labeling. (m) TinfSat05-4 $(CATA)_n$ repeats. NA lineage (MI): hybridization signals on the heterochromatic regions of three bivalents. (n) TinfSat05-4 $(CATA)_n$ repeats. A lineage (MI): hybridization signals on the heterochromatic regions of four bivalents and weak signals in other five bivalents.

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lineages are very different for satDNAs located on the heterochromatic regions. As above commented, non-Andean lineage bears C-heterochromatin in less autosome pairs than the Andean lineage (Fig 2A, 2B, 2C and 2D). As consequence the number of autosomal bivalents that show positive hybridization is greater in the Andean lineage than in the non-Andean lineage (compare Fig 2E with 2F, 2G, 2H, 2I and 2J or 2M and 2N). The hybridization patterns for satDNAs located in the euchromatic regions are more similar between both lineages, since hybridization signals are spread over all chromosomes, less the heterochromatic Y chromosome. We recognized three satDNA family types according to their chromosomal location: (a) satDNA families (TinfSat01-33 and TinfSat03-4) located on C-heterochromatic regions of the autosomes and both sex chromosomes (Fig 2E, 2F, 2I and 2J). (b) satDNA families (TinfSat02-79 and TinfSat05-4) located on the C-heterochromatic regions of the autosomes but not on the Y chromosome (Fig 2G, 2H, 2M and 2N) (c) satDNA families located on the euchromatin regions of the autosomes and the X chromosome (TinfSat04-1000; TinfSat06-180; TinfSat07-10; TinfSat08-239; TinfSat09-113; TinfSat10-53; TinfSat11-85) (Figs 2K, 2L and 3A–3F). As the X chromosome in non-Andean lineage lacks of heterochromatin, no hybridization signals were observed with the satDNA families belonging to the first two types.

Discussion

Total repetitive DNA content (40% mean between both lineages) is in the same range reported for other hemipteran species, as in the pest rice *Nilaparvata lugens* (48.6%) [23] or the pea aphid *Acyrthosiphon pisum* (33.3%) [24]. However, *T. infestans* repeatomes in both lineages are mostly composed by satDNA, representing about the 30% of the total genome in comparison with the 6.4% of *N. lugens*. In this last species as well in the pea aphid, the main repetitive fraction consists in transposable elements (TEs) (39% and 38% respectively) unlike the 5–7% observed in *T. infestans*. Other non-hemipteran insects with holocentric chromosomes as the lepidopteran *Bombyx mori* has in its genome a 43.6% of highly repetitive sequences, also mainly composed by TEs [25].

T. infestans is divided in two main lineages, Andean and non-Andean, with significant differences in their genome size (1.936 Gbp and 1.487 Gbp per haploid genome, respectively) [7,8]. Our results showed that both lineages have similar quantity of non-repetitive sequences, without statistically significant differences (Fig 1). The results obtained with RepeatExplorer showed that *T. infestans* repeatomes in both lineages are mostly composed by satDNA. Furthermore, Andean lineage presents almost twice more satDNA than the non-Andean lineage (630.78 Mbp and 376.05 Mbp per haploid genome, respectively) (Fig 1B), being the main responsible for the genome size differentiation between both lineages. The analysis of this genome fraction shows that satDNA families are conserved between lineages. Thirty-four of the 42 satDNA families described were found in both lineages, one family was only detected in Andean lineage and seven only in non-Andean lineage (S1 Table). One possible hypothesis is

that the differentiation of both lineages has been accompanied also by changes in the satDNA composition. Another hypothesis is that all satDNA families are present in both lineages but some of them have not been detected by the RepeatExplorer analyses. The obtained data support the second hypothesis. First, the exclusive families present very low frequencies and second, one of these families, TinfSat39-5, is the telomeric repeat found in several triatomine species [26]. It is hardly unlikely that telomeric repeats are absent in Andean lineage. So, although satDNA families are the same in both lineages, the reason for such a huge difference in DNA content lies on the amount of these same families in each sample (Table 1, S1 Table). This is in accordance with the "library" hypothesis, which predicts that related species share an ancestral set of different conserved satDNA families, which may be differentially amplified in each species due to stochastic mechanisms of concerted evolution [27,28]. In fact, most of the difference between *T. infestans* lineages is just due to five satDNA families (TinfSat01 to 05), especially the TinfSat01-33 that varies more than six times between lineages (Table 1). FISH results on both *T. infestans* lineages showed four of these families located on the heterochromatin (Table 1, Fig 2). Three of them were previously reported on heterochromatic chromosomes only in Andean lineage by Bardella *et al.* [20]. Their results show that the TinfSat01-33 satDNA is located in the heterochromatic regions in four bivalents and on the X chromosome. The Andean individuals analyzed in this work show hybridization in a greater number of bivalents (Fig 2F). However in the non-Andean lineage, where the number of autosomes with heterochromatic regions is lower, we found hybridization signals only in two bivalents (Fig 2E). We did not find hybridization on the X chromosome; probably due to the fact that the X chromosome lacks heterochromatic regions in non-Andean lineages. A similar pattern were

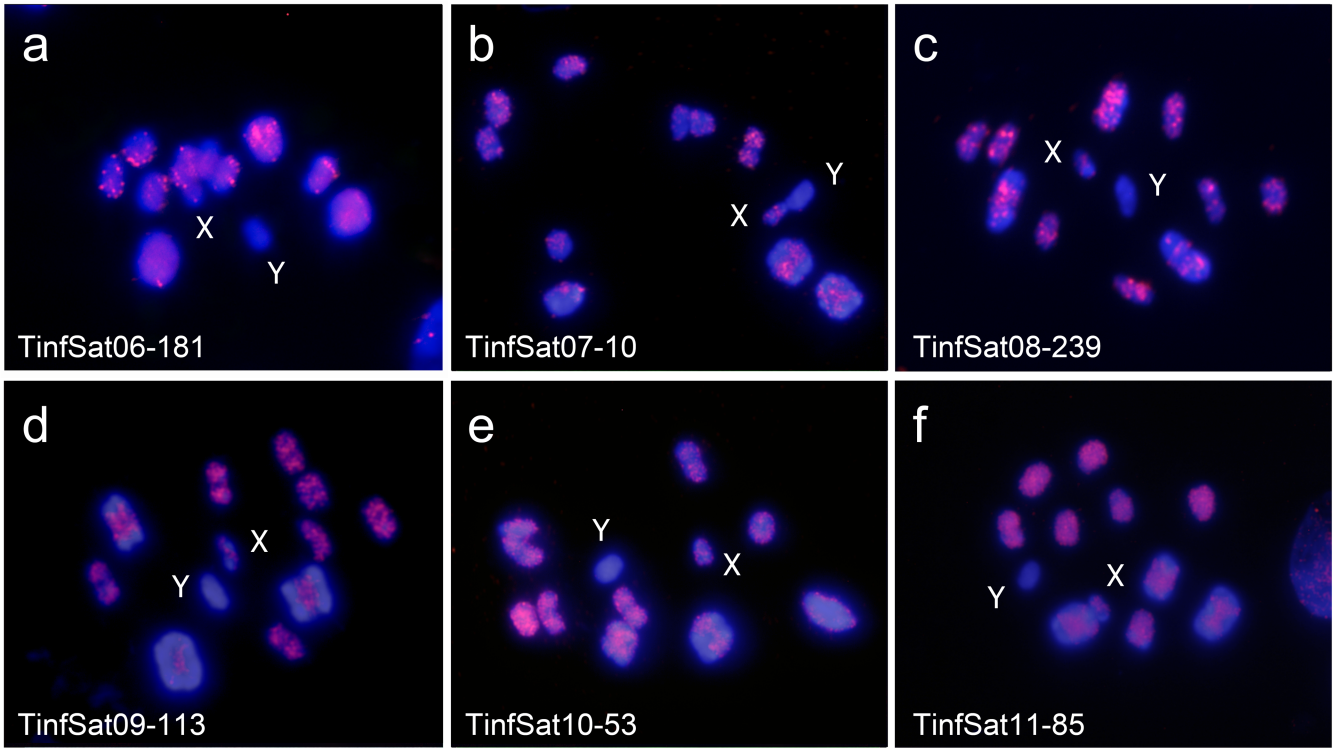

Fig 3. SatDNA families' hybridization observed in *Triatoma infestans* non-Andean lineage. (a) TinfSat06-181; (b) TinfSat07-10; (c) TinfSat08-239; (d) TinfSat09-113; (e) TinfSat10-53; (f) TinfSat11-85: All images in first meiotic metaphase. Hybridization signals on euchromatic regions of all autosomes and X chromosome, while that the autosomal heterochromatic regions and the Y chromosome appeared labeled free.

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observed for the TinfSat02-79 family, that was present in four bivalents in individuals from Andean regions (Fig 2H) and only in three bivalents in non-Andean lineages (Fig 2G). Similar results are also obtained with the TinfSat03-4, were the number of bivalents showing hybridization signals is greater in Andean individuals (Fig 2I) than in non-Andean individuals (Fig 2J). These three satDNA families constitute 984.95 Mbp in the Andean lineage genome and only 507.35 Mbp in the non-Andean lineage genome (Table 1). Hence, genome size differentiation is due to the satDNA, mainly by the higher amount of satDNA families located on heterochromatic regions. Similar conclusion was previously suggested regarding the variation in the number of chromosomes bearing C-bands [9,10].

Previous GISH (genomic *in situ* hybridization) results reported that the heterochromatic Y chromosome is constituted by highly repeated sequences, which are conserved among several species of the Triatomini tribe [29]. Present results show that *T. infestans* Y chromosome harbors two satDNA families: TinfSat01-33 and TinfSat03-4 (GATA repeats). However, unpublished results of our group on other *Triatoma* species have demonstrated that GATA repeat is present on the Y chromosome but not the TinfSat01-33 family. So, GATA repeats would be the only satDNA family that is shared on the Y chromosome in all species of Triatomini tribe. This $(GATA)_n$ motif is reported to be extended on Y or W chromosomes of several vertebrates, including human, mouse and snakes, and invertebrates species [30–32]. Furthermore, $(GATA)_n$ motif (included in Bkm repeats with $(GACA)_n$ motifs) could be required for sex determination and differentiation as well as to participate in the higher order chromatin organization and function, particularly in the formation of heterochromatin [33–35].

Although it is assumed that satDNA is mainly located on the heterochromatin, our data show that there is an important satDNA fraction located on euchromatic chromosomal regions. In *T. infestans*, FISH reveals that seven out of eleven analyzed DNA families are located on euchromatic regions of the autosomes and the X chromosome (Figs 2K, 2L and 3A–3F). These results agree with those obtained by microdissection of the X chromosomes, where repeat sequences of these chromosomes were also located on the autosomal euchromatic regions in several triatomine species, including *T. infestans* [36]. SatDNA characterization by genome sequencing suggests that their presence on the euchromatic regions is not uncommon, as has been reported in some insect species as *Tribolium castaneum* [37] and *Locusta migratoria* [16], as well as in plant species [38]. For these satDNA families it is probable that classical techniques for satDNA isolation fail due to their lower amount in comparison with the families located in the heterochromatin.

Unfortunately, satDNA comparison with the only Triatominae sequenced species, *Rhodnius prolixus*, is not possible, since data about satDNA were not mentioned at all. For other repeat sequences as TEs (including LTR, non-LTR and class II) the total amount is very similar, being the 6% in *R. prolixus* genome [39,40] and 5–7% in *T. infestans*.

Although the possible function of the repetitive DNA is still controversial, the satellite DNA has been related to the reproductive isolation and therefore with the appearance of new species [12]. Its importance in genome integrity and in karyotypic evolution has also been highlighted [19]. The chromosome number in Triatominae is highly conserved, indicating that species differentiation seems not to be accompanied by chromosome rearrangements which alter the chromosome number. Our results show that the main genomic change between both lineages is the variation in the amount of satellite DNA. These changes in the genome composition or organization could be related with the species diversification in Triatominae or other animal species groups with low karyotypic variation.

Supporting information

S1 Table. SatDNA families' complete data of the three sequenced genomes, including genome abundance (%), A+T content (%) and consensus sequences.
(DOCX)

S2 Table. Used primers for satDNA molecular and cytogenetic analyses.
(DOCX)

S1 Fig. Aligment of the four regions of the consensus monomeric unit of the TinfSat09-113 satDNA showing internal similarities that could suggest that this satDNA is really a HOR with four subrepeats.
(DOCX)

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Author Contributions

Conceptualization: Sebastián Pita, Francisco Panzera, Pedro Lorite.

Data curation: Sebastián Pita, Francisco Panzera, Pablo Mora, Jesús Vela, Ángeles Cuadrado, Antonio Sánchez, Pedro Lorite.

Formal analysis: Sebastián Pita, Francisco Panzera, Ángeles Cuadrado, Teresa Palomeque, Pedro Lorite.

Funding acquisition: Francisco Panzera, Antonio Sánchez, Teresa Palomeque, Pedro Lorite.

Investigation: Sebastián Pita, Francisco Panzera, Pablo Mora, Jesús Vela, Ángeles Cuadrado, Antonio Sánchez, Pedro Lorite.

Methodology: Sebastián Pita, Francisco Panzera, Pedro Lorite.

Writing – original draft: Sebastián Pita, Francisco Panzera, Antonio Sánchez, Teresa Palomeque, Pedro Lorite.

Writing – review & editing: Sebastián Pita, Francisco Panzera, Antonio Sánchez, Teresa Palomeque, Pedro Lorite.

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PLOS ONE

RESEARCH ARTICLE

Comparative repeatome analysis on *Triatoma infestans* Andean and Non-Andean lineages, main vector of Chagas disease

Sebastián Pita[1]*, Francisco Panzera[1], Pablo Mora[2], Jesús Vela[2], Ángeles Cuadrado[3], Antonio Sánchez[2], Teresa Palomeque[2], Pedro Lorite[2]

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SUPPLEMENTARY MATERIAL

S1Table: SatDNA families' complete data of the three sequenced genomes, including genome abundance (%),A+T content (%) and consensus sequences.

SatDNA Family	Andean	Non-Andean	A+T	Consensus sequences	Acc. number
TinfSat01-33	11.77	3.54	66.7	TTTCCATAAGTCTATTACTTCGTAATTACTGCG	KY242431
TinfSat02-79	8.99	10.04	59.5	CCCTAAAATCGGCGTTTCAAACGTAAGAGTGAGTTTTCGAGCAACACTCACTTCAGTTGGTTGTAAG GTTCAAGAAAAT	KY242402
TinfSat03-4	4.57	4.48	74.6	GATA (GATAGTTA) (GATAGATTA) (GATAGGTA)	KY242432
TinfSat04-1000	2.48	4.26	66.8	ATCGTCGGAGCCATTTTTGAGAAAATCGCAAAAAAGTAAAAAAAAAACAACTAGTAAAGTGGTACTTC CGGTTGAGGAATTTTGACAGATAACGTACTGTCAGATCCCATGGTGATACCTAAACGGAATATCAAG TTTCAACTTTCTACGGTTTTTCGTTTTTGAGCTATGCTGTTCACATACATACAT-(AC)$_{6-20}$- TTTGCTAAAAACCACTTTTTTGGACTCAGGGGACCTCAAAACGGATATTTCCGGTGAAAACTCGATA TCGAAAATTTGACACGATTACAATACTTCCTCTTACTAGGAGTAAGAGAAAGTAAAAAAAAAAAATC TGGTGTGAAACACTCACACAACTTTCTCTTACTCCAGTTCTCAAAATTATAATTGACAAATTTTATA GTTTGTGTACCAAATTTAATGAAATTCGTCGAAAACTGAAAAAAACTGTTCAGTAAAAGCGCACTTC CGGTTTACTAATTTTCTTGAAACTCGGAATTTTGGCCATCTATTTATTTCTAATTAATTTTGTTTGG ATGATAGAATTGTATAATCAACTATGATAGCACTTTAGAGAAAAACATAACCCCACCCCTCCCCYAA AAGTGCCCTTAAATGAATTTTCCCAGAGAAATGTTTTAAATAAAAGTTGTAGATCTTTGTATGTGTA GTTTATATAGGAAGTTTCAAGAAAATCGTCGGAGCCATTTTTGAGAAAATCGCGAAAAAGTRRAAAA AATCATTTAGTAAAGTGGTAAAGTAAACGCACTTCCGGTTATCCGATTTTTTTCAAACTCGGAATTT CAACAACATATTTAATTCTAATTAACATTATTTAGATGAYAGAATTGTCTAATAAACTGAAATAGCA CTTTAGATGAAAACCTAACCCCACCCCTCCCCCAAAAGTGCCCTTAAATGAATTTTCCCRGAGAAAT GTTTCAAATAAAAGTTGTAGCTCTTTGTATGTGTAGTTGATATACCAAGTTTAAAGAAA	KY242403
TinfSat05-4	1.42	0.94	75	CATA (CAATACATACATACATA)	KY242433
TinfSat06-181	0.23	0.18	75.7	CGGCTCAAAAACAATATTAAAGTCCTTAGAATGTGTTTAGCTAAAATATTATAAGTGTAAACATAAT TTAAACATACATAAAAAAAAAACAAAGTCTTTGTTCATAGTATTGGATGATAATATATTTAATGTTTT TAGCTAAATATTATAAAATATTAGCCCTCGTCTTGGATGTCATCTCA	KY242404
TinfSat07-10	0.13	0.04	57.2	RCATACTCGK	KY242434
TinfSat08-239	0.08	0.07	59	TCCCGCGCGAACCTATTCTAAAAACTAAATTCTCTGAAATAAAAGTAAAAGTAAAGAGTGCCTATTT TTTCAGGCTGACGTAACATTCAACTAACAATTAGGTATCGTGGTAGGGGGCACAAACTCCTACTTTA	KY242405

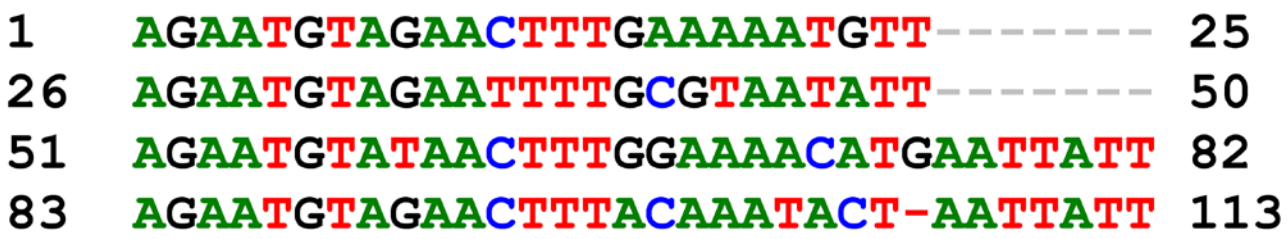
				CTATTAACATTTTCCATACCGATATAAAATTTAGCAACTCGACGCGGGACCCGAACTGGGGACCTCC CGCGTTCGAGTCCATGCTCACAACCACTAATGAAATAA	
TinfSat09-113	0.07	0.22	81.3	AGAATGTAGAACTTTGAAAAATGTTAGAATGTAGAATTTTGCGTAATATTAGAATGTATAACTTTGG AAAACATGAATTATTAGAATGTAGAACTTTACAAATACTAATTATT	KY242406
TinfSat10-53	0.05	0.03	58.5	AACGGTTTTGGTTATACTATTTTTCCAAACCCGCAATACACGTTTGCCCCTTC	KY242407
TinfSat11-85	0.03	0.03	70.6	TAAATGCGTGATTCAGCAATTAACATTCCAAGTTATCAACCACTTTACAGATATTTATAAACCATTT TCCTCTCAAATTGAACAA	KY242408
TinfSat12-84	0.03	0.02	65.5	AAAAAACATATGCGAACACATACAGGCGAGAAGCCATATAAATGTAGTGAATGTGATTACAGTTGTA CACAAtCTGGAAATCTT	KY242409
TinfSat13-147	0.02	0.02	58.5	GTTATCTGGTTGGCTTTTTTCCTGCGTTTCAGATTTTGATTCCTTTTCTGCCGCAGTTTTTGATCCC GCTTGAATGAATGGTGGGATTGGCGGAACATCGTCTATTTCAGATATCGATTTGTCAGCATCTTCAC CTTCATTACTTGA	KY242410
TinfSat14-147	0.02	0.03	63.9	CCAAGAAAGTTCATTTTATGGCGTTTTGGGTGGGGGGTAAGAGGCAAGCTTTTCCCAAAATAGTGGT TTTTATTGTTTAGATTCTCCCACTGATGTAAAATTGAAGACAACTAAACATCTAGTGAGAGAATTAG ACTTTTGAAAGTT	KY242411
TinfSat15-99	0.02	0.01	68.7	TACGTATTGCGTCATACCGTGCAACATGACATGTCTCAACATGTTCATTTTAATTTATTTCTTACTC TTGCTAAAACATCGAAAAAAATACTAGATTTG	KY242412
TinfSat16-49	0.02	<0.01	73.5	GAAGTTGTACAATAGTCAATTAGAATTAACAGAAAATTATGTGAAAGGT	KY242435
TinfSat17-118	0.02	<0.01	60.2	ATCGCAACACTTCCTTTTACTGCTACAACAGAATATAAACTACATGGTATACACCACTTACACCACA TTCAGTGCAGAGGGACCTCATTAGACATGTTTCCTAAATAGCTCAATTCCG	KY242413
TinfSat18-102	0.02	<0.01	62.7	GTCGCGGAAAAAAGTTCCCAATAAAAGTGACGGCATTTGCATTCACAGTTACTGTAGGACGTTTAGT GAAATCCGTTGCCATTTTTACTAAATAAATCAACT	KY242414
TinfSat19-104	0.02	0.01	64.5	GCTTAGTCAGTTCAATTATACATTTAGTTGGCTGAATGGTGAGCAACCTAATGTTTGTATTTATTAG CGAGAATGTGATGATAGGTTAGAGTGTGCTTGTATGG	KY242415
TinfSat20-46	0.02	<0.01	69.6	TCTACACCAGTACATACCTATCATTTTGTTTGAACTTTGTATACTA	KY242436
TinfSat21-38	0.01	<0.01	63.2	AAACACTACTTCCCAAACTTGATGTTTCCTTTAGACAC	KY242437
TinfSat22-64	0.01	<0.01	78.1	AATGTGCAAAATCTAAGTAATATTTATGACAAAGTTCTACATTCTAATACTAATATAAAATGGA	KY242416
TinfSat23-51	0.01	<0.01	66.7	AGATCTATCGCTATTATTTGTTAATGAACCACCCCAACTACAAATATACAC	KY242417
TinfSat24-112	0.01	0.01	58	TCCTGCGATGTTGGGGGGTTCCTCCCCCCGCTCTCTGTAGACATAGTGTAACTACACTATTGGCAGT TATATAGTTTTGTCACAATGAAAATGACTTATCAATTTGATGTTC	KY242418
TinfSat25-62	0.01	0.02	56.5	TTTTCTGCGATTTTCTCCATAACGGTGGACTAGAAGTGCTACTTCACTGGTGAAGTTCTTCG	KY242419
TinfSat26-53	0.01	<0.01	62.3	CAGTAAGAGAAAGTGGGGAAATGTATCACATTCAGTTGGCAAGAACTAAATTG	KY242420
TinfSat27-47	0.01	0.01	61.7	TAGGTAGTGCTAACACCAACAGAATAATTAATATTACCCCACCACTC	KY242438
TinfSat28-46	0.01	<0.01	65.2	ATGTACAGCCGATACAGAAAGTTTAAATAAAGTTGTCGCTCTTTGT	KY242439

TinfSat29-87	0.01	0.01	64.4	GTGTACAATGTGTGGCACTGACACAAAAACATTTTTTGGGGAGTTGTAAAGTAAGGTTGCTTTACAC TTACCTCTAAAGTATTACTT	KY242421
TinfSat30-58	0.01	<0.01	65.5	TAATCTGAGTTGGAACTATCCATTGTAGTGAACCACATATTTGTTACTATCAGCGTAT	KY242422
TinfSat31-71	0.01		70.4	CTAGACTGTATTTAGTTTGTAAAAATATTTTTGTCTGTTGGTCTTGGCCACGTATATAATTATTCGT TTGT	KY242423
TinfSat32-52	0.01	0.01	59.6	CACCTCCTACAGCACTAAGAGAAAGTCGTGTGATTACACATCAGATTTGTAT	KY242424
TinfSat33-372	0.01	0.02	55.8	TAGTGCTGCGTTTGTGCCGGCGATTCACCAAATTTCATCGTTTGTGCCGTAAAATTGACAAAGATAT TAAGGAAAAACTGTTCGTCGTACTTGAAAACCAAAGGCCATGGCTGCGAGTAACATTTGAATGTGCG CGTGCTCCACTGACCGCCAACGGCCCTTATCAGGGCCAGCAACTAAAATGTACTGGCCCTCGATTTC TAATCAAAATTCTGGAATTTTTTCTTCGCTATTCTTAAGTGTTTCATTTGTGCCAGCGACCCACCAA ATTTCAGCGTTTGTGCTGCAAAATTGACAAAGTTATTGAGGCGTCAAGTTAGACTGGCCACCAATTT CTATTCACTTTTTTAAACTTTTCTCTTTGCCAATCGT	KY242425
TinfSat34-28	0.01	0.02	60.7	TACACCTACATTATAGTACACCTTCTGG	KY242440
TinfSat35-35	0.01	0.01	68.6	ACTCATAGGTAGATGATTTAAAACAAAACACCAGT	KY242441
TinfSat36-10	--	0.06	55	GAGTATGTMC	KY242442
TinfSat37-314	--	0.04	44.6	GCATATATATGGCTAGCCAGAGTGGTCGTGGGCTGAAAGCCCGCTTAAAACTGGCTGATTGGGCTAC TGAGGTTCAATCTGCTGACTATGCCATCACTGCCGGGGCGGCGGTGCCGGAGAAATCGGCGATTAAG GTCGATAACACCACCTATACGGCAGGTAATGACATGACCGTTAGTGTGACGCTGAAGGATGCGCAGG GTAATGGGGTTACTGGCCAAGCTGCTGCCTTAACGTCCCAGGCGGTAACGGTGGCCAATGCCAGTGA GAAAGACGGTGTCACGTGGATCGATAACGGCGAGGCACCTATAGCC	KY242426
TinfSat38-315	--	0.03	61.6	ACAATTTATAAGGCCGAGACTGTAGGTGAAAACCTGAAAGCGACTGTAAAGCTTCCAGATTGGAAGG GTACAACTGAATCGGCTAATTACGCAATTACTACCGGTAACCCTGCGTATATGAAGTCAACGATTAC GTTAGATAAAGACATCTATCCCGCAAATAGTGATATGAAGGTTACGGTAACCTTGAAAGATTTGTAT GGTAATAGCGTAACTGGAAAAGTTGCAGAATTAACTGATGCGGTAGTAACAGTACCAAATGCAAAAA TGAAAATAGGTGAAAAATGGAAAGAAAATGAAGCTGGGACCTATACT	KY242427
TinfSat39-5	--	0.03	60	TTAGG	KY242443
TinfSat40-73	--	0.02	60.3	CCATTTGGGAAACAAGTCGGGCCACCAATACATAGTGAACTAGTAACAAAACGGAACGTTGAAGATT TTGTAA	KY242430
TinfSat41-101	--	0.01	67.3	AATATATTACAGTGAACACCTATCCTGCGCTGTACTAATGACAAGTTCGAAATAGATGTCTAACAAA ATTGTAAAAGAGCTGGGTCTAAAATTATAGCAAT	KY242428
TinfSat42-112	--	0.01	64.5	GACACTAAGTTATCTGTCAAAATTCCACAACCGGAACAAACACAACCACAACAAAAACAAAAATCGC AGAAAATTCAAACTTGACATTCTGTTTAGGTATCACCGTGGGTTC	KY242429

S2Table: Used primers for satDNA molecular and cytogenetic analyses.

SatDNA Family	Oligonucleotides	
TinfSat01-33	TinfSat01-33 5´- TTTCCATAAGTCTATTACTTCGTAATTACTGCG	
TinfSat02-79	TinfSat02-79-F 5´- TTGTAAGGTTCAAGAAAATCCC	TinfSat02-79-R 5´- CTCACTCTTACGGTTGAAACGC
TinfSat03-4	(GATA)5 5´ - GATAGATAGATAGATAGATA	
TinfSat04-1000	TinfSat04-1000-F 5'- GATATCGAAAATTTGACACG	TinfSat04-1000-R 5'- ATGTATGTGAACAGCATAGC
TinfSat05-4	(CATA)5 5´- CATACATACATACATACATA	
TinfSat06-181	TinfSat06-181 5´- CGGCTCAAAAACAATATTAAAGTCC	
TinfSat07-10	TinfSat07-10 5´- rCATACTCGkrCATACTCGk	
TinfSat08-239	TinfSat08-239-F 5´- GTTCGAGTCCATGCTCAC	TinfSat08-239-R 5´- AATTTTTAGAATAGGTTCGC
TinfSat09-113	TinfSat09-113 5´- AGAATGTAkAACTTTG	
TinfSat10-53	TinfSat10-53-F 5´- CGGTTTTGGTTATACTATTTTTCCA	TinfSat10-53-R 5´- GAAGGGGCAAACGTGTATT
TinfSat11-85	TinfSat11-85-F 5´- CCATTTTCCTCTCAAATTGAAC	TinfSat11-85-R 5´- ATGTTAATTGCTGAATCACGC

S1Figure: Aligment of the four regions of the consensus monomeric unit of the TinfSat09-113 satDNA showing internal similarities that could suggest that this satDNA is really a HOR with four subrepeats.



Capítulo V:

Secuencias teloméricas

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RESEARCH ARTICLE

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The presence of the ancestral insect telomeric motif in kissing bugs (Triatominae) rules out the hypothesis of its loss in evolutionarily advanced Heteroptera (Cimicomorpha)

Sebastián Pita[1], Francisco Panzera[1], Pablo Mora[2], Jesús Vela[2], Teresa Palomeque[2], Pedro Lorite[2]

1 *Sección Genética Evolutiva, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay*
2 *Departamento de Biología Experimental, Área de Genética, Universidad de Jaén, Jaen, Spain*

Corresponding author: *Pedro Lorite* (plorite@ujaen.es)

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Abstract

Next-generation sequencing data analysis on *Triatoma infestans* Klug, 1834 (Heteroptera, Cimicomorpha, Reduviidae) revealed the presence of the ancestral insect $(TTAGG)_n$ telomeric motif in its genome. Fluorescence *in situ* hybridization confirms that chromosomes bear this telomeric sequence in their chromosomal ends. Furthermore, motif amount estimation was about 0.03% of the total genome, so that the average telomere length in each chromosomal end is almost 18 kb long. We also detected the presence of $(TTAGG)_n$ telomeric repeat in mitotic and meiotic chromosomes in other three species of Triatominae: *Triatoma dimidiata* Latreille, 1811, *Dipetalogaster maxima* Uhler, 1894, and *Rhodnius prolixus* Ståhl, 1859. This is the first report of the $(TTAGG)_n$ telomeric repeat in the infraorder Cimicomorpha, contradicting the currently accepted hypothesis that evolutionarily recent heteropterans lack this ancestral insect telomeric sequence.

Keywords

Cimicomorpha, kissing bugs, holocentric chromosomes, telomeres, NGS, $(TTAGG)_n$

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Introduction

Telomeres, the physical ends of eukaryote chromosomes, are defined as specialized DNA-protein structures essential for chromosome replication, meiotic pairing and chromosome stability. In most organisms, telomeric DNA is composed by simple G-rich sequences repeats that extend for tens of base pairs (bp) as much as 150 kb, depending on the organism. Although telomeric repeats are diverse in their DNA sequence composition among different organisms (Zakian 1995), several taxonomic groups possess highly conserved motifs. Vertebrates, including bony fishes, reptiles, amphibians, and mammals exhibit the $(TTAGGG)_n$ repeat (Meyne et al. 1989) while the $(TTTAGGG)_n$ sequence appears highly conserved in the plant kingdom (Watson and Riha 2010). Extensive studies in arthropods have revealed that the predominant telomeric sequence is a pentanucleotide sequence repeat $(TTAGG)_n$, which has been considered as the ancestral telomeric motif in phylum Arthropoda, including insects (Sahara et al. 1999, Frydrychová et al. 2004, Vítková et al. 2005). However, numerous studies contradict this claim. For example several insect groups do not exhibit this telomeric repeat, such as Diptera, Ephemeroptera, Odonata, Dermaptera, Siphonaptera, Mecoptera, Raphidioptera and parasitic Hymenoptera. In addition, Coleoptera, Neuroptera and Hemiptera orders include species with and without the ancestral $(TTAGG)_n$ telomeric motif (Frydrychová et al. 2004, Gokhman et al. 2014, Korandová et al. 2014). In these insect groups, the ancestral telomeric motif is replaced by other alternative telomeric sequences such as $(TCAGG)_n$ in some coleopteran species (Mravinac et al. 2011), non-long terminal repeat (LTR) retrotransposons in *Drosophila* Fallén, 1823 (Mason et al. 2008), arrays of long satellite repeats in Culicomorpha dipteran (Walter et al. 2001), or by unknown sequences as in damselflies, mayflies and some aphid species (Frydrychová et al. 2004, Vítková et al. 2005). The most illustrative example of the variability of the telomeric sequences was observed in Coleoptera where ancestral $(TTAGG)_n$ has been lost at least eight times during the evolution of this insect group (Frydrychová and Marec 2002, Mravinac et al. 2011).

Among Hemiptera, the ancestral motif is present in the suborder Sternorrhyncha (coccids and aphids with some exceptions) (Mohan et al. 2011, Monti et al. 2011, Novotná et al. 2011), in several genera of Auchenorrhyncha (Frydrychová et al. 2004, Maryańska-Nadachowska et al. 2013, Golub et al. 2014, Kuznetsova et al. 2015a) and Coleorrhyncha (Kuznetsova et al. 2015b) suborders. In the suborder Heteroptera, only two species of the basal infraorders Nepomorpha and Gerromorpha show the ancestral telomeric motif (Kuznetsova et al. 2012, Mason et al. 2016). On the contrary, the most derived and specious heteropteran infraorders (Cimicomorpha and Pentatomomorpha) do not show the classic insect motif (for review see Grozeva et al. 2015, Mason et al. 2016). A recent survey of several sequenced genomes of these groups, including the triatomine *Rhodnius prolixus*, confirms the lack of the ancestral telomeric repeat and these groups are regarded as having a defective version of telomerase gene (Mason et al. 2016). Mason et al. (2016) have suggested the occurrence of a single loss event of the telomeric repeat, sometime before the Cimicomorpha and Pentatomomorpha divergence, and after their separation from Nepomorpha.

Kissing bugs (Triatominae, Reduviidae) are included within the infraorder Cimicomorpha (Heteroptera), constituting a group of medical relevance because they act as vectors of Chagas disease, also known as American trypanosomiasis. This subfamily includes 150 species, of which more than 80 have been cytogenetically studied (Panzera et al. 2010), having holocentric chromosomes. The current data, as above mentioned, suggest a high heterogeneity in insect telomere composition. One should also take into consideration that loss of the insect ancestral repeat in Cimicomorpha has been reported (Mason et al. 2016). For all these reasons it is important to explore for the first time in Triatominae the presence of $(TTAGG)_n$ motif, using next-generation sequencing (NGS) analysis tools and fluorescence *in situ* hybridization (FISH) in four triatomine species from three different genera. The results presented in this paper are in clear contradiction to the loss of ancestral telomeric repeats hypothesis in evolutionarily advanced Heteroptera.

Materials and methods

Material

Four species where analyzed, involving three different genera from the two principal tribes of the subfamily: Triatomini (*Dipetalogaster maxima*, *Triatoma infestans*, and *T. dimidiata*) and Rhodniini (*Rhodnius prolixus*). The last three species are the main vectors of Chagas disease. Origin and cytogenetic traits of each species are detailed in Table 1.

Telomere detection by genome sequencing

A *Triatoma infestans* (non-Andean lineage) specimen collected in Tacuarembó (Uruguay) was used for sequencing. Approximately 3 μg of genomic DNA were employed in a low coverage Illumina® Hiseq™ 2000 paired-end sequencing. Graph-based clustering analysis was carried out using RepeatExplorer (Novák et al. 2013), implemented within the Galaxy environment (http://repeatexplorer.umbr.cas.cz/) (Novák et al. 2010). RepeatExplorer also allow quantifying the abundance of the repeated sequences in the genome in base to the number of reads in each cluster.

Telomere detection by FISH

Chromosome preparations for FISH analyses were obtained from male gonads. Testes were removed from live adult insects, fixed in an ethanol–glacial acetic acid mixture (3:1) and stored at -20°C. Squashes were made in a 50% acetic acid drop, coverslips were removed after freezing in liquid nitrogen and the slides were air dried and then stored a 4°C.

Table 1. Geographical origin and male diploid chromosome number in the four species here analyzed. A = autosomes.

Species	Geographical origin	Male diploid chromosome number (2n)
Tribe *Rhodniini*		
Rhodnius prolixus	Guatemala, Quezaltenango, Insectary CDC (USA)	22= 20A + XY
Tribe *Triatomini*		
Dipetalogaster maxima	Baja California, Mexico	22= 20A + XY
Triatoma dimidiata	Jutiapa, Guatemala	23= 20A + X_1X_2Y
Triatoma infestans	Tacuarembó, Uruguay	22= 20A + XY

Telomeric TTAGG probe generation and FISH assays were carried out following Lorite et al. (2002) and Mora et al. (2015). Telomeric probes were generated by PCR using the primers $(TTAGG)_6$ and $(TAACC)_6$, following a similar procedure as described by IJdo et al. (1991). PCR was performed in 100 µl using 100 pmol of each primer and 2.5 units of Taq polymerase, in the absence of a template. PCRs were carried out using the following cycling profile: 30 cycles at 95°C (60 sec), 50°C (1 min), 72°C (3 min), with a final elongation step of 72°C for 10 min. PCR generated fragments (between 200 bp and 1 kb) were purified and labeled with biotin-16-dUTP (Roche) out using the Nick Translation Kit (Roche), following manufacturer's instructions. The labelled probe was precipitated and dissolved in 50% formamide.

Previously to hybridization, slides were treated with RNase A, pepsin and formaldehyde and dehydrated in 70%, 90% and 100% ethanol for 5 min each. Hybridization was performed applying 25 µl of DNA labelled solution to each slide, which was heated for 3 min at 80°C to denature the DNA, and immediately chilled on ice for 3 min. The hybridization mix consisted of (final concentrations) 50% formamide, 2xSSC, 50 mM sodium phosphate, 0.1 mg/ml sonicated salmon sperm DNA, 0.1 mg/ml yeast RNA, and 5 ng/ml labeled telomere probe. The slides were transferred to a moist chamber humidified with formamide (50%) and incubated overnight at 37°C. After incubation, the slides were washed in 50% formamide at 37°C, three times, 3 min each; followed by 2xSSC, 0.05% Tween-20, pH 7.5, three times, 5 min each. Fluorescence immunological detection was performed using the avidin-FICT/ anti-avidin-biotin system with four rounds of amplification. Slides were mounted with Vectashield (Vector). DAPI in the antifade solution was used to counterstain chromosomes.

Results and discussion

The data obtained from the *T. infestans* genome sequencing were analyzed with RepeatExplorer (Novák et al. 2013). One of the obtained clusters was formed by a telomeric sequence TTAGG array. In order to test if this repeat represents the putative telomere, FISH was carried out using the TTAGG repeat as probe. Hybridization signals were clearly seen at the ends of the mitotic chromosomes (Fig. 1A), revealing that telomeres in this species are really composed by this ancestral insect motif. The cluster of

the $(TTAGG)_n$ sequences was estimated for about 0.0266% of the total genome size, i.e. 395.5 kb. Considering that the haploid genome content in *T. infestans* is 1.52 pg (1.487 Mb) (Panzera et al. 2007, 2010) and that the chromosome number is 2n=22, the average telomeres length motifs in each chromosome end would be almost 18 kb long. This value is in the range of the telomere length observed in other insects with the ancestral motif or a variant of this repeat such as *Tenebrio molitor* Linnaeus, 1758 (15 kb) (Richards et al. 2008) but higher than the observed in other species with holocentric chromosomes as lepidopteran species (6-9 kb) (Okazaki et al. 1993, Mandrioli 2002), or in the homopteran coccid *Planococcus lilacinus* Cockerell, 1905 (6.4 kb) (Mohan et al. 2011).

Furthermore, we tested the telomeric motif presence by FISH in other three triatomine species with $(TTAGG)_n$ probe. Hybridization signals were clearly seen on the chromosomal ends of mitotic and meiotic chromosomes (Fig. 1B–D), revealing that Triatominae telomeres are composed by the ancestral insect motif. FISH technique in triatomines is highly sensitive to material fixation conditions. Cytoplasmic remnants in the slides represent the greatest challenge because it hinders the access of the telomeric probes to the chromosomes. This can be partially avoided using recently extracted gonads. In addition, access of the telomeric probes to the chromosome and its visualization are very sensitive to the chromosomes being on the same plane. As a result, differences in hybridization signals can be observed in the same slide or even within chromosomes of the same cell (Fig. 1).

Given our positive FISH hybridization results on *R. prolixus* chromosomes, we additionally conducted a BLAST search of telomeric sequences in the published genome of this species, available at https://www.vectorbase.org/. Similar as reported by Mason et al. (2016), we did not find $(TTAGG)_n$ repeats, so that these tandem sequences and probably others repeated sequences are not included in the published genome of *R. prolixus* (Mesquita et al. 2015). This reveals the difficulty of the repetitive DNA fraction assembly, as has been reported in different organisms including the well-studied human genome, making that many repetitive sequences have been omitted from the reference assembly and from most genome-wide analyses (Altemose et al. 2014).

Heteroptera or true bugs are a hemipteran suborder comprising seven infraorders and 40,000 species. All phylogenetic studies agreed that the infraorders Cimicomorpha and Pentatomomorpha are the most evolutionarily derived groups, with a common ancestor and involving about 80% of heteropteran species (Weirauch and Schuh 2011). Until now, the detection by FISH, Southern and/or dot-blot hybridization of telomeric repeat motif $(TTAGG)_n$ in Heteroptera has been unsuccessful in nine genera from five families of the infraorders Cimicomorpha and Pentatomomorpha (Sahara et al. 1999, Kuznetsova et al. 2011, Frydrychová et al. 2004, Grozeva et al. 2011, Golub et al. 2015). Only two heteropteran species from the basal infraorders Nepomorpha and Gerromorpha exhibit the ancestral telomeric motif (Kuznetsova et al. 2012, Mason et al. 2016). The $(TTAGG)_n$ motif was suggested to be lost in the early evolution being and secondarily replaced by another motif or an alternative telomerase-independent mechanism of telomere maintenance (Frydrychová et al. 2004, Lukhtanov and Kuznetsova 2010). Although several authors have suggested the loss of TTAGG repeat in all

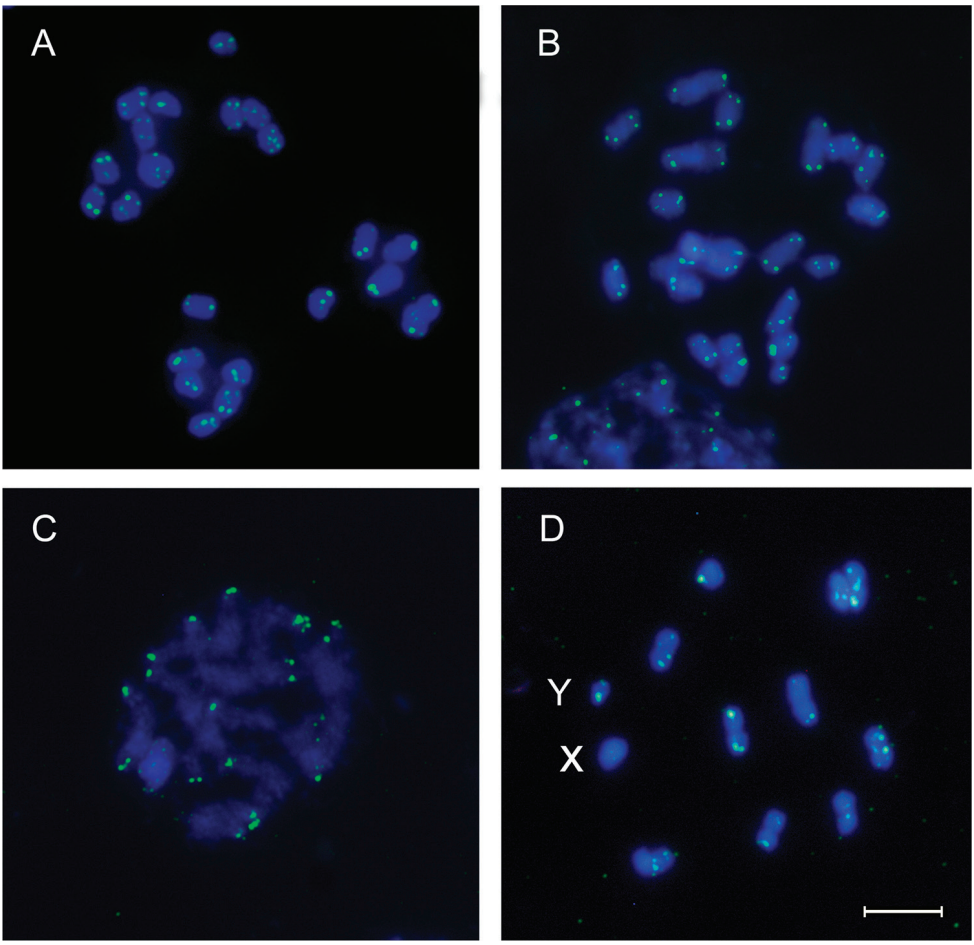

Figure 1. Fluorescence *in situ* hybridization with $(TTAGG)_n$ telomeric probe (green signals) on mitotic and meiotic chromosomes (counterstained with DAPI in blue) of four Triatominae species. **A** *Triatoma infestans* (2n=22), spermatogonial prometaphase **B** *Triatoma dimidiata* (2n=23), spermatogonial prometaphase **C** *Dipetalogaster maxima* (2n=22), pachytene stage **D** *Rhodnius prolixus* (2n=22), first meiotic division showing 10 bivalents and two sex chromosomes (X and Y). Scale bar: 5 μm.

Cimicomorpha species (Grozeva et al. 2015, Mason et al. 2016), the results presented here clearly contradict this hypothesis. According to the most comprehensive phylogeny of assassin bugs, the subfamily Triatominae is the youngest within Reduviidae, having evolved in the Oligocene, approximately 32 million years ago (24–38 Ma) (Hwang and Weirauch 2012). Whereas, a new acquisition of telomeric repeat in this recent evolutionary group seems unlikely, probably this lack of detection in Cimicomorpha and Pentatomomorpha is due to a methodological problem of the telomeric probe rather than a loss process during their evolution. Detailed analyses of the genomes repetitive fraction as well as exhaustive bioinformatics search on genomic databases might clarify the existence of these repeat sequences in other heteropteran groups.

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Discusión

A nivel citogenético los triatominos presentan cromosomas holocéntricos caracterizados por presentar un centrómero no localizado o difuso. La falta de constricción primaria, el pequeño tamaño cromosómico y su homogeneidad en el número cromosómico (entre 21 y 25 cromosomas) dificultan considerablemente la detección de reordenamientos cromosómicos y el análisis de la evolución cariotípica en estos insectos. Sin embargo nuestro grupo ha logrado revelar, por medio de diferentes abordajes metodológicos, una extensa variabilidad en los genomas de los triatominos. Esta variabilidad se refleja en la localización, tamaño y cantidad de regiones de heterocromatina constitutiva (Panzera et al. 2010) y en la localización cromosómica de las regiones del organizador nucleolar (NOR) que contienen los genes ribosomales 45S (Panzera et al. 2012; Pita et al. 2013).

Capítulo I. Genes ribosomales y otras familias multigénicas

En la presente tesis, hemos ampliado el análisis sobre la localización cromosómica de las regiones NOR de 71 a 89 especies, incluyendo también a nuevas poblaciones de especies ya conocidas, especificadas en el Anexo 1. Parte de los resultados obtenidos han sido publicados en 3 artículos (Panzera et al. 2015; Dujardin et al. 2015; Pita et al. 2016a). El análisis de esta gran cantidad de especies y poblaciones reafirmó las conclusiones previamente sugeridas en anteriores publicaciones de nuestro grupo: *i*) exceptuando a *T. infestans,* todos las especies de triatominos presentan las regiones NOR en uno o dos loci con cuatro patrones de localización cromosómica: en un par autosómico, en el cromosoma X, en dos cromosomas sexuales o en un par autosómico y el cromosoma X; *ii*) el cariotipo ancestral de la familia constaría de 20 pares autosómicos y un sistema sexual simple XX/XY con los NORs localizados en un par autosómico; *iii*) la localización de las regiones NOR no estaría relacionada con variaciones en el número de autosomas o de sexuales, ni tampoco aparece preferencialmente asociada con la heterocromatina constitutiva. Además los nuevos datos nos han permitido sugerir que la localización de las regiones NOR puede ser una herramienta valiosa para establecer relaciones de parentesco entre distintas especies (Pita et al. 2016a). Especies evolutivamente próximas presentan en general una misma localización cromosómica de los genes ribosomales. Por este motivo hemos utilizado a este marcador para reordenar la integración de las especies del género *Triatoma* en varios subcomplejos, contrastando dichos agrupamientos con datos de secuencias, tanto nucleares como mitocondriales. Las modificaciones comprendieron a los subcomplejos Maculata, Matogrossensis, Rubrovaria y Sordida (Pita et al. 2016a). Por otra parte, análisis del ADNr 45S junto con la caracterización de la heterocromatina constitutiva nos ha permitido identificar especies crípticas en poblaciones de *T. sordida, T. maculata y Panstrongylus rufotuberculatus*, habiendo publicado hasta ahora sólo las variaciones encontradas en *T. sordida* (Panzera et al. 2015). En este trabajo hemos propuesto que en lo que hasta ahora era considera una sola especie en realidad involucra al menos tres especies diferentes: *T. sordida Brasil, T. sordida Argentina* y *T. sordida Bolivia*, las cuales presentan distinta incidencia como vectores de la Enfermedad de Chagas.

Por último, el análisis de la evolución cariotípica de la localización de los NORs nos llevó a sugerir que su transferencia desde un autosoma hacia uno o dos cromosomas sexuales crea rápidamente una barrera reproductiva, ya que

involucra cambios en la regulación génica, en las tasas de recombinación y en los efectos de la selección natural. Además hemos planteado que la localización de los genes ribosomales en los cromosomas sexuales no es sólo un carácter derivado sino que también sería un estado de "*dead end*". Basados en reconstrucciones filogenéticas, los episodios de cambio del patrón autosómico hacia los cromosomas sexuales ocurren siempre en esta dirección y no a la inversa, ya que no se ha observado en ningún grupo de triatominos (Pita et al. 2016a).

Durante estos últimos años hemos logrado definir un modelo de evolución cariotípica para el ADNr 45S, el cual incluye una hipótesis de ancestralidad autosómica, la existencia de diferentes tasas de cambios en los distintos grupos de triatominos, y sugerir cuáles serían los mecanismos más probables para la transferencia de los genes ribosomales (Panzera et al. 2012; Panzera et al. 2015; Pita et al. 2013; Pita et al. 2016a). Sin embargo, todavía quedan tribus y/o géneros completos de las cuales no existe ningún tipo de información citogenética y que podrían ser importantes para poseer un panorama más amplio de la evolución cariotípica de toda la subfamilia.

Uno de los objetivos planteados inicialmente en esta tesis fue el análisis mediante FISH de nuevas familias génicas. Si bien logramos el aislamiento por PCR de las secuencias de los genes ADNr 5S y de la histona H4, en los ensayos de FISH no se obtuvieron resultados positivos. Probablemente, ésta pueda deberse a un bajo número de copias de estos genes en los clústeres que integran y/o que éstos se encuentren muy dispersos en el genoma, por debajo del nivel de resolución del microscopio óptico. Recientemente, se ha publicado el análisis de éstas y otras familias como los *small nuclear DNA U2* en heterópteros de las familias Coreidae y Pentatomidae, ambas del infraorden Pentatomomorpha (Bardella et al. 2016a). En colaboración con este grupo tampoco hemos tenido resultados positivos sobre estas familias multigénicas en triatominos.

Capítulo II. Diferenciación de los cromosomas sexuales

Uno de los objetivos iniciales de esta Tesis era determinar el grado de diferenciación de los cromosomas sexuales X e Y entre sí y con los autosomas usando para ello sondas obtenidas por microdisección cromosómica de ambos cromosomas sexuales: X e Y. Sin embargo, a pesar de los numerosos intentos de obtener sondas de los cromosomas Y no fue posible lograrlas. Sin embargo obtuvimos sondas de los cromosomas X_1 y X_2 de *T.* (*Mepraia*) *spinolai*, las cuales hibridamos sobre cuatro especies de Triatomini y sobre *R. prolixus* (Rhodniini) (Pita et al. 2017a). Los ensayos de FISH sobre las 4 especies de Triatomini mostraron que la sonda de los cromosomas Xs hibridaba exclusivamente sobre eucromatina, tanto de los autosomas como de los cromosomas sexuales. Las regiones de heterocromatina del cromosoma Y así como la de los autosomas siempre aparecen libres de marcaje. Los resultados obtenidos sobre los cromosomas de *R. prolixus* son completamente distintos de los observados en las especies de Triatomini. Las señales de hibridación son pequeñas y están dispersas sobre toda la cromatina, no mostrando marcación preferencial sobre ninguna región cromosómica incluyendo a los propios cromosomas X. Estos resultados concuerdan con los obtenidos por *Genomic in situ Hybridization* (GISH) (ver más adelante) (Pita et al. 2014; Pita et al. 2017b) y bandeo fluorescente (Bardella et al. 2016b), los cuales sugieren una clara diferenciación en la composición de secuencias repetidas de ambos cromosomas sexuales X e Y en la tribu Triatomini. Además, en este trabajo publicado, demostramos una diferenciación del cromosoma Y entre las tribus Triatomini y Rhodniini. Por último, nuestros resultados también apoyan que cada cromosoma sexual en Triatominae ha evolucionado de forma

independiente a partir de diferentes pares autosómicos, similar a lo descrito en otros grupos de insectos. Hasta ahora, no existían datos sobre la conformación de las secuencias de los cromosomas X en Triatominae, ni tampoco en otras especies del orden Hemiptera.

Capítulo III. Evolución cariotípica de las secuencias de ADN repetido

Con el objetivo de analizar la evolución cariotípica de la subfamilia Triatominae, el proyecto de Tesis originalmente planteado suponía el análisis comparativo de secuencias de ADN repetido entre distintas especies utilizando la técnica de GISH. Se elaboraron 6 sondas genómicas de 4 especies distintas: *T. infestans* (linajes Andino y no-Andino), *T. delpontei* (macho y hembra), *T. dimidiata* y *T. rubrofasciata*. Estas sondas fueron hibridadas sobre sus propios cromosomas (*self*-GISH) y sobre una docena de especies incluidas en seis géneros diferentes pertenecientes a las dos principales tribus de la subfamilia: Triatomini y Rhodniini.

De estos análisis resultaron 2 publicaciones (Pita et al. 2014; Pita et al. 2017b), cuyas principales conclusiones resumimos a continuación:

- Las secuencias de ADN repetido en las especies estudiadas de *Triatoma* poseen dos formas de distribución: dispersa y clusterizada.
- El cromosoma Y heterocromático está constituido principalmente por secuencias de ADN repetido, compartidas en todas las especies de la tribu Triatomini, por lo sugerimos que se trata de un carácter ancestral de este grupo.
- Las regiones autosómicas heterocromáticas están constituidas por secuencias compartidas (probablemente ADNsat) entre especies de *Triatoma* cercanas, pero son diferentes entre especies filogenéticamente más distantes. Estos resultados sugieren que los procesos de especiación en el género *Triatoma* ha involucrado la amplificación de distintos tipos de repetidos autosómicos.

Capítulo IV. Determinación del repeatoma de *T. infestans*

El último objetivo específico inicialmente planteado en esta Tesis era la búsqueda, identificación y mapeo cromosómico de secuencias de ADN repetido (elementos transponibles y familias génicas) mediante *Fluorescence in situ hybridization* (FISH). Sin embargo considerando los resultados obtenidos con las otras técnicas, creímos que la mejor estrategia para cumplir con nuestro objetivo general de analizar la evolución cariotípica de la subfamilia Triatominae, era caracterizar la totalidad del ADN repetido en un genoma (repeatoma) y la localización cromosómica de las principales secuencias repetidas.

La elección de la especie recayó en *T. infestans* dado que su genoma está constituido por una gran cantidad de secuencias repetidas. Por otro lado, el análisis comparativo del ADN repetido entre los linajes Andino y No-Andino, con cantidades diferenciales en su contenido de ADN genómico, nos permitiría determinar qué tipos de secuencias diferencian a ambos linajes.

La determinación del repeatoma en ambos linajes mostró que la cantidad de ADN repetido en *T. infestans* varía entre 630,78 Mpb (Andino) y 376,05 Mpb (No-Andino) por genoma haploide, representando el 44% y el 34% del genoma respectivamente (Pita et al. 2017c). Considerando que ambos linajes presentan la misma cantidad de ADN no repetido podemos concluir que las diferencias en el contenido genómico de ambos linajes se deben a la cantidad de ADN repetido que poseen.

Además, dentro del ADN repetido, determinamos que las secuencias de ADN satélite (ADNsat) son las únicas que reflejaron una diferencia significativa. Por lo tanto, las secuencias de ADNsat son las responsables de la variación en el contenido de ADN entre ambos linajes de *T. infestans*.

Dada la importancia de las secuencias de ADNsat en el genoma de *T. infestans* procedimos a realizar un análisis más exhaustivo de ellas. En total encontramos 42 familias, la gran mayoría de ellas presentes en ambos genomas. Este análisis además mostró que la diferencia significativa entre los linajes es debida a una mayor abundancia de solo unas pocas familias de ADNsat. La determinación de la localización cromosómica por FISH de aquellas familias cuya abundancia era superior al 0.03% nos permitió determinar que estas 11 familias de ADNsat estaban localizadas tanto en regiones heterocromáticas (4 familias) como regiones eucromáticas (7 familias). Finalmente, pudimos concluir que las diferencias de ADN repetido entre ambos linajes son debidas principalmente a cantidades diferenciales de las familias de ADNsat localizadas en la heterocromatina autosómica.

Por otra parte, identificamos que el cromosoma Y heterocromático está constituido principalmente por 2 familias de ADNsat: TinfSat01-33 (familia más frecuente del genoma de *T. infestans* constituida por monómeros de 33 pb) y TinfSat03-4 (repetidos GATA). Análisis preliminares sobre otras especies de *Triatoma* nos permiten afirmar que, de estas dos familias de ADNsat, únicamente el repetido GATA está conservado en el cromosoma Y de las especies del género *Triatoma*, no así la familia TinfSat01-33. Por lo cual podemos concluir que las secuencias repetidas GATA constituyen las secuencias compartidas en todos los cromosomas Y de las especies de la tribu Triatomini.

Capítulo V. Secuencias teloméricas

Los telómeros, en la mayoría de las especies eucariotas están constituidos por la repetición de secuencias cortas ricas en guanina y timina. Las secuencias teloméricas son de entre los ADNs repetitivos los más conservados entre especies. Se considera que el telómero en todos los vertebrados está formado por la repetición del hexanucleótido TTAGGG (Meyne et al. 1989) o TTTAGGG en las plantas superiores (Fuchs et al. 1995). El análisis de estas secuencias no estaba inicialmente incluido en el proyecto de Tesis. Numerosos estudios han revelado que la secuencia telomérica predominante en los artrópodos es el pentanucleótido $(TTAGG)_n$, considerado como ancestral tanto en artrópodos como en la clase Insecta (Vítková et al. 2005). Sin embargo, numerosos órdenes de insectos no presentan este repetido, siendo sustituido por otros motivos repetidos, retrotrasposones o por secuencias aún no determinadas (Mason et al. 2016). El repetido $(TTAGG)_n$ no había sido detectado en muchas especies de Heteroptera y especialmente en los infraórdenes evolutivamente derivados como Pentatomomorpha y Cimicomorpha (el cual incluye a los triatominos), habiendo sido descrito en solo dos especies de hemípteros basales (*Lethocerus patruelis* /infraorden Nepomorpha y *Gerris buenoi* /infraorden Gerromorpha) (Kuznetsova et al. 2012). Por lo cual se había propuesto que en los infraórdenes derivados habría ocurrido una pérdida del motivo $(TTAGG)_n$ (Mason et al. 2016).

Uno de los ADNs repetitivos encontrados con la secuenciación de genoma de *T. infestans* estaba formado por la repetición del pentanucleótido TTAGG. Mediante hibridación in situ se pudo confirmar que esta secuencia constituía el telómero genuino de esta especie, así como en otras especies de los géneros *Triatoma*, *Dipetalogaster* y *Rhodnius*. La presencia de esta secuencia ancestral en los telómeros de triatominos se opone a la hipótesis antes mencionada (Pita et al. 2016b). Nosotros sugerimos que la adquisición del repetido telomérico en Triatominae sería muy poco factible, por lo que probablemente su falta de detección en Cimicomorpha y Pentatomomorpha sería un problema metodológico más que un proceso de pérdida durante la evolución.

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Conclusiones

- En Triatominae, los genes ribosomales (ADNr 45S) se encuentran en uno o dos loci por genoma haploide (excepto en *T. infestans*), similar a lo observado en otros hemípteros. Sin embargo, presenta una extensa variabilidad en su localización cromosómica con cuatro patrones distintos, no observada en ninguna otra subfamilia de Hemiptera.

- La velocidad de cambio en la localización cromosómica de los genes ribosomales es distinta en los diferentes grupos de especies. Sin embargo, especies evolutivamente próximas tienden a conservar la misma ubicación de los genes ribosomales, por lo que esta característica puede ser utilizada como marcador filogenético.

- La localización de los NORs, junto con otros marcadores cromosómicos, ha demostrado ser una herramienta valiosa para identificar especies crípticas.

- Los triatominos poseen telómeros formados por el repetido ancestral para insectos $(TTAGG)_n$, hasta ahora no detectadas en el infraorden Cimicomorpha.

- El ADN repetido posee dos tipos de localización en el genoma: dispersa en todo el cromosoma y/o clusterizada en regiones específicas.

- Los cromosomas sexuales X e Y presentan entre sí una gran diferenciación en su composición de secuencias repetitivas, por lo que probablemente deriven de pares autosómicos distintos.

- El cromosoma X contiene secuencias repetitivas dispersas, similares a las observadas en la eucromatina autosómica.

- En las especies de las tribus Triatomini y Rhodniini los cromosomas Y presentan difierente composición en secuencias repetitivas.

- En la tribu Triatomini, el cromosoma Y heterocromático está constituido principalmente por secuencias repetitivas clusterizadas. Por el contrario, las especies de la tribu Rhodniini presentan un cromosoma Y con una composición distinta, aún sin determinar.

- En la tribu Triatomini, el principal ADN repetitivo localizado en el cromosoma Y es $(GATA)_n$, siendo probablemente un carácter ancestral de las especies de esta tribu.

- El ADN satélite es el mayor componente del repeatoma de *T. infestans*, estando presente tanto en la heterocromatina como en la eucromatina.

- Los bloques heterocromáticos en *T. infestans* están compuestos por pocas familias de ADN satélite, siendo la diferencia en la cantidad de estos ADNs la principal responsable de la diferenciación del contenido de ADN genómico entre ambos linajes de esta especie.

- A nivel evolutivo, especies próximas comparten secuencias de ADNsat, mientras que en especies más alejadas el ADNsat está constituido por familias diferentes y exclusivas de cada especie.

Perspectivas

Con el objetivo de seguir profundizando en conocer los mecanismos de variación genómica en los triatominos, pretendemos realizar los siguientes análisis:

- Extender el estudio del ADNr 45S a tribus no abordadas (Alberproseniini, Cavernicolini) y a las especies de *Triatoma* exclusivas de Asia. Igualmente, se quiere ampliar el estudio grupos donde hemos analizado pocas especies, como la tribu Bolboderini.

- Abordar el estudio de las secuencias repetidas mediante la técnica de ND-FISH (*non-denaturing* FISH), que permite localizar cromosómicamente ADNs repetitivos integrados por monómeros pequeños, de 1 a 5 pb.

- Continuar con el análisis de la diferenciación de la heterocromatina, utilizando sondas genómicas de diferentes especies pertenecientes a distintos grupos de Triatominae, tales como:

    - Especies de la tribu Rhodniini, de la cual se carece de información acerca de sus ADNs repetitivos.

    - Especies del género *Panstrongylus*, por ser un grupo variable en el número de autosomas, cromosomas sexuales y heterocromatina, además de ser un grupo aparentemente parafilético.

    - Especies de otros subcomplejos de *Triatoma* del Sur como *T. brasiliensis* y *T. sordida Brasil*, que permitirían conocer con mayor profundidad la evolución de las secuencias autosómicas repetidas en el grupo Infestans.

- Determinar el repeatoma de otras especies de triatominos, lo cual permitiría comprender, de manera más exhaustiva, la evolución del ADN repetitivo en la subfamilia Triatominae, tales como:

    - *T. delpontei*, especie hermana de *T. infestans* que presenta el mayor contenido de ADN genómico en la subfamilia Triatominae. Su análisis permitirá identificar las secuencias repetitivas responsables del gran aumento en el tamaño de su genoma.

    - ~~o~~ Determinar el repeatoma en especies de *Triatoma* de Norte América (grupo Rubrofasciata) tales como *T. dimidiata* y *T. rubrofasciata,* lo cual permitiría compararlas con el obtenido en *T. infestans* y obtener así un panorama más claro sobre la evolución genómica en el género *Triatoma*.

    - Determinar el satelitoma de *R. prolixus* (no identificado en el análisis posterior a la secuenciación de su genoma) que nos brindaría información esencial sobre la evolución genómica en la tribu Rhodniini.

Metodología

Obtención de preparaciones citológicas

Las gónadas, previamente fijadas en 3:1 son maceradas en ácido acético al 50%. Las preparaciones citológicas se obtuvieron mediante el método deaplastado. Se observó al microscopio de contraste de fase paraseleccionar aquellas con mayor cantidad, adherencia y calidad de materialcromosómico (elevado número de células en metafases de ambas divisionesmeióticas). Finalmente los cubreobjetos fueron levantados de las preparaciones seleccionadas con nitrógeno líquido y se conservaron en freezer a –20ºC hasta su uso.

Hibridación in situ

Para la marcación de las sondas generadas se utilizaron diferentes protocolos. Para las sondas del gen ribosomal, de ADN genómico, de las familias de ADN satélite que fueron clonadas en plásmidos y la sonda telomérica, se llevó a cabo una marcación por *Nick-Translation* por medio de kits comerciales. Para la sonda del gen ribosomal se utilizó el fluorocromo Cy-3 dUTP y en los otros dos casos se marcó con biotina-16-dUTP. La marcación de la sonda generada por microdisección fue marcada por DOP-PCR utilizando un cebador parcialmente degenerado (CCGACTGCAGNNNNNNATGTGG), y biotina-16-dUTP. Las sondas de familias de ADN satélite pequeñas, fueron marcadas utilizando la enzima transferasa terminal y biotina-16-dUTP. Por último, para generar la sonda con el repetido telomérico se utilizó la reacción de PCR con los cebadores 5´ $(TTAGG)_6$ y 5´ $(TAACC)_6$, sin el uso de ADN molde.

Todas las hibridaciones in situ siguieron el mismo protocolo. La única diferencia radicó en la detección, ya que el gen ribosomal fue marcado con fluorescencia directa y el resto de los casos se utilizó detección indirecta mediante el sistema biotina-avidina con fluoresceína (FITC).

El protocolo de pre-tratamiento e hibridación se detalla a continuación:

1. Pre-tratamiento de las láminas.

- Tratamiento con RNAasa [100µg/ml], en cámara húmeda a 37ºC por 1 hora.
- 3 lavadosde 5 minutos de 2X SSC
- Tratamiento con pepsina [0,01 mg/ml] en cámara húmedaa 37ºC por 20 minutos
- 3 lavadosde 5 minutos de 2X SSC
- Fijación en 3,7 % de formaldehído por 10 minutos
- 3 lavadosde 5 minutos de PBS 1X
- Deshidratación seriada en etanol 70 %, 95% y absoluto por 3 minutos

2. Hibridación.

- Solución de hibridación:Formamida 50%, Dextran Sulfato 10%, 2X SSC y 50 µg/µl de sonda marcada.
- Desnaturalización de la solución de hibridación con las sondas a 75ºC por 10 minutos
- Desnaturalización las preparaciones con la solución de hibridación en bloque térmico a 80ºC por 3 minutos
- Incubación en cámara húmeda toda la noche a 37ºC

Protocolo para uso de detección directa:

1. Lavados post-hibridación

- 2 lavados de 2X SSCa 42ºC por 5 minutos
- 2 lavados de 0,1X SSC a 42ºC por 5 minutos
- 2 lavados de 2X SSCa 42ºC por 5 minutos

2. Coloración y montaje.

- Colocar en cada preparado 1 gota de VectaShield®H1000con DAPI incluido.

Protocolo para uso de detección indirecta:

1. lavados post- hibridación y bloqueo

- Lavados con 2X SSC y 4T (4X SSC y tween 20 al 0,05%) a temperatura ambiente en agitador
- Bloqueo con 4M (4X SSC y agente de bloqueo comercial).

2. Amplificación y detección

- Incubación con avidina-fluoresceína durante 30 minutos
- Lavados con 4T
- Incubación con antiavidina-biotina durante 30 min.
- Lavados con 4T
- Segunda incubación con avidina-fluoresceína.
- Lavados con 4T y PBS 1X
- Deshidratación seriada
- Montaje con Vectashield® H1000.

Para la sonda telomérica se realizaron 3 rondas de incubación.

Análisis de secuencias repetidas utilizando RepeatExplorer

El ADN genómico extraído mediante kit comercial fue enviado a Hong Kong (BGI Americas®) para su secuenciación en la plataforma Illumina® Hi- seq® 2000. Los datos obtenidos fueron analizados utilizando el *pipeline* RepeatExplorer. Este programa agrupa lecturas con al menos un 80%de identidad en un clúster. Posteriormente representa en un grafo las conexiones entre lecturas, lo cual nos puede dar una idea sobre la estructura de ese elemento en el genoma. Finalmente ensambla estas lecturas generando contigs que anota con Repbase y opcionalmente con una base de datos generada por nosotros. Con el protocolo estándar de RepeatExplorer podemos caracterizar secuencias repetidas, incluido el ADN satélite. Sin embargo, a la gran mayoría de los clústeres generados no se les asigna anotación por lo que se realizó un análisis de BLAST (http://www.blast.ncbi.nlm.nih.gov)para determinar su identidad.

La caracterización pormenorizada de las familias de ADN satélite se realizó mediante el alineamiento y el análisis más detallado de los contigs con el programa MEGA (http://www.megasoftware.net) y dotmattcher (http://www.bioinformatics.nl/cgi-bin/emboss/dotmatcher). Aquellas familias que poseían monómero de más de 20 pares de bases fueron amplificadas por PCR y clonadas en vectores plasmídicos.

Los análisis estadísticos de χ^2(chi cuadrado) fueron realizados utilizando el programa PAST (http://palaeo-electronica.org/2001_1/past/issue1_01.htm).

Anexo 1. Resumen de la localización cromosómica de los genes ribosomales (45S) en la subfamilia Triatominae.

ESPECIE	2n	ORIGEN GEOGRAFICO	REFERENCIA
TRIBU RHODNIINI			
Linaje robustus			
Rhodnius domesticus	20A + XY	Brasil (Santa Catarina)	Panzera et al 2012 CGR
R. milesi	20A + XY	Brasil (Pará)	Pita et al 2013 MIOC
R. montenegrensis	20A + XY	Brasil (Rondonia)	**NO PUBLICADA TESIS DOCTORADO**
R. nasutus	20A + XY	Brasil (Pará)	Pita et al 2013 MIOC
R. neglectus	20A + XY	Brasil (Minas Gerais)	Pita et al 2013 MIOC
R. neivai	20A + XY	Venezuela (Maracay)	Pita et al 2013 MIOC
R. prolixus	20A + XY	Colombia (Casanare); Guatemala (Las Palmas)	Panzera et al 2012 CGR
	20A + XY	Venezuela (Cojedes); Colombia (Magdalena)	Pita et al 2013 MIOC
R. robustus	20A + XY	Peru (Loreto)	Pita et al 2013 MIOC
Psammolestes tertius	20A + XY	Brasil (Ceará)	Panzera et al 2012 CGR
Linaje pallescens			
Rhodnius colombiensis	20A + XY	Colombia (Tolima)	Panzera et al 2012 CGR
R. ecuadoriensis	20A + XY	Ecuador (Malabí)	Pita et al 2013 MIOC
	20A + XY	Perú (La Libertad)	Pita et al 2013 MIOC
R. pallescens	20A + XY	Colombia (sin determinar)	Morielle & Azeredo 2007
	20A + XY	Colombia (Caldas)	Panzera et al 2012 CGR
	20A + XY	Panamá (Chilibre); Colombia (Magdalena y Sucre)	Pita et al 2013 MIOC
Linaje pictipes			
Rhodnius brethesi	20A + XY	Brasil (sin determinar)	**NO PUBLICADA TESIS DOCTORADO**
R. pictipes	20A + XY	Brasil (Pará)	Pita et al 2013 MIOC
R. stali	20A + XY	Bolivia (La Paz)	Pita et al 2013 MIOC
TRIBU BOLBODERINI			
Género *Belminus*			
B. herreri	20A + X1X2Y	Colombia (Santander)	NO PUBLICADA

TRIBU TRIATOMINI			
Género *Dipetalogaster*			
D. maxima	20A + XY	México (Baja California)	Panzera et al 2012 CGR
Género *Eratyrus*			
E. cuspidatus	20A + X1X2Y	Colombia (Sucre)	Panzera et al 2012 CGR
E. mucronatus	20A + X1X2Y	Bolivia (La Paz)	NO PUBLICADA
	20A + X1X2Y	Brasil (Pará)	**NO PUBLICADA TESIS DOCTORADO**
Género *Mepraia*			
M. spinolai	20A + X1X2Y	Chile (Región III)	Panzera et al 2012 CGR
	20A + X1X2Y	Chile (Región IV)	**NO PUBLICADA TESIS DOCTORADO**
M. gajardoi	20A + X1X2Y	Chile (Region XV)	Panzera et al 2012 CGR
	20A + X1X2Y	Chile (Reg. XV y Reg. III)	**NO PUBLICADA TESIS DOCTORADO**
T. breyeri	20A + X1X2X3Y	Bolivia (Cochabamba)	NO PUBLICADA
T. eratyrusiformis	20A + X1X2X3Y	Argentina (Neuquen)	NO PUBLICADA
Género *Panstrongylus*			
P. chinai	20A + X1X2Y	Perú (Lambayeque)	Panzera et al 2012 CGR
	20A + X1X2Y	Ecuador (La Loja)	**NO PUBLICADA TESIS DOCTORADO**
P. geniculatus	20A + X1X2Y	Colombia (Santander y Antioquia)	NO PUBLICADA
	20A + X1X2Y	Colombia (Antioquia); Venezuela	**NO PUBLICADA TESIS DOCTORADO**
P. howardi	20A + X1X2Y	Ecuador (Manabí)	NO PUBLICADA
P. lignarius/herreri	20A + X1X2Y	Perú (San Martín)	Panzera et al 2012 CGR
	20A + X1X2Y	Perú (Amazonas)	**NO PUBLICADA TESIS DOCTORADO**
P. megistus	18A + X1X2Y	Brasil (sin determinar)	Morielle & Azeredo 2007
	18A + X1X2Y	Brasil (Minas Gerais)	Panzera et al 2012 CGR
P. rufotuberculatus	20A + X1X2Y	Colombia (Guajira y Antioquia)	NO PUBLICADA
P. rufotuberculatus n. sp.	20A + XY	Bolivia (La Paz)	NO PUBLICADA

Género *Triatoma*			
Grupo Infestans			
Subcomplejo brasiliensis			
T. brasiliensis	20A + XY	Brasil (sin determinar)	Bardella et al. 2010
T. brasiliensis brasiliensis	20A + XY	Brasil (Pernambuco)	Panzera et al 2012 CGR
T. bras. macromelanosoma	20A + XY	Brasil (Pernambuco)	NO PUBLICADA
T. bahiensis	20A + XY	Brasil (Bahia)	**NO PUBLICADA TESIS DOCTORADO**
T. juazeirensis	20A + XY	Brasil (Bahia)	NO PUBLICADA
T. lenti	20A + XY	Brasil (Bahia)	NO PUBLICADA
T. melanica	20A + XY	Brasil (Bahia)	**NO PUBLICADA TESIS DOCTORADO**
T. petrochiae	20A + XY	Brasil (Rio Grande do Norte)	NO PUBLICADA
T. sherlocki	20A + XY	Brasil (Bahia)	Panzera et al 2012 CGR
Subcomplejo rubrovaria			
T. carcavalloi	20A + XY	Brasil (Rio Grande do Sul)	Panzera et al 2012 CGR
T. circummaculata	20A + XX	Uruguay (Cerro Largo); Brasil (Matto Grosso do Sul)	Pita et al 2016 IGE
T. klugi	20A + XY	Brasil (Rio Grande do Sul)	Pita et al 2016 IGE
T. rubrovaria	20A + XY	Brasil (sin determinar)	Bardella et al. 2010
	20A + XY	Uruguay (Artigas)	Panzera et al 2012 CGR
T. pintodiasi	20A + XY	Brasil (Rio Grande do Sul)	**Pita et al 2016 IGE TESIS DOCTORADO**
Subcomplejo matogrosenssis			
T. baratai	20A + XY	Brasil (Matto Grosso do Sul)	**Pita et al 2016 IGE TESIS DOCTORADO**
T. costalimai	20A + XY	Brasil (Tocantins)	Pita et al 2016 IGE
T. guazu	20A + XY	Brasil (Matto Grosso y Pará)	**Pita et al 2016 IGE TESIS DOCTORADO**
T. jatai	20A + XY	Brasil (Tocantins)	**Pita et al 2016 IGE TESIS DOCTORADO**
T. jurbergi	20A + XY	Brasil (Matto Grosso)	Pita et al 2016 IGE
T. matogrossensis	20A + XY	Brasil (Matto Grosso do Sul)	Panzera et al 2012 CGR
	20A + XY	Brasil (sin determinar)	Bardella et al 2010
T. vandae	20A + XY	Brasil (Matto Grosso)	Panzera et al 2012 CGR
T. williami	20A + XY	Brasil (Matto Grosso)	**Pita et al 2016 IGE TESIS DOCTORADO**

Subcomplejo sordida			
T. sordida sensu stricto	20A + XY	Brasil (Matto Grosso)	Panzera et al 2012 CGR
		Brasil (Piaui y Minas Gerais); Bolivia (Santa Cruz); Paraguay (Concepción)	**Panzera et al 2015 P&V TESIS DOCTORADO**
T. sordida La Paz	20A + XY	Bolivia (La Paz)	**Panzera et al 2015 P&V TESIS DOCTORADO**
T. sordida Argentina	20A + XY	Argentina (Corrientes, Chaco, Stgo. Estero, Formosa); Bolivia (Cochabamba); Paraguay (Paraguarí y S.Pedro).	**Panzera et al 2015 P&V TESIS DOCTORADO**
T. sordida HIBRIDOS	20A + XY	Bolivia (Santa Cruz)	**Panzera et al 2015 P&V TESIS DOCTORADO**
T. garciabesi	20A + XY	Argentina (Salta, Rivadavia, S.)	Panzera et al 2012 CGR
		Argentina (Stgo Estero, Chaco, Formosa); Paraguay (Pte. Hayes); Bolivia (Santa Cruz)	**Panzera et al 2015 P&V TESIS DOCTORADO**
T. guasayana	20A + XY	Bolivia (Santa Cruz, Cochabamba); Argentina (Córdoba)	**Panzera et al 2015 P&V TESIS DOCTORADO**
T. patagonica	20A + XY	Argentina (Stgo. Del Estero, Santa Fe, San Luis, Rio Negro, La Pampa)	**Pita et al 2016 IGE TESIS DOCTORADO**
Subcomplejo maculata			
T. maculata	20A + XY	Brasil (Roraima)	Panzera et al 2012 CGR
	20A + XY	Venezuela (Roraima)	**Pita et al 2016 IGE TESIS DOCTORADO**
T. maculata n. sp.	20A + XY	Venezuela (Anzoategui); Colombia (Bolivar, Casanare, Magdalena)	**NO PUBLICADA TESIS DOCTORADO**
T. pseudomaculata	20A + XY	Brasil (Ceará)	Panzera et al 2012 CGR
	20A + XY	Brasil (Minas Gerais)	Pita et al 2016 IGE
T. wygodzinsky	20A + XY	Brasil (São Paulo)	Panzera et al 2012
	20A + XY	Brasil (São Paulo)	Pita et al 2016 IGE
Subcomplejo infestans			
T. infestans	20A + XY	Argentina, Brasil, Bolivia, Chile, Paraguay y Uruguay	Panzera et al 2012; 2014; IGE
T. platensis	20A + XY	Sin determinar	Severi & Azeredo 2005
	20A + XY	Argentina (Córdoba); Uruguay (Paysandú)	Panzera et al 2012 CGR
T. delpontei	20A + XY	Argentina (Salta)	Panzera et al 2012 CGR
	20A + XY	Bolivia (Potosí y Santa Cruz)	NO PUBLICADA

GRUPO RUBROFASCIATA			
Complejo Lecticularia			
T. gerstaeckeri	20A + X1X2Y	USA (sin determinar)	**NO PUBLICADA TESIS DOCTORADO**
T. lecticularia	20A + XY	USA (Oklahoma)	Panzera et al 2012 CGR
T. recurva	20A + X1X2Y	USA (sin determinar)	**NO PUBLICADA TESIS DOCTORADO**
T. sanguisuga	20A + X1X2Y	USA (sin determinar)	**NO PUBLICADA TESIS DOCTORADO**
Complejo Protracta			
T. barberi	20A + X1X2Y	México (Queretaro)	NO PUBLICADA
T. nitida	18A + X1X2Y	Guatemala (Quiché)	Panzera et al 2012 CGR
T. protracta	20A + X1X2Y	USA (sin determinar)	Severi & Azeredo 2005
	20A + X1X2Y	USA (California)	Panzera et al 2012 CGR
Complejo Rubrofasciata			
T. rubrofasciata	22A + X1X2Y	Brasil y Vietnam	**Dujardin et al 2015 Acta Trop TESIS DOCTORADO**
Complejo Phyllosoma			
Subcomplejo Phyllosoma			
T. bassolsae	20A + X1X2Y	México (Puebla)	NO PUBLICADA
T. longipennis	20A + X1X2Y	México (Zacatecas)	NO PUBLICADA
T. mazzottii	20A + X1X2Y	México (Oaxaca)	Panzera et al 2012 CGR
T. pallidipennis	20A + X1X2Y	México (Morelos)	Panzera et al 2012 CGR
T. phyllosoma	20A + X1X2Y	México (Oaxaca)	Panzera et al 2012 CGR
T. picturata	20A + X1X2Y	México	NO PUBLICADA
T. ryckmani	20A + X1X2Y	Guatemala (El Progreso)	NO PUBLICADA
Subcomplejo Dimidiata			
T dimidiata maculipennis	20A + X1X2Y	México (Oaxaca)	Panzera et al 2012 CGR
		México (San Luis Potosí)	NO PUBLICADA
T. dimidiata dimidiata	20A + X1X2Y	Guatemala (Jutiapa)	Panzera et al 2012 CGR
		Guatemala (Chiquimula, Alta Verapaz, Petén); Nicaragua (Nueva Segovia); Honduras (Tegucigalpa)	NO PUBLICADA
T. dimidiata capitata	20A + X1X2Y	Colombia (Magdalena)	Panzera et al 2012 CGR
		Colombia (Boyacá); Panamá (Veraguas); Costa Rica (Heredia)	NO PUBLICADA
T. spp. 1 affin dimidiata	20A + X1X2Y	Guatemala (Petén, Ruinas Yaxhá)	NO PUBLICADA

T. spp. 2 affin dimidiata	20A + X1X2Y	Belice (Cueva Rio Frio)	NO PUBLICADA
T. hegneri	20A + X1X2Y	México (Isla Cozumel)	NO PUBLICADA
Complejo Flavida			
T. flavida	20A + X1X2Y	Cuba (Peninsula Guanahacabibes)	Panzera et al 2012 CGR
T. bruneri	20A + X1X2Y	Cuba (sin determinar)	**NO PUBLICADA TESIS DOCTORADO**
GRUPO DISPAR			
T. boliviana	20A + XY	Bolivia (La Paz)	Panzera et al 2012 CGR
T. carrioni	20A + XY	Perú (Piura)	Panzera et al 2012 CGR
SIN ASIGNAR			
T. tibiamaculata	20A + X1X2Y	Brasil (sin determinar)	Severi & Azeredo 2005
	20A + X1X2Y	Brasil (Pará)	Panzera et al 2012 CGR
T. vitticeps	20A +X1X2X3Y	Brasil (Rio de Janeiro)	Severi & Azeredo 2005
	20A +X1X2X3Y	Brasil (Rio de Janeiro)	Panzera et al 2012 CGR
T. melanocephala	20A +X1X2X3Y	Brasil (Bahia)	**NO PUBLICADA TESIS DOCTORADO**