Research paper

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

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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

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

Table 1 (continued)

Species	Geographical origin	45S rDNA location
		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.
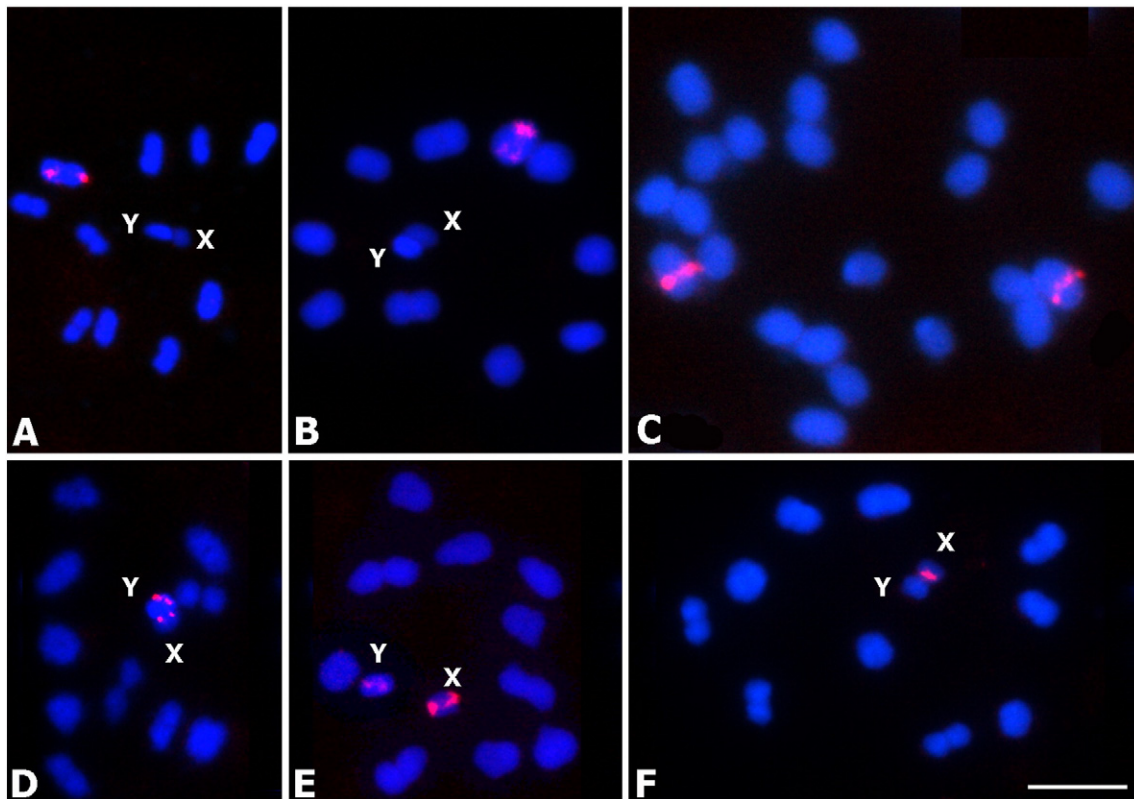
Fig. 1. Localization of 45S ribosomal DNA by fluorescent in situ hybridization (FISH) in male meiosis and spermatogonial prometaphase in different *Triatoma* species from South America, using 18S rDNA as probe. All species showed a diploid chromosome number of 22 chromosomes (2n = 20 autosomes plus XY 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.

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).

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.

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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