Chromosomal divergence and evolutionary inferences in Rhodniini based on the chromosomal location of ribosomal genes

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In this study, we used fluorescence in situ hybridisation to determine the chromosomal location of 45S rDNA clusters in 10 species of the tribe Rhodniini (Hemiptera: Reduviidae: Triatominae). The results showed striking inter and intraspecific variability, with the location of the rDNA clusters restricted to sex chromosomes with two patterns: either on one (X chromosome) or both sex chromosomes (X and Y chromosomes). This variation occurs within a genus that has an unchanging diploid chromosome number (2n = 22, including 20 autosomes and 2 sex chromosomes) and a similar chromosome size and genomic DNA content, reflecting a genome dynamic not revealed by these chromosome traits. The rDNA variation in closely related species and the intraspecific polymorphism in Rhodnius ecuadoriensis *suggested that the chromosomal position of rDNA clusters might be a useful marker to identify recently diverged species or populations. We discuss the ancestral position of ribosomal genes in the tribe Rhodniini and the possible mechanisms involved in the variation of the rDNA clusters, including the loss of rDNA* loci *on the Y chromosome, transposition and ectopic pairing. The last two processes involve chromosomal exchanges between both sex chromosomes, in contrast to the widely accepted idea that the achiasmatic sex chromosomes of Heteroptera do not interchange sequences.*

Key words: chromosomal evolution - Chagas disease vectors - Triatominae - holocentric chromosomes - rDNA variability

The subfamily Triatominae (Hemiptera: Reduviidae) currently comprises 142 species of blood-sucking insects, assembled in five-six tribes and 15-19 genera (Galvão et al. 2003, Schofield & Galvão 2009, Rosa et al. 2012). The Rhodniini tribe is the second largest and includes two genera: *Rhodnius* (18 species) and *Psammolestes* (3 species). Although the phylogenetic origin of many triatomine tribes and genera remains problematic (Hwang & Weirauch 2012), the Rhodniini tribe is considered monophyletic, comprising two groups, namely the robustus and pictipes lineages, with the *Psammolestes* genus integrating the robustus lineage (Monteiro et al. 2000, 2002). Rhodniini species display different degrees of synanthropic behaviour from sylvatic to highly domestic (Abad-Franch et al. 2009). The domestic species are of great medical interest for their role as vectors of Chagas disease, particularly *Rhodnius prolixus* in Venezuela and Colombia (and also in parts of Central America) (Hashimoto & Schofield 2012), *Rhodnius pallescens* in Panama, Colombia, Costa Rica and Nicaragua (Calzada et al. 2010), *Rhodnius ecuadoriensis* in Ecuador and northern Peru (Cuba Cuba et al. 2002), *Rhodnius stali* in Bolivia (Matias et al. 2003) and several species in the Amazon Region (Aguilar et al. 2007).

As in other Heteroptera, the Triatominae possess holocentric chromosomes characterised by a diffuse or non-localised centromere (Hughes-Schrader & Schrader 1961). The main source of karyotype variation in this subfamily involves changes in chromosome number, differences in C-heterochromatin (such as the number, size and location of C-blocks) and genomic DNA content (Panzera et al. 2007, 2010). However, Rhodniini karyotypes seem to be highly conserved, with no known variations either in the number of autosomes (20) or sex mechanism (XY in males, XX in females). In addition, Rhodniini species exhibit chromosomes of similar size and possess the lowest genomic DNA content of all the triatomines (Panzera et al. 2007). Only a few Rhodniini species present C-heterochromatin (*Rhodnius colombiensis*, *Rhodnius nasutus*, *R. pallescens* and *Rhodnius pictipes*) (Dujardin et al. 2002), with only one of these species exhibiting C-band polymorphisms (Gómez-Palacio et al. 2008, 2012).

Several authors have applied fluorescence in situ hybridisation (FISH) using rDNA probes to reveal chromosomal changes in the holocentric chromosomes of Heteroptera (Kuznetsova et al. 2011). Previous studies in Triatominae have demonstrated that the 45S rDNA clusters show remarkable variation in their chromosomal location, particularly in the genus *Triatoma* (Panzera et al. 2012).

The aim of this study was to determine whether the Rhodniini genus displays karyotype homogeneity, which involves the chromosomal position of active genes, such as ribosomal genes. We used FISH to analyse the chro-

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mosomal location of 45S rDNA clusters in the robustus and pictipes lineages and performed population analyses of the most important vector species (*R. prolixus, R. pallescens* and *R. ecuadoriensis*). The data obtained using this marker correlated with the evolutionary relationships established using molecular analyses.

MATERIALS AND METHODS

Materials - We studied the chromosomal location of 45S rDNA clusters in 10 *Rhodnius* species representing the two phylogenetic groups of the genus (Abad-Franch et al. 2009, Schofield & Galvão 2009): the robustus lineage (*Rhodnius milesi, R. nasutus, Rhodnius neglectus, Rhodnius neivai, R. prolixus* and *Rhodnius robustus*) and pictipes lineage (*R. ecuadoriensis, R. pallescens, R. pictipes* and *R. stali*). At least two male individuals were analysed for each species and individuals collected from natural populations were used when possible (Table). For the species analysed at the population level, at least three individuals were studied per location (Table). For *R. prolixus*, we studied specimens from additional localities to complement our previous results (Panzera et al. 2012). For *R. ecuadoriensis*, we studied populations from different countries, i.e., Peru and Ecuador. For *R. pallescens*, we included new populations from Colombia and Panama, representing the two evolutionary groups of the species recognised by morphometric, molecular and cytogenetic differences, as reported by Gómez-Palacio et al. (2008, 2012) (Table).

FISH - The FISH assays were conducted using squashed gonad preparations that had previously been fixed in 3:1 ethanol:acetic acid, as described by Panzera et al. (2012). A DNA plasmid harbouring an 807-bp region of the 18S rDNA gene of *Triatoma infestans* (accession Y18750) was used as a probe; the probe was labelled using the Nick Translation System (Invitrogen, Carlsbad, California, USA) with Cy3-dUTP (GE Healthcare, Life Sciences, Oregon, USA). The slides were mounted using Vectashield H-100 (Vector Laboratories, Inc, Burlingame, California, USA) containing 2 µg/mL 4',6-diamidino-2-phenylindole.

Microscopy and imaging - The slides were analysed using a Nikon Eclipse 80i epifluorescence microscope. The images were obtained with a Nikon DS-5Mc-U2 digital, cooled camera using Nikon Nis Elements 3.1 Advanced Research software and processed with Adobe Photoshop® software. At least 30 meiotic metaphases (I or II) or diplotene stages were studied for each individual.

RESULTS

The mapping results are summarised in Table and are discriminated by species and populations; previous reports on rDNA cluster location in Rhodniini are also included. The 45S rDNA clusters have one or two chromosome *loci* per haploid genome, showing two chromosome location patterns: either on both sex chromosomes (X and Y chromosomes) or only on the X chromosome (Figs 1, 2). In all cases, the hybridisation signals were located in a terminal or subterminal chromosomal position. In all the species (except *R. stali*) carrying two 45S rDNA *loci*, great differences in the signal intensities were observed and the intensity for the Y chromosome was reduced. Both chromosomal location patterns were observed in each phylogenetic group, as described below.

Robustus lineage - In *R. neivai, R. neglectus* and *R. milesi*, the rDNA clusters were located on both sex chromosomes (Fig. 1A, B, C, respectively), whereas the rDNA signal was detected on the X chromosome in *R. nasutus* and *R. robustus* (Fig. 1D, E). The *R. prolixus* population analyses did not reveal variability in the individuals from the new localities studied, with all presenting rDNA clusters on the X chromosome (Fig. 1F).

Pictipes lineage - In *R. pictipes* and *R. stali*, the rDNA clusters were located on the X and Y sex chromosomes (Fig. 2A, B, respectively). The same pattern was observed for *R. pallescens* among the individuals from the four populations studied, with the rDNA clusters located on the X and Y sex chromosomes, even in specimens with different amounts of C-heterochromatin (Fig. 2C). However, we observed striking intraspecific variability in *R. ecuadoriensis*. The population from Ecuador showed ribosomal genes located on both sex chromosomes (Fig. 2D), whereas the hybridisation signal was detected only on the X chromosome in the Peruvian population (Fig. 2E).

DISCUSSION

Interspecific variability of the 45S rDNA cluster location - Based on the FISH analyses performed on 13 Rhodniini species to date, eight of which are described for the first time in the present study, one or two 45S rDNA *loci* per haploid genome were observed. Two localisation patterns were revealed: either on both sex chromosomes (X and Y chromosomes) (8 species) or only on the X chromosome (4 species), with one species presenting both patterns (*R. ecuadoriensis*) (Figs 1, 2, Table). The number of rDNA *loci* was consistent with other Triatominae species, with the detection of up to two *loci* (see Panzera et al. 2012 for a review). In the Triatomini tribe, or within the genus *Triatoma*, the ribosomal genes showed four different chromosomal positions, including autosomes bearing 45S rDNA (Panzera et al. 2012). The Rhodniini tribe showed far less variability (rDNA *loci* restricted to the sex chromosomes), which might reflect their monophyletic origin, in contrast to the putative polyphyletic origin of the genus *Triatoma* (Hypsa et al. 2002, Hwang & Weirauch 2012). However, the Rhodniini variation in the ribosomal cluster position occurred despite their karyotype homogeneity, reflecting a genome dynamic that is not revealed by other chromosome traits. Different authors have proposed that changes in C-heterochromatin could affect the position and number of rDNA *loci* in several insect groups (Hirai et al. 1996, Criniti et al. 2005, Roy et al. 2005, Oliveira et al. 2010). Nevertheless, the position of rDNA *loci* in Rhodniini species and other triatomines does not appear to be associated with the presence, absence and/or variations in the amount of autosomal constitutive heterochromatin (i.e., *R. pallescens*) (Table).

TABLE

Geographical origin and available data on 45S rDNA chromosomal location in Rhodniini species obtained by fluorescent in situ hybridization

Species	45S rDNA chromosomal location	Geographic origin	References
Robustus lineage			
Rhodnius domesticus	X and Y chromosomes, small signal in Y chromosome	Brazil, Santa Catarina (S)	Panzera et al. (2012)
Rhodnius nasutus (Fig. 1D)	X chromosome	Insectary Evandro Chagas Institute (IEC) (Pará, Brazil). Origin: ND	Present paper
Rhodnius neglectus (Fig. 1B)	X and Y chromosomes, small signal in Y chromosome	Brazil, Minas Gerais, Uberaba (S)	Present paper
Rhodnius neivai (Fig. 1A)	X and Y chromosomes, small signal in Y chromosome	Insectary Fiocruz (Rio de Janeiro, Brazil). Origin: Venezuela, Maracay (P)	Present paper
Rhodnius milesi (Fig. 1C)	X and Y chromosomes, small signal in Y chromosome	Insectary IEC (Brazil). Origin: Brazil, Pará, Bragança (S)	Present paper
Rhodnius prolixus (Fig. 1F)	X chromosome	Insectary CDC (Atlanta, USA). Origin: Colombia	Panzera et al. (2012)
		Colombia, Casanare (S)	Panzera et al. (2012)
		Guatemala, Las Palmas (D)	Panzera et al. (2012)
		Colombia, Magdalena, Santa Marta, Kasakúmake (D)	Present paper
		Insectary Fiocruz (Brazil). Origin: Venezuela, Cojedes (S)	Present paper
Rhodnius robustus (Fig. 1E)	X chromosome	Insectary Fiocruz (Brazil). Origin: Perú, Loreto (S)	Present paper
Psammolestes tertius	X and Y chromosomes, small signal in Y chromosome	Insectary Araraquara (São Paulo, Brazil). Origin: Brazil, Ceará, Espírito Santo (S)	Panzera et al. (2012)
Pictipes lineage			
Rhodnius colombiensis	X chromosome	Colombia, Tolima, Coyaima, Totarco (S)	Panzera et al. (2012)
Rhodnius ecuadoriensis (Fig. 2D, E)	X and Y chromosomes, X chromosome	Insectary Univ. Antioquia (Colombia). Origin: Ecuador, Manabí (S)	Present paper
		Peru, La Libertad, Gran Chimú (D)	Present paper
Rhodnius pallescens (Fig. 2C)	X and Y chromosomes, small signal in Y chromosome	Origin: ND	Morielle-Souza and Azeredo-Oliveira (2007)
		Colombia, Norcasia, Caldas (S)	Panzera et al. (2012)
		Colombia, Magdalena, Santa Marta, San Sebastián de Buena Vista (S)	Present paper
		Colombia, Magdalena,Santa Marta, Mendihuaca (S)	Present paper
		Colombia, Sucre, Galeras (S)	Present paper
		Panama, Chilibre (S)	Present paper
Rhodnius pictipes (Fig. 2A)	X and Y chromosomes, small signal in Y chromosome	Insectary IEC (Brazil). Origin: Brazil, Pará, Bacarena (S)	Present paper
Rhodnius stali (Fig. 2B)	X and Y chromosomes	Insectary Fiocruz (Brazil). Origin: Bolivia, La Paz, Alto Beni (P)	Present paper

all of them have the same diploid chromosome number: 2n = 22 chromosomes (20 autosomes plus XY in males, XX in females). D: domiciliary; ND: not determined; P: peridomiciliary; S: sylvatic.

Intraspecific variation of rDNA chromosomal location - Population studies were performed on the three *Rhodnius* species which serve as the main vectors of Chagas disease (Table). No intraspecific variation was revealed in the *R. prolixus* and *R. pallescens* populations. The uniformity of the ribosomal gene location in *R. prolixus*, including the sylvatic and domestic populations, was consistent with the molecular studies indicating low variability in this species (Monteiro et al. 2003). In *R. pallescens*, the two evolutionary groups with morphometric and genetic divergence (Gómez-Palacio et al. 2008, 2012) also presented the same rDNA chromosome location. However, for *R. ecuadoriensis*, we detected striking differences in the rDNA location between the individuals from Peru and Ecuador (Fig. 2E, F). Molecular comparisons with mitochondrial sequences (cytochrome b) also showed markedly divergent haplotypes in the populations from these countries, suggesting that these populations represent discrete groups or incipient species (Abad-Franch et al. 2003, Abad-Franch & Monteiro 2005). Considering that both populations show different affinities for domestic and sylvatic habitats (Grijalva et al. 2012), their identification as distinct genetic groups has important epidemiological consequences for vector control programmes (Abad-Franch et al. 2001). Further FISH studies with other Peruvian and Ecuadorean populations from different habitats and experimental crosses among them are crucial to determine whether the observed genetic variations reflect intraspecific polymorphisms or involve different species.

Intraspecific variation of rDNA cluster location is fairly common in plants (Pedrosa et al. 2006), is rare in such vertebrates as amphibians (Andronico et al. 1985) and fish (Castro et al. 2001), but is unusual in insects, with only one case reported in species with holocentric chromosomes (Panzera et al. 2012). In Orthoptera and Coleoptera (Cabrero et al. 2003a, b, Cabral-de-Mello et al. 2011b, respectively), intraspecific variation involves a polymorphism in the number of chromosomes that harbour ribosomal clusters. With regard to holocentric chromosomes, the variation recently described in *T. infestans* populations involves the complete transfer of the rDNA *locus* from an autosomal position to the X chromosome (Panzera et al. 2012). The polymorphism in *R. ecuadoriensis* described here is similar to that observed in Orthoptera and Coleoptera because it also represents a variation in the number of chromosomes carrying ribosomal genes (Fig. 2D, E). The intraspecific polymorphisms in the ribosomal cluster position in these two Triatominae species suggests that this character varies at a faster rate than that observed in other insects groups, particularly those with holocentric chromosomes.

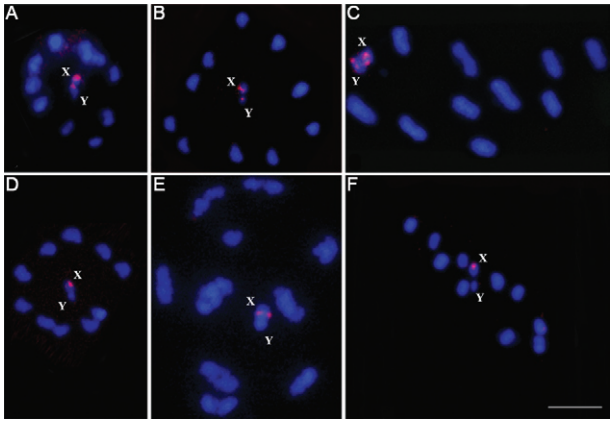

Fig. 1: tribe Rhodniini: robustus lineage. Interspecific variability in the chromosome location of the 45S rDNA by fluorescent in situ hybridization in male meiotic cells. All species have the same diploid chromosome number: 2n = 22 chromosomes (20 autosomes plus XY in males). The rDNA signals are located either on both sex chromosomes (A-C) or just on one sex chromosome (X chromosome) (D-F). A: *Rhodnius neivai,* metaphase II; B: *Rhodnius neglectus*, metaphase II; C: *Rhodnius milesi,* metaphase I; D: *Rhodnius nasutus*, metaphase II; E: *Rhodnius robustus,* diplotene; F: *Rhodnius prolixus,* metaphase II. Bar = 10 µm.

Taxonomic and evolutionary features - According to the FISH results obtained for the 46 triatomine species studied to date, the ribosomal gene chromosomal location is a species-specific characteristic (Panzera et al. 2012, this paper). Both cases of intraspecific variation detected in triatomines reflect recent divergence processes, which could lead to incipient speciation (*R. ecuadoriensis*) or a

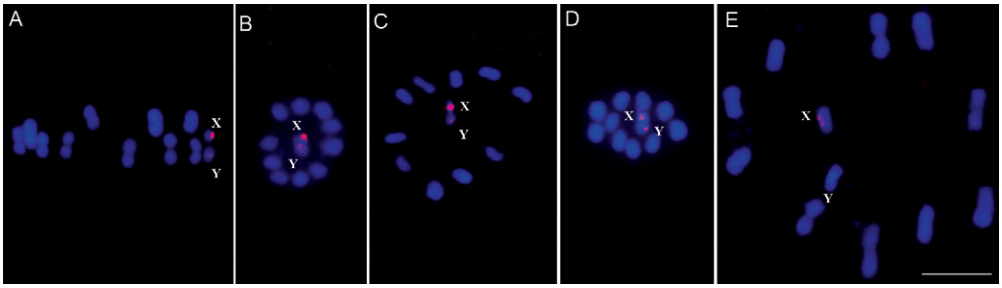

Fig. 2: tribe Rhodniini: pictipes lineage. Interspecific and intraspecific variability in the chromosome location of the 45S rDNA by fluorescent in situ hybridization in male meiotic cells. All species have the same diploid chromosome number: 2n = 22 chromosomes (20 autosomes plus XY in males). The rDNA signals are located on both sex chromosomes (A-D) and only in one sex chromosome (X chromosome) (E). A: *Rhodnius pictipes,* metaphase II; B: *Rhodnius stali,* metaphase II; C: *Rhodnius pallescens,* metaphase II, D: *Rhodnius ecuadoriensis* from Ecuador, metaphase II; E: *Rhodnius ecuadoriensis* from Peru, metaphase I. Bar = 10 µm.

marked population differentiation (*T. infestans*). In addition, closely related species, such as *R. colombiensis* and *R. pallescens* (Abad-Franch & Monteiro 2005), can be differentiated using this marker (Table), suggesting that the 45S chromosomal localisation of the ribosomal clusters could be an appropriate marker for the recognition and detection of recently diverged species or populations. FISH analyses of *R. robustus* (Monteiro et al. 2003) and/or *Triatoma sordida* (Noireau et al. 1999) cryptic species could help to test this hypothesis.

The determination of the chromosomal location of rDNA clusters is important to understanding karyotypic evolution in different insect taxa (Hirai et al. 1996, Roy et al. 2005, Cabrero & Camacho 2008, Oliveira et al. 2010), including lepidopteran species, which, similar to Heteroptera, also possess holocentric chromosomes (Nguyen et al. 2010). In several closely related species of Triatominae, such as the protracta and dispar complexes within the genus *Triatoma*, or in all *Panstrongylus* species, the chromosomal location of rDNA clusters is an evolutionarily conserved cytogenetic trait and closely related species display the same chromosomal positions (Panzera et al. 2012). However, this character is not evolutionarily conserved in the Rhodniini tribe, as each of the two genetic lineages shows both patterns of chromosomal location (Table).

Considering the monophyletic origin of the Rhodniini tribe, we can hypothesise which of the two ribosomal genes locations is the ancestral form. Given the number of *Rhodnius* species with ribosomal *loci* on both sex chromosomes (8/13 species), we suggest that this pattern represents the primitive form; thus, species with rDNA clusters only on the X chromosome could reflect a loss of the Y chromosome ribosomal *locus*. In support of this idea, *Rhodnius domesticus* and *R. neivai*, which are considered to be ancient isolated species, present the X-Y pattern (Abad-Franch & Monteiro 2007). Additional evidence for the X-Y pattern as the ancestral location is provided by the variation observed in the present study for the *R. ecuadoriensis* populations from Peru. These populations exhibit rDNA clusters only on the X chromosome (Fig. 2F) and are believed to have derived from ancestral populations in central Ecuador (Abad-Franch et al. 2001, 2009, Grijalva et al. 2005), which show ribosomal genes on both sex chromosomes (Fig. 2E). An ancestral configuration of rDNA *loci* on both sex chromosomes has also been suggested in other insects, such as melanogaster group, whereby some species have also subsequently lost the ribosomal genes located on the Y chromosome (Roy et al. 2005).

Nevertheless, the alternative hypothesis, i.e., an ancestral location of the ribosomal genes on the X chromosome, should not be excluded, considering that a significant excess of gene movement from the X chromosome has been demonstrated in most species with the XY sex system (see Pease & Hahn 2012 for a review). This hypothesis would imply either a partial transfer of ribosomal genes from the X to the Y chromosome, as mediated through the transposition of mobile elements (Schubert & Wobus 1985) or chromosomal rearrangements promoted through ectopic recombination (homologous recombination between repetitive sequences of non-homologous chromosomes) (Hanson et al. 1996). In almost all of the Rhodniini species described in this paper (Figs 1, 2), the Y chromosome ribosomal signal was less intense than that observed for the X chromosome, indicating that the ribosomal cluster copy number is significantly lower on the Y chromosome. This low copy number supports the mechanism proposed by Dubcovsky and Dvorak (1995), which implies the dispersion of a single rDNA copy and the successive amplification of the copy number, suggesting an ancestral location of ribosomal genes on the X chromosome. In many organisms, rDNA clusters behave as mobile genetic elements due to the presence of transposable elements adjacent to the ribosomal genes (Schubert & Wobus 1985, Zhang et al. 2008). Several transposable elements have been described in Triatominae (Gilbert et al. 2010), including a non-long terminal repeat retrotransposon inserted inside the 28S rDNA sequence of *R. prolixus* (Jakubczak et al. 1991). Thus, we can envisage an rDNA cluster with associated transposable elements moving through genomes, as previously postulated for other insects (Cabrero & Camacho 2008, Nguyen et al. 2010, Cabral-de-Mello et al. 2011a). Such species as *T. infestans* and *R. ecuadoriensis,* which are polymorphic for the location of rDNA, would be excellent models for the identification of transposable elements and the analysis of their dispersal mechanisms.

Ectopic recombination and rDNA transposition indicate a heterologous association between sex chromosomes. As a rule, heteropteran sex chromosomes are considered asynaptic and achiasmatic during meiotic division and do not form synaptonemal complexes (Solari 1979, Ueshima 1979, Pigozzi & Solari 2003). In all triatomine species, sex chromosomes are intimately associated from early meiotic prophase until diakinesis, forming a positive heteropycnotic body (Panzera et al. 2010). This close spatial proximity between sex chromosomes is an essential prerequisite for ectopic recombination and rDNA transposition. Thus, the existence of ribosomal *loci* on two sex chromosomes in 13 triatomine species from four different genera (*Rhodnius*, *Psammolestes, Eratyrus* and *Triatoma*) (Panzera et al. 2012, this paper) suggests that chromosomal exchanges between achiasmatic sex chromosomes are more common than previously suspected, at least in this insect group.

Moreover, regardless of which ribosomal gene location is ancestral, the inter and intraspecific rDNA variation in Rhodniini indicates that rDNA transposition is independent of taxonomic units. Furthermore, the variation suggests that the three proposed mechanisms of rDNA change, i.e., the loss of rDNA *loci* on the Y chromosome, rDNA transposition and ectopic pairing between both sex chromosomes, could have occurred several times during the karyotypic evolution of the Rhodniini tribe.

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