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Research Article

Chromosome mapping of ribosomal genes and histone H4 in the genus *Radacridium* (Romaleidae)

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Abstract

In this study, two species of Romaleidae grasshoppers, *Radacridium mariajoseae* and *R.nordestinum*, were analyzed after CMA_3/DA/DAPI sequential staining and fluorescence *in situ* hybridization (FISH) to determine the location of the 18S and 5S rDNA and histone H4 genes. Both species presented karyotypes composed of *2n* = 23, X0 with exclusively *acrocentric chromosomes.* CMA_3^+ blocks were detected after CMA_3/DA/DAPI staining in only one medium size autosome bivalent and in the X chromosome in *R. mariajoseae*. On the other hand, all chromosomes, except the L1 bivalent, of *R. nordestinum* presented CMA_3^+ blocks. FISH analysis showed that the 18S genes are restricted to the X chromosome in *R. mariajoseae*, whereas these genes were located in the L_2, S_9 and S_{10} autosomes in *R. nordestinum*. In *R. mariajoseae*, the 5S rDNA sites were localized in the in L_1 and L_2 bivalents and in the X chromosome. In *R. nordestinum*, the 5S genes were located in the L_2, L_3, M_4 and M_5 pairs. In both species the histone H4 genes were present in a medium size bivalent. Together, these data evidence a great variability of chromosome markers and show that the 18S and 5S ribosomal genes are dispersed in the *Radacridium* genome without a significant correlation.

Keywords: cytogenetics, FISH, fluorochromes, grasshoppers, rDNA.

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Introduction

The Romaleidae family accounts for more than 200 species of grasshoppers comprising three subfamilies: Romaleinae, Aucacrinae and Trybliophorinae. It is the second most diverse family of the superfamily Acridoidea occurring from semiarid regions to tropical rainforests (Carbonell, 1977; Roberts and Carbonell, 1982; Carbonell, 1984, 1986, 2002). The genus *Radacridium* is dispersed across the Northeast region of Brazil, where it seems to be well-adapted to the severely arid conditions (Carbonell, 1984). Two species are found in the state of Pernambuco: *Radacridium mariajoseae*, which is typical of the Agreste region, and *Radacridium nordestinum,* typical of the Caatinga biome (Carbonell, 1984, 1996).

Cytogenetic studies involving representatives of Romaleidae have revealed a great karyotypic conservation (Mesa *et al.*, 1982, 2004; Souza and Kido, 1995; Rocha *et al.*, 1997; Pereira and Souza, 2000). Most of analyzed species, including *R. mariajoseae* and *R. nordestinum,* presented karyotypes with 2n = 23 male, 24 female, an X0/XX sex chromosome system and exclusively acrocentric chromosomes. The constitutive heterochromatin (CH) in Romaleidae is predominantly located in the pericentromeric regions of all chromosomes, as observed in *Xyleus angulatus*, *Brasilacris gigas*, *Chromacris nuptialis*, *Radacridium nordestinum* e *Phaeoparia megacephala* (Souza and Kido, 1995; Rocha *et al.*, 1997; Pereira and Souza, 2000). Analyses with DAPI and CMA_3 in romaleids showed a predominance of GC-rich (CMA_3^+) regions. In *Xyleus angulatus* and *Xestotrachelus robustus*, CMA_3^+ blocks were observed in all chromosomes (Souza *et al.*, 1998; Souza *et al.*, 2003). In contrast, few GC-rich CH regions were observed by Loreto *et al.* (2005) in two *Chromacris* species. *C. nuptialis* had only one bivalent (M_6) with a pericentromeric CMA_3^+ block, whereas *C. speciosa* presented CMA_3^+ blocks in two autosomal bivalents, a proximal one in M_6 and a telomeric one in L_2.

A large part of the eukaryotic genome is formed by repetitive DNA, including tandem sequences mainly comprising satellite DNA and multigene families. Ribosomal DNA and histone gene families include a variable number of copies with various genome locations (Charlesworth *et al.*, 1994; Nei and Rooney, 2005). Fluorescence *in situ* hybridization (FISH) with rDNA and histone genes probes has proven useful for mapping and their location and in clarifying the genome organization of several organisms. Among invertebrates, rDNA gene mapping has been used

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in several groups, including worms (Vitturi *et al.*, 2002), mollusks (Colomba *et al.*, 2002), insects (Cabrero and Camacho, 2008; Cabral-de-Mello *et al.*, 2011a; Panzera *et al.*, 2012), echinoderms **(**Caradonna *et al.*, 2007) and others. The studies involving histone genes mapping in grasshoppers included some species of the Acrididae family (Cabrero *et al.*, 2009; Oliveira *et al.*, 2011) and four species of the Proscopiidae family (Cabral-de-Mello *et al.*, 2011b) were mapped.

This study aimed at understanding the pattern of organization of multigene families in two species of *Radacridium*. Our results showed a great variability in the number and location of rDNA clusters in both species, whereas the histone H4 genes were highly conserved in number. These results are compared with data from other grasshopper species and are discussed based on the possible mechanisms involved in repetitive DNA diversification.

Material and Methods

We analyzed ten individuals of *R. mariajoseae* from Gravatá (08º12'04" S; 35º33'53" W) and Bezerros (08º14'00" S; 35º47'49" W) and ten adult males of *Radacridium nordestinum* from Surubim (07º49'59" S; 35º45'17" W), all located in the Agreste region of the state of Pernambuco, Brazilian Northeast. Specimens were processed and their testes were fixed in Carnoy solution (3:1 ethanol:acetic acid). Chromosome preparations were obtained by the testicular follicles squashing technique, in one drop of 45% acetic acid. The coverslips were removed after liquid nitrogen immersion.

CMA_3/DA/DAPI sequential staining was performed according to Schweizer (1976). The slides were stained with CMA_3 (0.5 mg/mL) for one hour, washed with distilled water and stained with DA (Distamicine A, 0.1 mg/mL) for 45 min. The slides were rewashed and stained with DAPI (2 µg/mL) for 20 min and mounted in glycerol/McIlvaine buffer/$MgCl_2$.

Probes were obtained by PCR performed according to Ayres (2002), with modifications, using genomic DNA from both species and the primers: 18S DNAr - Sca18S1F (F 5' - CCC CGT AAT CGG AAT GAG TA - 3'); Sca18S1R (R 5'- GAG GTT TCC CGT GTT GAG TC - 3'), 5SrDNA F(5'- AAC GAC CAT ACC ACG CTG AA - 3'); R (5'- AAG CGG TCC CCC ATC TAA GT - 3'), Histone H4 F-1 (5' - TSC GIG AYA ACA TYC AGG GIA TCA C - 3') and R-1 (5' - CKY TTI AGI GCR TAI ACC ACR TCC AT - 3'). The PCR products analyzed after electrophoresis in a 1% agarose gel had the expected sizes. The 18S rDNA and histone H4 probes were labeled with biotin-16-dUTP and the 5S rDNA probe, with digoxigenin-11-dUTP.

FISH was performed according to Cabral-de-Mello *et al.* (2011c), with some modifications. The chromosome preparations were submitted to an alcohol series pretreatment and then treated with RNase and pepsin. The hybridization mix contained 1 µL of probe and the denaturation was performed in a humid chamber at 75 °C, followed renaturation overnight at 37 °C. Immunodetection was performed with mouse anti-biotin (M743, DAKO) and rabbit anti-mouse-TRITC (R270, DAKO) for biotin and sheep anti-digoxigenin (Roche 1 207 741) and sheep anti-rabbit-FITC (DAKO-F0135) for digoxigenin. Chromosomes were counterstained with 4,6 diamidine-2-phenyl indole (DAPI) and the slides were mounted with Vectashield (Vector). Images were obtained in a Leica epifluorescence microscope, captured with the CW4000 program (Leica) and adjusted in Adobe Photoshop CS5.

Results

Radacridium mariajoseae and *R. nordestinum* presented 2n = 23(male), a sex determination mechanism of the X0(male) type and exclusively acrocentric chromosomes. Chromosomes of both species were grouped in three large (L_1-L_3), five medium (M_4-M_8) and three small (S_9-S_{11}) pairs. The X chromosome was a medium size acrocentric in both species. CMA_3/DA/DAPI sequential staining showed GC-rich (CMA_3 positive) constitutive heterochromatin (CH) blocks in the interstitial region of only one medium-sized bivalent (M_5) and in the pericentromeric region of the X chromosome in *R. mariajoseae* (Figure 1a). In *R. nordestinum*, CMA_3^+ blocks were present in the interstitial region of the L_2 bivalent and in the pericentromeric region of all chromosomes, except the L_1 bivalent (Figure 1c). DAPI staining was homogeneous in both species (Figure 1b and d).

After FISH, the 18S rDNA probe labeled a single site in the pericentromeric region of the X chromosome in *R. mariajoseae* (Figure 2a) and three pericentromeric sites, in the bivalents L_2, S_9 and S_{10} of *R. nordestinum* (Figure 2b). The 5S rDNA genes were located in the pericentromeric region of the two largest bivalents (L_1 and L_2) and in the X chromosome of *R. mariajoseae*, (Figure 2c) and in the bivalents L_2, L_3 M_4 and M_5 of *R. nordestinumm* (Figure 2d). The histone H4 probe was mapped in a proximal location in a medium-sized autosome bivalent (M_5) in both species (Figure 2e and f).

Discussion

The karyotypic similarities detected after conventional analysis between the two species of *Radacridium* did not extend to their CMA_3 and DAPI staining patterns. Our results showed GC-richness CH heterogeneity. In *R. mariajoseae* only one autosome pair and the X chromosome presented CMA_3^+ blocks, which resembled the scarcity in GC-rich regions of other Romaleidae, as two species of *Chromacris* with a single CMA_3^+ block (Loreto *et al.*, 2005). The presence of a large number of CMA_3^+ blocks is more frequent in Romaleidae, as observed in *Xyleus*

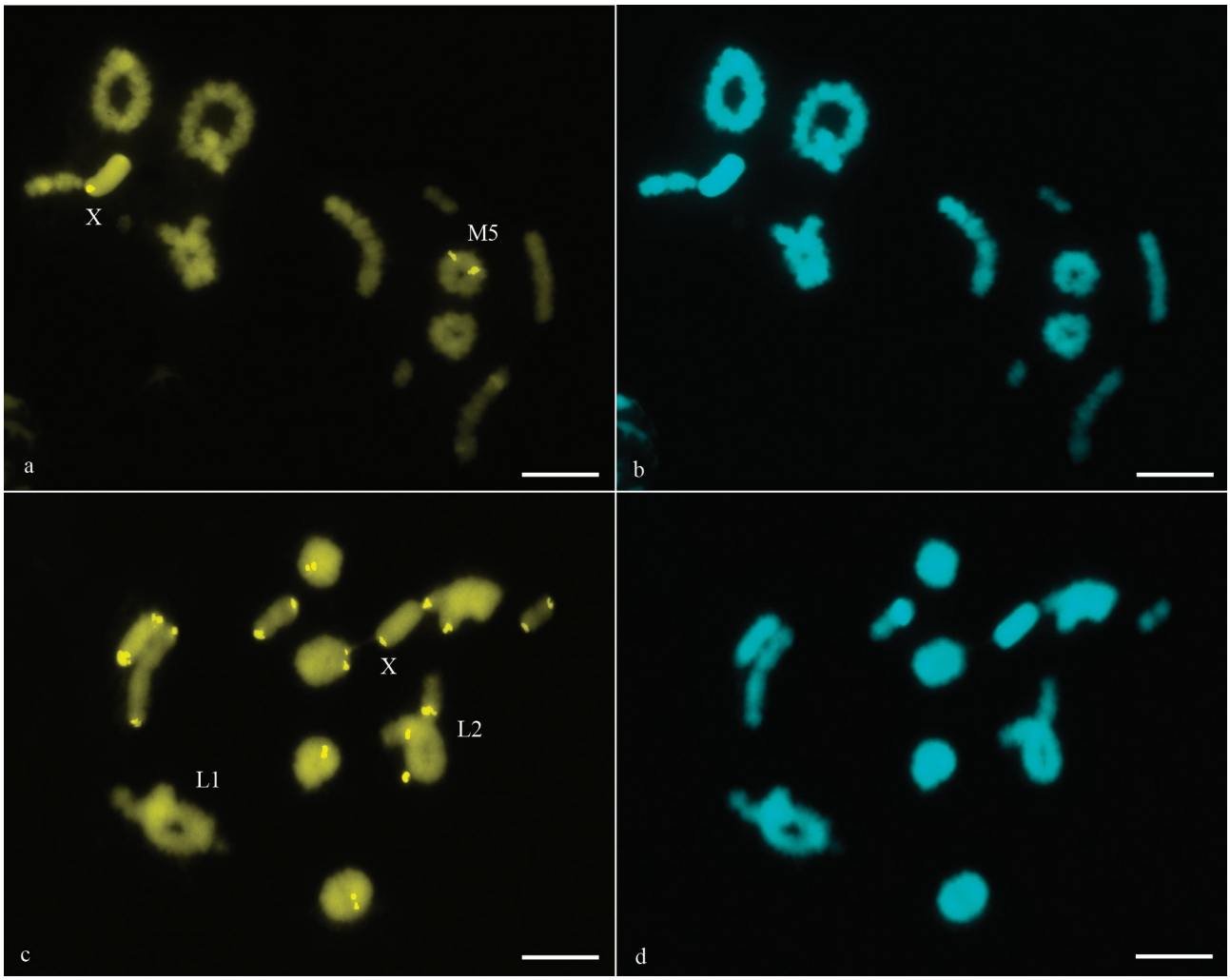

Figure 1 - CMA_3/DA/DAPI sequential staining in *R. mariajoseae* (a-b) and *R. nordestinum* (c-d). In (a) CMA_3^+ blocks are only present in the X chromosome and in the M_5 bivalent. In (c) all chromosomes, including the X chromosome, show CMA_3^+ blocks, except the L_1 bivalent. DAPI staining was homogeneous for both species (b-d).Scale bar = 10 μm.

angulatus, *Phaeoparia megacephala* and *Xestotrachelus robustus*, in which all CH was shown to be GC-rich (Souza *et al.*, 1998; Pereira and Souza, 2000; Souza *et al.*, 2003).

A great difference in the 18S and 5S rDNA distribution was observed between the species studied. *R. mariajoseae* presented a single 18S rDNA site in the X chromosome, confirming the finding of only one active nucleolus organizer region (NOR) after silver nitrate impregnation ($AgNO_3$) by Rocha *et al.* (1997). Although three autosome bivalents showed 18S hybridization signals in *R. nordestinum*, only the L_2 pair had a corresponding active NOR identified by Rocha *et al.* (1997). These data indicate a possible dispersion of the 18S rDNA sequences, which may have been caused by structural chromosome rearrangements, ectopic recombination and transpositions (Cabrero and Camacho, 2008). These kind of events have been observed in other insects (Nguyen *et al.*, 2010; Cabral-de-Mello *et al.*, 2011c). In the genus *Radacridium*, the ancestral rDNA site could have been present in the M_9 bivalent in a common ancestor. Two points support this hypothesis: the presence of a 18S rDNA site in this pair in *R. nordestinum*, which was considered as a megameric chromosome by Rocha *et al.* (1997) and the preferential localization of NORs is in this type of chromosome in several species of grasshoppers (Rufas *et al.*, 1985). The 18S rDNA probe location in the X chromosome of *R. mariajoseae* could have resulted from a chromosome rearrangement, such as a translocation or transposition, that moved it from M_9 to the X, as a meiotic association between this chromosome and the megameric one has been described (Loreto *et al.*, 2008a).

18S rDNA sites restricted to autosomes, as observed in *R. nordestinum*, were also detected in *Xestotrachelus robustus*, *Chromacris nuptialis* and *C. speciosa* (Souza *et al.*, 2003; Loreto *et al.*, 2005). On the other hand, 18S rDNA sites located both in autosomes and in the sex chromosome have been described, for example, in *Xyleus discoideus angulatus* (Souza *et al.*, 1998; Loreto *et al.*,

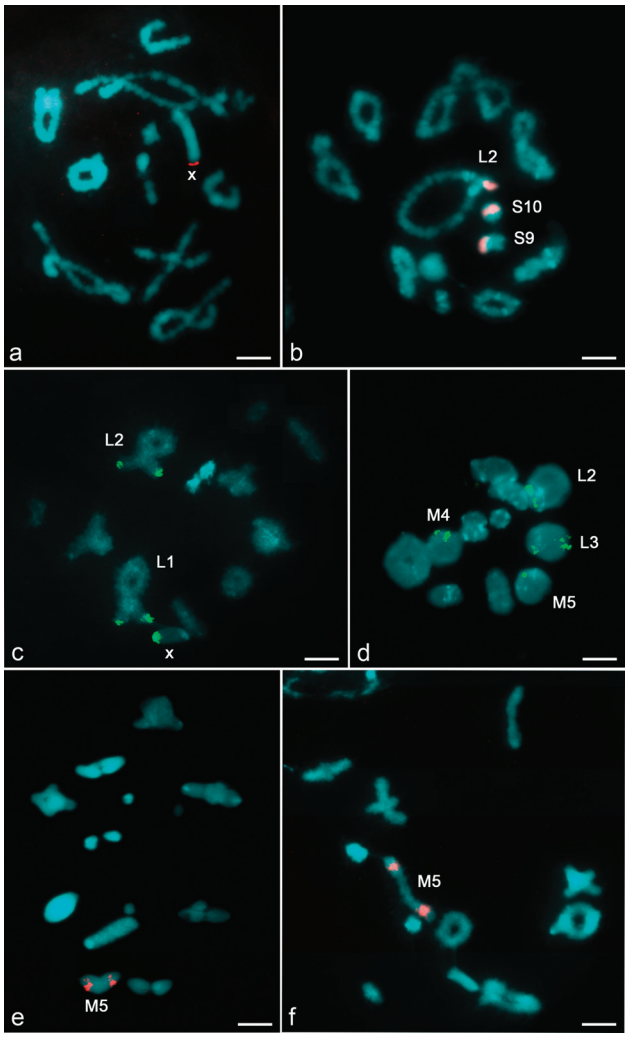

Figure 2 - Fluorescence *in situ* hybridization of 18S, 5S rDNAs and histone H4 in *R. mariajoseae* (a-c-e) and *R. nordestinum* (b-d-f). (a) The 18S rDNA in *R. mariajoseae* is present in a single site on the X chromosome, whereas in (b) the 18S rDNA in *R. nordestinum* is present at three sites. (c-d) 5S rDNA in *R. mariajoseae* and *R. nordestinum*, respectively, showing an extensive variation. (e-f) Note the same histone H4 hybridization pattern in both species. Scale bar = 10 μm.

2008b). 18S rDNA sites have been frequently observed associated with GC-rich regions in Romaleidae (Pereira and Souza, 2000; Loreto *et al.*, 2005), Acrididae (Loreto and Souza, 2000; Rocha *et al.*, 2004), Proscopiidae (Souza and Moura, 2000) and Ommexechidae (Carvalho *et al.*, 2011), a pattern also observed in the two species analyzed herein.

The histone H4 genes were located in a single chromosome pair (M_5) in both species. A single chromosome pair bearing histone genes was also described in other species of grasshoppers. Cabrero *et al.* (2009) analyzed the location of the histone H3 and H4 genes in 35 species of grasshoppers from the Acrididae family. They observed that the great majority of species analyzed showed only one histone site, located in an autosome pair. In the same work, double FISH performed in 11 randomly chosen species revealed that, in all cases, both genes were present in the same chromosome site, indicating a great conservation of histone gene location in Acrididae. Cabral-de-Mello *et al.* (2011b), using a histone H3 probe in four species of Proscopiidae (*Tetanorhynchus silvai*, *Scleratoscopia protopeirae*, *S. spinosa* and *Stiphra robusta*), observed a single site in the M4 bivalent in all the species. Oliveira *et al.* (2011) observed multiples sites of histone H3 in *Rhammatocerous brasiliensis* (Acrididae) and concluded that this would be a derived condition and the presence of a single histone site, as observed in both species studied herein, would be the ancestral form.

The 5S and the 18S genes co-localized in the L_2 chromosome of *R. nordestinum* and in the X chromosome of *R. mariajoseae*. Similarly to what we observed in both *Radacridium* species, an extensive variation in 5S rDNA distribution has been found in others grasshoppers, in which species presenting single sites and extending to all chromosome pairs have been described (Loreto *et al.*, 2008b; Cabral-de-Mello *et al.*, 2011a).

The results obtained in this study indicate a great level of karyotypic differentiation between *R. mariajoseae* and *R. nordestinum*. These data reinforce the fact that the high conservation observed at the chromosome level, including chromosome number and morphology, in *Radacridium* and other romaleids, is not reflected at the genomic level. Our results also contribute to the understanding of chromosome evolution patterns in the family Romaleidae.

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