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A comparative cytogenetic analysis between the grasshopper species *Chromacris nuptialis* and *C. speciosa* (Romaleidae): constitutive heterochromatin variability and rDNA sites

Vilma Loreto[1,†], Eva Stadtler[1], Natoniel F. de Melo[2] & Maria José de Souza[1]
[1]*Departamento de Genética, CCB, Universidade Federal de Pernambuco (Phone: +55-81-2126-8520; E-mail: vloreto@zipmail.com.br),* [2]*Embrapa Semi-árido, Petrolina, Brasil,* [†]*Av. Prof. Moraes Rego s/n Cidade Universitária, Recife, PE, 50732-970, Brasil*

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Abstract

The chromosomes of *Chromacris nuptialis* and *C. speciosa* were comparatively analyzed using different cytogenetic techniques, in order to determine the level of karyotypic similarities and differences between the species. The results show similarities in chromosome number ($2n = 23$,X0) and acrocentric morphology. In some *C. nuptialis* individuals meiotic irregularities were detected involving the L_2 bivalent. This bivalent was delayed and presented anaphasic bridges and other aberrations. Differences in constitutive heterochromatin (CH) patterns and composition were observed through C-banding and fluorochromes staining. Silver nitrate staining revealed a single medium nucleolar organizer regions (NORs) pair, per species. Differences were also observed in NORs location, which was pericentromeric in *C. nuptialis* and proximal in *C. speciosa*. FISH using an rDNA probe confirmed the existence of ribosomal sites coinciding with active regions visualized by silver nitrate. The possible implications of the karyotype differences observed between both species are discussed.

Introduction

Grasshoppers of the genus *Chromacris* are restricted to the Neotropical region and are found from Mexico to Argentina. These insects present strong colors with yellow or red stains, on their wings. Based on those characteristics, Roberts and Carbonell (1982) divided the genus *Chromacris* in two groups: one called trogon, that includes *C. trogon*, *C. psittacus*, *C. icterus* and *C. peruviana*, and another called colorata that consists of *C. colorata*, *C. minuta*, *C. miles*, *C. speciosa* and *C. nuptialis*. The latter taxon is related to *C. speciosa*, which was initially considered to be a variant form. However, the study of some morphological traits has indicated that it is a distinct species (Roberts & Carbonell, 1982).

Romaleidae family has shown a marked predominance of conserved karyotypes ($2n = 23,24$ X0:XX) (Mesa, Ferreira & Carbonell, 1982). Nevertheless, analysis of constitutive heterochromatin (CH) by C-banding has revealed both variations in terms of the quantity and location of CH blocks, among different species. Pericentromeric location has been the most frequent having been observed in seven out of eight species studied so far (Vilardi, 1986; Souza & Kido, 1995; Pereira & Souza, 2000; Souza, Haver & Melo, 2003). Additionaly, ocurrence of interstitial and distal blocks has been reported for *Xyleus angulatus* (Souza & Kido, 1995; Souza, Rufas & Orellana, 1998), *Radacridium mariajoseae*, *R. nordestinum* (Rocha, Souza & Tashiro, 1997), and *Chromacris speciosa* (Souza & Kido, 1995). Polymorphisms for supernumerary heterochromatic segments have been described for *X. angulatus* (Souza & Silva-Filha, 1993) and *R. nordestinum* (Rocha, Souza & Tashiro, 1997) and B chromosomes have been observed in *X. angulatus*

(Souza & Kido, 1995) and *Zoniopoda tarsata* (Vilardi, 1986), proving the intensive variability in CH, among representatives of this family.

As to cytogenetic characteristics, knowledge about the genus *Chromacris* is limited to the studies of Mesa, Ferreira and Carbonell (1982) who described the karyotype of three species (*C. speciosa*, *C. peruviana* and *C. miles*) as $2n = 23,X0$ and $2n = 24,XX$ and Souza and Kido (1995) that analyzed the variability in CH distribution in *C. speciosa* by C-banding.

Some studies have shown that comparative cytogenetic analysis of grasshoppers can contribute to the understanding of the speciation process (Bella et al., 1990). Bridle et al. (2002) emphasized the close karyotype similarity between *Chorthippus brunneus* and *C. jacobsi*, when analyzing C-banding patterns and CH composition by fluorochrome labeling. However, Rodríguez-Iñigo, Bella and García de la Vega (1993), analyzing two species of the genus *Dociostaurus*, and Camacho and Cabrero (1983), analyzing the species *Acrotylus insubricus* and *A. patruelis*, showed differences in the patterns and composition of CH between related species, indicating that both loss or acquisition of heterochromatin might be involved in karyotype evolution.

In the present study, we performed a comparative cytogenetic analysis between *C. nuptialis* and *C. speciosa* by conventional analysis, C-banding, fluorochrome and silver nitrate staining, and fluorescence *in situ* hybridization (FISH) using a 45S rDNA probe, in order to determine the level of similarities and differences between these species.

Material and methods

A total of 16 adult male specimens of *C. nuptialis* collected in the locality of Exu (7°26′0″ S 39°50′36″ W) and 25 specimens of *C. speciosa* collected in Moreno (8°7′7″ S 35°5′32″ W) (15 males and 3 females) and Igarassú (7°50′3″ S 34°54′23″ W) (5 males and 2 females), State of Pernambuco, Brazil, were studied. The grasshoppers were brought to the laboratory, dissected and the testes and ovaries were fixed in ethanol:acetic acid (3:1). Females were injected with 0.1% colchicine for 6 h. Slides were prepared using the classical testicular and ovarian follicles squashing technique, followed by staining with 2% lacto-acetic orcein for conventional chromosome analysis. For the different banding techniques, the slides were prepared by the addition of one drop of 45% acetic acid and were covered with coverslips that were removed after freezing in liquid nitrogen.

C-banding and CMA_3/DA/DAPI staining

C-banding was performed according to Sumner (1972). Slides aged for 2 days were treated with 0.2 N HCl for 30 min, 5% barium hydroxide (60°C) for 45 s, and 2× SSC (60°C) for 45 min. After drying, the preparations were stained with Giemsa 5% diluted in phosphate buffer, pH 7.0, for 3 min. For CMA_3/DA/DAPI staining (Schweizer et al., 1983), the slides were aged for 3 days, stained with CMA_3 (0.5 mg/ml in McIlvaine buffer, pH 7.0) for 1 h, washed in distilled water, stained with Distamycin A (DA) (0.1 mg/ml) for 45 min, washed again, and stained with DAPI (0.5 µg/ml) for 20 min.

Silver nitrate staining ($AgNO_3$) and fluorescence in situ hybridization (FISH)

Cytological preparations were pretreated with 2× SSC at 60°C for 10 min, washed, dried, stained with one drop of $AgNO_3$ solution (1:1) in distilled water (with the pH adjusted with formic acid), and then incubated in a humid chamber at 70°C for 3 min (Rufas, Esponda & Gosálvez, 1985). Some preparations were counterstained with Giemsa before mounting.

For FISH, a probe containing fragments of the 45S ribosomal genes (18S–5.8S–25S) of *Arabidopsis thaliana* (Unfried, Stocker & Gruendler, 1989; Unfried & Gruendler, 1990) was used to localize rDNA sequences. The probe was labeled with biotin–11-dUTP by nick translation, and the preparations were incubated with RNAse, proteinase K, $MgCl_2$/PBS and paraformaldehyde/PBS solutions, and finally dehydrated in an increasing alcohol series (70–90–100%, 5 min each) according to Moscone, Matzke and Matzke (1996). Hybridization was performed at 80°C using 5 µl of the mixture. The slides were kept at 37°C overnight for renaturation. The probe was detected with an anti-biotin-rhodamine antibody. The chromosomes were counterstained with DAPI and the slides were mounted in Antifade Vectashield (Vector) medium.

Photographs were taken with a Leica microscope using Kodak ISO 25 films, TMAX–400 and Fuji Film ISO–400 for FISH, and printed on Kodak Kodabrome Print F3 paper.

Results

The diploid number of *Chromacris speciosa* and *C. nuptialis* was $2n = 23,X0$, in males and $2n = 24,XX$, in females. Both species present acrocentric chromosomes which were grouped in two large sized one (L_1–L_2), six medium (M_3–M_8) and three small pairs (S_9–S_{11}). The X chromosome was medium sized (Figure 1a-f). In both species, the X presented a variable heteropycnotic behavior, positive from leptotene to diplotene and negative during diakinesis and metaphase I (Figure 1a–c).

Our cytogenetics studies in the 16 individuals of *C. nuptialis* confirmed that six of them have meiotic irregularities likely the stickiness. The L_2 pair presented anaphasic delay and atypic bridges formation through the union of the homologous chromatids, in terminal position (Figure 2a–b). In that region a block of constitutive heterochromatin (CH) was observed close to the sticky point (Figure 2b). In some cells the behavior of the laggard pair may indicate that it has not been incorporated to the new formed nucleus (Figure 2c). The presence of chromosomal fragments (Figure 2d) might have originated from the separation of telophasic cells

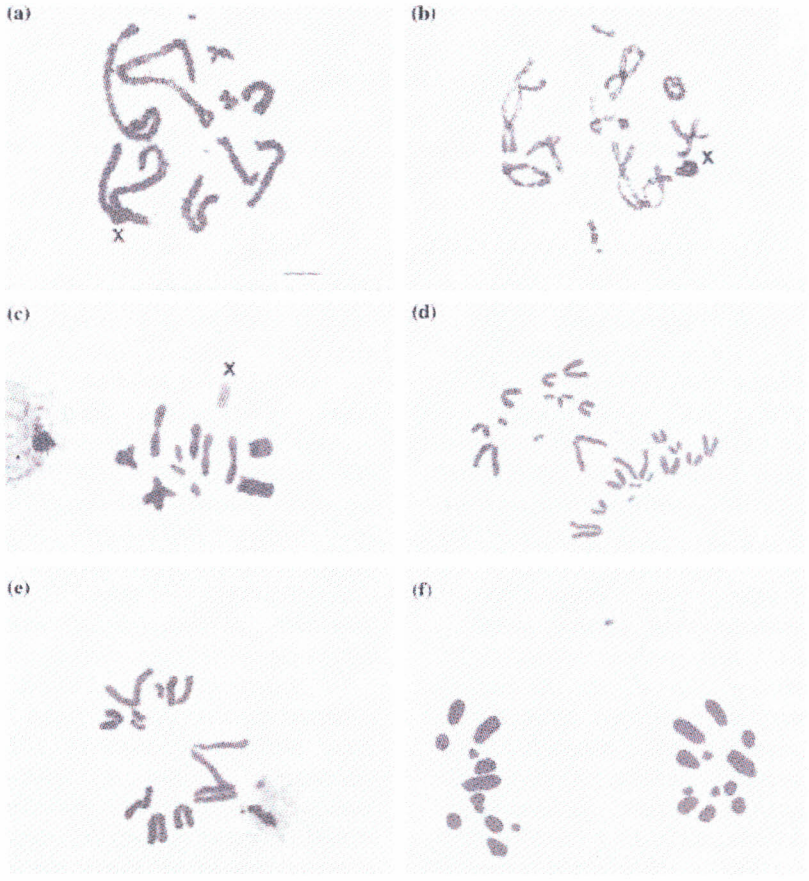

Figure 1. Meiotic cells of *Chromacris nuptialis* (a,b,d,f) and *C. speciosa* (c and e). (a) Pachytene, (b) Diplotene, (c) Metaphase I, (d) Anaphase I, (e) Metaphase II with 11 chromosomes, and (f) Anaphase II with 12 chromosomes. Note the X chromosome heteropycnotic positive (a and b) and negative (c). Bar = 10 μm.

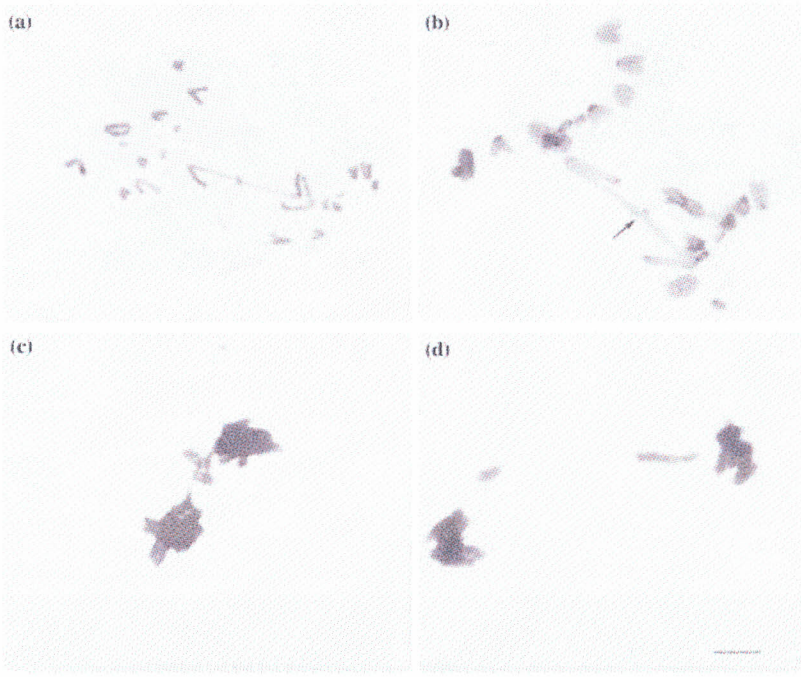

Figure 2. Irregularities in the meiosis I of *C. nuptialis*. (a and b) Anaphase showing the bridge formation among homologous chromatids of bivalent L_2. Note in (b) the terminal block of CH in L_2 (arrow). (c and d) Telophase showing the delayed pair and the union among chromatids resulting in fragments. Bar = 10 μm.

and the breaking of bridges. Out of the 647 cells in anaphase and telophase I from three individuals analyzed, 15 (2.3%) presented bridge formation and 632 (97.7%) were normal.

C-banding allowed the visualization of distinct CH distribution patterns in the two species. *C. nuptialis* exhibited CH blocks in the pericentromeric region of most chromosomes, while the small pairs presented variable CH blocks in location and size. It was visualized an interstitial, a telomeric and a small block and apparently pericentromeric in the S_9, S_{10} and S_{11} bivalents, respectively. A block was also observed in the telomeric region of the L_2 pair (Figure 3a).

On the other hand, *C. speciosa*, presented a highly variable CH, in terms of size and block position. C-banding of mitotic metaphases revealed pericentromeric CH in all chromosomes. However, on M_3, M_4, M_5 and M_6 pairs CH blocks covered a marked portion of the proximal part of the long arm. A telomeric C-band was observed on the L_1 and L_2 pairs, and a large interstitial block was seen in the M_8 pair. A small intersticial block was also noted on the M_5 and M_7 pairs, as well as in the X chromosome (Figure 3c). This pattern was detected in meiotic cells (Figure 3b).

The CMA_3/DA/DAPI staining revealed different patterns in the species. Whereas *C. nuptialis* presented only one bivalent (M_6) with a CMA_3^+ block in the pericentromeric region (Figure 3d), *C. speciosa* had two CMA_3^+ blocks, one in the proximal portion of M_6 and another in the telomeric region of L_2 (Figure 3f). DAPI staining was homogenous in the two species (Figure 3e,g).

Silver nitrate staining identified only one NOR, in the medium pair (M_6) of both species (Figure 4a, c). *In situ* hybridization with the rDNA probe confirmed the existence of only one pair of ribosomal sites per species (M_6) and these sites were located in different regions of the chromosomes, with a pericentromeric location being observed for *C. nuptialis* (Figure 4b) and a proximal location for *C. speciosa* (Figure 4d). In both cases, the nucleolar organizer regions (NORs) coincided with the CMA_3^+ heterochromatin regions.

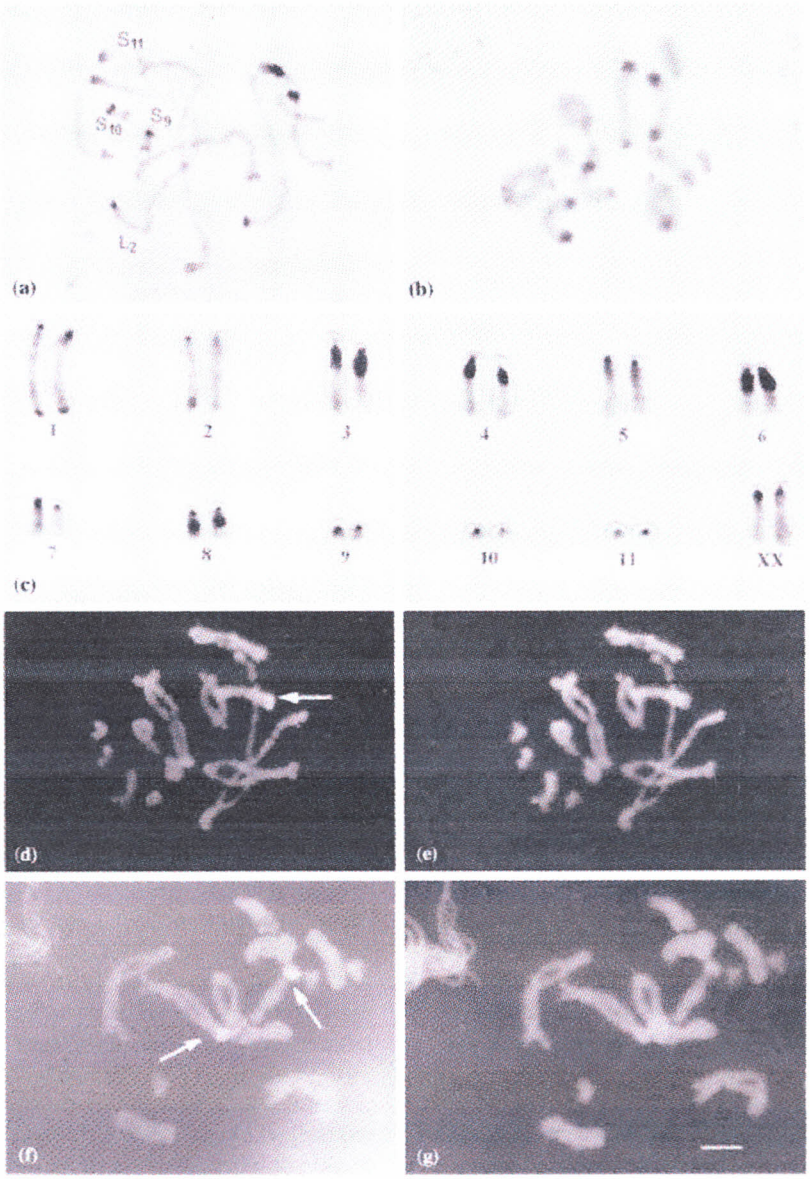

Figure 3. C-banding pattern in *C. nuptialis* (a) and *C. speciosa* (b and c). Note the CH blocks on the small pairs and in L_2. CMA_3/DA/DAPI staining of diplotene cells from *C. nuptialis* (d and e) and *C. speciosa* (f and g). The arrows indicate the CMA_3^+ blocks. Bar = 10 μm.

Discussion

Karyotypes of *C. nuptialis* and *C. speciosa* resemble those reported for other *Chromacris* species and for most romaleid species, as to chromosome number ($2n$ = 23,X0 and $2n$ = 24,XX) and morphology (acrocentric) (Mesa, Ferreira & Carbonell, 1982; Souza & Kido, 1995; Rocha, Souza & Tashiro, 1997). One exception to this uniformity has been described for *Xestotrachelus robustus* ($2n$ = 23,X0 and $2n$ = 24,XX), a species taxonomically related to *Chromacris*, where the S_9 and S_{10} pairs are meta-submetacentric and probably originated from a pericentric inversion, since the rearrangement did not change the chromosome number of the species (Souza, Haver &

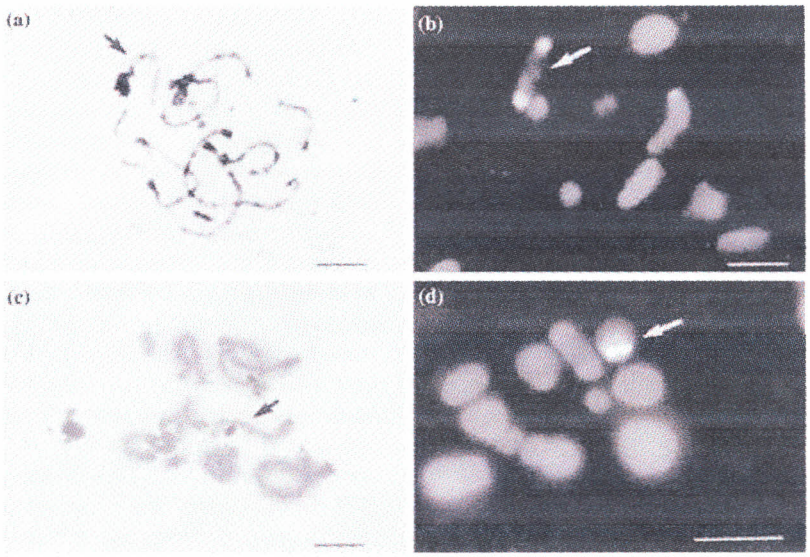

Figure 4. Identification of nucleolar organizer regions (NORs) and rDNA sites in *C. nuptialis* and *C. speciosa* by silver nitrate staining (a and c) and FISH (b and d), respectively. The arrows indicate the M_6 pair with NORs in the pericentromeric region of *C. nuptialis* (a and b) and in the proximal region of *C. speciosa* (c and d). Bar = 10 μm.

Melo, 2003). Indeed, romaleid species have shown a highly conserved karyotype, with only a few cases of rearrangements being known (X-autosome and autosome–autosome fusion), what lead to a reduction in chromosome number (Mesa, Ferreira & Carbonell, 1982).

Cellular events as paracentric inversion (Koehler et al., 2002), cohesion loss among chromatid-sisters (Cimini et al., 2003) and failure in the checkpoint of the metaphase/anaphase control (LeMaire-Adkins, Radke & Hunt, 1997) has been described in some organisms. However, the detailed analysis of meiotic aberrations in *C. nuptialis* does not seem to indicate any of those events. A possible explanation for the abnormalities would be that the sticky among homologous chromatids to the block of CH of L_2 is maintained by proteins connection in the heterochromatin. Stickness has been described in polytene chromosomes of *Drosophila* and *Chironomus*, in mitotic chromosomes of maize callus culture (Fluminhan, Aguiar-Perecin & Dos Santos, 1996) and in *Ornithogalum longibracteatum* (Pedrosa et al., 2001), where the existence of proteic factors, associated with CH, was admitted.

The karyotype similarity detected by conventional analysis between romaleid species was not observed when comparing CH patterns. No similar pattern has been identified between the few species studied thus far. Despite a predominance of pericentromeric CH, a wide variability in size, block locations (telomeric, interstitial and proximal) and the presence of an extra CH as supernumerary segments and B chromosomes, have been reported (Vilardi, 1986; Souza & Kido, 1995; Rocha, Souza & Tashiro, 1997; Pereira & Souza, 2000; Souza, Haver & Melo, 2003). When CH patterns of *C. nuptialis* and *C. speciosa* were compared with those of *Xestotrachelus robustus*, a closer similarity was observed between *C. nuptialis* and *X. robustus* due to the predominance of pericentromeric CH. However, differences were detected in the small pairs. Whereas *C. nuptialis* possesses an interstitial block in S_9, a telomeric in S_{10} and a pericentromeric one in S_{11}, in *X. robustus* the S_9 and S_{10} chromosomes are entirely heterochromatic (Souza, Haver & Melo, 2003). *Chromacris speciosa* showed even a more particular pattern consisting of large pericentromeric CH blocks, on the large and medium bivalents, and a diversity of proximal, telomeric and interstitial blocks. This divergence of C-banding pattern in *Chromacris* indicates that heterochromatin rearrangements (amplification for example) may be involved in the karyotypic evolution of the group.

Comparison of the results obtained by CMA_3/DA/DAPI staining for *C. nuptialis* and *C. speciosa*

also revealed some differences. *Chromacris nuptialis* had only one CMA_3^+ pericentromeric block, while two blocks (a proximal and a telomeric one) were observed in *C. speciosa*. This scarcity of GC base pairs in CH contrasts with data reported for other romaleid species, in which CMA_3^+ blocks predominate (Souza, Rufas & Orellana, 1998; Pereira & Souza, 2000; Souza, Haver & Melo, 2003). John et al. (1985), analyzing ten Australian acridid grasshoppers, identified distinct classes of CH, when cytological preparations of these species were stained with CMA_3 and DAPI fluorochromes. In *Recitropis* sp.1 a part of CH was CMA_3^+, while the other was homogenous for these fluorochromes. On the other hand, *Ursina* sp. showed three classes, CMA_3^+, $DAPI^+$ and a third one without specificity. Two categories of CH could be distinguished in the species analyzed here, one GC-rich and the other, a more frequent, one without any specific richness.

The location of NORs in representatives of the Romaleidae family can be divided into three categories: those restricted to autosomes, as observed in *Xestotrachelus robustus* (pericentromeric region of M_5) (Souza, Haver & Melo, 2003), *Radacridium nordestinum* (interstitial region of the L_2 bivalent) (Rocha, Souza & Tashiro, 1997) and *Phaeoparia megacephala* (proximal region of M_6) (Pereira & Souza, 2000); those located on the sex chromosome, as in *Radacridium mariajoseae* (proximal site of the X) (Rocha, Souza & Tashiro, 1997); and those with NORs on both autosomes and allosomes, as in *Xyleus angulatus*, with proximal sites on the L_3 and M_4 bivalents and the X chromosome (Souza, Rufas & Orellana, 1998).

Chromacris nuptialis and *C. speciosa* had only one nucleolar organizer pair, as observed for most romaleid species analyzed, but they differed in terms of the NOR position, which was pericentric in *C. nuptialis* and proximal in *C. speciosa*. Since the NOR is located on the same pair (M_6) in those species, we may suppose that some event (paracentric inversion, heterochromatin amplification or unequal crossing-over) might have led to the modification of the original pericentric position to the proximal one, observed in *C. speciosa* during the course of its evolution. Paracentric inversion was hyphothesized as the rearrangement involved in karyotypes differences for NORs location in two fish species. The location was in pericentromeric and subtelomeric region, in *Umbra pygmaea* and *U. limi*, respectively (Rab et al., 2002). Moreover, amplification was pointed out as the first event, in the different NORs location in Triticeae genomes (Dubcovsky & Dvorak, 1995).

FISH using 45S rDNA probe confirmed the existence of only one nucleolar organizer pair in the two species and helped to clarify the position of rDNA sites, which are sometimes hidden by nucleolar remnants, when visualized by silver nitrate. This result is similar to the one reported by Souza, Haver and Melo (2003) for *X. robustus* showing only one sign on M_5 pair. Since FISH coincided with the proximal region of the M_6 bivalent (CMA_3^+) of *C. speciosa*, and no sign was observed in the CMA_3^+ telomeric region of L_2, the CH in this region, although GC-rich, is likely to show no functional relationship with rDNA sites, and it might be organized differentially from the other CH regions.

Our results indicated a significant degree of chromosome differentiation between *C. nuptialis* and *C. speciosa*, when comparing the distribution pattern and composition of CH, as well as the position of the rDNA sites. The karyotype differences observed indicate a distinction in taxonomic status between species, confirming the analysis of some morphological traits, performed by Roberts and Carbonell (1982), who demonstrated that *C. nuptialis* is not a variant form of *C. speciosa*. However, only cytogenetic and molecular analysis of other members of colorata and trogon groups will determine whether the differences observed are frequent among these representatives, or whether *C. speciosa* and *C. nuptialis* can be included in distinct groups.

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