Constitutive heterochromatin, NOR location and FISH in the grasshopper *Xyleus angulatus* (Romaleidae)

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SUMMARY - C-banding, fluorochrome staining, silver staining and fluorescence *in situ* hybridization (FISH) were used to characterization of the meiotic chromosomes in the grasshopper *Xyleus angulatus*. The C-banding pattern showed perincentromeric heterochromatin blocks in all chromosomes, interstitial blocks in the L_1 , L_2 and L_3 pairs and distal blocks in medium and small-sized pairs. CMA_3/DA staining revealed that these heterochromatic regions were characterized by high content of GC bases pairs. The ribosomal DNA probe (pTa71) showed strong hybridization signal in the pericentromeric regions at bivalents L_3, M4 and X chromosome, corresponding to the silver-stained chromosomal positions of active NORs and the heterochromatic area CMA_3 + of these chromosomes.

Key words: grasshopper, heterochromatin, NORs, FISH.

INTRODUCTION

In recent years many studies have been performed on the banding patterns in the chromosomes of several grasshopper species, by means of different fluorochromes. The use of specific fluorochromes for regions rich in AT base pairs (DAPI) and GC base pairs (chromomycin A_3) has allowed a better characterization of the heterochromatin heterogeneity (SCHWEIZER 1976; GOSALVEZ *et al.* 1988; BELLA *et al.* 1993). Heterochromatic genomic parts revealed by conventional C-banding can also be further differentiated by double staining with chromomycin A_3 and distamycin A (CMA_3/DA). C-banding data, in turn, has shown that the constitutive heterochromatin in grasshoppers has different chromosome locations and different reactions to this technique (JOHN and KING 1977; KING and JOHN 1980; CAMACHO *et al.* 1984; SUJA *et al.* 1993) revealing the variability of these regions. The aim of this paper was to report observations on the primary spermatocytes after Cbanding and fluorescence banding (CMA_3/DA), and silver staining. We also

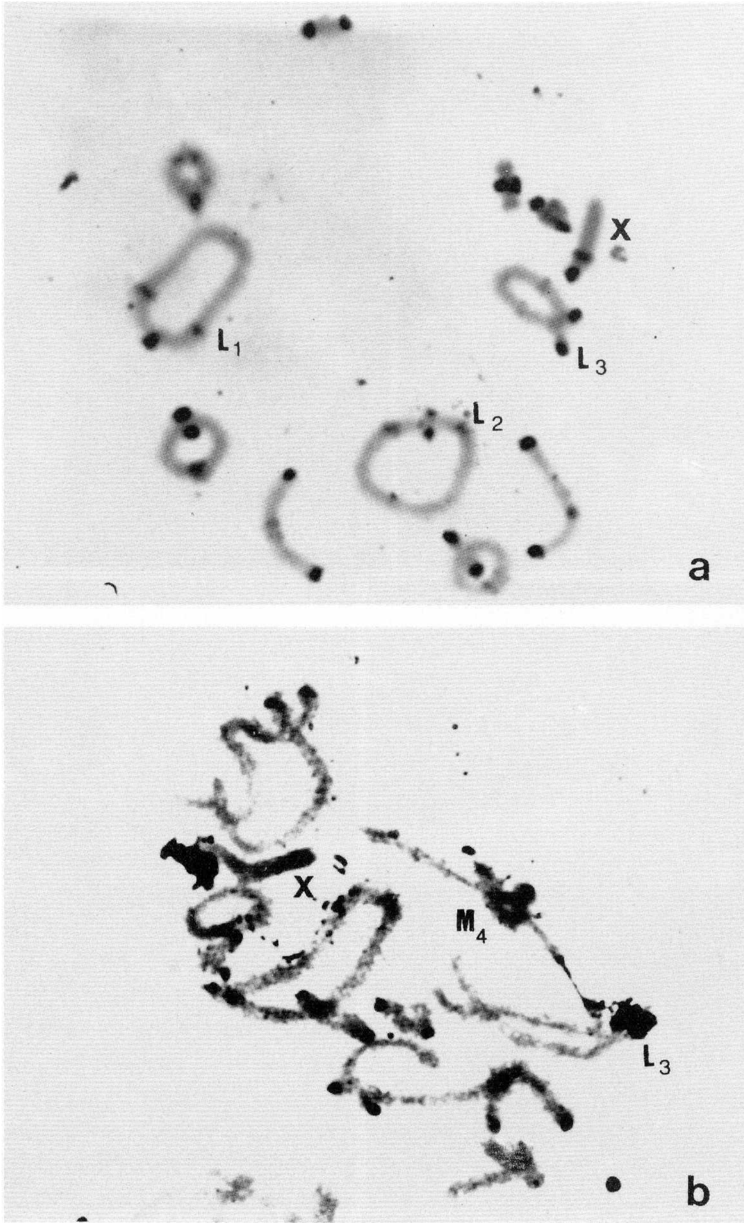
presents the first results of a molecular investigation obtained by the fluorescence *in situ* hybridization (FISH) for rDNA sequences in X. *angulatus.*

MATERIAL AND METHODS

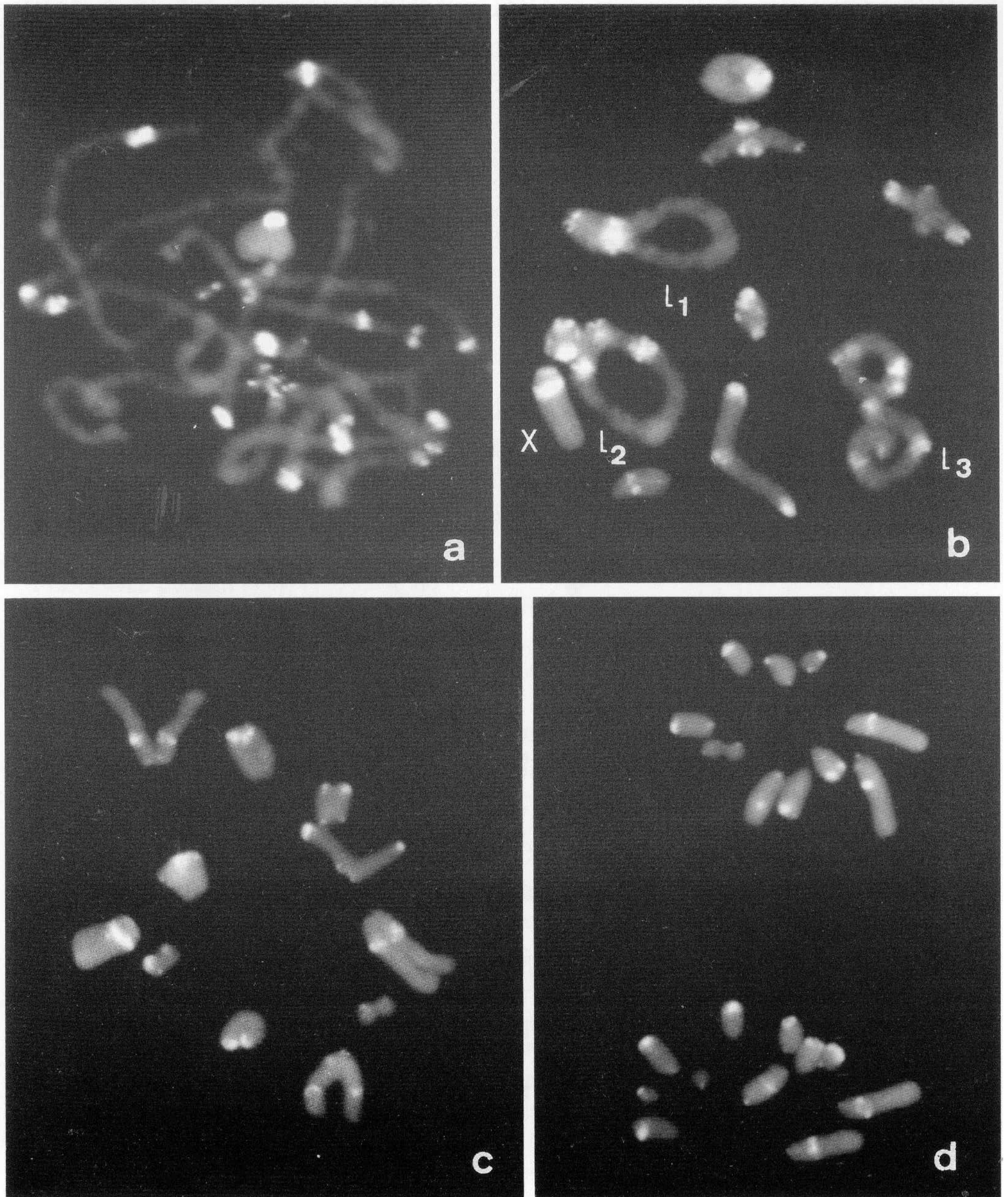
The present data were obtained by karyotypic analysis using C-banding, silver nitrate staining, fluorochrome CMA_3 staining and fluorescence *in situ* hybridization in *Xyleus angulatus.* The individuals for this study were collected in Igaraçu District, State of Pernambuco, Northeast Region of Brasil. A total of 20 males of this species were investigated. Testes of adult males were fixed in 3: 1 ethanol:acetic acid and cytologic preparations were obtained by the classical testicular follicle squashing technique. The C-banding method (SUMNER 1972) was used for constitutive heterochromatin localization. Silver stained preparations were prepared according to the method of RUFAS *et al.* (1985). Fluorescence analysis was carried out using chromomycin A_3 (CMA_3) and distamycin (DA) according to the method developed by SCHWEIZER *et al.* (1983). The probe pTa71 containing a single wheat 185 265 rDNA gene repeat unit (8.9 kb) and intergenic spacers (GERLACH and BEDBROOK 1979) was labeled with digoxigenin 11dUTP (Boehringer Mannheim) by nick translation according to the manufacturer' s instructions and used at a concentration of 10ngful in the hybridization mixture. *In situ* hybridization immunological detection of the probe was carried out as described by FERNANDEZ-CALVIN *et al.* (1995).

RESULTS

Autosomal complement of *Xyleus angulatus* comprises three large pairs (L_1-L), five medium pairs (M_4-M_8) and three small pairs (S_9-S_{11}), all of them acrocentrics, plus a single X chromosome in males. The X is also acrocentric and comparable in size to the pairs 3 or 4 (SOUZA and SILVA FILHA 1993).
The constitutive heterochromatin of X. *angulatus* is easily identified by C-banding and is present in all chromosomes of the complement but at different sites. With this technique pericentromeric blocks are constantly detected, whereas interstitial and distal blocks are more difficult to observe (Fig. la). C-bands in *Xyleus angulatus* seem to be highly guanine-cytosine rich, judging by response to the chromomycin A_3/distamycin (CMA_3/DA) staining. The use of the fluorochrome chromomycin A_3, counterstained with distamycin A, in turn allowed the localization of heterochromatin blocks in the different chromosomal positions (Figs. 2a-d). All chromosomes in the complement of X. *angulatus* showed pericentromeric heterochromatin with a CMA_3/DA + pattern. Furthermore, the interstinal blocks of bivalents L_1, L_2 and L_3 and distal blocks in medium-and small-sized pairs were also positive for this staining. The supernumerary heterochromatin in bivalent S9 is rich in CG base pairs and is intensevely stained by CMA_3/DA. In general, the interstitial and distal CMA_3/DA + blocks were larger in size when compared to the pericentromeric ones. Chro-



Figs. la-b. - Meiotic chromosomes from*Xyleus angulatus.* (a) typical C-banded chromosomes; (b) silver-stained nucleolar organizer regions on bivalents L_3 M_4 and X chromosome in diplotene.



Figs. 2a-d. - Chromomycin A_3/Distamycin (CMA3/DA) staining on meiotic chromosomes of *Xyleus angulatus.* Note the existence of large guanine/cytosine rich at the heterochromatic bands in all the chromosomes of the complement. (a) pachytene, (b) diplotene, (c) metaphase II and (d) anaphase II.

mosome X, in turn, presented two CMA_3/DA + blocks, a proximal one and a pericentromeric one, the latter coinciding with the NOR of this chromosome (Fig. 2b).

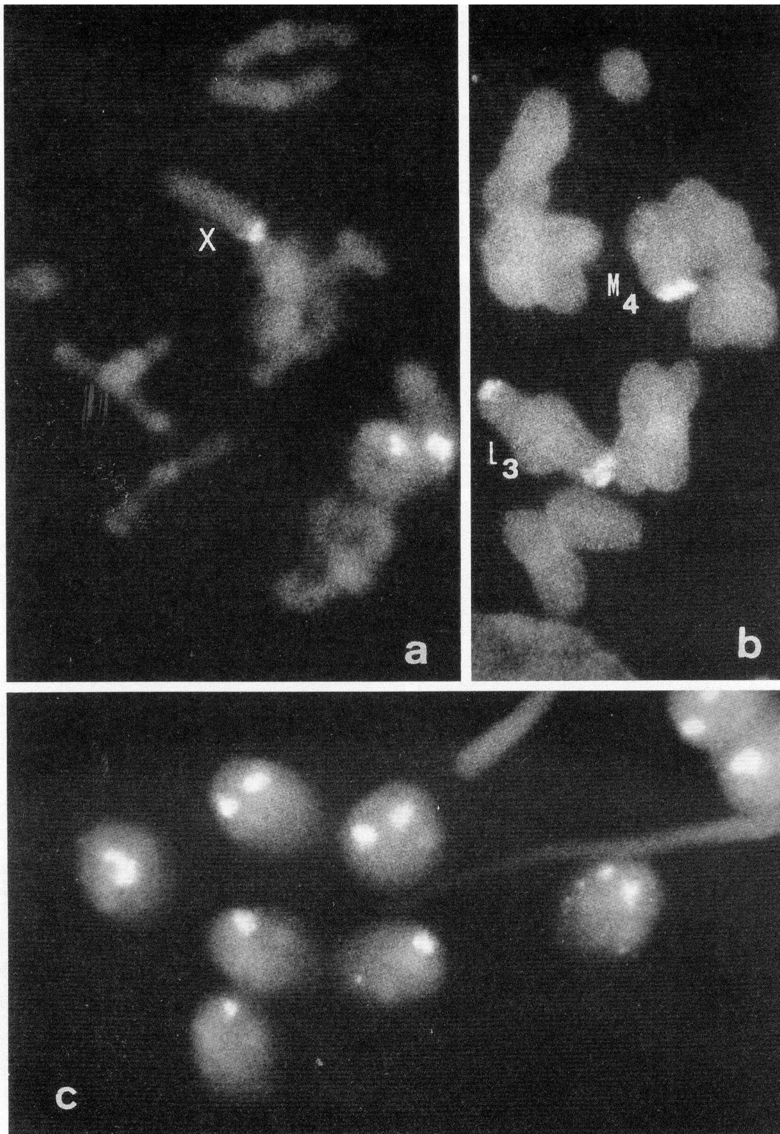
Silver staining of primary spermatocytes of *Xyleus angulatus* showed the presence of nucleolar masses associated to the proximal regions in two autosomal bivalents, probably pairs L_3 and M_4, and in the X chromosome. The nucleoli on the autosomes and X chromosome are small and desintegrate early at first meiotic prophase (in early pachytene) which makes it difficult to localize them precisely. Fig. 1b shows the labelling of L_3, M_4 and X.

In situ hybridization with ribosomal DNA probe (pTa71) showed that this sequence hybridize in the proximal regions in the bivalents L_3, M_4 and X chromosome where nucleolar organizer regions is sited (Figs. 3a-b). The rRNA genes are located in specific chromosome regions, the nucleolar organizers (NORs). These regions have been cytologically identified by silver staining in X. *angulatus.* Results of the *in situ* hybridization analysis with rDNA probe are shown in Figs. 3a-b.

DISCUSSION

The karyotype, C-banding pattern and a extensive polymorphism for supernumerary heterochromatin in *Xyleus angulatus* have been described by SOUZA and SILVA FILHA (1993). That variability includes extra heterochromatic segments in the bivalents M_5, M_6, M_7 and M_{11}, in specimens of different populations, that happen in the State of Pernambuco. Chromosomes B were also found in different specimens (SOUZA and Krno 1995). In the present study, heterochromatic chromosome regions of this species were comparatively analyzed by C-banding and by CMA_3/DA staining. The combined use of these two methods permitted a much more precise characterization of heterochromatin regions. The chromosome of X. *angulatus* presented a large amount of heterochromatin rich in GC base pairs, as shown by their response to CMA_3/DA staining. Small CMA_3/DA + blocks were located in the pericentromeric regions of all chromosomes. This species presented a complete coincidence of C-band blocks and CMA_3/DA + blocks in the entire chromosome complement. Studies on C-banding pattern and distribution in acridids (KING and JOHN 1980) have emphasized the validity of the model of heterochromatin equilocality, but have pointed out that this model, in its definition (HEITZ 193 3) takes into account only the position and not the size or composition of this heterochromatin of equilocal distribution. Fluorescence data (JOHN *et al.* 1985) have shown that different sites inside the same complement tend to show different fluorescence properties. In *Xyleus angulatus* it was possible to distinguish the pericentromeric heterochromatic blocks from the interstitial and distal blocks by the staining behavior of these sites. Of special distinction are the three

autosomes L_1, L_2 and L_3, which exhibited interstitial blocks of C-band positive material what fluoresce brightly when treated with chromomycin A_3 and the proximal block of the X chromosome.



Figs. 3a-c. - Fluorescence *in situ* hybridization *ot* meiotic nuclei of *Xyleus angulatus* with the ribosomal probe pTa71. (a) (b) the probe hybridizes with the pericentromeric regions of the bivalents L_3, M_4 and X chromosome; (c) strong hybridization signals in the spermatids.

The karyotype of the Romaleidae family have been investigated by conventional orcein staining (MESA *et al.* 1982) but have been scarce studies to date on this family that have employed banding techniques. To date, few banded karyotypes have been reported for romaleid species. C-banded karyotype were performed on the *Zoniopoda tarsata* (VILARDI 1986, 1988), *Xyleus angulatus* (SOUZA and SILVA FILHA 1993) *Brasilacris gigas* and *Chromacris speciosa* (SOUZA and Krno 1995). We have now conducted a fluorescent banding study using the dye combination chromomycin A_3/distamycin A (CMA_3/DA) which proved useful in generation of banding patterns with good definition of pericentromeric, interstitial and distal CMA_3/DA positive bands in the karyotype of X. *angulatus.*
In this species, large and potentially active rDNA clusters were present in the pericentromeric regions of the bivalents L_3, M_4 and X chromosome. These regions showed bright fluorescence with CMA_3, with GC rich DNA sequences. In X.*angulatus* the rDNA is localized within of the pericentromeric heterochromatin (C-bands). The size of the signals of the hybridization is similar in size of the corresponding C-bands. The similarities in intensity of the fluorescent signals between the bivalents L_3, M_4 and X chromosome probably suggests that the number of copies of rDNA genes no varies from one location to another . Little attention has been dispensed to the *in situ* hybridization data of particular families of highly repetitive DNA sequences in grasshoppers (JOHN *et al.* 1986; RODRIGUEZ-INIGO *et al.* 1996) in contrast to the studies concerning other animal and plant species. Localization of rRNA genes on the chromosome complement has also been found in the grasshopper *Eyprepocnemis plorans* by non isotopic *in situ* hybridization using the rDNA probe pTa71. In this species, however the ribosomal DNA showed weak hybridization signals in the centromeric regions of chromosomes 1-8 but strong signals in the X, 9, 10, 11 and B chromosomes (LOPEZ-LEON *et al.* 1994).
In contrast to many other grasshopper species (RUFAS *et al.* 1985) that present NORs located in medium and small chromosomes, X.*angulatus* has at least two NOR in large chromosomes (L_3, X chromosome). However, the nucleolar remnants of this species are visible only at the beginning of meiotic prophase (early pachytene) when the bivalents are still barely differentiated. This impairs a more precise identification of the correct number of nucleolar organizer chromosomes by silver staining. On the other hand, our FISH analysis confirmed the location of suspected rDNA sites in chromosome X, L_3 and M_4.

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