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Karyotype, C- and fluorescence banding patterns, NOR location and FISH in the grasshopper *Xestotrachelus robustus* (Romaleidae)

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Abstract - Different techniques involving fluorochrome staining, C-banding, NOR location and FISH were used in order to characterize the karyotype and to determine the characteristics of the constitutive heterochromatin in the genome of grasshopper *Xestotrachelus robustus*. This species presents uniform karyotype in terms of chromosome number ($2n$=23,XO in males) but differed in the morphology of some chromosomes of the complement. Fluorescence *in situ* hybridization (FISH) was applied to the location of 45S genes. The results of FISH are compared with those coming from classical cytogenetics (C, $AgNO_3$ and CMA_3) banding procedures.

Key words: constitutive heterochromatin, NOR, C-banding, fluorochromes, FISH.

INTRODUCTION

Xestotrachelus is an exclusively Neotropical grasshopper. This genus is taxonomically related to *Chromacris* that is polytypic and has a wide distribution from Mexico to Argentina (ROBERTS and CARBONELL 1982). The *Xestotrachelus* phallic structures are similar to those in *Chromacris*.

One of the most frequent categories of chromosome differences in grasshoppers involves changes in the amount and localization of constitutive heterochromatin (JOHN and KING 1983; JOHN *et al.* 1985). C-banding offers information concerning karyotype structure and reveals differences in the distribution of heterochromatin. It has been argued that heterochromatin is the most dynamic of chromosome components and this genomic material is subject to both qualitative and quantitative variation. Furthermore, the assortment of heterochromatin and euchromatin into different chromosomal domains appears to play also an important role of chromosomal structure (WALLRATH 1998). Fluorescent DNA-banding dyes of different specificity have made it possible to characterize heterochromatin regions in a much more precise manner. These fluorochromes have been used whether they display AT-specificity (DAPI) or GC-specificity (CMA_3) (SCHWEIZER 1976, 1981). Fluorochrome brightness is normally a function of DNA composition, modified by interaction with the chromosome structure (SCHWEIZER 1981).

Nucleolus organizer regions (NORs) are important markers for the study of chromosome evolution. The number and location of NORs are usually characteristic of species or population within different insect species (RUFAS *et al.* 1987; BEDO 1991; BELLA *et al.* 1993). In this organisms, the rRNA genes are clustered in one or several nucleolus organizer regions (NORs) and usually located in heterochromatin.

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The cytogenetics of the Neotropical acridoid grasshoppers of the family Romaleidae has been studied by some authors (VILARDI 1986, 1988; SOUZA and SILVA FILHA 1993; SOUZA *et al.* 1998; PEREIRA and SOUZA 2000). In most species heterochromatic C-positive bands were localized in the pericentromeric regions of the chromosomes although examples of non-centromeric location were also detected in some species (SOUZA and KIDO 1995; ROCHA *et al.* 1997). This paper describes the characteristics of constitutive heterochromatin regions in meiotic and mitotic chromosomes of *Xestotrachelus robustus* using C-banding, specific fluorochrome banding, NOR location, as well as FISH of rRNA genes.

MATERIAL AND METHODS

A total of thirty individuals (21 males and 09 females) of *Xestotrachelus robustus* were collected in the states of Pernambuco and Bahia in the Northeast Region of Brazil. Chromosome preparations were

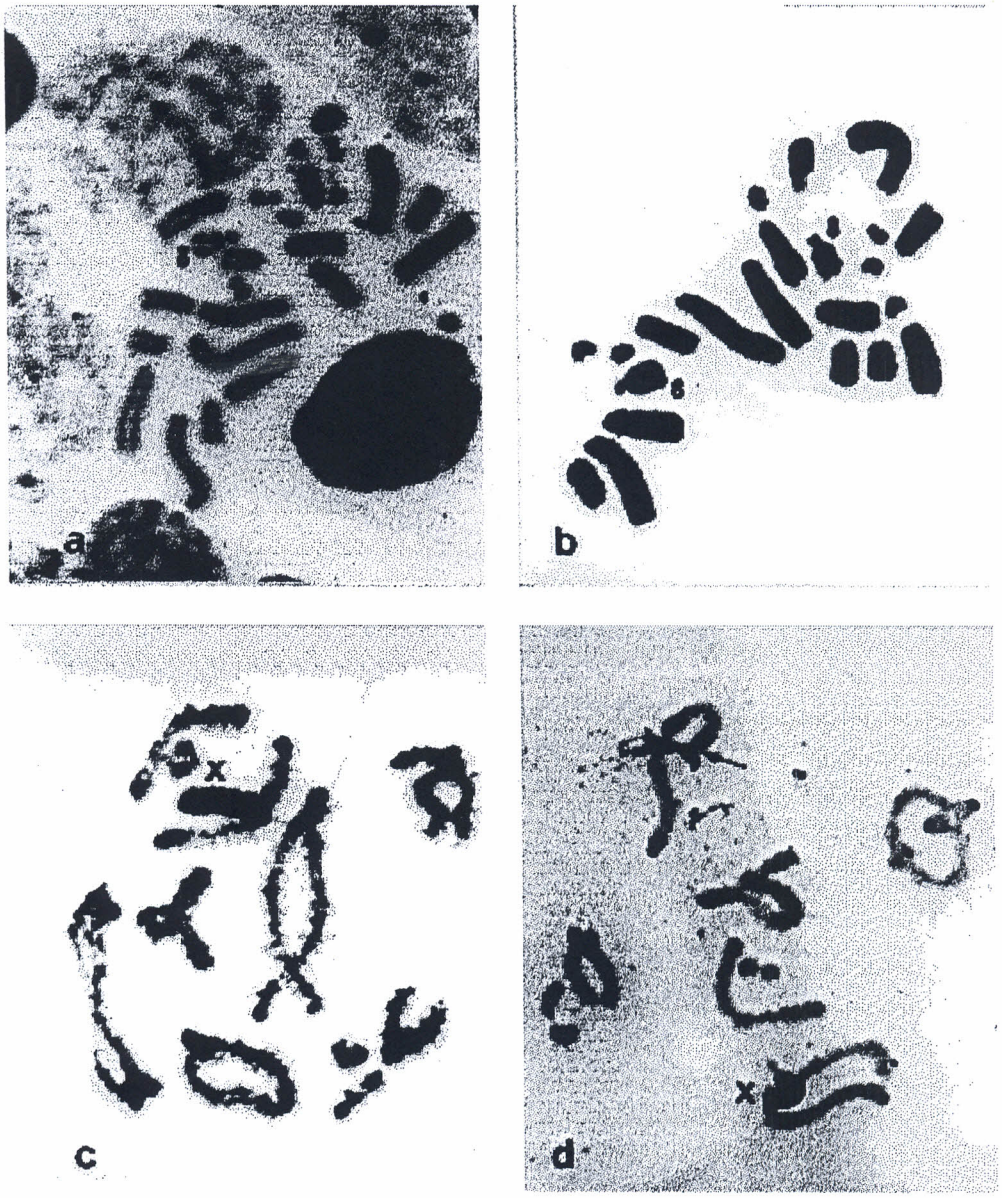

Fig. 1 – Standard karyotype and the pericentric inversion in the M_8 chromosome of *X. robustus*. Mitotic metaphase from ovariole cell and spermatogonial metaphase (a,b), respectively, (c) diplotene, (d) silver-stained diplotene from males. All bivalents and X univalent show silver-stained kinetochores. Arrow indicates NOR.

made from testes of adult males fixed in 3:1 ethanol:acetic acid. Females were treated with colchicine (0.1% in insect saline solution) in the proportion to 0.1 ml/3g body weight. Cytological preparations were made by the classical follicle squashing method and chromosomes were stained with 1% lactoacetic orcein. The C-banding method was carried out as described by SUMNER (1972). Silver staining was performed according to RUFAS *et al.* (1985). Fluorescence patterns (CMA_3/DA/DAPI) were analyzed using the techniques developed by SCHWEIZER *et al.* (1983) with minor modifications. For sequential staining ($AgNO_3$/CMA_3) the slides were stained with silver nitrate, photographed, destained, stained with CMA_3, washed and mounted in a medium supplemented with glycerol. A probe containing fragments of ribosomal genes 45S (18S-5.8S-25S) from *Arabidopsis thaliana* (UNFRIED *et al.* 1989; UNFRIED and GRUENDLER 1990) was used. *In situ* hybridization procedure was followed according to MOSCONE *et al.* (1996). The probes was labelled by nick translation (Life Technologies) using biotin-11-dUTP and detected using anti-biotin-rhodamine. The slides were counterstained with DAPI and mounted in Vectashield (Vector Laboratories). Photographs were taken in a Leitz Orthoplan microscope using Kodak T-MAX 400 and 400 ISO Fuji Films. Copies were taken using Kodak Kodabrome Print F_3 paper.

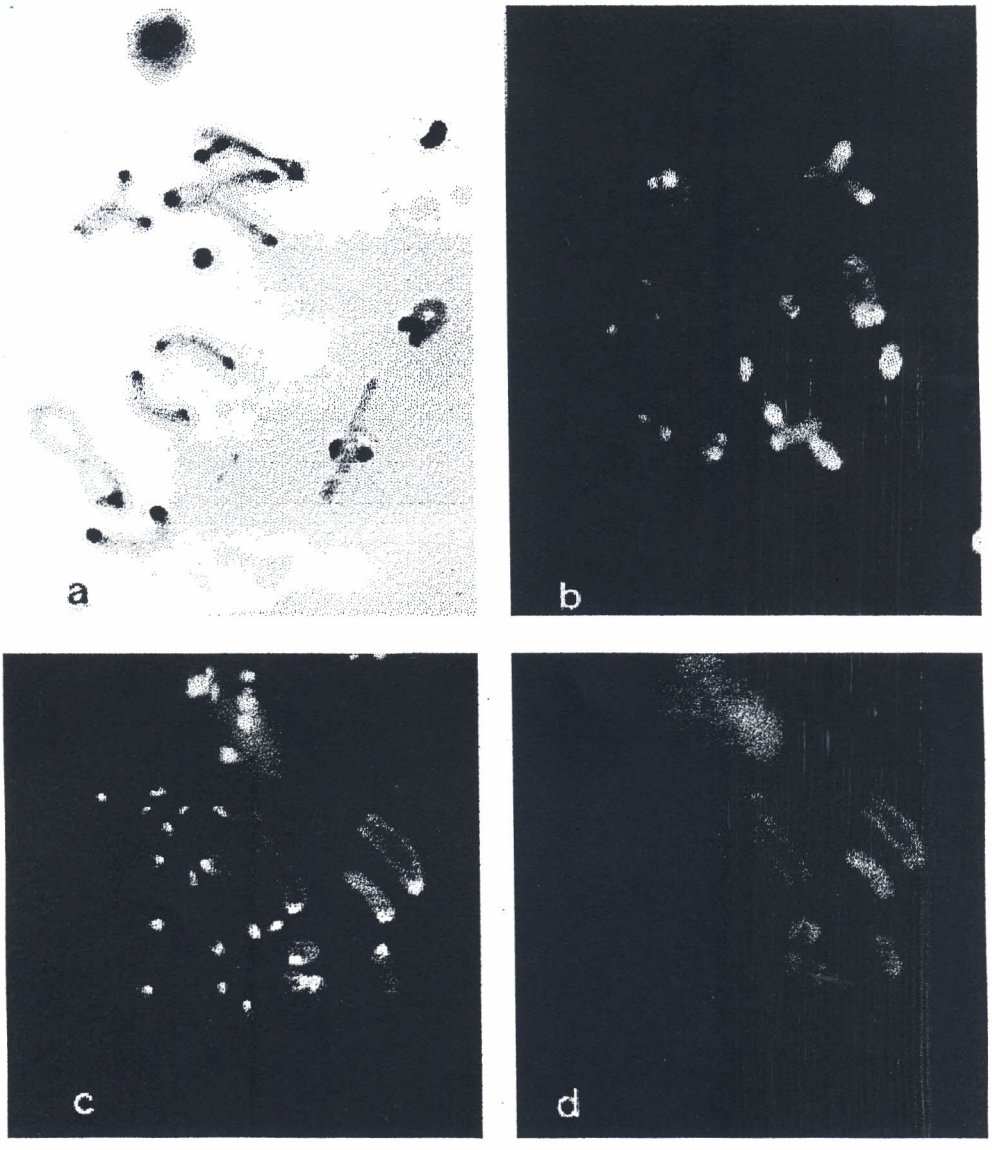

Fig. 2 – C-banding and fluorochrome staining in *X. robustus*. (a) C-banding plus Giemsa in diplotene. (b) C-banding stained with acridine orange on diplotene. Triple staining CMA_3/DA/DAPI in spermatogonial metaphase (c,d). Note in (c) the pattern of CMA_3 positive in the entire chromosome complement.

RESULTS

The conventional chromosome complement of *Xestotrachelus robustus* consists of $2n=23$, XO in the males and $2n=24$, XX in the females. The autosomes of this species were divided into three large (L_1-L_3) and five medium (M_4-M_8) acrocentric pairs, two small meta-submetacentric pairs (S_9-S_{10}) and one acrocentric pair (S_{11}). The X chromosome is the 4th in decreasing size (Fig. 1c). Three out six females and two males from Chapada Diamatina state of Bahia were heterozygous to a probably pericentric inversion in the M_8 pair. One male and two females from Buique state of Pernambuco were homozygous for this inversion. Thus, while one of the both M_8 chromosome appeared acrocentric, the other was submetacentric (Fig. 1a,b). The C-banding showed that the chromosomes of *X. robustus* have heterochromatin restricted to pericentromeric regions of all chromosomes of the complement (Fig.2a). In addition, the pairs S_9 and S_{10} were almost completely heterochromatic. The pattern of fluorescent bands using acridine orange was the same of that found with Giemsa C-bands (Fig. 2b).

The triple staining CMA_3/DA/DAPI revealed numerous bright CMA_3 blocks distributed throughout the pericentromeric regions of all

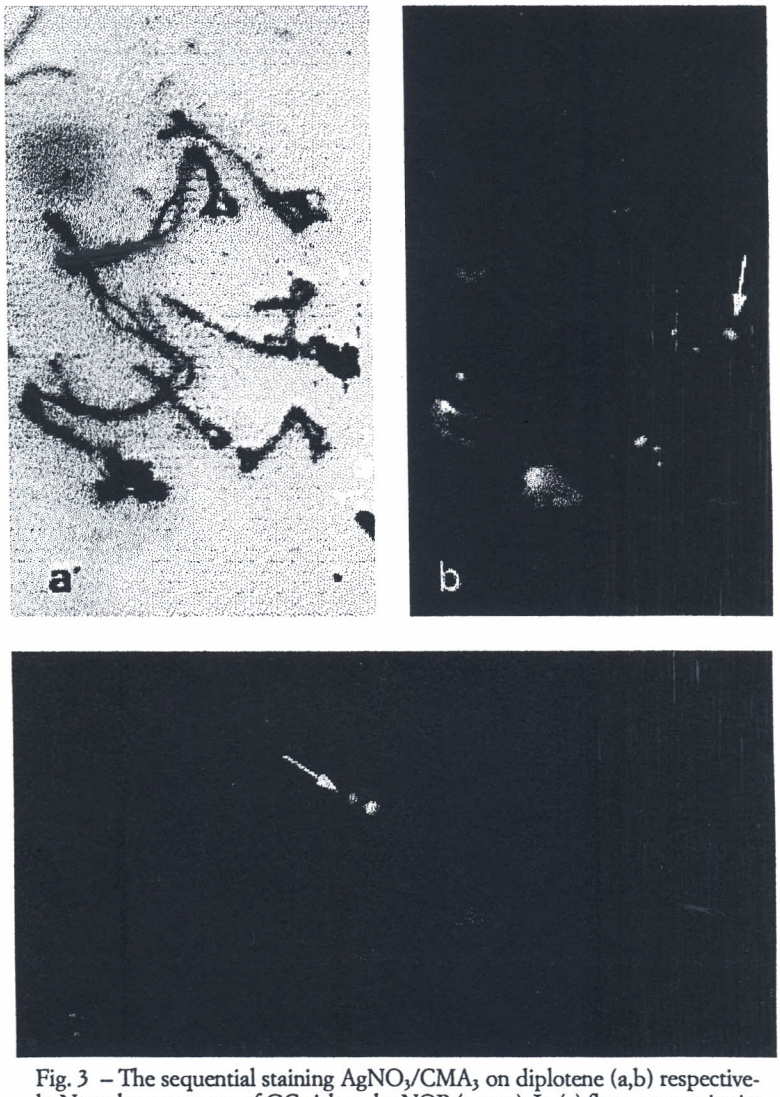

Fig. 3 – The sequential staining $AgNO_3$/CMA_3 on diplotene (a,b) respectively. Note the occurence of GC-rich at the NOR (arrow). In (c) fluorescence *in situ* hybridization of meiotic nuclei of *X. robustus* with the rDNA 45S probe. The probe hybridize with the pericentromeric region of the bivalent M_5 (arrow).

chromosomes (Fig. 2c,d). *X. robustus* showed a complete coincidence of C-band patterns and CMA_3+ bands in the entire chromosome set. No bright DA/DAPI fluorescence was evident in the chromosome complement (Fig. 2d). In mitotic metaphase of females all chromosomes presented the maximal band resolution. One nucleolus was observed throughout the first meiotic prophase (Fig. 1d). The nucleolar remnants appeared associated to pericentromeric region of the M_5 bivalent. Early diplotene cell in figure 1d showed silver-positive kinetochores in all bivalents and in the single X chromosome. The FISH analysis with the probe 45S rDNA revealed the intense of rDNA sequences in the pericentromeric region of bivalent M_5. This label (Fig. 3c) corresponds to the active NOR, located in this bivalent. The sequential staining $AgNO_3/CMA_3$ showed (Fig. 3a,b) that the heterochromatin associated with active NORs appears bright with CMA_3 in this species.

DISCUSSION

Romaleidae is a very diversified Neotropical family, with many species inhabiting arid or subarid areas. Most cytological data on this family was obtained through conventional analysis (MESA *et al.* 1982). The karyotype of *X. robustus* comprises nine pairs of acrocentric chromosomes (L_1-L_3, M_4-M_8 and S_{11}) and two meta-submetacentric pairs (S_9-S_{10}) plus a single X chromosome in males and two X in females. The karyotypes of romaleid species are remarkably uniform with regard to the number and morphology of their chromosomes. Furthermore, these apparent similarities in conventionally techniques disappear as more advanced methods of analysis are employed. In *X. robustus* all heterochromatic regions show DA/DAPI homogeneous staining and DA/CMA_3 bright fluorescence. These cytological data showed that the centromeric heterochromatin of the chromosomes is GC-rich. In this and other species, fluorescence staining techniques, provide an important method for mapping heterochromatin of specific DNA composition. This approach has been also demonstrated in several other species of grasshoppers (GOSALVES *et al.* 1987; BELLA *et al.* 1993). It permits both the characterization of heterochromatin and the study of changes during the chromosome evolution in this group of organisms. C-banding patterns have been observed in few species of the family Romaleidae. However, they have revealed that significant intra and inter-specific differences in the distribution and amount of constitutive heterochromatin (VILARDI 1986, 1988; SOUZA and SILVA FILHA 1993; SOUZA and KIDO 1995; SOUZA *et al.* 1998; PEREIRA and SOUZA 2000). Furthermore, *Xestotrachelus* and *Chromacris* are two genera closely related. *Chromacris speciosa*, on the other hand, have large pericentromeric and telomeric blocks of constitutive heterochromatin (SOUZA and KIDO 1995), by comparison with X. *robustus* that has basically small pericentromeric blocks in all chromosomes in the complement. In most extensively characterized groups of acridid grasshoppers there are examples where closely related species with similar karyotype differ in the heterochromatin patterns (JOHN and KING 1983; JOHN *et al.* 1985). Variation in the C-banding patterns among congeneric grasshopper are also reported by SANTOS *et al.* (1983) for *Calliptamus, Oeidopoda* and *Euchorthippus* and by ROCHA *et al.* (1997) for *Radacridium.*

In *X. robustus* the 45S rRNA genes are clustered on single chromosomal site, located in the pericentromeric regions on bivalent M_5. The NORs on this bivalent were found to be $AgNO3/CMA_3$ positive and marked to C-banding. This indicates that the pericentromeric regions of this chromosome pair are mainly composed of constitutive heterochromatin. In contrast, a different pattern of localization of the rRNA genes has been found in the other romaleid species (*Xyleus angulatus*) analysed (SOUZA *et al.* 1998). With respect to pericentric inversion in M_8 of *X. robustus* this chromosomal rearrangement occurs as a polymorphism. Evidences it does not lead in reproductive isolation and the meiotic pairing process is not disturbed. The small number of specimens, and the limited amount of testicular material examined does not allow to determine if the presence of the heterozygous inversion had any effect on crossing-over frequency and location. On the other hand, small chromosomes (S_9-S_{10}) have also apparently undergone pericentric inversions without reduction in the usual chromosome number ($2n$=23, XO in males). However, the probable pericentric inversions in this small chromosome have led to a modification of the chromosome morphology. These rearrangements could play a role in the speciation processes of *X. robustus*. Likewise,

pericentric inversion polymorphisms have been described in Australian morabines (WHITE 1973; HEWITT 1979). It is known that this kind of inversion also exists as fixed differences between species and may be involved in speciation (KING 1993). Our results indicate that studies of a great number of populations of *X. robustus* covering the whole range of this species are needed in order to understand the role of the polymorphism for pericentric inversion observed.

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