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# Karyotype, constitutive heterochromatin and nucleolar organizer regions (NORs) in *Belosacris coccineipes* (Acrididae-Leptysminae)

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*Abstract*

Several techniques including C-banding, fluorochromes and silver staining were used to obtain information about heterochromatin patterns in the grasshopper *B. coccineipes*. Conventional staining showed a karyotype with 2n = 23 chromosomes in males and 2n = 24 in females, as well as XO:XX sex determination and acrotelocentric chromosomes. The medium-sized X chromosome was heteropycnotic positive at the beginning of prophase I and negative in metaphase I. C-banding revealed heterochromatic blocks in the pericentromeric regions of all chromosomes. Silver nitrate staining in this species showed three small bivalents ($S_9$-$S_{11}$) as nucleolar organizers with NORs located in the pericentromeric regions. $CMA_3$-positive blocks were seen in pericentromeric regions of pairs $M_6$, $S_9$, $S_{10}$ and $S_{11}$. Sequential staining with $CMA_3/AgNO_3$ revealed homology between the $CMA_3$-positive bands and NORs of the bivalents $S_9$, $S_{10}$ and $S_{11}$. The $CMA_3$-positive block of the bivalent $M_6$ could represent a latent secondary NOR. The results obtained permit us to distinguish two categories of the constitutive heterochromatin in *B. coccineipes*.

## INTRODUCTION

Although the family Acrididae has a worldwide distribution, some subfamilies are exclusively neotropical, as is the case for Leptysminae (Carbonell, 1977). *Belosacris* is a genus of restricted occurrence, being found in Mexico, Paraguay, Brazil and Argentina. In Brazil, two species have been described: *B. coccineipes*, distributed from Pará to São Paulo and Mato Grosso, and *B. stali*, found in Mato Grosso and Goiás (Roberts, 1978).

There are few cytogenetic studies of the Leptysminae, most of them being limited to conventional analysis (Mesa *et al.*, 1982; Colombo, 1989b). Mesa *et al*. (1982) made an extensive revision of the karyology of neotropical grasshoppers in which 15 of the 17 species of Leptysminae analyzed had karyotypes with 2n = 23/24 chromosomes, XO:XX sex determination and acrotelocentric chromosomes. However, a karyotype of 2n = 21 was found in *Stenopola pallida* males, whereas *Tetrataenia surinama* had 2n = 19, with two pairs of metacentric and two of submetacentric chromosomes. *Leptysma argentina* possesses a highly polymorphic karyotype, with centric fusions involving pairs 3 and 6. In this species, polymorphism of the B chromosome and a complex of translocations involving autosomes are particularly frequent (Bidau and Hasson, 1984; Colombo, 1989a, 1990).

The use of special techniques such as silver nitrate staining, for the identification of nucleolar organizer regions (NORs), and C-banding, for the detection of constitutive heterochromatin, allows for better karyotypic analysis. These techniques have been extensively used to characterize several species of grasshoppers of the family Acrididae (King and John, 1980; Rufas *et al.*, 1985). Different fluorochromes capable of detecting specific segments of DNA base pairs have been used to analyze the composition of heterochromatic segments with a homogeneous appearance. DAPI (4'-6-diamidino-2-phenylindol) is specific for AT-rich regions, whereas $CMA_3$ (chromomycin $A_3$) is specific for GC-rich regions (Schweizer, 1980).

In the present study, we examined the karyotype of *Belosacris coccineipes*, and identified the NORs and the C-banding pattern. The composition of the constitutive heterochromatin was also analyzed using the fluorochromes $CMA_3$, DAPI and acridine orange.

## MATERIAL AND METHODS

Adult males and females of *Belosacris coccineipes* were collected from natural populations in the cities of Cabo and Recife, State of Pernambuco, Brazil. Fifteen specimens (11 males and 4 females) from the first population and five males from the second were analyzed. The cytological preparations were obtained from testes or ovarioles. The latter were pretreated with colchicine (0.1%) for 6 h. The material was fixed in 3:1 ethanol-acetic acid. Slides were prepared by the classic squashing technique followed by staining with 2% lacto-acetic orcein for the conventional chromosomal analysis.

C-banding was done as described by Sumner (1972). The material was treated with 0.2 N HCl, 5% barium hydroxide and 2 x SSC. The temperature of the last two solutions was 60°C. Some slides pretreated for C-banding were also

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stained for 30 s with acridine orange (Bella *et al.*, 1986). The silver nitrate staining was done by the method of Rufas *et al.* (1987), the slides being pretreated with 2 x SSC (60°C) for 10 min and followed by staining with silver nitrate (1 g/ml) at 70-80°C. For triple staining with $CMA_3$/DA/DAPI (Schweizer *et al.*, 1983), the slides were aged for three days and stained with $CMA_3$ (0.5 mg/ml in McIlvaine buffer, pH 7.0, containing 10 nM $MgCl_2$) for 60 min, washed with distilled water, stained with distamycin A (0.1 mg/ml) for 40 min, washed again and stained with DAPI (0.5 µg/ml) for 20 min. Sequential staining with $CMA_3/AgNO_3$ was also used to characterize the NORs.

The slides were photographed with Agfa Copex Pan film. Fluorescence photomicrographs were taken with a Leitz Orthoplan microscope using Kodak T-MAX 400 film. Copies were made using Kodak Kodabrome Print F3 paper.

## RESULTS

The karyotype of *B. coccineipes*, stained with lacto-acetic orcein, consisted of 11 autosomal pairs and a simple X chromosome in males (2n = 23, XO). The females had a karyotype of 2n = 24, XX. The autosomes were acrotelocentrics and could be arranged according to their size as large ($L_1$-$L_2$), medium ($M_3$-$M_8$) and small ($S_9$-$S_{11}$) chromosomes. The X chromosome was of medium size, also acrotelocentric and had variable heteropycnotic behavior during meiosis I. At the beginning of prophase I, the X chromosome was heteropycnotic positive and was more condensed than the autosomes. However, in metaphase I this condition reversed and the X chromosome was heteropycnotic negative (Figure 1A-D).

C-banded preparations of *B. coccineipes* showed

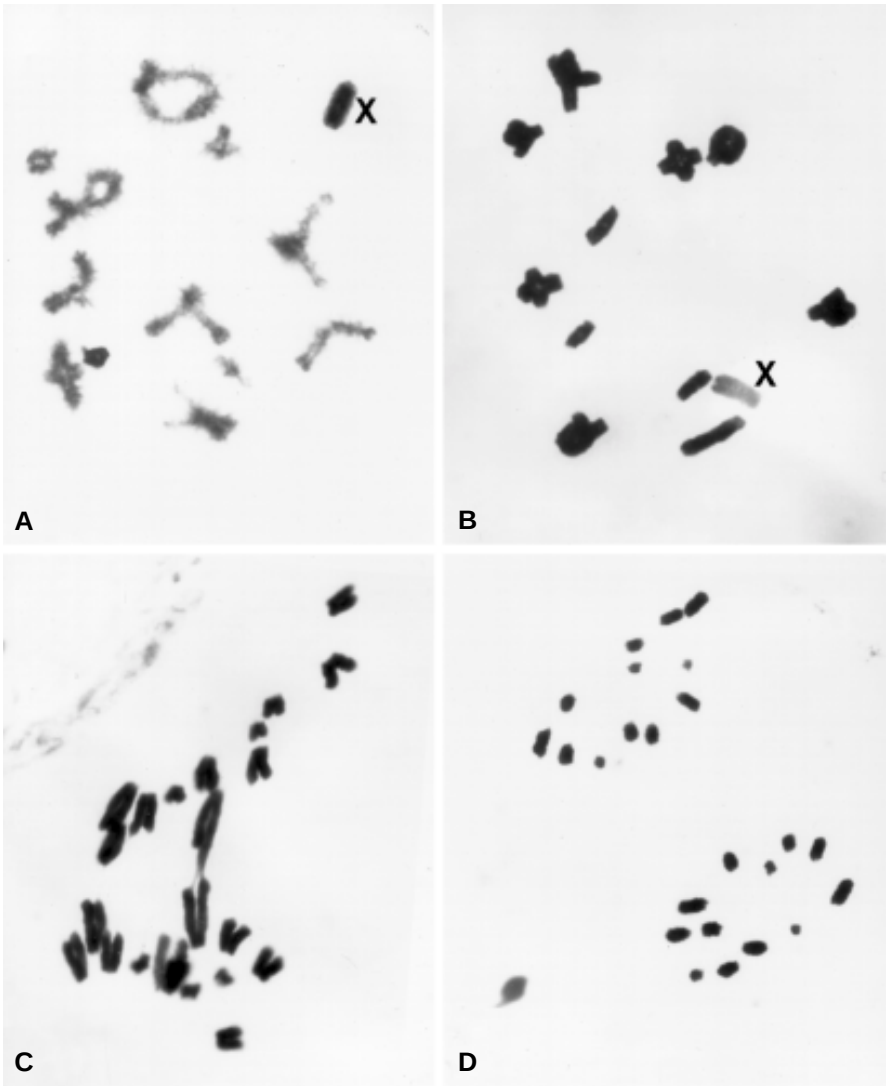


**Figure 1** - Conventional staining with lacto-acetic orcein of meiotic cells of *Belosacris coccineipes*. **A**) Diplotene, **B**) metaphase I, **C**) anaphase I and **D**) anaphase II with 12 chromosomes. Note the X chromosome which is heteropycnotic positive in A and negative in B.

positive staining in the pericentromeric regions of all chromosomes (Figure 2A,B). When acridine orange was used after C-banding, the chromosomes showed the same pattern seen with C-bands stained with Giemsa. Silver staining was used to locate active NORs and three nucleolar remnants were identified in zygotene and pachytene cells. These remnants were located in pericentromeric regions of the small bivalent autosomes ($S_9$-$S_{11}$) (Figure 2C,D).

The triple stain $CMA_3$/DA/DAPI revealed four $CMA_3$-positive blocks located in the pericentromeric regions of a medium-size chromosome ($M_6$) and in three small ones ($S_9$-$S_{11}$) (Figure 3A-B). This label was seen better in pachytene cells. DAPI produced essentially homogeneous staining with no particular positive or negative blocks. Sequential staining with $CMA_3$/$AgNO_3$ showed that the $CMA_3$ positive blocks in the small chromosomes ($S_9$-$S_{11}$) corresponded to the NORs (data not shown).

## DISCUSSION

According to Amedegnato (1974), the subfamily Leptysminae is represented in the neotropical regions by 22 genera. On the other hand, the Leptysmini tribe possesses eight genera (including *Belosacris*) and 42 species, about nine of them have been studied chromosomically. The family Acrididae has a very uniform karyotype with 2n = 23, XO and 2n = 24, XX. The subfamily Leptysminae (Mesa *et al.*, 1982; Bidau and Hasson, 1984) is also characterized by this uniformity in chromosome complement.

The C-banding patterns in several species of grasshoppers provide important clues on the changes that have occurred in the patterns of constitutive heterochromatin during the evolution of the group. King and John (1980) and Santos *et al.* (1983) observed numerous variations of the C-banding patterns of representatives of the Acrididae.

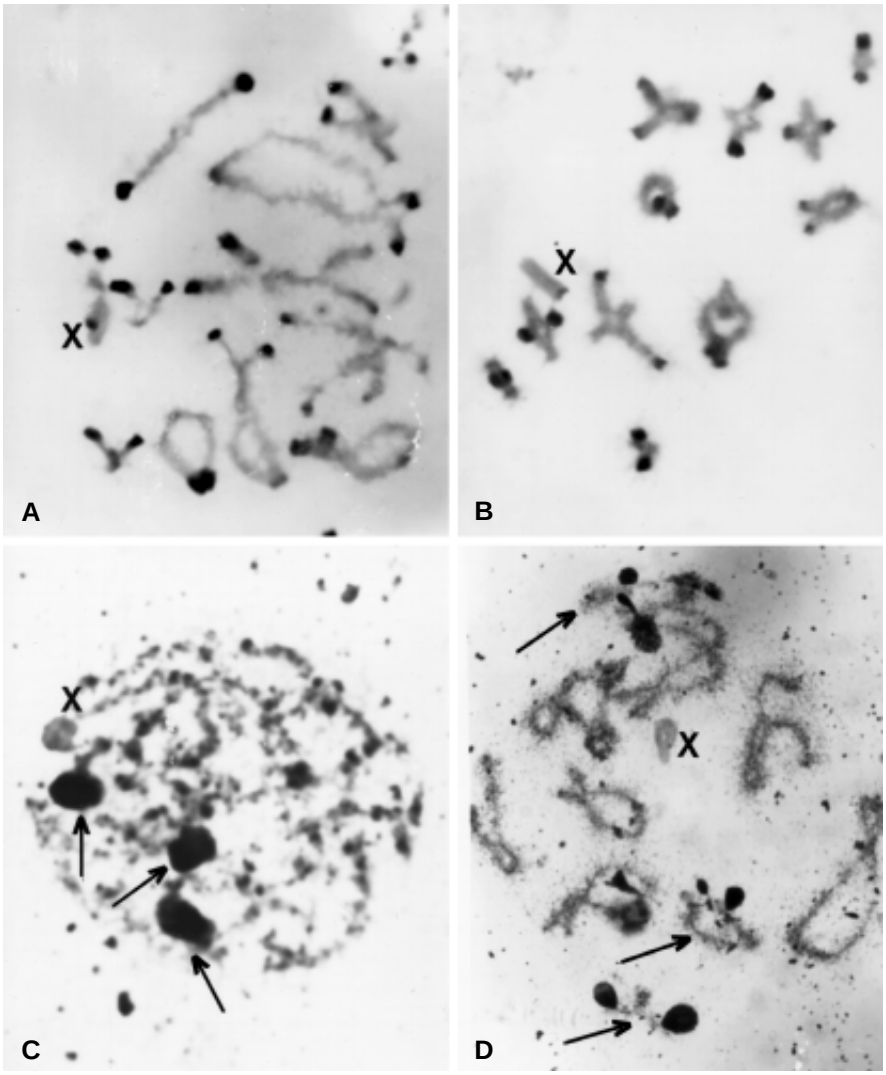


**Figure 2** - **A,B**) Cells in diplotene showing the C-banding pattern of *B. coccineipes.* **A**) Initial diplotene and **B**) final diplotene. **C,D**) Nucleolar organizer regions (NORs) stained with silver nitrate in meiotic chromosomes, **C**) zygotene and **D**) initial diplotene. The nucleolar remnants associated with the bivalents $S_9$, $S_{10}$ and $S_{11}$ are indicated by arrows. X represents the X chromosome.

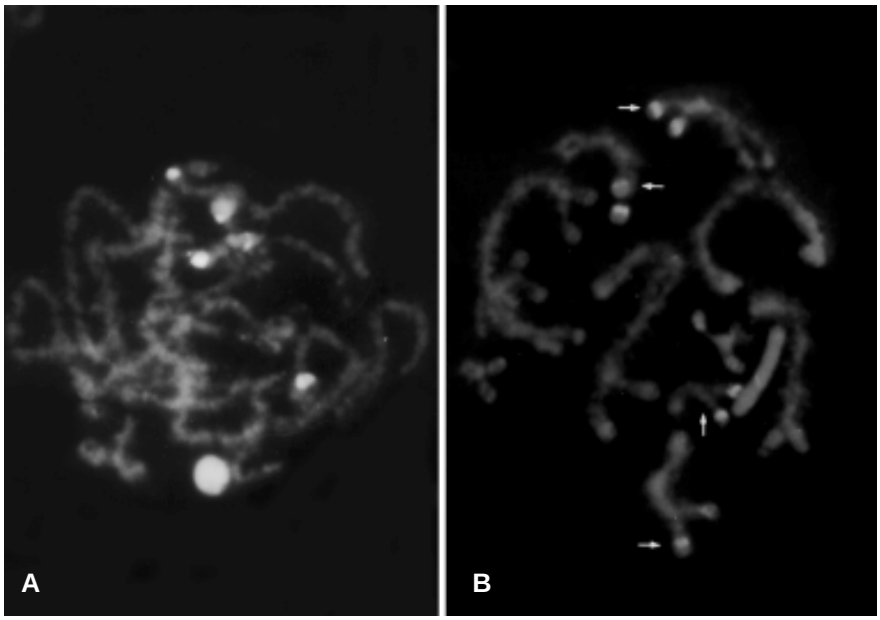


**Figure 3** - $CMA_3$/DA staining in meiotic cells of *B. coccineipes.* A) Zygotene and B) pachytene. $CMA_3$-positive blocks are visible in the pericentromeric regions of $M_6$, $S_9$, $S_{10}$ and $S_{11}$, as indicated by the arrows in B.

Polymorphisms for extra segments (Camacho and Cabrero, 1982; Navas-Castillo *et al.*, 1987) and B chromosomes (Henriques-Gil *et al.*, 1984) are also frequent. The Leptysminae *Cylindrotettix obscurus*, *C. santaroseae* and *Leptysma argentina* have B chromosomes and, in the latter species, C-banding shows heterochromatin blocks in the centromeric regions of all chromosomes and in the interstitial regions of three medium pairs (Bidau and Hasson, 1984). Although C-banding has been used in several species of Acrididae, only a few species of the subfamily Leptysminae (endemic to the neotropics) have been investigated with this approach. The C-banding pattern we observed in *B. coccineipes* was very uniform, with pericentromeric blocks in the entire chromosomal set.

Silver nitrate staining has been used to study nucleolar activity and the distribution and position of NORs in different species of grasshoppers. Rufas *et al.* (1985) showed that the most common location of NORs was at proximal sites in medium and small chromosomes, distributed in pairs throughout the genome of most of the species. However, in *B. coccineipes*, the NORs were restricted to autosomes**,** located in pericentromeric areas of the small chromosomes ($S_9$-$S_{11}$). Other species such as *Ramburiella hispanica*, *Chortippus apicalis* (Rufas *et al.*, 1985) and *Xyleus angulatus* (Souza *et al*., 1998) have NORs distributed both in the autosomes and in the sex chromosomes. In *Radacridium mariajoseae* (Rocha *et al.*, 1997), the NOR is restricted to the X chromosome. On the other hand, Rufas *et al.* (1985) suggested that the presence of NORs in the X chromosome would be an ancestral condition for the family Acrididae. A similar situation has also been found in the Romaleidae (Rocha *et al.*, 1997).

In *B. coccineipes*, the $CMA_3$-positive blocks and the homogeneous DAPI staining could mean the absence of AT-rich regions. In insects, especially grasshoppers, chromosomal regions with differential fluorescence may be rich in GC base pairs. This pattern has been found in *Chorthippus parallelus parallelus* and *C. p. erythropus* (Bella *et al*., 1993), *Podisma pedestris* and *P. ignatii* (Bella *et al*., 1990) and *Xyleus angulatus* (Souza *et al*., 1998). However, in other species such as *Arcyptera fusca*, *A. tornosi* (Bella and Gosálvez, 1991) and *Dociostaurus genei* (Rodríguez-Iñigo *et al*., 1993), AT-rich regions have been identified. The affinity of $CMA_3$ for chromosome regions that contain rDNA in some organisms is attributable to the high GC content of these regions (Schweizer *et al.*, 1983). The use of $CMA_3$ to identify rDNA regions has an advantage over silver staining in that $CMA_3$ binds preferentially to GC-rich DNA independent of whether it is active or not in the preceding interphase.

Of the four $CMA_3$-positive labels seen in *B. coccineipes*, only three corresponded to NORs (pericentromeric regions of $S_9$, $S_{10}$ and $S_{11}$) after silver staining, while in the $M_6$ chromosome no NOR was seen with silver nitrate. Teixeira *et al.* (1997) showed that in *Schistocerca pallens* (Acrididae - Cyrtachantacridinae) small $CMA_3$-positive blocks were located at the interstitial position on $L_3$ and $M_6$ and at a proximal position on $M_7$ after triple staining with $CMA_3$/DA/DAPI. Silver staining confirmed the homology between $CMA_3$-positive bands and the label in the bivalents $L_3$ and $M_6$. The $CMA_3$-positive label of the bivalent $M_7$ could represent a latent secondary NOR. A similar phenomenon can also explain the $CMA_3$-positive label in bivalent $M_6$ of *B. coccineipes*. Other studies using *in situ*

hybridization with rDNA probes could elucidate whether the $CMA_3$-positive block in chromosome $M_6$ is indeed a latent secondary NOR that may be expressed, or whether it is a GC-rich region that bears no relationship with an NOR. In conclusion, we can distinguish two categories of constitutive heterochromatin in *B. coccineipes*: the constitutive heterochromatin positive for $CMA_3$, restricted to the NORs, and constitutive heterochromatin that has no specificity to AT or GC base pairs. This information could help elucidate the evolution of this type of chromatin within the Leptysminae.

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## RESUMO

Algumas técnicas incluindo bandeamento C, fluorocromos e coloração com nitrato de prata foram utilizadas visando obter informações sobre os padrões de heterocromatina no gafanhoto *B. coccineipes*. A análise convencional mostrou um cariótipo com 2n = 23 cromossomos nos machos e 2n = 24 nas fêmeas, sistema XO de determinação do sexo e cromossomos acro-telocêntricos. O cromossomo X de tamanho médio mostrou-se heteropicnótico positivo no início da prófase I e negativo em metáfase I. O bandeamento C revelou blocos positivos nas regiões pericentroméricas de todos os cromossomos. A coloração com nitrato de prata nesta espécie evidenciou 3 bivalentes pequenos ($S_9$-$S_{11}$) portadores de nucléolos com as NORs localizadas nas regiões pericentroméricas. Blocos $CMA_3$-positivos foram visualizados nas regiões pericentroméricas dos pares $M_6$, $S_9$, $S_{10}$ e $S_{11}$. Pela coloração seqüencial $CMA_3/AgNO_3$ observamos homologia entre as bandas $CMA_3$-positivas e as NORs dos bivalentes $S_9$, $S_{10}$ e $S_{11}$. A marcação $CMA_3$-positiva do bivalente $M_6$ poderia representar uma NOR secundária latente. Os resultados obtidos permitiram distinguir duas categorias de heterocromatina constitutiva em *B. coccineipes*.

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