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Differential characterization of holocentric chromosomes in triatomines (Heteroptera, Triatominae) using different staining techniques and fluorescent *in situ* hybridization

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ABSTRACT. A comparative study of holocentric chromosomes in the triatomine species *Panstrongylus megistus*, *Rhodnius pallescens* and *Triatoma infestans* was carried out in order to characterize heterochromatin, rDNA active sites and nucleolar proteins. Cytological preparations of seminiferous tubules were stained by silver impregnation, C banding, fluorochromes CMA_3/DA and DAPI/DA, and fluorescent *in situ* hybridization (FISH) with *Drosophila melanogaster* 28S rDNA probe. Our results showed interesting aspects of the organization of chromatin and chromosomes in the meiotic cells of these insects. In *R. pallescens*, sex chromosomes (X, Y) were distinct from autosomes, when submitted to silver impregnation, C banding, CMA_3 staining, and FISH, confirming that these chromosomes bear nucleolar organizer regions (NORs). In *P. megistus*, two of the three sex chromosomes were CMA_3/$DAPI^-$; at early meiotic prophase and at diakinesis, silver impregnation corresponded with FISH signals, indicating that in this species, two chromosomes (probably a sex chromosome and an autosome) bear NORs. In *T. infestans*, silver nitrate and FISH also stained corresponding areas on meiotic chromosomes. Our data suggest that in triatomines, in general, the number and location of NORs are species-specific. These regions may be considered important

chromosome markers for comparative studies to improve the understanding of evolutionary mechanisms in these hematophagous insects.

Key words: Holocentric chromosomes, Nucleolus organizer region, CMA_3, DAPI, Fluorescent *in situ* hybridization, Triatomines

INTRODUCTION

Triatomines, hematophagous insect vectors of the protozoon *Trypanosoma cruzi* and causative agents of Chagas' disease, are of great importance, not only in regard to the investigation of their biological control, but also because of their uncommon cytogenetic characteristics which include the presence of holocentric chromosomes and an unusual meiotic behavior with sex chromosomes segregating post-reductionally (Hughes-Schrader and Schrader, 1961; De Vaio et al., 1985).

Until not very long ago, most cytogenetic studies on heteroptera holocentric chromosomes focused exclusively on heterochromatin and nucleolar organizer regions (NORs). However, molecular cytogenetic techniques, including fluorescent banding and fluorescent *in situ* hybridization (FISH) with specific probes, have allowed a better understanding of the constitution of chromatin in invertebrates, and thus have furthered the advance of studies in this group of animals.

Although some heterochromatin is present on most eukaryotic chromosomes, its staining patterns and the nature of the DNA it contains vary widely within and among species. In addition, heterochromatin blocks are generally heteromorphic in relation to size and sometimes staining pattern. Differences among species with regard to heterochromatin location and amount are very frequent (Sumner, 1990). In some species, constitutive heterochromatin content varies greatly from individual to individual, and this variation may play an important role in speciation (Clark and Wall, 1996).

In some organisms, such as fish and anurans, the number and location of rDNA/AgNOR are species-specific and important karyotypic markers. Indeed, silver impregnation, as well as FISH, have been demonstrated to be able to detect genomic NORs in these individuals (Fujiwara et al., 1998; Lourenço et al., 1998).

Since information about the structure and base composition of some specific regions of triatomine holocentric chromosomes is relevant to the understanding of triatomine molecular structure, the aim of this study was to examine meiotic cells in species of the genera *Panstrongylus*, *Rhodnius and Triatoma*, by differential chromosome banding techniques (chromomycin A_3/distamycin (CMA_3/DA) and 4'6-diamycin-2-phenylindol-dihydrochloride/distamycin (DAPI/DA)), FISH with *Drosophila melanogaster* 28S rDNA probe, C banding and silver impregnation which reveal differential heterochromatin pattern and locate NORs as well as ribosomal DNA sites.

MATERIAL AND METHODS

Seminiferous tubules from adult insects of the following genera and species were used: *Panstrongylus megistus* ($9A + X_1X_2Y$), *Rhodnius pallescens* ($10A + XY$), and *Triatoma infestans* ($10A + XY$). Following the spread and fixation of the biological material (Imai et al., 1988), the slides were randomly submitted to the following analyses: silver impregnation (Howell and Black, 1980) for discrimination of nucleolar proteins; C banding (Sumner, 1972); double staining

by fluorochromes CMA_3/DA and DAPI/DA (Schmid, 1980) after pre-treatment with RNAse for heterochromatic discrimination, and FISH with *D. melanogaster* 28S-12-kb rDNA probe (Viegas-Péquignot, 1992) for rDNA localization.

Photomicrographs were obtained using a Zeiss-Jenaval microscope for cytochemical techniques, and a Zeiss-Axioskop and Olympus BX-FLA for molecular cytogenetic techniques (CMA_3/DA, DAPI/DA and FISH).

RESULTS

Panstrongylus megistus

Silver-stained meiotic cells showed two chromosomes with NORs in spermatogonial metaphases. In nuclei at early meiotic prophase I, a prominent nucleolar region containing several dispersed nucleolar fragments was observed. In nuclei at diplotene-diakinesis, NORs were seen on an autosomal chromosome and on a sex chromosome (Figure 1a-c).

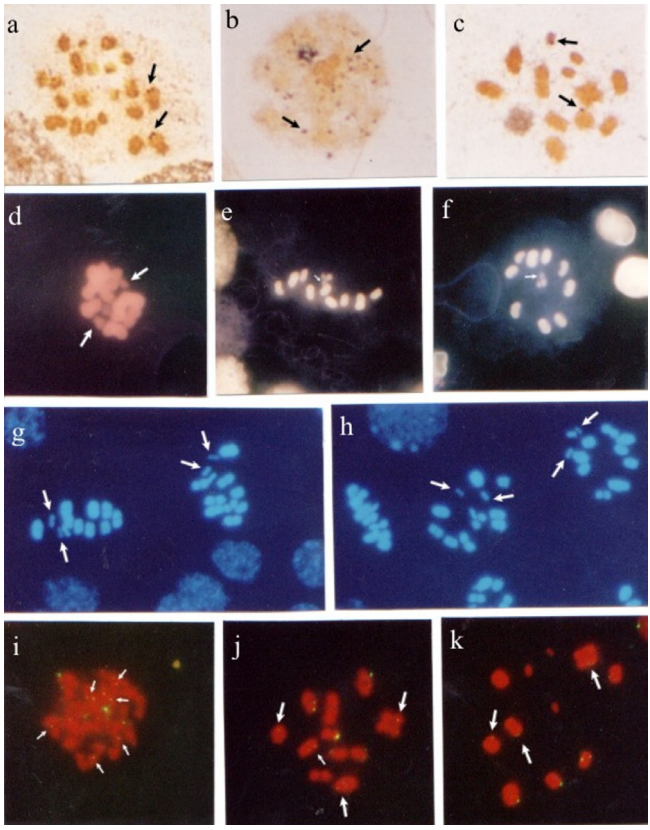

Figure 1. *Panstrongylus megistus* seminiferous tubules submitted to silver impregnation (a-c); double staining with fluorochromes CMA_3/DA (d-f) and DAPI/DA (g,h), and fluorescent *in situ* hybridization (FISH) with *Drosophila melanogaster* 28S rDNA probe (i-k). Spermatogonial metaphases (a,d). Arrows indicate nucleolar organizer regions (NORs) in *a*, and CMA^- chromosomes in *d*. Nuclei at meiotic prophase I (b,i). Arrows indicate silver-impregnated regions in *b*, and rDNA sites in *i*. Nuclei at diplotene-diakinesis (c,j,k). Arrows indicate NORs in *c*, and hybridization regions in *j*,*k*. First meiotic division metaphases (e,g,h). Arrows indicate a CMA^- in *e*, and two $DAPI^-$ sex chromosomes in *g*,*h*. Second meiotic division metaphases (f), where the arrow indicates a CMA^- sex chromosome. Bar = 10 µm.

In testicular cells submitted to CMA_3/DA staining, spermatogonial metaphases exhibited some CMA_3^- chromosomes. At first meiotic division metaphase (MI) and second meiotic division metaphase (MII), a CMA_3^- sex chromosome (Figure 1d-f), and two $DAPI^-$ sex chromosomes (Figure 1g,h) were observed.

FISH with a 28S-12-kb rDNA probe stained several chromosomal regions in nuclei at meiotic prophase I. In nuclei at diplotene-diakinesis, hybridization with the rDNA probe was observed on some autosomal chromosomes (Figure 1 i-k).

Rhodnius pallescens

In seminiferous tubule cells at MII, two sex chromosomes were silver-stained (Figure 2a,b). In the meiotic cells at MII, C banding showed evidence of two heterochromatic chromosomes, which were sometimes both sex chromosomes and in other cases a sex chromosome and the interstitial region of one of the autosomes (Figure 2c,d).

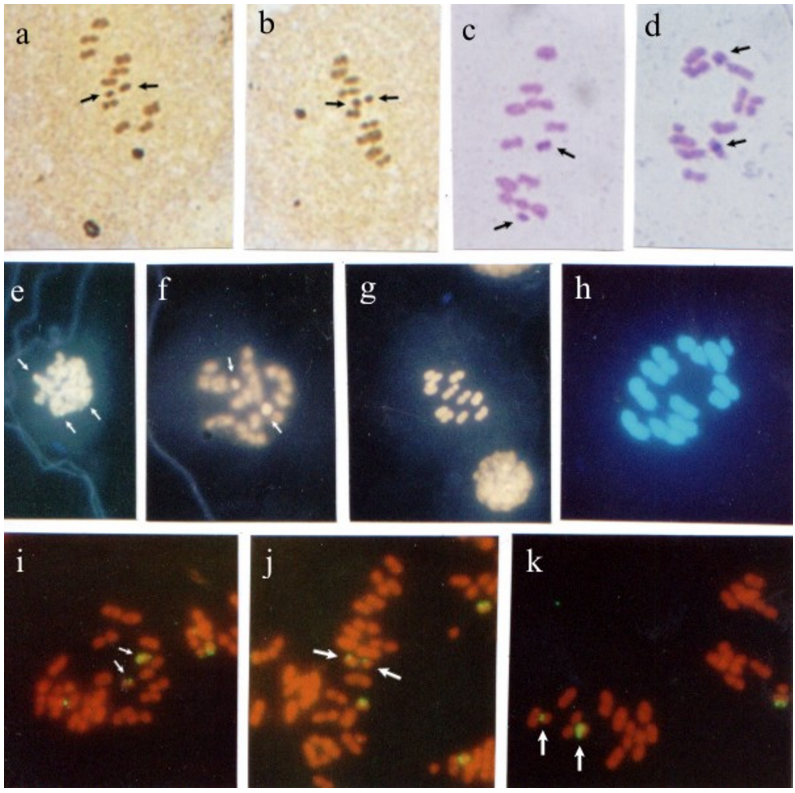

Figure 2. *Rhodnius pallescens* seminiferous tubules submitted to silver impregnation (a,b); C banding (c,d), double staining with fluorochromes CMA_3/DA (e-g) and DAPI/DA (h), and fluorescent *in situ* hybridization (FISH) with *Drosophila melanogaster* 28S rDNA probe (i-k). Spermatogonial metaphases (e); arrows indicate CMA^+ chromosomes. Nuclei at diplotene-diakinesis (f), where arrows indicate two CMA^+ regions. First (c,d,g,h,i,j,k) and second (a,b) meiotic division metaphases; arrows indicate NORs on sex chromosomes X and Y in *a*,*b*, and heterochromatic chromosomes and regions in *c*,*d*. In *c*, both sex chromosomes (X, Y) are heterochromatic, while in *d* one of the sex chromosomes (X) is heterochromatic, and one of the autosomes shows a heterochromatic block in the median region of the chromosome. In *g*,*h*, the chromosomes stained with fluorochromes CMA_3/DA and DAPI/DA, respectively, showed no distinct regions. Hybridization signals with rDNA probe on the sex chromosomes (X, Y) were observed in *i*-*k* (arrows). Bar = 10 µm.

In meiotic cells submitted to CMA_3/DA staining, spermatogonial metaphases showed some CMA_3^+ regions which could have been the sex chromosomes. At MI, the use of fluorochromes CMA_3/DA and DAPI/DA did not yield chromosome differentiation (Figure 1e-h).

FISH with the 28S-12-kb rDNA probe allowed the observation of hybridization signals on both sex chromosomes (X, Y).

Triatoma infestans

Meiotic cells impregnated with silver exhibited some silver-stained chromosomes in the nuclei at diplotene-diakinesis. At anaphase I, some chromosomal regions showed staining (Figure 3a-c).

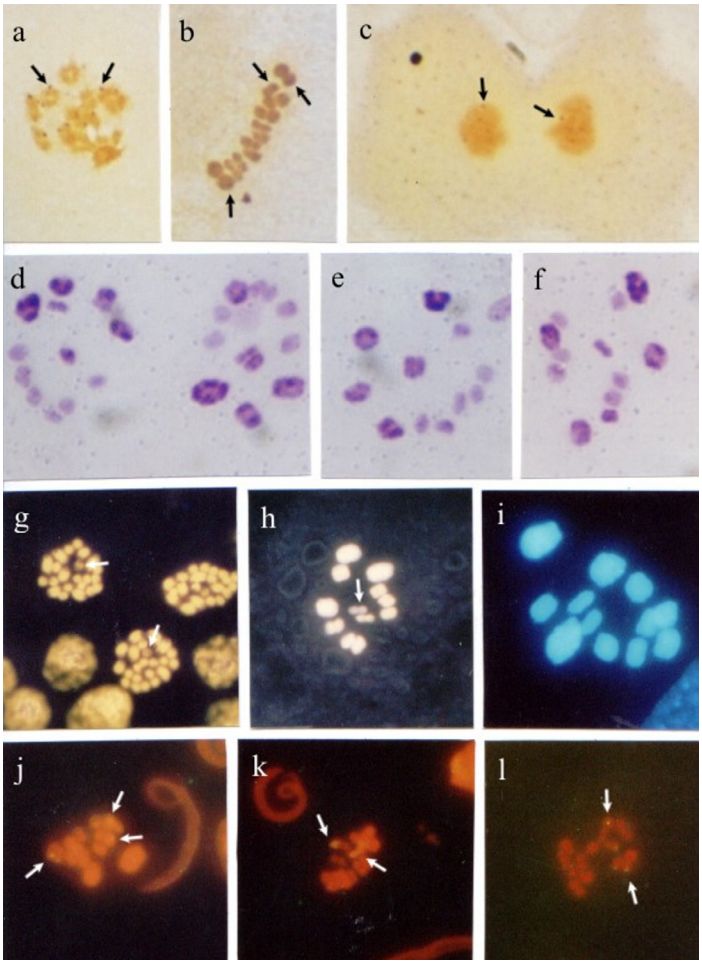

Figure 3. *Triatoma infestans* seminiferous tubules submitted to silver impregnation (a-c); C banding (d-f), double staining with fluorochromes CMA_3/DA (g,h) and DAPI/DA (i), and fluorescent *in situ* hybridization (FISH) with *Drosophila melanogaster* 28S rDNA probe (j-l). Spermatogonial metaphases (g). Nuclei at diplotene-diakinesis (a,j). First (d,e,f,i) and second (h,k) meiotic division metaphases. Anaphases (c,l). Arrows indicate chromosomes with silver-impregnated regions (a-c). In *d-f*, four autosomal chromosomes and both sex chromosomes (X, Y) are heterochromatic. Arrows indicate CMA^- chromosomes in *g,h*. In *i*, DAPI/DA staining showed no distinction among chromosomes. In *j-l*, arrows indicate regions hybridized by the probe. Bar = 10 µm.

The use of C banding differentiated four autosomes at first division metaphase: one was completely heterochromatic; another had heterochromatic telomeric regions; another had two heterochromatic regions (one telomeric and the other interstitial), and one other had a heterochromatic interstitial region. Both sex chromosomes also showed heterochromatic interstitial regions (Figure 3d-f).

In meiotic cells submitted to CMA_3/DA staining, CMA_3^- chromosomes were seen in the spermatogonial metaphases, while a CMA_3^- sex chromosome was observed at MI. DAPI/DA did not discriminate chromosomes at MI (Figure 3g-i).

FISH with a 28S rDNA probe showed some chromosomes with a hybridization signal in the nuclei at diplotene-diakinesis, as in MI and meiotic anaphases (Figure 3j-l).

DISCUSSION

Heterochromatin is considered to be responsible for creating new genes, maintaining chromosomes, recognizing chromosomes in the pairing of homologs and non-homolog chromosomes at meiosis, regulating recombination and segregation, and also, restoring telomeric stability after centric fission (John, 1988; Imai et al., 1988; Irick, 1994). In a variety of organisms, heterochromatin is completely eliminated from somatic cells. However, it is always retained in cells of the germinative strain. In meiotic cells, heterochromatin seems to be related to the formation of chiasmata, as crossing-over rarely occurs in heterochromatic regions (John and Miklos, 1979; Sumner, 1990).

The characterization and identification of Heteropteran holocentric chromosomes is usually hindered by the fact that they are difficult to be morphologically differentiated and display a high chromosome condensation. In this study, however, both sex chromosomes (X, Y), or sometimes just one of them, along with several autosomes were observed to be heterochromatic in *R. pallescens* at MI.

The analysis of *T. infestans* at MI demonstrated that four autosomal chromosomes (3 with telomeric blocks and 1 with an interstitial block) and both sex chromosomes (X, Y) (1 with a telomeric block and 1 with an interstitial block) are heterochromatic. These findings are not consistent with some classical reports, which describe a distinct pattern in *T. infestans*, where only three autosomal chromosomes (with telomeric chromosome blocks) and the Y sex chromosome are heterochromatic (Solari, 1979; Panzera et al., 1992). The difference in heterochromatin pattern found in this species could be related to the long-term maintenance of the colonies in laboratories, in specific crystallizers, which favors crossing-over. This is likely to have somehow influenced the heterochromatic pattern of the chromosomes in the specific case of the strain used in this study.

Differences in heterochromatin amount and location on chromosomes of different species of the same genus and among individuals of the same species are very common. Thus, closely related species may differ in number of C^+ bands, as well as in heterochromatin amount and location (Sumner, 1990; Clark and Wall, 1996).

Panzera et al. (2000) analyzed several *Triatoma* species and suggested that the rate of chromosomal evolution is related to adaptation to different environments or to some properties intrinsic to the chromosome complement. These authors observed morphologic, ecologic and molecular differences among the chromatin forms of the species, indicating that the populations

were undergoing differentiation processes that did not involve chromosome organization. Dujardin et al. (1999) supported this hypothesis by reporting that the differentiation that occurs among populations of a given species may lead to a speciation process that in Triatominae seems to be brief and, above all, induced by ecological factors. The hypothesis raised by these investigators supports the notion that the increase in heterochromatic blocks is due to the crossing-over caused by confining the *T. infestans* strain used in this study in crystallizers.

Our results show that, when CMA_3/DA and DAPI/DA were employed, the *P. megistus* species exhibited some CMA^- chromosomes at spermatogonial metaphases, and two CMA_3^+/$DAPI^-$ sex chromosomes (X_1X_2Y) at meiotic metaphases (MI and MII), confirming the presence of regions rich in CG bases. These findings are consistent with the C banding study conducted by Tartarotti and Azeredo-Oliveira (1999) in *P. megistus* meiotic chromosomes.

The species *T. infestans* studied herein showed some CMA_3^- chromosomes at spermatogonial metaphases, including one of the sex chromosome (X, Y) at MI, which indicated few CG regions. These findings support the results obtained by Mello and Recco-Pimentel (1987). DAPI banding failed to display heterochromatin regions. In *R. pallescens*, both spermatogonial metaphases and nuclei at meiotic prophase showed some chromosome regions rich in CG bases (CMA_3^+). Chromosomes at MI showed no heterochromatic regions when submitted to CMA_3/DA and DAPI/DA as the chromosomes were homogeneously stained.

In the species under study, the analyses of meiotic cells with the techniques of silver-impregnation and FISH with *D. melanogaster* 28S-12-kb rDNA probe revealed some correspondence between NORs detected by silver staining and the regions indicated by the rDNA probe. Some nonspecific staining was observed, as in *P. megistus*, but it did not affect data analysis. *R. pallescens* sex chromosomes (XY) were distinct from autosomes when submitted to silver impregnation and FISH, confirming that these chromosomes bear NORs. In *P. megistus*, the nuclei at early prophase I and at diakinesis exhibited corresponding staining when the techniques of silver impregnation and hybridization were used. In this species, NORs were observed on autosomal chromosomes at spermatogonial metaphase, and a sex chromosome, as well as an autosome bearing NORs were seen at diakinesis. In *T. infestans*, correspondence between silver staining and hybridization spots was demonstrated at diakinesis and at anaphase.

In a study of four triatomine species of the genus *Triatoma*, the use of *D. melanogaster* 28S-12-kb rDNA probe revealed that in two of these species (*T. tibiamaculata* and *T. protacta*), some of the autosomes exhibited hybridization spots. In *T. tibiamaculata*, such spots were telomeric and found in only one bivalent. These spots seemed to coincide with CMA_3^+ regions. In *T. platensis*, a fluorescent spot seen in one of the sex chromosomes (probably the X chromosome) coincided with the silver-stained area. In *T. vitticeps*, hybridization was detected in two of the three sex chromosomes (Severi-Aguiar and Azeredo-Oliveira, 2005; Severi-Aguiar et al., 2006).

The results obtained in the present study indicate that in triatomines, in general, the number and location of NORs are species-specific and, as in other animal groups, important chromosome markers that facilitate comparative studies for a better understanding of the evolutionary mechanisms of these important hematophagous insects.

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