



## C-banding and fluorescent *in situ* hybridization with rDNA sequences in chromosomes of *Cycloneda sanguinea* Linnaeus (Coleoptera, Coccinellidae)

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### Abstract

The aim of this study was to describe mitotic and meiotic chromosomes of *Cycloneda sanguinea* using C-banding, fluorescent *in situ* hybridization (FISH) rDNA probes, and sequential FISH/Ag-NOR staining. The chromosome number was  $2n = 18 + XX$  for females and  $2n = 18 + Xy$  for males. The X chromosome was metacentric and the Y chromosome was very small. During meiosis, the karyotypic meioformula was  $n = 9 + Xy_p$ , and sex chromosomes configured a parachute at metaphase I. At the beginning of pachytene, bivalents were still individualized, and sex chromosomes were associated end-to-end through the heteropycnotic region of the X chromosome. Later in pachytene, further condensation led to the formation of a pseudo-ring by the sex bivalent. All chromosomes showed pericentromeric heterochromatin. FISH and sequential FISH/Ag-NOR staining evidenced the location of the nucleolar organizer region in one pair of autosomes (at spermatogonial metaphase). During meiosis, these genes were mapped to a region outside the sex vesicle by FISH, although  $Xy_p$  was deeply stained with silver at metaphase I. These results suggest that these argyrophilic substances are of a nucleolar protein nature, and seem to be synthesized by a pair of autosomes and imported during meiosis (prophase I) to the sex pair, during the association of the sex chromosomes.

**Key words:** *in situ* hybridization, rDNA sequences, sequential FISH/Ag-NOR staining, C-banding, *Cycloneda sanguinea*.

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### Introduction

The lady beetle *Cycloneda sanguinea* belongs to the family Coccinellidae, a cosmopolitan taxon that comprises 5000 species, 2000 of which are Neotropical species (Iabhlkoff-Khuzorian, 1982). Many species of this family are highly active predators and are intensively used for biological control (Borror and De Long, 1988; De Bach, 1964; Gordon, 1985; Levins and Wilson, 1980).

Cytogenetic analyses of the family Coccinellidae are scarce, and the karyotypes of only 4% of the species are known. All of them are characterized by meiosis with an  $n = 9 + Xy_p$  in males. Sex chromosome association during metaphase shows the typical "parachute" shape that characterizes Coleoptera. This peculiar type of association has been traditionally explained as being due to the presence of the nucleolar organizer region (John and Lewis 1960;

Smith and Virkki 1978; Virkki *et al.* 1991), and fluorescent *in situ* hybridization (FISH) using 18S-28S rDNA gene probes has corroborated this theory for some Australian species of *Cicindela* (Galián and Hudson, 1999) and for *Olla v-nigrum* (Maffei *et al.*, 2001a). However, the nucleolar theory was questioned by other investigators, who found nucleoli in a pair of autosomes in other species (Drets *et al.*, 1983; Postiglioni and Brum- Zorrilla 1988; Postiglioni *et al.* 1991).

Banded karyotypes of Coccinellidae are almost unknown, except for C-banding in six species of the genus *Chilocorus*, *Epilachna paenulata*, *Eriopis connexa* and seven species of lady-birds from Central Europe (Ennis, 1974; Drets *et al.*, 1983; Maffei *et al.*, 2000; Rošek and Holecová, 2002).

The aim of the present study was to describe the mitotic and meiotic chromosomes in females and males of *Cycloneda sanguinea*, establishing their C-banding pattern and physical mapping of rDNA sequences using FISH and sequential FISH/Ag-NOR staining.

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## Material and Methods

The *Cycloneda sanguinea* L. specimens were collected on the campus of the Universidade Federal de Viçosa (UFV). Mitotic and meiotic analyses were carried out in 12 prepupal larvae and 40 male adults, with a sample size of 10 mitotic metaphases per individual. Cytogenetic preparations of mitotic metaphase chromosomes (cerebral ganglion) and meiosis analysis were carried out as described by Imai *et al.* (1988) and adapted for Coccinellidae (Maffei *et al.*, 2000).

C-banding was performed as described by Sumner (1972) with modifications (Maffei *et al.*, 2000). Slides were subjected to hydrolysis with 0.2 N HCl for 4 min at room temperature, washed in distilled water and incubated with 5% barium hydroxide at 60 °C for 8 min, then washed in 0.2 N HCl at room temperature for 30 s, incubated with 2xSSC at 60 °C for 10 min, and stained with 6.6% Giemsa in Sørensen buffer pH 6.8 for 50 min.

Sequential FISH/Ag-NOR staining followed the technique described by Maffei *et al.* (2001a). One drop of 2% gelatin (2 g gelatin diluted in 100 mL distilled water and 0.5 mL formic acid) and four drops of aqueous 50% Ag-NO<sub>3</sub> solution (Merck) were added onto slides already hybridized with rDNA probes. The slides were then covered with a coverslip and incubated in a moist chamber at 38% humidity for 25 min. Finally, the material was thoroughly rinsed with a squirt bottle for approximately 5 min, air-dried and mounted with Entellan (Merck).

Ribosomal DNA sequences were mapped using FISH (Viegas-Péquignot, 1992). The rDNA probes (pDm 238) containing the 18S, 28S and 5.8S genes were labeled with biotin by nick translation using the Bionick kit (Gibco). Slides with fixed meiotic chromosomes were incubated with RNase (100 µg/mL) for 1 h in a moist chamber containing 70% formamide at 37 °C, followed by three incubations in 2xSSC for 3 min each, and dehydrated in 50%, 75% and 100% alcohol for 3 min each. After denaturation of the chromosomal DNA in 70% formamide for 2 min at 70 °C, the slides were dehydrated in ice-cold 2xSSC for 2 min and immersed in 50%, 75% and 100% alcohol for 2 min each. In parallel, the probe was incubated at 100 °C for 2 min and maintained on ice. Ten microliters of the probe (diluted in hybridization mixture) per hybridization area were then added onto the slides, which were incubated in a 70% formamide moist chamber at 37 °C for 36 h. After incubation, the slides were immersed twice in 50% formamide at 37 °C for 2 min each, and washed with 2xSSC at 37 °C for 2 min each, followed by two washes with PBT for 2 min each.

## Immunological detection

The slides were incubated with anti-biotin (Vector SP3000) and anti-goat IgG-FITC (Vector FI5000). The DNA was counterstained with 100 µL propidium iodide for 1 min and washed with PBS. The slides were mounted in

13 µL Vectashield and photographed under an Olympus BX60 photomicroscope using appropriate fluorescence filters.

## Results

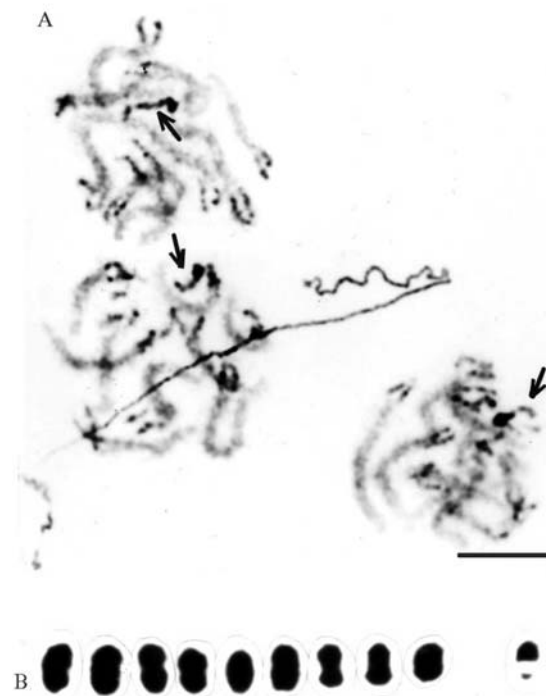
### Meiosis and mitosis

*C. sanguinea* cells in meiosis showed a large, single regular heteropycnotic chromocenter at prophase I (zygotene). At the beginning of pachytene, the bivalents were individualized and the sex chromosomes showed linear end-to-end association. As pachytene progressed, DNA condensation led to the formation of a pseudo-ring by the sex bivalents (Figure 1A). At metaphase I, the meioformula was  $n = 9 + Xy_p$  (Figure 1B), followed by normal chromosome segregation at anaphase I.

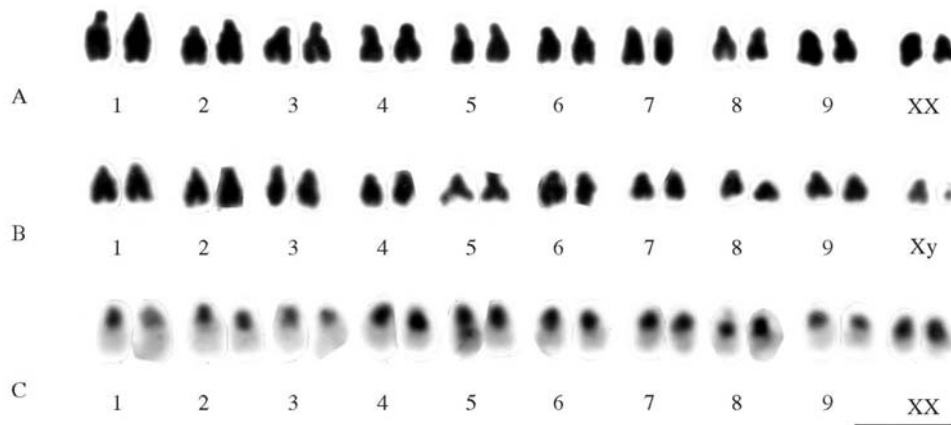
The analysis of mitotic cells with standard staining showed a chromosome number of  $2n = 18 + XX$  in 10 *C. sanguinea* females and of  $2n = 18 + Xy$  in two males. The chromosomes of this species were mainly submetacentric and the y chromosome was very small (Figures 2A and 2B). C-banding revealed heterochromatin in the pericentromeric regions of all chromosomes, including the short arms (Figure 2C).

### Location of rDNA genes and Ag-NOR banding

The location of rDNA sequences was evidenced by FISH as a large block at prophase I (early zygotene)



**Figure 1** - Meiosis of a *Cycloneda sanguinea* male. A) Pachytene showing the X and y chromosomes aligned end-to-end until the formation of a pseudo-ring. B) Metaphase I karyotype. Bar = 5 µm.



**Figure 2** - Karyotype of *Cycloneda sanguinea*. A) Female:  $2n = 18 + XX$ . B) Male:  $2n = 18 + Xy_p$ . C) C-banding pattern obtained for one female. Bar = 5  $\mu\text{m}$ .

(Figure 3A). Sequential FISH/Ag-NOR staining showed that the rDNA sequences were located outside the sex vesicle (Figures 3B and 3C). Sequential FISH/Ag-NOR treatment showed a marked silver staining of both the sex vesicles (prophase I) and the lumen of the sex chromosomes associated in a parachute format (metaphase I).

## Discussion

### Mitosis and meiosis

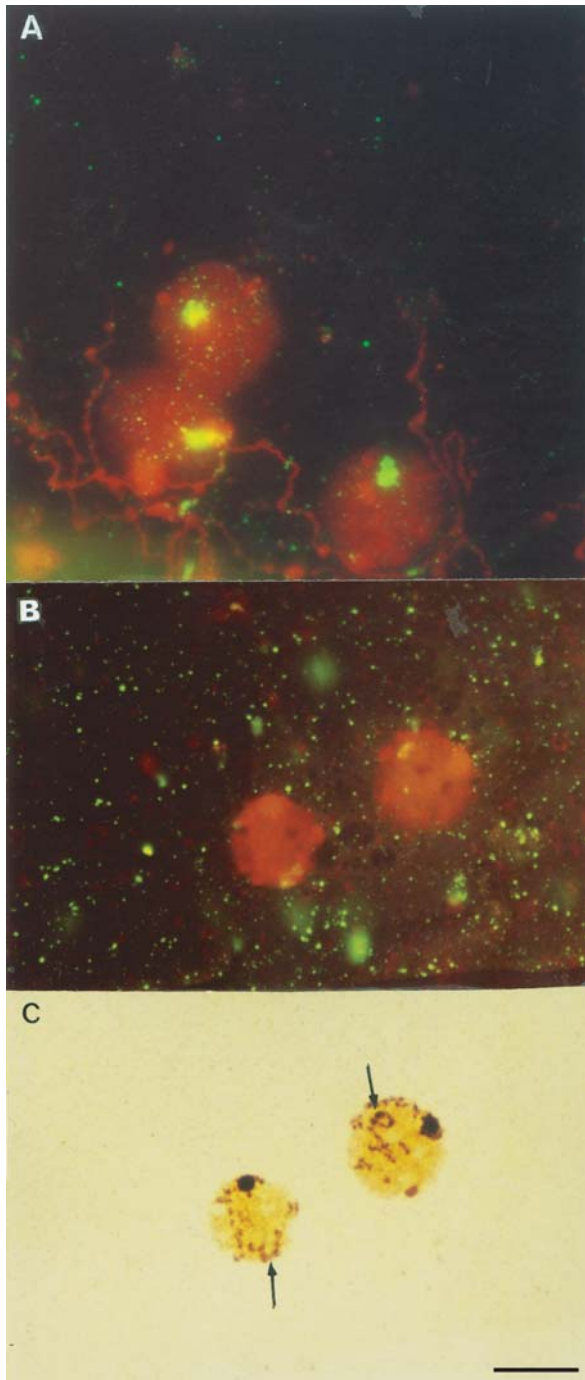
The lady-beetle *Cycloneda sanguinea* has a chromosome number of  $2n = 18 + XX$  for females and a meioformula of  $n = 9 + Xy_p$  for males, characteristic of most Coleopteran species. This may also represent the basic (ancestral) karyotype, especially in the suborder Polyphaga (Smith, 1950; Smith and Virkki, 1978). As recently reported for *Eriopis connexa* (Maffei *et al.*, 2000) and *Olla v-nigrum* (Maffei *et al.*, 2001a), all members of the family Coccinellidae (42%) apparently share the same chromosomal behavior: at prophase I (pachytene) of the male meiosis, sex chromosomes appear associated end-to-end, with the short arm of the X chromosome joining the y chromosome in a linear fashion followed by a pseudo-ring and, finally, a parachute is visible at metaphase I, with the expanded chromatin of the short arm of the X chromosome contributing to the parachute. These results are in agreement with the model of  $Xy_p$  sex chromosome association proposed by Drets *et al.* (1983) for *Epilachna paenulata* (Coleoptera, Coccinellidae). In this species, C-banding analysis revealed a sex chromosome association different from other Coleopteran species studied, due to the extensive role played by constitutive heterochromatin segments in chromosome association and  $Xy_p$  formation. The authors proposed a model where the parachutes consist of three different segments: two corpuscles showing intense heterochromatin upon C-banding and a euchromatic segment that forms the parachute. The euchromatic segment is

acknowledged as the long arm of the X chromosome in the parachute, and the formation of the parachute itself is the result of the association between the X and y chromosomes. It has been suggested that this association is not mediated by the nucleolar organizer region, since no nucleolar material associated with the sex chromosomes was detected. The observations made by Virkki *et al.* (1991) on the behavior of  $Xy_p$  association in six Coleopteran species by silver staining supported the interpretation of Drets *et al.* (1983).

In the present study, the heterochromatin of *C. sanguinea* was mainly located in the pericentromeric region of all chromosomes and in the short arms, as observed for most Coleopterans studied with C-banding technique (Ennis, 1974; Angus, 1982, 1983; Drets *et al.*, 1983; Juan and Petitpierre, 1989; Holecová *et al.*, 1997; Rošek and Holecová, 2000; 2002).

### Location of rDNA sequences (FISH) and gene activity

Meiotic chromosomes (prophase I) and NOR-banding analysis in spermatogonial metaphase of *C. sanguinea* are described for the first time in the present work. In an earlier study using NOR-banding techniques in mitotic cells of adult males, Maffei *et al.* (2001b) reported active regions as restricted to one pair of autosomes. They also indicated that rDNA sequences occur in a region outside the sex vesicle (prophase I). Sequential FISH/Ag-NOR confirmed the result obtained by silver staining regarding the  $Xy_p$  bivalent. FISH using rDNA probes has been little applied in Coleoptera. Juan *et al.* (1993) applied rDNA FISH to *Tenebrio molitor* and *Misolampus goudoti*, both bearing  $2n = 20$  chromosomes and an  $Xy_p$  sex chromosome system. In *T. molitor*, mitotic metaphases showed that the nucleolar organizer regions were located on two pairs of autosomes and on one pair of sex chromosome, thus supporting the classic hypothesis of the nucleolar origin of the



**Figure 3** - A) Mapping of the rDNA sequences by FISH during prophase I (early zygotene). B) and C) Sequential FISH/Ag-NOR staining. Arrows show the location of the rDNA sequences outside the sex vesicle. Bar = 5 µm.

$Xy_p$  association. In contrast, in *M. goudoti*, the rDNA sequences were restricted to one pair of autosomes. In *Cicindela melancholica*, FISH mapped the rDNA sequences to one of three X chromosomes and to the y chromosome (multiple sex system). However, in *Cicindela*

*paludosa* (a related species), which has an X0 sex determination system, the rDNA sequences were restricted to one pair of autosomes (Galián *et al.*, 1995). FISH for the 18S-28S rDNA genes was applied to other *Cicindela* species (Cicindelini tribe), and in three species of them, *Cicindela cardinalba*, *Cicindela* sp. (saetigera group) and *Cicindela gillesensis*, these genes were mapped to two of the four sex chromosomes that contributed to the sex vesicle. On the other hand, in *Megacephala whelani*, fluorescent hybridization during meiosis and mitosis revealed that the rDNA sequences were located on three pairs of autosomes (Galián and Hudson, 1999). This result does not support the classic nucleolar theory. The detection of argyrophilic substances in *C. sanguinea* during mitosis and meiosis by FISH and sequential FISH/Ag-NOR suggests that these substances are nucleolar proteins synthesized by a pair of autosomes and imported at prophase I, during sex chromosome association.

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