Sequential FISH analysis with rDNA genes and Ag-NOR banding in the lady beetle *Olla v-nigrum* (Coleoptera: Coccinellidae)

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We have characterized the meiosis of *Olla v-nigrum* by standard analysis, performed a NOR study using NOR banding, FISH of rDNA genes and sequential FISH/AgNOR analysis, and adapted the FISH methodology to Coccinellidae. The chromosome number determined at metaphase I was $n = 9 + Xy_p$. At zygotene it was possible to identify the sex vesicle which presented a deeply stained heteropycnotic block. Chromosome X is much larger than the y and the two combine, forming a "parachute" in metaphase I. FISH analysis using a probe of rDNA genes 18S, 28S and 5.8S of *D. melanogaster* was used to map the genes in the sex vesicle. The NOR band showed high gene activity in this region. These results were confirmed using sequential FISH/Ag NOR analysis. The data obtained for *Olla v-nigrum* agree with the classical hypothesis raised to explain the type of sex chromosome association in a parachute format (Xy_p) as being due to the presence of nucleolar material. The chromosome number and parachute configuration during metaphase I in this species agree with the basic karyotype of most Coleopterans. The major adaptation of the FISH method was the simultaneous denaturation and hybridization that permitted preservation of chromosome morphology, an essential factor when the chromosomes are small.

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The melanic form of the lady beetle *Olla v-nigrum* originates from the New World. The family Coccinellidae comprises approximately 4000 species. Most of them are effective predators mainly of aphids (DE BACH 1964; GORDON 1985). Many species have been efficiently used in programs of pest management and for this reason these beetles are intensively studied (DE BACH 1964; SMITH and REYNOLDS 1966; LEVINS and WILSON 1980; CHAVES 1991; MAJERUS 1994). The method of fluorescent in situ hybridization (FISH) first developed by LANGER-SAFER et al. (1982) has become an efficient and versatile technique for both the localization and mapping of specific sequences and for the identification of chromosomal DNA alterations in different organisms (MALUSZYNSKA and HESLOP-HARRISON 1991). The efficiency of the technique is high even when the chromosomes are of small size (MOYZIS et al. 1988; TRASK 1991; HAMILTON et al. 1992), as is usually the case for Coleoptera (SMITH and VIRKKI 1978; JUAN et al. 1993). There are relatively few reports of FISH applications in insects (HIRAI et al. 1996; PETITPIERRE 1996). The FISH technique with rDNA genes differs from NOR banding since it reveals the number of chromosome pairs of a given taxon that carry rDNA genes, while silver staining reveals active NORs in a given stage of the cell cycle (GALIÁN et al. 1995). However, in the ant *Tapinoma nigerrimum* NOR-banding results have shown that several chromosomes were stained with silver and the FISH technique mapped the rDNA genes on a single chromosome pair (LORITE et al. 1997).

The application of both methods is of particular interest for the study of beetles of the suborder Polyphaga, especially to clarify the non-chiasmatic associations of sex chromosomes such as the sex system of the Xy_p type in Coleoptera (PETITPIERRE 1996). JOHN and LEWIS (1960) proposed the nucleolar theory, suggesting that the association of sex chromosomes was due to the presence of nucleoli associated with these chromosomes. Several authors agree with this theory (SMITH and VIRKKI 1978; VIRKKI and SEPÚLVEDA 1990; VIRKKI et al. 1990). However, the nucleolar theory has been questioned in cases where nucleoli are located in an autosomal chromosome pair (WEBER 1971; DRETS et al. 1983; VIRKKI 1983; POSTIGLIONI and BRUM-ZORRILLA 1988; POSTIGLIONI et al. 1991). The subject is controversial because in both the cases described above the sex vesicle forms during meiosis (prophase I) in Coleoptera males with the Xy_p, sex system and is stained with silver when NOR banding is performed.

The FISH method has not been, so far, applied to any Coccinellidae species. The objectives of the present study were to map the rDNA genes 18S, 28S and 5.8S by FISH during meiosis in *Olla v-nigrum*, and to compare this localization with those of silver-stained NORs.

MATERIALS AND METHODS

Fifteen *Olla v-nigrum* males were collected in Viçosa-MG, Brasil. Slides to be submitted to FISH were prepared by the method of MAFFEI et al. (2000) without using colchicine. The testes were removed in Ringer and transferred to a slide to which several drops of fixative I (4 water:3 ethanol:3 acetic acid) were applied. The testes were dissociated with a stylet and two drops of fixative 2 (1 ethanol:1 acetic acid) were added, followed by three drops of fixative 3 (100 % acetic acid).

The in situ hybridization fluorescence (FISH) was based on the methods of VIÉGAS-PÉQUIGNOT (1992) and JUAN et al. (1993), with adaptations. The rDNA probes (pDm 238) containing ribosomal genes 28S, 18S and 5.8S of *D. melanogaster* were labeled with biotin by nick translation according to the instruction manual of the BIONICK kit (GIBCO). Slides with the fixed meiocytes were incubated with RNase (100 µg/ml in 2 × SSC) for 1 h in a chamber humidified with water at 37°C, treated with 0.005 % pepsin in 10 mM HCl at 37°C for 10 min, and then treated with three 2 × SSC baths for 3 min per bath. The material was dehydrated in 50 %, 75 % and 100 % alcohol for 3 min per treatment.

Simultaneous hybridization and denaturation

Five µl of the probe (1 µl probe diluted in 4 µl of hybridization mixture) was added to each hybridization area and the slide was covered with a 20 × 20 mm coverslip and incubated at 80°C for 3 min. Overnight incubation was then performed at 37°C.

After incubation, the slides were treated with two 50 % formamide baths of 2 min each at 37°C and then with two PBT baths of 2 min each.

Immunological detection

The slides were treated with anti-biotin (VECTOR:SP3000) and anti-goat IgG-FITC (VECTOR:FI5000). DNA was counterstained with 100 µl propid iodide, the slides were washed with PBS for 1 min and mounted with 13 µl Vectashield. The material was photographed with an Olympus BX60 photomicroscope using an appropriate fluorescence filter.

NOR banding was performed as described by MAFFEI et al. (2001). Two parts of developer (2 g of gelatine dissolved in 100 ml of distilled water and 1 ml of formic acid) and four drops of $AgNO_3$ (Merck) in 50 % aqueous solution were mixed on slides. Then, the material was covered with a coverslip, transferred to Petri dishes, overlaid with a moist filter paper and incubated at 38°C for 25 to 30 min, until the staining solution turned golden brown. The coverslip was rinsed off with tap water and the slide was then squirted vigorously with a 20 ml syringe. After the drying process the slide was mounted on Entellan (Merck). After placing slides in a warm oven (37°C) for 24 h, metaphases were photographed with a light microscope.

RESULTS

The specimens of the melanic form of *Olla v-nigrum* submitted to standard staining (meiosis) showed the $9 + Xy_p$ meioformula in metaphase I. During prophase I, two more intensely stained points (heteropycnotic) were observed in leptotene (Fig. 1A). In zygotene it was possible to identify the sex vesicle which presented a deeply stained large heteropycnotic block (Fig. 1B). In pachytene, the bivalents were already individualized and chromosome association was observed in the heteropycnotic block (Fig. 1C). Chromosome X was much larger than the y and the two combined to form a parachute in metaphase I (Fig. 1D and E). Few cells with anaphase bridges were observed (Fig. 1F). FISH analysis using a probe of rDNA genes of *D. melanogaster* mapped the genes in the sex vesicle (Fig. 2A). After sequential FISH/Ag NOR analysis, the sex vesicle was stained with silver, indicating gene activity. We observed that in the region where the probe hybridized completely there was little or no silver staining (Fig. 2B). The figure 3A illustrates a block of rDNA genes mapped in the sex vesicle (FISH). NOR banding alone revealed large silver-stained blocks which combined to form the sex vesicle (Fig. 3B, C and D) and a large single block was strongly stained with silver in pachytene (Fig. 3E).

DISCUSSION

Olla v-nigrum males have $2n = 20$ and a meioformula $9 + Xy_p$. This chromosome number and parachute configuration during metaphase I observed in this species agree with most coleopterans. In the family Coccinellidae, approximately 42 % of the karyotypes are of this basic type (SMITH and VIRKKI 1978). Recently, our group (MAFFEI et al. 2000) characterized the karyotype (mitosis, meiosis and constitutive heterochromatin) of both sexes of *Eriopis connexa* in a population from Viçosa, MG, Brazil, and detected a $9 + Xy_p$ formula in male meiosis and one small-

Fig. 1A–F. Meiosis of *Olla v-nigrum* males. **A** Leptotene. **B** Zygotene. **C** Pachytene. The arrowheads indicate the sex vesicle. **D** and **E** Chromosome X was much larger than chromosome Y and the two combined to form a parachute in metaphase I. The arrows indicate the chromosome association. **F** Few cells formed anaphase bridges. Bar = 5 μm.

sized supernumerary chromosome restricted to females.

The analysis of meiosis (prophase I) using only NOR banding indicated that the meiocytes of *Olla v-nigrum* had an active NOR region in the sex bivalent. Recently, in the lady-beetle *Cycloneda sanguinea* the nucleolar organizer region was located on an acrocentric autosome pair using Ag-NOR (MAFFEI et al. 2001). POSTIGLIONI and BRUM-ZORRILLA (1988) and POSTIGLIONI et al. (1991) described one pair of NOR with an autosomal location in the chrysomelid *Chelymorpha variabilis* using Ag-NOR staining, orange acridine fluorescence, and microspreading techniques. The results of their work led to a rejection of the hypothesis of JOHN and LEWIS (1960).

FISH of rDNA genes mapped this region in the sex vesicle (prophase I) and sequential Ag-NOR staining confirmed gene activity in the same region. However, silver staining was reduced due to the use of pepsin. The results described for *Olla v-nigrum* agree with the classical hypothesis suggested by some investigators to explain that the association of the sex chromosomes (Xy_p) may be due to the presence of nucleolar material (JOHN and LEWIS 1960; SMITH and VIRKKI 1978).

Fig. 2A–B. *Olla v-nigrum.* **A** FISH rDNA genes were mapped to the sex vesicle. **B** Sequential FISH/Ag NOR, with the sex vesicle being stained with silver. Bar = 5 μm.

The FISH technique using an rDNA probe has rarely been applied to Coleoptera. JUAN et al. (1993) applied FISH with rDNA probes to *Tenebrio molitor* and *Misolampus goudoti*, both with 2n = 20 and an Xy_p sex determination system. Analysis of mitotic metaphase of *T. molitor* showed that the NORs are located in two autosomal chromosome pairs and in the sex pair, supporting the classical hypothesis of a nucleolar origin of the Xy_p association. However, in *M. goudoti* the rDNA genes were mapped only on an autosome pair. FISH of rDNA genes was applied to *Cicindela melancholica* and the results revealed that the rDNA genes are located in one of the three X chromosomes and in the y (multiple sex system). In contrast, in *Cicindela paludosa* (a related species) which has an XO sex system, the rDNA genes were mapped on a pair of autosomal chromosomes (GALIÁN et al. 1995). FISH of the rDNA genes 18S–28S was applied to other *Cicindela* species (Cicindelini tribe) and in three species, *Cicindela cardinalba*, *Cicindela* sp. (saetigera group) and *Cicindela gillesensis*, the genes were mapped on two of the four sex chromosomes that formed the sex vesicle. In *Megacephala whelani*, hybridization during meiosis and mitosis showed, however, that the rDNA genes were localized in three autosome pairs (GALIÁN and HUDSON 1999). DE LA RUA et al. (1996) reported the 18S rDNA genes in the largest autosome pair in 12 species of *Carabus* and two of *Calosoma* (Carabini tribe) and in three related species of *Ceroglossus chilensis* (Ceroglossini tribe). The results suggested the existence of a conservative pattern in these genera. However, in *Cychrus* (carabids, tribe Cychrini), the ribosomal cistrons were localized in two medium-sized autosome pairs (DE LA RUA et al. 1996). FISH data obtained for seven species of chrysomelids of the genus *Timarcha* revealed that in all species except one with the neoXy sex-determination system, the rDNA genes were in an autosomal pair, even though Xy_p was strongly silver stained (GÓMEZ-ZURITA and PETITPIERRE, pers. com.).

The major adaptation of the methodology was simultaneous denaturation and hybridization, as first proposed by JUAN et al. (1993), which permitted the preservation of chromosome morphology, an essential factor especially when the chromosomes are of small size, as is usually the case for Coleoptera (SMITH and VIRKKI 1978; JUAN et al. 1993). Usually, mitotic metaphase chromosomes are employed for FISH analysis. However, other phases can be used, such as prophase I of meiosis which has the following advantages: 1) cells free of cell debris which might interfere with probe penetration, 2) chromatin that is little condensed in this phase and is highly accessible to the probe, and 3) considerably voluminous cells showing a ten-fold increase in size, permitting high resolution mapping by FISH (PETERSON et al. 1999).

These rDNA and others, used as probes, could probably identify marker chromosomes for evolutionary studies, and for the larger rDNA loci, the level of correspondence between the results of FISH and Ag-NOR staining is also pertinent for measuring gene activity at NOR sites and possible shifts during evolution (PETITPIERRE, 1996). This was reported, for example, for the Australian primitive ant group of *Myrmecia pilosula* complex, in which great changes in diploid numbers are paralleled by increases in the number of NORs (HIRAI et al. 1994). Numerical and structural polymorphism in rDNA genes was reported by VITTURI et al. (1999) for the coleopteran *Thorectes intermedius* using FISH. The results showed numerical and structural inter and intraindividual polymorphism in NOR number and size. FISH was recently used in Coleoptera to map the loci of the 18S rDNA genes in 19 taxa of the genus

Fig. 3A–E. *Olla v-nigrum*. **A** FISH of rDNA genes at zygotene. **B**, **C** and **D** NOR banding (alone) revealed large silver-stained blocks which associated to form the sex vesicle. **E** Pachytene showing a large strongly silver-stained single block. Bar = 5 μm.

Zabrus (2n = 47 – 63). Variations were detected both in the number of chromosomes bearing rDNA gene clusters (2–12) and in the signals of hybridization, which varied from small points up to the entire chromosome arm (SANCHEZ-GEA et al. 2000).

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