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Chromosomal localization and partial sequencing of the 18S and 28S ribosomal genes from *Bradysia hygida* (Diptera: Sciaridae)

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ABSTRACT. In insects, ribosomal genes are usually detected in sex chromosomes, but have also or only been detected in autosomal chromosomes in some cases. Previous results from our research group indicated that in *Bradysia hygida*, nucleolus organizer regions were associated with heterochromatic regions of the autosomal C chromosome, using the silver impregnation technique. The present study confirmed this location of the ribosomal genes using fluorescence *in situ* hybridization analysis. This analysis also revealed the partial sequences of the 18S and 28S genes for this sciarid. The sequence alignment showed that the 18S gene has 98% identity to *Corydalus armatus* and 91% identity to *Drosophila persimilis* and *Drosophila melanogaster*. The partial sequence analysis of the 28S gene showed 95% identity with *Bradysia amoena* and 93% identity with *Schwenckfeldina* sp. These results confirmed the location of ribosomal genes of *B. hygida* in an autosomal chromosome, and the partial sequence analysis of the 18S and 28S genes demonstrated a high percentage of identity among

several insect ribosomal genes.

Key words: *Bradysia hygida*; 28S rDNA gene; 18S rDNA gene; Sciarid; Fluorescence *in situ* hybridization

INTRODUCTION

Ribosomal gene (rDNA) analysis is a useful tool for studying variability and divergence within and among species (Nguyen et al., 2010), and has been widely used in phylogenetic studies (Stage and Eickbush, 2007; Marvaldi et al., 2009). In eukaryote genomes, the multigene families of ribosomal RNA (rRNA) genes are tandemly arrayed in clusters located in one or several chromosomes that are organized in multiple copies of cistrons (Cabral-de-Mello et al., 2011a). Each cistron contains an intergenic spacer (IGS) that is not transcribed, an external transcribed spacer (ETS), the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second internal transcribed spacer (ITS2), and the 28S gene. The ETS is part of the primary transcription unit, but is removed after RNA processing. The gene for 5S rRNA is moderately repetitive (Paques et al., 1995); it is not associated with nucleolar organizer regions (NORs), and it is not necessarily located on the same chromosome as other ribosomal genes. Furthermore, unlike the transcription unit of the 18S, 28S, and 5.8S, this gene is transcribed by RNA polymerase III. In insects, rDNA is most commonly found in sex chromosomes (Gerbi, 1971; Javier et al., 2000; Madalena et al., 2007), although it has also been localized in autosomal chromosomes (Hales, 1989; Madalena et al., 2007), and in some cases (e.g., *Diabroctis mimas* and *Rhynchosciara americana*), in both sex and autosomal chromosomes (Pardue et al., 1970; Bione et al., 2005). In general, sciarid adults are simply considered to be a nuisance without causing major problems (Cloyd, 2000); however, the larvae of some species have been reported to cause major plant damage once they feed on their roots (Cloyd et al., 2007). *Bradysia impatiens* has been reported to be an economically problematic species in Florida (Mead, 1978), *Bradysia* species are known major insect pests in greenhouses (Meers and Cloyd, 2005), and *Bradysia odoriphaga* Yang and Zhang is the most serious pest of Chinese chive (Li et al., 2007).

Bradysia hygida, first described by Sauaia and Alves (1968), is a sciarid that has been extensively used as a model system to study developmentally regulated gene transcription and amplification (Laicine et al. 1984; Monesi et al. 1995; Fiorini et al., 2001; Basso Jr et al., 2002; Candido-Silva and Monesi, 2010; Garcia et al., 2011; Passos et al., 2012). In this sciarid, the 5S ribosomal gene is localized in the autosomal A chromosome. Its localization was determined by fluorescence *in situ* hybridization (FISH) and gene sequencing (Ribeiro and Fernandez, 2004). The 18S/28S rDNA unit was detected by Ag-DAPI staining and was associated to the constitutive heterochromatin in the end of the autosomal C chromosome (Gaspar et al., 2002). However, the silver impregnation method is not as specific as the combination of FISH for rRNA genes and silver impregnation, which constitutes an excellent option for both locating the chromosomes carrying these genes and for ascertaining their phenotypic expression by the presence of an attached nucleolus (Cabrero and Camacho, 2008). In the present work, we confirmed the rDNA gene localization in the *B. hygida* autosomal C chromosome for both mitotic, as well as polytene chromosomes, by FISH. In order to confirm the clones used and to add information about *B. hygida* chromosome structure, partial sequencing of the 18S and 28S genes is also described.

MATERIAL AND METHODS

Biological material

B. hygida was reared at 22°C in dry potato foliage fermented with yeast extract. A detailed description of its life cycle and culture methods was previously reported (Laicine et al., 1984; Silva and Fernandez, 2000).

Mitotic and polytene chromosomes preparation

Mitotic chromosomes were obtained from larval brains, according to methods described in Borges et al. (2000), and polytene chromosome preparations were obtained from the salivary gland S1 region as described by Laicine et al (1984). After dissection, the material was immediately squashed in 45% acetic acid, the slides were frozen in liquid nitrogen, and the coverslip was flipped off with a razor blade. The slides were fixed in Alfac solution (methanol:formaldehyde:acetic acid 85:10:5) for 1 h, followed by immersion in 70% ethanol at 4°C until processing, when they were air-dried.

Preparation of the probes

Two cosmid clones, *Bh2a12G* and *Bh2b11C* (kindly provided by Dr. Maria Luisa Paçó-Larson from Faculdade de Medicina de Ribeirão Preto, FMRP, USP), which were cloned in c2XMCS, and a clone previously detected by p*Ra*-1.4 from *R. americana* (Zaha et al., 1982) were utilized. The cosmid DNA was extracted by the CBAT method (Del et al., 1989) and labeled with biotin-16-dUTP (Boehringer).

FISH

FISH was performed as described by Ribeiro and Fernandez (2004) at 37°C for 18 h. Layers of fluoresceinated avidin conjugated biotin-antiavidin D (Vector Laboratories) detected the hybridization sites. Slides were observed under the Axioskop MC 100 (Carl Zeiss) microscope with the 450 to 490 nm filter for fluorescein and the 515 to 560 nm filter for the propidium iodide. Some of the images were obtained in the confocal LSM 510 Zeiss microscope.

DNA extraction for sequencing

DNA from adult flies was extracted according to methods described in Monesi et al. (1995). DNA from the *B. hygida* ribosomal clones (*Bh2a12G* and *Bh2b11C*) were inoculated into Luria-Bertani (LB) medium (Sambrook et al., 1989) supplemented with 50 μg/mL ampicillin and incubated with constant agitation at 250 rpm at 37°C for 16-18 h. The cosmidial DNA was obtained using the HiSpeed plasmid extraction kit (Qiagen) according to manufacturer recommendations.

Primer design

Two primer pairs for the 18S segment were designed using the PCR FAST program

(Ruslan Kalendar 05/03/30 version) from a ribosomal gene sequence of *Drosophila melanogaster* deposited in GenBank (M21017.1) (Bh18SP1 Forward: 5'-GAA CCT TAT GGG ACG TGT GC-3'; Bh18SP1 Reverse: 5'-AGC TTT GGT TTC CCG GAA GC-3'; Bh18SP2 Forward: 5'-TTC TAA CCA TAA ACG ATG CCA GC-3'; Bh18SP2 Reverse: 5'-TGA TCC TTC CGC AGG TTC AC-3'), and three pairs for the 28S segment were designed (using the same program) from a ribosomal gene sequence of *Bradysia amoena* deposited in GenBank (FJ040522.1) (Bh28SP1 Forward: 5'-GTT GAT GCC ATA GCG GTG AC-3'; Bh28SP1 Reverse: 5'-AAA AGT GGC CCA CTG GGC AC-3'; Bh28SP2 Forward: 5'-AGT GTT TGG CGT GAG CCT GC-3'; Bh28SP2 Reverse: 5'-CCC AAT CAA GCC CGA CAA TC-3'; Bh28SP3 Forward: 5'-GGA TTC GTC TAT GCG CGA TTG-3'; Bh28SP3 Reverse: 5'-GAT TTC GGG CAC CAG CAT CG-3').

Sequencing

Polymerase chain reaction (PCR) was performed using the designed primers (Bh-18SP1; Bh18SP2; Bh28SP1; Bh28SP2; Bh28SP3), template DNA from the *Bh2a12G* and *Bh2b11C* clones, and total DNA from adult flies. The reaction conditions included an initial denaturation of 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, annealing temperature for 1 min (Bh18SP1 = 59°C; Bh18SP2 = 61°C; Bh28SP1 = 60°C; Bh28SP2 = 60°C; Bh28SP3 = 61°C), 72°C for 1 min, followed by a final extension of 10 min at 72°C.

The amplified regions were linked in pGEM (Promega) according to manufacturer recommendations. Transformation by heat shock in DH5α thermo competent bacteria was carried out according to routine protocols (Sambrook et al., 1989). White colonies were selected and inoculated into LB medium supplemented with 50 µg/mL ampicillin, and incubated with constant agitation at 70 rpm at 37°C for 16-18 h. The plasmid DNA was extracted by the CTAB method (Del et al., 1989). The PCR for sequencing was performed using the Dynamic Mega BACE ET Dye Terminator kit (Amersham Biosciences). The reaction conditions were 25 cycles of 95°C for 30 s, 55°C for 15 s, and 60°C for 1 min with the M13 forward or reverse primers, according to manufacturer specifications. Sequencing was performed using the MegaBACE 1000 automated DNA sequencing system and data was analyzed in the GenBank database, using the BLAST program. Sequences were aligned using the online ClustalW program (EMBL-EBI).

RESULTS

The NOR sites in *B. hygida* were previously found to be associated with the heterochromatic region in a terminal position in the autosomal C chromosome based on Ag-DAPI staining (Gaspar et al., 2002). In the present study, FISH analysis with the clones *Bh2a12G* and *Bh2b11C* confirmed this localization in mitotic chromosomes (Figure 1). Optical sections in confocal microscopy with 0.25 µm intervals (Figure 2) indicated that the rRNA genes were most likely contained in chromatin that is associated with or near the nuclear membrane.

Polytene chromosomes are a result of a process in which the DNA is replicated over a thousand times, with no mitoses or separation of homologous chromosomes; therefore, they are considered an important tool for the physical localization of genes. FISH of polytene chromosomes of *B. hygida* detected the same terminal position in chromosome C, but it also detected a small band at an internal portion (Figure 3). Given that the clones used as probes also contain internal spacers, the detected band could represent repetitive sequences.

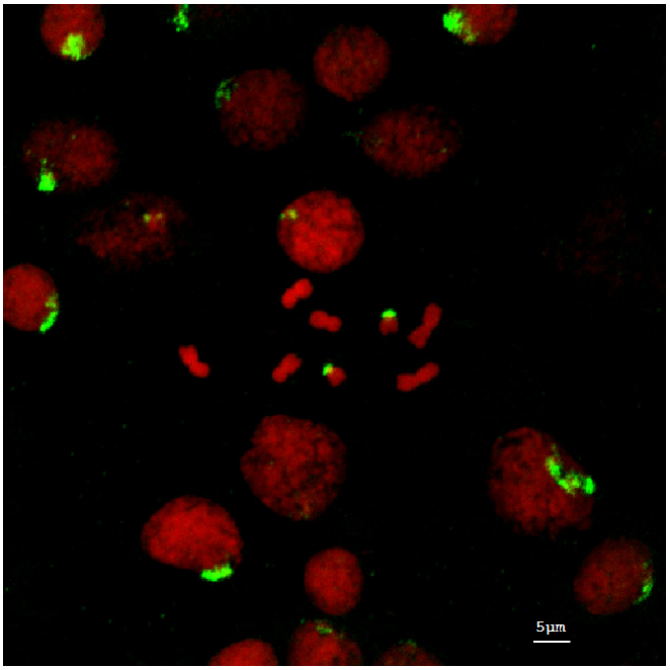

Figure 1. *Bradysia hygida* mitotic chromosome ribosomal gene localization. Fluorescence *in situ* hybridization of mitotic chromosomes was performed using larvae neuroblasts with the *Bh2b11C* clone. The hybridization was detected in the neuroblast nucleus, and the C chromosome pairs showed in the metaphase plate.

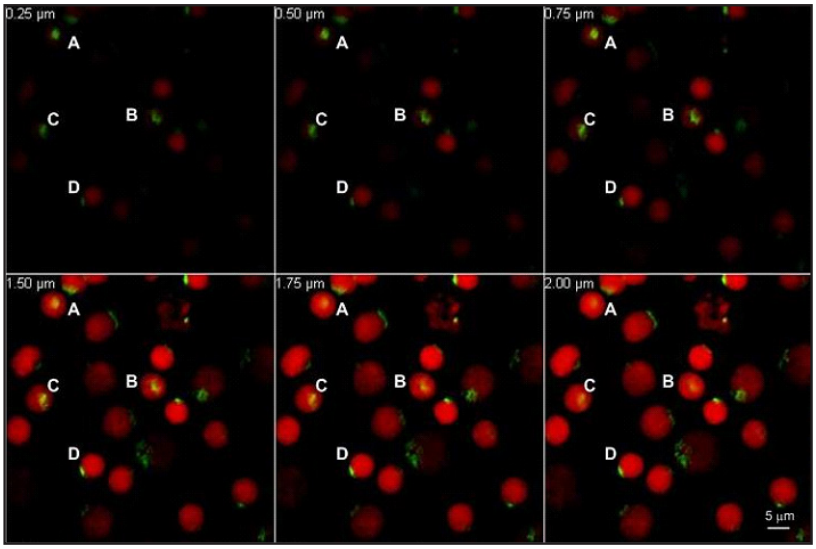

Figure 2. Optical sections of *Bradysia hygida* neuroblast nucleus. Fluorescence *in situ* hybridization of mitotic chromosomes was performed using larva neuroblasts with the *Bh2b11C* clone. The interval between each optical section was 0.25 µm.

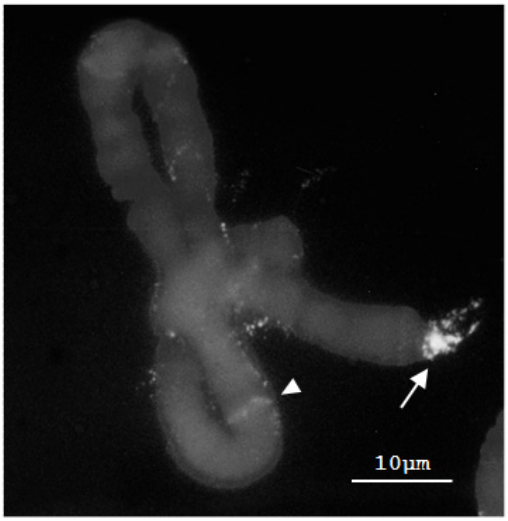

Figure 3. *Bradysia hygida* polytene chromosome ribosomal gene localization. Fluorescence *in situ* hybridization was performed using the *Bh2b11C* clone with polytene chromosomes from the *Bradysia hygida* salivary gland S1 region. The hybridization in the C10 regions of the C chromosome is marked with an arrow; a faint signal was also observed at the C6 region (arrowhead).

Previous results (data not shown) showed that the *Bh2a12G* clone contains both the 28S and 18S portions, whereas the *Bh2b11C* clone contains only the 28S portion. Using the primer pairs designed for the 18S and 28S regions, different regions of the *Bh2a12G* and *Bh2b11C* clones were amplified along with genomic DNA. For the 28S region, all three pairs amplified all three templates (*Bh2a12G*, *Bh2b11C*, and genomic DNA). For the 18S portion, the first primer pair did not amplify in any template, while the second pair amplified the *Bh2a12G* clone and the genomic DNA. Partial sequencing revealed a 3547-bp fragment for the 28S portion and a 1327-bp fragment for the 18S portion (GenBank accession Nos. JQ652461 and JQ652462 respectively). Alignments of these 28S and 18S sequences with ribosomal genes from other flies showed that this region is highly conserved. The 28S portion has 95% similarity with *B. amoena* (FJ040522.1) and 93% identity with *Schwenckfeldina* sp (FJ040523.1). The first 890 bases of the 18S portion has 98% with *Corydalus armatus* (EU815238.1) and 91% identity with *Drosophila persimilis* (XR048244.1) and *D. melanogaster* (M21017.1). Furthermore, for the final portion of 437 bases, a similarity of 73% with an internal spacer of *Sciara coprophila* (V01341.1) was found between positions 165 to 298 in our sample and 23 to 145 in *S. coprophila* (Figure 4).

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B. hygida      165  AGAAACGGAAGCACTTTGATATAGTGTCTTTGGGTACTTATTTTAAATAAGTATAATCGA  224
                    || || ||||||||||||||| | |   |||||||||||| |||||    |||||| |||
S. ooprophila  23   AGGAATGGAAGCACTTTGATAGAATTATTTTGGGTACTTACTTTAA----GTATAAACGA  78

B. hygida      225  CCAGG-TCGTCGAGACGTATATACACGCCCAAGCCTCACACTTAAAATCATTGTGTTTAC  283
                    | |   | |||| ||      ||  |||||||  |  | |  | ||||||||||||||||
S. ooprophila  79   CGAACCTTGTCGTGA------TATTCGCCCAAAACAAATA--TGAAATCATTGTGTTTAC  130

B. hygida      284  AAACCCTTTGTCGTA  298
                    |||||||  |||||||
S. ooprophila  131  AAACCCTCTGTCGTA  145
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Figure 4. Sequence alignment between the 3' end *Bradysia hygida* 18S sequence and *Sciara coprophila* (V01340.1) is shown.

DISCUSSION

The characterization of chromosomes is a very useful tool that has been widely used in phylogenetic studies, as well as for species identification. Insect rDNA genes have been shown to be localized in autosomal and/or sex chromosomes. In coleopterans, the autosomal NOR pattern appears to be the most stable evolutionary condition in species of the two suborders studied (Adephaga and Polyphaga), and the presence of NORs on autosomal and sex chromosomes could be indicative of karyotypic differentiation (Schneider et al, 2007). Virkki and Denton (1987) described NORs associated with sex chromosomes in different coleopteran species: *Alagoasa bicolor*, *Omophoita cyanipennis*, and *Omophoita albicollis.* In *Diabroctis minas*, NORs were detected in autosomal and sex chromosomes, whereas in *Digitonthophagus gazella* and *Isocopris inhiata* they were in autosomal chromosomes only, and in *Eurystermus caribaeus*, variability in the 18S rDNA cluster size was observed between the X and Z chromosomes (Bione et al., 2005; Cabral-de-Mello et al., 2011b).

Although research related to rRNA in diptera is scarce, we found that in many species of culicidae (Rafael et al., 2006) and tephritidae (Drosopoulou et al., 2012), the rRNA genes are usually localized in sex chromosomes; however, in some cases (e.g., *Rhagoletis pomonella*), it can be found in autosomal chromosomes (Procunier and Smith, 1993).

Ribosomal genes have been studied in only a few sciarid species (Gerbi 1971; Madalena et al., 2007). In *S. coprophila*, both chromosome types (polytene and mitotic) were studied, and rDNA was also found in the X chromosomes. In *Trichosia pubescens*, the rDNA was found to be localized in the X chromosome and in micronucleoli. In *R. americana* and *Rynchosciara milleri*, it was found in the X chromosome only, whereas in *Schwenkfeldina* sp, it was detected in the autosomal C chromosome.

Our results provide important information regarding chromosome structure in *B. hygida.* C banding analyses in metaphasic chromosomes revealed heterochromatic regions in the centromeric regions of chromosomes A and C and in the pericentromeric and interstitial regions of chromosomes B and X (Gaspar et al., 2002). Based on its transcriptional activity, the rDNA gene was determined to be localized on autosomal C chromosomes (Gaspar et al., 2002) associated to the heterochromatin. Using the FISH technique, we confirmed these findings in both mitotic and polytene chromosomes.

rDNA genes are expressed mainly in the X chromosomes, as there are indications that the X and Y rDNA arrays evolve independently and only the X chromosome is under selection pressure (Clark et al., 1991). Some sciarids show a dosage compensation mechanism (da Cunha et al., 1994), in which there is hypertranscription in the male soma in order to compensate for the lack of one chromosome. Since the sexual system of *B. hygida* is XX/X0 (Borges et al., 2000), this chromosome hypertranscription would also be expected to occur in this species. Interestingly, the localization of the rDNA genes remained outside of the sex chromosomes, enabling exchanges between homologous chromosomes, which could result in homogenization of multigene families in the population.

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