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A comparative cytogenetic study of *Drosophila* parasitoids (Hymenoptera, Figitidae) using DNA-binding fluorochromes and FISH with 45S rDNA probe

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Abstract Karyotypes of *Leptopilina boulardi* (Barbotin, Carton et Keiner-Pillault, 1979) (n = 9), *L. heterotoma* (Thomson, 1862) (n = 10), *L. victoriae* Nordlander, 1980 (n = 10) and *Ganaspis xanthopoda* (Ashmead, 1896) (n = 9) (Hymenoptera, Figitidae) were studied using DNA-binding ligands with different base specificity [propidium iodide (PI), chromomycin A_3 (CMA_3) and 4′,6-diamidino-2-phenylindole (DAPI)], and fluorescence in situ hybridization (FISH) with a 45S rDNA probe. Fluorochrome staining was similar between the different fluorochromes, except for a single CMA_3- and PI-positive and DAPI-negative band per haploid karyotype of each species. FISH with 45S rDNA probe detected a single rDNA site in place of the bright CMA_3-positive band, thus identifying the nucleolus organizing region (NOR). Chromosomal locations of NORs were similar for both *L. heterotoma* and *L. victoriae*, but strongly differed in *L. boulardi* as well as in *G. xanthopoda*. Phylogenetic aspects of NOR localization in all studied species are briefly discussed.

Keywords Hymenoptera · Figitidae · Karyotypes · DNA-binding fluorochromes · FISH · 45S rDNA

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Introduction

The Hymenoptera are one of the largest, most taxonomically complicated and economically important insect orders, with its world fauna containing more than 150,000 described species (Aguiar et al. 2013). They are also very diverse in terms of bionomics, since members of the main groups of this order can be herbivores, parasitoids, predators as well as pollinators and nectar feeders (Gauld and Bolton 1988). Considerable efforts are currently underway to sequence a number of insect genomes, especially under the i5K initiative (Evans et al. 2013). However, to date, just a few Hymenoptera genomes are fully sequenced. For example, data on full genomes of only twenty members of this order are available at present through the Hymenoptera Genome Database (Elsik et al. 2016). Of these, the genome of the single parasitoid species, *Nasonia vitripennis* (Walker) (Chalcidoidea, Pteromalidae), has been studied in detail (The *Nasonia* Genome Working Group 2010).

Wasps which belong to the genera *Leptopilina* Förster, 1869 and *Ganaspis* Förster, 1869 (Cynipoidea, Figitidae) are solitary larval/pupal endoparasitoids of various *Drosophila* Fallen, 1823 species (Melk and Govind 1999; Allemand et al. 2002). Since some of these parasitoid/host assemblages represent good models for investigating many general aspects of biology and physiology of insect parasitism, genomes of these wasp species are under intensive investigation. Transcriptome analyses of these species (Colinet et al. 2013; Goecks et al. 2013; Heavner et al. 2013; Mortimer et al. 2013) are being followed by genome sequencing efforts. In this regard, studying parasitoid karyotypes can provide important information for genome research (Gokhman 2009).

Morphometric study of routinely stained chromosomes and genome sizes of three *Leptopilina* and one *Ganaspis*

species, i.e. *L. boulardi* (Barbotin, Carton et Keiner-Pillault, 1979), *L. heterotoma* (Thomson, 1862), *L. victoriae* Nordlander, 1980 and *G. xanthopoda* (Ashmead, 1896), were recently reported by Gokhman et al. (2011). Here, we extend these observations and present data on the location of the multicopy 45S ribosomal gene sites, also referred to as the nucleolus organizing regions (NORs). NORs contain clusters of rDNA genes that are generally difficult to map via DNA sequencing as well as other multicopy genes (Treangen and Salzberg 2011), but nevertheless provide useful physical chromosomal landmarks. Since rDNA clusters in eukaryotic organisms (including parasitic wasps) are rich in GC-base pairs (see e.g. Bolsheva et al. 2012), we performed chromosome staining of above-mentioned species using DNA-binding fluorochromes and correlated the banding patterns to binding of 45S rDNA probe in order to identify the location of NORs.

Materials and methods

Origin of parasitic wasps

All parasitoid species studied in the present paper were cultured on the *y w* strain of *Drosophila melanogaster* Meigen, 1830 at the City College of the City University of New York (see Gokhman et al. 2011). Host egglays took place at 25 °C in vials containing standard yeast/cornmeal/agar fly food that had been sprinkled with dry yeast. Stock egglays were allowed to take place for 2 to 8 h. Larvae were exposed to parasitoid females beginning from 48 h after the initiation of the egglays; exposure period was 24 h as described in Small et al. (2012).

Preparation of chromosomes

Chromosomal preparations were obtained from cerebral ganglia of prepupae generally following the protocol developed by Imai et al. (1988). Ganglia were extracted from insects dissected in 0.5 % hypotonic sodium citrate solution containing 0.005 % colchicine. The extracted ganglia were then transferred to a fresh portion of hypotonic solution and incubated for 30 min at room temperature. The material was transferred onto a pre-cleaned microscope slide using a Pasteur pipette and then gently flushed with Fixative I (glacial acetic acid: absolute ethanol: distilled water 3:3:4). The tissues were disrupted using dissecting needles in an additional drop of Fixative I. Another drop of Fixative II (glacial acetic acid: absolute ethanol 1:1) was applied to the center of the area, and the more aqueous phase was blotted off the edges of the slide. The slides were then dried for approximately half an hour and stored for a few months at −20 °C.

Fluorochrome staining

Chromosome spreads were stained with combinations of several fluorochromes, i.e. chromomycin A_3 (CMA_3)/4′,6-diamidino-2-phenylindole (DAPI) (Schweizer and Ambros 1994) and propidium iodide (PI)/DAPI (Kim et al. 2002).

CMA_3/DAPI staining

The slide was flooded with chromomycin staining solution (0.5 mg/ml in McIlvaine's buffer (pH 7.0) containing 5 mM $MgCl_2$), covered with a coverslip, and incubated at room temperature in the dark overnight. The coverslip was then removed, and the slide was briefly rinsed with distilled water and air-dried. The slide was then flooded with DAPI solution (1 μg/ml in McIlvaine's buffer), covered with a coverslip, and stained in the dark at room temperature for 15 min. The coverslip was then removed, and the slide was briefly rinsed with distilled water before being air-dried. The preparation was then mounted in a 1:1 mixture of glycerol/McIlvaine's buffer containing 2.5 mM $MgCl_2$ and sealed with rubber cement. The slide was aged prior to examination by storing in the dark at 30–37 °C for a minimum of one day.

PI/DAPI staining

The slide was stained with a PI and DAPI mixture (1 mg/ml and 0.5 mg/ml respectively in McIlvaine's buffer) for 20 min with 10 min of pre- and post-incubation in McIlvaine's buffer. The slide was then briefly rinsed with distilled water, air-dried and mounted in VECTASHIELD anti-fading medium (Vector Laboratories).

Fluorescence in situ hybridization (FISH)

Plasmid pTa 71 containing the full DNA sequence of the 45S rRNA gene of wheat (Gerlach and Bedbrook 1979) was used as the probe for visualizing ribosomal genes. This probe was labeled using Biotin-Nick Translation Mix (Roche). FISH with rDNA probes was carried out as described previously (Bolsheva et al. 2012). Chromosome slides were pretreated with 1 mg/ml RNAse A (Roche) in 2 × SSC at 37 °C for 1 h, washed three times for 10 min in 2 × SSC, dehydrated in a series of 70, 85, and 96 % ethanol solutions, and then air-dried. The hybridization mixture contained 50 % de-ionized formamide, 10 % dextran sulfate, 1 % Tween 20, and 2 × SSC. Fifteen microliters of hybridization mixture containing 40 ng of biotin-labeled DNA probe was added to each slide. Samples were then covered with coverslips, sealed with rubber cement and denatured at 74 °C for 5 min. The hybridization was carried out in a moisture chamber at 37 °C

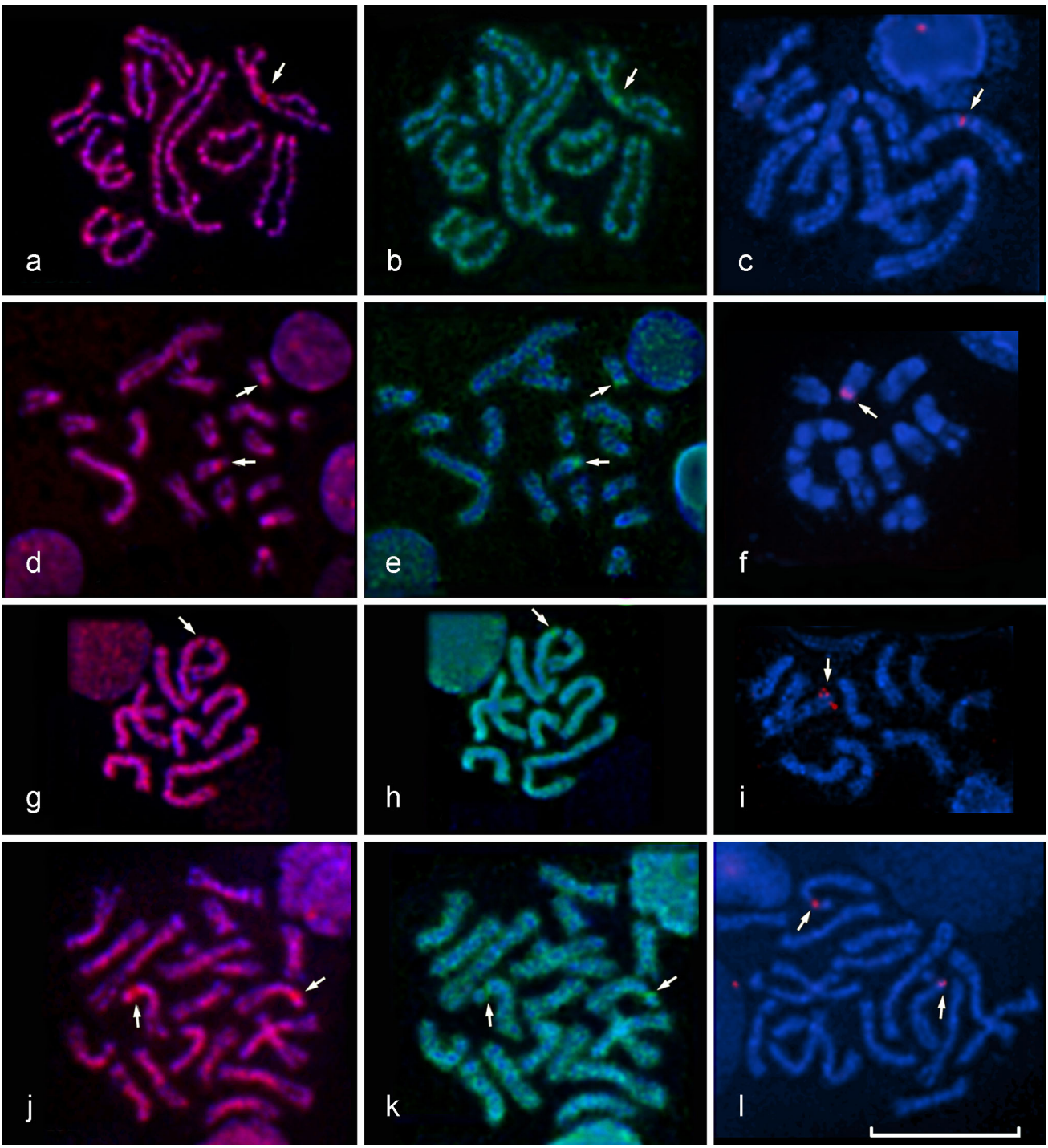

Fig. 1 Metaphase plates of Figitidae. **a–c** *Ganaspis xanthopoda*, male; **d**, **e** *Leptopilina boulardi*, female; **f** *L. boulardi*, male; **g–i** *L. heterotoma*, male; **j–l** *L. victoriae*, female. **a**, **d**, **g**, **j** PI/DAPI staining; **b**, **e**, **h**, **k** CMA_3/DAPI staining; **c**, **f**, **i**, **l** FISH with 45S rDNA probe (*red* hybridization signals), counterstaining with DAPI. *Arrows* indicate DAPI-negative, PI- and CMA_3-positive NORs as well as hybridization signals on chromosomes. *Scale bar* 10 μm

overnight. After removing the coverslips, the slides were washed twice with 0.1 × SSC at 44 °C for 10 min, followed by washing with 2 × SSC at 44 °C for 2 × 5 min and with 2 × SSC at room temperature for 5 min. Biotin was detected using avidin-Texas Red (Vector Laboratories). The slides were mounted in VECTASHIELD antifading medium (Vector Laboratories) containing 1.5 μl of DAPI (Sigma-Aldrich).

Chromosomal analysis

Metaphase plates were studied and photographed using an Olympus BX-61 epifluorescence microscope, fitted with Cool Snap black-and-white CCD camera (Roper Technologies). The obtained images were processed using Adobe Photoshop CS6.

Results and discussion

Three to six individuals were examined for every studied species; however, no intraspecific chromosomal variation was observed. PI stained all chromosomes without revealing any substantial gaps (Fig. 1a, d, g, j). Nevertheless, certain brighter segments, usually confined to pericentromeric and telomeric regions, were visualized. DAPI showed the same general pattern on the chromosomes, but it also visualized a single distinct gap per haploid karyotype in each species. These DAPI-negative regions corresponded to CMA_3-positive bands (Fig. 1b, e, h, k). In all studied species, strong signals of hybridization with 45S rDNA probe were also found in these DAPI-negative/CMA_3-positive regions (Fig. 1c, f, i, l). A single CMA_3-positive segment was visualized in the pericentromeric region of the second bi-armed chromosome in the karyotype of *G. xanthopoda* with n = 9 (Fig. 1b). However, in the karyotype of *L. boulardi* with the same chromosome number, the 45S rDNA cluster was localized in the pericentromeric region of the smaller subtelocentric/acrocentric chromosome (Fig. 1f). In both other closely related species of the genus *Leptopilina*, i.e. *L. heterotoma* and *L. victoriae* with the similar karyotype structure, the 45S rDNA sites occupied the subterminal region of the medium-sized bi-armed chromosome (Fig. 1i, l).

Although PI stains all chromosomal DNA regardless of its base content, it seems possible that at least some brighter segments point to chromosomal regions with higher spiralization (e.g. pericentromeric and telomeric heterochromatin). This is also true for DAPI except for a single gap, or negative band, per haploid karyotype of each studied species. However, this gap is selectively stained with CMA_3 which generally reveals GC-rich chromosomal segments. Moreover, all these CMA_3-positive bands also co-localized with strong hybridization signals visualized by FISH with 45S rDNA probe, and their nature as NORs was therefore confirmed. The pattern of one NOR per haploid genome, observed in all four species studied here, is consistent with our previous observations on the only other studied member of the superfamily Cynipoidea, *Diplolepis rosae* (Linnaeus, 1758) (Cynipidae) as well as on some other relatively advanced groups of parasitic wasps (Gokhman et al. 2014).

Of the three *Leptopilina* species studied here, phylogenetic analysis places *L. heterotoma* and *L. victoriae* in the *heterotoma* species group which excludes *L. boulardi*, which in turn belongs to the *boulardi* species group (Allemand et al. 2002). It is therefore not surprising that the karyotypes of *L. heterotoma* and *L. victoriae* are indistinguishable as analyzed by these fluorochromes and their NORs map to virtually identical positions on the chromosomes in the context of chromosomal banding patterns. *L. boulardi* shares the same chromosome number, n = 9, with *G. xanthopoda*, although they have very different karyotypes structures (see also Gokhman et al. 2011) as well as NOR locations. These features support the independent origins of the same chromosome number, n = 9, in *L. boulardi* and *G. xanthopoda*, an idea that is consistent with the main pathway proposed for karyotype evolution in parasitoid Hymenoptera (Gokhman 2009). Such trends in the reduction of chromosome numbers via chromosomal fusions occurring independently across different genera can explain the appearance of large bi-armed chromosomes, reported here for both *G. xanthopoda* and *L. boulardi* karyotypes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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