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Analysis of the nucleolar organizing regions in the ant *Tapinoma nigerrimum* (Hymenoptera, Formicidae)

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This study analyses the NORs of *Tapinoma nigerrimum*, a species that, as known from previous studies, has various chromosomes which carry a NOR site. The analysis was made by a combination of three methods: silver nitrate staining, *in situ* hybridization with fluorescein- or digoxigenin-labelled probes, and staining with the CG-specific fluorochrome chromomycin A_3. The silver staining technique showed an Ag-positive region on chromosome 6 and on various other chromosomes. However, the application of *in situ* hybridization techniques showed only one positive signal in the proximal region of the short arm of chromosome 6 of *T. nigerrimum*. Similar results were observed by CMA banding. The absence of rDNA genes or the presence of only a small number of these, not detectable with the above probes, might explain the absence of hybridization signal in the remaining chromosomes.

Keywords: Ag-NORs, ants, fluorescent CMA bands, *in situ* hybridization.

Introduction

In eukaryotic genomes the genes (the rDNA) for ribosomal RNA are arranged as clusters of tandemly repeated units. It is well known that profiles of the chromosomal distribution of RNA, such as the number of chromosomal loci and the number of genes at each locus, vary among species, among populations and among individuals. These features of the NORs have been demonstrated either by silver staining techniques or by *in situ* hybridization. The latter technique can detect all clusters of rDNA, whereas the former specifically identifies transcriptionally active rDNA cistrons, the so-called silver stainable nucleolar organizer regions (Ag-NORs). It is also well known that there are two different types of rDNA, active and inactive, although the mechanism controlling the difference is not known.

In a previous article (Palomeque *et al.*, 1988) we have reported the existence in *Tapinoma nigerrimum* of an Ag-positive region (or Ag-NOR) in chromosomes 6 and 8. Amongst the remaining chromosomes, the number of chromosomes bearing an Ag-positive region varied between individuals and populations. In this article we report an analysis of the nucleolar organizing regions of *T. nigerrimum* using silver nitrate, *in situ* hybridization with fluorescein- or digoxigenin-labelled probes, and chromomycin A_3 (CMA) staining. The silver staining technique showed an Ag-positive region on chromosome 6 and on various other chromosomes. However, only one positive signal in the proximal region of the short arm of chromosome 6 was observed using the *in situ* hybridization techniques. A similar result to the *in situ* hybridization was obtained by CMA banding techniques.

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Materials and methods

The samples analysed were collected in different sites in Almería (Southern Spain).

Chromosome preparations

Chromosome preparations were made from germ cells of early male and female pupae using the technique described by Meredith (1969).

Ag-stain

Silver nitrate stainings were performed according to the techniques described by Howell & Black (1980) and Rufas *et al.* (1982).

DA/CMA, DA/DAPI stain

Staining with DA/CMA and DA/DAPI was performed according to the techniques of Schweizer *et al.* (1979) and Schweizer (1980).

DNA probe

For Southern hybridization and *in situ* hybridization we used a plasmid containing the *Drosophila* rDNA gene (pDm r.a 51#1) provided by Dr S. A. Endow from Duke University Medical Center. This plasmid consists of a noninterrupted 11.5 kb repeat unit and encodes noninterrupted rRNA genes (18S and 28S; Endow, 1982).

Endonuclease digestion and Southern hybridization

Isolated nuclear DNAs, 10 μg, were digested with restriction enzymes *Eco*RI and *Sma*I according to the manual of the supplier (Boehringer Mannheim). Digestion fragments were separated on a 0.8 per cent agarose gel, then blotted onto Hybond-N membranes (Amersham) under standard conditions.

The Southern hybridization was performed according to the technique described by Sambrook *et al.* (1989). The probe was labelled with digoxigenin-11-dUTP using a random primer DNA labelling kit (Boehringer Mannheim). The hybridization conditions were set at high stringency, in a solution containing 5 × SSC overnight at 68°C. Detection of hybridization was performed with a DIG-detection kit from Boehringer-Mannheim.

In situ *hybridization*

One μg of DNA probe was labelled with digoxigenin-11-dUTP using a random primed DNA labelling kit (Boehringer Mannheim) for 2 h at 37°C, or with fluorescein-12-dUTP by a nick-translation procedure (Sambrook *et al.*, 1989). The labelled probe was precipitated with sodium acetate 3 M and ethanol at −20°C and dissolved in 100 μL of 50 or 30 per cent formamide and stored at 4°C until used directly for hybridization. Prior to hybridization, slides were treated with RNase A, pepsin and formaldehyde and dehydrated in 70 per cent, 90 per cent and 100 per cent ethanol for 5 min each. Hybridization was performed applying 25 μL of DNA-labelled solution to each slide, which was heated for 5 min at 80°C to denature the DNA, and immediately chilled on ice for 3 min. The slides were transferred to a moist chamber humidified with formamide (30 per cent or 50 per cent) and incubated overnight at 37°C. After incubation, the slides were washed in 50 or 30 per cent formamide for 10 min at 37°C, washed twice in 2 × SSC for 5 min each and once in TNT (0.1 M Tris-HCl, 0.15 M NaCl, 0.05 per cent Tween-20, pH 7.5) for 5 min. After the washing step, the slides with fluorescein-labelled probe were dehydrated and mounted with 50 μL of a propidium iodide solution (1 μL propidium iodide in PBS/mL of Vectashield) and observed with a fluorescence microscope, using an excitation filter of 460–490 nm, IB (Olympus Optical Co. Ltd., Tokyo, Japan). Photographs were taken with a B&W Kodak Technical Pan. The slides with a digoxigenin-labelled probe were washed in TNT, and then blocked with 100 μL of TNB (0.1 M Tris-HCl, 0.15 M NaCl, 1 per cent Boehringer Mannheim blocking reagent) at 37°C for 30 min. Hybridization was detected using the DIG-detection kit from Boehringer Mannheim, and observed with an optical phase-contrast microscope.

Results and discussion

The chromosome number of *T. nigerrimum* is $n = 9$ in haploid males and $2n = 18$ in diploid females and workers. The haploid karyotype formula is $n = 5m + 2sm + 2st$ (Palomeque *et al.*, 1988).

Previous articles reported the existence in *T. nigerrimum* of an Ag-positive region (or Ag-NOR) in all chromosomes of the complement, although there were interpopulation differences in relation to the number of chromosomes bearing an Ag-positive region. Chromosomes 6 and 8 showed an Ag-NOR in all populations studied. Amongst the remaining chromosomes, the number of chromosomes bearing an Ag-positive region varied between individuals and populations. In all cases, the positive Ag-regions were adjacent to the respective centromeric regions and coincident with positive C-banding sites (Palomeque *et al.*, 1988, 1990).

In this study the analysis of NORs was made by a combination of three methods: silver nitrate staining, *in situ* hybridization with fluorescein- or digoxigenin-labelled probes, and staining with the fluorochrome chromomycin A_3. *In situ* hybridization techniques were carried out on chromosome preparations of haploid males and diploid females.

The silver staining technique showed an Ag-positive region in chromosome 6 and on various other chromosomes (Fig. 1). These results are similar to those obtained in other populations (Palomeque *et al.*, 1990).

In situ hybridization techniques were carried out using *Drosophila* rDNA as a probe. As shown in Fig. 2a, nucleotide sequences homologous to the

rDNA of *Drosophila* were detected in the *Tapinoma* nuclear genome. This result was obtained under high stringency conditions. Homologous nucleotide sequences between *Drosophila* rDNA and the nuclear genome of the *Myrmecia* ant have also been detected (Imai *et al.*, 1992; Hirai *et al.*, 1994).

In situ hybridization using fluorescein-labelled probes showed only one positive signal situated in the short arm of chromosome 6, near the centromeric region (Fig. 2b). A similar result was obtained by *in situ* hybridization using digoxigenin-labelled probes (Fig. 2c). Two positive signals occurring in the two homologous chromosomes 6 were identified in the chromosome preparations of diploid females (Fig. 2d). In both hybridization techniques, similar results were obtained under low and high stringency conditions. The hybridization technique using digoxigenin-labelled probes was also applied under overexposure conditions (data not shown). Only one positive signal on chromosome 6 was obtained.

Only the proximal region of the short arm of chromosome 6 showed a differential fluorescence after staining with CMA, indicating that it is particularly rich in C-G base pairs, like the majority of the NORs in eukaryotic genomes (Schmid & Guttenbach, 1988; Cerbah *et al.*, 1995; Reed & Phillips, 1995). A CMA-negative region coincident with a DAPI-positive region (Lorite *et al.*, 1996) is also observed in this chromosome (Fig. 2e).

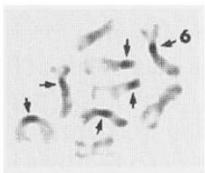

Fig. 1 *Tapinoma nigerrimum*. Haploid metaphase plate showing an Ag-positive region on chromosome 6 and on various other chromosomes (arrows).

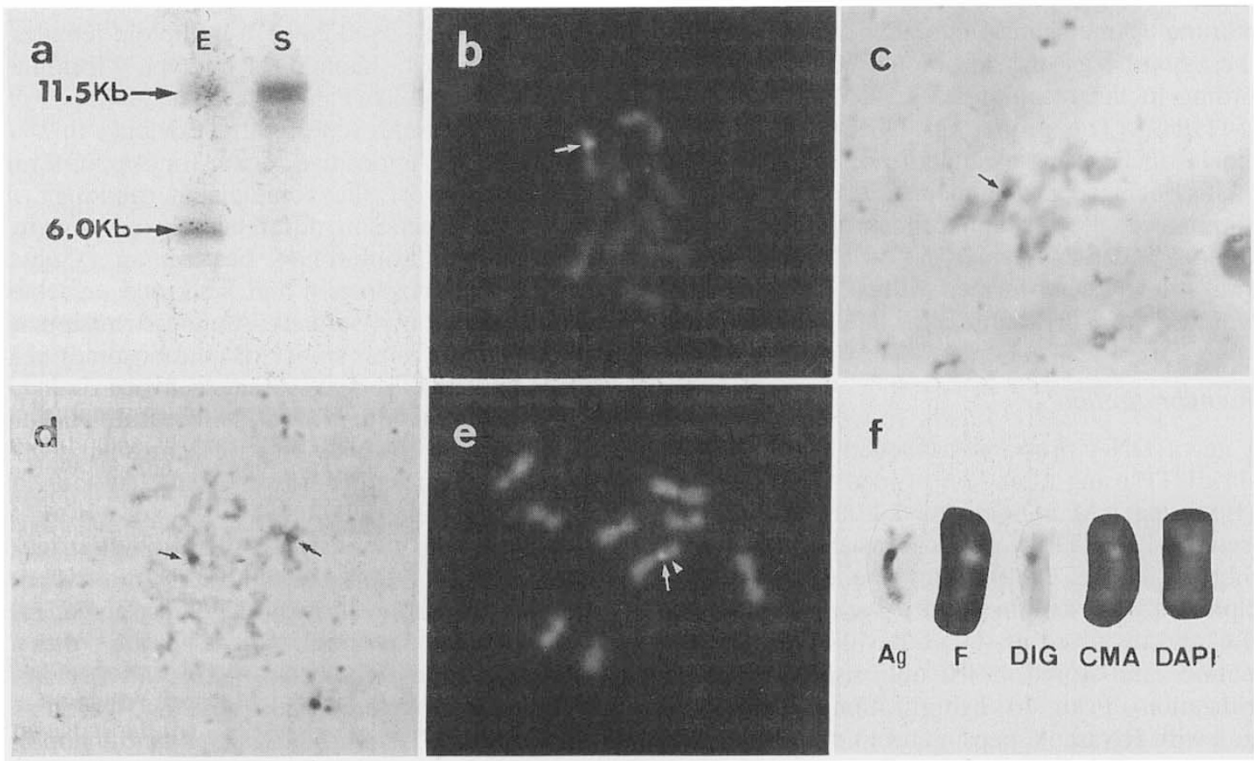

Fig. 2 (a) Southern hybridization of *Tapinoma nigerrimum* genomic DNAs with *Drosophila melanogaster* rDNAs. DNAs digested with *Eco*RI (E) and *Sma*I (S) were separated on 0.8 per cent agarose gel. (b–d) *In situ* hybridization with rDNA; (b) haploid metaphase using fluorescein-labelled probes, showing only one positive signal in the short arm of chromosome 6 (arrow); (c, d) haploid and diploid metaphases after *in situ* hybridization using DIG-labelled probes and detected by alkaline phosphatase, showing also one site of hybridization on chromosomes 6 (arrows). (e) Haploid metaphase plate showing only one region with differential CMA staining on the short arm of chromosome 6 (arrow) adjacent to one CMA-negative region (arrowhead). (f) Chromosome 6 from different metaphases observed after silver nitrate staining (Ag), *in situ* hybridization with fluorescein (F) and digoxigenin (DIG)-labelled probes, CMA banding and DAPI banding.

In accordance with all the results shown here, the rDNA seems to be present only on chromosome 6. The rDNA genes are in an Ag-positive, CMA-positive, DAPI-negative chromosomal region and are coincident with the positive signal obtained by hybridization techniques (Fig. 2f).

In situ hybridization to chromosomal DNA is a technique which allows specific detection of single nucleic acid sequences in morphologically preserved biological specimens. Fluorescence *in situ* hybridization (FISH) is an easy, sensitive nonradioisotopic method for localizing probes in different chromosomes (Larin *et al.*, 1994). In addition to many practical advantages, the sensitivity of FISH to metaphase chromosomes is now nearly equivalent to that of *in situ* hybridization using radioactive probes (Lemieux *et al.*, 1992). A significant improvement in fluorescence *in situ* hybridization enabling the detection of single-copy genes as small as 500 bp directly on banded chromosomes has been well reported (Lemieux *et al.*, 1992).

In spite of this, the absence of rRNA genes, or the presence of only a small number of these, not detectable with the probes used here, might explain the absence of signal in the remaining chromosomes. On the other hand, other authors have shown that silver also stains chromosomal regions other than NORs. In rye, for example, silver nitrate stains not only the NOR sites but also all regions of constitutive heterochromatin (Murray *et al.*, 1992). Sánchez *et al.* (1995) have also observed that silver stains the NORs and the heterochromatic blocks in hedgehogs. Silva *et al.* (1995) have reported that in *Aegilops umbellulata* and *Secale cereale* the silver banding pattern is coincident with the banding pattern obtained after FISH, using as a probe a repetitive sequence isolated from rye.

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