Cytogenetic characterization of the genome of mealybug *Planococcus citri* (Homoptera, Coccoidea)

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SUMMARY - Mealybugs although being agriculturally harmful insects have been very poorly studied by modern cytogenetics techniques, and no cytotaxonomic criteria to distinguish between closely related species is available yet. In the mealybug *Planococcus citri (2n = 10)* male and female individuals are both diploid, however in males, at the stage of blastula, the haploid chromosome set of paternal origin becomes heterochromatic, even though its complete inertia has been considered questionable. Here we present data on the cytogenetic characterization of the chromosomes of *Planococcus citri.* We report on (i) the fluorescence karyotype (D287/170), which to our knowledge is the first banded karyotype of a mealybug to be described; (ii) the chromosome localization of constitutive heterochromatin; (iii) the chromosome localization of rDNA sites; (iv) NORs activity. Our data also show, for the first time, that in the heterochromatic chromosome set ribosomal genes are still active.

INTRODUCTION

Mealybugs (Homoptera, Coccoidea, Pseudococcidae) are agriculturally harmful insects which are present all over the world with more than 2000 species. Nevertheless, they have been very poorly studied by modern cytogenetics techniques possibly because of their very small and holocentric chromosomes. No chromosome banding of any species of Coccids nor cytotaxonomic criteria to distinguish between closely related species has been available yet. Their cytogenetic characterization is therefore of most importance as the preliminary step toward the application of cytogenetics to the problem of cytotaxonomy of these species. In the mealybug *Planococcus citri* male and female individuals are both diploid, however in males, at the stage of blastula, one entire haploid set becomes heterochromatic in most of the tissues. Males also present an inverted meiotic sequence with euchromatic chromosomes segregating from their homologs, and only those cells receiving the euchromatic set proceed to form sperm. (HUGHES-SHRADER 1948). Some Authors (BROWN and NUR 1964) have suggested the possibility of some residual activity in the

inactivated chromosome set, however further investigations have never been attempted. Interestingly, the heterochromatized chromosome set is invariably that of paternal origin (BROWN and NELSON-REES 1961), implying that, at the blastula stage, the paternal and the maternal sets should be differentially imprinted.Mealybugs should therefore provide plenty of scope for further investigations on such fundamental biological phenomena as heterochromatization mechanisms and chromosome imprinting. In this paper we report for the first time on the banded karyotype of the mealybug P. *citri (2n = 10)* after chromosome staining with the A T -specific compound D287/170. Moreover, we describe the chromosome localization, by C-banding, of constitutive heterochromatin and its characterization by fluorescent banding with A T - and GCspecific fluorochromes, and by H33258-induced site-specific chromosome undercondensation. Finally, we report on the localization of ribosomal genes obtained by fluorescent *in situ* hybridization of a rDNA probe from *Drosophila melanogaster,* and about the activity of r-gene clusters and demonstrate that, in males, also the ribosomal genes located in the heterochromatic chromosome set are active.

MATERIALS AND METHODS

Chromosome preparations - The chromosome spreads were obtained by air drying according to the technique described by ODIERNA*et al.* (1993) with minor modifications. Ovaries containing fertilized eggs were removed from females dissected in 0,9%NaCl and placed in a drop of colchicine 10-JM in H2O for 2 hours. Successively they were transferred to an Eppendorf-like tube and centrifuged at 5000 rpm for 5 min. After discarding the supernatant, 200l of fixative (methanol: acetic acid, 3: 1) was added and the tissues homogenized through a 20G syringe gauge. The material was centrifuged again at 7000 rpm for 8 min, and after the supernatant was discarded and freshly made fixative added, it was passed again repeatedly through the syringe gauge. 60- 70 ìl of the suspension were dropped on each slide. In some experiments, immediately after dissection, the material was placed in a drop of Hoechst 33258 (Sigma) 50ìg/ml in 0,7% NaCl at 26°C for 10-30 hours.

 C-banding- Slides were treated at 30°C with a saturated solution of Ba(OH)2 for time lengths ranging from 10 to 30 min. After washing in distilled water the slides were incubated in 2xSSC at 60°C for 60 min, then stained in a 5%Giemsa solution.

Fluorochrome staining - DAPI. Slides were hydrated in 2xSSC for 5 min, stained in DAPI (Boehringer) 0.2 ìg/ml in 2xSSC for 10 min in the dark, then washed in 2xSSC *Hoechst* 33258 (H33258). Slides were hydrated in 0.15M NaCl, 0.03M KC1, 0.01M disodium phosphate, pH 7, for 5 min and stained in Hoechst 33258 (Sigma) 0.5 ìg/ml in the above solution for 5 min. *Chromomycin A3* (CMA3). Slides were hydrated in 0.14M phosphate buffer, pH 6.8, containing 500 ìM MgCL, for 10 min. Then, 3-4 drops of CMA3 Boehringer) (0,5mg/ml), made in phosphate buffer without MgCI2

were placed on the slides. After 1 hour of staining the slides were washed in 0.15M NaCl/0.005M Hepes buffer (GIBCO) and counter stained in 100~M methyl green (Merck) dissolved in the above buffer, for 15 min. *D287/170.* A few drops of D287/170 staining solution (50ìg/ml in McIlvaine buffer) were placed over slides for 20-30 min at room temp. in the dark, then rinsed in deionized water (SCHNEDL *et al.* 1981).

"In situ hybridization" - rDNA 28S: a plasmid containing a 1.3 kb *BgI II-Hind* II fragment from the 28S rDNA gene of *Drosophila melanogaster* (LOHE and ROBERTS 1990); this fragment crosshybridizes in Southern hybridization with *P. citri* DNA, giving a 1.8 kb single band (not shown). Biotynilated dUTP was incorporated into probe DNA by nick-translation. Slides were treated with 0.08 ìg/ml Proteinase K, at 37°C, for time lengths of 5-10 min, and RNase (200~gfml) at 37°C for 1 hour. Chromosomal DNA was denaturated by incubation in 70% formamide in 2xSSC at 70°C for two min. Hybridization was carried out overnight at 37°C in 50% formamide in 2xSSC. Post-hybridization washes were 3x5 min at 37°C in 50% formamide in 2xSSC and 3x5 min at 42°C in 2xSSC. For the detection the slides were incubated with blocking solution (4xSSC, 30 mgfml BSA, 0.1% Tween 20) at 37°C for 30 min, then with Avidine-FITC (Vector laboratories) diluted 1:300 in 4xSSC, 10 mg/ml BSA, 0.1% Tween 20, at 37°C for 30 min. After counterstaining in DAPI fluorescent images were captured with a CCD camera (Photometrics 01) using IPLab software (Signal Analytic Corporation) and processed with a Macintosh Power 7100 using Adobe Photoshop software.

NOR-banding. Slides were treated with a mixture of gelatin solution/AgNO3 solution (50%) at 70°C for 2-3 min, rinsed with distilled water, then stained with Giemsa (HOWELL and BLACK 1980).

RESULTS

Our initial effort was to obtain preparations with a high number of mitotic figures with elongated chromosomes showing a good morphology. In Figure la, prophase male cells are shown with different degrees of condensation between the maternal, euchromatic (E), and the paternal, heterochromatic (H), chromosome sets. As indicated in the diagram, the difference decreases as the cell proceeds towards metaphase, where all chromosomes are equally condensed. In Fig. 1b, two female cells at mid and late prophase but showing no difference between the two haploid sets are shown.

Chromosome distribution of AT- and GC-rich DNA regions. - In order to identify the five pairs of homologs and to characterize the chromosomes on the base of composition along their length, chromosome preparations were stained with A T -specific (H33258, DAPI, D287/170) and GC-specific (CMA3) fluorochromes, or gently dissected ovaries were treated, before fixing, with a solution of Hoechst 33258. When added to "living" cells H33258 binds to AT rich DNA regions inhibiting their condensation at mitosis.
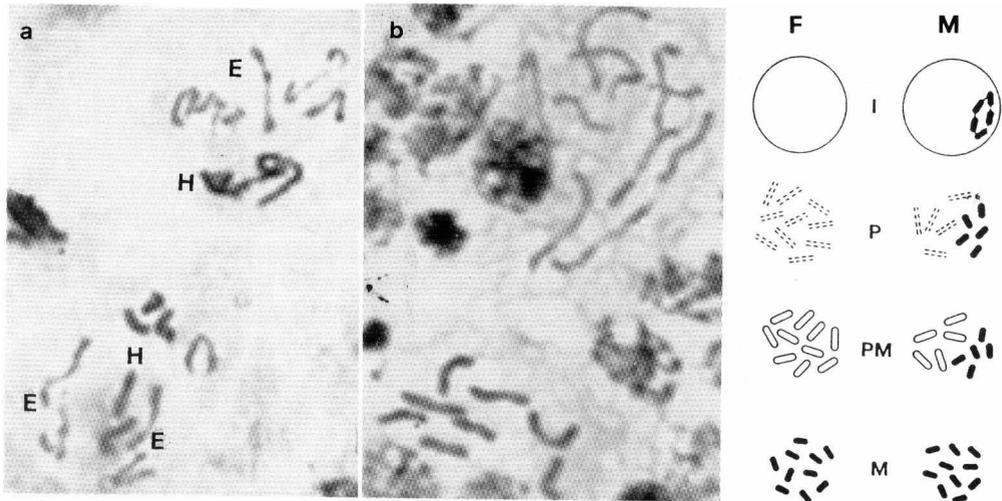
Figs. la,b. - Morphological differences between male and female cells chromosomes. (a) male: the chromosome sets of maternal (E) and paternal (H) origin show quite different degrees of condensation. (b) female cells: all the chromosomes always show the same degree of condensation. The variations of male and female chromosome condensation from interphase to metaphase are schematized at far right of the figure, I = interphase, p = prophase, PM = prometaphase, M = metaphase.

Fluorochrome staining. - After H33258 and DAPI staining the chromosomes appeared roughly uniformly fluorescent (not shown). However, after staining with D287/170, a clear banding pattern was obtained which allowed the unequivocal identification of homologous chromosomes (Fig. 2). In each chromosome pair, a difference in the fluorescence intensity between the two homologs was appreciable. Furthermore, in metaphases with more elongated chromosomes a difference also in the degree of condensation was evident. In addition, CMA3 produced a banding pattern, also if less detailed than the one obtained after D287/170, that was quite similar to the one observed after C banding.

C-banding. - Higly condensed chromosomes have C-bands mainly confined to the telomeric regions while in more elongated chromosomes some intercalary C-bands become evident. This is clearly shown in Fig. 3 where three haploid chromosome sets, at different degrees of chromosome condensation are vertically arranged. Corresponding-number chromosomes from each metaphase are horizontally aligned and lines are traced to follow the splitting of C-bands in more elongated chromosomes.
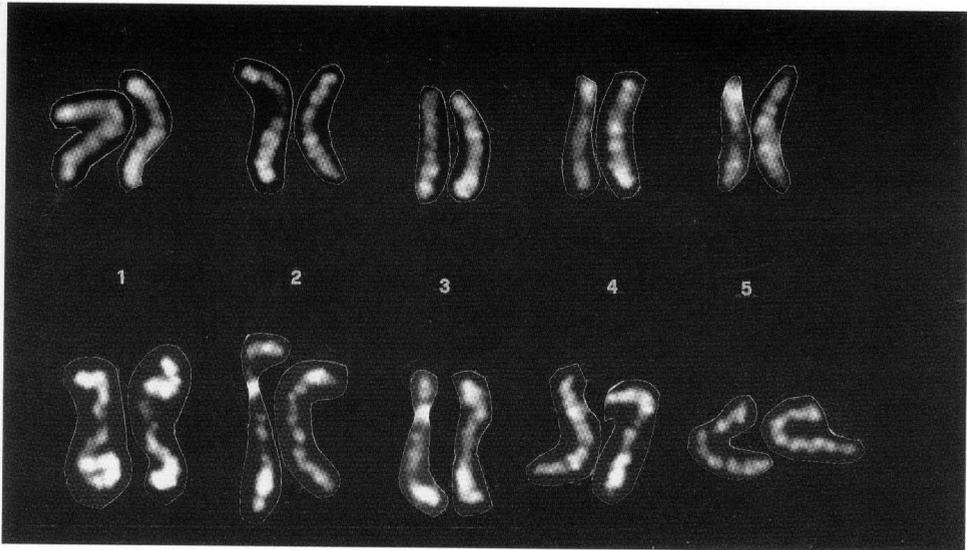
Fig. 2. - Karyotype of two cells after staining with D287/170. Note that in each chromosome pair one of the two homologs shows a higher fluorescence intensity, and that, particularly in more elongated chromosomes, the two members of each pair of homologs show different degrees of condensation.

H33258-induced undercondensation. - In Fig. 4 the five euchromatic chromosomes from three male cells treated for C-banding (the first of the three), stained with CMA3 (the second of the three), and after incorporation of H33258 (the third of the three) are shown arranged by groups. From the comparison of the three differently treated chromosomes emerges a good correspondence between C-bands> CMA3-positive areas, and the undercondensation pattern.

Ribosomal genes localization and activity.

In situ hybridization. - The results of fluorescence in situ hybridization with a 28S rDNA probe from *Drosophila melanogaster* are shown in Fig 5. Fluorescent signals were constantly observed at four sites, two at a more or less median position (Fig.5, arrows) and two at a more distal location. Additional hybridization spots were also observed, although not constantly, reaching a maximum of six.

Ag staining. - Two different types of silver spots, namely a large "ball-shaped" and a small very sharp dot were observed both in male and female cells. From the comparison of the same metaphases sequentially stained by D287/170 and
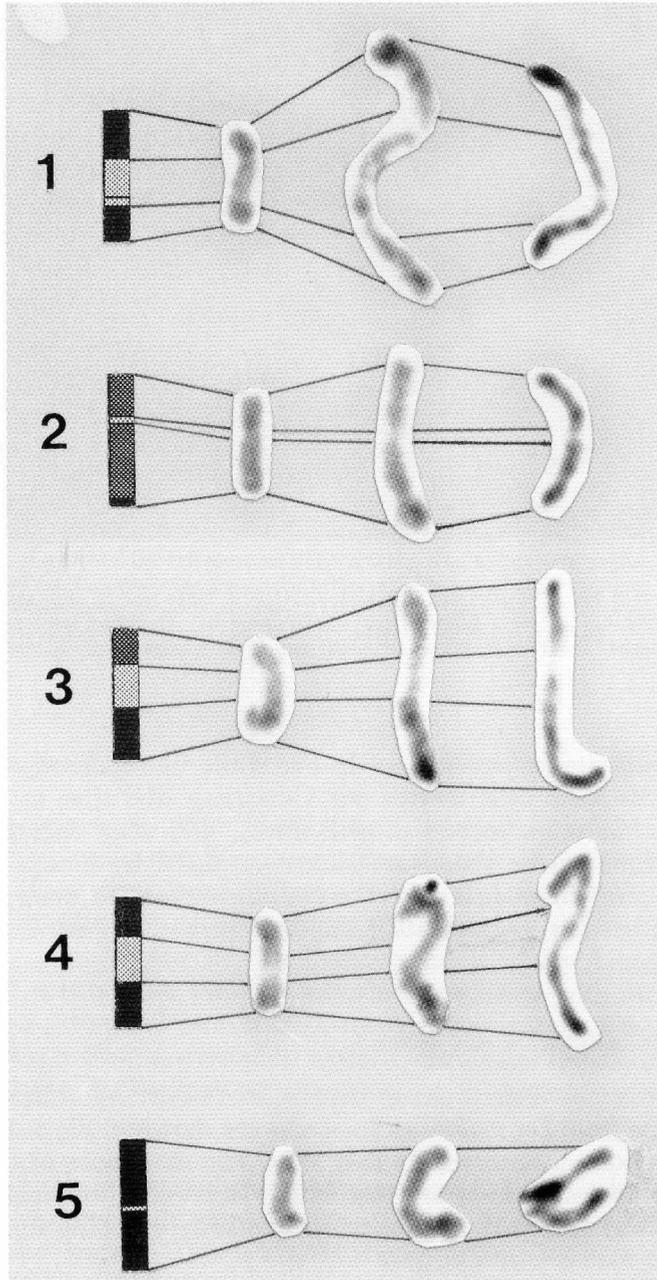
Fig. 3. - C-banding pattern. C-banded haploid chromosome sets from three cells at different degrees of condensation are shown. From a band idiogram for each chromosome, lines are traced to follow the splitting of C-bands in more elongated chromosomes
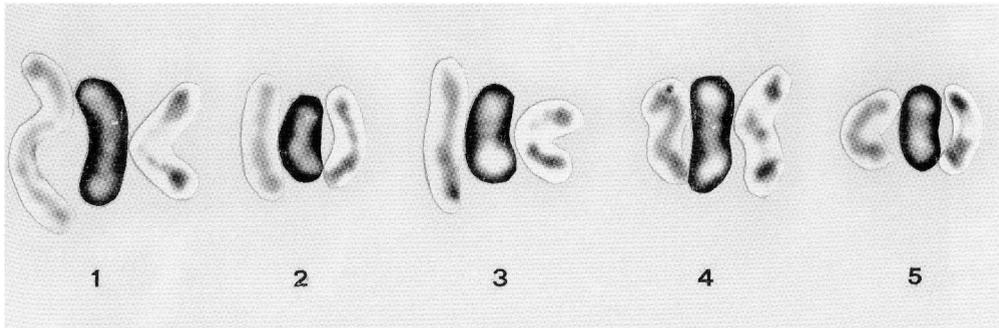
Fig. 4 - Comparison of patterns obtained for each chromosome after C-banding (first), CMA3 staining (second), and H33258 incorporation (third). In each group the three patterns show a good correspondence.

by silver (Fig. 6) it was possible to check the location of the two different types of silver spots. The large spots (arrows) were always located at a more or less median position of the first pair of homologs at the site on the chromosomes corresponding to a faintly fluorescent band after staining with D287/170. The small spots (arrowheads) were observed at subterminal position in the chromosome pairs number 2, number 3 and number 4. Occasionally, a silver spot was observed at telomeric position of a chromosome identified as chromosome number 5. The number of silver spots observed in a metaphase cell varied from a minimum of 2 to a maximum of 5. Two silver stained chromosomes were usually present in prometaphase male cells, those being chromosomes number 1 and number 4 (Fig. 6 upper row). Moreover, in cells where it was possible to distinguish between the euchromatic and the heterochromatic homologs, it has been found that both the eu- and the heterochromatic homologs could be silver stained (Fig. 7).

DISCUSSION

The chromosomes of *Planococcus citri,* like all other Coccid species, are very small and holocentric. This feature has made their identification quite difficult and it is probably the main reason why a banded karyotype of none of these species has been described yet. A modification of the air-drying technique for insects described by ODIERNA *et al.* (1993) allowed us to obtain metaphases showing well elongated chromosomes and the use of both A T-specific (H33258, DAPI, D287/170) and GC-specific (CMA3) fluorochromes allowed us to characterize DNA base composition variations along P. *citri* chromosomes, and to describe its banded karyotype by D287/170 (Fig. 2) . This
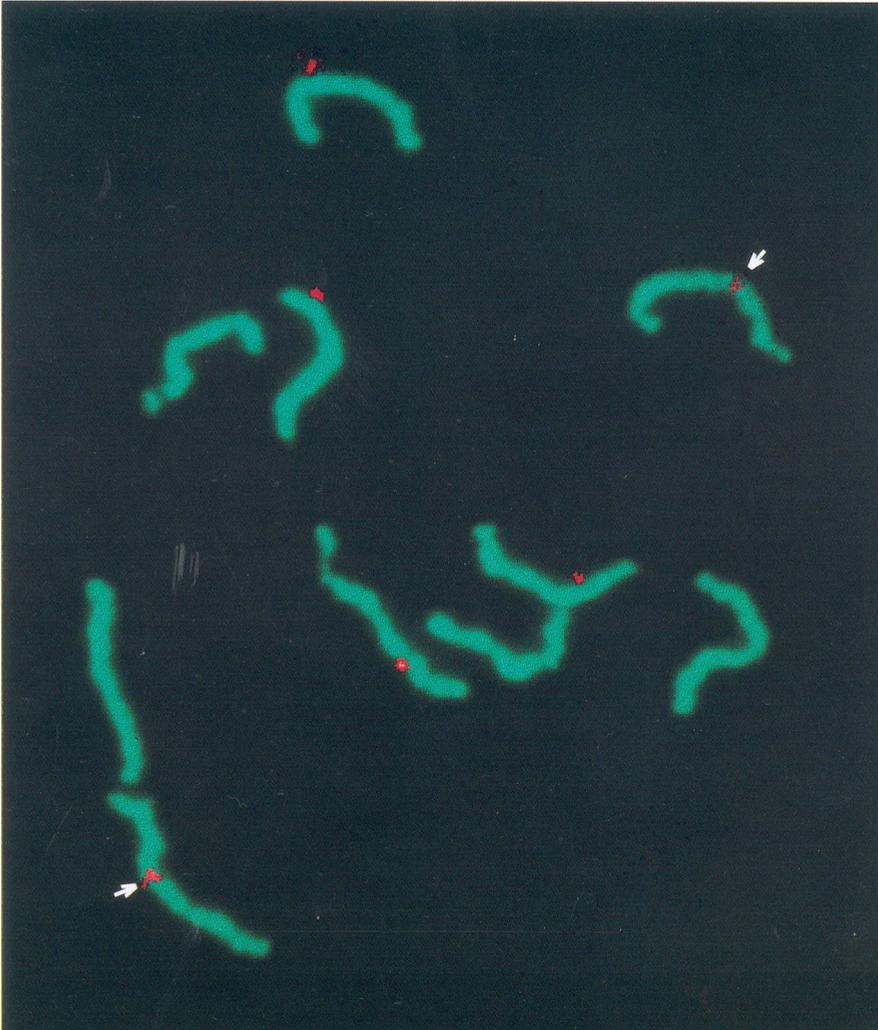
Fig. 5 - Localization of 28S rDNA by fluorescence in situ hybridization. The two fluorescent spots at the more median position (arrows) and two of those more distally located are always present. Six hybridization spots, as seen in the figure, are only seldom visible. Note that the hybridization spots are more often seen as a single lateral signal.

is a compound derived from a modification of DAPI considered as having affinity for A T -rich DNA, as suggested by the Q-banding pattern produced on mouse chromosomes when used at low concentrations. Moreover, the restricted distribution of positive sites when used at high concentration suggests that this fluorochrome does not simply show specificity for A T -rich DNA, but may also show sequence specificity (SCHNEDL et al. 1981a). Indeed, it has been used

to distinguish certain classes of heterochromatin in different species (SCHNEDL*et al. 1981b;* BABU and VERMA 1986; MAYR *et al.* 1986). Our results therefore both confirm the indications about the peculiarity of this compound - see the lack of chromosome differentiation after H33258 and DAPI staining - and suggest the existence of discrete stretches of specific AT - rich sequences along P.*citri* chromosomes.

The analysis of karyotypes evidenced that homologous chromosomes consistently show different fluorescence intensities, and that, especially in metaphases with more elongated chromosomes, the two members of each chromosome pair show a different degree of condensation (Fig. 2, bottom row). This could possibly be caused by differences in the level of genetic activity between the two homologs, the different degree of compaction being a way of dosage compensation in female cells in response to the heterochromatization of a whole haploid set in males. In another example of dosage compensation, namely the X-chromosome inactivation in mammalian female cells, the phenomenon is also accompanied by a higher degree of compaction of the inactive homolog (LATT 1973; KEREM*et al.* 1983).

The application of the C-banding technique evidenced a defined pattern of bands on all the five chromosome pairs (Fig.3). The staining pattern obtained after CMA3-Methyl Green, showing that the more fluorescent regions correspond to C-band positive areas, indicated that in P*citri* the constitutive heterochromatin is at least partially GC-rich. This suggestion was confirmed by the experiments of H33258 binding to "living" chromosomes. It is known that, when added to culture cells, H33258 binds to AT-rich DNA before mitotic condensation thus inducing the appearance, along mitotic chromosomes, of these regions as undercondensed areas (HILWIG and GROPP 1973, 1975; ROCCHI *et al.* 1976; PIMPINELLI *et al.,* 1976). Indeed, the incorporation of H33258 resulted in a banding pattern of chromosomes close to that obtained after C-banding (Fig. 4). The failure to find a dull appearance of these regions after staining with the A T -specific fluorochromes, could be explained by the fact that in the more condensed constitutive heterocromatin there is more DNA per unit length than in the euchromatin. However, some of the telomeric regions being positive to D287/170, we can hypotesize the presence of AT-rich sequences embedded in predominantly GC-rich areas.

Fig. 6. - Identification of the chromosomes showing active ribosomal gene clusters. Three karyotypes from cells sequentially stained with D280/170 and silver are shown. The first karyotype is unequivocally from a male cell while for the two others it is not possible to say if they are made from a male- or a female-embryo cell since both are present in the ovary, and at metaphase all chromosomes are equally condensed also in males. Chromosomes of the first pair (always Ag +) show a large dot (arrows), while dots on other chromosomes of the same cell, when present, are always quite smaller (arrowheads).
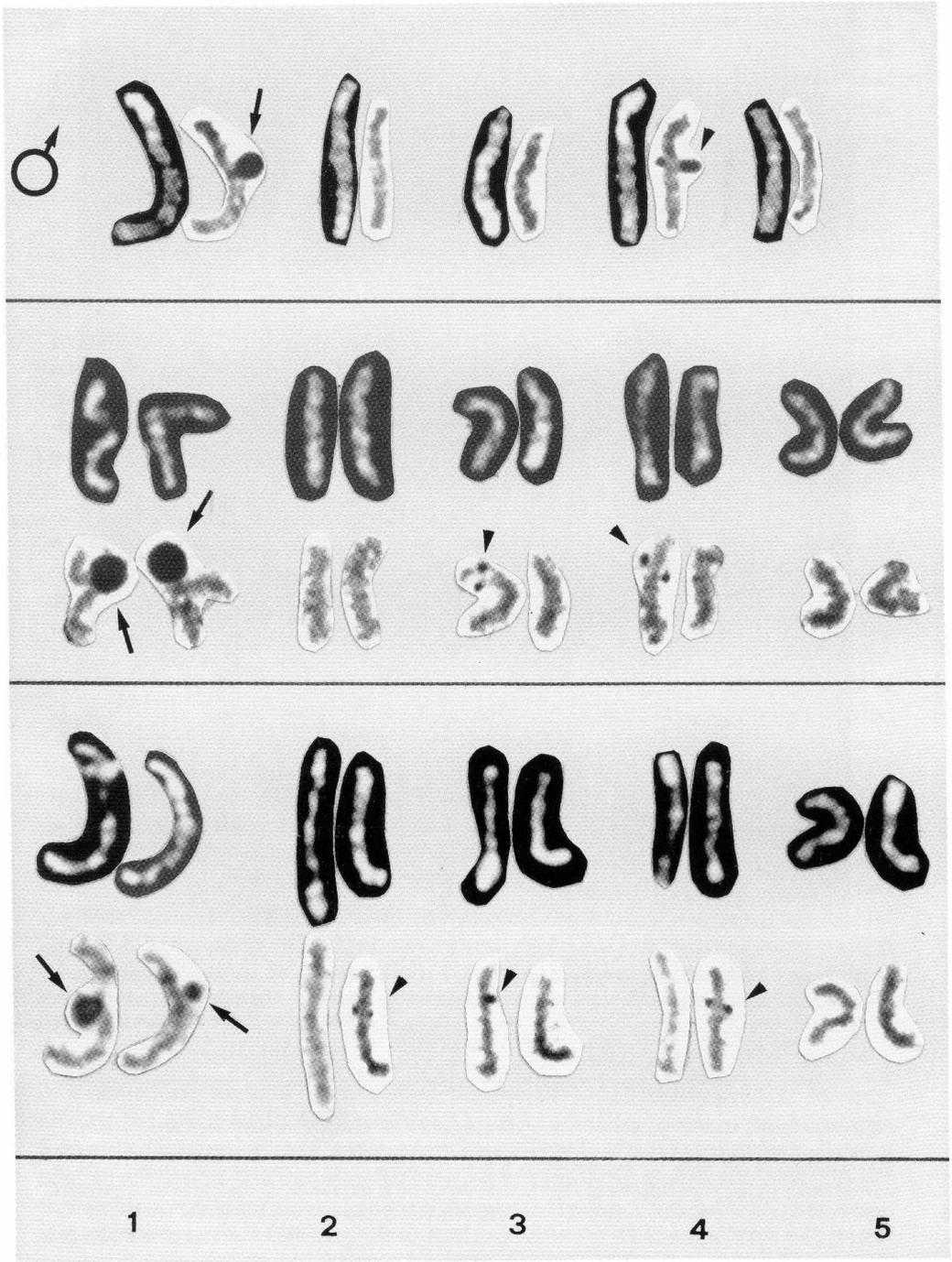
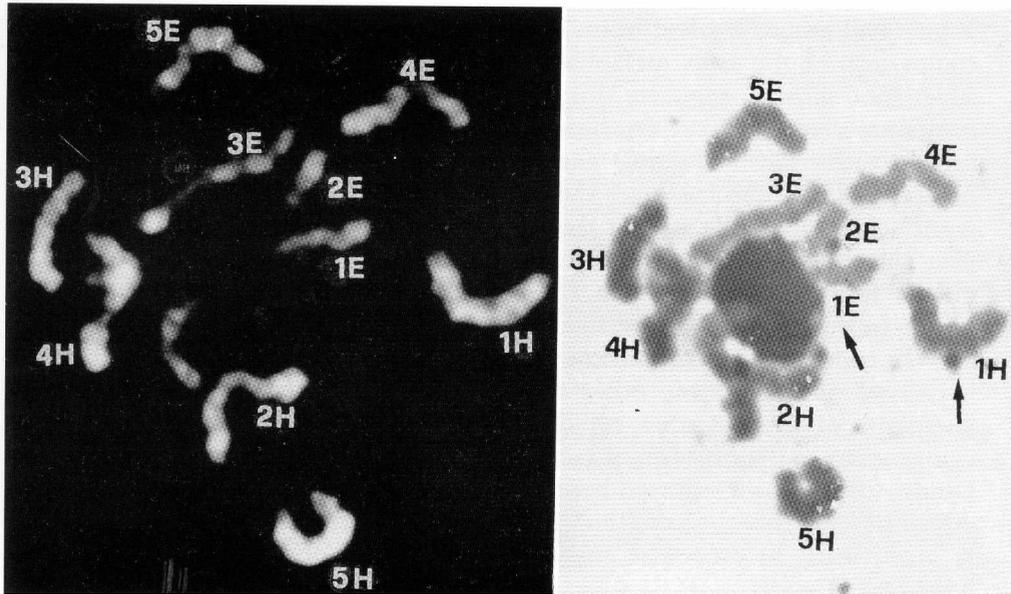Fig. 7. - Demonstration of ribosomal genes activity in the heterocromatic chromosome set. A male metaphase sequentially stained with D280/170 and silver where for each chromosome pair is possible to distinguish between the eu- and the heterochromatic homologs is shown. A silver spot is visible lying on one heterochromatic chromosome (IH, arrow) while its euchromatic homolog (IE), is also stained by silver and still associated with chromosome 2E.

As to ribosomal genes, while it is true that the only certain method for identifying sites of NORs is in situ hybridization to ribosomal genes, silver staining is nevertheless remarkably specific for NORs. In particular, silver staining has been observed as indicative of active ribosomal genes for vertebrates, plants and several species of insects (SUMNER 1990).

Additionally, when homologous rDNA probes are not available, we consider particularly appropriate the combined evaluation of the data obtained from in situ hybridization and silver staining. In p .citri, as suggested by the finding of silver stained chromosomes belonging to all but one chromosome pair, eventhough not all at the same time, r-gene clusters are present on four pairs of chromosomes. The fact that eight positive sites have never been found after in situ hybridization could be explained by the incomplete homology of the probe used, which with smaller clusters could be a highly, or even completely, limiting factor for an adequate annealing, thus resulting under the threshold of Fluorescent *in situ* hybridization (FISH) resolution. This hypothesis is supported by the observation that two of the sites which are always positive after in situ hybridization correspond to the ones showing the

large "ball-shaped" silver spots, constantly found in all metaphases. The comparison of the same metaphases first stained with Giemsa and then with silver (not shown) , supports this hypothesis since the silver stained chromosomes are very often the ones associated with the nucleolus. Finally, in male cells, positive silver staining was clearly detected both on euchromatic and heterochromatic chromosomes therefore demonstrating that in the heterochromatic set of male cells at least the ribosomal genes are active. The fact that r-gene clusters in a heterochromatic background can still function is nevertheless known for many species, like Drosophila melanogaster and man, where the NORs indeed map in constitutive heterocromatic regions.

In conclusion, (i) to our knowledge, this is the first time when a banded karyotype of this or any other coccid species has been made. The banded karyotype of these species could be a first step toward the application of cytogenetics to the problem of taxonomy which is particularly relevant for this family. (ii) The data reported here about ribosomal genes, gives the first direct demonstration that, indeed, in P. citri the heterochromatic set is not completely inactivated, as otherwise suggested by indirect observations by NELSON-REES (1962).

Finally, we would like to stress that, chromosome imprinting being a phenomenon whose relevance is becoming apparent in most organisms> including man, mealybugs could be a useful system to study fundamental biological phenomena as the heterochromatization process (see the indications coming from the differences both in fluorescence intensity and condensation of homologous chromosomes) and the chromosome imprinting.

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