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In situ hybridization of rDNA on chromosomes of the honeybee, *Apis mellifera* L.

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Abstract. DNA probes containing the repeated rDNA region of *Drosophila melanogaster* (coding for e.g. 28S and 18S rRNA) hybridized in situ to distinct regions of two heterologous mitotic chromosomes of the honeybee, identifying the nucleolus organizing regions (NORs). The method allows a rapid establishment of a physical map of *Apis mellifera* using other DNA probes of *Drosophila*. This is the first report on well-defined chromosomal markers in the honeybee.
Key words. Apis mellifera; fluorescence in situ hybridization; rDNA; chromosome.

Despite the ecological and economic importance of *Apis mellifera*, the genetics of the honeybee is less well understood than that of other domestic animals[1]. Although 27 phenotypic mutations have been described[2,3], a linkage map of the honeybee genome has not been established. Three linkage groups have been discovered, but we do not know on which chromosomes they are located, and we are far from having map-like information[4]. The chromosome set of honeybees is large ($n = 16$)[5], and their mating system complex. These factors may have made progress in understanding honeybee genetics slow in comparison with other organisms. Honeybee queens mate in flight with up to 17 drones[6], and controlled matings can only be achieved through artificial insemination.

Looking at the great potential of honeybees for applied breeding and basic genetic research, there is a clear need for a detailed genetic linkage map, and preferably a physical gene map similar to that available for other insects (e.g. *Drosophila melanogaster*). Utilizing DNA probes of *D. melanogaster*[7] that are already available should enable us to map various regions of the honeybee genome with in situ hybridization techniques. Here we report on the first fluorescence in situ hybridization (FISH) in honeybees, using the well known and sequenced rDNA probe[8] of *D. melanogaster*. In fruit flies these genes occur at about 250 tandem repeats on the X chromosome and in about 200 copies on the Y chromosome. Similar tandem repeat gene structures have been identified in many other organisms[9], which makes this conserved probe ideal for physical gene mapping through in situ hybridization.

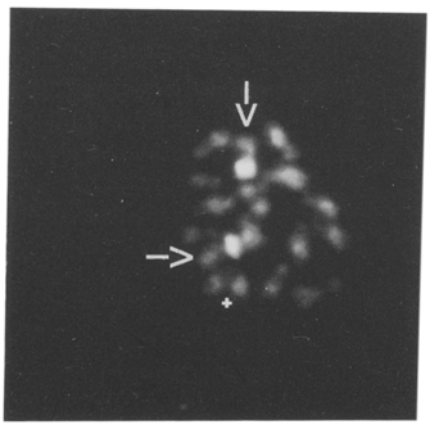
Material and methods

We used the *Drosophila melanogaster* clone pD103 containing the repeated rDNA[8] coding for 28S, 18S, 5.8S, and 2S rRNA, including intergenic and transcribed spacers in a cosmid pML2. Probe DNA was labeled by nick-translation[10] with biotin-dATP (BioNick System/GIBCO) according to the instructions of the supplier. Labeling of the probe and successful cross-hybridization to honeybee DNA isolated from adult bees[11] were tested by dot blot analysis onto a nylon membrane. The detection was performed using streptavidin conjugates according to the instructions of the PhotoGene-System supplied by GIBCO.

Testes of drone honeybee larvae (*Apis mellifera carnica*) (9–10 d after oviposition) were carefully excised under a dissection microscope, incubated for 2 h in Grace's Insect Medium with 0.6 µg/ml colcemid and centrifuged at 1000 × g. The pellet was suspended and incubated for 15 min in 1% Na-citrate for tissue swelling. Metaphase chromosomes were obtained using a standard 3:1 (v/v) methanol/acetic acid fixation protocol[12]. Fixation was repeated 6 times with ice-cold fixative to obtain plasma free preparations. After the tissue had been macerated in 60% acetic acid, the suspension was spread on microscope slides and fixed on a hot plate (60 °C) for 2 min. The preparations were dehydrated in an ascending ethanol series, air dried and incubated at 37 °C for 3 days. Subsequently the chromosome preparations were digested with RNase A (100 µg/ml) for 1 h at 37 °C, rinsed with 2 × SSC (0.1 M NaCl, 0.03 m Na_3-citrate) and digested with pepsin (1 µg/ml 0.01 M HCl) for 10 min at 37 °C[12]. After rinsing 3 times in PBS (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4) preparations were fixed for 10 min in 1% formalin-PBS, again rinsed in PBS, and dehydrated in an ascending ethanol series.

In situ hybridization and label detection. The hybridization protocol of Pinkel et al.[14] was slightly modified. 50 ng probe DNA and 10 µg of sheared herring sperm DNA were dissolved in 20 µl hybridization solution (50% formamide, 2 × SSC, and 10% dextran sulfate). After denaturation (75 °C for 5 min), the DNA mixture was ice-chilled, and added to the chromosome prepara-

tions. The slides were sealed with a cover slip using rubber cement, and were heated for DNA denaturation at 80 °C for 2 min. After 24-h hybridization at 37 °C, unhybridized DNA was removed by washing 4 times in 0.05 × SSC at 42 °C for 5 min. The slides were preincubated with blocking solution (2% BSA [bovine serum albumin] in BT-buffer: 0.15 M $NaHCO_3$, 0.05% Tween 20, 0.6 mM Na_3-citrate, pH 8) for 5 min at room temperature. The biotinylated DNA probe was detected using FITC (fluorescein isothiocyanateavidin 2.5 µg/ml) conjugates[14]. Incubation was performed for 1 h in a 37 °C warm moist chamber. The slides were washed 3 times in BT-buffer at 37 °C. The fluorescence signal was amplified using a biotinylated anti-avidin antibody (2.5 µg/ml BT-buffer), followed by an FITC-avidin incubation to label the biotinylated antibodies[14]. Incubation time in these steps was reduced to 30 min and washing was carried out as above. After adding antifade solution (0.2 M DABCO (1,4-diazabicyclo-2,2,2-octane), 20 mM $NaHCO_3$ (pH 8), 75% v/v glycerol[13]) and counterstaining with propidiumiodide (1 µg/ml) and DAPI (4,6-diamidino-2-phenylindole, 0.5 µg/ml), the labeled chromosome regions were analyzed with an epifluorescence microscope using a 530 nm barrier-filter and slide films.



rDNA probe *Drosophila melanogaster* hybridized to a haploid set of metaphase chromosomes of the drone honeybee. In spite of the contrast decrease through the black and white photographic reproduction, the homologous sites on the two different chromosomes are clearly visible (arrows). The largest chromosome, No. 1 according to the nomenclature of Hoshiba and Kusangi[16], is labeled with an asterisk. The other chromosomes cannot be classified corresponding to size (or various banding techniques), and are not numbered.

Results and discussion

The figure shows the result of a successful in situ hybridization to a haploid set of chromosomes. Hybridization signals are clearly visible on telomeric positions of a metacentric and a submetacentric chromosome. The same hybridization pattern was obtained in chromosome preparations of more than 15 different drones of various unrelated colonies, suggesting that this is a typical result for *Apis mellifera carnica*. The two clear and very strong signals suggest a high copy number of rDNA in *Apis mellifera*, probably representing two NOR sites in haploid cells. This compares with the two sites containing tandem repeats on the X and Y chromosome known from *D. melanogaster*[15].

Although this is the first report on a physical location of a gene in the honeybee genome, the most significant finding is the feasibility of the FISH technique in the system *Apis mellifera*. We are now in a position to rapidly map the honeybee genome without tedious crossing and back-crossing experiments, and can avoid the problems with G- and C-banding for chromosome characterization[16,17].

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