

## Distribution of heterochromatin and rDNA on the holocentric chromosomes of the aphids *Dysaphis plantaginea* and *Melanaphis pyrararia* (Hemiptera: Aphididae)

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**Abstract.** The structure of the holocentric chromosomes of the rosy apple aphid, *Dysaphis plantaginea* ( $2n = 12$ ), and pear-grass aphid, *Melanaphis pyrararia* ( $2n = 8$ ), was studied using C-banding, NOR, Giemsa and fluorochrome staining, and fluorescent in situ hybridization (FISH). Contrary to the equilocal distribution of heterochromatin typical of monocentric chromosomes, in both species C-banding evidenced a tendency of highly repetitive DNAs to be restricted to the X chromosomes. Silver staining and FISH, using a 28S rDNA probe, located rDNA genes on one telomere of each X chromosome, the only brightly fluorescent C-positive sites revealed by CMA<sub>3</sub> staining, whereas all other heterochromatic C-bands were DAPI positive. Both species showed a noticeable amount of rDNA heteromorphism. Mitotic recombination is proposed as a possible mechanism responsible for the variation in size of rDNA.

### INTRODUCTION

Most eukaryotes possess monocentric chromosomes characterised by a single localized centromere, which, under a microscope, appears as a primary constriction in the chromosome. At anaphase, in species with monocentric chromosomes, microtubules attach to the centromeres and bring the chromosomes to the pole, with the centromere leading. By contrast, holocentric chromosomes lack a distinct primary constriction and centromeric activity is spread along the entire length of the chromosomes. During mitosis, microtubules become attached to almost the entire length of each chromosome and as a result they each move in the form of a straight rod towards a pole (Hughes-Schrader & Schrader, 1961). However, during meiosis kinetic activity is restricted to the telomeric regions and the chromosomes can be regarded as telokinetic (Nekkala, 1985).

Compared with many species with monocentric chromosomes, very few have holocentric chromosomes. Nevertheless, species with holocentric chromosomes are recorded in both the plant and animal kingdoms, including insects and nematodes, which is thought to be a result of convergent evolution (Dernburg, 2001). Notwithstanding their uniqueness, holocentric chromosomes have not been intensively studied, except in the nematode worm *Caenorhabditis elegans* (for a review see Maddox et al., 2004).

Mitotic chromosomes can be easily observed in aphid embryonic tissues. For this reason, aphids represent a suitable insect model for determining the differences and

similarities in the structure and activity of holocentric and monocentric chromosomes. The identification of chromosomal markers in organisms possessing holocentric chromosomes is hampered by the lack of a simple morphological index (i.e. a centromere) and of clear-cut banding patterns (Blackman, 1987; Hales et al., 1997; Manicardi et al., 2002). Indeed, the development of cytogenetic tools in aphid species is becoming crucial for mapping and positional cloning of orthologous genes coming from the genome project of the pea aphid *Acyrtosiphon pisum* which is currently under the annotation step ([www.hgsc.bcm.tmc.edu/projects/aphid/](http://www.hgsc.bcm.tmc.edu/projects/aphid/)). These resources will provide novel opportunities for investigating many peculiar features of aphid biology, such as aphid-plant interactions and insecticide resistance in aphid species of agricultural importance.

The present paper reports the results of a cytogenetic study of two pests of pome orchards: the rosy apple aphid, *Dysaphis plantaginea* (Passerini), which is an important pest in western European apple orchards and particularly troublesome in organic apple production (Cross et al., 2006), and the brown pear aphid, *Melanaphis pyrararia*, which in addition to causing direct damage also transmits barley yellow dwarf luteovirus (El Yamani & Hill, 1991).

### MATERIAL AND METHODS

Aphids used in this study originated from field populations near Reggio Emilia (Northern Italy). Specimens of *Dysaphis plantaginea* were collected from apple trees and *Melanaphis pyrararia* from pear orchards.

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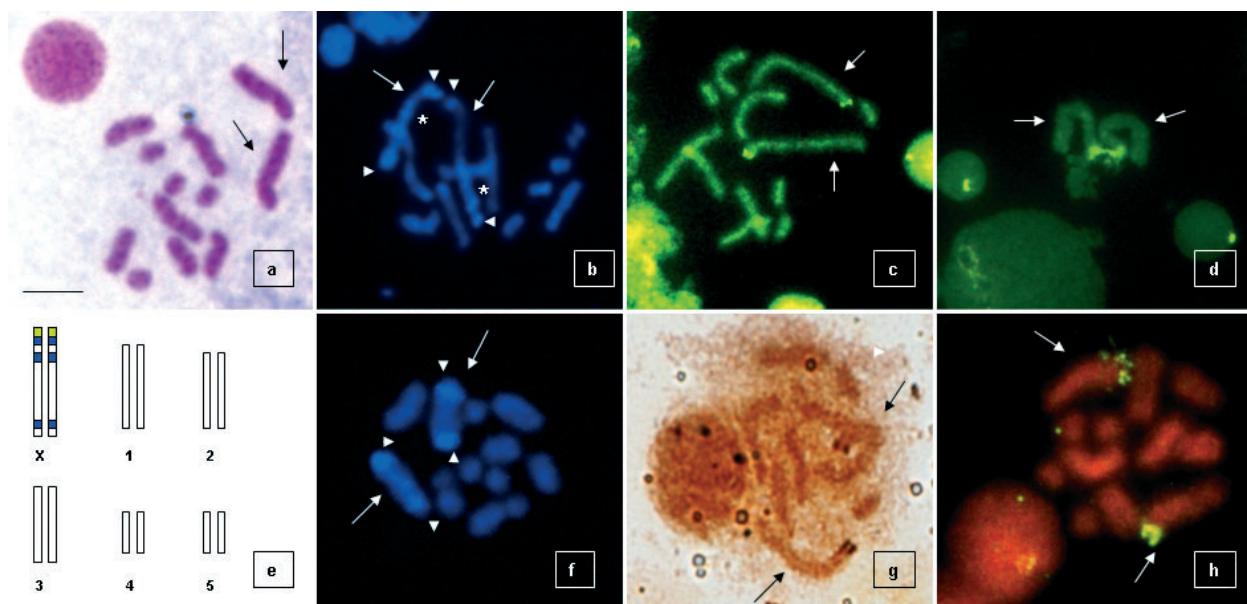


Fig. 1. Mitotic chromosome complements of *Dysaphis plantaginea* at metaphase after Giemsa staining (a); late prophase after DAPI-staining (b) and metaphase (f) after C-banding; late prophase after CMA<sub>3</sub> staining (c, d); metaphase after silver staining (g); metaphase after FISH with the 28S rDNA probe (h). The ideogram (e) summarises the C-banding pattern in the *D. plantaginea* karyotype, AT-rich (blue) and GC-rich (green) chromatin. Arrows indicate X chromosomes; arrowheads and asterisks indicate telomeric and intercalary C-bands, respectively. Bar = 10 µm.

Chromosome spreads of embryonic cells obtained from hundreds of parthenogenetic females were prepared as previously described by Manicardi et al. (1996). C-banding treatment was performed according to the procedure described in Sumner (1972). After this treatment, some slides were stained with 5% Giemsa solution in Sørensen buffer, pH 6.8, for 10 min. Chromomycine A<sub>3</sub> (CMA<sub>3</sub>) staining was carried out as described in Schweizer (1976) and the 4'-6'-diamidino-2-phenylindole (DAPI) treatment as described by Donlon & Magenis (1983). Silver staining of nucleolar organizing regions (NORs) was performed following the technique of Howell & Black (1980).

DNA extraction from aphid embryos was carried out as described in Bizzaro et al. (1996). Two species specific 28S rDNA probes were obtained by PCR amplification of *D. plantaginea* and *M. pyrraria* genomic DNAs, respectively, using two primers, forward (5'-AACAAACAACCGATACGTCCG-3') and reverse (5'-CTCTGTCCGTTTACAACCGAGC-3'), designed according to the coding 28S sequence of the aphid *Acyrtosiphon pisum* (GenBank acc. no. X66419) (Amako et al., 1996). The amplification mix contained 100 ng genomic DNA, 1 mM of each primer, 200 mM dNTPs and 2 U of DyNA-Zyme II DNA polymerase (Finnzymes Oy, Espoo, Finland). The amplification was performed with a thermocycler Hybaid (Hybaid Ltd, Basingstoke, UK). PCR amplification conditions were 2 min of denaturation at 94°C, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, with final extension step of 72°C for 10 min. Probe labeling and fluorescent in situ hybridization (FISH) were performed according to Bizzaro et al. (1996).

## RESULTS

*Dysaphis plantaginea* metaphases, observed in embryonic cells, revealed a chromosome number of  $2n = 12$  (Fig. 1a). *Melanaphis pyrraria* metaphases revealed a chromosome number of  $2n = 8$  (Fig. 2a).

DAPI staining of C-banded chromosomes of *Dysaphis plantaginea* revealed two telomeric and two faint intercalary bands on the X chromosomes (Fig. 1b). C-banding treatment in *Melanaphis pyrraria* revealed one main heterochromatic band located in a telomeric position on the X chromosomes. The same chromosomes also have a number of slim intercalary bands, which are not always distinguishable (Fig. 2b). In both species, the telomeric C-positive bands are always visible, independent of the degree of X chromosome condensation, whereas, in the more condensed metaphases intercalary bands are indistinguishable (Figs 1f and 2f). Both in *D. plantaginea* and *M. pyrraria*, autosomes lack any kind of longitudinal differentiation (Figs 1b, f and 2b–f).

CMA<sub>3</sub> staining of C-banded mitotic metaphases in *D. plantaginea* and *M. pyrraria* revealed a GC rich DNA region located at one telomere on each X chromosome (Figs 1c and 2c–f). In both species, the ends of the X chromosome that were brightly labelled with CMA<sub>3</sub> fluorescence also stained with AgNO<sub>3</sub>, indicating remnants of argentophilic proteins (Figs 1g and 2g). After FISH, using the 28S rDNA probes, there were strong hybridization signals at the ends of the same X-chromosome (Figs 1h and 2h). This suggests that in both *D. plantaginea* and *M. pyrraria* this is the nucleolar organizing region (NOR), containing actively transcribed rDNA genes. In both species, we observed a substantial amount of heteromorphism between homologous rDNA sites after CMA<sub>3</sub> and FISH using a rDNA probe (Figs 1c, h and 2c, h). In some mitotic plates the two X chromosome were connected by their NOR bearing telomeres (Figs 1d and 2d).

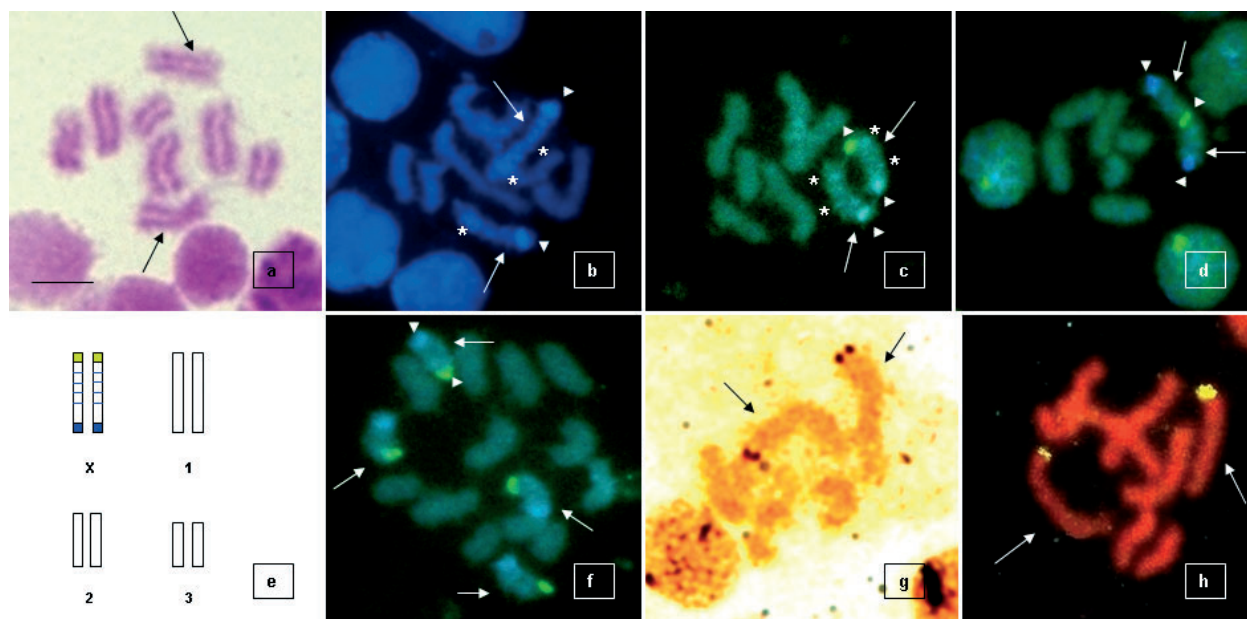


Fig. 2. Mitotic metaphase chromosomes of *Melanaphys pyrraria* after Giemsa staining (a); DAPI staining (b) and combined DAPI-CMA<sub>3</sub> staining (c, d, f) after C-banding; silver staining (g); and FISH with the 28S rDNA probe (h). The ideogram (e) summarises the C-banding pattern in the *M. pyrraria* karyotype, AT-rich (blue) and GC-rich (green) chromatin. Arrows indicate X chromosomes; arrowheads and asterisks indicate telomeric and intercalary C-bands, respectively. Bar = 10  $\mu$ m.

## DISCUSSION

In spite of the holocentric nature of aphid chromosomes, a high variation in chromosome number is recorded within the genus *Amphorophora* (Blackman, 1980) and *Trama* (Blackman et al., 2000), whereas most aphid genera have a remarkably stable number of chromosomes. For example, all of the *Dysaphis* species so far examined cytologically have  $2n = 12$ . When chromosomal rearrangements occur, particularly fragmentations and translocations, they most commonly affect the autosomes, whereas the Xs, with few exceptions (Blackman, 1986; Hales, 1989), are the two longest chromosomes. The analysis of mitotic metaphases revealed diploid chromosome numbers for *Dysaphis plantaginea* ( $2n = 12$ ) and *Melanaphys pyrraria* ( $2n = 8$ ) that accord with those previously recorded in the literature (Blackman & Eastop, 2000).

From a cytogenetic point of view, the most important result is that heterochromatic bands were only found on the X chromosomes in these two species. However, it must be stressed that C-banding highlights only large blocks of highly repetitive DNA and, therefore, the presence of middle repetitive DNA at other sites of the chromosome complement can not be excluded. A different localization of C-positive heterochromatin on non homologous chromosomes of the same genome, which clearly contradicts "the equilocal distribution of heterochromatin" typical of monocentric chromosomes (Schweizer & Loidl, 1987), is reported in other aphid species (Manicardi et al., 2002) but an exclusive localization on the X chromosomes has so far only been reported for two species of the *Aphis* genus, *Aphis sambuci* and *A. pomi* (Manicardi et al., 1998; Criniti et al., 2005). It has not always been possible to localize the heterochromatin

in other organisms with holocentric chromosomes (Collet & Westerman, 1984) but when possible, C-positive bands occur generally in telomeric and sometimes intercalary positions on the whole chromosome complement, (Camacho et al., 1985; Papeschi, 1988; Grozeva & Nokkala, 2003; Guerra & García, 2004). This suggests that the preferential, and in some case the exclusive localization of heterochromatin on X chromosomes is not a consequence of the holocentric structure but must be considered a peculiar feature of the aphid genome. Since heterochromatic blocks may influence the order of separation of sister chromatids in different chromosomes at anaphase (Vig, 1987), we hypothesize that the heterochromatic blocks restricted to X chromosomes are involved in the delay of X chromosome separation occurring during the maturation of the parthenogenetic oocyte, which is responsible for male sex determination in aphids (Blackman, 1987).

Sites of ribosomal rDNA genes (rDNA) can be identified by Ag staining, which visualize active NORs, by fluorescent staining with the antibiotic CMA<sub>3</sub>, which binds preferentially with GC-rich chromatin segments characteristically present at rDNA sites in aphids (Manicardi et al., 2002) and/or by in situ hybridization with rDNA probes. The constrained localization of rDNA cistrons on only one X telomere seems to be a general feature of aphids, since few exceptions are reported (Blackman & Spence, 1996). This specific location could be the result of a strong evolutionary constraint related to the peculiar XO male determination, which involves the pairing of the X chromosomes by their NORs during prophase of the maturation division (Blackman & Spence, 1996). In *D. plantaginea* and *M. pyrraria*, both CMA<sub>3</sub> and FISH using 28S rDNA probes revealed a noticeable

variation in the size of rDNA on homologous X chromosomes. This finding strongly suggests that changes in intrachromosomal structure, involving rDNA loci, are responsible for the observed heteromorphism.

Variation in ribosomal gene copy number between homologous chromosomes has been attributed to various mechanisms, including unequal crossing over, transposition, tandem amplification and other rearrangements involving homologous segments, yielding structural modification of NORs (Castro et al., 2001). Connections between X chromosomes via their NOR-bearing telomeres were repeatedly observed in mitotic plates (Figs 1d and 2d), which suggests that unequal recombination at mitosis could be involved in the NOR heteromorphism observed in the aphids studied.

Recombination in mitotic cells is reported for several species, ranging from placental mammals (Cornforth & Eberle, 2001; Svetlova et al., 2001) to insects (Stern, 1936; Bartsch et al., 1997) and yeast (Huang & Keil, 1995). For example, in the yeast *Saccharomyces cerevisiae*, the recombination hotspot *HOT1* initiates mitotic recombination when inserted into novel locations throughout the genome (Huang & Keil, 1995). Among aphids, the occurrence of unequal mitotic recombination seems to be the more likely explanation for the intraindividual NORs heteromorphism reported in a parthenogenetic lineage of *Megoura viciae* (Mandrioli et al., 1999).

However, the use of polymorphic microsatellite markers did not reveal recombinant genotypes in parthenogenetically produced males or females (Hales et al., 2002), thus suggesting that either recombination or non-equal exchange between homologues is a peculiarity of rDNA sites rather than a general process affecting the whole X chromosome.

Further research is required to answer the intriguing question on the presence/benefit of mitotic recombination in aphids, whose reproductive strategy is largely characterized by apomictic parthenogenesis, which reduces (holocyclic strains) and in some cases excludes (obligate parthenogenetic strains) the possibility of genetic recombination by meiosis.

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