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X-linked heterochromatin distribution in the holocentric chromosomes of the green apple aphid *Aphis pomi*

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Abstract

Chromatin organization in the holocentric chromosomes of the green apple aphid *Aphis pomi* has been investigated at a cytological level after C-banding, NOR, Giemsa, fluorochrome staining and fluorescent *in situ* hybridization (FISH). C-banding technique showed that heterochromatic bands are exclusively located on X chromosomes. This data represents a peculiar feature that clearly contradicts the equilocal distribution of heterochromatin typical of monocentric chromosomes. Moreover, silver staining and FISH carried out with a 28S rDNA probe localized rDNA genes on one telomere of each X chromosome; CMA_3 staining reveals that these silver positive telomeres are the only GC-rich regions among *A. pomi* heterochromatin, whereas all other C-positive bands are DAPI positive thus containing AT-rich DNA.

Introduction

To date, almost all studies concerning chromatin structure and organization have been focused on monocentric chromosomes whereas data regarding holocentric ones have been neglected. These chromosomes are also termed holokinetic because, during mitotic anaphase, they behave as if the spindle attachment is not localized, so that chromatids move apart in parallel and do not form the classical V shaped figures usually observed during the movement of monocentric ones (Blackman, 1987). Aphids, because of the easiness with which mitotic chromosomes could be obtained from embryonic tissues, represent a useful tool for a better understanding of holocentric/holokinetc chromosomes architecture, in order to work out differences and/or similarities to monocentric ones. The identification of chromosomal markers in organisms possessing holocentric chromosomes is extremely important since the lack of a primary conscription together with the difficult in obtaining a clear-cut banding pattern have greatly hampered cytogenetic studies in species possessing such a peculiar chromatin organization (Blackman 1987, Hales et al., 1997, Manicardi et al., 2002). The interest of a cytogenetic approach towards this taxon is also emphasized by the lymph-sucking feeding of these insects which represent a serious problem for agriculture, not only in view of their parasitic action against crops, but also because they represent active vectors of crop viruses.

This work is aimed to search for the localization and the DNA composition of heterochromatin in the holocentric chromosome of the green apple aphid *Aphis pomi* a small yellow-green aphids widely distributed in Europe, Middle East, throughout North America. Apple and Pear are the primarily host (Blackman & Eastop, 1984) on which *A. pomi* can cause direct damage to apple fruit (Oatman & Legner, 1961), but in most cases the damage is indirect through reductions in general

vigor of the apple tree (Hamilton, Swift & Marini, 1986). Green apple aphids are therefore important indirect pests of apple that require monitoring and, often, insecticide treatments (Carroll & Hoyt, 1984, Pfeier, Brown & Varn 1989).

Material and methods

Colonies of *Aphis pomi* were collected on apple trees close to Reggio Emilia (Northern Italy).

Chromosome spreads of embryo cells obtained from parthenogenetic females were prepared as previously described by Manicardi et al. 1996.

C-banding treatment was performed according to Sumner's technique (1972). After the treatment, some slides were stained with 5% Giemsa solution in Soerensen buffer pH 6.8, for 10 min. Chromomycin A_3 (CMA_3) staining was made in accordance to Schweizer (1976), whereas 4′-6′-diamidino-2-phenylindole (DAPI) treatment was carried out as described by Danlon and Magenis, (1983). Silver staining of nucleolar organizing regions (NORs) was performed following the technique of Howell and Black, (1980).

DNA extraction from aphid embryos was carried out as described in Bizzaro, Manicardi & Bianchi, 1996. The 28S rDNA probe was obtained by PCR amplification of *A. pomi* genomic DNA carried out using two primers, F (5′-AACAAAC-AACCGATACGTTCCG) and R (5′-CTCTGT-CCGTTTACAACCGAGC), designed according to the coding 28S sequence of the aphid *Acyrthosiphon pisum* (GenBank X66419) (Amako, Kwon & Ishikawa, 1996). The amplification mix contained 100 ng genomic DNA, 1 μM of each primer, 200 μM dNTPs and 2 U of DyNAZyme II DNA polymerase (Finnzymes Oy). The amplification was performed with a thermocycler Hybaid at an annealing temperature of 60°C for 1 min and extension at 72°C for 1 min. Probe labelling and fluorescent *in situ* hybridisation (FISH) were performed according to Bizzaro, Manicardi & Bianchi, 1996.

Results

Aphis pomi metaphases, obtained from parthenogenetic females, revealed a chromosome number of $2n = 8$ (Figure 1(a)). Occasionally plates containing 7 or 9 chromosomes have been observed (Figure 1(b and c)). These aneuploidies have never been observed in a whole specimen but they were always surrounded by plates containing the classical $2n = 8$ karyotype. Moreover, in some cases polypolid plates have been evidenced (Figure 1(d)).

Giemsa staining of C-banded chromosomes put in evidence four heterochromatic bands limited to the X chromosomes both at telomeric and interstitial sites, whereas autosomes lack any kind of differential staining (Figure 2(a)). Staining of C-banded chromosomes with fluorochromes shows that most of the *A. pomi* heterochromatin was brightly fluorescent after DAPI staining and therefore AT rich (Figure 2(b)), whereas only the C-band located at one telomere of each X chromosome contains CMA_3 positive, GC rich DNA (Figure 2(c)). Banding pattern on X chromosomes is always visible independently from the degree of X chromosome condensation even if, in more condensed metaphases, X chromatin results generally heavily stained in respect to the autosome one (Figure 2(e)).

The CMA_3 positive X telomeres were also argentophilic after $AgNO_3$ staining (Figure 2(g)) and brightly fluorescent after FISH experiments carried out using a 28S rDNA as a probe (Fig. 2(h)), thus demonstrating that these telomeres are the NORs, containing actively transcribed rDNA genes. A variable heteromorphism between homologous NORs was observed with all the technique utilised (Figure 2(c, g and h)) and, occasionally, plates in which the rDNA genes result fully concentrated on only one of the X chromosomes were observed (Figure 2(i)).

In addition to the NORs, silver staining also revealed the presence of axial structures, running parallel along the chromatid axes, without point of intersection, as expected by the holocentric nature of aphid chromosomes (Figure 2(g)). This peculiar feature is highlighted in late metaphase plates in which the chromatids do not form the classical V shaped figures usually observed during the movement of monocentric chromosomes (Figure 2(f)).

Discussion

The foremost result evidenced in this paper regards the X restricted localization of all heterochromatic bands to *A. pomi* X chromosome. A preferential localization of C-positive

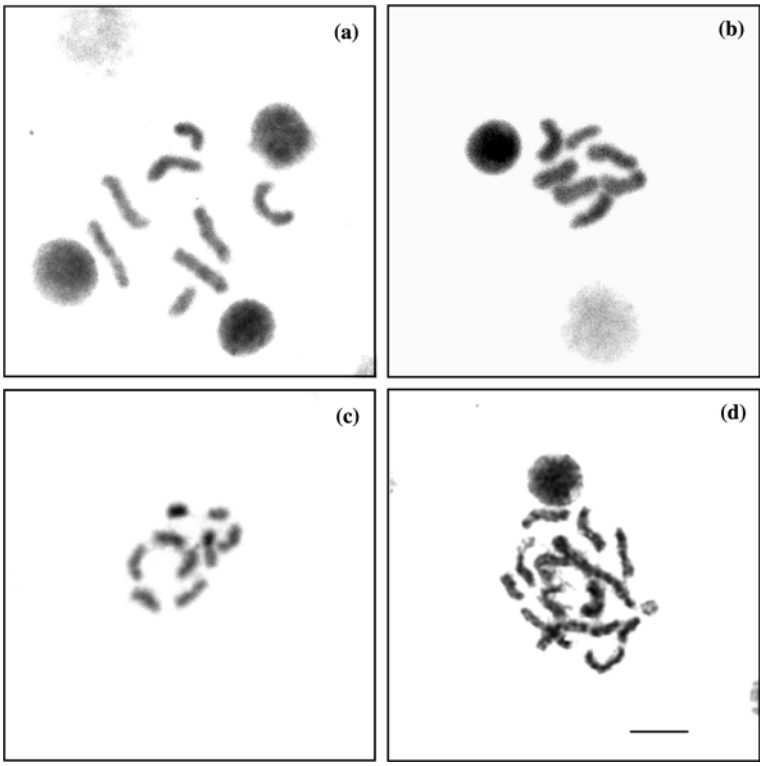

Figure 1. Giemsa staining of *Aphis pomi* metaphases with 8 (a), 7 (b), 9 chromosomes (c) and polyploid plates (d). Bar corresponds to 10 μm.

heterochromatin on X chromosomes, which clearly contradicts 'the equilocal distribution of heterochromatin' described in monocentric chromosomes (Schweizer & Loidl, 1987), has been already reported in other aphid species (Manicardi et al., 2002) but an exclusive localization on X chromosomes has been found only in another species of the *Aphis* genus, *Aphis sambuci* (Manicardi et al., 1998). The rare research carried out in order to analyse heterochromatin localization in organisms possessing holocentric chromosomes in some cases were unsuccessful (Collet & Westerman, 1984) but generally a telomeric and sometimes intercalary localization of C positive bands on whole chromosome complement has been described (Camacho, Belda & Cabrero, 1985, Papeschi, 1988, Groeva & Nokkala, 2003, Guerra & Garcia, 2004). These data as a whole suggest that the preferential, and in some case the exclusive localization of heterochromatin on X chromosome is not a consequence of the holocentrisms but must be considered a peculiar feature of aphid chromatin. In this connection, the heterochromatin blocks on X chromosomes could be involved in the delay of X chromosome separation occurring during maturation of aphid parthenogenetic oocytes, which is considered to be the basis of male sex determination in aphids (Orlando, 1974, Blackman, 1987). Moreover, an overload of heterochromation on X chromosomes could reduce the number of genes on these chromosomes and so lowering the need of dosage compensation between 2AXX females and 2AXO males.

The different pattern of CMA3 and DAPI staining after C-banding points out the heterogeneity of heterochromatic DNA composition in *A. pomi* genome. Indeed, GC-rich NOR associated heterochromatin differs from all other heterochromatic bands that are characterized by AT-rich DNA. This pattern of heterochromatin heterogeneity seems to be a general feature of aphid genome, since it has been described in all species investigated so far (Manicardi et al. 2002).

The location on one X telomere of NORs in *A. pomi* complement fits what has been evidenced

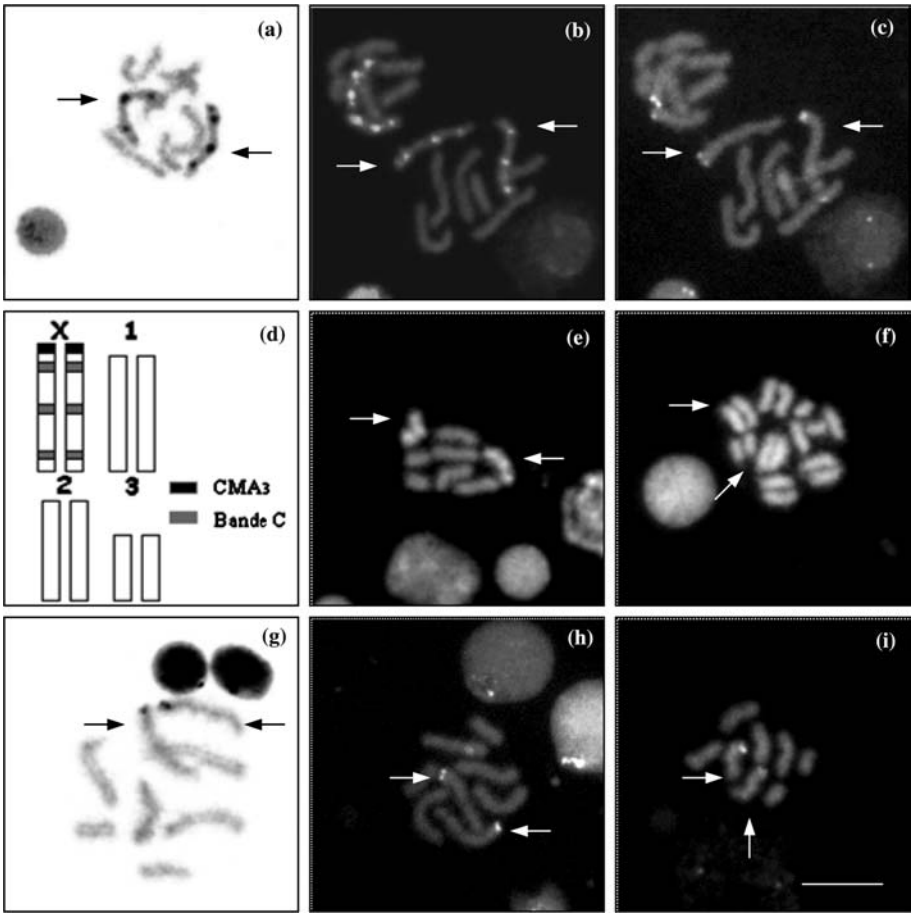

Figure 2. Giemsa (a), DAPI (b, e), and CMA_3 staining (c) of *A. pomi* C-banded chromosomes; the idiogram in (d) summaries C-banding pattern. DAPI staining of late metaphase (f). Silver-staining (g) and FISH with a rDNA probe (h i). Arrows indicate X chromosomes. Bar corresponds to 10 μm.

in all the species of aphids, with the exclusion of the cases of *Schoutedenia ralumensis* and *Maculachnus submacula* the NORs of which are located on the autosomes (Hales, 1989; Blackman & Spence, 1996). Another exception has been found in *Amphoropora idaei* that, according to Blackman (1980), could have the NORs in an intermediate X position, due to a chromosome rearrangement. It can, therefore, reasonably be inferred that in this taxon, the chromosomal location of NORs has been extremely retained and this leads to suppose that the location of the rDNA genes of aphids may have been subjected to a strong evolutive constraint. In fact, since male sex determination appears to be dependent on the capability of the X chromosomes to associate through the telomeres, where the NOR regions are located (Orlando, 1974, 1983, Blackman & Hales, 1986), the necessity of maintaining the NORs in a well defined single site, could have fixed their location in the whole taxon.

All techniques utilised to study rDNA distribution in *A. pomi* genome, evidence a detectable level of heteromorphism of NOR regions, another feature which seems to be spread in aphid X chromosomes (Manicardi et al., 2002).

The capability to expand and to contract the number of rDNA copies is a well recognized property of rDNA. Several different mechanisms can be invoked to explain DNA expansion, in particular the unequal crossing-over both meiotic and mitotic, and unequal sister chromatid exchanges (SCE). Considering the characteristics of the biological cycle in aphids, in which meiosis

is, in any case, a rather rare phenomenon, we are inclined to suggest that phenomena such as unequal mitotic crossing over and unequal SCE may be the main cause of the rDNA heteromorphism. The presence of χ sequences in the IGS of *Acyrthosiphon pisum* strongly (Mandrioli et al., 1999) supports the hypothesis that unequal crossing-over between rDNA genes can be at the basis of NOR heteromorphism described in different aphid species. The occurrence of somatic recombination between homologous and non homologous rDNA sites resulting in asymmetric exchanges of these tandemly repeated DNA sequences has been found to be particularly diffused in yeasts (Huang & Keil, 1995). The same phenomenon also described in number of eukaryotes suggests that the genes for ribosomal RNA can operate also as hotspot of mitotic ricombination (Birky, 1996).

The occurrence of NOR heteromorphism in parthenogenetic lineages of aphids is particularly interesting because it suggests a possible presence of mitotic crossing-over, at least at certain recombination hotspots within rDNA arrays, during parthenogenetic generations, when it is generally believed that no recombination occurs (Carvalho et al., 1991).

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References

Amako, D., O.Y. Kwon & H. Ishikawa, 1996. Nucleotide sequence and presumed secondary structure of the 28S rRNA of pea aphid: implication for diversification of insect rRNA. J. Mol. Evol. 43: 469–475.

Birky, C.W., 1996. Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. Genetics 144: 427–437.

Bizzaro, D., G.C. Manicardi & U. Bianchi, 1996. Chromosomal localization of a highly repeated *Eco*RI DNA fragment in *Megoura viciae* (Homoptera, Aphididae) by nick-translation and fluorescence in situ hybridization. Chromosome Res. 4: 392–396.

Blackman, R.L., 1980. Chromosome numbers in the Aphididae and their taxonomic significance. Syst. Entomol. 5: 7–25.

Blackman, R.L., 1987. Reproduction, cytogenetic and development, pp. 163–195 in Aphids: Their Biology, Natural Enemies and Control, Vol. A, edited by A.K. Minsk P. Harrewijn. Elsevier, Amsterdam.

Blackman, R.L. & V.F. Eastop, 1984. Aphids on the World's Crop: An Identification and Information Guide. John Wiley and Sons, Chichester.

Blackman, R.L. & D.F. Hales, 1986. Behaviour of the X chromosomes during growth and maturation of parthenogenetic eggs of *Amphorophora tuberculata* (Homoptera, Aphididae), in relation to sex determination. Chromosoma 94: 59–64.

Blackman, R.L. & J.M. Spence, 1996. Ribosomal DNA is frequently concentrated on only one X chromosome in permanently apomiptic aphids, but this does not inhibit male determination. Chromosome Res. 4: 324–320.

Camacho, J.P.M., J. Belda & J. Cabrero, 1985. Meiotic behaviour of the holocentric chromosomes of *Nezara viridula* (Insecta, Heteroptera) analyzed by C-banding and silver impregnation. Can. J. Genet. Cytol. 27: 490–497.

Carrol, D.P. & S.C. Hoyt, 1984. Augmentation of European earwigs (Dermaptera: Forficulidae) for biological control of apple aphid (Homoptera: Aphididae) in an apple orchard. J. Econ. Entomol. 77: 738–743.

Carvalho, G.R., N. Maclean, S.D. Wratten, R.E. Carter & J.P. Thurston, 1991. Differentation of aphid clones using DNA fingerprints from individual aphids. Proc. R. Soc. Lond. B 243: 109–114.

Collet, C. & M. Westerman, 1984. Interspersed distribution patterns of C-bands and satellite DNA in the holocentric chromosomes of *Luzula flaccida* (Juncaceae). Genetica 63: 175–179.

Danlon, T.A. & R.E. Magenis, 1983. Methyl green is a substitute for distamycin A in the formation of distamycin A/DAPI C-bands. Hum. Genet. 65: 144–146.

Grozeva, S. & S. Nokkala, 2003. C-heterochromatin and extra (B) chromosome distribution in six species of the Nabis (Heteroptera, Nabidae) with the modal male karyotype $2n = 16XY$. Folia Biol. (Krako'w) 51: 13–22.

Guerra, M. & M.A. García, 2004. Heterochromatin and rDNA sites distribution in the holocentric chromosomes of *Cuscuta approximata* Bab. (Convolvulaceae). Genome 47: 134–140.

Hales, D.F., 1989. The chromosome of *Schoutedenia lutea* (Homoptera, Aphididae, Greenideinae), with an account of meiosis in the male. Chromosoma 98: 295–300.

Hales, D.F., J. Tomiuk, K. Wohrmann & P. Sunnucks, 1997. Evolutionary and genetic aspects of aphid biology: A review. Eur. J. Entomol. 94: 1–55.

Hamilton, G.C., F.C. Swift & R. Marini, 1986. Effect of *Aphis pomi* (Homoptera: Aphididae) density on apples. J. Econ. Entomol. 79: 471–478.

Howell, W.M. & D.A. Black, 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015.

Huang, G.S. & R.L. Keil, 1995. Requirements for activity of the yeast mitotic recombination hotspot HOT1: RNA polymerase I and multiple cis-acting sequences. Genetics 141: 845–855.

Mandrioli, M., D. Bizzarro, M. Giusti, G.C. Manicardi & U. Bianchi, 1999. NOR heteromorphism within a parthenogenetic lineage of aphid *Megoura viciae*. Chromosome Res. 7: 157–162.

Manicardi, G.C., D. Bizzaro, E. Galli & U. Bianchi, 1996. Heterochromatin Heterogeneity in the holocentric X chromatin of *Megoura vicine* (Homoptera, Aphididae). Genome 39: 465–470.

Manicardi, G.C., M. Mandrioli, D. Bizzarro & U. Bianchi, 1998. Silver staining as a new bandin tecnique to identify aphid chromosome. Chromosome Res. 6: 55–57.

Manicardi, G.C., M. Mandrioli, D. Bizzarro & U. Bianchi, 2002. Cytogenetic and molecular analysis of heterochromatic areas in the holocentric chromosomes of different aphid species, pp. 47–56 in Some Aspects of Chromosome Structure and Functions, edited by R.C. Sobti, G. Obe, R.S. Athwal. Narosa Publishing House, New Delhi.

Oatman, E.R. & E.F. Legner, 1961. Bionomics of the apple aphid *Aphispomi* DeGeer, on young nonbearing apple trees. J. Econ. Entomol. 54: 1034–1037.

Orlando, E., 1974. - Sex determination in *Megoura viciae* Bukton (Homoptera, Aphididae). Monit. Zool. Ital. 8: 61–70.

Orlando, E., 1983. Chromosomal abnormalities in male producing-eggs: a study in *Megoura viciae* (Homoptera, Aphidiae). Genetica 62: 55–59.

Papeschi, A.G., 1988. C-banding and DNA content in three species of *Belostoma* (Heteroptera) with large differences in chromosome size and number. Genetica 76: 43–51.

Pfeiffer, D.G., M.W. Brown & M.W. Varn, 1989. Incidence of spirea aphid (Homoptera: Aphididae) in apple orchards in Virginia, West Virginia, and Maryland. J. Entomol. Sci. 24: 145–149.

Schweizer, D., 1976. Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58: 307–324.

Schweizer, D. & J. Loidl, 1987. A model for heterochromatin dispersion and the evolution of C-band patterns. Chromosome Today 9: 61–74.

Sumner, A.T., 1972. A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. 75: 304–306.