Hereditas 131: 185–190 (1999)

Cytogenetic analysis of the holocentric chromosomes of the aphid *Schizaphis graminum*

M. MANDRIOLI, S. GANASSI, D. BIZZARO and G. C. MANICARDI

Dipartimento di Biologia Animale, Università di Modena e Reggio Emilia, Modena, Italy

Mandrioli, M., Ganassi, S., Bizzaro, D. and Manicardi, G. C. 1999. Cytogenetic analysis of the holocentric chromosomes of the aphid *Schizaphis graminum*. — *Hereditas 131*: 185–190. Lund, Sweden. ISSN 0018-0661. Received June 28, 1999. Accepted November 2, 1999

Chromatin organization in the holocentric chromosomes of the aphid *Schizaphis graminum* has been investigated at a cytological level after C-banding, NOR, Giemsa, DAPI and CMA_3 staining. C-banding technique showed the presence of numerous C bands on the two X chromosomes both in telomeric and intercalary regions, whereas autosomes show a small number of heterochromatic bands. Contrary to the results with other aphid species, in *S. graminum* the C-banding pattern is peculiar to each chromosome pair, thus allowing the identification of homologues and the reliable reconstruction of a karyotype. These cytogenetic data could be useful for the identification of chromosomal rearrangement eventually occurred between different *S. graminum* biotypes. Moreover, silver staining and fluorescent in situ hybridization (FISH) with a 28S rDNA probe localized rDNA genes on one telomere of each X chromosome; these are the only brightly fluorescent C-positive regions revealed after CMA_3 staining, whereas all other heterochromatic bands are DAPI positive.

Gian Carlo Manicardi, Dipartimento di Biologia Animale, Viale Berengario 14, 41100 Modena, Italy. E-mail: manicardi.giancarlo@unimo.it

Aphids are plant sap-sucking insects very important for agricultural entomology in view of their direct parasitic action against crops and for the role they play as active vectors of crop viruses (BLACKMAN 1980). At a cytogenetic level, aphids represent an interesting model because they possess holocentric chromosomes showing a centromeric activity along the whole chromosomal axis (HUGHES-SCHRADER and SCHRADER 1961; BLACKMAN 1987). 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).

The holocentric nature of aphid chromosomes has represented a great obstacle for cytogenetic studies, as homologues, in view of the absence of primary and/or secondary constrictions, can be paired on the basis of their size only (BLACKMAN 1980).

In view of the great interest of such a taxon, an extensive survey at cytogenetic and molecular level regarding the structure of the holocentric chromosomes of several aphid species, such as *Megoura viciae*, *Acyrthosiphon pisum*, *Aphis sambuci*, *Myzus persicae*, *Tetraneura nigriabdominalis* and *Tetraneura ulmi*, has been carried out in our laboratory (MANICARDI et al. 1991a,b, 1992, 1994, 1996, 1998a,b; MANICARDI and GAUTAM 1994; BIZZARO et al. 1996; GALLI and MANICARDI 1998; MANDRIOLI et al. 1999a,b,c). This experimental approach allows the development of cytogenetic markers which could be useful for the taxonomic identification of aphids, as species-specific morphological characters are often difficult to find.

The aim of this work is to carry out a cytogenetic analysis of the holocentric chromosomes of the aphid *Schizaphis graminum*, an important pest of wheat and several other crops.

MATERIAL AND METHODS

The specimens of *Schizaphis graminum* used for this research were obtained from a laboratory clone collected several years ago on mais plants near Modena. Aphids are maintained in our Department at 19°C with 16 hours of light and 8 of darkness on *Zea mais* plants. Chromosome preparations of parthenogenetic females were made by spreading embryo cells, as previously described by MANICARDI et al. 1996.

C-banding treatment was performed according to SUMNER, 1972. After the treatments, some slides were stained with 5% Giemsa solution in Soerensen buffer pH 6.8, for 10 min. Chromomycin A_3 (CMA_3) staining was performed according to SCHWEIZER (1976), whereas 4′-6′diamidino-2-phenylindole (DAPI) treatment was carried out as described by DONLON and MAGENIS (1983). Silver staining of NORs was performed following the technique of HOWELL and BLACK (1980).

DNA extraction from aphid embryos was performed as described in MANDRIOLI et al. 1999a. The 28S rDNA probe was obtained by PCR amplification of *S. graminum* genomic DNA carried out using two primers, F (5'-AACAAACAACCGATACGTTCCG) and R. (5'CTCTGTCCGTTTACAACCGAGC), designed according to the coding 28S sequence of the aphid *A. pisum* (GenBank X66419) (AMAKO et al. 1996). The amplification mix contained 100 ng genomic DNA, 1 μM of each primer, 200 μM dNTPs and 2 U of DyNAZyme II 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 FISH were performed according to BIZZARO et al. 1996.

Images of 50 chromosomal plates were acquired using a CCD camera Sony and image analysis of C-banded chromosomes was done with the software Matrix Quantavision, according to VENORA et al. 1995.

RESULTS

S. graminum metaphases, obtained from parthenogenetic females, revealed a chromosome number of $2n = 8$. C-banding technique showed the presence of numerous heterochromatic bands on the X chromosomes both at telomeric and interstitial sites, whereas autosomes had fewer heterochromatic bands (Fig. 1a). The localization of C bands on chromosomes was unique to each chromosome pair, allowing identification of homologous chromosomes and construction of a reliable karyotype (Fig. 1d). C bands were variable both in number and staining intensity, depending on the degree of chromosome condensation; this effect was particularly evident on X chromosomes as they showed more heterochromatic bands than the autosomes (Fig. 2).

PC-based image analysis showed that the average relative amount of heterochromatin in *S. graminum* is about $42.3\% \pm 7.8$ of the genome and that $48.4\% \pm 6.9$ of the total heterochromatin is located on X chromosomes.

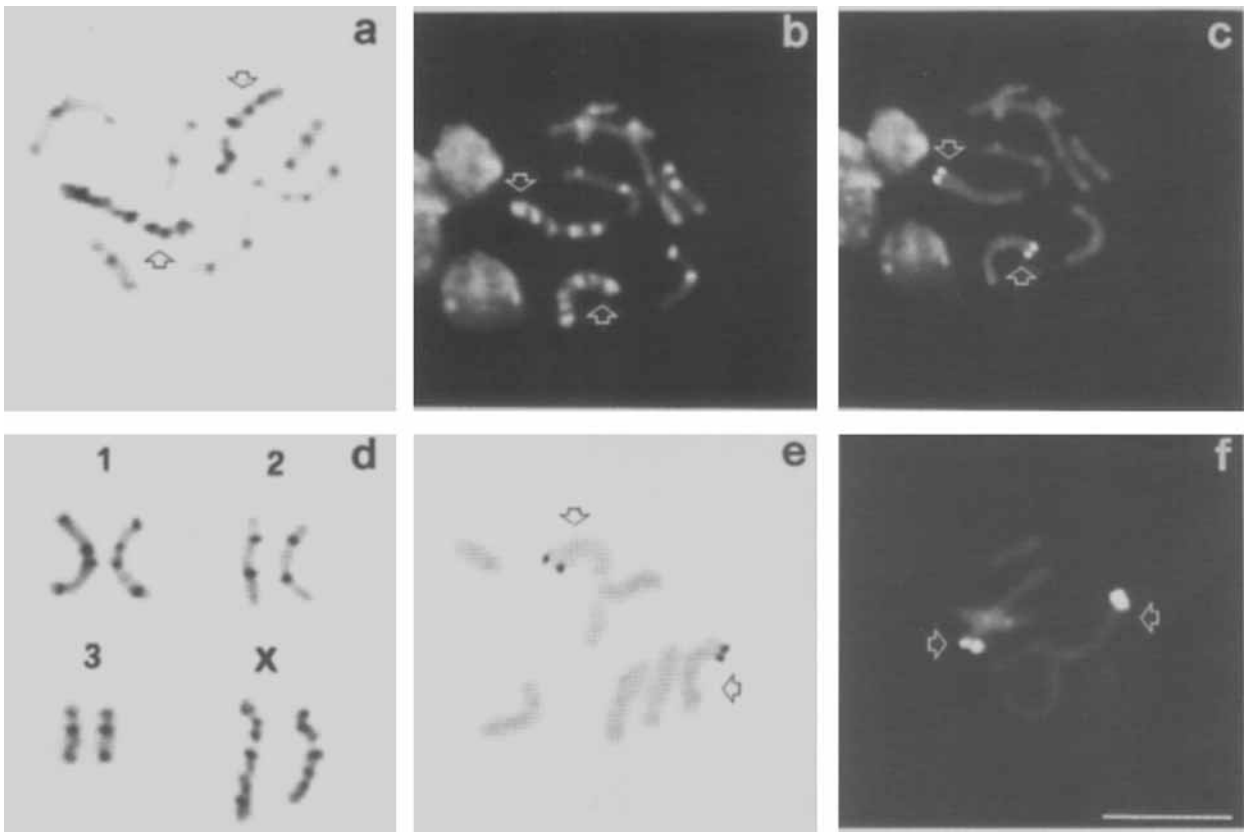

Fig. 1. a–f. C-banded metaphase chromosomes of *S. graminum* females after Giemsa (**a**), DAPI (**b**) and CMA_3(**c**) staining. C-banding technique allows homologues pairing and karyotype reconstruction (**d**). Silver staining (**e**) and FISH with a rDNA probe (**f**) show that the CMA_3 brightly fluorescence on X telomeres is due to the localization in these region of rDNA genes. Arrows indicate X chromosomes. Bar = 10 μm

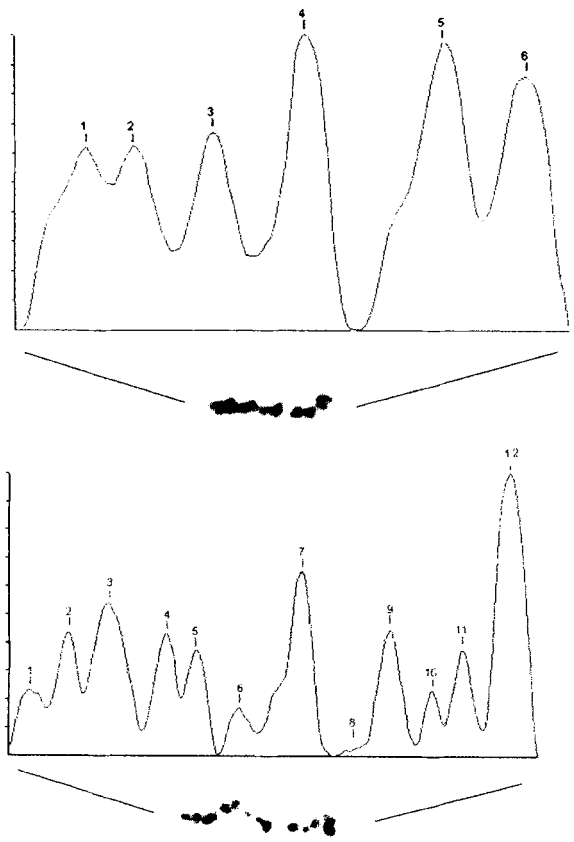

Fig. 2. C-banded X chromosomes of *S. graminum* females after Giemsa staining at different condensation degree. Image analysis clearly shows that the number of chromosomal bands is linked to the condensation degree of chromosomes since longer X chromosome shows a larger number of heterochromatic bands (12 in longest one in contrast to 6 in the shortest one).

Staining of C-banded chromosomes with fluorochromes gave the same banding pattern as after C-banding and Giemsa staining. Most of the *S. graminum* heterochromatin was brightly fluorescent after DAPI staining and therefore AT rich (Fig. 1b), whereas only the C-band located at one telomere of each X chromosome contains CMA_3 positive, GC rich DNA (Fig. 1c).

The CMA_3 positive X telomeres were also argentophilic after staining with $AgNO_3$ (Fig. 1e) and intensely fluorescent after FISH experiments carried out utilizing a 28S rDNA sequences as a probe, thus demonstrating that these telomeres are the NOR, containing actively transcribed rDNA genes (Fig. 1f). A slight difference between homologous NORs was observed both after silver staining and FISH.

DISCUSSION

Banding techniques have been primarily developed for the identification of homologous chromosomes. In higher vertebrates G-, Q- and R-banding techniques, which can give a detailed pattern of bands throughout the length of the chromosomes, are used for this purpose (COMINGS et al. 1973; SUMNER 1990). In all other organisms such banding patterns can not generally be induced so that identification of homologous chromosomes has been frequently obtained using patterns of C-bands or other bands which represent subsets of heterochromatin (SUMNER 1990).

C-banding has been rarely used up to now in insect species possessing holocentric chromosomes (BLACKMAN 1976, 1990). In some cases the attempts to localize constitutive heterochromatin were unsuccessful (COLLET and WESTERMANN 1984), whereas a telomeric localization of the C-bands have been described in different Heteroptera species (MURAMOTO 1976; SOLARI 1979; CAMACHO et al. 1985; PAPESCHI 1988).

C-banding technique applied to *S. graminum* mitotic metaphases and followed by Giemsa staining showed the presence of numerous C-bands on the X chromosomes both at telomeric and interstitial locations. Autosomes possess few heterochromatic bands and the banding pattern is unique for each chromosome pair. Such localization argues against the theory of the "equilocal distribution of heterochromatin" which states that, in any species, the constitutive heterochromatin is located at similar sites on non-homologous chromosomes (HEITZ 1933; SCHWEIZER and LOIDL 1987). In the holocentric chromosomes of *S. graminum*, the distribution of heterochromatin clearly contradicts this statement, since C-positive bands have been found to be located in different regions along the chromosomes. An interspersed distribution of heterochromatin amongst the euchromatin, similar to that observed in aphids, has been reported for holocentric chromosomes in plant species belonging to the genus *Luzula* (RAY and VENKETESWARAN 1978; COLLET and WESTERMANN 1984).

The tendency of heterochromatin to be concentrated on the X chromosomes is not a unique feature of *S. graminum*, as it has been repeatedly described in the holocentric chromosomes of other aphid species (MANICARDI et al. 1991b, 1992, 1996; MANDRIOLI et al. 1999a). In these studies, focused on the description of heterochromatin localization and distribution, aphid heterochromatin was not quantified. In the present paper we report the amount of heterochromatin on X chromosomes evaluated by means of image analysis. These values are not based on a densitometric analysis, but on a geometric one, as a direct correlation between areas and DNA content has been reported in different organisms (REES and HAZARIKA 1969; RAINA and REES 1983; MARTINEZ

and GINZO 1985; VENORA et al. 1995). The use of computer-based analysis provides an objective and therefore highly reproducible evaluation of the distribution of heterochromatin in chromosomes (VENORA et al. 1995).

The preferential localization of heterochromatin on aphid X chromosomes could be due to a functional constrains which direct heterochromatin on these chromosomes. In particular, the large heterochromatic 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).

DAPI staining of *S. graminum* C-banded chromosomes indicates that heterochromatin is essentially AT rich, whereas CMA_3 staining induced a bright fluorescence limited to one telomere of each X chromosome. The different response to CMA_3 and DAPI staining after C-banding points out the heterogeneity of heterochromatic DNA composition in *S. graminum* genome. This pattern of heterochromatin heterogeneity seems to be a general characteristic of aphid chromatin since it has been described in all species investigated at cytogenetic level so far (MANICARDI et al. 1996; 1998b; MANDRIOLI et al. 1999a). The heterogeneity of C-bands have been previously observed in numerous organisms (ROCCHI 1982).

The C-banding pattern obtained both after Giemsa and DAPI staining evidenced that the numbers of heterochromatic bands on each chromosome pair is not constant but it is strongly connected to the degree of chromosome condensation. Decondensed prometaphase chromosomes showed in fact a large number of heterochromatic bands which fuse together during chromosome condensation and give rise to a smaller number of large C-bands at metaphase. This behaviour was particularly evident on X chromosomes since they have the higher heterochromatic content. This peculiar feature, already described both in animals and plants with holocentric chromosomes (RAY and VENKETESWARAN 1978; COLLET and WESTERMANN 1984; MANICARDI et al. 1991a; 1996), fits the hypothesis that in holocentric chromosomes, heterochromatic areas are widely scattered along the chromosomal axis, in contrast to the high heterochromatin concentration near the centromere found in monocentric chromosomes (LAGOWSKY et al. 1973; COLLET and WESTERMANN 1984; GODAY and PIMPINELLI 1984).

Silver staining and FISH experiments, carried out utilising a 28S rDNA sequence as a probe, clearly indicate that, in *S graminum*, CMA_3 heterochromatic bands on X chromosomes coincides with the nucleolar organizing region (NOR). The CMA_3 bright fluorescence of these regions is therefore due to the high GC content typical of genes coding for ribosomal RNA (rDNA) (AMAKO et al. 1996). Ribosomal genes have been already located on aphid chromosomes using various techniques such as CMA_3 staining (MANICARDI et al. 1992, 1996, 1998b), silver staining (BLACKMAN and HALES 1986; HALES 1989; SEN and KHUDA-BUKHSH 1992; MANICARDI et al. 1992, 1996, 1998b) and FISH with rDNA probes (FENTON et al. 1994; BLACKMAN and SPENCE 1996; MANICARDI et al. 1998b). Generally in aphids rDNA genes are arranged as tandemly repeated clusters located at one telomeric region of each X chromosome (BLACKMAN and SPENCE 1996). Exceptions include the interstitial position of rDNA genes in *Amphorophora idaei* (FENTON et al. 1994), and the autosomal location of NORs in *Schoutedenia lutea* (HALES 1989). CMA_3 staining, silver staining and FISH in *S. graminum* fully confirms the localization of rDNA genes at one telomere of each X chromosome. Using these staining techniques it has been possible to detect the presence of an intra and inter individual heteromorphism both in size and number of active NORs in several aphid species (BLACKMAN and SPENCE 1996; GALLI and MANICARDI 1998; MANDRIOLI et al. 1999b,c).

The identification of chromosomal markers in organisms with holocentric chromosomes is extremely important since the lack of a primary constriction, together with the difficult in obtaining a clear-cut banding pattern, has greatly hampered cytogenetic studies in organisms possessing such a peculiar chromatin organization (SPENCE and BLACKMAN 1998). Moreover, it must be emphasised that the structure of holocentric-holokinetic chromosomes allows for karyotype rearrangements as all fragments produced by X-ray irradiation, are conserved in a chromosomal set (KHUDA-BUSH and DATTA 1980; KUZNETZOVA and SAPUNOV 1987). This may explain the high diffusion of chromosomal polymorphism known for many aphid species (BLACKMAN 1978, 1980, 1990; KHUDA-BUSH and KAR 1990). For example, in *S. graminum* mitotic plates ranging from $2n = 6$ and $2n = 8$ have been observed (RUBIN DE CELIS et al. 1997). Moreover, a variation in chromosome length among five *S. graminum* biotypes has been reported (MAYO et al. 1988). The use of C-banding technique on *S. graminum* chromosomes, by allowing a reliable identification of all homologues will provide an useful tool for the study of karyotype evolution within this taxon.

ACKNOWLEDGEMENTS

We are greatly indebted to Prof. Umberto Bianchi and Dr. RL Blackmann for their critical review of the manuscript. This work was supported by grants from the Italian Consiglio Nazionale delle Ricerche (CNR).

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