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# Composition and Epigenetic Markers of Heterochromatin in the Aphid *Aphis nerii* (Hemiptera: Aphididae)

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## Key Words

Aphid · Epigenetics · Heterochromatin · Holocentric chromosomes · Satellite DNA

## Abstract

A detailed karyotype analysis of the oleander aphid *Aphis nerii* focusing on the distribution, molecular composition and epigenetic modifications of heterochromatin was done in order to better understand the structure and evolution of holocentric/holokinetic chromosomes in aphids. The female karyotype ( $2n = 8$ ) consisted of 3 pairs of autosomes and a pair of X chromosomes that were the longest elements in the karyotype and carried a single, terminally located nucleolar organizer region. Males showed  $2n = 7$  chromosomes due to the presence of a single X chromosome. Heterochromatin was located in the X chromosomes only and consisted of 4 satellite DNAs that have been identified. *A. nerii* constitutive heterochromatin was enriched in mono-, di- and trimethylated H3 histones and HP1 proteins but, interestingly, it lacked DNA methylation that was widespread in euchromatic chromosomal regions. These results suggest that aphid heterochromatin is assembled and condensed without any involvement of DNA methylation.

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Holocentric/holokinetic chromosomes show centromeric activity spread along the whole chromosomal axis so that at anaphase chromatids move apart in parallel and do not form the classical V-shaped figures typically observed in monocentric chromosomes [Hughes-Schrader and Schrader, 1961; Blackman, 1987]. They have been described in almost all the higher eukaryote taxa so far studied, with the exception of echinoderms and chordates and they are present in each principal node of the eukaryote phylogenetic tree [Wrensch et al., 1994; Dernburg, 2001; Manicardi et al., 2002].

Even though aphids are extremely useful for the study of holocentric/holokinetic chromosomes in view of the ease mitotic chromosomes are obtained from their embryonic tissues, until now their chromosomes have not been deeply studied [Bizzaro et al., 1996, 2000; Mandrioli et al., 1999a, b; Manicardi et al., 2002; Mandrioli and Borsatti, 2007; Criniti et al., 2009]. Moreover, the relationship between their chromatin structure and epigenetics has been just lightly touched upon [Mandrioli and Borsatti, 2007] making aphids effectively uncharted biological models for epigenetic studies. At the same time, a cytogenetic analysis of aphids is very helpful since the description of species-specific chromosomal markers could make easier the identification of species that is,

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at present, quite difficult [Blackman, 1980; Rakauskas, 1998].

Aphid karyotypes are frequently described in the literature as highly variable in view of the holocentric/holokinetic nature of their chromosomes which stabilize rearrangements and fragmentations [Blackman, 1980; Blackman et al., 2000]. Surprisingly, the species of the genus *Aphis* present a very stable chromosome number, as observed in *A. gossypii*, *A. verbasci*, *A. spiraecola*, *A. affinis*, *A. clematidis*, *A. sambuci*, *A. pomi*, *A. solanella* and *A. fabae* [Khuda-Bukhsh and Pal, 1985; Manicardi et al., 1998a, b; Criniti et al., 2005; Rivi et al., 2009] making this taxon intriguing in terms of karyotype constancy.

In the present paper, a detailed cytogenetic analysis of the oleander aphid *Aphis nerii* has been done allowing us to identify and localize on chromosome 4 satellite DNAs that constitute *A. nerii* heterochromatin. Moreover, the species specificity of *A. nerii* satellite DNAs has been evaluated in *Aphis gossypii* and *Aphis spiraecola* by dot blotting, whilst the epigenetic state of *A. nerii* heterochromatin has been studied by looking for the presence of DNA methylation, H3 histone methylation at lysine 9 and heterochromatic protein 1 (HP1). Lastly, other repetitive DNAs and multigenic DNA families (including telomeric sequences and 28S and 5S ribosomal DNA genes) have been identified and localized on chromosomes.

## Material and Methods

Specimens of *A. nerii* were collected in Modena (Italy) and maintained at 22°C with 16:8 h light/darkness on *Hoya carnosa* plants. Male aphids were obtained by exposing parthenogenetic females to short photoperiods (8:16 h light/darkness) according to Crema [1979]. *A. gossypii* and *A. spiraecola* were collected on *Hibiscus* sp. plants in Modena (Italy).

Chromosome preparations were made from parthenogenetic female embryos by spreading as previously described [Mandrioli et al., 1999a]. Male chromosomes have been obtained by squash preparation of single embryos as reported by Manicardi et al. [1991].

C-banding treatment was performed according to Sumner [1972]. After treatment, slides were stained with chromomycin A<sub>3</sub> (CMA<sub>3</sub>) according to Schweizer [1976] and with 4,6-diamidino-2-phenylindole (DAPI), as described by Donlon and Magenis [1983]. Silver staining was performed according to Manicardi et al. [1998a].

In situ nick translation (NT) experiments were done on fresh chromosome spreads digested with 0.6 U/μl of the endonucleases in the supplier's buffer for 1 h at 37°C. NT was carried out for 30 min according to Sumner et al. [1990], except that extravidin-FITC was used as a label, in place of streptavidin alkaline phosphatase, to reveal the incorporation of biotin-dUTP.

In situ immune-staining experiments were performed with a mouse anti-*Drosophila melanogaster* HP1a antibody diluted

1:500 (kindly provided by Rebecca Kellum) [Huang et al., 1998], with rabbit antibodies against mono- and di-methylated-K9 histone H3 (Me9H3) (Upstate, Cambridge, UK) and with rabbit anti-tri-methylated-K9 histone H3 (Me9H3) (Active Motif, Carlsbad, Calif., USA) diluted 1:250 according to Bongiorno et al. [2001].

In situ immuno-detection of 5-methylcytosine residues was performed using a mouse anti-5-methylcytosine monoclonal antibody (Epigentek Inc., Brooklyn, N.Y., USA) following the protocol reported in Pfarr et al. [2005].

Random priming probe digoxigenin-labeling was performed according to the Roche protocol, whereas fluorescent in situ hybridization (FISH) was done according to Mandrioli et al. [1999a].

The presence of the telomeric (TTAGG)<sub>n</sub> repeat has been evaluated in *A. nerii* by in situ hybridization with a telomeric probe obtained by PCR amplification using the 2 primers F (TTAGG)<sub>5</sub> and R (CCTAA)<sub>5</sub> in the absence of template, as described by Ijdo et al. [1991].

Immuno-fluorescent preparations and FISH slides were observed using a Zeiss Axioplan epifluorescence microscope equipped with a 100 W mercury light source. Photographs of the fluorescent images were taken using a CCD camera (Spot, Digital Instrument, Madison, Wisc., USA) and using the Spot software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View, Calif., USA).

DNA extraction followed a standard phenol-chloroform protocol [Mandrioli et al., 1999a], whilst Southern blot hybridization, cloning and restriction enzyme digestion were done as described in Bizzaro et al. [2000]. SatDNA clones have been sequenced at BMR Genomics, whereas sequence alignments and search for internal repeats were done using the GCG software (GCG Computer Group, Madison, Wisc., USA). The curvature-propensity plot was calculated with DNase I parameters of the bend.it server ([http://www2.icgeb.trieste.it/~dna/bend\\_it.html](http://www2.icgeb.trieste.it/~dna/bend_it.html)) according to Gabrieli et al. [1996].

RNA extraction and reverse-transcription PCR (RT-PCR) were performed with the SV Total RNA Isolation System (Promega Corporation, Madison, Wisc., USA) and the Access RT-PCR System (Promega), respectively, according to the supplier's protocols.

The β-tubulin gene was amplified using the primers F<sub>TUB</sub> (5'ACTAGCATAGCAATTCGG) and R<sub>TUB</sub> (5'GCCTTGACCA-TTACGGACG). Amplification served us as positive control for RNA samples in RT-PCR experiments.

The 28S rDNA probe was obtained by PCR amplification of a 400 bp fragment of the 28S rDNA gene using the 2 primers, F (5'AACAAACAACCGATACGTTCCG) and R (5'CTCTGTCC-GTTTACAACCGAGC), designed according to the insect 28S rRNA sequences available in GenBank. 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). Amplification was performed using a Hybaid thermal-cycler at an annealing temperature of 60°C for 1 min with an extension time of 1 min at 72°C.

The 5S rDNA repeat unit of *A. nerii* was amplified by PCR using 2 primers, F (5'TGCACGTAGTGTCCCAAGC) and R (5'ACGACCATAACCAGTTGAATAC), derived from the insect 5S rRNA sequences available in GenBank. The 2 primers were designed so that primer cross-hybridization with other pol III-controlled genes was prevented [Geiduschek and Tocchini-Valentini, 1988]. The amplification mix contained 100 ng ge-

omic DNA, 1  $\mu\text{M}$  of each primer, 200  $\mu\text{M}$  dNTPs and 2 U of DyNAzyme II polymerase (Finnzymes Oy). Amplification was performed as above except that the annealing temperature was 59°C for 30 s and the extension time 45 s at 72°C.

## Results

According to DAPI, CMA<sub>3</sub> and silver staining (fig. 1A–C), *A. nerii* female karyotype consists of 8 chromosomes including 6 autosomes and 2 X chromosomes, easily identified as they are usually the longest chromosomes and bear the unique nucleolar organizing regions (NORs) [Manicardi et al., 2002]. To confirm X chromosome identification, the male karyotype was examined evidencing 7 autosomes and only one NOR-bearing X chromosome (fig. 1D–F).

For a detailed analysis of the *A. nerii* karyotype, we performed C-banding followed by DAPI staining showing large AT-rich heterochromatic regions at one telomere and in 5 intercalary bands of the X chromosomes (fig. 1G). No heterochromatic bands were detected in the autosomes (fig. 1G, Q).

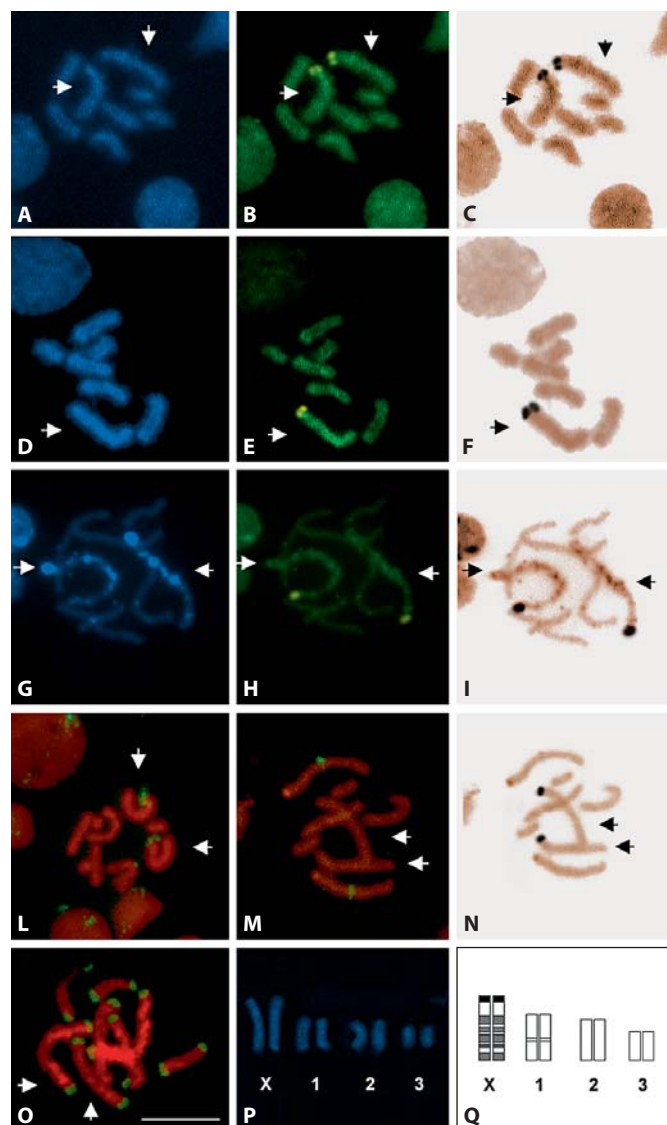
CMA<sub>3</sub>-stained mitotic metaphases showed a single GC-rich heterochromatic region located at one telomere of both X chromosomes (fig. 1H) indicating that most of the aphid heterochromatin is AT-rich. This GC-rich band was highly positive after silver staining (fig. 1I) and FISH carried out with the 28S rDNA probe (fig. 1L) assessing that it corresponds to NOR-containing actively transcribed rDNA genes.

The chromosomal localization of 5S rDNA genes revealed a single hybridization interstitial signal located in autosome pair 1 (fig. 1M), as deduced by the silver staining of the same metaphase plate (fig. 1N).

FISH experiments with the (TTAGG)<sub>n</sub> telomeric probe revealed bright signals at each telomere of all chromosomes, whilst no interstitial signals were detected (fig. 1O).

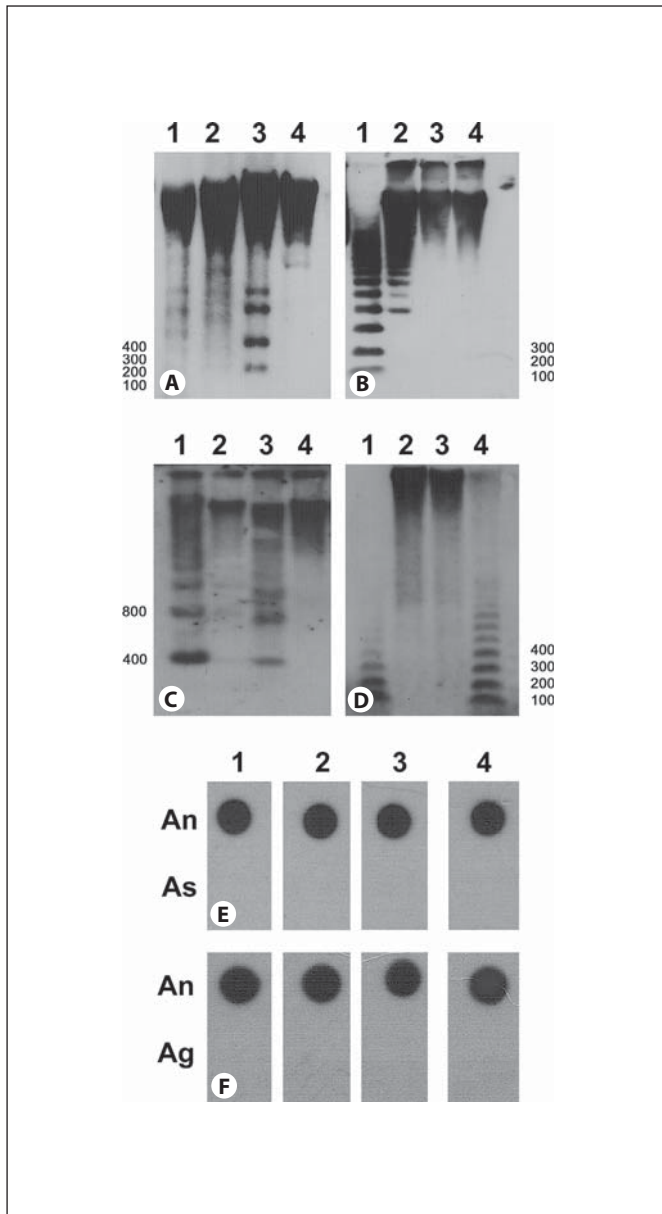
The composition of heterochromatin was studied by the digestion of *A. nerii* genomic DNA with 30 endonucleases (*MspI*, *HpaII*, *BamHI*, *ScaI*, *XmaI*, *SmaI*, *AvaI*, *MboI*, *SacI*, *SacII*, *DraI*, *BglI*, *SphI*, *FokI*, *RsaI*, *CfoI*, *KpnI*, *AluI*, *EcoRI*, *NotI*, *Sall*, *HhaI*, *XbaI*, *EcoRV*, *ApaI*, *NdeI*, *HaeIII*, *HinfI*, *PstI* and *Scal*) searching for satellite DNAs that are generally common components of constitutive heterochromatin. Four restriction enzymes (*MboI*, *RsaI*, *EcoRI* and *AluI*) showed electrophoretic band ladders that are a typical feature of clustered satellite DNAs.

Monomeric fragments of the *MboI*, *RsaI*, *EcoRI* and *AluI* ladders have been used as hybridization probes in



**Fig. 1.** Mitotic chromosomes of *Aphis nerii*: DAPI (blue) (A, D), CMA<sub>3</sub> (yellow-green) (B, E) and silver staining (C, F, P) of *A. nerii* female (A–C, P) and male (D–F) unbanded chromosomes. DAPI (G), CMA<sub>3</sub> (H) and silver staining (I) of female chromosomes after C-banding. FISH with fluorescein-labeled (green) 28S rDNA (L), 5S rDNA (M) and telomeric probes (N) on *A. nerii* female chromosomes stained with propidium iodide (red). DAPI-stained karyotype (O) and karyogram (Q) showing the distribution of GC-rich heterochromatin/NORs (black band), AT-rich heterochromatin (dark gray) and 5S cluster (light gray) of *A. nerii*. Arrows indicate X chromosomes. Bar corresponds to 10  $\mu\text{m}$ .

Southern blotting experiments showing a regular ladder of multimers of basic length, which is typical of clustered satellite DNA confirming that these restriction enzymes isolated highly repeated and clustered DNA sequences (fig. 2A–D).



**Fig. 2.** Southern blotting with the AluSAT (A), RsaSAT (B), EcoSAT (C) and MboSAT (D) probes showed that these sequences are highly repeated and clustered in *A. nerii* genome. A Digestion of *A. nerii* genomic DNA with *KpnI* (1), *HindIII* (2), *AluI* (3) and *ScaI* (4) and hybridized with the AluSAT probe. B Digestion of *A. nerii* genomic DNA with *RsaI* (1), *HindIII* (2), *AluI* (3) and *ScaI* (4) and hybridized with the RsaSAT probe. C Digestion of *A. nerii* genomic DNA with *EcoRI* (1), *ScaI* (2), *NotI* (3) and *SalI* (4) and hybridized with the EcoSAT probe. D Digestion of *A. nerii* genomic DNA with *Sau3A* (1), *SalI* (2), *ScaI* (3) and *MboI* (4) and hybridized with the MboSAT probe. E, F Dot blot experiments have been performed in order to verify the species specificity of *A. nerii* (An) satellites showing that AluSAT (1), RsaSAT (2), EcoSAT (3) and RsaSAT (4) are not present in *A. spiraecola* (As, E) nor in *A. gossypii* (Ag, F) genomes. Molecular weight is indicated near each blot in panels (A–D).

Both in situ nick translation, performed after digestion of chromosomes with the endonucleases (fig. 3A, D, G, L), and in situ hybridization using the satellite DNAs as probes (fig. 3C, F, I, N) showed that the 4 highly repeated sequences co-localized with heterochromatic areas located in the X chromosomes, whilst no signals were detected on autosomes. In particular, MboSAT showed a bright labelling of the heterochromatic AT-rich telomere and in some of the interstitial heterochromatic bands of the X chromosomes (fig. 3A–C). EcoSAT was mainly located in a single intercalary heterochromatic band (fig. 3D–F), whereas both AluSAT (fig. 3G–I) and RsaSAT (fig. 3L–N) were present in all the heterochromatic bands of the *A. nerii* X chromosomes.

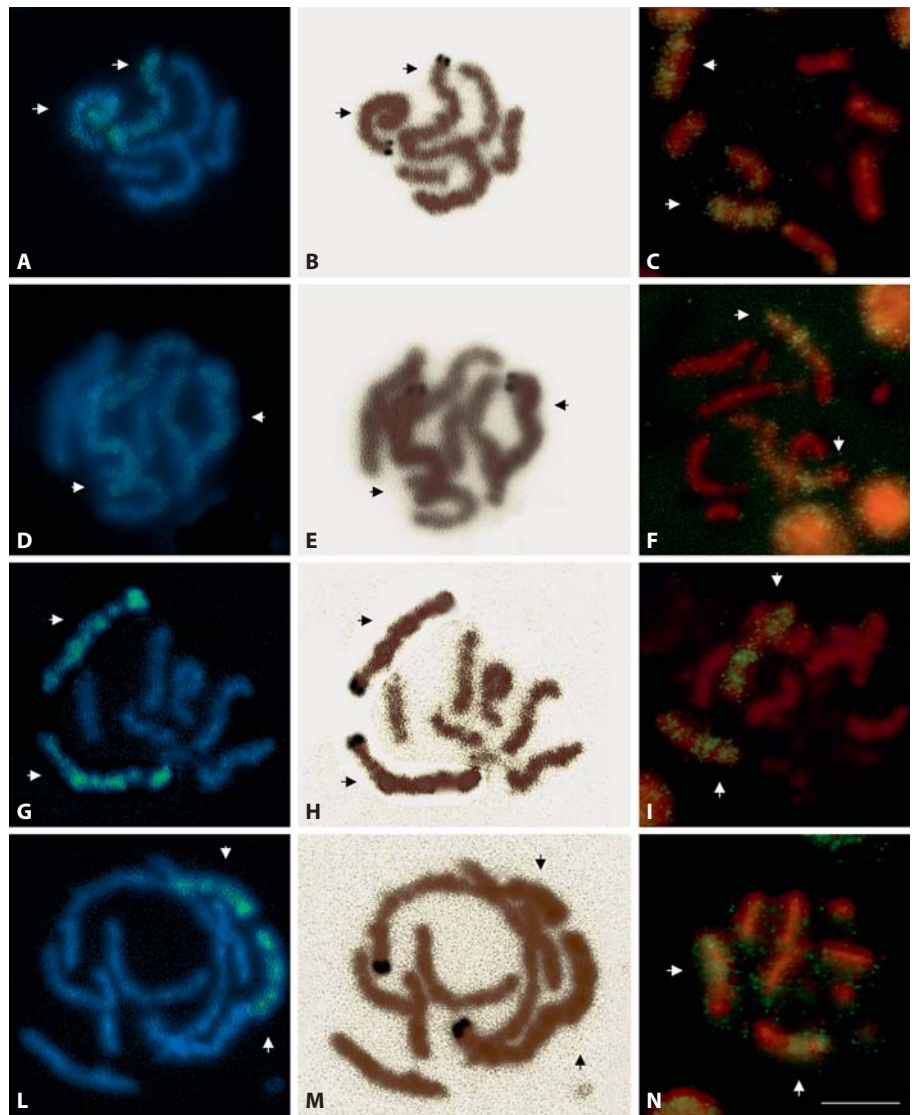
Dot blotting in 2 other aphids (*A. gossypii* and *A. spiraecola*) indicated that the 4 *A. nerii* satellite DNAs were not present in these 2 co-generic species (fig. 2E–F).

*A. nerii* satDNAs have been cloned (fig. 4) and 5 clones sequenced for each satellite DNA (table 1). Bioinformatic analyses showed that all the satDNAs presented conserved sequences whose similarities ranged from 93 to 100% (table 1). The 4 satellite DNAs presented a high AT content (from 60.1 to 66.4%) with the only differences due to nucleotide mutations, whereas no sequence rearrangements were detected. A search for homology with other DNA sequences in GenBank and EMBL databases yielded no significant results with all 4 satDNA consensus sequences. Similarly, no significant direct or inverted repeats were found.

The curvature-propensity plot, calculated with the DNase I parameters of the bend.it server, clearly showed that the 4 satDNAs presented at least one region with a high curvature propensity value, whose magnitude roughly corresponds to the value calculated for a highly curved motif described in a DNA satellite of the pigeon *Columba risoria* (GenBank ID: CRBENSAT).

A transcription assay, carried out by RT-PCR together with the  $\beta$ -tubulin gene (utilized in order to confirm both the presence and integrity of the mRNAs), did not give any product suggesting that the 4 satellite DNAs were not transcribed in *A. nerii* (data not shown).

In order to further characterize the *A. nerii* heterochromatin, we also verified the presence of HP1 proteins, methylated H3 histones and methylcytosine residues. In situ immuno-detection clearly indicated that HP1 proteins were not scattered along all *A. nerii* chromosomes, but mainly located on both X chromosomes which showed several bands that overlap with heterochromatic regions (fig. 5A). The same heterochromatic areas were also labeled with antibodies against mono-, di- and tri-



**Fig. 3.** Localization of the 4 *A. nerii* satellite DNAs evaluated by in situ nick translation carried out after digestion of chromosomes with the endonucleases *MboI* (A), *EcoRI* (D), *AluI* (G) and *RsaI* (L); fluorescence in situ hybridization using MboSAT (C), EcoSAT (F), AluSAT (I) and RsaSAT (N) as fluorescein-labeled probes (green) on *A. nerii* female chromosomes stained with propidium iodide (red). The identification of X chromosomes has been performed using silver staining (B, E, H, M). Arrows indicate X chromosomes. Bar corresponds to 10  $\mu$ m.

**Table 1.** Sequence analysis of the four satDNAs of *A. nerii*

SatDNA	Length	Sequence similarity	AT content	Curvature propensity	Curved region	Similarity in databases	Sequence ID in GenBank
EcoSAT	406 bp	96–100%	60.1%	yes	100–150	none	HM467647
AluSAT	198 bp	95–99%	63.6%	yes	50–100	none	HM467648
RsaSAT	164 bp	95–100%	66.4%	yes	30–50/55–75/ 85–105/108–138	none	HM467649
MboSAT	107 bp	93–99%	64.4%	yes	65–105	none	HM467650

The analysis of the four satDNAs of *A. nerii* allowed to determine the length of the consensus sequence, the variability in sequence for each satDNA, the AT content, the curvature propensity, the localization of the curved satDNA portion in each sequence, the presence of similar sequence in GenBank and EMBL and the ID of the satDNA consensus sequences.

#### EcoSAT

```
GGAAAAACGTTGTTGCGCCTTCCTAGTCTGGAGCCTCAGTAGTTCTCAA  
CAATCAGACTGATTGGAATATAAATATTGACAAAGGGAATGGGTAGAT  
TATATAACAAAAATAGTGAAGAATGTAGCTTAATAGTCTAGGGAGAGC  
AGGATAATCTTCGTTTTTGTTCATTAACGGCAATTGTATCCTGTTTCGT  
CAATCAGTGTACATGATGTCCATTGCTCGAGATATGCATTAGTTCGGC  
ACTTCTAATCTTAAACCATTCTTCACTCTTCCAGGAATATTCTTCGCA  
TTCCTCCTTGTGAAATATTCCATTACCCGTGACGTTGATGAAAGAC  
GTGAAAAACCAAGACCTCAATTGGCAACTGTGGAAGTTCTCCTTTTGA  
AGCTGCACATCAGGTCTGCTTA
```

#### AluSAT

```
CAAGATAAGACATATTAAGTGTGGCATAACGTTTTGTGAAGCTCTCAC  
ATCCACAAACATAATGCAAAAGTCATGAAAATTCTCTTGGCATCTGG  
TATAGATTTGCCAATGATGCTACTAAAATGTATTCTGAGGAATACAT  
GGCGTTGAGTATTCGGCTCATGATATTTAAGTCTGTGGATGCTTTTTA  
CTGTCA
```

#### RsaSAT

```
TTGCTTTCATCGATCTCTATAAATCATTTTATGAAGGAATGTACCCTA  
AGTTTGGAGAACACGATATCGAAAACCCGAAGATATCAATTAATATCA  
ATCATGCAACAGACCTTGTAAGAGCTAAGTTTGCATCGGTGCACCTA  
TCAAGAAGATAAATATTATA
```

#### MboSAT

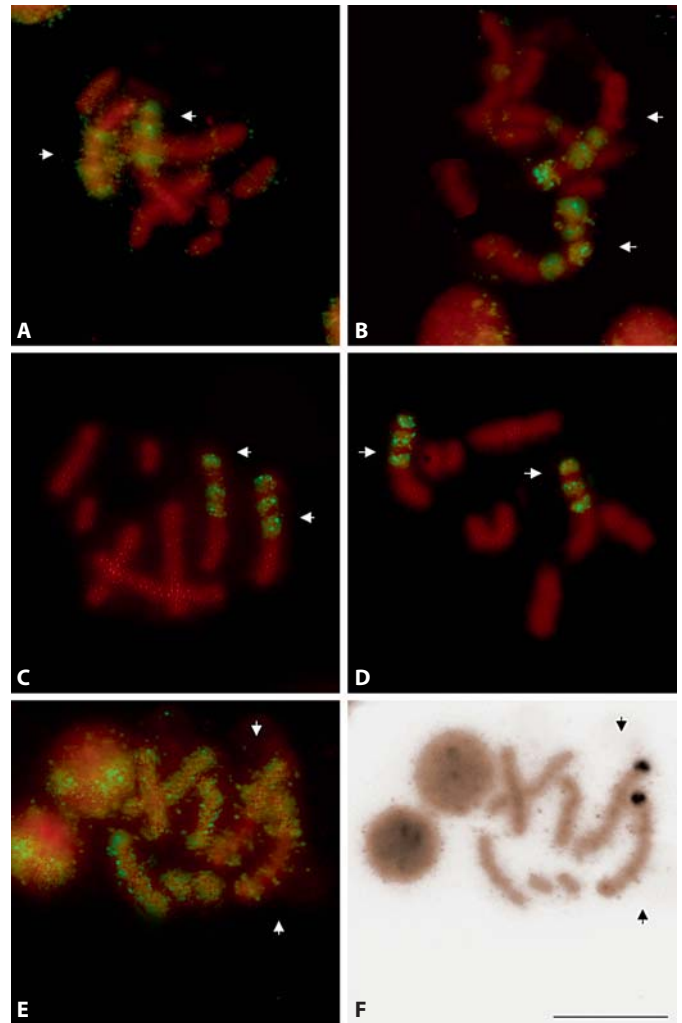
```
AGCGGGAGTTAAAAAGACGCTAACTTCATAGTAAATCTCTAGAAGAG  
ATACTGTACGTATAAAAATTGCTTCCAGTTATCTCAAAAACGTTTCCT  
ATCTCTGCACA
```

**Fig. 4.** Consensus sequences of the *A. nerii* satellite DNAs: EcoSAT, AluSAT, RsaSAT and MboSAT.

methylated-K9 histone H3 (fig. 5B–D). On the contrary, heterochromatin was not labeled after immuno-detection of methylated cytosines using mouse anti-5-methylcytosine antibodies (fig. 5E). In particular, the X chromosomes presented bright labeling limited to a large area close to the NOR-bearing telomere (as deduced by silver staining the same plate) (fig. 5F). According to previous analyses with C-banding, these portions of the X chromosomes are euchromatic. A widespread occurrence of methylcytosine residues has been also observed in autosomes (fig. 5E).

## Discussion

Even if aphid chromosomes are highly variable in number in view of their holocentric nature [Blackman, 1980; Blackman et al., 2000], our analysis of *A. nerii* metaphases evidenced the presence of 8 chromosomes which is in accordance with a previous estimation published by Khuda-Bukhsh and Pal [1985] confirming that aphids of the *Aphis* genus present a constant chromosome



**Fig. 5.** *A. nerii* chromosomes (stained in red with propidium iodide) analyzed by immuno-staining with fluorescein-labeled antibodies against HP1 (A), monomethylated-K9 H3 histones (B), dimethylated-K9 H3 histones (C), trimethylated-K9 H3 histones (D) and methylcytosine residues (E). In order to verify if the large band labeled after immuno-labeling with the anti-methylcytosine antibodies was near the NOR-bearing telomere, silver staining of the same plate has been done (F). Arrows indicate sex chromosomes. Bar corresponds to 10  $\mu$ m.

number [Khuda-Bukhsh and Pal, 1985; Manicardi et al., 1998a, b; Criniti et al., 2005; Rivi et al., 2009] with only one exception, *A. farinose* [Blackman, 1980]. *A. nerii* also presents a stable chromosome number among different geographic populations, as demonstrated by the comparison of our data with that reported for *A. nerii* strains collected more than 20 years ago in India [Khuda-Bukhsh and Pal, 1985].

The production of males in *A. nerii* is noteworthy since this species has frequently been reported as being obligate parthenogenetic in Europe [e.g. Groeters and Dingle, 1989; Blackman and Eastop, 2006]. However, the occurrence of sexual generations of *A. nerii* has already been reported in the literature [Takada and Miyazaki, 1992] suggesting that this species could be a mosaic of holocyclic and obligate parthenogenetic populations, as reported in other aphid species [Blackman and Eastop, 2007].

C-banding and FISH utilizing 5SrDNA as a probe allow clear-cut identification of X and chromosomes 1 within the *A. nerii* complement thus improving the construction of a non-ambiguous karyotype. Indeed, holocentric chromosomes lack primary constriction, whereas attempts to identify homologue chromosomes only on the basis of their length can be misleading due to the phenomenon of allocydia (see fig. 1N and in particular fig. 1M in which the autosome pair 1 appears longer than the X chromosome),

Aphid X chromosomes present strong structural constraints since all the aphid species analysed to date have both a large load of heterochromatin at the telomeric and interstitial level and a single rDNA cluster located at one telomere (with the unique exceptions of *Schoutedenia rulumensis* and *Maculachnus submacula* that present autosomal NORs and *Amphorophora idaei* showing interstitial NORs at X chromosomes) [Mandrioli et al., 1999a, b; Manicardi et al., 2002]. This peculiar feature of X chromosomes could be related to the sex determination mechanism, which, in aphids, is generally based on the presence of females with 2 X chromosomes (XX) and males with only one X chromosome (X0). Aphid males are produced by parthenogenetic females as a consequence of an X chromosome loss occurring in the course of a single maturation division [Blackman and Spence, 1996]. Indeed, the large blocks of heterochromatin may be involved in the delayed separation of X chromosomes during maturation of parthenogenetic aphid oocytes [Orlando, 1974; Blackman, 1987].

Furthermore, all the parthenogenetic eggs during prophase present a connection between the 2 NOR-bearing telomeres of the X chromosomes [Schrader, 1940; Orlando, 1974; Hales and Mitler, 1983; Blackman and Hales, 1986] that is quickly lost in females, whereas in male generating eggs the X chromosomes remain attached by sticky NORs and undergo a sort of non-canonical reductional division [Blackman and Hales, 1986]. At the end of this peculiar division the egg has one X chromosome only and is thereby determined as male. Aphid rDNA genes

contain specific sequences that present high similarity with the consensus core region of human hypervariable minisatellites [Jeffreys et al., 1985] and with the  $\chi$  sequence of *Escherichia coli* [Smith, 1983] that are involved in rDNA pairing, as also previously reported in *D. melanogaster* [Ault et al., 1982; Park and Yamamoto, 1995]. The occurrence of X chromosome pairing at NORs is supported by the frequent observation (also in *A. nerii*, data not shown) of intra- and inter-individual NOR heteromorphism, already reported in several aphid species [Mandrioli et al., 1999b, c] as the result of unequal crossing over between the 2 X chromosomes.

NOR number and position have been reported as highly variable in insects and rDNA genes have been frequently found also (or mostly) on autosomes, including several species with multiple NORs [e.g. Postiglioni and Brum-Zorrilla, 1981, 1988; Juan et al., 1993; Mandrioli, 2002; Rocha et al., 2002; Loreto et al., 2008; Nguyen et al., 2010] making aphids an interesting exception among insects.

FISH experiments with 5S rDNA probe revealed a single cluster located on autosome pair 1 indicating that 5S localization is not linked to the other rDNA genes. This is not surprising considering that the absence of linkage between the 5S and 28S rDNA genes has already been reported in other insects [Drouin et al., 1992]. Up till now, 5S rDNA localization in aphids has only been evaluated in *A. pisum* [Bizzaro et al., 2000], where the 5S genes are located in 2 interstitial clusters on X chromosomes indicating that, contrarily to what is observed for the 28S rDNA genes, 5S gene localization in the aphid chromosomes is not under constraints.

The telomere of each chromosome consisted of the telomeric (TTAGG)<sub>n</sub> repeat, as already reported not only in aphids, but also in other insects [Lorite et al., 2002; Robertson and Gordon, 2006; Frydrychová et al., 2004; Vítková et al., 2005]. In accordance with the stability of the chromosome number observed in *A. nerii*, no interstitial (TTAGG)<sub>n</sub> sequence was detected. However, more sensitive FISH procedures would have to be used in order to verify if no chromosome fusion occurred in *A. nerii*, or alternatively, if fusions occurred in the past so that interstitial telomeric sequences have been progressively eroded.

Several satellite DNAs have been isolated in organisms possessing monocentric chromosomes, whereas only a few studies were published on highly repeated DNAs in invertebrates with holocentric chromosomes with the exception of nematodes [Grenier et al., 1997]. To date, satellite DNAs have been isolated in only 2 aphid species, *My-*

*zus persicae* [Spence et al., 1998; Mandrioli et al., 1999a] and *Megoura viciae* [Bizzaro et al., 1996], making difficult the identification of common properties of aphid satellite DNAs.

The sequences of the *A. nerii* satDNAs were highly conserved and this could be due to processes of 'concerted evolution' determined by molecular drive [Dover et al., 1982; Dover and Tautz, 1986] or to the presence of constraints due to the interaction of satDNAs with specific proteins involved in heterochromatin formation [Talbert et al., 2004; Palomeque and Lorite, 2008]. Sequence variability of satDNAs depends on the ratio between mutation and homogenization/fixation rates [Dover, 2002] and in insects falls in the range 1–13% even if very similar satDNAs are more frequent than variable ones [Lopez-Leon et al., 1995; Mandrioli et al., 1999a; Landais et al., 2000].

The 4 sequenced satDNAs possess a conserved length and this feature was related to a possible role of the satellite DNAs in the nucleosome phasing necessary during the process of heterochromatin condensation [Henikoff et al., 2001]. At the same time, however, the absence of internal repeats in all the *A. nerii* satDNAs could also explain this constancy since internal direct and inverted repeats are correlated to satellite DNA rearrangements [Palomeque and Lorite, 2008].

The presence of a high AT content is a general feature of satellite DNAs identified in *A. nerii* and it is consistent with the AT richness reported in satDNA of several other animals [Palomeque and Lorite, 2008]. Besides this, the high AT content of the *A. nerii* satDNAs fits our cytological data which showed their localization into DAPI fluorescent C bands after FISH. Indeed, in situ hybridization showed that they co-localize with heterochromatic bands of the X chromosomes, whereas no hybridization signals have been observed on autosomes. This distribution is similar to that observed in *M. persicae* and *M. viciae*, where the satellite DNAs labeled only intercalary heterochromatic bands of the 2 X chromosomes [Bizzaro et al., 1996; Mandrioli et al., 1999a].

The transcription of insect satDNAs has been studied in a few species and just some of them present RNAs related to these repetitive DNA. RNAs related to the *A. nerii* satDNAs have been searched by RT-PCR without positive results. However, in insects satDNA transcription is related to cell type, developmental stage and sex so that it may not be so easy to detect the transcripts [Palomeque and Lorite, 2008]. In addition, at least in *D. melanogaster* [Palomeque and Lorite, 2008], satellite DNA-related transcripts consist of small RNAs that cannot be detected by

RT-PCR so that an exhaustive assessment of satellite transcription in aphids is worthy of further investigations.

As a whole, *A. nerii* satDNAs present several features that are typical of the other satellite DNAs isolated in the aphids since (i) they present a high degree of similarity among the sequenced repeats both in length and sequence (the percentage of similarity is greater than 90%), (ii) they show a high AT content (at least 60%), (iii) they contain an internal region with a high curvature propensity and localize in AT-rich heterochromatic regions of the X chromosomes.

These data suggest that, in holocentric chromosomes also, constitutive heterochromatin is principally made of satellite DNAs in agreement with data repeatedly described in monocentric chromosomes [John and Miklos, 1979; John, 1988].

According to previous studies in different animals, including the holocentric chromosomes of the coccid *Planococcus citri* [Cowell et al., 2002; Bongiorno and Pranter, 2003; Kourmouli et al., 2004], *A. nerii* constitutive heterochromatin is enriched in both bi- and trimethylated H3 histones and HP1 proteins. On the contrary, data about the localization of H3K9me1 are in contrast with the high-resolution profiling of histone methylations in the human genome which revealed that the higher H3K9me1 levels were mainly detected near the 5' end of actively transcribed genes [Barski et al., 2007]. These results, previously observed in the aphid *Acyrtosiphon pisum* [Mandrioli and Borsatti, 2007], strongly resemble those reported for DNA methylation that is present both in silent and actively transcribed genes, and this double function is due to the different effector proteins that interact with DNA methylation at the promoter (where DNA methylation suppresses transcription) [Mandrioli, 2004] and inside the coding regions (where DNA methylation suppresses the transcriptional background of active genes) [Mandrioli, 2004]. Similarly, H3K9me1 could be involved both in silencing and activation of transcription through a different location and the recruitment of different proteins. Our cytogenetic data do not allow us to give a proper reply to this question since immunolabelling did not furnish information at a molecular level but at a chromosomal level only. Further studies could be therefore prompted by our results.

Our experiments showed that *A. nerii* heterochromatin lacks DNA methylation since methylated cytosine residues are widespread in euchromatic chromosomal regions. This is very interesting since the results reported



in *A. pisum* and in the coccid *Planococcus citri* [Bongiorno et al., 1999] indicate that insect heterochromatin is poor in terms of DNA methylation, whereas euchromatin can be highly methylated. Hence, taken together with data in other insects and in the nematode *Caenorhabditis elegans* [Mandrioli, 2004; Mandrioli and Borsatti, 2006, 2007], it emerges that in invertebrates, contrarily to what is reported in plants and mammals [Mandrioli and Borsatti, 2005], heterochromatin is assembled and condensed without any involvement of DNA methylation.

This suggestion, even if apparently surprising in comparison to the canonical view of DNA methylation, is not unexpected in insects where it has been previously suggested that methylated genes can be actively transcribed [Field et al., 2004; Mandrioli, 2004; Walsh et al., 2010]. In particular, it has been suggested that DNA methylation has been recruited in insects not for silencing gene transcription, but to avoid transcription initiation from spurious promoters [Field et al., 2004; Mandrioli, 2004]. On the basis of this presumed function, DNA methylation in insects should be present in euchromatin rather than in

heterochromatin, a hypothesis confirmed in the case of *A. nerii*.

In view of such a scenario, it could be very intriguing to study the expression of genes coding for DNA methyltransferase and histone methyltransferase in the aphid genome in order to better understand the roles played by these epigenetic processes and their interactions. The availability of the whole sequenced *A. pisum* genome (which has been recently annotated) will surely provide important replies to these questions, thereby making aphids good models for studying the evolution of histone and DNA methylation in insects.

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