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ORIGINAL RESEARCH

Distribution and molecular composition of heterochromatin in the holocentric chromosomes of the aphid *Rhopalosiphum padi* (Hemiptera: Aphididae)

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Abstract In order to study the structure of holocentric chromosomes in aphids, the localization and the composition of *Rhopalosiphum padi* heterochromatin and rDNA genes have been evaluated at cytogenetic and molecular level. In particular, heterochromatin resulted located on all the chromosomes both in intercalary and telomeric positions. Moreover, enzymatic digestion of *R. padi* genome put in evidence a *Dra*I satellite DNA which has been isolated, cloned and sequenced. FISH experiments showed that this satellite DNA clusters in an intercalary C-positive band on the two X chromosomes.

Keywords Aphid · Holocentric chromosomes · Heterochromatin · Satellite DNA

Introduction

Satellite DNAs (satDNAs) constitute a considerable portion of the eukaryotic genome and represent the major components of heterochromatin (John and Miklos 1979; John 1988; Charlesworth et al. 1994; Cesari et al. 2003; Palomeque and Lorite 2008). They generally form long tandem arrays in which monomer sequences are repeated in a head-to-tail fashion. In view of this organization, the digestion of the satDNA arrays with the appropriate restriction enzyme gives rise to a characteristic ladder of bands (multimers of the basic satellite DNA repeat unit) after agarose gel electrophoresis (e.g. Spence et al. 1998; Mandrioli et al. 1999a).

SatDNAs have been described in different eukaryotic species and they present some common properties (for a review see Palomeque and Lorite 2008). One of the most widespread features of satDNAs is their bent structure that is related to a sequence-dependent property of the DNA molecule (Gabrielian et al. 1996). The role of satDNA curvature is not well established, but it seems to be related to the chromatin organization and to the tight winding of DNA in heterochromatin as well as to the binding of specific proteins to heterochromatic regions (Lobov et al. 2001). The degree of curvature has been related to the richness in adenine (A) and thymine (T) of the satellite DNAs as well as to the presence of clustered AT dinucleotides periodically spaced in the satDNA sequence (Plohl et al. 1990; Ugarkovic et al. 1992).

Species-specific satDNAs have been reported in several insects, such as in *Gryllus bimaculatus* (Yoshimura et al. 2006), but satellite sequences shared among different species were also identified (e.g. Bachmann and Sperlich 1993; Spence et al. 1998). At a cytogenetic level, satDNAs may be dispersed on all chromosomes (e.g. Lorite et al. 2001; Spence et al. 1998) or they can be specific to some chromosomes, as reported in the aphid *Myzus persicae* for the *Hind*III satellite DNA, whose presence is limited to the X chromosomes (Mandrioli et al. 1999a).

Satellite DNAs may be transcribed and these transcripts could be involved in the RNA interference processes and in the heterochromatin formation (as reviewed in Bernstein and Allis 2005). Interestingly, small-interfering RNAs cognate to several types of repetitive DNAs have been isolated in *Drosophila melanogaster* suggesting that they

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are involved in the process of chromatin modification (Aravin et al. 2003; Usakin et al. 2007).

At present, the few studies carried out on satellite DNA sequences in organisms possessing holocentric chromosomes were mostly focused on nematodes (Lagowsky et al. 1973; Roth 1979; Collet and Westerman 1987; Naclerio et al. 1992; Grenier et al. 1996, 1997; Castagnone-Sereno et al. 1998a, b). In order to improve our knowledge about the structure of holocentric chromosomes, in the present paper we investigated the localization and the DNA composition of the heterochromatic bands in the holocentric chromosomes of the aphid *Rhopalosiphum padi* and isolated a satellite DNA that has been cloned, sequenced and localized by fluorescent in situ hybridization.

Materials and methods

Rhopalosiphum padi represents one of the major pest of cereal crops on a world scale (Wiktelius et al. 1990; Halbert and Voegtlin 1995). Indeed, *R. padi* may damage wheat plants via direct feeding and by transmitting viruses, which causes an economically important disease of small grains throughout the world (Irwin and Thresh 1990). Holocyclic *R. padi* specimens were collected in Modena and maintained at 20°C with 16 h of light and 8 h of darkness on *Zea mais* plants.

Chromosome preparations of parthenogenetic females were made by spreading embryo cells, as previously described (Mandrioli et al. 1999a).

C-banding treatment was performed according to Sumner's technique (1972). After the treatments, slides were stained with chromomycin A$_3$ (CMA$_3$) according to Schweizer (1976) and with 4′,6-diamidino-2-phenylindole (DAPI), as described by Donlon and Magenis (1983). Silver staining has been performed according to Mandrioli et al. (1999b).

DNA extraction was performed using a standard protocol given in Mandrioli et al. (1999c). Southern blotting hybridization, dot blotting and restriction enzyme digestion were carried out as described in Mandrioli et al. (1999a). Densitometric analysis of the dot blots has been performed with the freely available software Image J (http://rsb.info.nih.gov/ij/).

Random priming probe digoxigenin-labeling was performed according to Roche protocol, whereas fluorescent in situ hybridization (FISH) was carried out as described by Mandrioli et al. (1999a).

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, USA) and using the Spot software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Sequence alignments and search for open reading frame and internal repeats were performed using the GCG software (GCG Computer Group, Madison, 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) according to Gabrielian et al. (1996).

RNA extraction and RT–PCR were performed with the SV Total RNA Isolation System (Promega Corporation, Madison, USA) and with the Access RT–PCR System (Promega), respectively, according to the supplier's suggestions. The 5S rDNA repeat unit was amplified by PCR using two primers, F (5′-TGCACGTAGTGTTCCCAAGC) and R (5′-ACGACCATACCACGTTGAATAC), derived from the insect 5S 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 as above except that the annealing temperature was 59°C for 30 s and the extension time 45 s at 72°C.

β-Tubulin gene was amplified using the primers F$_{TUB}$ (5′-ACTAGCATAGCAATTCGG) and R$_{TUB}$ (5′-GCCTTGACCATTACGGACG). This amplification served us as positive control for RNA samples in RT–PCR experiments. Amplification was performed making the primer annealing at 57°C for 30 s and the extension at 72°C for 45 s.

The 28S rDNA probe was obtained by PCR amplification of a 400 bp long fragment of the 28S rDNA gene using the two primers, F (5′-AACAAACAACCGATACGTTCCG) and R (5′-CTCTGTCCGTTTAC AACCGAGC), 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 at an annealing temperature of 60°C for 1 min with an extension time of 1 min at 72°C.

Results

DAPI, CMA$_3$ and silver staining of *R. padi* chromosomes indicated that female karyotype consists of 8 chromosomes including six autosomes and two X chromosomes, in accordance with previous literature data (De Battista 1992; Lazzari and Voegtlin 1993). X chromosomes have been easily identified as, on the basis of previous studies on aphids, they are the longest and the unique nucleolar organizing region (NOR)-bearing chromosomes (Fig. 1a–c). C banding, followed by DAPI staining, showed several AT-rich heterochromatic regions located both on the X chromosomes and autosomes (Fig. 1d, f). Interestingly, all

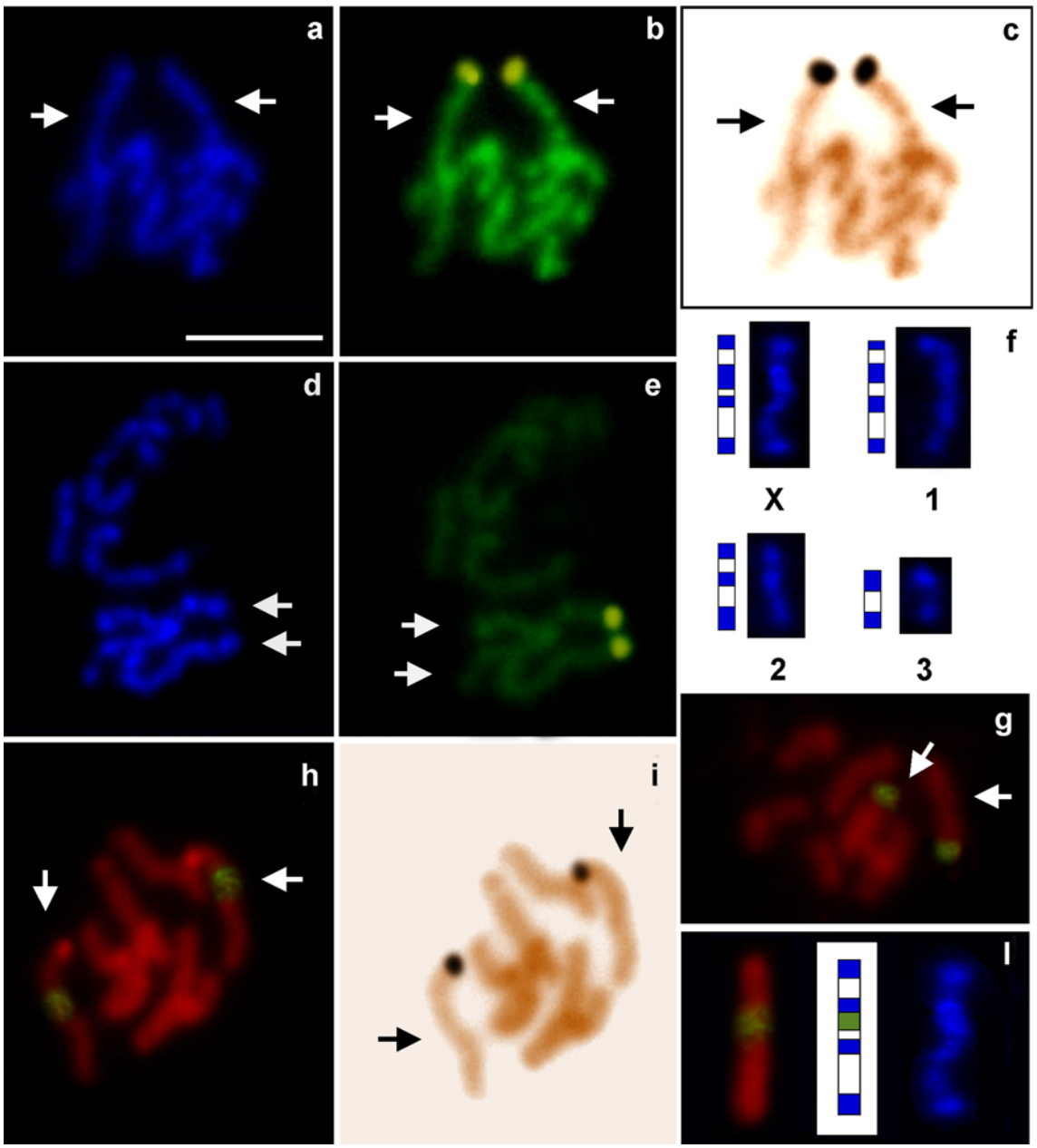

Fig. 1 DAPI (a, d, f), CMA3 (b, e) and silver staining (c, i) of R. padi female unbanded (a–c) and C-banded (d–f) chromosomes. FISH with 28S rDNA (g) and *Dra*I satDNA (h) probes on R. padi chromosomes showed that both sequences localize on X chromosomes, as assessed by silver staining (i). The ideogram (l) summarises C-banding pattern, whereas the X karyogram (h) allows to map *Dra*I satDNA (on the *left*) and C band (on the *right*) showing the *Dra*I is localized on a AT-rich intercalary heterochromatic band. *Arrows* indicate X chromosomes. Bar corresponds to 10 μm

the chromosomes possessed heterochromatic telomeres (Fig. 1d, f).

CMA_3 staining of C banded chromosomes put in evidence a unique GC-rich heterochromatic region located at one telomere of the two X chromosomes (Fig. 1e). These X telomeres were highly positive also after in situ hybridization with a 28S rDNA probe (Fig. 1g) indicating that they contain rDNA genes, as evidenced also with silver staining (Fig. 1c).

In view of the occurrence of large and numerous heterochromatic blocks on the whole chromosome complement, *R. padi* genomic DNA has been digested with endonucleases (*Eco*RI, *Alu*I, *Kpn*I, *Not*I, *Mbo*I, *Hind*III, *Cfo*I, *Bam*HI, *Sau*3AI, *Sal*I, *Fok*I, *Dra*I, *Sca*I, *Cla*I, *Dde*I, *Sac*I, *Sac*II, *Apa*I, *Not*I, *Sph*I, *Bgl*II, *Sss*I and *Xba*I) searching for satellite DNAs. Gel electrophoresis after *Dra*I digestion showed a faint band ladder (Fig. 2), typical feature of clustered satellite DNAs, prompting us to use the

monomeric fragments of the *Dra*I ladder as hybridization probe in Southern blotting experiments and FISH. Southern blotting evidenced a regular ladder of multimers of basic length, which is typical of clustered satellite DNA confirming that *Dra*I isolated highly repeated and clustered DNA sequences (Fig. 2).

Dot blot experiments (Fig. 2), combined with densitometric analyses, allowed us to estimate in more than 400 copies the number of the *Dra*I satellite DNA in the *R. padi* genome by comparison with the 5S rDNA genes whose amount has been estimated in 100–120 copies per haploid genome in insects (Paques et al. 1995).

In situ hybridization performed on *R. padi* chromosomes using the *Dra*I satellite DNA as a probe (Fig. 1h, l) showed that this highly repeated sequence is localized on a intercalary heterochromatic band on the X chromosomes, which can be easily identified by silver staining (Fig. 1g). No signals were detected on other X heterochromatic regions neither on autosomes.

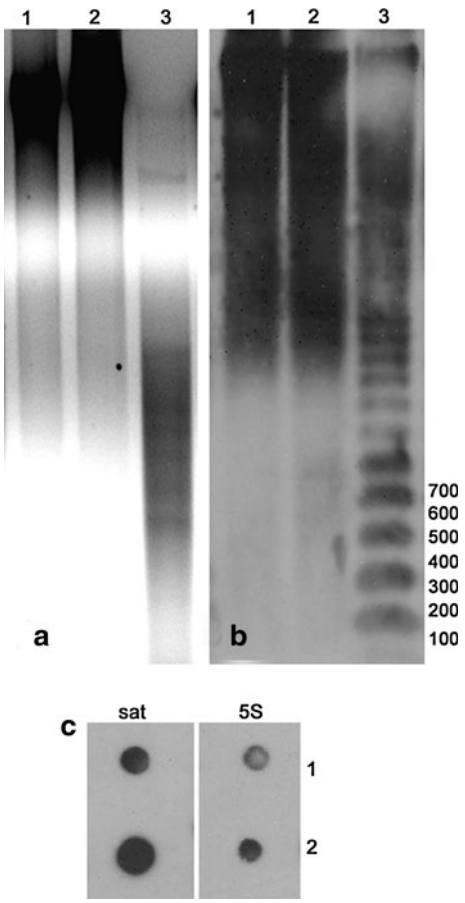

Fig. 2 Digestion of *R. padi* genomic DNA with *Msp*I (*lane 1*), *Hpa*II (*lane2*) and *Dra*I (*lane 3*) (a) and successive Southern blot hybridization with the *Dra*I satDNA probe (b) revealed that *Dra*I isolate a highly repeated and clustered satellite DNA. The molecular weight of the bands was deduced using a 100 bp ladder marker. Dot blot experiments (c) performed in order to verify if *Dra*I satDNA was repeated in the *R. padi* genome showed that this satDNA (sat) is more repeated than the multigenic 5S rRNA gene family (5S). DNA samples of 1 μg (*1*) and 4 μg (*2*) have been, respectively, spotted on the membrane

*Dra*I satellite DNA has been cloned and twenty clones were sequenced and aligned. They showed a sequence similarity ranging from 89.1 to 100%, the only differences being due to nucleotide mutations, whereas no sequence rearrangements have been detected (Fig. 3). The length of the consensus sequence is 177 bp with an AT content of 67.42%. A search for homology with other DNA sequences within GenBank and EMBL databases yielded no significant results. Similarly, no significant direct or inverted repeats have been found.

The curvature-propensity plot, calculated with DNase I parameters of the bend.it server, presented a region between nucleotide 60 and 100 with a curvature propensity value whose magnitude roughly corresponds to the value calculated for a highly curved motif described in a DNA satellite of *Columba risoria* (GenBank ID: CRBENSAT) (Fig. 4).

Transcription of the *Dra*I satDNA carried out by RT–PCR together with β-tubulin gene (utilized in order to confirm both the presence and the integrity of the mRNAs) did not evidenced any result, thus demonstrating that *Dra*I satDNA is not transcribed in *R. padi* (Fig. 5).

Discussion

In the last two decades the distribution of heterochromatin has been evaluated in several aphid species showing a tendency to accumulate heterochromatin on the X chromosomes (Manicardi et al. 1991, 1996, 1998). Heterochromatin amount on autosomes may vary significantly since it could be absent (as reported in *Aphis pomi* by Criniti et al. 2005), located in different intercalary positions (as in *Myzus varians*) (Bizzaro et al. 1999) or restricted to telomeres (as in *M. persicae*, Mandrioli et al. 1999a). Interestingly, *R. padi* showed large blocks of heterochromatin on all the chromosome complement, including large heterochromatic bands at both the telomeres of all the chromosomes. The rare researches carried out in order to analyse heterochromatin localization in other taxa possessing holocentric chromosomes, in some cases were unsuccessful (Collet and Westerman 1987), but generally a telomeric and sometimes intercalary localization of C positive bands on whole chromosome complement was described (Papeschi 1988; Grozeva and Nokkala 2003). This distribution substantially differs from what observed in monocentric chromosomes, where the heterochromatic regions typically occupy specific zones of all chromosomes, corresponding to centromeres (Schweizer and Loidl 1987).

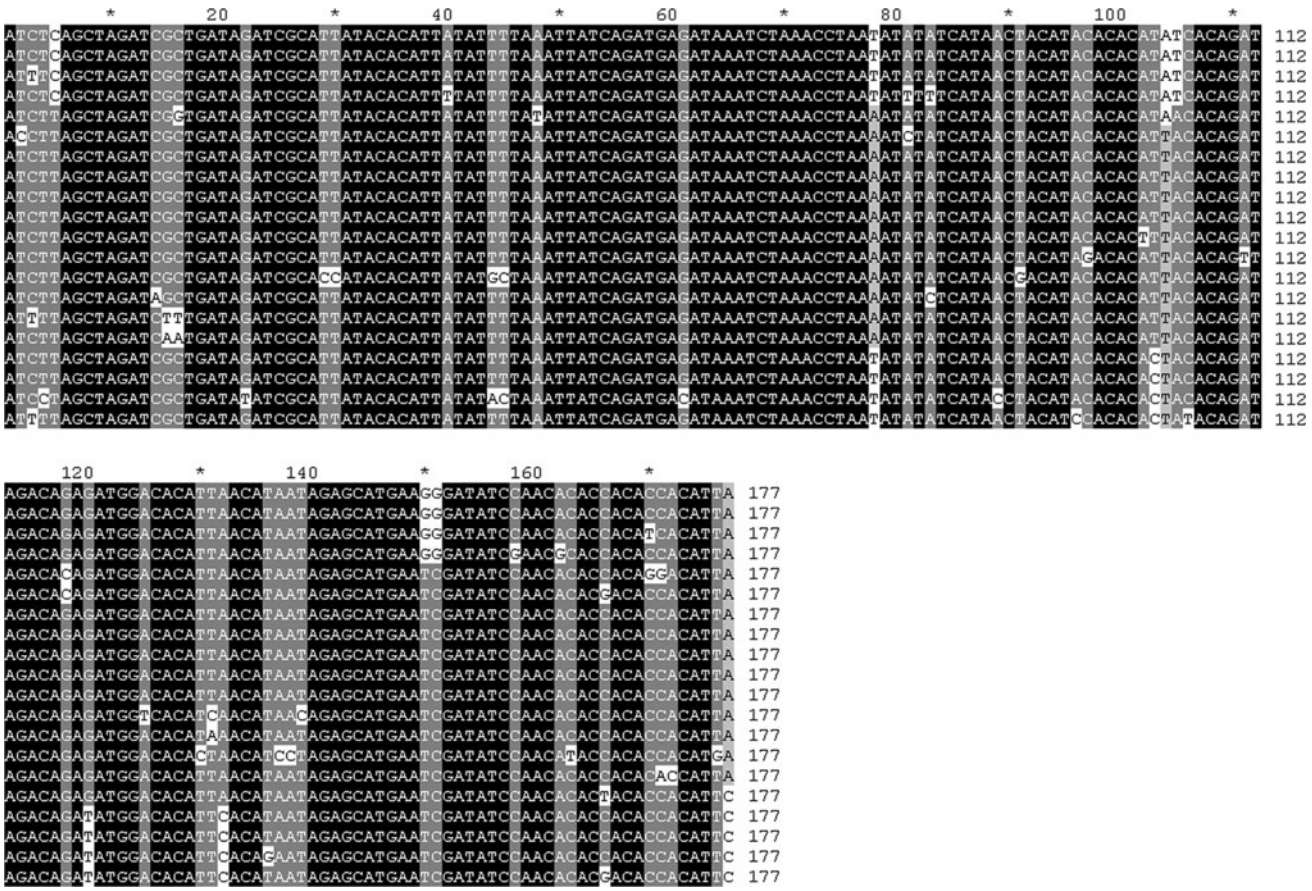

Fig. 3 Sequence alignment of the 20 sequenced *Dra*I DNA satellite. Sequence identity decreases from *black* to *white*

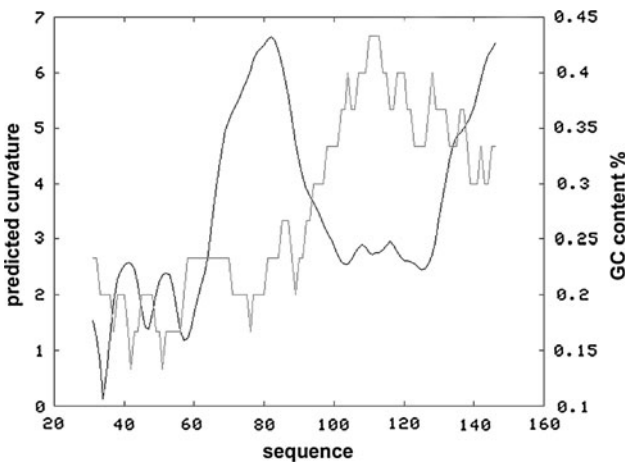

Fig. 4 Curvature-propensity plot of *Dra*I DNA satellite showing the presence of a high value of curvature propensity in the region betwee nucleotide 60 and 100. *Black line* plots the curvature propensity in the *Dra*I satDNA sequence, whereas the *grey line* estimates the GC content

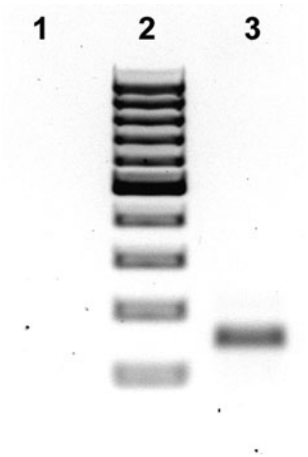

Fig. 5 Search for transcription of the *Dra*I satDNA by electrophoresis of the RTÐPCR products failed to evidence any satellite transcripts in *R. padi* (*lane 1*) suggesting that this sequence is not transcribed. The *β*-tubulin gene was ampliÞed in order to conÞrm both the presence and the quality of mRNAs in the RNA samples used for RTÐPCR experiments (*lane 3*). *Lane 2* shows the 100 bp ladder DNA molecular marker

C banding of *R. padi* chromosomes conÞrmed the occurrence of heterogeneity in the composition of heterochromatin since all C-bands resulted AT-rich with the

exception of a GC-rich region corresponding to the NOR-bearing telomere on both the X chromosomes. It must be noted that rDNA genes have been located on one X chromosome telomere in all the species analysed to date (Manicardi et al. 2002) with the only exceptions of *Schoutedenia ralumensis* and *Maculachnus submacula* that present autosomic NORs and *Amphorophora idaei* which shows interstitial NORs on X chromosomes (Blackman 1987). This specific location could be the result of a strong evolutionary constrain related to the peculiar mechanism of X0 male determination which involve pairing of the X chromosomes by their NORs during prophase of the maturation division (Blackman and Spence 1996).

To date, the studies carried out on satellite DNA sequences in organisms possessing holocentric chromosomes are scanty and generally centred on nematodes (Grenier et al. 1997). In aphids, only three repeated DNA sequences have been described so far: (1) a 600 bp repeat unit located in the intercalary heterochromatin of *Megoura viciae* X chromosomes (Bizzaro et al. 1996); (2) the 169 bp tandem repeat occurring at a subtelomeric location on all chromosomes of three members of the *M. persicae* group of species (Spence et al. 1998); (3) a 189 bp long *Hin*dIII satDNA located on intercalary heterochromatin of the two X chromosomes in *M. persicae* (Mandrioli et al. 1999a).

At a sequence level, *Dra*I satDNAs resulted conserved both in length and sequence with differences due to nucleotide substitutions only. At present the intra-specific sequence variability in insect satDNAs falls in the range 1–13% even if very similar satDNAs are more frequent than variable sequences (Lopez-Leon et al. 1995; Mandrioli et al. 1999a; Landais et al. 2000). This conservation could be due to a process of "concerted evolution" determined by molecular drive (Dover et al. 1982; Dover and Tautz 1986), but may also be related to the presence of constrains due to the interaction of satDNAs with specific proteins involved in heterochromatin formation trough a direct binding or via RNA interference (Talbert et al. 2004).

*Dra*I satDNAs is also characterised by a strong conservation in their length. In other species, this feature has been related to a possible role of satellite DNA in the nucleosome phasing necessary during the process of heterochromatin condensation (Henikoff et al. 2001).

The evolutionary turnover of satDNA is usually very fast so that in closely related species non-orthologous satDNAs are often found at the same chromosomal locations (Csink and Henikoff 1998). However, other satDNA families evolve more slowly and are represented in several closely related species (Mantovani et al. 1997; Cesari et al. 2003). Consequently, some satDNAs may be valuable taxonomic tools for species determination, while others might be informative in phylogeny (Mantovani et al. 1997; Cesari et al. 2003). Molecular studies addressed questions regarding levels of genetic diversity found in *Rhopalosiphum* populations (Hales and Cowen 1990; Bulman et al. 2005; Valenzuela et al. 2007). Using a combination of mitochondrial DNA sequences and microsatellite data Delmotte et al. 2003 put in evidence the presence of two haplotypes within *R. padi* populations. The identification of the *Dra*I satellite DNA could contribute to reveal phylogenetic relationships among different *R. padi* populations and investigate putative hybrid origin of cryptic taxa.

*Dra*I satDNA presented a high AT content that fits our cytological data which showed its localization into a bright fluorescent C band after FISH. Indeed, in situ hybridization showed that *Dra*I satDNA is located limitedly to an intercalary heterochromatic band of the two X chromosomes, whereas no hybridization signals have been observed in the other heterochromatic bands of the X chromosomes neither on autosomes. This distribution is similar to that observed in *M. persicae* and *M. viciae*, where the satellite DNAs labelled only intercalary heterochromatic bands of the two X chromosomes (Bizzaro et al. 1996; Mandrioli et al. 1999a).

Bioinformatic analyses showed that an internal portion of the *Dra*I satDNA possesses a high curvature propensity suggesting that this satellite DNA could be involved in the tight winding of DNA in heterochromatin, as previously suggested for other satDNAs (Palomeque and Lorite 2008).

The transcription of insect satDNAs has been studied in few species and just some of them present RNAs related to these repetitive DNA. RNAs related to the *Dra*I satDNA have been searched in *R. padi* by RT–PCR without any positive result. However, in insects satDNA transcription has been related to cell type, developmental stage and sex so that it could be not so easy to detect the transcripts (Renault et al. 1999; Lorite et al. 2002; Palomeque and Lorite 2008). Moreover, at least in *D. melanogaster* satellite DNA-related transcripts consist in small RNAs that cannot be detected by RT–PCR (Aravin et al. 2003; Usakin et al. 2007) so that an exhaustive assessment of satellite transcription in aphids require further investigations.

As a whole, *R. padi Dra*I satDNAs present several features common to the two satellites isolated in the aphid *M. persicae* since they are long less that 200 bp, present a high degree of similarity among the sequenced repeats in both length and sequence (the percentage of similarity is greater than 90%), show an high AT content (about 60%), contain an internal region with a high curvature propensity and localize in AT-rich heterochromatic regions. This is interesting considering that *M. viciae* and *M. persicae* belong to the tribe Macrosiphini, whereas *R. padi* belongs to the tribe Aphidini suggesting that aphid satellite DNAs present common properties despite the relative phylogenetic distance of the studied species.

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