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Highly GC-rich heterochromatin in chili peppers (*Capsicum*-Solanaceae): A cytogenetic and molecular characterization

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ABSTRACT

Capsicum comprises 35 species of chili peppers and five of them are cultivated worldwide as spices or vegetables. Diploid karyotypes based in x = 12 and x = 13 are common in *Capsicum* and the constitutive heterochromatin (cHet) is of particular interest in the genus since it is largely variable, particularly its highly GC-rich fraction. However, the repetitive DNA components of this heterochromatic regions are unknown. Given the co-localization of rDNA loci with the CMA$^+$DAPI– heterochromatic bands, we tested the hypothesis that the highly GC-rich cHet fraction is composed of the whole 18S-25S ribosomal DNA (rDNA) unit or some of its components. Here we report on a novel satellite for *Capsicum* and Solanaceae composed of the complete rDNA unit. We physically mapped six *Capsicum* derived specific 18S-25S rDNA probes that covered the entire span of the rDNA unit and analysed a *Dra*I restriction product on eight chromosomally different taxa of *Capsicum,* representative of the major phylogenetic clades of chili peppers. The co-localization of every gene and spacer probes of the 18S-25S rDNA unit suggest their structural function as a major repetitive component of the highly GC-rich cHet in *Capsicum* species with x = 12. In addition, analyses of the clones derived from restriction assays in *C. pubescens* suggested that the differential functional status of 18S-25S rDNA loci (nucleolar organizer regions -NORs- or cHet) in this species is related to a divergence in a short sequence upstream the regulatory transcription initiation site (TIS) of the intergenic spacer (IGS). The results here provided evidence that an rDNA mega satellite played a significant role in the evolution of the karyotype features of x = 12 *Capsicum* species. The finding of a mega satellite family derived from the whole rDNA unit is a novelty for plant genomes.

1. Introduction

Capsicum is a small American genus of Solanaceae with about 35 species that can be grouped in eleven major phylogenetic clades (Carrizo García et al., 2016). Five of these species are worldwide cultivated as spices (hot chili peppers) or vegetables (sweet peppers) and their high nutritional contents fruits are important constituents of the human diet. In addition, the *Capsicum* spices have medical and ornamental applications (Moscone et al., 2007).

Diploid karyotypes based in x = 12 and x = 13 are common in *Capsicum* and the cHet fraction is of particular interest in the genus. Detailed Giemsa-C and CMA/DA/DAPI fluorescent chromosome banding karyotypes embracing the five cultivated species (x = 12) and 18 wild taxa of *Capsicum* revealed a large variability in the nucleotide composition, number, size and distribution of those cHet regions among taxa. As a whole, the banding patterns of these species are a valuable tool to assess taxonomic identity (Moscone et al., 1993, 1996a, 2007; Scaldaferro et al., 2013).

The highly GC-rich (CMA$^+$DAPI–) cHet fraction, although universal in *Capsicum*, is the most variable among taxa. Hence, there are species with almost half of their karyotypes composed bythis cHet type, while in others this fraction covered only about 2% of the complement length (Moscone et al., 2007). Moreover, in most species the bands are highly equilocalized among the non-homologous chromosomes of the

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karyotype (Scaldaferro et al., 2013). Therefore, the analysis of the amount and patterns of distribution of the highly GC-rich cHet in the genus is of great interest in studying the evolution of karyotypes and nuclei architecture.

In chili peppers, two to eight active NOR sites per diploid complement were reported using silver staining and the NOR-associated cHet is highly GC-rich (Moscone et al., 1995, 1996a, 2007; Scaldaferro et al., 2013, 2016). Notably, fluorescent *in situ* hybridization (FISH) using heterologous probes derived from wheat, *Arabidopsis*, pea and soybean (Tanksley et al., 1988; Park et al., 1999; Scaldaferro et al., 2006, 2016; Kwon and Kim, 2009) revealed in some *Capsicum* taxa a larger number of 18S-25S rDNA loci aside of the NORs. Moreover, the patterns of 18S-25S rDNA loci observed in those species reflect, to different extents, those of the highly GC-rich cHet. Despite this general congruency, the patterns of rDNA loci reported for each species by those authors were different, and in some cases inconsistent. A further cytogenetic analysis using 25S rDNA gene and incomplete IGS probes derived from *Capsicum* species found that the IGS loci surpassed in number those of the 25S rDNA sites (Jo et al., 2011). On this basis, the authors proposed the existence of a genus specific satellite DNA with homology to the rDNA IGS portion that hybridized to the GC-rich heterochromatic blocks in *Capsicum*. However, that analysis was insufficient to assess whole diversity of the 18S-25S rDNA derived satellites and their role in the evolution of the GC-rich heterochomatin in *Capsicum*.

In this report we examine the distribution of complete 18S-25S rDNA unit and its individual components and make inferences about the impact that each portion had in the evolution of the highly GC-rich cHet in *Capsicum* by means of a deep cytogenetic analysis. For this purpose we constructed a set of probes with the different genic and spacer regions of the 18S-25S rDNA by PCR that in sum covered the entire span of the rDNA unit, as well as a set of repetitive probes derived from clones isolated from restriction assays in *C. pubescens*. The probes were mapped by FISH in different combinations onto eight taxa of *Capsicum* that are chromosomally divergent and representative of seven out of the eleven major phylogenetic clades of the genus.

2. Materials and methods

2.1. Plant materials

Eight taxa of *Capsicum* were selected for a molecular cytogenetic analysis according to their differences in basic chromosome number, karyotype symmetry, type, amount, number, distribution and size of cHet blocks, detailed in depth in Moscone et al. (2007) and Scaldaferro et al. (2013). All the taxa were identified by Dr. Gloria E. Barboza (Instituto Multidisiplinario de Biología Vegetal, IMBIV, Córdoba, Argentina) and their respective names, provenance, voucher specimen, herbarium, status, chromosome features and use are detailed in Table 1.

2.2. Construction of specific rDNA probes derived from different Capsicum species by PCR

Partial probes for the genic and intergenic regions of rDNA used in FISH experiments were planned to cover the entire span of the unit. Moreover, since two major types of IGS were described for *Capsicum* species (Grabiele et al., 2012), probes were designed to enable the analysis of the representativeness and distribution of each version in the karyotypes. A summary of the *Capsicum* specific rDNA FISH probes, their attainment and major features is presented in Table 2. Total DNA was isolated and purified from fresh leaves according to Rogers and Bendich (1994). DNA was evaluated for quality by agarose gel electrophoresis and quantified by spectrophotometry. PCR primers were designed from rDNA consensus sequences of Solanaceae at Genbank. In the PCR reactions, the *Taq* DNA Polymerase “Sequencing Grade” (Promega, USA) was used (10 ng of template DNA; 0.5 pmoles of each primer; 200 mM of dNTPs; 5 µl of 10X buffer; 1 unit of polymerase). PCR products were electrophoresed in 1.4% agarose (Fig. 1a), gel isolated, purified by the GFX kit (Amersham Pharmacia, USA), cloned in pCR2.1-TOPO and transformed into “TOP10 One Shot” *E. coli* (Invitrogen, USA) according to manufacturer instructions. Clones were subsequently grown in LB media with proper antibiotics and the obtained cultures were subjected to plasmidic DNA minipreparations using the Wizard-Plus Minipreps DNA Purification System (Promega, USA). Plasmidic DNAs were then digested with *Eco*RI (NEB, USA) according to manufacturer instructions and visualized in 1% agarose gel to check the stability of the inserts. Selected clones were sequenced at Macrogen (Korea) and Genbank BLASTN homology searches were conducted to confirm their identities prior to FISH analysis.

2.3. Construction of cHet probes derived from C. pubescens restriction assays

To isolate probable satellite DNA sequences from *Capsicum*, genomic DNA from *C. pubescens*, a taxa with high heterochromatin content, was digested with several restriction endonucleases, i.e. *Apa*I, *Bam*HI, *Eco*RV, *Hind*III, *Pvu*II, *Xho*I, *Eco*RI and *Dra*I. Restriction endonucleases products were gel electrophoresed (Fig. 1b), isolated, purified and used as a bulk sequence pool in FISH experiments. For further analysis and characterization, the *Dra*I restriction band was cloned in pBluescript KS + (Stratagene inc.) and transformed into *E. coli* DH-5α. Clones were grown and purified as described above and the plasmidic DNAs were digested with *Pvu*II (NEB, USA) according to manufacturer instructions and visualized in 1% agarose gel (Fig. 1c). FISH experiments were conducted with those clones and a selected clone, pCp-200/33, was sequenced at Macrogen (Korea).

Table 1
Summary of the *Capsicum* material, their use in this study and major features.

Taxon	Provenance	Voucher specimen and herbarium	Status	2n	Karyotype	Use
C. annuum L. var. *annuum*	México	NMSU ID10272. CORD	Cultivated	24	20m + 2sm + 2 st	A
C. frutescens L.	Brasil, Minas Gerais	GE Barboza 795. CORD	Cultivated	24	22m + 2 st	A, B
C. pubescens Ruiz et Pav. cv. locoto rojo	Argentina, Salta	EA Moscone 256. CORD	Cultivated	24	22m + 2 st	A, B
C. baccatum L. var. *pendulum* (Willd.) Eshbaugh cv. cayenne	Argentina, Salta	EA Moscone et al. 211. CORD	Cultivated	24	22m + 2 st	B
C. praetermissum Heiser et Smith	Brasil, São Paulo	E Forni Martins 05/17. CORD	Wild	24	22m + 2sm	B
C. eximium Hunz.	Argentina, Salta	EA Moscone 255. CORD	Wild	24	22m + 2sm	B
C. flexuosum Sendtn.	Argentina, Misiones	GE Barboza et al. 1034. CORD	Wild	24	22m + 2 st	B
C. recurvatum Witas	Brasil, Paraná	GE Barboza et al. 915. CORD	Wild	26	20m + 4sm + 2 st	B
C. rhomboideum (Dunal) Kuntze	Venezuela, Táchira	Y Sánchez García 19. CORD	Wild	26	20m + 4sm + 2 st	B

NMSU = New Mexico State University, USA. CORD = Herbarium of Museo Botánico de Córdoba, Argentina. var. = variety. cv. = cultivar. 2n = chromosome number. A = FISH probe construction. B = FISH analysis.

Table 2
Summary of the *Capsicum* specific rDNA FISH probes, their attainment and major features.

PCR template	PCR primer sequence (5` to 3`)	PCR conditions	PCR product	Size (Kbp)	Clone name	Genbank accession
1	Fw GCATATCAATAAGCGGAGGAAAAGAAARv TCAGTGGATCGTGGCAGCAAGGCCACTC	36 cycles; 94 °C 1 min, 60 °C 1 min, 72 °C 3 min	25S rDNA entire region	3.2	Ca25S-29	JF766708, JF766709
2	Fw TCATATGCTTGTCTCAAAGATTAAGCCATRv CGATCTCCTTCCTCTAAATGATAAGGTT	36 cycles; 94 °C 1 min, 60 °C 1 min, 72 °C 3 min	18S rDNA entire region	1.8	Cf18S-17	JF766710, JF766711
3	Fw TCGAAACCTGCAAAGCAGAACGACRv CGGAGCGCCTGAGCGCGGA	36 cycles; 94 °C 1 min, 55 °C 50 sec, 72 °C 1 min	ITS1/5.8S/ITS2 rDNA entire region	0.7	CpITS1,2/5.8S-1	
3	Fw TAAATACGGACGGGGTATTGTAARv GACTACTGGCAGGATCAACCAGGT	36 cycles; 94 °C 1 min, 57 °C 1 min, 72 °C 2 min	IGS rDNA entire region[5]	1.8[5]	CpIGS-A3[6]	FJ460246
				2.0[5]	CpIGS-B4[7]	FJ460247
4	Fw CATATGTTGGGACGGTTTTCRv TTTCGGTTTGGCCACATGTG	36 cycles; 94 °C 1 min, 55 °C 50 sec, 72 °C 1 min	IGS rDNA internal region	0.5	CpIGS-B4rd[8]	

min = minutes. sec = seconds. 1 = *C. annuum* var. *annuum*. 2 = *C. frutescens*. 3 = *C. pubescens*. 4 = CpIGS-B4. 5, two distinct PCR products. 6 and 7, non-functional and functional IGS variants from *C. pubescens*, respectively. These clones share a whole sequence similarity of ca. 80%, however CpIGS-3A lacks an internal region of 0.5 Kbp that contains the putative transcription initiation site (TIS) and diverse regulatory elements (Grabiele et al., 2012). 8, this clone contains the internal region of CpIGS-B4 that is completely lacking in CpIGS-A3.

2.4. Characterization of the pCp-200/33 cHet derived clone by PCR and sequence analysis

PCR primers specific to each end of this sequence (5′TAATTTAAG GCTATTA3′ and 5′AAATATTTCAATGTT3′) were designed to examine its presence and amplification pattern in *C. pubescens*. PCR reactions proceeded as described above and 36 cycles (94 °C 1 min, 45 °C 1 min, 72 °C 1 min) were performed. PCR products were electrophoresed in 1.4% agarose gel (Fig. 1d).

In house and Genbank BLASTN homology searches were directed to reveal the identity of this clone. The editing and multiple alignments of the nucleotide sequences were performed in Geneious Pro 8.1.6 (Biomatters Ltd.). Secondary structure analysis were performed in UNAFold (Markham and Zuker, 2008) and the RNAfold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

2.5. Chromosome mapping of the rDNA and heterochromatin derived probes

Mitotic arrest of root tips by p-dichlorobenzene, fixation, and enzyme maceration are detailed in Moscone et al. (1993). FISH procedure followed that of Moscone et al. (1996b). Clones and purification products were labeled by nick translation with digoxigenin-11-dUTP or biotin-11-dUTP following the manufacturer instructions (Enzo, USA) to generate distinct DNA probes. Slide preparations were subjected to RNAse and Proteinase K pretreatments, followed by steps of denaturalization, probe hybridization, blocking, probe detection by means of antibodies linked to fluorochromes [anti-digoxigenin to fluorescein (FITC) and anti-biotin to rhodamine (TRITC)] (Dako, USA), washing, and DAPI staining for contrast. In particular, during the hybridization and washing of restriction derived probes, different stringency conditions were assayed, i.e. 87%, 74% and 63%.

Metaphase chromosomes and interphase nuclei were viewed and photographed with a Leica DMLB fluorescence microscope equipped with a computer-assisted digital camera system. At least three individuals from each taxon and 30 metaphases for each individual and FISH procedure were considered to assess the number and position of FISH signals in selected cells. Images were captured in black and white using appropriate filters for FITC, TRITC and DAPI excitation. Digital images were compiled with Photoshop CS6 (Adobe, USA) for final processing.

3. Results

3.1. PCR derived partial rDNA probes specific for Capsicum

Six distinct rDNA probes i.e. Ca25S-29, Cf18S-17, CpITS1,2/5.8S-1, CpIGS-B4, CpIGS-B4rd and CpIGS-A3, that in sum covered the entire span of the 18S-25S rDNA unit in *Capsicum*, were constructed to be used in FISH experiments. The product size and the sources of the templates are detailed in Table 2. The target loci and coverage of the probes are depicted in Fig. 2.

Three of the probes (Ca25S-29, Cf18S-17, CpITS1,2/5.8S-1) covered the rDNA genes and the internal transcribed spacers -ITS1 and ITS2- of the ribosomal unit (Fig. 2). Three probes covering different regions of the IGS were obtained to analyze the representativeness and distribution of the different types of IGS described for *Capsicum* species. The probe CpIGS-B4 covered the entire B-type IGS region (SRI-VII; 2 Kbp) (Fig. 2). The CpIGS-B4rd probe embraced only the structural regions SRIII-VI (0.5 Kbp) of the B-type IGS. This probe included the putative TIS and diverse regulatory elements largely conserved in Solanaceae. By contrast, the probe CpIGS-A3 (1.8 Kbp) was a probe designed to covered the entire A-type IGS (SRI-VII), which lacks the TIS and regulatory elements (SRIII-VI), but displays an extended portion of the repetitive SRII region (Fig. 2).

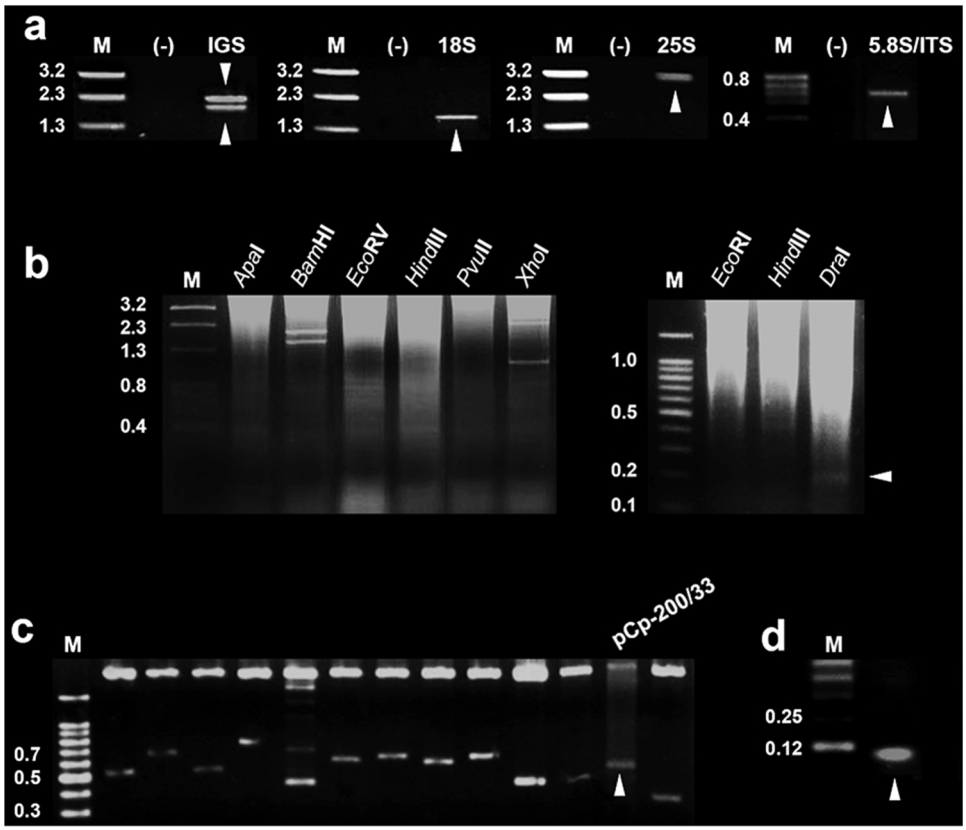

Fig. 1. (a) PCR amplification products of 18S-25S rDNA regions in *Capsicum*. (b) Restriction assays and obtained *Dra*I band in *C. pubescens*. (c) Restriction assay to clones of *Dra*I; note pCp-200/33. (d) PCR amplification product of pCp-200/33 in *C. pubescens*; note the absence of an amplification ladder pattern of this clone. M, marker (Kbp); (-), negative control.

3.2. FISH analysis with specific PCR derived 18S-25S rDNA probes

The six distinct rDNA probes were hybridized to each of the eight *Capsicum* species. All the rDNA probes assayed by FISH, irrespective to its nature (genic or intergenic), showed a similar pattern of loci (in the number, size and position) within each taxon. Figs. 3 and 4 illustrate key examples of the hybridization patterns in representative taxa and the results are summarized in Table 3. These results are represented graphically in the idiograms of Fig. 5.

However, the patterns of the rDNA loci were very different among the species with different basic chromosome numbers. In all taxa with x = 12, all (six) rDNA-derived probes hybridized to the active NORs and most of the bands composed of highly GC-rich heterochromatin (Table 3, Figs. 3 and 4). Notably, the detected loci were distributed equilocally among heterologous chromosomes. By contrast, in the two taxa with x = 13 here analyzed, the rDNA probes hybridized exclusively onto the NORs but not in the highly GC-rich heterochromatin (Table 3).

3.3. Characterization of the restriction assay derived probes from C. pubescens and FISH analysis

From all the restrictions assays, only after *Dra*I digestion a unique band of ca. 150 bp was obtained (Fig. 1b). FISH to somatic chromosomes and interphase nuclei of *C. pubescens* revealed that the bulk *Dra*I band probe co-localized with most of the heterochromatic blocks of this taxon, visualized here as DAPI enhanced regions that appear after the FISH procedure (Fig. 6a–f). The hybridization pattern of this probe was completely consistent with the pattern revealed by the PCR derived probes of the 18S-25S rDNA.

In an attempt to characterize the sequence components that account for the repetitive FISH pattern, thirteen clones obtained after the cloning of *Dra*I band were used independently as probes for FISH assays (data not shown). None of them reproduced the FISH pattern of *Dra*I bulk in metaphase chromosomes of *C. pubescens*. However, the clone pCp-200/33 hybridized to 22 sites out of the 26 revealed by the *Dra*I bulk probe. The double FISH to mitotic chromosomes of *C. pubescens* evidenced a wide co-localization of the pCp-200/33 and 18S rDNA probes along the heterochromatic bands except in the NORs of pairs no.

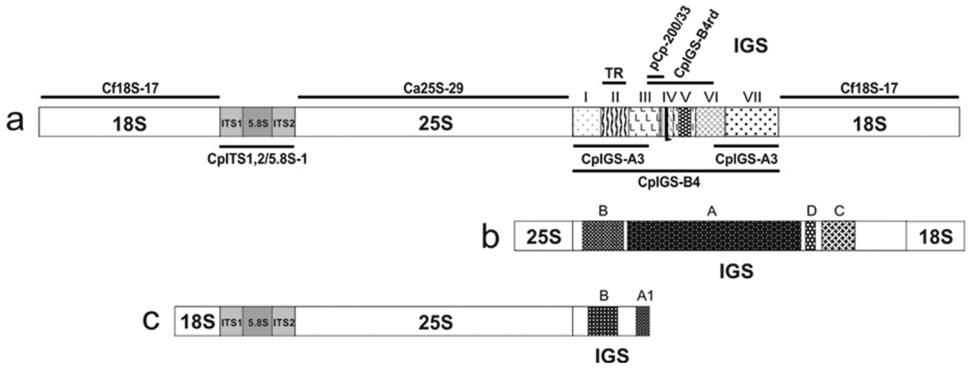

Fig. 2. 18S-25S rDNA FISH probes used in chili peppers. (a) *Capsicum* rDNA unit and derived probes (underlined); I-VII: IGS structural regions (Grabiele et al., 2012); II: repetitive block; III: AT-rich region upstream the transcription initiation site (TIS) in IV; IV-VII: external transcribed spacer (ETS). TR, probe of Jo et al. (2011). (b) pTa71 -GenBank Acc. no. X07841- (B, A, C, D: IGS repetitive blocks). (c) R2 -assembled from X16077, X52320 and X15550- (A_1, B: IGS repetitive blocks). Sequence similarity among pTa71, R2 and *Capsicum* derived probes exclude their respective IGSs.

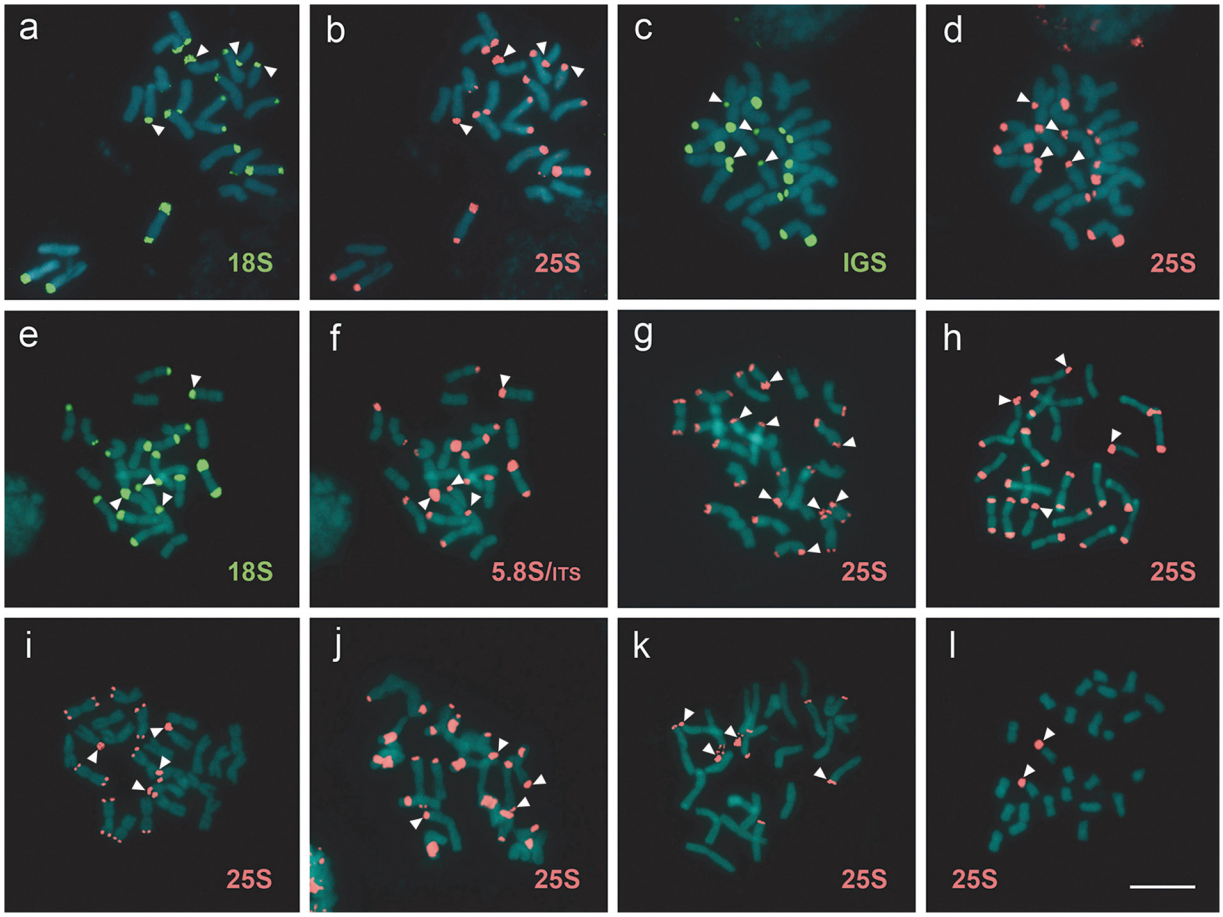

Fig. 3. FISH to DAPI stained metaphase chromosomes with *Capsicum* specific rDNA probes. (a–f) *C. pubescens*. (g) *C. baccatum* var. *pendulum*. (h) *C. praetermissum*. (i) *C. frutescens*. (j) *C. flexuosum*. (k) *C. eximium*. (l) *C. rhomboideum*. Arrowheads point out active NOR loci. Scale bar is 10 µm.

10 and 12. In those chromosome pairs, the pCp-200/33 probe did not hybridize (Fig. 6g–j), regardless of stringency conditions assayed (i.e. 87%, 74% and 63%).

Concerning the spatial distribution of the loci in interphase nuclei, the analysis in *C. pubescens* experiments using the pCp-200/33 probe, alone and together with 18S rDNA probe, showed that both probes hybridized to all the chromocenters at the nuclear periphery. However, in those chromocenters that corresponded to the nucleoli associated heterochromatin, that usually had a more central position in the nuclei, only the 18S rDNA probe hybridized (Fig. 6k and l).

3.4. Characterization of the pCp-200/33 cHet derived clone by sequence analysis

The clone pCp-200/33 was 94 bp in length and 70.2% AT-rich. The sequence shared 80% of identity with the SRIII of the functional IGS variant (FJ460247; B-type, Grabiele et al., 2012) of *C. pubescens* (ca. 140 bp upstream of its TIS), and 88–92% with the equivalent IGS region of several chili peppers (Figs. 2 and 7). This sequence contained a 79 nucleotides inverted repeat that corresponds to a region which could fold into a tentative hairpin in FJ460247. However, the occurrence of 15 nucleotide differences mainly at the stem region may inhibit the folding into a hairpin in pCp-200/33 (Fig. 7). Interestingly, the non-functional IGS variant of *C. pubescens* FJ460246 lacks this homologous region (Fig. 7).

4. Discussion

Highly GC-rich cHet is universal in *Capsicum* and it was proposed as one of the most important components that modulated the karyotypes during the evolution of the genus (Moscone et al., 2007). In the present study, we report on the isolation and characterization of a novel DNA satellite composed of the entire 18S-25S rDNA unit in *Capsicum*. Remarkably, the complete rDNA unit amplified, dispersed and arranged in tandem repeats in the genomes of x = 12 species, becoming the major component of the highly GC-rich cHet of these taxa but not of those with x = 13 here analyzed. We additionally provide hints to the mutations that may have inactivated the rDNA units that form the heterochromatic bands. To our knowledge, the finding of a mega satellite family derived from the whole rDNA unit, comprising coding sequences and spacers, is a novelty for plant genomes. These results add important information to the understanding of the karyotype evolution through changes in the heterochromatic patterns in *Capsicum*.

4.1. Organization of the rDNA satellite and its linkage with the highly GC-rich heterochromatin in Capsicum

The occurrence of rDNA-derived satellites in plant genomes, particularly IGS-like repetitive sequences has been previously described in few genus like *Vigna* (Unfried et al., 1991) and *Phaseolus* (Falquet et al., 1997). In Solanaceae, IGS derived satellites werereported in potatoes (Stupar et al., 2002), tomatoes (Jo et al., 2009) and tobacco (Lim et al., 2004) and was also proposed to occur in *Capsicum* (Jo et al., 2009). However, in our study, the co-localization of the six probes embracing genes and spacers detected by FISH in the x = 12 chili peppers demonstrated that each rDNA-associated locus is composed of the entire rDNA unit. Thus, the results evidenced that the highly GC-rich cHet of the x = 12 *Capsicum* species is composed of a mega satellite DNA

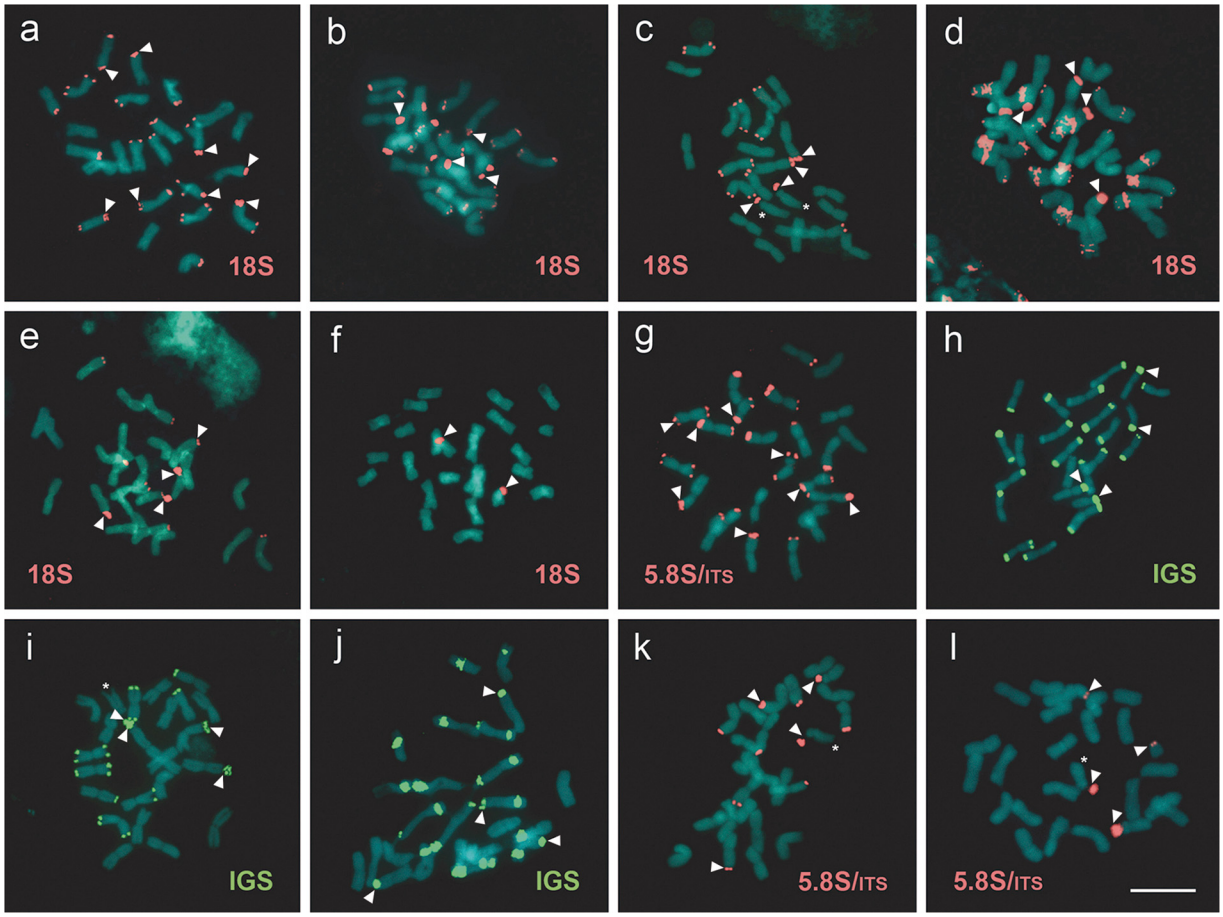

Fig. 4. FISH to DAPI stained metaphase chromosomes with *Capsicum* specific rDNA probes. (a, g) *C. baccatum* var. *pendulum*. (b, h) *C. praetermissum*. (c, i) *C. frutescens*. (d, j) *C. flexuosum*. (e, k) *C. eximium*. (f) *C. rhomboideum*. (l) *C. recurvatum*. Arrowheads point out active NOR loci while asterisks the owner of those distant signals. Scale bar is 10 µm.

sequence, the complete rDNA unit (7.8 Kbp).

These results are in disagreement with the proposal of the existence of sole IGS-derived sequences in *Capsicum* as the main satellite associated to heterochromatin (Jo et al., 2011). Using probes derived from *C. annuum*, those authors reported that the 25S rDNA gene probe hybridized only to the secondary constrictions (two to four) of the three species analyzed (*C. annuum*, *C. frutescens* and *C. baccatum*, all x = 12), while the IGS derived probe revealed highly polymorphic (in number and size) patterns of loci mainly associated to the distal blocks of heterochromatin. The disparity between our cytogenetic results and those of Jo et al. (2011) is difficult to explain since the 25S rDNA gene is conserved and because both reports used probes derived from *Capsicum* species.

By contrast, the results here obtained are consistent with those reported for *Capsicum* species using pTa71 (derived from wheat) and R2 (derived from *Arabidopsis thaliana*) as probes for FISH experiments

Table 3
Summary of cytological landmarks in studied taxa of *Capsicum*.

Taxon	18S-25S rDNA loci	Active NOR loci[A]	Highly GC-rich cHet loci[B]
C. frutescens	16 distals (p: 1, 3, 8, 12; q: 3, 4, 8, 9)	4 distals (p: 1, 12)	16 distals (p: 1, 3, 8, 12; q: 3, 4, 8, 9)
C. pubescens cv. locoto rojo	26 distals (p: 1, 2, 3, 6, 10, 12; q: 2, 3, 4, 5, 6, 8, 11)	4 distals (p: 10, 12)	36 distals (p: 1, 2, 3, 4, 6, 7, 8, 10, 11, 12; q: 2, 3, 4, 5, 6, 8, 9, 11)
C. baccatum var. *pendulum* cv. cayenne	32 distals (p: 1, 2, 3, 5, 7, 8, 10, 12; q: 3, 4, 5, 6, 8, 9, 10, 12)	8 distals (p: 1, 3, 10, 12)	32 distals (p: 1, 2, 3, 5, 7, 8, 10, 12; q: 3, 4, 5, 6, 8, 9, 10, 12)
C. praetermissum	22 distals (p: 1, 2, 3, 4, 6, 7, 9, 11, 12; q: 2, 10); 6 interstitials (p: 10, q: 3, 5)	4 distals (p: 6, 12)	22 distals (p: 1, 2, 3, 4, 6, 7, 9, 11, 12; q: 2, 10); 6 interstitials (p: 10, q: 3, 5)
C. eximium	10 distals (p: 2, 5, 12; q: 7, 11)	4 distals (p: 12; q: 7)	10 distals (p: 2, 5, 12; q: 7, 11)
C. flexuosum	12 distals (p: 2, 5, 9; q: 4, 6, 7); 18 interstitials (q: 1, 3, 4, 5, 6, 8, 9, 12a, 12b)	4 distals (p: 2, 5)	10 distals (p: 2, 5, 9; q: 4, 6); 18 interstitials (q: 1, 3, 4, 5, 6, 8, 9, 12a, 12b)
C. recurvatum	4 distals (p: 11, 13)	4 distals (p: 11, 13)	42 distals (p: 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13; q: 1, 2, 3, 5, 6, 7, 8, 9, 10, 12); 6 interstitials (p: 1, 8; q: 1)
C. rhomboideum	2 distals (p: 9)	2 distals (p: 9)	30 distals (p: 2, 3, 5, 6, 7, 8, 9, 10, 12; q: 1, 2, 4, 8, 10, 12)

p, q = short and large chromosome arm, respectively (numbers refers to chromosome pair).
[A] Revealed here as secondary constrictions associated to macrosatellites and by silver staining (Moscone et al., 1995, 2007; Scaldaferro et al., 2016).
[B] According to Moscone et al. (1996a, 2007) and Scaldaferro et al. (2013).

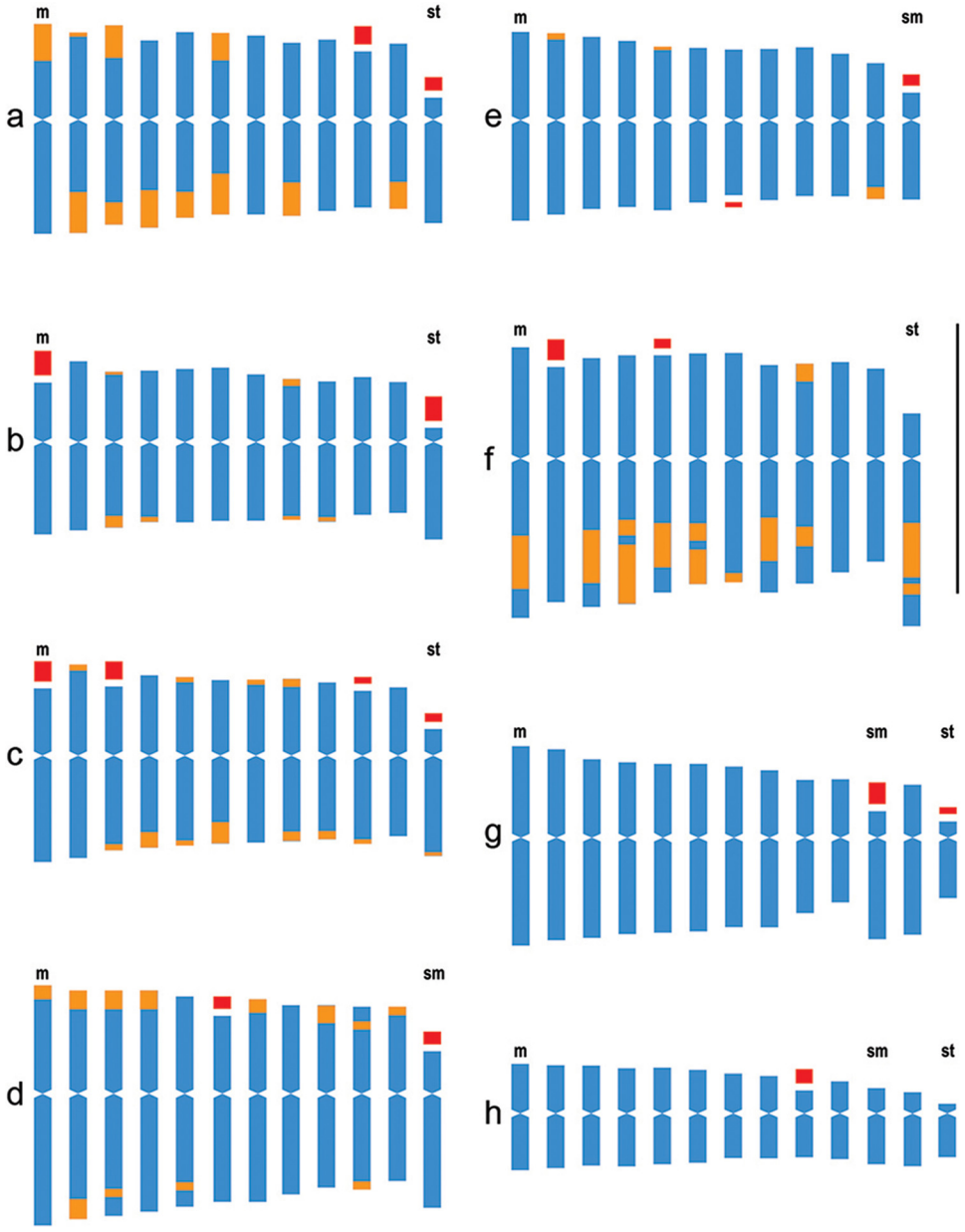

Fig. 5. Chromosomal distribution of the active NOR (red) and inactive NOR (orange) 18S–25S rDNA locus in *Capsicum*. (a) *C. pubescens*. (b) *C. frutescens*. (c) *C. baccatum* var. *pendulum*. (d) *C. praetermissum*. (e) *C. eximium*. (f) *C. flexuosum*. (g) *C. recurvatum*. (h) *C. rhomboideum*. Scale bar is 10 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Park et al., 1999; Scaldaferro et al., 2006, 2016). Particularly, the patterns obtained with the R2 probe are significant, since that probe comprises the whole 25S and 5.8S rDNA genes as well as the ITS1 and ITS2 spacers, but small portions of the 18S rDNA gene and of the IGS (777 bp of the IGS with less than 40% homology to the IGS of *Capsicum*). According to the sequence composition of the R2 probe, the hybridization (80% stringency) of this probe onto the highly GC-rich heterochromatic bands of the x = 12 species should be mainly attributed to the 25S rDNA gene sequence, less to the ITS spacers, 5.8S and 18S rDNA gene sequences, while the IGS is not expected to contribute to the signals. Under this expectation, the reported patterns of hybridization of R2 (Scaldaferro et al., 2006, 2016) on most of the highly GC-rich cHet support our results that the complete rDNA unit is the major satellite that compose the heterochromatin in the x = 12 species of *Capsicum*.

The FISH analysis here performed confirmed and expanded the existence of a great variability in the number of loci that hybridized with 18S-25S rDNA probes among chili peppers. The number of active NORs revealed previously by silver staining (Moscone et al., 1995, 2007; Scaldaferro et al., 2016) and here as extended secondary constriction hybridized with rDNA-derived probes, varied from four to eight among species. All the additional hybridization signals observed onto the highly GC-rich, $CMA^{+}DAPI–$, heterochromatin (Table 3; Moscone et al., 1996a, 2007; Scaldaferro et al., 2013, this report) in the x = 12 species may be considered as inactive rDNA loci composed mainly of the rDNA mega satellite described here. By contrast, the hybridization of the rDNA probes exclusively to the NORs in all the x = 13 species here analyzed suggests that the main component of the highly GC-rich heterochromatin in those species is other different from the rDNA sequences.

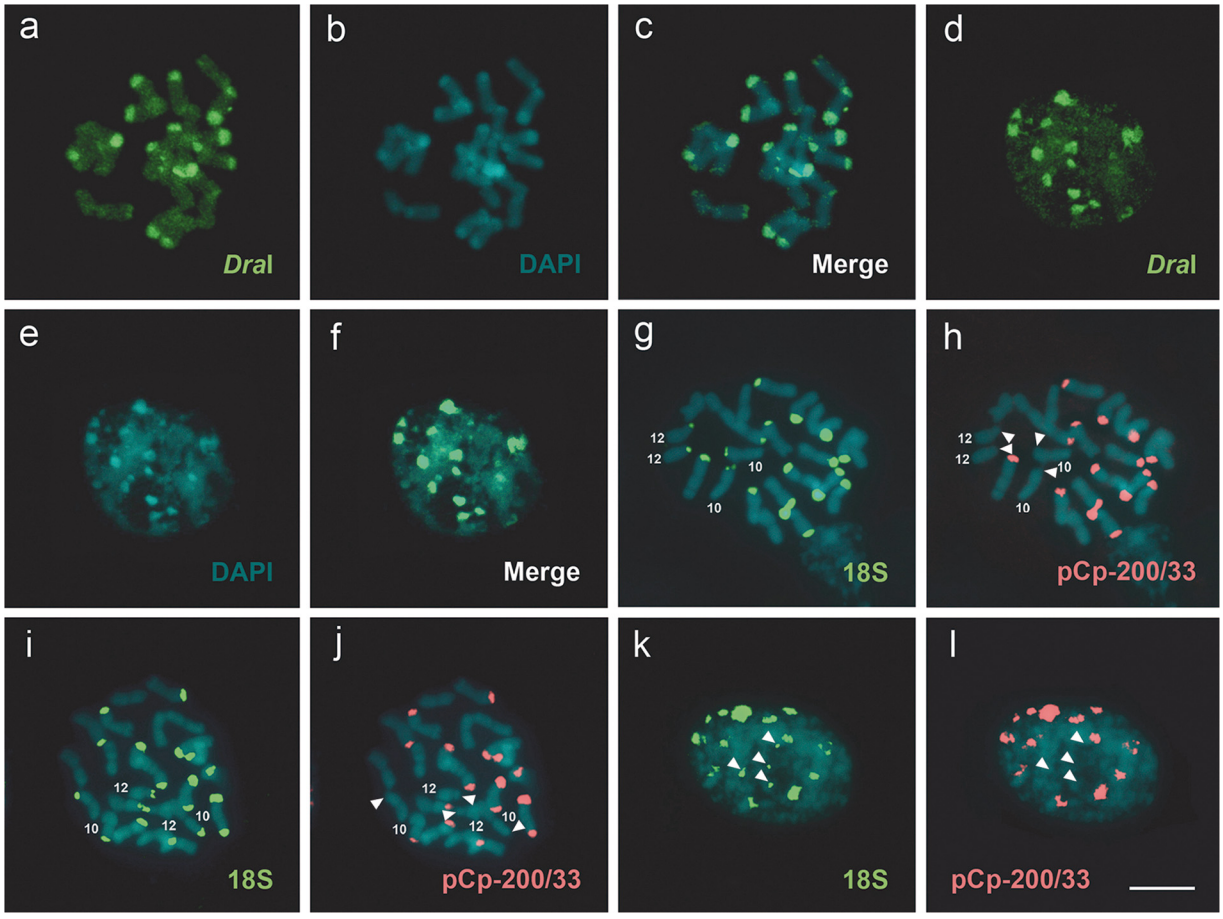

Fig. 6. FISH to DAPI stained interphase nuclei and metaphase chromosomes in *C. pubescens*. (a–f) Co-localization of *Dra*I and heterochromatic blocks. (g–l) Co-localization of 18S and pCp-200/33, excluding active NOR pairs nos. 10 and 12 and knobs, which are pointed out with arrowheads, respectively. Scale bar is 10 µm.

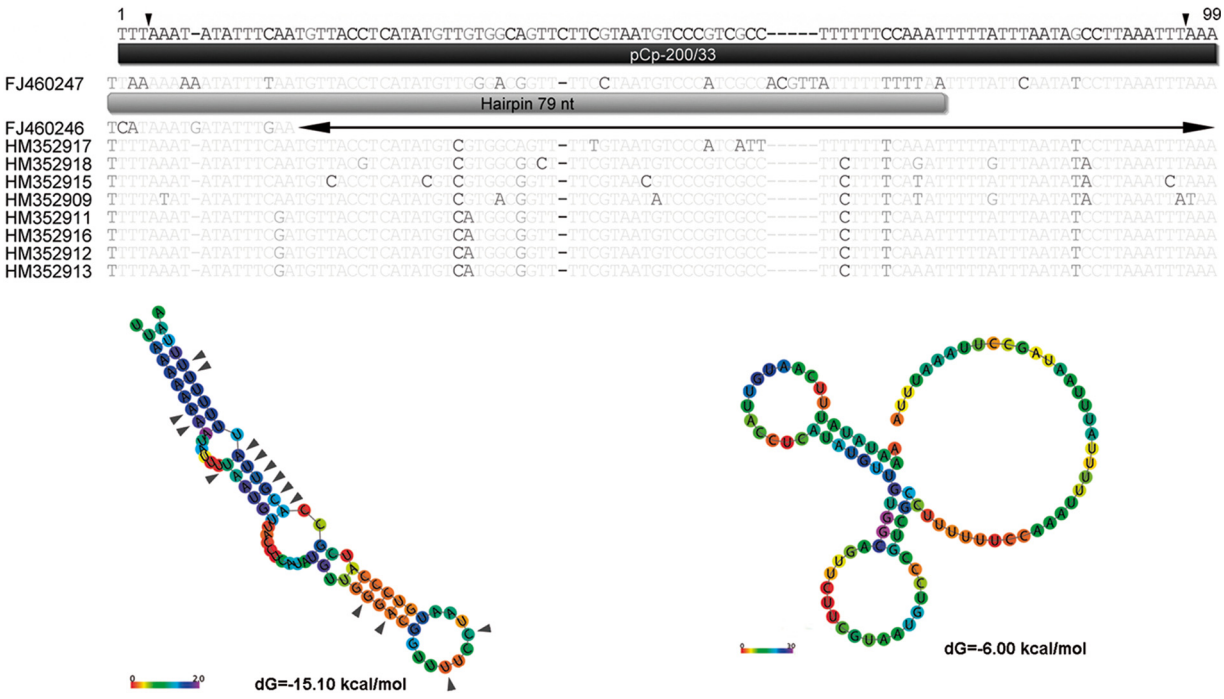

Fig. 7. Alignment of pCp-200/33 with homologous IGS regions in *Capsicum*. FJ, GenBank Acc. nos. from Grabiele et al. (2012) and HM from Jo et al. (2011). Note that FJ460246 lacks this region. (left) FJ460247 hairpin and nucleotide differences (arrowheads) that prevent this folding in pCp-200/33 (right).

4.2. The DraI restriction clone pCp-200/33 provides hints on the rDNA derived mega satellite inactivation in C. pubescens

The sequencing and hybridization of different isolates from the restriction *Dra*I product in *C. pubescens* shed light on the differences that may explain the differential behavior of the rDNA unit observed in the NOR loci and in the highly GC-rich cHet loci. The hybridization of pCp-200/33 to the cHet blocks but not to the NORs evidenced that the rDNA units that compose the heterochromatin have different nucleotide sequence in the IGS. Indeed, the differential hybridization patterns may be explained by the 20% of nucleotide differences detected between the clone pCp-200/33 and the homologous region of the functional IGS.

Secondary structure analysis of the IGS rDNA showed that the nucleotide differences found in the clone pCp-200/33 could be associated to a potential thermodynamical instability of the region that may impede the DNA to fold in the predicted local hairpin conformation. This inability to form a hairpin may be linked to the inactivation of the rDNA units in the cHetregions. This hypothesis is supported by the fact that the pCp/200-33 sequence corresponds to a fragment upstream the TIS of the functional IGS. It has been reported that several IGS hairpins, and particularly those inside the TIS region, take part in the regulation of the rDNA units transcription (Mayer et al., 2006; Preuss et al., 2008; Santoro et al., 2010; Jacob et al., 2012). Particularly, the IGS transcripts derived from regions upstream the TIS are involved in the heterochromatinization of the unit at the NORs. An emergent regulatory pathway based on these hairpins may be determinant to the inactivation of the rDNA units, a plausible scenario in units that composed the highly GC-rich cHet of *C. pubescens*.

4.3. Final considerations

To date, fluorescent heterochromatic banding has been applied to members of nine out of the eleven supported phylogenetic clades of chili peppers, and highly GC-rich cHet revealed universal in *Capsicum*, occurring at taxa with x = 12 and x = 13 of both Andean and Atlantic forest clades (Moscone et al., 2007; Scaldaferro et al., 2013; Carrizo García et al., 2016). In this scenario, the fact that 18S-25S rDNA is the essential component of those highly GC-rich cHet regions in x = 12 taxa could be extensive to x = 13 clades, considering other members than studied here. To shape a broader evolutionary picture of the reported 18S-25S rDNA mega satellite in *Capsicum*, the approach outlined here should be extended in the genus of chili peppers.

Declaration of interest

None.

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