



FISH-mapping of the 5S rDNA locus in chili peppers (*Capsicum*-Solanaceae)

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ABSTRACT

We present here the physical mapping of the 5S rDNA locus in six wild and five cultivated taxa of *Capsicum* by means of a genus-specific FISH probe. In all taxa, a single 5S locus per haploid genome that persistently mapped onto the short arm of a unique metacentric chromosome pair at intercalary position, was found. 5S FISH signals of almost the same size and brightness intensity were observed in all the analyzed taxa. This is the first cytological characterization of the 5S in wild taxa of *Capsicum* by using a genus-derived probe, and the most exhaustive and comprehensive in the chili peppers up to now. The information provided here will aid the cytomolecular characterization of pepper germplasm to evaluate variability and can be instrumental to integrate physical, genetic and genomic maps already generated in the genus.

Key words: wild and cultivated *Capsicum*, 5S rRNA gene, *Capsicum*-derived probe, FISH-mapping.

INTRODUCTION

Capsicum L., or chili peppers, (Solanaceae) is a New World genus distributed from Mexico to Argentina that comprises 31 species, several of which are of considerable economic importance. Five *Capsicum* species (*C. annuum* L. var. *annuum*, *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L. var. *pendulum* (Willd.) Eshbaugh and var. *umblicatum* (Vellozo) Hunz. et Barboza, and *C. pubescens* Ruiz et Pav.) were domesticated by American natives and are now cultivated as part

of the human diet (Moscone et al. 2007). In fact, after tomato, peppers are the most cultivated fruit vegetable species in the world (FAOSTAT 2014).

In plants, 5S rRNA genes are organized in one or several loci, arranged as tandem repeats of thousand to hundreds of thousand copies per genome, with each copy comprising a coding region (CR; ~120 bp) and a non-transcribed intergenic spacer (NTS; ~100-900 bp) (Cloix et al. 2001 and references therein).

Previous fluorescent *in situ* hybridization (FISH) assays revealed the loci number and localization of the 5S rRNA gene in only a few *Capsicum* species, by means of different probes,

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some of them derived from unrelated taxa (Park et al. 1999, 2000, Scaldaferrero et al. 2006, Kwon and Kim 2009). Similarly, other economically important members of the Solanaceae family such as tomato, tobacco and petunia have been studied to reveal the presence of 5S sites (Garcia et al. 2012) but information on potato and eggplant is still absent. In addition, more comprehensive approaches led to the development of several pepper maps (Wu et al. 2009 and references therein) and recently, to the genome sequencing of wild and cultivated species of chili peppers (Kim et al. 2014, Qin et al. 2014). However, despite 45S rRNA genes have been mapped onto a pepper genetic linkage map (Wu et al. 2009), information regarding 5S rDNA genes in *Capsicum*, is still lacking.

The main purpose of this contribution is to achieve a physical mapping of the 5S rDNA locus in wild and cultivated taxa of *Capsicum* by means of a genus-specific FISH probe. The cytological 5S rDNA marker is highly useful in the identification of individual chromosomes for further germplasm characterization. In addition, the information reported here, not only broadly expands the limited knowledge of 5S rDNA mapping in *Capsicum*, but will also be undoubtedly useful to integrate physical, genetic and genomic maps already generated in the genus (Tanksley et al. 1988, Kang et al. 1997, Wu et al. 2009, Qin et al. 2014, Kim et al. 2014).

MATERIALS AND METHODS

PLANT MATERIAL

Five cultivated in addition to six wild taxa of *Capsicum* were employed to cytologically map the 5S rRNA gene. Taxa, provenances, herbarium specimens and status of the studied material are summarized in Table I. Plant material was determined by Dr. Gloria E. Barboza.

CHROMOSOME PREPARATIONS

Chromosomes of somatic metaphases were observed in root tip squashes obtained from seed

germination. Seeds were placed on moisturized filter paper in Petri dishes and germinated at room temperature (about 25 °C). Roots were collected when they reached 2 cm in length and were immediately pretreated with *p*-dichlorobenzene saturated solution for 3 h at room temperature and then fixed in Farmer solution (absolute ethanol : glacial acetic acid, 3 : 1) for a minimum of 12 h at 4 °C. Somatic chromosome spreads were prepared following the procedure described by Schwarzacher et al. (1980). Root tips were macerated in 2% (m/v) cellulase (Onozuka R-10 from *Trichoderma viridae*; Serva, Heidelberg, Germany) plus 10% (v/v) pectinase (from *Aspergillus niger*; Sigma, St. Louis, Missouri, USA), dissolved in 40% glycerol in 0.01 mol/L citric acid/sodium citrate buffer, pH 4.8, at 37 °C for 2 h, and then squashed in 45% acetic acid. After removal of the coverslip with CO₂, slides were air dried, incubated for 1–2 days at room temperature, and then kept at 4 °C until use.

DNA ISOLATION AND 5S PROBE PREPARATION

Genomic DNA was isolated and purified from fresh leaves of *C. pubescens* by using the CTAB method, with minor modifications (Rogers and Bendich 1994). An additional step of DNA purification using phenol-chloroform and a RNase A treatment (Invitrogen, USA) was added, followed by DNA precipitation with ethanol (Sambrook et al. 1989). Sample quality was checked by electrophoresis in agarose 0.8% gel, and by measurements of Abs. 260 nm/280 nm index, to confirm DNA integrity and absence of RNA contamination. The 5S rDNA unit of *C. pubescens* was amplified through polymerase chain reaction (PCR) by using two oligonucleotide primers, P1 5'-GATCCCATCAGAACTCC-3' (17mer) and P2 5'-GGTGCTTTAGTGCTGGTAT-3' (19mer) from Park et al. (2000). Both primer sequences bind to the highly conserved sections in the coding region of the 5S gene (Fig. 1). The PCR reaction mixture comprised 10 ng of template DNA from

TABLE I

Summary of 5S rDNA FISH in *Capsicum*. CORD = Herbarium from Museo Botánico de Córdoba, Argentina; Status: c = cultivated; w = wild. Collectors: GEB = GE Barboza; YSG = Y Sánchez García; EAM = EA Moscone; Abb = Abbreviation; $2n$ = somatic chromosome number; p = short chromosome arm; di = intercalary band distance to the centromere index; CL = chromosome length in μm ; CI = centromeric index; RCL = relative chromosome length expressed as percentage of the genome size in μm .

Taxa, provenance, herbarium and status	Abb	$2n$	Karyotype formula	# loci	Position	CL	CI	RCL
<i>C. chacoense</i> Hunz. Bolivia. GEB et al. 1793-CORD (w)	cha	24	22m+2st	1	4p di=73.68	5.60	47.50	8.70
<i>C. cardenasii</i> Heiser et Smith Bolivia. CORD 1135 (w)	car	24	22m+2sm	1	9p di=57.89	5.53	48.10	7.25
<i>C. eximium</i> Hunz. Argentina. EAM 255-CORD (w)	exi	24	22m+2sm	1	6p di=67.50	5.80	48.28	8.29
<i>C. flexuosum</i> Sendtn. Argentina. GEB et al. 1034-CORD (w)	fle	24	22m+2st	1	9p di=59.38	8.17	43.70	7.85
<i>C. rhomboideum</i> (Dunal) Kuntze. Venezuela. YSG 19-CORD (w)	rho	26	20m+2sm+4st	1	3p di=50.59	3.50	48.57	8.68
<i>C. baccatum</i> L. var. <i>praetermissum</i> Heiser et Smith Brasil. GEB et al. 1646-CORD (w)	pra	24	22m+2sm	1	7p di=59.57	6.71	49.03	8.56
<i>C. annuum</i> L. var. <i>annuum</i> . México. NMCA 10272-CORD (c)	ann	24	20m+2sm+2st	1	3p di=69.52	6.07	48.11	8.67
<i>C. chinense</i> Jacq. Brasil. GEB 807-CORD (c)	chi	24	22m+2st	1	3p di=74.40	5.23	47.80	8.52
<i>C. frutescens</i> L. Brasil. GEB 806-CORD (c)	fru	24	22m+2st	1	8p di=77.24	5.86	49.49	8.32
<i>C. baccatum</i> L. var. <i>pendulum</i> (Willd.) Eshbaugh. Argentina. EAM et al. 211-CORD (c)	pen	24	22m+2st	1	5p di=76.19	6.23	47.19	8.40
<i>C. pubescens</i> Ruiz et Pav. Argentina. EAM 256-CORD (c)	pub	24	22m+2st	1	3p di=56.86	7.35	48.57	9.16

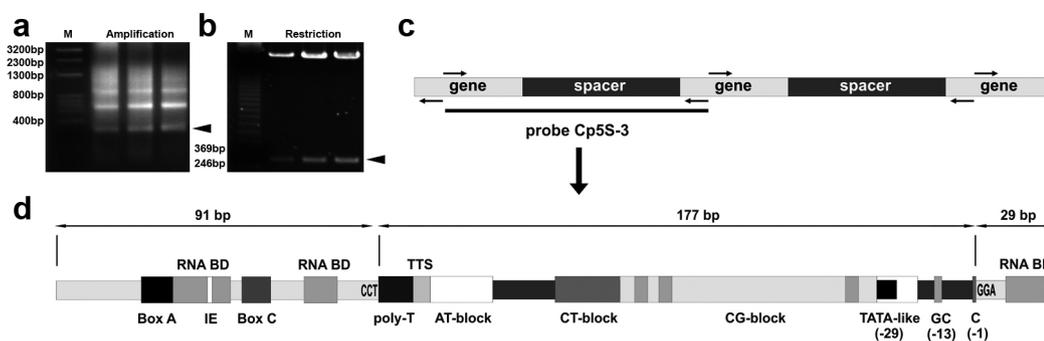


Figure 1 - Development and molecular characterization of the 5S rDNA probe from *C. pubescens*. (a-b) Gel electrophoresis of the PCR amplification products and clones, respectively, with that related to the FISH probe marked with arrowheads; (c) Amplification strategy according to the 5S rDNA unit organization and primers P1/P2 from Park et al. (2000); (d) Core features of the FISH probe Cp5S-3; in the gene, the internal control region (ICR) formed by the motives Box A, Box C and IE (Cloix et al. 2001) and the RNA binding domains (RNA BD) to the TFIIIA (Kellogg and Appels 1995) are shown; additional features correspond to the non transcribed spacer (NTS); TTS, transcription termination site.

C. pubescens, 0.5 pmol of each primer, 200 mM of each dNTP, 5 µl of 10X reaction buffer and 1 unit of *Taq* DNA polymerase “Sequencing Grade” (Promega, USA). Thirty six amplification cycles were performed, each involving denaturation at 94 °C for 1 min, primer annealing at 57 °C for 1 min, and extension at 72 °C for 1 min. Amplified 5S rDNA fragments were separated in agarose 1.4% gel and then were excised from the gel and purified by using the GFX kit (Amersham Pharmacia, USA). Amplified fragments were cloned into the pCR2.1-TOPO vector (Invitrogen, USA) and transformed in *Escherichia coli* “TOP10 One Shot” (Invitrogen, USA), following the manufacturer’s instructions. The obtained clones were grown in LB liquid selective medium. Plasmidic DNA was isolated through mini-preparations, digested with restriction enzymes and visualized in agarose 1% gel to check the insert stability. The nucleotide sequences of the clones were determined (Macrogen, Korea) and Cp5S-3 was characterized (Fig. 1) and selected for further FISH analysis.

FLUORESCENCE *in situ* HYBRIDIZATION

FISH experiments were undertaken according to Moscone et al. (1996). The obtained clone Cp5S-3 was labeled by nick translation with digoxigenin-11-dUTP following the manufacturer instructions (Enzo, USA) to generate a ribosomal DNA probe. 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)] (Dako, USA), washing, and DAPI staining for contrast.

FLUORESCENCE MICROSCOPY AND IMAGE ACQUISITION

Chromosomes were viewed and photographed with an Olympus BX50 fluorescence microscope equipped with a computer-assisted digital camera system. Images were captured in black and white

using appropriate filters for FITC and DAPI excitation, respectively. Digital images were combined in Photoshop 7.0 (Adobe, San Jose, California, USA) for final processing.

KARYOTYPE ANALYSIS

At least five metaphase plates per taxa were used for chromosome measurements. The centromeric index (CI = short arm length x 100/chromosome length) was used to classify the chromosomes according to Levan et al. (1964) in metacentric (m; CI = 50-37.51), submetacentric (sm; CI = 37.50-25.10) and subtelocentric (st; CI = 25.00-12.51). Different lengths of the same arm (and band/locus, where applicable) from homologous chromosomes were combined to mean values and represented in the haploid complements of the idiograms. Intercalary markers were mapped using the index $d_i = d \times 100/a$ (d = distance of band center from the centromere; a = length of the corresponding chromosome arm) according to Greilhuber and Speta (1976).

RESULTS

GENUS SPECIFIC PROBE DEVELOPMENT AND CHARACTERIZATION

The generation and molecular characterization of the 5S rDNA probe from *C. pubescens* is displayed in Figure 1a-d. Clone Cp5S-3 is 297 bp in length and exhibits, at the sequence level, all the specific features of functional 5S rDNA units.

FISH EXPERIMENTS

A *Capsicum*-derived rDNA probe (Cp5S-3) was used in FISH experiments in order to cytologically map the 5S rDNA locus in the chili peppers. The somatic chromosome number added to the karyotype formula and the number and position of the 5S rDNA loci per diploid complement in the analyzed taxa are summarized in Table I. Additional features associated to the chromosome that carries

the 5S rDNA are also provided in Table I, i.e. the chromosome length (CL), the centromeric index (CI) and the relative chromosome length (RCL). All the studied taxa are diploid and have $2n = 24$, except for *C. rhomboideum* (Dunal) Kuntze which has $2n = 26$ chromosomes. Karyotype formula varied among taxa but symmetry is a core feature in *Capsicum*.

LOCI NUMBER, SIZE AND DISTRIBUTION

FISH experiments revealed a unique chromosome pair hybridized with the Cp5S-3 probe (green dots) in the eleven examined taxa (Fig. 2a). Polymorphisms for these loci within individuals or between taxa were not observed. Therefore, a single 5S locus per haploid genome was found in *Capsicum*. 5S FISH signals of almost the same size and brightness intensity were observed in the studied taxa of *Capsicum* (Fig. 2a, b). In the analyzed taxa, the 5S rDNA locus persistently mapped onto the short arm of a unique metacentric chromosome pair, at intercalary position (Fig. 2a). Four species, i.e. *C. rhomboideum*, *C. annuum* var. *annuum*, *C. chinense* and *C. pubescens*, carried the 5S locus on the chromosome pair #3. *Capsicum chacoense* Hunz. exhibited the 5S locus onto pair #4. In addition, *C. baccatum* var. *pendulum*, *C. eximium* Hunz., *C. baccatum* var. *praetermissum* Heiser et Smith and *C. frutescens* bore the 5S locus in pairs #5, 6, 7 and 8, respectively. In *C. cardenasii* Heiser et Smith and *C. flexuosum* Sendtn. 5S signals were located on pair #9. The distance of the 5S locus to the centromere, measured as the intercalary distance index (di), averaged 65.71 (± 9.19) and ranged from 50.59 (in *C. rhomboideum*) to 77.24 (in *C. frutescens*) (Table I and Fig. 2b).

DISCUSSION

FISH PROBE CHARACTERIZATION

According to genic and non transcribed spacer (NTS) motives, the FISH probe derived clone

Cp5S-3 is fairly representative of functional 5S segments. Gene features, i.e. the internal control region (ICR) formed by the motives Box A, Box C and IE of Cloix et al. (2001) and the RNA binding domains (RNA BD) to the TFIIA (Kellogg and Appels 1995), were identified in the primary sequence and are represented in Figure 1d. Further structures in the NTS, i.e. the transcription termination site (TTS) and the TATA-like box (-29), the GC dinucleotides (-13) and the C (-1) at 3' end region, are also associated to functionality (Cloix et al. 2001).

FISH EXPERIMENTS

Cytological mapping of the 5S has been previously performed in several chili peppers. Scaldaferrero et al. (2006) used a probe derived from *Beta vulgaris* (pXV1) on wild and cultivated taxa. Furthermore, Park et al. (1999, 2000) used a wheat derived-probe (pScT7) and a *Capsicum*-related one, respectively, on cultivated taxa, the same as Kwon and Kim (2009). However, ours is the first report on the cytological characterization of the 5S in wild taxa of *Capsicum* by using a genus-specific FISH probe. Moreover, our results in *C. eximium*, *C. flexuosum*, *C. rhomboideum* and both wild and cultivated varieties of *C. baccatum* (*praetermissum* and *pendulum*) are novel in the genus. Furthermore, considering the number of analyzed taxa, our contribution is the most exhaustive and extensive characterization of the 5S locus in chili peppers.

LOCI NUMBER AND DISTRIBUTION

Our results on the FISH location in the wild and cultivated taxa of *Capsicum* revealed a single 5S locus per haploid genome. This locus is regularly mapped onto the short arm of a unique metacentric chromosome pair of approximately the same size among taxa, with the exception of the CL of *C. flexuosum* and *C. rhomboideum*. All the analyzed taxa exhibited the 5S locus at an intercalary position, which is closer to the distal portion of the short

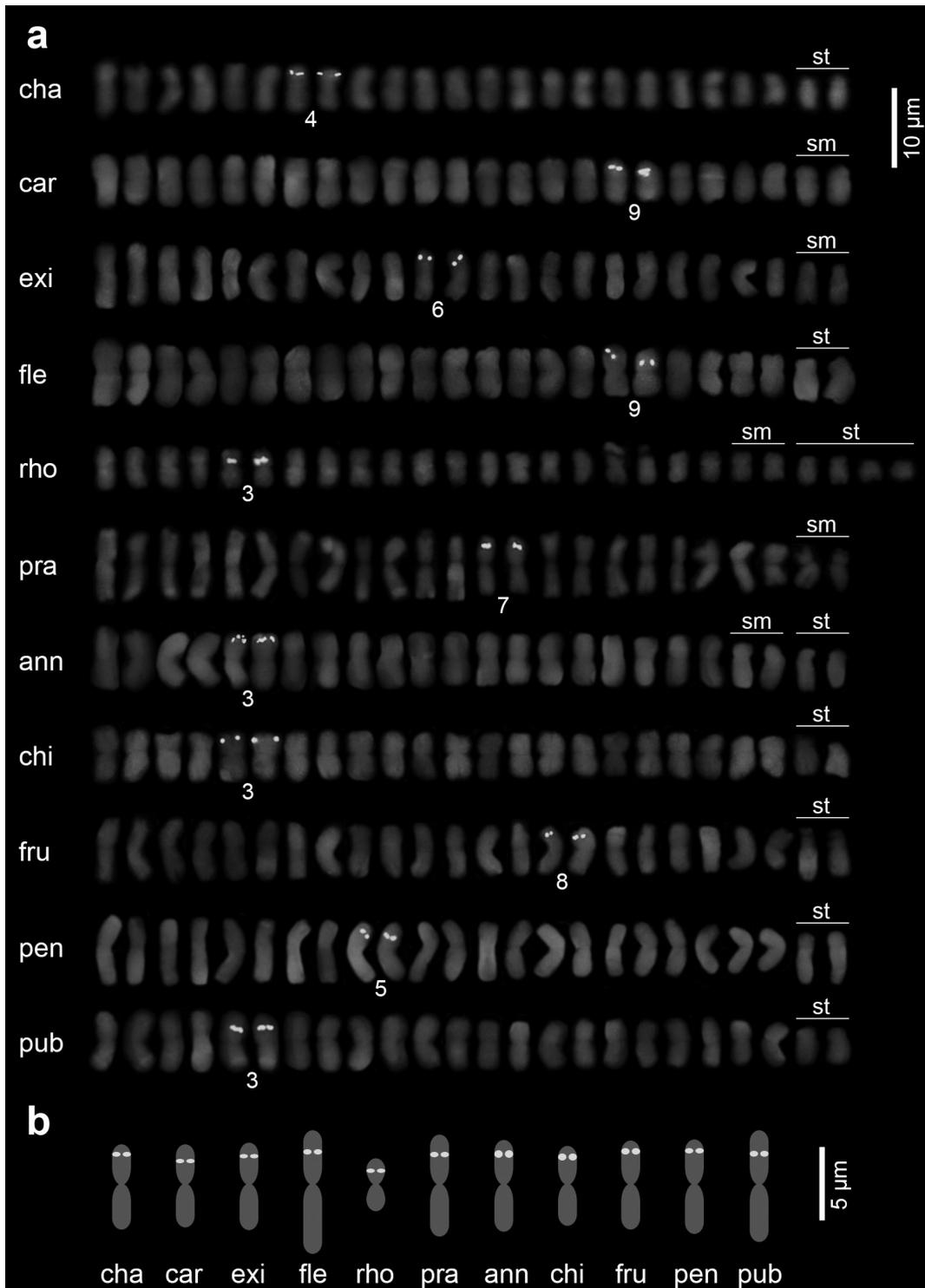


Figure 2 - Physical mapping of the 5S rDNA locus in the wild and cultivated *Capsicum* taxa by means of FISH. **(a)** Well-arranged karyograms derived from DAPI (blue) stained somatic metaphase plates showing the number and position of the 5S rDNA locus (green dots) added to further elementary cytological features; chromosomes other than metacentric (m) are solely marked (sm, st); **(b)** Schematic depiction of the chromosomes that carry the 5S rDNA locus considering the mean values described in Table I; green dots size relates to the mean fluorescence intensity. (See the colors in the on-line version).

arm, rather than to the centromere, except for the di value of *C. rhomboideum*. The metacentric chromosome pair that carries the 5S locus resulted homeologous among these taxa. These results are in agreement with previous reports in *Capsicum*. A sole and short arm intercalary 5S rDNA locus (two FISH signals) was also observed in *C. annuum* var. *annuum*, *C. chinense*, *C. frutescens*, *C. baccatum* var. *umblicatum*, *C. pubescens*, *C. chacoense* and *C. cardenasii*, all of them of distinct provenance regarding our plant material (Park et al. 1999, 2000, Scaldaferrero et al. 2006, Kwon and Kim 2009). Altogether, the combined results evidence the universality of this cytological feature in *Capsicum*. Physical location of the 5S in the same chromosome pair regarding our accessions and those of Scaldaferrero et al. (2006), was also found in *C. cardenasii* (#9), *C. pubescens* (#3) and cultivated varieties of *C. baccatum* (#5). In contrast to the latter authors who found the 5S loci at the chromosome pair #6 of *C. annuum* var. *annuum*, we detected it at #3. According to Park et al. (2000), the 5S loci in the cultivated taxa of *Capsicum* was assigned to chromosome 1 by the synteny relationship with the corresponding linkage group of tomato, of Lapitan et al. (1991). Furthermore, Kwon and Kim (2009) assigned the 5S loci of *C. annuum* to the linkage group 7 of the genetic map of Kang et al. (1997). However, the 5S loci was not mapped in the recent contributions of the genome of chili peppers (Qin et al. 2014, Kim et al. 2014).

LOCI SIZE

5S FISH signals of almost the same size and brightness intensity were observed in the studied taxa of *Capsicum*. In essence, this feature suggests similar ribosomal copy number in the 5S per genome in the genus. However, Giemsa-C and fluorescence bandings revealed a Giemsa+ and CMA+DAPI- constitutive heterochromatic region (Het) located at the short arm of a unique metacentric chromosome pair at intercalary position,

in diverse chili peppers (Moscone et al. 2007 and references therein; Scaldaferrero et al. 2013), that in fact co-localize with the 5S loci. This Het is polymorphic among taxa, regarding presence and size, suggesting variability in the 5S ribosomal copy number in *Capsicum*. In this regard, the expression level of the 5S rRNA gene is, in part, regulated by transcriptional inactivation, associated to cytosine methylation, mediated by siRNAs, as occur in the 18S-25S rRNA and other repetitive sequences. The heterochromatic state of those loci is more conspicuous at higher copy numbers (Neves et al. 2005, Pontes et al. 2006, Daxinger et al. 2009). In addition, the only report on the copy number of the 5S in *Capsicum* is that of Kwon and Kim (2009) in extended DNA fibers of *C. annuum*, who estimated it in 1453 copies per haploid genome.

5S IN SOLANACEAE

In addition to chili peppers, the family contains other economically important species such as tomato, potato, tobacco and eggplant. Considering all Solanaceae in the rDNA Plant database (www.plantrdnadatabase.com), a single 5S rDNA locus per haploid genome is common in diploid taxa, with some exceptions (Garcia et al. 2012). Particularly, the cultivated taxa exhibit a single (tomato), a single or double (petunia) and a double (tobacco, a polyploid taxon) 5S rDNA locus per haploid genome, however, this information is not available for the 5S of potato and eggplant (Garcia et al. 2012).

CONCLUSIONS

The main purpose of this contribution was to achieve a physical mapping of the 5S rDNA locus in wild and cultivated taxa of *Capsicum* by means of a genus-specific FISH probe. This cytological marker is useful to identify individual chromosomes in order to assist the cytomolecular characterization of pepper germplasm by the evaluation of inter and

intraspecific cytogenetic variability. Additionally, the information reported here can be used to integrate physical, genetic and genomic maps already generated in the genus which undoubtedly will represent a valuable tool for breeding purposes of chili peppers.

RESUMO

Neste trabalho, apresentamos o mapeamento físico do locus 5S rDNA em seis taxa silvestres e cinco cultivados de *Capsicum*, obtido através do uso de uma sonda FISH gênero-específica. Em todos os taxa, um único locus 5S por genoma haplóide foi persistentemente mapeado no braço curto de um único par de cromossomos metacêntricos na posição intercalar, e foram observados sinais 5S de FISH de tamanho e intensidade de brilho quase semelhantes. Esta é a primeira caracterização citológica dos 5S nos taxa silvestres de *Capsicum* usando uma sonda gênero-específica e o estudo sobre o grupo das pimentas mais exaustivo e abrangente realizado até ao momento. A informação aqui fornecida irá ajudar a caracterização citomolecular do germoplasma das pimentas, permitindo avaliar a sua variabilidade e podendo ser usada para integrar mapas físicos, genéticos e genômicos já realizados no gênero.

Palavras-chave: *Capsicum* cultivados e silvestres, gene de rRNA 5S, sonda derivada do *Capsicum*, mapeamento FISH.

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