Mol. Cells *27*, 205-209, February 28, 2009
DOI/10.1007/s10059-009-0025-z

Molecules and Cells

Localization of 5S and 25S rRNA Genes on Somatic and Meiotic Chromosomes in *Capsicum* Species of Chili Pepper

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The loci of the 5S and 45S rRNA genes were localized on chromosomes in five species of *Capsicum,* namely, *annuum*, *chacoense, frutescens, baccatum,* and *chinense* by FISH. The 5S rDNA was localized to the distal region of one chromosome in all species observed. The number of 45S rDNA loci varied among species; one in *annuum*, two in *chacoense, frutescens,* and *chinense,* and four in *baccatum*, with the exceptions that 'CM334' of *annuum* had three loci and 'tabasco' of *frutescens* had one locus. 'CM334'-derived BAC clones, 384B09 and 365P05, were screened with 5S rDNA as a probe, and BACs 278M03 and 262A23 were screened with 25S rDNA as a probe. Both ends of these BAC clones were sequenced. FISH with these BAC probes on pachytenes from 'CM334' plant showed one 5S rDNA locus and three 45S rDNA loci, consistent with the patterns on the somatic chromosomes. The 5S rDNA probe was also applied on extended DNA fibers to reveal that its coverage measured as long as 0.439 Mb in the pepper genome. FISH techniques applied on somatic and meiotic chromosomes and fibers have been established for chili to provide valuable information about the copy number variation of 45S rDNA and the actual physical size of the 5S rDNA in chili.

INTRODUCTION

Chili pepper or chili in short is one of the most important vegetable crops worldwide. Its genetic map, which includes 12 linkage groups, has been constructed by restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) markers (Kang et al., 1997; Lee et al., 2009). One-hundred thirty-nine polymorphic EST-SSRs were mapped onto a pepper linkage map created from an interspecific cross of *C. annuum* TF68 x *C. chinense* habanero (Yi et al., 2006).

Most *Capsicum* species have the same chromosome number, 2n = 24, composed of 11 pairs of metacentric chromosomes of similar length and a small chromosome with a satellite. Additionally, one of the metacentric chromosomes has a secondary constriction with a nucleolar organizing region (NOR). The difference in quantity or distribution of heterochromatin may permit critical discrimination among similar chromosomes by Giemsa C-banding (Moscone et al., 1993). The fluorochrome chromosome banding patterns differ among cytotypes, species, and groups of five cultivated *Capsicum* species (Moscone et al., 1996).

Ribosomal RNA genes, rDNAs, consisting of 5S and 18S-5.8S-25S (45S), are tandemly arrayed. In plants, investigation of the distribution of rDNA has provided information on relationships (Maluszynska and Heslop-Harrison, 1993), evolution (Cai et al., 2006; Kang et al., 2008; Pedrosa-Harand et al., 2006) and location (Hasterok et al., 2006; Kamisugi et al., 1994; Schondelmaier et al., 1997) of genes in interspecies comparative studies.

Using 5S and 18S-26S rDNA probes, five species of chili were karyotyped by Park et al. (1999). They reported that the loci of 18S-26S rDNA were variable in *Capsicum* species, and in *C. annuum* they were two, one on chromosome 4 and the other on chromosome 12. The 5S rDNA was localized on chromosome 1 according to the chromosome synteny in tomato (Tanksley et al., 1988).

The fluorescence in situ hybridization (FISH) technique has been widely used as a tool for investigating the complex genomes of animal and plant species. FISH is a useful technique for physical mapping of rDNA (Murata et al., 1997; Schondelmaier et al., 1997), molecular markers (Ohmido et al., 1998), and single- (Franze et al., 1996b) or low-copy sequences (Ohmido et al., 1998) and is an important part of many genome mapping projects. FISH has higher sensitivity and specificity and gives greater resolution of chromosome aberrations than is possible by banding analysis (karyotyping).

In the present study we show that 5S and 45S rDNAs can be localized on metaphase chromosomes of inter- and intra-species of *Capsicum*, meiotic pachytene and extended DNA fibers of pepper. The variability in the 5S and 45S rDNA localization patterns in five species (*C. annuum*, *C. chacoense, C. frutescens, C. baccatum,* and *C. chinense*) of *Capsicum* was investigated by simultaneous use of 5S and 25S rDNA probes.

MATERIALS AND METHODS

Plant materials and chromosome preparation

Seeds of five pepper species (Table 1) were germinated for

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Received October 27, 2008; revised November 25, 2008; accepted November 27, 2008; published online February 20, 2009

Keywords: BAC, fiber-FISH, FISH, rDNA, pachytene

Table 1. The five species of *Capsicum* used in this study and total numbers of 5S and 45S rDNA loci from FISH

Species	Cultivars or lines	Number of 45S rDNA loci (n = 12)	Number of 5S rDNA loci (n = 12)	Sources
C. annuum	chilsungcho	1	1	PMGB[1]
	hot piment	1	1	″
	CM334	3	1	″
	ECW123R	1	1	″
C. chinense	habanero	2	1	″
C. baccatum	PBC81	4	1	PBI[2]
	PI260549	4	1	″
C. frutescens	3CA124	2	1	NHRI[3]
	3CA125	2	1	″
	3CA129	2	1	″
	tabasco	1	1	″
C. chacoense	3CA87	2	1	″

[1]PMGB, Center for Plant Molecular Genetics and Breeding Research
[2]PBI, Pepper and Breeding Institute
[3]NHRI, National Horticultural Research Institute

one week on moist filter paper in the dark at 25°C in a growth chamber. When root length reached 1-2 cm, root tips were sliced and then transferred to 0.8% (v/v) bromonaphthalene for 4 h under the same conditions and fixed in ethanol-glacial acetic acid (3:1) at 4°C. Flower buds were directly fixed in the same fixative at least for 24 h at 4°C and transferred to 70% ethanol at 4°C if longer storage was required. Then root tips were washed three times in distilled water. Meristematic regions were excised and digested at 37°C for 50 min in an enzyme mixture of 2% cellulase (Sigma, USA), 1% pectinase (Sigma, USA) and 0.5% pectolyase (Sigma, USA). One or two root tips were put on each clean slide and fixed with 1:1 ethanol-glacial acetic acid. Pachytene chromosomes in meiosis were prepared from early stage anthers with one hour digestion in the same enzyme mixture and fixation in 60% acetic acid.

Nuclear isolation and preparation of extended fibers

Pepper nuclei were isolated as described by Franze et al. (1996a). Fresh young leaves were ground to a fine powder in liquid nitrogen. The nuclei were isolated with nuclei isolation buffer (10 mM Tris-HCl pH 9.5, 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 4 mM spermidine, 1 mM spermine, and 0.1% 2-mercaptoethanol) followed by filtration through 150, 120, 50, and 20 μm nylon mesh series. The nuclei were resuspended in PBS and deposited onto one end of a clean slide and air dried for 10 min at room temperature. Sixty microliters of nuclei lysis buffer (0.5% sodium dodecylsulfate, 5 mM EDTA, and 100 mM Tris pH 7.0) was added to the nuclei for 5 min at room temperature. The slides were tilted and air dried for 30 min. The extended DNA fibers were fixed with ethanol:acetic acid (3:1) for 2 min and then incubated for 30 min at 60°C. Isolated nuclei and DNA fibers were observed by staining with propidium iodide (PI; 0.2 mg/ml) or 1.5 g/ml 4-,6-diamidino-2-phenylindole (DAPI) made in Vectashield antifade solution (Vector Laboratories Inc., USA).

Probe labeling and BAC screening

The 3.4 kb 25S rDNA from soybean was labeled with digoxygenin 11-dUTP (Roche, Germany) by nick translation. The 5S rDNA probes were amplified by PCR from total pepper genomic DNA using 5S-specific primers 5′-GATCCCATCAGAACTCC-3′ and 5′-GGTGCTTTAGTGCTGGTAT-3′. The PCR products were visualized by agarose gel electrophoresis, extracted from the gel and sequenced with an ABI Prism 377 sequencing system to verify the sequences of the 5S rDNA. The 5S rDNA sequences were analyzed using the BLAST network service at the National Center for Biotechnology Information (NCBI). For FISH, the 5S rDNA was labeled with biotin 16-dUTP (Roche, Germany) by nick translation.

The pepper BAC library was hybridized with 5S rDNA and 25S rDNA genes as described by Yoo et al. (2003). Screened BAC clones that gave positive signals were selected and their ends sequenced by an ABI 3700 automatic DNA sequencer. The sequenced BAC ends were analyzed using BLAST. BAC clone DNA (1 μg) was labeled with either biotin 16-dUTP or digoxygenin 11-dUTP by nick translation using the manufacturer's protocol (Roche, Germany).

FISH

FISH was carried out as described by Ohmido and Fukui (1996) with minor modifications. The hybridization mixture contained 50% formamide (w/v), 10% dextran sulfate (w/v), 5 ng/μl of sheared salmon sperm DNA and probe DNA labeled with biotin 16-dUTP or digoxygenin 11-dUTP (Roche, Germany) in 2× SSC. After hybridization over night, the slides were washed twice in 2× SSC for 5 min, once in 50% formamide/2× SSC for 10 min, twice in 2× SSC, and once in 4× SSC /0.2% Tween 20 at 42°C. To detect the probes, fluorescein avidin DCS (Vector Laboratories Inc., USA) and anti-avidin-D (Vector Laboratories Inc., USA) or rhodamine anti-digoxygenin (Roche, Germany) and Texas Red anti-sheep IgG (Vector Laboratories Inc., USA) were added on slides for 1 h at 37°C in 1% bovine serum albumin or goat serum. After counterstaining with DAPI (1 mg/ml), slides were examined with a fluorescence microscope containing a Cool SNAP CCD camera (Delta Vision, USA).

All images were pseudo-colored and improved for optimal brightness and contrast using Adobe Photoshop. For fiber length analysis, we measured the chromosome length using Softworks S/W (Delta Vision, USA).

RESULTS

The loci of the 5S and 45S rRNA genes were localized on somatic chromosomes of five species of chili pepper, *Capsicum annuum*, *C. chacoense, C. frutescens, C. baccatum,* and *C. chinense*, to estimate the variation in the number and position of those genes. The results were compared among interspecies as well as intraspecies of *Capsicum*. The cultivars or lines of *Capsicum* species used in the study and the numbers of 45S and 5S FISH signals are summarized in Table 1. Figure 1 shows the location of 5S and 45S rDNAs on metaphase chromosomes for each species. The 5S rDNA (green signal) from pepper was located on the distal region of middle chromosomes in all samples (Fig. 1). Most of the loci of 45S rDNA were at the ends of chromosomes and at the NOR as described by Park et al (1999). In order to compare variation of rDNA loci within species, FISH was conducted to position the ribosomal RNA genes on metaphase chromosomes of four accessions of cultivated *C. annuum*. In *C. annuum* (Fig. 1, A1-A4), one pair of intense 5S signals (green) was detected on chromosome 1-related linkage group 5. The signals for 45S rDNA (Fig. 1) were detected on secondary constrictions, known as NORs, as red fluorescence from the rhodamine and Texas Red-conjugated antibodies. However, for *C. annuum* CM334,

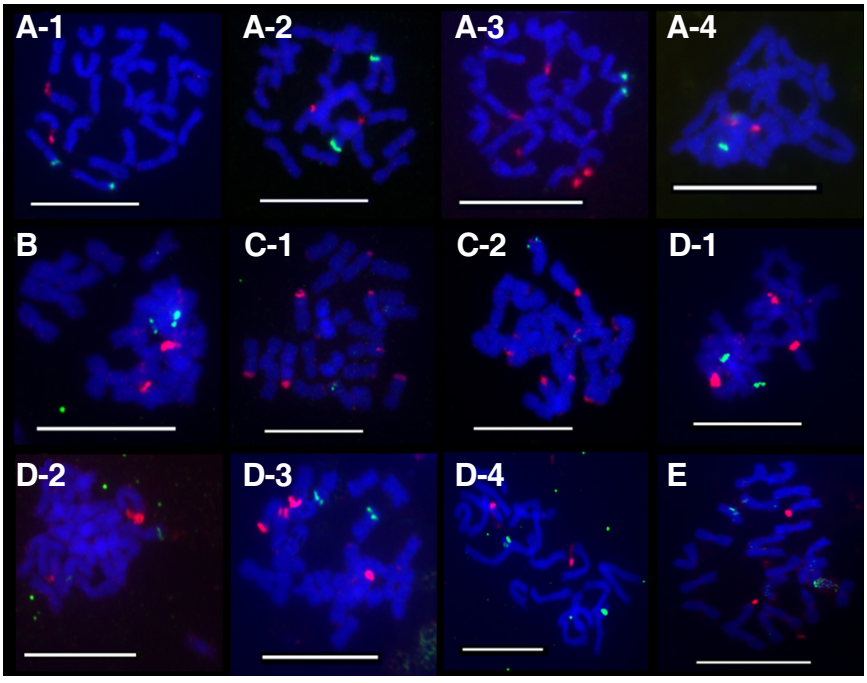

Fig. 1. FISH with 5S (green) and 25S (red) ribosomal DNA probes to somatic metaphase chromosomes of *C. annuum* (A1: chilsungcho, A2: hot piment; A3: CM334 and A4: ECW123R), *C. chinense* (B: habanero), *C. baccatum* (C1: PBC81 and C2: PI260549), *C. frutescens* (D1: 3CA124, D2: 3CA125, D3: 3CA129 and D4: tabasco), *and C. chacoense* (E: 3CA87). The scale bar = 15 μm.

the 45S rDNA loci were also on the proximal region in the long arms in two chromosomes and NOR region of a telocentric chromosome.

In *C. chinense* (Fig. 1B), two pairs of 45S rDNA loci were detected; one at the NOR site of its bearing chromosome and another at the distal region of one arm of a chromosome. In *C. baccatum* (Fig. 1, C1-C2), four pairs of 45S rDNA loci were detected; one at the NOR region of its bearing chromosome and the others at the distal regions of one arm of three different chromosomes. In *C. frutescens* (Fig. 1, D1-D4), two pairs of 45S rDNA loci were detected; one at the NOR region of its bearing chromosome and another at the distal region of one arm of a chromosome, but 'tabasco' showed only one pair of 45S rDNA loci. In *C. chacoense*, two pairs of 45S rDNA loci were detected; one at the NOR region of its bearing chromosome and another at the telocentric region of a chromosome. The 45S rDNA FISH signal patterns from five *Capsicum* species differed even among cultivars within *C. annuum* and *C. frutescens*.

Using 5S rDNA as a probe, two BAC clones (384B09 and 365PO5) were selected by screening a *C. annuum* 'CM334'-derived BAC library. BAC end sequencing showed that BACs 384B09 and 365P05 consisted of a 302-bp tandem repeat of 5S rDNA, which showed a 84% sequence homology to a 346-bp repeat of 5S gene from *C. annuum* 'Geumtap'. Using 25S rDNA as a probe BAC clones 278M03 and 262A23 were selected and were demonstrated to contain part of the 45S rDNA.

Pachytene chromosome preparation has been established in this study for rather recalcitrant chili pepper. Almost 80% of the pachytenes were heterochromatic regions as they were stained bright with DAPI, while the euchromatic regions at the end of pachytenes were stained weakly. FISH signals with 5S rDNA-containing BAC to spreads of pachytene and diakinesis in meiosis showed the same pattern with those of mitotic metaphase chromosomes (Figs. 2A-2D). A single green signal was detected on a specific spot of a diakinesis chromosome (Fig. 2A). A strong green signal the size of which was approximately 2.66 μm was located on a pachytene which was 39.38 μm in length (Figs. 2B-2D). This FISH signal encompassed about 6.57% of this pachytene chromosome length.

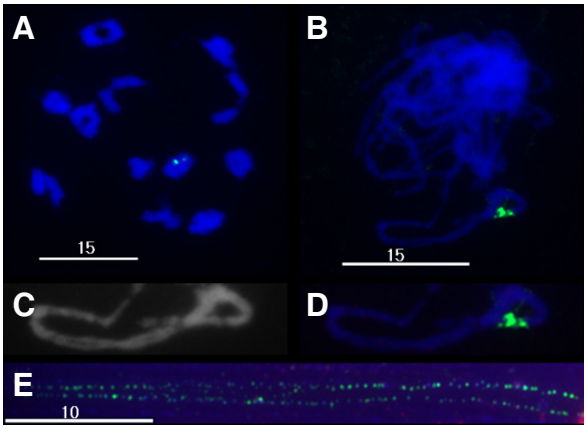

Fig. 2. FISH with 5S rDNA BAC 384B09 (green) to meiotic chromosomes and extended DNA fiber of *C. annuum* 'CM334'. (A) diakinesis, (B-D) pachytene, (C) DAPI and (E) extended DNA fiber.

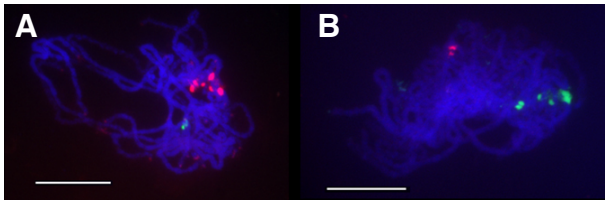

Fig. 3. (A) FISH with 5S rDNA genes (green), 25S rDNA genes (strong red signal) and rice telomere BAC clone (weak red signal on telomere) on pachytene of *C. annuum* 'CM334'. (B) FISH with 5S rDNA BAC clone 384B09 (red) and 45S rDNA BAC clone 278M03 (green). The scale bar = 15 μm.

Single extended DNA fibers were prepared to estimate the size of the 5S rDNA in pepper genome. The signals from a 5S rDNA probe labeled with biotin were observed as green fluorescent strings (Fig. 2E). Fiber FISH with the 5S rDNA probe showed an average signal size of 134.27 μm (STDEV = 14.83, N = 12). Assuming a stretching degree of approximately 3.27 kb/μm (Franze et al., 1996a), the size of the 5S rDNA was estimated to be approximately 0.439 Mb in the pepper genome. Thus these cytological methods provide valuable information about the actual physical size of 5S ribosomal RNA genes in chili pepper.

One 5S rDNA site represented by a green signal (Fig. 3A) on pepper pachytenes were detected, but 45S rDNA sites represented by strong red signals (Fig. 3A) were located on a wider area. The ends of pachytenes were confirmed with a rice telomere BAC, represented by weak red signals on the telomeres.

FISH with BAC 384B09 obtained by screening with 5S rDNA and BAC 278M03 obtained by screening with 25S rDNA is shown in Fig. 3B. The patterns of signal using these BAC clones were similar to ones obtained by FISH with those rDNA genes.

DISCUSSION

Karyotyping of *Capsicum* species has revealed that chromosomal morphology is highly conserved within the genus. For example, most of the chromosomes have similar lengths and arm ratios, so karyotyping of *Capsicum* has been difficult (Moscone et al., 1996; Park et al., 1999). The use of multicolored FISH has facilitated the identification of homologous chromosomes and the inference of genome evolution among plant species. In this study, multi-colored rDNA FISH facilitated visualization of rDNA loci on somatic chromosomes from five pepper species, meiotic pachytene, and extended DNA fibers of pepper. All experimental conditions such as types of rDNA, hybridization conditions, and washing stringency were identical. In this study, only strong signals were counted and ambiguous end signals were avoided.

FISH on chromosomes of four cultivars of *C. annuum* demonstrated that there was intraspecies variability in 45S rDNA loci, especially with 'CM334', a cultivar possessing broad resistance to virus and fungi. The sizes of chromosomes and karyotypes were similar in *C. annuum* cultivars with one 45S rDNA locus except 'CM334' which contained three loci on three different chromosomes.

The 45S rDNA loci of *C. chinense, C. frutescens* and *C. chacoense* were present in two pairs, while those of *C. baccatum* were in four pairs and *C. annuum* were in single pair (Fig. 1, Table 1). The phylogenetic relationship of 5S rDNA sequences among the five *Capsicum* species are in agreement with the evolution of the *Capsicum* species (Park et al., 2000). *C. chinense, C. frutescens,* and *C. annuum* form one lineage. *C. baccatum* is an intermediate species. It has been suggestive that the 45S rDNA in *Capsicum* is a rich source of copy number variation along the course of evolution and might offer an excellent experimental system for studying genome rearrangements accompanying its functional divergence.

The 5S rDNA in chili pepper was located on the distal region in one arm of a chromosome, and the variations in inter- and intraspecies were not shown as well as in pachytene and diakinesis. However, the 45S rDNA loci varied in inter- and intraspecies of *Capsicum* and located at the ends of chromosomes. Hanson et al. (1996) reported that species with terminal 45S rDNA sites would show a greater degree of concerted evolution, a higher number of loci and more variability in locus number and size between and within species than species with interstitial loci in cotton. The terminal position of the 45S rDNA loci is an important condition in evolution, because it allows frequent rearrangement to occur without disrupting other gene linkages (Hanson et al., 1996). Differentiation in 45S rDNA distribution patterns among *Capsicum* species and within a species might be caused by several mechanisms such as chromosome inversion and/or translocation, or amplification of satellite DNA or dispersion of rDNA repeats.

We screened a fraction of the pepper BAC library with 5S and 25S rDNA probes to select BAC clones. The BAC clones selected with the 5S rDNA probe generated distinct signals from a single pair of somatic metaphase chromosomes (data not shown) or a single meiotic pachytene chromosome (Fig. 2B) and diakinesis chromosome (Fig. 2A). This FISH signal provided a cytological marker for chromosome identification in chili pepper. The 1 μm size of FISH signals on 11.2 μm somatic metaphase chromosomes constitutes about 8.9%. The average 2.66 μm signal of 5S rDNA on a 39.38 μm pachytene constitutes about 6.75% (Figs. 2C-2D), which is suggestive of improvement of resolution.

Resolution of the 5S rDNA size can be enhanced greatly the extended DNA fibers (Fig. 2E). An average FISH signal size 134.27 μm of the 5S rDNA stretch in the fiber can be translated into about 439 kb of the 5S rDNA stretch which constitutes about 0.016% of the pepper haploid genome size of 2,702 Mbp/C (Moscone et al., 2003). The 302 bp tandem 5S rDNA repeats found in the 'CM334' in the 439 kb stretch means that there are about 1,453 copies of the 5S rDNA repeat units in pepper haploid genome. With regard to the 25S rDNA signal, such measurements and calculations were difficult due to the variations in size and length of the signals in fiber FISH.

Pepper karyotypes have been reported based on somatic metaphase chromosomes (Moscone et al., 1999; Park et al., 1999), but not pachytene chromosomes probably due to pepper genome complexity and plant characteristics. DAPI binds to AT-rich regions and, therefore, is the most common dye to stain heterochromatic regions. But in pepper it is difficult to accurately measure the length of DAPI-stained pachytene based on DAPI staining alone. Euchromatic regions of pepper staining weakly with DAPI are not only located at the ends of pachytenes but also dispersed in between heterochromatic regions. In contrast, tomato with haploid genome size 980 Mb contain long, contiguous stretches of euchromatin at the distal ends of most chromosomes and heterochromatic regions flank the centromeres (De Jong, 1998; Peterson et al., 1998).

The tandem arranged 5S rDNA of chili pepper was typically observed as a ladder banding pattern. Polymorphic fragments were visible with *Dra*I-digested DNA. For linkage analysis, Southern hybridization was done on 103 F_2 plants of a mapping population. The 5S and 25S rDNA were used as dominant RFLP markers. Linkage analysis revealed that the 45S rDNA locus was mapped to a cluster of AFLP and RFLP markers on linkage group 5 in genetic map (Kang et al., 1997). The 5S rDNA localized to linkage group 7 on the same genetic map. The 5S rDNA and 45S rDNA were located by FISH on the chromosomes consistent with the genetic map.

Physical and genetic maps are basic tools needed to understand a genome. The physical map displays the physical location of genes and other physical markers along the length of the chromosomes. On the other hand, the genetic map is based on chromosome linkage groups on which phenotypic traits, RFLP, random amplified polymorphic DNA (RAPD), AFLP, microsatellite and other markers are located. Genetic map distance is given by genetic or recombination distance between the mark-

ers on each chromosome. Even though physical and genetic locations are known, there is often little correlation between the separation distances of markers on the two types of maps. Knowledge of the physical distances is important for cloning strategies involving cloning or walking between an RFLP marker and a linked gene. Currently physical mapping technologies are beginning to link the maps more closely and to answer questions about gene localization.

Karyotyping will be facilitated by developing cytological-specific markers to distinguish among the chromosomes by BAC FISH using screened BAC clones containing molecular markers. In future studies BAC clones and RFLP markers will be localized on the chromosomes by FISH. Once enough FISH markers are located for each individual chromosome, alignment of the linkage map of the DNA markers with cytological markers will greatly be facilitated.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Myeong Cheoul Cho, National Horticultural Research Institute, and Dr. Jae Bok Yoon, Pepper and Breeding Institute, for seeds of *Capsicum* species. We also thank the National Instruments Center for Environmental Management for sequencing and imaging. This study was supported by grants from the Korea Science and Engineering Foundation and the Korea Ministry of Education, Science and Technology through the Center for Plant Molecular Genetics and Breeding Research.

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