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ORIGINAL PAPER

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A high-resolution karyotype of *Brassica rapa* ssp. *pekinensis* revealed by pachytene analysis and multicolor fluorescence in situ hybridization

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Abstract A molecular cytogenetic map of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*, 2*n*=20) was constructed based on the 4′-6-diamino-2-phenylindole dihydrochloride-stained mitotic metaphase and pachytene chromosomes and multicolor fluorescence in situ hybridization (McFISH), using three repetitive DNA sequences, 5S rDNA, 45S rDNA, and C11-350H. The lengths of mitotic metaphase chromosomes ranged from 1.46 μm to 3.30 μm. Five 45S and three 5S rDNA loci identified were assigned to different chromosomes. The C11-350H loci were located on all the mitotic metaphase chromosomes, except chromosomes 2 and 4. The pachytene karyotype consisted of two metacentric (chromosomes 1 and 6), five submetacentric (chromosomes 3, 4, 5, 9 and 10), two subtelocentric (chromosomes 7 and 8), and one acrocentric (chromosome 2) chromosome(s). The mean lengths of ten pachytene chromosomes ranged from 23.7 μm to 51.3 μm, with a total of 385.3 μm, which is 17.5-fold longer than that of the mitotic metaphase chromosomes. In the proposed pachytene karyotype, all the chromosomes of *B. rapa* ssp. *pekinensis* can be identified on the basis of chromosome length, centromere position, heterochromatin pattern, and the location of the three repetitive sequences. Moreover, the precise locations of the earlier reported loci of 5S rDNA, 45S rDNA, and Chinese cabbage tandem DNA repeat C11-350H were established using McFISH analysis. We also identified a 5S rDNA locus on the long arm of pachytene bivalent 7, which could not be detected in the mitotic metaphase chromosomes in the present and earlier studies. The deduced karyotype will be useful for structural and functional genomic studies in *B. rapa*.

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Introduction

The genus *Brassica* is one of the genera in the subtribe Brassicinae and includes a number of crops with wide adaptation and uses. Of the different cultivated *Brassica* species, *B. rapa* consists of a number of vegetables such as *Pak-choi*, Chinese cabbage, etc. Chinese cabbage (*B. rapa* ssp. *pekinensis*) is one of the most widely used and versatile vegetable crops (dried, pickled or cooked).

Determination of chromosome number of *B. rapa* (2*n*=20) by Takamine (1916) made the beginning of cytogenetic research in *Brassicas*. U (1935) investigated and established the relationship among *B. campestris*, *B. oleracea*, *B. nigra* and their amphidiploids. Since then, chromosomes of *Brassica* species have been extensively studied. Earlier, karyotypes of *B. rapa* were based on simple morphometric measurements of Giemsa-stained mitotic metaphase or pro-metaphase chromosomes (Nishibayasahi 1992; Olin-Fatih and Heneen 1992; Olin-Fatih 1994; Cheng et al. 1995). However, the small size of Brassica chromosomes makes it difficult to identify homologues and distinguish different chromosomes to generate a karyotype. These limitations were partially overcome, and prometaphase chromosomes were characterized by their condensation pattern profiles and fluorescence in situ hybridization (FISH) pattern, using a 45S rDNA probe. This led to the development of a karyotype of *B. rapa* by Fukui et al. (1998). Subsequently, Snowdon et al. (2002) used the FISH technique for chromosomal localization of 25S rDNA and 5S rDNA loci and proposed a karyotype of *B. rapa*.

Several researchers have used FISH to map DNA sequences on *B. rapa* chromosomes (Snowdon et al. 1997;

Fukui et al. 1998; Iwano et al. 1998; Hasterok et al. 2001; Snowdon et al. 2002). The majority of these reports pertain to map DNA sequences on mitotic metaphase chromosomes. FISH analysis, using mitotic metaphase chromosomes, has low resolution and provides limited details regarding cytological structure of the *B. rapa* genome, although it is easy to prepare a large number of mitotic chromosome spreads. Moreover, the material (root tips) for mitotic analysis can be obtained any time by germinating seeds in contrast to pachytene analysis, which requires fixing of meiocytes during the short period of flowering (Sadder and Weber 2001). On the other hand, the extended bivalents at pachytene provide excellent material to develop a physical map for species with small chromosomes (Fransz et al. 1998, 2000; Cheng et al. 2002). Successful application of FISH to pachytene chromosomes has been reported in tomato (Zhong et al. 1996), rice (Cheng et al. 2001), and *Medicago truncatula* (Kulikova et al. 2001). However, molecular cytogenetic studies using pachytene chromosome have not been reported in *B. rapa*.

Recently, it has been proposed to embark on the Brassica Genome Sequencing Project (http://brassica.bbsrc.ac.uk/brassica_genome_sequencing_concept_htm). In this multinational project, the genome of *B. rapa* ssp. *pekinensis*, representing the A genome, will be sequenced. A BAC library has already been constructed in inbred line 'Chiifu'. Here, we present a pachytene karyotype of *B. rapa*. The chromosomes were stained with 4′-6-diamino-2-phenylindole dihydrochloride (DAPI) and their fluorescence images used for karyotype analysis on the basis of morphometric data and heterochromatin patterns. In addition, a pericentromeric repeat C11-350H, isolated by *Hin*dIII restriction digestion of *B. rapa* BAC clone KBrH77C11, and 45S and 5S rDNAs were used as probes in multicolor fluorescence in situ hybridization (McFISH) to facilitate chromosome identification.

Materials and methods

Plant material

B. rapa spp. *pekinensis* inbred line 'Chiifu' was used for analyzing mitotic metaphase and pachytene chromosomes. Seeds were germinated on moist filter paper in petri dishes at 28°C for 48 h. About 1-cm-long root tips were harvested from the germinating seeds, pre-treated in saturated 1-bromonaphthalene at room temperature for 2 h, and fixed in a 1:3 (v/v) acetic acid:ethanol for 1–2 days.

Chromosome preparation

Fixed root tips were washed in distilled water for 10 min. The meristematic portions of the root tips were excised and incubated in 2% cellulase (Onozuka, cat. no. 201064), 1.5% macerozyme (Yakult, cat. no. 202025), 1% pectolyase (Sigma), and 1 mM EDTA at 37°C for 40 min, followed by washing in distilled water for 20 min. These root tips were then transferred to a clean glass slide and macerated with a drop of ethanol:acetic acid (3:1, v/v) using a fine-pointed forceps. After air drying, the slides were stored at −20°C.

For separating pollen mother cells (PMCs), the method of Fransz et al. (1998) was used with some modifications. Immature flower buds were fixed in ethanol:acetic acid (3:1) for 2 h and stored at 4°C. The flower buds were rinsed in distilled water and incubated in an enzyme mix (0.3% pectolyase, 0.3% cytohelicase, and 0.3% cellulase) in citrate buffer (10 mM sodium citrate, pH 4.5) for 2 h. Each bud was softened in 60% acetic acid on a non-coated and ethanol-cleaned microscopic slide kept at 45°C on a hot plate. The contents were smeared on the slide, fixed with ice-cold ethanol:acetic acid (3:1) and dried.

McFISH

A clone, pTa71, containing a 9.1-kb fragment of 18S–5.8S–25S rRNA genes (45S rDNA) of *Triticum aestivum* L. (Gerlach and Bedrock 1979) was labeled with biotin16-dUTP, using the Nick Translation kit (GIBCO–BRL). The 5S rDNA probe was amplified with a specific primer pair (Koo et al. 2002) and labeled with digoxigenin-11-dUTP (Roche). The clone C11-350H, containing a 350-bp tandem repeat, was isolated from *B. rapa* ssp. *pekinensis* BAC clone KBrH77C11 after restriction digestion with *Hin*dIII (D.H. Koo et al., unpublished data), and labeled with digoxigenin-11-dUTP or biotin 16-dUTP by PCR with M13 primer (Invitrogen).

The FISH procedure applied to both mitotic and meiotic chromosomes was the same as previously reported by Koo et al. (2002). In brief, chromosomal DNA on the slides was denatured with 70% formamide at 70°C for 2.5 min, followed by dehydration in a 70, 85, 95 and 100% ethanol series at −20°C for 3 min each. The probe mixture, containing 50% formamide (v/v), 10% dextran sulfate (w/v), 5 ng/μl salmon sperm DNA, and 50 ng/μl of labeled probe DNA, was heated at 90°C for 10 min and kept on ice for 5 min. A volume of 20 μl of this mixture was applied to the denatured chromosomal DNA and covered with glass coverslip. Slides were then placed in a humid chamber at 37°C for 18 h. Probes were detected with avidin-FITC and anti-digoxigenin Cy3 (Roche). Chromosomes were counterstained with 1 μg/μl DAPI (Sigma). The signals were detected with a Cooled CCD Camera (CoolSNAP, Photometrics). Images were processed with software (Meta Imaging Series, version 4.6), using Leica epi-fluorescence microscope equipped with FITC-DAPI two-way or FITC-Rhodamine-DAPI three-way filter sets (Leica, Japan). The final printed images were prepared with the Adobe Photoshop, version 7.0, program.

Karyotype preparation

Mitotic analysis

Mitotic metaphase preparations with well-spread chromosome were probed with three repetitive DNA sequences (45S rDNA, 5S rDNA, and C11-350H) and counterstained with DAPI. These preparations were also used for repeated probing, i.e., after the first round of probing and image capturing, the slides were soaked in a 4× SSC/0.2% Tween 20 to remove the coverslips. The slides were then air-dried, denatured in 70% formamide at 70°C for 2 min, dehydrated in cold ethanol series, and incubated with another probe for FISH analysis. Nine mitotic metaphase spreads were used for measuring the lengths of chromosomes. The captured images of the chromosomes were measured directly on the screen, using FISH Image System (Meta Imaging Series). The homologues of mitotic chromosomes were identified based on FISH signal and chromosome lengths, and the chromosomes were ordered according to their decreasing lengths.

Pachytene analysis

In order to identify the pachytene chromosomes of *B. rapa*, PMCs were analyzed by DAPI staining and McFISH with 45S rDNA, 5S rDNA and C11-350H. The images of six to ten pachytene bivalents, nearly at the same stage, were captured from different PMCs to study the distribution of heterochromatin and position of FISH signals and lengths of pachytene chromosomes, using FISH Image System (Meta Imaging Series), without discriminating heterochromatin and euchromatin.

The pachytene karyotype was prepared based on the data on morphological parameters of chromosomes, coupled with localization of the repetitive DNA probes on mitotic metaphase chromosomes. Chromosome morphology was established following Levan et al. (1964). The chromosomes were arranged in order of decreasing length to make the karyotype.

Table 1 Average lengths of mitotic metaphase chromosomes of *B. rapa* ssp. *pekinensis*

Chromosome no.	Average length (μm±SD)
1	3.30±0.21
2	2.89±0.25
3	2.53±0.17
4	2.35±0.16
5	2.20±0.21
6	2.00±0.20
7	1.84±0.12
8	1.73±0.16
9	1.62±0.15
10	1.46±0.14

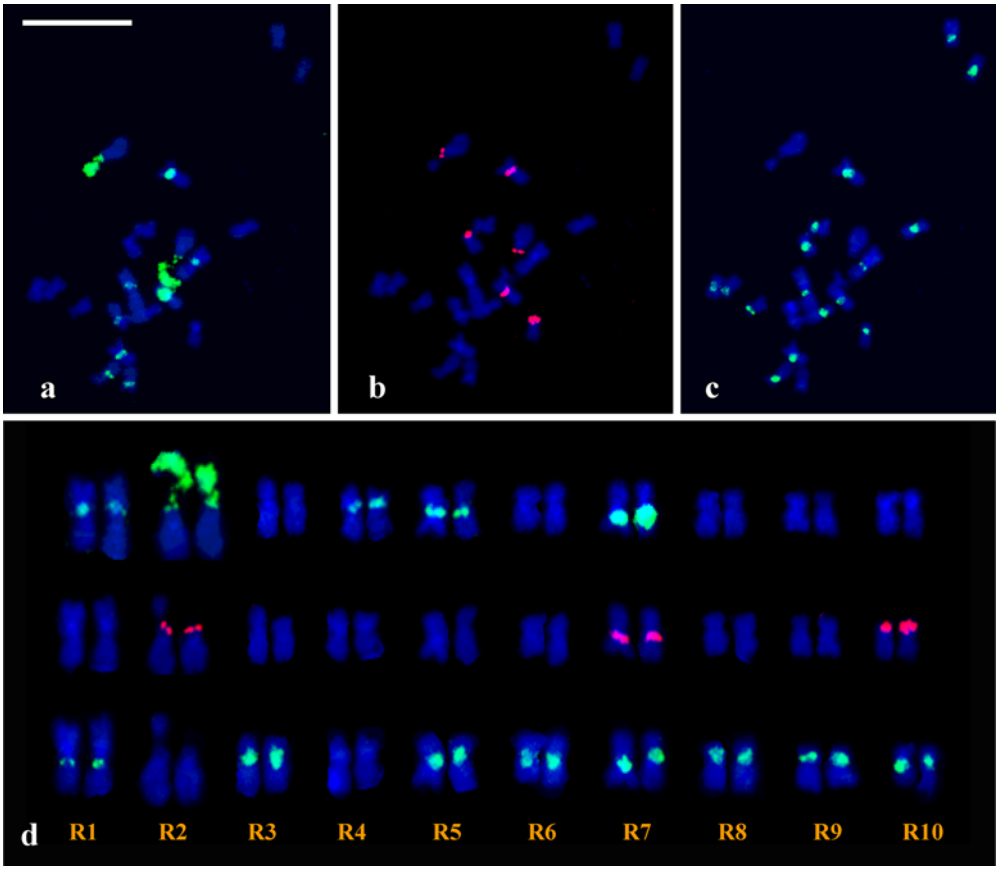

Fig. 1a–d Fluorescence in situ hybridization (FISH) mapping of 45S rDNA, 5S rDNA, and the C11-350H repeat on the mitotic metaphase chromosomes of *B. rapa* spp. *pekinensis*. Mitotic metaphase chromosomes hybridized with 45S rDNA (**a**), 5S rDNA (**b**), and C11-350H (**c**). Karyotype of mitotic metaphase chromosomes showing FISH pattern with 45S rDNA (*Row 1*), 5S rDNA (*Row 2*), and C11-350H (*Row 3*) (**d**). *Bar* represents 10 μm

Results and discussion

Mitotic analysis

A partial karyotype of *B. rapa* was constructed based on the analysis of mitotic metaphase chromosomes, which were hybridized with three repetitive sequences (45S rDNA, 5S rDNA, and C11-350H) and counter-stained with DAPI. The average lengths of the mitotic metaphase chromosomes ranged from 1.46 μm to 3.30 μm (Table 1). However, we could not measure the chromosome arms-length ratios, because the precise location of the centromeres could not be established. The small chromosome size of mitotic metaphase chromosomes of *B. rapa* has precluded intensive studies of chromosome morphology. Previously, Cheng et al. (1995) reported that length of mitotic prometaphase of *B. rapa* chromosomes ranged from 2.2 μm to 5.2 μm. However, Kim et al. (1998) found that lengths of mitotic metaphase chromosomes ranged from 1.01 μm to 2.06 μm.

We performed FISH, using three different repetitive DNA probes, 45S rDNA, 5S rDNA, and C11-350H. The signals for 45S rDNA were detected in the pericentromeric regions of chromosomes 1, 2, 4, 5, and 7 (Fig. 1a). The same number of 45S rDNA loci on mitotic prometaphase chromosomes was earlier reported by Fukui et al. (1998) and Snowdon et al. (2002). Fukui et al. (1998) could also assign the localization of 45S rDNA loci to long/short chromosomal arms. The 5S rDNA loci were located on chromosomes 2, 7, and 10 (Fig. 1b). These results are in agreement with those of Snowdon et al. (2002). A tandem repeat, C11-350H, associated with pericentromeric heterochromatin, was derived by *Hin*dIII restriction digestion of *B. rapa* ssp. *pekinensis* BAC clone, KBrH77C11. The sequencing of this 350-bp restriction fragment revealed 87% homology to pBcKB4 (data not shown). The latter was demonstrated to map on the centromeric region of seven to nine mitotic metaphase chromosomes of Chinese cabbage (Harrison and Heslop-Harrison 1995). The 350-bp fragment (C11-350H) used in the present study gave signals on all the chromosomes of *B. rapa* ssp. *perkinensis*, except chromosomes 2 and 4 (Fig. 1c). However, Iwabuchi et al. (1991) reported the localization of another repeat sequence, pBT 11 (homologous to pBcKB4), in the pericentromeric region of all the mitotic metaphase chromosomes of *B. rapa*. It is pertinent to mention that FISH-based localization of loci of three repetitive DNA elements were deduced by using the same mitotic metaphase spreads. The karyotype deduced by arranging the chromosome on the basis of their decreasing lengths and depicting the distribution of 45S rDNA, 5S rDNA, and C11-350H loci on different chromosomes is shown Fig. 1d.

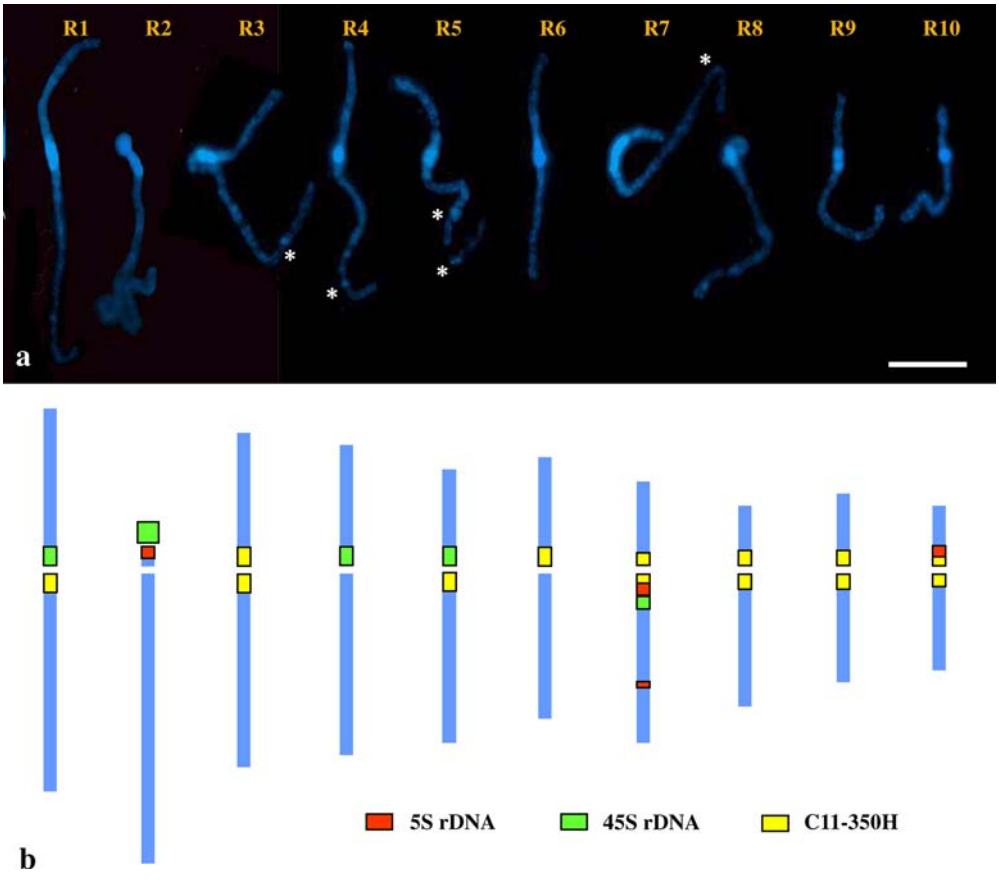

Fig. 2 **a** Karyotype of *B. rapa* spp. *pekinensis*, showing representative pachytene bivalents of 1–10 chromosomes, digitally sorted out from 4′-6-diamino-2-phenylindole dihydrochloride-stained pachytene spreads. Brightly stained regions in each bivalent represent pericentromeric heterochromatin. The short arm of chromosome 2 contains a nucleolus organizing region site. *Asterisks* denote location of heterochromatin regions. The *bar* represents 10 μm. **b** Idiogram showing the physical locations of the 5S rDNA, 45S rDNA, and C11-350H loci

Pachytene analysis

Chromosome measurements

Generally, it is difficult to obtain well-spread pachytene preparations in *Brassica* species. Although ten brightly stained, pericentromeric heterochromatic blocks were occasionally observed in single cell, it was difficult to identify individual pachytene bivalents. Nevertheless, one to three bivalents are frequently separated in most of the pachytene preparations. Furthermore, contours such bivalents could be traced from one end to the other in pachytene preparations. These characteristics were helpful in identifying individual pachytene chromosomes in different meiocytes and preparing molecular cytogenetic map.

Figure 2a shows representative pachytene bivalents of the ten DAPI-stained chromosomes. The corresponding measurements of pachytene chromosomes are given in Table 2. The pachytene karyotype comprised two median, five submedian, two subtelocentric, and one acrocentric chromosome(s), with the corresponding centromeric index ranges of 38.8–41.0%, 29.5–36.7%, 17.4–20.2%, and 9.38%. The mean lengths of chromosomes at pachytene varied from 23.7 μm to 51.3 μm, with a total length of 385.3 μm. This is 17.5-fold longer in comparison to the length of mitotic metaphase chromosomes, which ranged from 1.46 μm to 3.30 μm in the present study. On the other hand, pachytene:metaphase chromosome length ratios are reported to be 10-fold longer in maize, 15-fold in tomato, 20-fold in *Arabidopsis thaliana*, and 40-fold in rice (de Jong et al. 1999). In addition, the chromosomes at pachytene exhibit differentiated heterochromatic and euchromatic regions (Zhong et al. 1996; Fransz et al. 1998; de Jong et al. 1999).

DAPI binds preferentially to AT-rich regions (Plader et al. 1998) and is the most common dye to study heterochromatin distribution. Heterochromatic segments are very distinct at the pachytene stage, when the homologues are fully paired, forming traceable linear structures (Fransz et al. 2003). Brightly fluorescing heterochromatic blocks were detected in the pericentromeric regions of all the chromosomes (Fig. 2a), although their lengths were different. In addition to the pericentromeric localization, small heterochromatic regions were observed on the long arms of chromosomes 3, 4, 5, and 7 (asterisks in Fig. 2a). These heterochromatic regions were not detected in the mitotic metaphase chromosomes. The total length of all heterochromatic regions was 38.2 μm, which is approximately 10% of the total length of pachytene chromosomes (Table 2). This indicates that the major part of the chromosomes consisted of euchromatin. These results are in close agreement with those obtained in *A. thaliana* (Fransz et al. 1998), where 7.4% of the total chromosome length was composed of heterochromatin. However, in *M. truncatula* (Kulikova et al. 2001) and tomato (de Jong et al. 1999) 15% and 24%, respectively, of the total pachytene chromosome length was heterochromatic.

McFISH analysis

Distinctly separated bivalents from pachytene cells were used for FISH analysis. The data on localization of three repetitive elements on mitotic metaphase chromosomes was useful in identifying the corresponding pachytene bivalents. The FISH results revealed that the 5S rDNA loci were located on pericentromeric region of short arm of chromosomes 2 and 10, and the long arm of chromosome 7 (Figs. 2b, R2, R7, R10; 3, R2, R7, R10). Moreover, the long arm of chromosome 7 had another 5S rDNA site

Table 2 Average lengths, centromeric index, heterochromatin (percentage), and positions of 5S rDNA, 45S rDNA, and C11-350H on pachytene chromosomes of *B. rapa* ssp. *pekinensis*

Chromosome no.[a]	Parameters						
	Chromosome length (μm)		Centromeric index[b]	Heterochromatin (%)	Fluorescence in situ hybridization signal[c]		
	Range	Mean			5S rDNA	45S rDNA	C11-350H
1	46.0–66.0	51.3±7.45	38.8±1.59	6.37±1.44	–	S	L
2	45.1–53.3	48.0±3.05	9.38±0.91	11.5±1.14	S	S	–
3	38.8–55.8	47.1±6.20	29.5±2.78	6.37±0.78	–	–	L+S
4	37.9–47.7	41.4±3.64	30.4±3.26	6.04±0.66	–	S	–
5	34.2–47.8	40.8±6.16	32.3±2.18	14.7±1.84	–	S	L
6	34.0–40.4	36.8±2.49	41.0±4.66	13.5±1.07	–	–	S
7	32.4–42.2	36.8±4.41	17.4±4.73	16.9±1.57	L[d]	L	L+S
8	28.8–37.6	32.7±3.18	20.2±2.08	7.65±0.93	–	–	L+S
9	25.6–33.2	27.0±2.62	36.7±1.94	9.26±1.02	–	–	L+S
10	18.5–27.0	23.7±2.97	32.9±2.43	7.38±1.13	S	–	L+S

[a]Chromosomes arranged in order of decreasing length
[b]Centromeric index is the percentage of short arm/total chromosome length (Levan et al. 1964)
[c]*S* short arm, *L* long arm, – no locus
[d]Two loci
± indicates SD

(Fig. 3, R7) that could not be detected in the mitotic metaphase spreads (Fig. 1b). Extended pachytene chromosomes have been reported to provide a higher resolution of adjacent FISH sites relative to mitotic metaphase chromosomes (Armstrong et al. 1998; Ziolkowski and Sadowski 2002). However, it is possible that the two closely linked 5S rDNA loci found on chromosome 7 in this study could not be resolved earlier because of the lower resolution of FISH on mitotic metaphase chromosomes.

FISH hybridization revealed localization of 45S rDNA loci on pericentromeric regions of the short arm of chromosomes 1, 2, 4, and 5, and the long arm of chromosome 7 (Figs. 2b, R1, R2, R4, R5, R7; 3, R1, R2, R4, R5, R7). FISH signals of C11-350H were observed around the centromere in all the chromosomes except chromosomes 2 and 4 (Figs. 2b, R2, R4; 3, R2, R4). These signals were localized on the proximal region of both arms of chromosomes 3 and 7–10. Further, the signals were detected in the proximal region of long arms of chromosomes 1 and 5, and the short arm of chromosome 6 (Figs. 2b, R1, R5, R6; 3, R1, R5, R6).

Karyotypes of *B. rapa* based on mitotic metaphase chromosomes have been proposed by different researchers. Nishibayasahi (1992), using Giemsa staining, reported a karyotype comprising six median, three submedian, and one subterminal chromosome(s). On the other hand, Kim et al. (1998) reported that the chromosome complement consisted of six median, two submedian and two

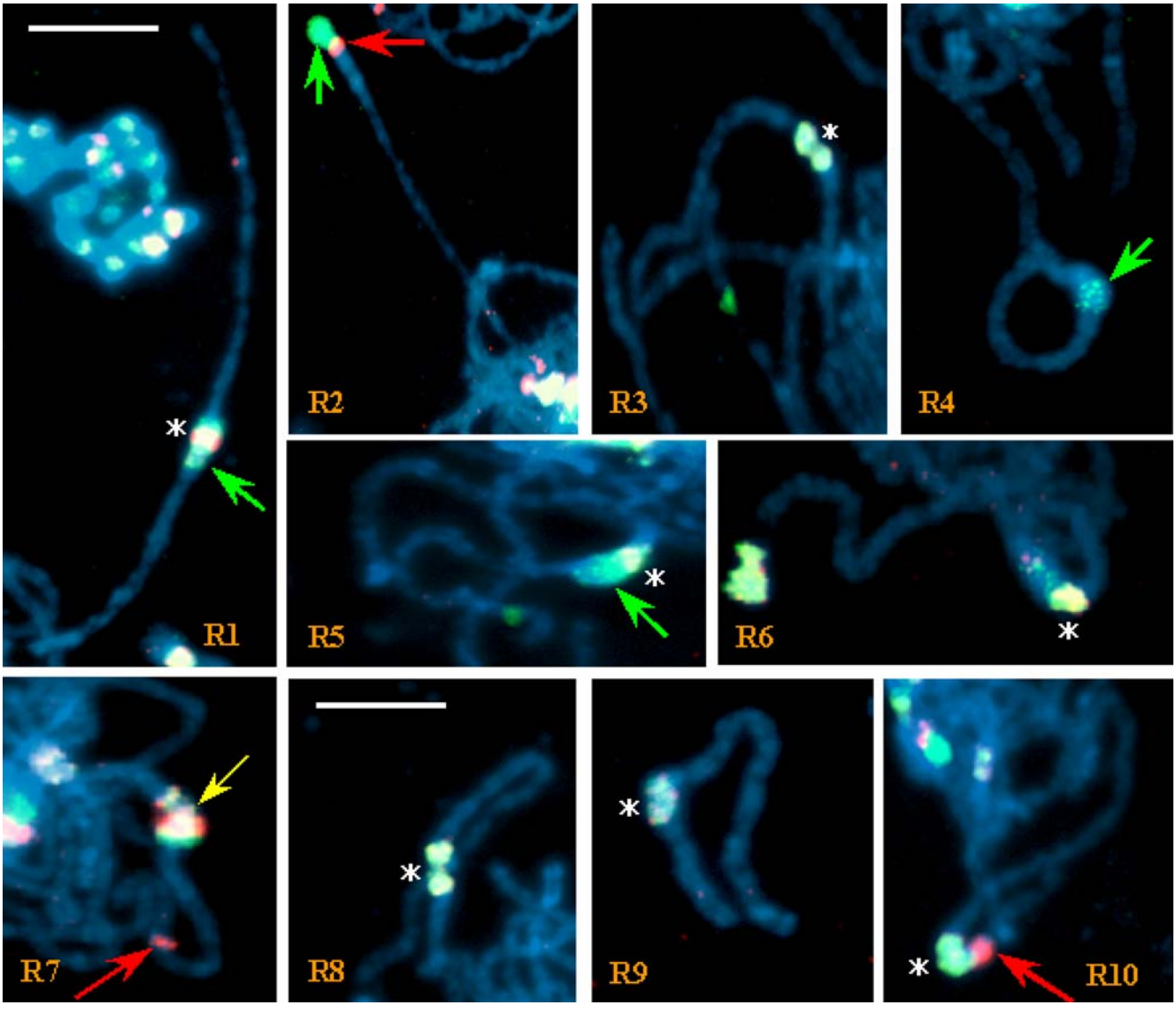

Fig. 3 Digitally sorted out representative pachytene bivalents of ten chromosomes from pachytene spreads showing multicolor fluorescence in situ hybridization (McFISH) signals of 5S rDNA, 45S rDNA, and the C11-350H repeat element (*R1–R10*). Each pachytene bivalent was identified based on morphology and McFISH signals. The digoxigenin-labeled 5S rDNA probe was detected with antidigoxigenin rhodamine conjugate (*red color*, *red arrows*). Biotin-labeled 45S rDNA probe was detected with avidin-FITC conjugate (*green colors*, *green arrows*). C11-350H was labeled with 50% biotin and 50% digoxigenin, which resulted in the pinkish white colors (*asterisks*). The *yellow arrow* indicates co-localization of 5S rDNA, 45S rDNA, and C11-350H loci. The *bars* represent 10 μm

subeterminal chromosomes. Because of less condensation at metaphase, Cheng et al. (1995) studied mitotic prometaphase chromosomes, using Giemsa stain and reported four median, four submedian, and two subterminal chromosomes. The karyotype proposed by Fukui et al. (1998) from mitotic prometaphase chromosomes, analyzed by imaging method, had the same number of median, submedian, and subterminal chromosomes as proposed by Cheng et al. (1995). Moreover, the former researchers could localize the distribution of 45S rDNA loci to individual chromosome arms.

The DAPI-stained pachytene karyotype presented in the present study provided details with respect to centromeric indexes, lengths of chromosomes and their arms, and size and distribution of heterochromatic regions. Furthermore, the combination of chromosome length, centromere position, and distribution of repetitive DNA sequences (5S rDNA, 45S rDNA, and C11-350H) enabled us to identify all the chromosomes. This karyotype will be useful for structural and functional genomic studies in *B. rapa*.

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