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Isolation and characterization of microsatellites in *Brassica rapa* L.

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Abstract We report here the isolation and characterization of microsatellites, or simple sequence repeats (SSRs), in *Brassica rapa*. The size-fractionated genomic library was screened with $(GA)_{15}$ and $(GT)_{15}$ oligonucleotide probes. A total of 58 clones were identified as having the microsatellite repeats, and specific primer pairs were designed for 38 microsatellite loci. All primer pairs, except two, amplified fragments having the sizes expected from the sequences. Of the 36 primer pairs, 35 amplified polymorphic loci in 19 cultivars of *B. rapa*, while monomorphism was observed in only one primer pair. A total of 232 alleles was identified by the 36 primer pairs in 19 cultivars of *B. rapa*, and these primer pairs were examined also in nine Brassicaceae species. Most of the 36 primer pairs amplified the loci in the Brassicaceae species. Segregation of the microsatellites was studied in an F_2 population from a cross of doubled-haploid lines DH27 × G309. The microsatellites segregated in a co-dominant manner. These results indicate that the microsatellites isolated in this study were highly informative and could be useful tools for genetic analysis in *B. rapa* and other related species.

Keywords *Brassica rapa* · Microsatellites · Molecular marker · Segregation analysis · Simple sequence repeats

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Introduction

The difficulties in plant breeding are: (1) to screen a large number of segregating individuals for desirable genes and traits, e.g. disease resistance; (2) to grow a population up to maturity, because various traits cannot be distinguished in younger stages; and (3) to distinguish desirable traits under various environments. Molecular markers are useful tools for identifying economically important major genes and quantitative trait loci (QTLs). Pre-selection using molecular markers can reduce the size of a population, and the markers can identify desirable genotypes in the early seedling stage without disturbance by environmental effects. Therefore, molecular markers are considered useful tools for plant breeding, and various kinds of molecular markers have been developed and used for plant breeding and genetic studies.

In recent years, microsatellites, or simple sequence repeats (SSRs), have been recognized as useful molecular markers in marker-assisted selection (MAS), the analysis of genetic diversity, population analysis, and other purposes in various species (Gupta and Varshney 2000). Microsatellites are short, tandemly repeated nucleotide motifs (1–6 bp) existing throughout the whole genome of an organism, especially in eukaryotes (Tautz and Renz 1984; Dib et al. 1996; Dietrich et al. 1996). They are abundant in most species and highly polymorphic, owing largely to variations in the number of repeat units (Tautz 1989; Weber and May 1989; Hancock 1995). They are inherited in a co-dominant manner (Morgante and Olivieri 1993) and can be analyzed by a convenient PCR-based method, which makes it easy to screen a large number of individuals. Above all, microsatellites are preferable to other molecular markers. For example, RFLPs are co-dominant molecular markers, but they require more-complex techniques, such as hybridization; RAPDs have low reproducibility and are not inherited in a co-dominant manner.

In spite of their usefulness, however, the development of microsatellite markers requires significant money and time, owing to the cloning and sequencing strategy.

Hence, their development in plant species is still limited to a small number of laboratories. But if microsatellite markers are developed, their use will be more-effective and more-convenient in various analyses.

Brassica rapa originated in the Mediterranean area and is now widely cultivated in the world as Chinese cabbage, turnip, and other leafy vegetable crops. It has a wide genetic diversity (Li 1981). A number of cultivars with wide variation are bred and released as Chinese cabbage. Variations include time of maturing, inside-head color, and head size and shape. Because most *B. rapa* cultivars are out-breeding, most Chinese cabbage cultivars are hybrids and thus require a complicated breeding procedure (Nishi 1980). Therefore, much of the genetic information on *B. rapa* is accumulated as breeders' knowledge, which has not been published because of a lack of detailed genetic analysis. Although a linkage map is a powerful tool for analyzing complex traits, the mapping study of *B. rapa* is not as advanced as that of *Brassica napus*. Some traits, such as flower induction, have already been mapped by using RFLP markers (Song et al. 1991; Chyi et al. 1992; Ajisaka et al. 2001). A large number of agronomic traits, such as heading and disease resistance, however, remained unmapped. Mapping of these agronomic traits requires many mapping populations, and the maps obtained should be integrated into one common map with anchor markers. Several research groups have isolated a number of microsatellites for molecular markers in *Brassica* species, mainly in *B. napus* (Kresovich et al. 1995; Truco et al. 1996; Uzunova and Ecke 1999), and have used them as anchor markers on an RFLP map. Microsatellite markers in *B. rapa* would be powerful tools for the resolution of difficulties in mapping studies.

The objective of this study are: (1) to isolate microsatellites from *B. rapa*, (2) to develop molecular markers based on them, and (3) to examine their usefulness as genetic markers in *B. rapa* and other related species.

Materials and methods

Plant material and extraction of genomic DNA

All plants used in this study were grown in a greenhouse. The doubled-haploid lines of *B. rapa*, developed at NIVTS, were A9709, G004, DH27 and G309. The morphologically diverse cultivars of *B. rapa* belong to seven varieties (Nishi 1980): var. *japonica* (Wase Mibuna), var. *chinensis* (Seppaku-taisai, Sangatsuman), var. *pekinensis* (Wase Nagasaki Hakusai, Osaka Shirona, Chousen, Nozaki 2, Matsushima Shin 2, Homei), var. *rapifera* (Kidaao, Someya Kanamachi, Hakata Suwari, Komatsuna, Siloga, Manchester Market, Toria), var. *parachinensis* (Kosaitai), var. *narinosa* (Chijimina), and var. *oleifera* (Kukitachina). All cultivars were held at NIVTS. Other Brassicaceae species used in this study are listed in Table 1. In addition, an F_2 population from the cross of two doubled-haploid lines, DH27 × G309 (Ajisaka et al. 2001), were screened for segregation analysis.

Genomic DNA was isolated from freeze-dried young leaves of a single plant of each line or accession by the CTAB method (Murray and Thompson 1980).

Isolation of microsatellites

A small-insert genomic library of line A9709 was developed for the isolation of microsatellites. Genomic DNA was digested completely by *Sau*3AI and fractionated in a 1.0% agarose gel. DNA fragments of about 200 to 1,000 bp were excised from the gel and ligated into the *Bam*HI site of lambda phage vector, ZAP express (Stratagene). The library was screened with digoxygenin-labeled synthetic $(GA)_{15}$ and $(GT)_{15}$ oligonucleotide probes. Plaque hybridization was carried out according to the manufacturer's instructions (DIG detection system, Roche) with a stringency wash at 60 °C in 0.5 × SSC containing 0.1% SDS. Positive clones were isolated after two screening steps and were converted into pBK-CMV phagemids by an in vivo excision system (Stratagene). The phagemids were sequenced with T3 and T7 promoter primers by an ABI377 sequencer (Applied Biosystems) to determine the existence of microsatellites.

PCR amplification of microsatellites

To amplify the microsatellite loci, the PCR primers were designed manually for regions upstream of and downstream from the repeat region. They had an about 50% GC content, and yield an expected product of about 100 to 250 bp. PCR was performed in a 20-µl vol containing 2 ng of genomic DNA, 250 nM of each primer, 0.25 mM of dNTPs, 1 × reaction buffer (Takara, Japan), and 1 unit of *Taq* polymerase (Takara). The reaction mixture was initially denatured at 94 °C for 2 min, followed by 30 cycles of amplification at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and final extension at 72 °C for 4 min in a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were fractionated in a 2.5% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and viewed with a UV illuminator. When polymorphism of the PCR products could not be identified clearly in the agarose gel, the products were fractionated in a 4.0% denatured polyacrylamide gel and stained with a SYBR Green I Nucleic Acid Gel Stain (Molecular Probes), and then made visible with FM BIO (Hitachi).

Statistical analysis

To refer to the informativeness of microsatellites, we employed the term polymorphism information content (PIC). The PIC value was calculated according to the formula:

$$PIC_i = 1 - \Sigma p_{ij^2},$$

Table 1 Plant materials for diversity of microsatellites

Species	Cultivar	Acc. no.	Source
B. nigra	–	141(439–1975)	Tohoku university, Japan
B. oleracea	FUJIWASE SHIN1	–	NIVTS, Japan
B. napus	TOWADA NATANE	JP No. 67870	Genebank, MAFF, Japan
B. carinata	–	101(182–1989)	Tohoku university, Japan
B. juncea	KAICHOY	JP No. 37394	Genebank, MAFF, Japan
R. sativus	MIURA	–	NIVTS, Japan
S. alba	–	25(954–1984)	Tohoku university, Japan
A. thaliana	Col-0	CS1092	ABRC, Ohio state, USA

Table 2 Numbers of microsatellite clones isolated from a small-insert genomic library of the *B. rapa* doubled-haploid line A9709

Core sequence	Number of clones	Repeat times
(GA/CT)	41	13–49
(GT/CA)	8	10–33
(GA/CT)+(GT/CA)	4	14–53
(GA) or (GT) + others	5	26–45

where p_{ij} is the frequency of the *j*th microsatellite allele for clone *i*. This value is referred to as heterozygosity and gene diversity (Weir 1990; Anderson et al. 1993).

Results

About 120,000 clones were screened for the presence of GA/CT and GT/CA repeats. After two screening steps, 81 clones proved to be positive and were sequenced. A total of 58 clones were identified as containing microsatellite repeats after sequencing (Table 2).

Primer pairs were designed for 38 of the 58 clones and were used to examine the amplification of microsatellite loci using genomic DNA of A9709 as a template (Table 3). Most of the primer pairs amplified single fragments of the expected size. However, no fragment was amplified with the two primer pairs BRMS-011 and BRMS-048 under various PCR conditions. Primer pairs could not be designed for the remaining 20 clones, because the microsatellite repeats were found at either end of the insert.

All primer pairs which amplified microsatellite loci in A9709 were then examined for polymorphism among the 19 cultivars of *B. rapa* (Table 3). Thirty five primer pairs amplified fragments and showed polymorphism. Among them, however, six pairs amplified no fragment in some cultivars. Null alleles are expected at these loci, although detailed a segregation study is required. Some polymorphism was demonstrated in agarose-gel electrophoresis (Fig. 1A). Other pairs looked monomorphic in the agarose gel, but clearly showed polymorphism in polyacrylamide-gel electrophoresis (Fig. 1B). One primer pair, BRMS-049, showed no polymorphism in 19 cultivars, even in the polyacrylamide gel. Most of the primer pairs examined here amplified one or two main fragments in each accession. These results show that the primer pairs designed detected only one locus in a co-dominant manner among each accession. PIC values of microsatellite markers varied from 0 to 0.918 with an average of 0.708. Most of them ranged from 0.5 to 0.9 (Table 3).

The 36 primer pairs were then examined for amplification in the other Brassicaceae species (Table 4, Fig. 2). All except one pair, BRMS-002, amplified microsatellite loci in most *Brassica* species, although some failures were observed in *Brassica nigra*. In addition, three-fourths of the primer pairs amplified microsatellite loci within *Raphanus sativus* and *Sinapis alba*, while about a half of the pairs could not amplify the loci in *Arabidopsis thaliana*.

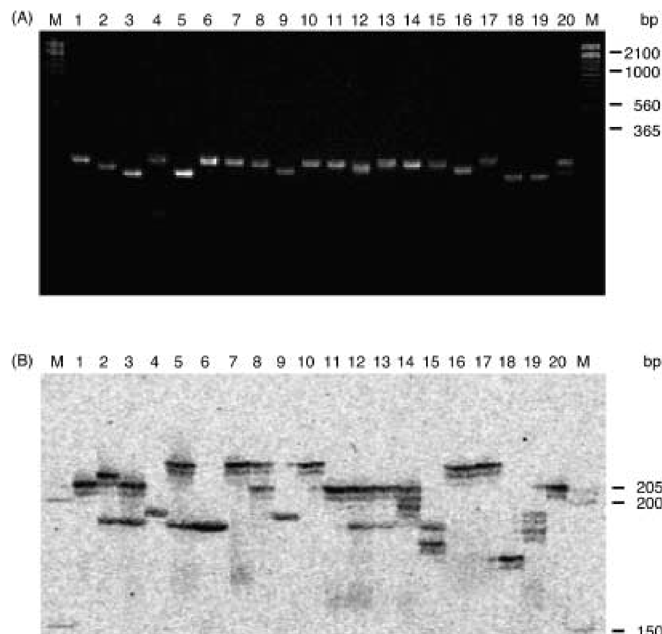

Fig. 1A, B Polymorphism of microsatellites among 19 cultivars of *B. rapa*. (**A**) BRMS-006 in 2.5% agarose gel and (**B**) BRMS-027 in 4.0% denatured polyacrylamide gel. *Lane 1*, A9709; *lanes 2–20*, 19 cultivars in the following order: Kukitatina, Komatsuna, Wase Mibuna, Seppaku-taisai, Sangatsuman, Kosaitai, Chijimina, Wase Nagasaki Hakusai, Osaka Shirona, Homei, Chousen, Nozaki 2, Matsushima Shin 2, Kidaao, Someya Kanamachi, Hakata Suwari, Siloga, Manchester Market and Toria

Fig. 2 Amplification of microsatellite BRMS-037 in Brassicaceae spesies. The fragments were separated in a 4.0% denatured polyacrylamide gel

To confirm the co-dominant nature of the microsatellites in *B. rapa*, we examined the segregation of 11 loci, which showed clear polymorphism in agarose gels of DH27 and G309, in an F_2 population from the cross (Table 5). All primer pairs detected single loci, and the amplified fragments segregated in a co-dominant manner, although some primer pairs amplified fragments showing slightly biased segregation.

Discussion

We isolated 58 microsatellites from *B. rapa*. Because we found no redundancies among the 58 clones, we think

Table 3 Microsatellite repeat motifs, primer sequences, expected allele size, amplification in line A9709 and polymorphism in 19 cultivars of *B. rapa*

Locus	Repeat motif	Primer sequence (5′–3′)	Expected size (bp)[a]	Number of alleles[b]	PIC
BRMS-001	$(GA)_{25}$	GGTGGCTCTAATTCCTCTGA ATCTTTCTCTCACCAACCCC	139	13	0.886
BRMS-002	$(CT)_{22}$	GATCTTCTCTCCAAAA TCCAAGCTAAATTACG	168	3	0.64
BRMS-003	$(CT)_{19}$	ACGAATTGAATTGGACAGAG CAGATGGGAGTCAAGTCAAC	192	4	0.695
BRMS-005	$(GA)_{13}$	ACCTCCTGCAGATTCGTGTC GCTGACCTTTCTTACCGCTC	162	12	0.901
BRMS-006	$(GA)_{34}$	TGGTGGCTTGAGATTAGTTC ACTCGAAGCCTAATGAAAAG	193	13	0.87
BRMS-007	$(CT)_{24}$	AAATTGTTTCTCTTCCCCAT GTGTTAGGGAGCTGGAGAAT	152	14	0.918
BRMS-008	$(TC)_{30}$	AGGACACCAGGCACCATATA CATTGTTGTCTTGGGAGAGC	145	11	0.86
BRMS-011	$(GA)_{18}$	GAACGGGCAACAACAAATAGTG CGCGTCACAATCGTAGAGAATC	184	–[c]	–
BRMS-014	$(TC)_{15}$	CCGTAAGGAATATTGAGGCA TTCCCAATTCTCAAACGGTA	156	9	0.85
BRMS-015	$(TG)_4$, $(GA)_{20}$	TCGCCAATAGAACCCAAAACTT CATCTCCATTGCTGCATCTGCT	263	3[d]	0.611
BRMS-016	$(TC)_{20}$	TCCCGTATCAATGGCGTAACAG CGATGGTGACATTATTGTGGCG	144	6[d]	0.808
BRMS-017	$(CA)_{33}$	GGAAAGGGAAGCTTCATATC CTGGAAAGCATACACTTTGG	209	3	0.591
BRMS-018	$(GA)_{45}$	TCCCACGCCTTCTAGCCTTC ACCGGAGCTTTTCTGTTGCC	168	9	0.85
BRMS-019	$(GT)_{10}$	CCCAAACGCTTTTGACACAT GGCACAATCCACTCAGCTTT	220	4	0.634
BRMS-020	$(TC)_{21}$	AACAAGAGAAGGAGAGCCACCG CGCTTATAAAATGGCAGTCGCA	143	2[d]	0.5
BRMS-024	$(GA)_{24}$	TGAATTGAAAGGCATAAGCA CAGCCTCCACCACTTATTCT	129	10	0.86
BRMS-025	$(GA)_{37}$	TGAAAACAAGCGCTACATGTGG CAAGCAAGCATGACAAGCAACA	163	4[d]	0.611
BRMS-026	$(CT)_{26}$	CCTATCCTCGGACTAATCAGAA GTGCTTGATGAGTTTCACATTG	122	6	0.81
BRMS-027	$(GA)_{17}$	GCAGGCGTTGCCTTTATGTA TCGTTGGTCGGTCACTCCTT	205	7	0.771
BRMS-029	$(GA)_{43}$	AACAAATGACACACACCACACT ATTGAAAATCTTAACCGTGAAG	232	11	0.88
BRMS-030	$(CT)_{14}$	TCAGCCTACCAACGAGTCATAA AAGGTCTCATACGATGGGAGTG	212	5	0.74
BRMS-031	$(TC)_{33}$	TGCCACCAATGACAATGACACTATC GATGCACTGGGACCACTTACATTTT	238	11	0.896
BRMS-033	$(CA)_{11}$	GCGGAAACGAACACTCCTCCCATGT CCTCCTTGTGCTTTCCCTGGAGACG	225	4	0.719
BRMS-034	$(GA)_{18}$	GATCAAATAACGAACGGAGAGA GAGCCAAGAAAGGACCTAAGAT	145	9	0.771
BRMS-036	$(CA)_{10}$, $(GA)_4$	GGTCCATTCCTTTTTGCATCTG CATGGCAAGGGGTAACAAACAT	136	6	0.768
BRMS-037	$(CA)_{10}$	CTGCTCGCATTTTTTATCATAC TACGCTTGGGAGAGAAAACTAT	154	3	0.445
BRMS-040	$(GA)_{49}$ $(GT)_4$	TCGGATTTGCATGTTCCTGACT CCGATACACAACCAGCCAACTC	283	9	0.829
BRMS-042	$(AAT)_4$, $(CT)_4$ $(T)_2$ $(CT)_4$	GGATCAGTTATCTGCACCACAA TCGGAATTGGATAAGAATTCAA	122	8	0.84
BRMS-042-2	$(GA)_4$, $(CT)_{26}$	AGCTCCCGACAGCAACAAAAGA TTCGCTTCCTTTTCTGGGAATG	220	3	0.595
BRMS-043	$(A)_{21}$, $(T)_{14}$ $(GT)_6$	GCGATGTTTTTTCTTCAGTGTC TTAATCCCTACCCACAATTTCC	319	4	0.683
BRMS-044	$(GA)_{27}$	AGGCGAGGAGAAGACAACACAA TACGGGTGGTTTGAATCAGCAG	355	4[d]	0.667
BRMS-046	$(GA)_8$ $(CA)_1$ $(GA)_6$	TTGGCCTTGCTATTACGAGCTG ATGCGCAAACCCTAATTTTCAC	156	3[d]	0.357
BRMS-048	$(TC)_{21}$	TGCCTCCTCTCATTTTTTCTCT TGACCGAGAGGTTCACAAGTAA	197	–[c]	–

Table 3 (continued)

Locus	Repeat motif	Primer sequence (5′–3′)	Expected size (bp)[a]	Number of alleles[b]	PIC
BRMS-049	$(CT)_{26}$	GATCTTCTCTCCAAAACTCTCT AAAGTCCAAGCTAAATTACAAA	177	1	0
BRMS-050	$(AAT)_4$, $(TC)_{19}$ $(TTC)_3$	AACTTTGCTTCCACTGATTTTT TTGCTTAACGCTAAATCCATAT	186	8	0.845
BRMS-051	$(TC)_{15}$	GGCCAAGCCACTACTGCTCAGA GCGGAGAGTGAGGGAGTTATGG	265	7	0.786
BRMS-054	$(GA)_{39}$	GAATCTCTGCAAGAAACAAATG TTCCTCAGCATCAAGTAACCTC	133	5	0.484
BRMS-056	$(GA)_{13}$	GATCAAGGCTACGGAGAGAGAG CGTGACGCTAGAGTAATCGAGT	216	3	0.531

[a] Calculated from the sequence in A9709
[b] Estimated from the amplified fragments in 19 cultivars of *B. rapa*
[c] No amplification in A9709 or the 19 cultivars
[d] Although null alleles were expected, they are not included in the number

Table 4 Detection of microsatellite loci in the Brassicaceae[a]

Primer pair	Genus *Brassica*						Other genera		
	B. rapa	*B. nigra*	*B. oleracea*	*B. napus*	*B. carinata*	*B. juncea*	*R. sativus*	*S. alba*	*A. thaliana*
BRMS-001	1	1	1	1	1	1	1	1	1
BRMS-002	1	–	–	–	–	–	–	–	–
BRMS-003	1	1	1	1	1	1	1	1	1
BRMS-005	1	1	1	1	1	1	1	–	–
BRMS-006	1	–	1	1	1	–	–	–	–
BRMS-007	1	1	1	1	2	1	1	2	–
BRMS-008	1	1	1	1	1	2	1	1	1
BRMS-011	1	–	1	1	1	1	–	–	–
BRMS-014	1	1	1	1	1	1	–	–	–
BRMS-015	1	–	1	1	1	1	–	1	1
BRMS-016	1	–	1	2	2	1	1	2	–
BRMS-017	1	1	1	1	1	1	1	–	–
BRMS-018	1	–	1	1	1	1	1	1	–
BRMS-019	1	1	1	1	1	1	1	1	1
BRMS-020	2	2	2	3	2	1	2	2	2
BRMS-024	1	1	1	1	1	1	–	–	–
BRMS-025	2	4	2	3	3	2	2	2	2
BRMS-026	1	1	1	1	1	1	–	–	–
BRMS-027	1	1	1	1	1	1	1	1	1
BRMS-029	1	1	1	1	1	1	1	1	1
BRMS-030	1	1	1	1	1	1	1	1	–
BRMS-031	1	1	1	1	1	2	–	1	–
BRMS-033	1	1	1	1	1	1	2	1	1
BRMS-034	1	1	1	1	1	1	–	1	–
BRMS-036	1	1	1	1	1	1	1	1	–
BRMS-037	1	1	1	1	1	1	1	1	1
BRMS-040	1	1	1	1	1	1	2	2	2
BRMS-042	1	1	1	1	1	1	1	1	–
BRMS-042-2	1	1	1	1	1	1	1	1	–
BRMS-043	1	–	–	1	1	1	1	1	–
BRMS-044	2	2	2	2	2	2	3	2	2
BRMS-046	1	1	1	–	1	1	1	1	–
BRMS-048	1	1	1	1	3	1	2	2	2
BRMS-049	1	1	1	1	1	1	2	1	1
BRMS-050	1	1	1	1	1	1	1	1	–
BRMS-051	1	1	1	1	1	1	2	1	1
BRMS-054	1	–	1	1	1	1	1	–	–
BRMS-056	1	1	1	1	1	1	1	1	–

[a] The number of amplified bands is listed, –; no amplification

Table 5 Segregation in an F_2 population of DH27 × G309

Locus	Segregation ratio[a]	$P(\chi^2)$
BRMS-031	32:35:20	0.036
BRMS-007	27:27:14	0.020
BRMS-008	20:43:24	0.83
BRMS-031	32:34:18	0.021
BRMS-037	23:41:18	0.74
BRMS-040	19:38:23	0.74
BRMS-042	20:40:11	0.18
BRMS-029	17:39:20	0.87
BRMS-036	10:43:29	0.011
BRMS-042-2	21:44:10	0.065
BRMS-050	21:44:11	0.10

[a] Excludes individuals which could not amplify fragments

that microsatellites are abundant in the *B. rapa* genome. Screening technology using an enriched genomic library has a tendency to catch redundant clones (Brondani et al. 1998; Rossetto et al. 1999, Rallo et al. 2000). In contrast, our screening strategy using an un-enriched library has the advantage of not catching the reduntant clones.

GA/CT repeats were approximately five-times more abundant than GT/CA repeats. These results are consistent with reports from *B. napus* (Uzunova and Ecke 1999) and other plants (Stallings 1992; Lagercrantz et al. 1993).

We preliminarily designed primers having 20 nucleotides to amplify the microsatellite loci. These primer pairs amplified additional fragments in some analyses; the amplification was more-sensitive to PCR conditions, e.g. annealing temperature. This is a common phenomenon in microsatellite analysis (Smith and Devey 1994; Rallo et al. 2000). Designing longer primers (23–25-mer), we found that they stably amplified single fragments in the homozygous doubled-haploid line, A9709. The fragment sizes were similar to those expected from the sequences. Thus, we think that the longer primers are more suitable for genetic analysis.

Among the primer pairs evaluated, 94.7% (36/38) amplified microsatellite loci among the 19 cultivars of *B. rapa*. Of the 36 pairs, 35 showed polymorphism. A total of 232 alleles were found among the 19 cultivars. Monomorphism was observed only in the case of one primer pair. PIC values obtained in the present study were higher than those reported in the *B. napus* varieties (Plieske and Struss 2001). These results indicate the highly informative nature of the microsatellites obtained here.

The degree of polymorphism detected by these primer pairs did not correlate with the number of repeats in the microsatellites. Although the relationship between the degree of polymorphism and the number of repeats has been reported in some species (Weber 1990; Saghai-Maroof et al. 1994; Fisher et al. 1998), theoretically the number of repeats is correlated with the mutation rate, and not with the degree of polymorphism (Brinkmann et al. 1998; Xu et al. 2000). Polymorphism may correlate with the product of the mutation rate and the generation term of the locus. Younger microsatellites would have less polymorphism because of less occasion for mutation, even if they have longer repeats. For this reason, our results would be logically acceptable in respect of of evolution of the microsatellites.

As shown in Table 4 and Fig. 2, most of microsatellite primer pairs amplified single fragments in both diploid and amphidiploid species. However, a small number of the primer pairs amplified two or more fragments. A part of these pairs may detect more than one microsatellite locus. The number of fragments amplified in amphidiploid species was only slightly higher than that in diploid species. While, Plieske and Struss (2001) reported that the microsatellite primers detected two or more alleles in *B. napus*, although information on the primer size and the sequence was not disclosed. The number of bands amplified in amphidiploids should be twice that in diploid species. The former was, however, only slightly higher than the latter in this study. Here, we employed rather-strict PCR conditions to detect a single locus. Competition of primer-binding sites in homoeologous loci of amphidiploids may reduce the amplified fragments, because we sometimes detected more than one band in amphidiploids using a less-stringent annealing temperature in the preliminary experiments.

It is noteworthy that the most of our primer pairs detected only one locus in the *B. rapa* genome. RFLP probes are extensively used as landmarks to join the linkage maps developed by different laboratories (Quiros et al. 1991). However, RFLP probes sometimes detected more than one locus in the case of *Brassica* (Song et al. 1991; Truco et al. 1996). Because the genomes of *B. rapa* and *Brassica oleracea* originated from a triplicated genome of *Arabidopsis* (Lagercrantz 1998), duplicated or triplicated segments may be scattered throughout the genomes. Therefore, detailed examination is required to determine the corresponding locus on two independently developed maps. In contrast, most of microsatellite primers designed here detect only one locus, and could therefore be useful in integrating a new map into existing linkage maps. We also found that the microsatellites of *B. rapa* are highly informative, and some of the polymorphism is detectable even with agarose-gel electrophoresis. Our results clearly indicate that the microsatellites are useful as landmarks for linkage-mapping in *B. rapa* and related species.

In the segregation analysis, all 11 primer pairs identified single loci and amplified co-dominant fragments (Table 5). Some loci, however, showed a slight bias in the Mendelian Mode. The observed distortion may be partly derived from the highly homozygous nature of both parents. Such homozygosity is unusual in outbreeding *Brassica* crops. As reported in other out-breeding species (Bentolila et al. 1992), doubled-haploid lines may accumulate undesirable alleles in homozygotes and these alleles may affect segregation in their progeny.

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