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The use of species-specific DNA markers for assessing alien chromosome transfer in *Brassica rapa* and *Brassica oleracea* monosomic additions of *Raphanus sativus*

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Abstract

Monosomic addition lines (MALs) are useful materials not only for cytogenetic and molecular genetic studies but also for plant breeding as gene sources. In our previous study, two MALs in the tribe Brassiceae were developed, one being *Raphanus sativus* lines with alien chromosomes of *Brassica rapa* (*B. rapa*-monosomic addition lines; BrMALs) and the second being those with alien chromosomes of *Brassica oleracea* (*B. oleracea*-monosomic addition lines; BoMALs). We developed species-specific DNA markers from the genomic sequences of *B. rapa* and *B. oleracea* comparing them with those of *R. sativus*, and identified chromosomes added in BrMALs and BoMALs using these markers. It was revealed that eight types of BrMALs have seven chromosomes of *B. rapa* and seven types of BoMALs have six chromosomes of *B. oleracea*. Furthermore, chromosome breakage and homoeologous recombination were suggested to have occurred in some MALs. The developed species-specific DNA markers are considered to be useful for producing MALs and also for assessing chromosome abnormality in MALs.

Keywords: monosomic addition line, species-specific DNA marker, chromosome breakage, homoeologous recombination

Introduction

Monosomic addition lines (MALs) developed by interspecific or intergeneric hybridization and repeated backcrossing with recipient parents are useful materials in plant breeding programs. An extremely long time is required to introduce a trait from related species to crop species by interspecific and intergeneric hybridizations, which are often hindered by hybridization barriers, such as interspecific incompatibility, hybrid embryo breakdown, hybrid necrosis, and hybrid sterility (Khush and Brar 1992). Therefore, sets of MALs having each chromosome of closely related species are useful for rapid introduction of desirable traits from related species to crop species.

Since a set of rye monosomic addition lines with wheat background was first developed by Leighty and Taylor (1924), many MALs have been produced in many crop species, e.g., wheat (Friebe et al. 2000), Japanease bunching onion (Shigyo et al. 1996), beet (van Geyt et al. 1988), rice (Multani et al. 1994, Tan et al. 2005), and *Brassica* crops (Peterka et al. 2004, Heneen et al. 2012). These MALs have contributed to successful introduction of some useful traits, such as resistance to biotic and abiotic stresses (Ren et al. 2009, Yang et al. 2012) and several functional ingredient (Masuzaki et al. 2006, Yaguchi et al. 2009) from related species into crops. Furthermore, MALs are useful in cytogenetic and molecular generic studies (Ma et al. 2011, Fu et al. 2013a, 2013b).

Although a number of studies on MALs have been reported, few reports have shown full sets of all alien chromosomes (Chang and de Jong 2005). MALs have often been selected on the basis of MAL-specific morphological phenotypes and karyotype analysis. In these methods, it is difficult to distinguish MALs having a full set of alien chromosomes from each other. An important tool to visualize alien chromosomes is genomic *in situ* hybridization (GISH; Schwarzacher et al. 1989). Although this method is the most accurate for identification of alien chromosomes in MALs, it requires elaborate techniques and is laborious. Moreover, there is a possibility of the occurrence of chromosome breakage and homoeologous recombination during the development of MALs through interspecific crossing and backcrossing (Finch et al. 1984, Ji et al. 2003). It is necessary to check the integrity of alien chromosomes for using MALs as genetic resources. Simpler and more reliable techniques are required for the development of a full set of MALs to identify alien chromosomes and to check their integrity.

In our previous study, we developed several MALs in *Brassica* crops, whose alien chromosomes were identified by morphological investigation and random amplified polymorphic DNA (RAPD) marker analysis (Kaneko et al. 1987, 2001, 2003). Early-bolting trait (Kaneko et al. 2000) and resistances to turnip mosaic virus (TuMV, Kaneko et al 1996) and clubroot (Akaba et al. 2009) were conveyed by alien chromosomes in these MALs, but a full set of MALs with all the chromosomes of related species has not yet been obtained.

In the present study, to identify alien chromosomes in *Brassica rapa*-monosomic addition lines (BrMALs) and *Brassica oleracea*-monosomic addition lines (BoMALs) with *Raphanus sativus* background (Kaneko et al 1987, 2001, 2003), we developed *B. rapa* and *B. oleracea* species-specific DNA markers, which are species-specific primers and probes for dot-blot analysis, and analyzed two sets of MALs using the developed markers. In addition, we assessed the alien chromosome abnormalities in BrMALs and BoMALs using these markers.

Materials and methods

Plant materials and preparation of genomic DNA

B. rapa-monosomic addition lines with *R. sativus* background (BrMALs; a - h type, 2*n*=19, Kaneko et al. 2001, 2003) and *B.*

oleracea-monosomic addition lines with *R. sativus* background (BoMALs; a-g type, 2*n*=19, Kaneko et al. 1987) maintained at the plant breeding laboratory of Utsunomiya University, Japan, were used. Two MALs have been developed from synthesized amphidiploid plants, *Raphanobrassica* (*R. sativus* × *B. rapa* cv. 'Kyo-mizuna', 2*n*=38 or *R. sativus* × *B. oleracea* cv. 'Murasaki-Habotan', 2*n*=36), by successive backcrossing with *R. sativus* cv. 'Shogoin-daikon' (2*n*=18) (Kaneko et al. 1987, 2001). BrMALs and BoMALs have been classified into eight types (a - h type) and seven types (a - g type), respectively, based on the morphological traits of their leaves, roots, inflorescences, and pods (Kaneko et al. 1987, 2001, 2003). *B. rapa* cv. 'Kyo-mizuna' (2*n*=20) and 'Shogoin-kabu', *B. oleracea* cv. 'Murasaki-Habotan' and a homozygous line with *S-23* haplotype of kale (2*n*=18), and *R. sativus* cv. 'Miura' and 'Shogoin-daikon' were used for developing species-specific DNA markers.

Genomic DNA was isolated by the modified CTAB method (Doyle and Doyle 1990). A 0.1 g piece of a leaf was pulverized in liquid nitrogen and suspended in 2 × CTAB solution (2% cetyltrimethyl ammonium bromide, 100 mM Tris-HCl buffer pH 8.0, 1.4 M NaCl, 20 mM EDTA). After chloroform/isoamylalcohol (24:1) extraction, DNA was precipitated by the addition of isopropanol. DNA was dissolved in 1 × TE buffer and treated with RNase.

Production of species-specific DNA makers

The *B. rapa*-specific DNA markers, which can detect sequences of only *B. rapa* genome, were screened from the SNP markers mapped on the *B. rapa* linkage map of Tonosaki et al. (2013). To develop new *B. rapa*-specific DNA markers, we searched syntenic regions between *R. sativus* and *B. rapa* using the *R. sativus* EST sequences in the Radish DB (http://radish.plantbiology.msu.edu/) and the *B. rapa* genome sequences in the *Brassica* database (BRAD, http://brassicadb.org/brad) by BLAST search, and identified *B. rapa*-specific sequences by comparing nucleotide sequences between these two species. We designed primers for amplification of a single-copy gene and selected 15- to 24-bp sequences for preparation of species-specific oligonucleotide probes from *B. rapa*-specific sequences (Supplemental Table 1). Similarly, *B. oleracea*-specific makers, which can detect chromosomes of only *B. oleracea*, were also developed. The syntenic regions between *B. oleracea* and *R. sativus* were searched using the *B. oleracea* genomic sequences in BRAD and the *R. sativus* EST sequences by the BLAST search, and *B. oleracea*-specific markers were designed (Supplemental Table 2). All species-specific DNA markers of *B. rapa* and *B. oleracea* were evenly distributed on each chromosome. Specificity of species-specific DNA markers was checked by BLAST search so as not to detect other regions having high homology in the same genome.

Species-specific oligonucleotide probes were prepared as bridge probes for indirect hybridization with a digoxygenin-labeled oligonucleotide probe according to Shiokai et al. (2010). These bridge probes consisted of a probe sequence for detection, a 6-bp spacer sequence of TATATT, and a sequence (5'-TACATTCGCAATTGAGGCTTCGT-3') complementary to the sequence of the digoxygenin-labeled oligonucleotide probe.

Dot-blot hybridization

DNA fragments were amplified by PCR using primer pairs (Supplemental Table 1 and 2). A PCR product was mixed with an equal volume of a denaturation solution containing 0.4 N NaOH and 10 mM EDTA and dot-blotted onto a nylon membrane (Hybond-N; GE Healthcare UK) by a Multi-pin Blotter (ATTO, Japan). After UV exposure (Bio-Rad Laboratories, USA), the membrane was hybridized with a species-specific probe for more than 2 h at the temperature shown in Supplemental Table 1 and 2. After hybridization, the membrane was washed twice in 2 × SSC containing 0.1 % sodium dodecyl sulfate (SDS) at room temperature for 5 min and then in the solution listed in Supplemental Table 1 and 2 at the same temperature as that for hybridization. A hybridized digoxygenin-labeled probe was detected by an anti-digoxygenin immunoglobulin (IgG) Fab fragment conjugated with alkaline phosphatase (Roche, Germany) followed by a chemiluminescent reaction (CSPD; Roche). Chemiliminescence was detected by exposure to X-ray film (Fuji, Japan).

Results

Development of chromosome-specific markers

Species-specific DNA markers were selected from these BrCL markers for identification of alien chromosomes in BrMALs. Among 79 markers on the *B. rapa* genetic map used for screening, 19 markers detected only *B. rapa* genomic DNA without detection of *R. sativus* genomic DNA (19/79 = 24.0%). Therefore, 70 *B. rapa*-specific markers (Brsp) were newly designed by comparison of nucleotide sequences between *B. rapa* and *R. sativus* in the syntenic regions. Out of these markers, 49 markers showed *B. rapa*-specific signals (49/70 = 70.0%) without detection of *R. sativus* genomic DNA (Fig. 1). In total, we obtained 68 markers (Supplemental Table 1) which can detect *B. rapa*-specific sequences. Chromosome A01, A02, A03, A04, A05, A06, A07, A08, A09 and A10 had 5, 5, 8, 7, 10, 10, 6, 3, 7 and 7 markers, respectively (Fig. 2).

We also developed 127 *B. oleracea*-specific DNA markers (Bosp) by comparison of the syntenic regions between *B. oleracea* and *R. sativus*. Eighty markers (Supplemental Table 2) detected only *B. oleracea* chromosome-specific signals (80/127 = 63.0%). Chromosome C01, C02, C03, C04, C05, C06, C07, C08 and C09 had 12, 8, 8, 10, 9, 6, 8, 12 and 7 markers, respectively (Fig. 3).

Identification of alien chromosomes in MALs

To identify alien chromosomes in the two sets of MALs, we

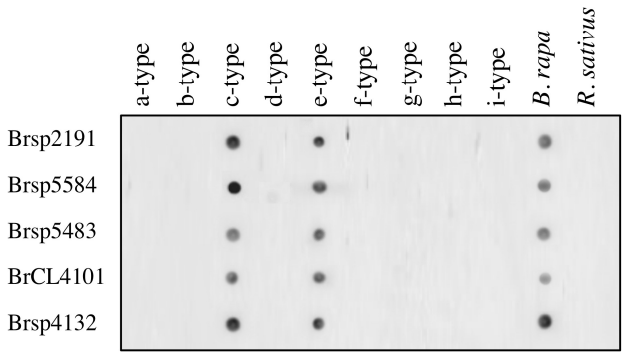


Fig. 1. Detection of specific signals by dot-blot hybridization using species-specific chromosome markers.
Five markers are A02 chromosome-specific markers. Genomic DNAs dot-blotted on a membrane are a- to i-type BrMALs, *B. rapa,* and *R. sativus*.

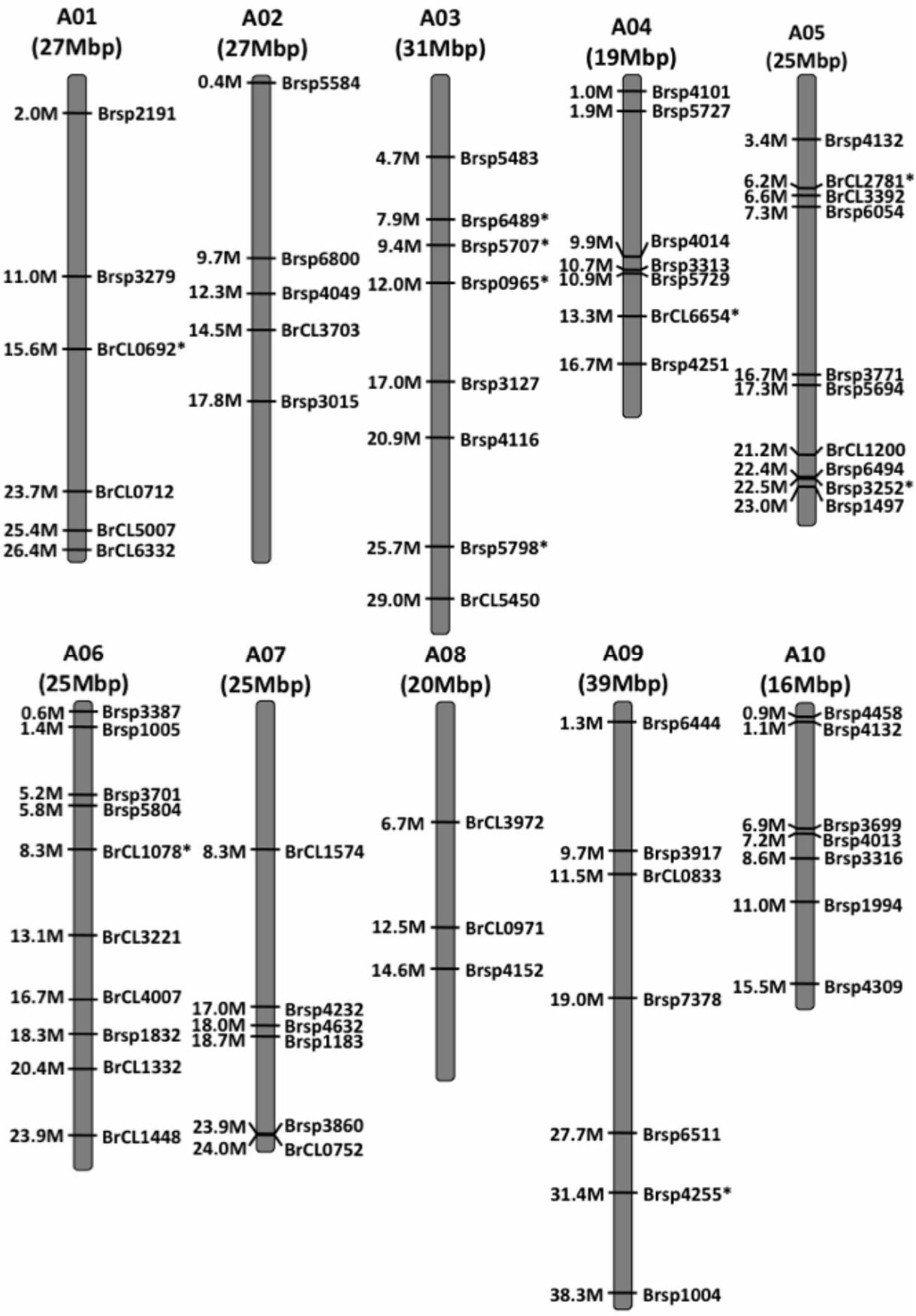


Fig. 2. Positions of *B. rapa*-specific DNA markers in the *B. rapa* genome.

The marker names and positions on *B. rapa* chromosomes are shown. BrCL markers were screened from the *B. rapa* linkage map (Tonosaki et al. 2013) and Brsp markers were newly developed. * indicates *B. rapa*-specific DNA markers detecting signals in BrMALs containing different alien chromosomes.

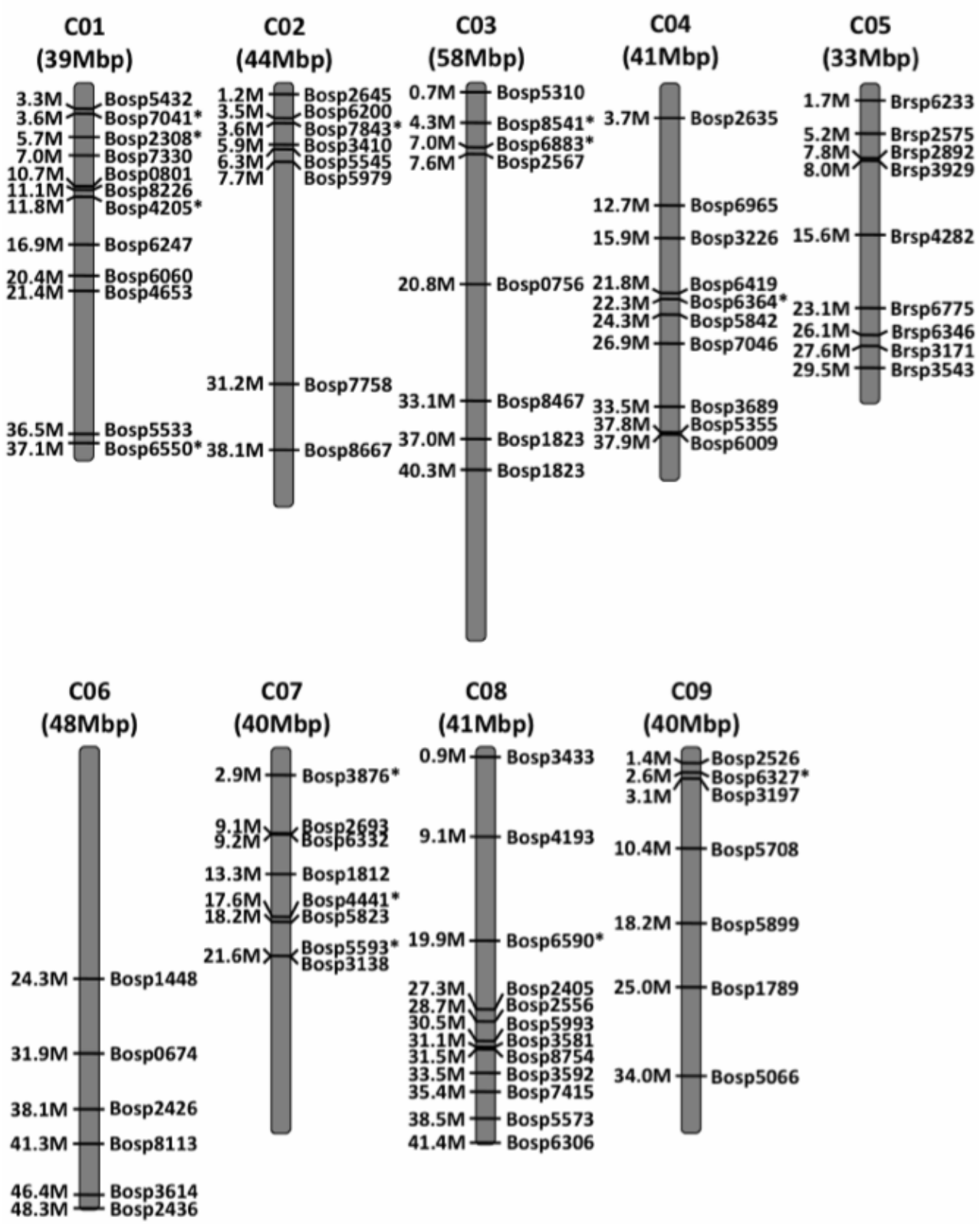


Fig. 3. Positions of *B. oleracea*-specific DNA markers in the *B. oleracea* genome.
The marker names and positions on *B. oleracea* chromosomes are shown. Bosp markers were newly developed. * indicates *B. oleracea*-specific DNA markers detecting signals in BoMALs containing different alien chromosomes.

analyzed eight types of BrMALs and seven types of BoMALs using species-specific DNA markers. Sixteen lines in eight different types (a-type to h-type) of BrMALs, which have distinctive traits and different DNA markers from each other (Kaneko et al. 2003), were analyzed using 68 *B. rapa*-specific DNA markers (Fig. 2). Fifty-five markers detected signals, but 13 markers showed no signals in any lines (Table 1). All A03 chromosome-specific markers detected specific signals in b-type BrMALs, and A06 and A07 chromosome-specific markers were detected in g-type BrMALs and f-type BrMALs, respectively. A04

Table 1. Brassica rapa-specific chromosome markers detected in BrMALs

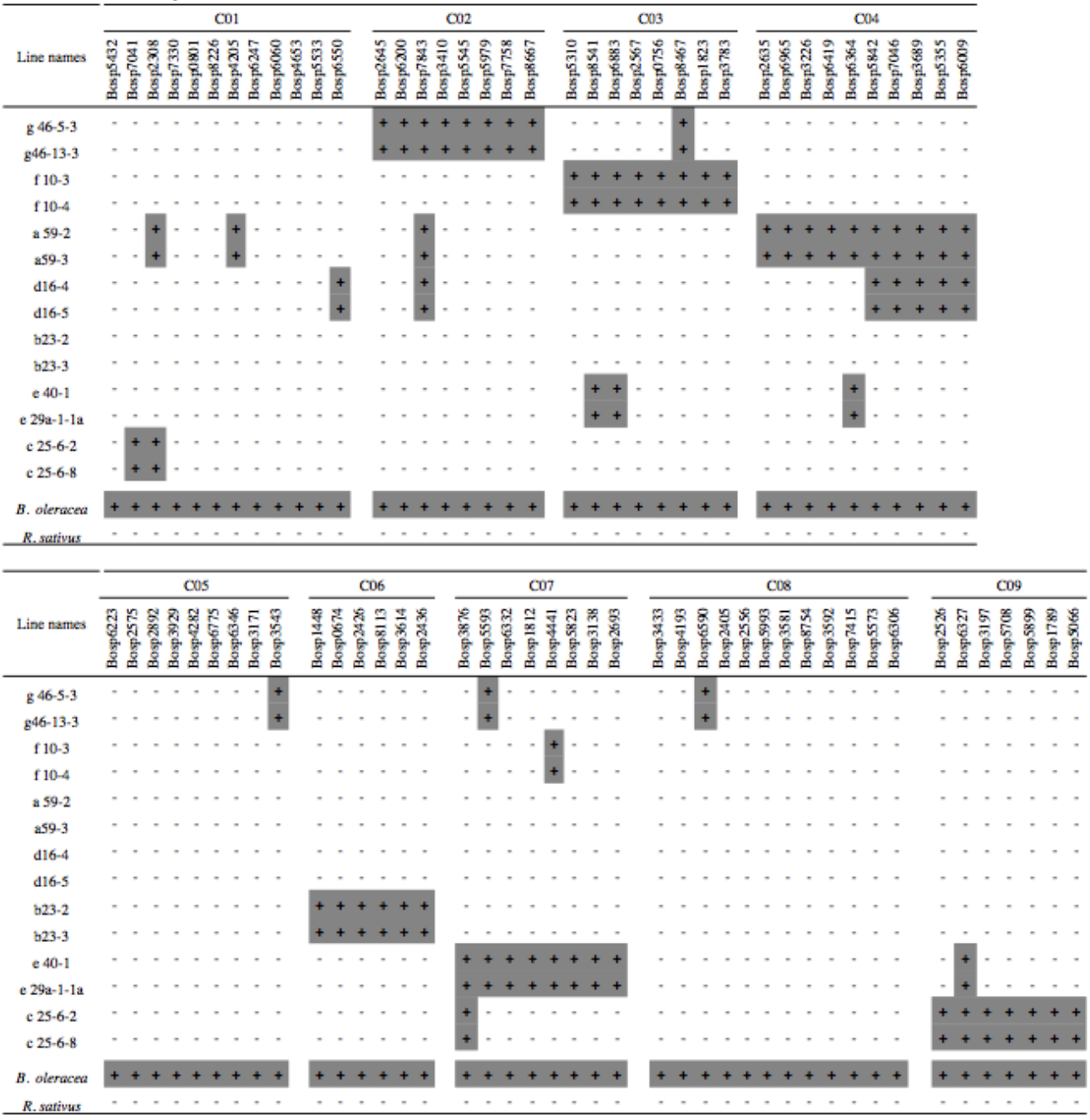
Line names	A01					A02					A03								A04							A05									
	Brsp2191	Brsp3279	BrCL0692	BrCL0712	BrCL5007	Brsp5584	Brsp6800	Brsp4049	BrCL3703	Brsp3015	Brsp5483	Brsp6489	Brsp5707	Brsp0965	Brsp3127	Brsp4116	Brsp5798	BrCL5450	Brsp4101	Brsp5727	Brsp6654	Brsp4014	Brsp3313	Brsp5729	Brsp4251	Brsp4132	BrCL2781	BrCL3392	Brsp6054	Brsp5771	Brsp5694	BrCL1200	Brsp6494	Brsp3252	Brsp1497
c17-19-22-9	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c17-19-22-14	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
e251-5	-	-	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
b197-1	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
b'197-6	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
d41-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
d41-15-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
h117-10-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
h117-10-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
e179-14	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+
g280-3-2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
g280-3-5	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
f300-8	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
f300-24-7	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
a5-1	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
a83-5	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
i88-3	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. rapa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
R. sativus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Line names	A06										A07						A08			A09						A10						
	Bro3387	Brsp1005	Brsp3701	Brsp5804	Brsp1078	Brsp3221	BrCL4007	Brsp1832	BrCL1332	Brsp1448	BrCL1574	Brsp4232	Brsp4632	Brsp1183	Brsp3860	Brsp0752	Brsp3972	BrCL0971	Brsp4152	Brsp6444	Brsp3917	BrCL0833	Brsp7378	Brsp6511	Brsp4255	Brsp1004	Brsp4458	Brsp4132	Brsp3699	Brsp4013	Brsp3316	Brsp1994	Brsp4309
c17-19-22-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c17-19-22-14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
e251-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
b197-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
b'197-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
d41-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
d41-15-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
h117-10-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
h117-10-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
e179-14	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
g280-3-2	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
g280-3-5	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
f300-8	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
f300-24-7	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
a5-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
a83-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
i88-3	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
B. rapa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
R. sativus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

chromosome-specific markers detected specific signals in both d- and h-type BrMALs, although d-type BrMALs and h-type BrMALs had been classified into different lines from each other (Kaneko et al. 2001, 2003). These results suggest that alien chromosomes of b-, g-, and f-type BrMALs are A03, A06, and A07 chromosome, respectively, and both d- and h-type BrMALs have the A04 chromosome.

A02 chromosome-specific markers detected signals in c-type BrMALs and 'e251-5', which had been classified into e-type BrMALs, and A05-specific markers did so in 'e179-14', which had

Table 2. Brassica oleracea-specific chromosome markers detected in BoMALs



also been classified into e-type BrMALs. Therefore, it was inferred that c-type BrMALs and 'e251-5' have the A02 chromosome, and 'e179-14' has the A05 chromosome. Thirteen markers located on A01, A08, and A09 chromosomes detected no specific signal. BrMALs containing A01, A08, and A09 are considered to be absent.

Eleven lines in seven different types (a-g type) of BoMALs were analyzed using 80 *B. oleracea* chromosome-specific markers (Fig. 3). Fifty-three markers detected signals, but 27 markers did not in any BoMALs (Fig. 3 and Table 2). C02, C03, C06, C07, and C09 chromosome-specific markers detected specific signals in g-, f-, b-, e-, and c-type BoMALs, respectively. All the C04 chromosome-specific markers detected signals in a-type BoMAL, and some of these markers also detected signals in d-type BoMALs. C01, C05, and C08 chromosome-specific markers did not detect specific signals in any lines. These results indicate that alien chromosomes of g-, f-, b-, e-, and c-type BoMALs are C02, C03, C06, C07, and C09 chromosomes, respectively. It is inferred that both a- and d-type BoMALs have the C04 chromosome. There were no BoMALs having C01, C05, or C08 chromosome.

Assessment of chromosome breakage

To investigate the chromosome breakage in MALs, we analyzed detected signals of the chromosome-specific markers in detail. Most species-specific DNA markers in the same chromosomes detected specific signals in the same line, indicating that chromosome breakage has seldom occurred in most alien chromosomes in BrMALs and BoMALs except the d-type BoMALs. Although C04 chromosome-specific markers detected specific signals in a-type BoMALs, only half of those markers (Bosp5842-Bosp6009) did so in d-type BoMALs, and the other markers (Bosp2635-Bosp6364) did not (Fig. 3 and Table 2). This result suggests that chromosome breakage has taken place in the alien C04 chromosome of d-type BoMALs during their

development.

Nine *B. rapa*- and fourteen *B. oleracea*-specific DNA markers also detected signals in some lines containing different alien chromosomes (Fig. 2, 3 and Table 1, 2). For instance, BrCL2781 of an A05 chromosome-specific marker showed a specific signal in f-type BrMALs containing A07 chromosome. Brsp5798 of an A03 chromosome-specific marker detected signals not only in b-type BrMALs but also in d- and h-type BrMALs having the A04 chromosome. These results suggest possibilities that some markers detect signals of similar sequences on different chromosomes of the donor species and that DNA fragments of the donor genome have been transferred into the *R. sativus* chromosomes or the alien chromosomes by meiotic recombination.

Discussion

Alien chromosomes in BrMALs and BoMALs, which have been classified by Kaneko et al (1987, 2001, 2003), were identified using species-specific DNA markers. In this analysis, we revealed that BrMALs contain seven chromosomes of *B. rapa*, and BoMALs have six chromosomes of *B. oleracea*. There were no BrMALs having A01, A08, and A09 chromosomes of *B. rapa* and no BoMALs having C01, C05, and C08 chromosomes of *B. oleracea* (Table 1 and 2). Although c-type and 'e-251-5' (e-type) of BrMALs, d-type and h-type of BrMALs, and a-type and d-type of BoMALs have been classified into different types of MALs, these pairs of the lines were revealed to have the same chromosomes. On the other hand, 'e179-14' and 'e-251-5' of BrMALs, which have been classified into the same e-type MALs, were found to contain different chromosomes. These results suggest difficulty of identification of alien chromosomes in MALs by morphological investigation and RAPD marker analysis (Kaneko et al 1987, 2001, 2003).

Chromosome abnormalities such as chromosome breakage and homoeologous recombination have often been observed in interspecific hybrids and their offspring, including MALs (Qi et al. 2007, Xiong et al. 2011). They have been verified by cytological analysis such as FISH or GISH (Ji et al. 2003, Fu et al. 2013b). In the present study, we showed the chromosome abnormality in both BrMALs and BoMALs. The chromosome breakage of alien chromosomes was found in the alien C04 chromosome of d-type BoMALs, in which specific signals of d-type BoMALs were detected by only half of the C04 chromosome-specific markers located in a region between 24.3 to 37.9 Mbp (Bosp5842-Bosp6009), while specific signals of a-type BoMALs were detected by all the C04 chromosome-specific markers. In a previous investigation (Kaneko et al 1987), it was difficult to distinguish a-type BoMALs from d-type BoMALs because of their morphological similarity. Plants of d-type BoMALs were vigorous, while those of a-type BoMALs were compact with round-shaped leaves. It can be suggested that addition of a whole chromosome has larger deleterious effect on plant morphology than that of a chromosome segment.

Although the species-specific DNA markers detected specific signals of the alien chromosomes in MALs, some markers also detected signals in MALs having different alien chromosomes. Out of the species-specific DNA markers, ones showing these additional signals in MALs accounted for 14.7 % (10/68) in *B. rapa*-specific DNA markers and 18.8 % (15/80) in *B. oleracea*-specific DNA markers. To examine the possibilities of low specificity of DNA markers resulting in nonspecific detection of the donor DNA fragments and homologous recombination between different chromosomes of the same donor genome, we analyzed homology of the nucleotide sequences of the DNA markers, i.e., primers and probes, with the published sequences of the chromosomes possessed by the MALs showing the additional signals. High homologies were not found between the markers and the alien chromosomes of these MALs. Furthermore, we investigated the presence of the same synteny blocks (Wang et al. 2011a and 2011b) on the chromosomes containing the DNA marker sequences and those possessed by the MALs showing the signals, but no common synteny blocks were found. These observations suggest that DNA fragments of *Brassica* species have been introduced into the *R. sativus* chromosomes probably by homoeologous recombination.

B. rapa-specific DNA markers located in a region from 7.9 to 12.0 Mbp (Brsp6489 -Brsp0965) of A03 chromosome detected signals in three or four different BrMALs. It is suggested that homoeologous recombination between the *Brassica* genome and the *R. sativus* genome may have occurred during the development of MALs and that *Brassica* genome sequences were introduced into and remained in the *R. sativus* genome. The 7.9 – 12.0 Mbp region of A03 chromosome might be a region amenable to homoeologous recombination. Further backcrossings are required to produce MALs having a clean background.

When the homoeologous recombination occurs, multivalent chromosomes can be observed in interspecific hybrids and their offspring (Kamstra et al. 1999). In BrMALs and BoMALs, multivalent chromosomes have been observed (Kaneko et al. 1987, 2001), but the frequencies of multivalent chromosomes were less than 4% and ca. 1% in BrMALs and BoMALs, respectively. In MALs obtained by interspecific crossing between *B. rapa* and *B. oleracea*, the frequency of multivalent chromosomes has been reported to be 22% (Hasterok et al. 2005), suggesting high frequency of homoeologous recombination. Such high frequency of multivalent chromosomes may be due to a close relationship between the parental species. In fact, resynthesized *Brassica napus* plants generally have complicated genome constructs (Szadkowski et al. 2010, Xiong et al. 2011). Alien chromosomes in BrMALs and BoMALs may have been maintained more stably than those in the MALs derived from hybrids between *B. rapa* and *B. oleracea*, and BrMALs and BoMALs are expected to be useful gene sources for plant breeding.

Species-specific DNA markers were able to identify alien chromosomes of two sets of MALs, including lines which are difficult to distinguish from each other by analyses of morphological traits and RAPD markers. Furthermore, these markers were also found to identify abnormalities of alien chromosomes and possible homoeologous recombination in MALs. It is suggested that the misclassification of MALs might be caused by those chromosome abnormalities. Identification of alien chromosomes in most MALs so far developed in many crops have been performed by using RAPD, amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) markers (Ji et al. 2003, Friebe et al. 2000, Tsukazaki et al. 2011, Gereta et al. 2012). Analyses with RAPD and AFLP markers are less reliable than those with other DNA markers. Electrophoretic analysis using SSR markers can detect only the differences of amplified DNA fragment sizes, and, therefore, polymorphism between species is difficult to distinguish from that within a species. Species-specific markers developed by careful investigation of published genome sequences, species specificities of which are secured by the sequence specificities of primers and/or probes, have not been used

for analysis of MALs. The species-specific DNA markers developed in the present study will be applicable to analysis of a large number of lines owing to their use in the procedure of DNA polymorphism analysis, i.e., dot-blot analysis (Shiokai et al. 2010), and can be an efficient tool for producing a full set of MALs.

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Supplemental Data

Supplemental Table 1. Sequences of primers and oligonucleotide probes of Brassica rapa-specific chromosome markers and conditions of hybridization and washing

Supplemental Table 2. Sequences of primers and oligonucleotide probes of Brassica oleracea-specific chromosome markers and conditions of hybridization and washing

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Supplemental Table1. Sequences of primers and oligonucleotide probes of *Brassica rapa*-specific chromosome markers and conditions of hybridization and washing

Marker names	Chromosomes		Primer sequences (5' → 3')		Probe sequences	Hybridization conditions	
	No.	Position (bp)	Forward	Reverse		Temperature	SSC concentration
Brsp2191	A01	2,090,469	GATCAGGGACAATCCCATTAGC	AGCCTTCAGTGTGCTAGGTTCA	TCTTTTGACGACTGTTT	40	1
Brsp3279	A01	11,024,087	TAAGCGTTCACGACTCATCCAT	GGTTCTCCTGAGCGTCAAAAAG	TCTGCATATACCAATCA	40	1
BrCL0692	A01	15,661,449	GAAATGTGTGGCTGAGACTCGT	CGTAGAAGGGAACCTCAATGCT	TGATGAGTCTTGCAAGC	45	0.5
BrCL0712	A01	23,724,563	TAGCCTGTGACACCAACGCTAT	GCAGTGCTGTTTGCTTGTTTCT	ACCAAGAACACCGACAA	45	0.5
BrCL5007	A01	25,441,801	CGAAGAAAAGAAACCCGAAG	GTATCCATAAGGCCATGGTTG	CCGAGGAGAAAAAACCA	40	0,2
Brsp5584	A02	1,736,612	AGCTAGAGCCAGGGTCAACA	TAGCCGTAAAGCCATCGTTC	CATCGAGCGGTGGTA	50	1
Brsp6800	A02	10,800,423	CTTTATGGCCTTGGTTCAGC	CCATTGAGCTTGGCGTAT	ACCTAGTTGATATTCCAT	40	0.5
Brsp4049	A02	12,362,659	AACTGGGTGGACTTCAGTGG	GCTTGCAAACGGCTAGTTTC	GGATCTGGTGAGTGAG	50	1
BrCL3703	A02	14,449,226	TTTCCCTCCATTTCAGGACCTA	TCTCGTTCTCACGTTCTCATGC	AGTTTTGGGAACGATAT	40	1
Brsp3015	A02	17,777,614	CAGCTGTTGTGGGTATTGGAAA	TCCTTGGTTGATCTGGAACTGA	AGGAGCTTAATGGCTTG	50	1
Brsp5483	A03	4,696,805	GGCGTCCTCAAGATCTTTTT	AGAAACCCAGAAACGCAAAC	CGCTGTTACCTACACC	40	0.5
Brsp6489	A03	7,926,432	ATATGCTCAAGCGCGGTTAC	TCTCAGCACACCAAATCTGC	GCTTAATTCCAGGTTT	50	1
Brsp5707	A03	9,447,615	GGGATAGCAATGTTGGATGG	GTTCAAGCATTTCCCCTGAG	GCAGGACTATCTGTTTG	50	1
Brsp0965	A03	11,957,491	GGTGAAGAAGAGGCATCTGG	TGTACCACACAAGGCACCAC	CGATCTTCTCAGCTGT	50	1
Brsp3127	A03	17,074,203	ATTGAGTGTGGTGTCACGGAAC	GGAGCTTGATCTCTCCCTTGAA	GAACTTAGGATCAGTGA	40	0.5
Brsp4116	A03	20,904,144	CCACGAGTTTCCACCTCTCTTT	AACACCGTACTTGATGGCAATG	ACTATAGATCAATCAAA	40	1
Brsp5798	A03	25,713,235	GAAATCCACGTCGAAAAGGAAC	CATAAACACACACACCCCCAAT	ACATATATAGAGATAAG	30	0.5
BrCL5450	A03	29,039,136	TAATCCAAGTGGCTCTGCTCTG	CAAACCCAATCTGAATCCTCCT	TCCCACATTGAAAACGA	45	0.5
Brsp4101	A04	1,073,997	TCGTTGTCAGCAGACTTCACAA	AGCCTGCAGTAGGGTCAAATTC	CAATAGGCACAAAGAAC	40	0.5
Brsp5727	A04	1,925,197	CTCCGCTGCTCGTGAATATCTA	ACCATTTACCGCATAACCCTTG	ACATGTTAATAAGAATG	40	1
BrCL6654	A04	7,800,998	GTCCCGTGCTCTCCATAAAAAC	GTGTTAAACGGAGCAGATGGTG	GAGACACTAATTCACTA	40	0.5
Brsp4014	A04	9,996,140	GCTCGTGAGTTGCTGAAACTTG	TGGTAGAACCACCAACAAGGAA	CCTTACAAAGAATGGGA	40	0.5
Brsp3313	A04	10,706,536	CCGATGATTCGTCCGTGTTA	CGAACTGCAATCAGCCATTC	TAAAAAAAATTAGATCT	30	0.5
Brsp5729	A04	10,948,425	ACAGAGACACGAAACCCAATGA	GAGAAACGAGGAAGGCAGTCTA	GCAACCGCACCCTCTCT	50	0.5
Brsp4251	A04	16,683,818	TGCGTAAAGCAGGATACAATGG	GTTGCGTTTTCAGAGAATGGTG	TGCACCACCAAACTC	50	1
Brsp4132	A05	3,434,120	AAGTGGCCGTATGGGATACA	GGTCCACTTTCTTTCCATGC	TGCAAGTGGTTGCAGT	50	0.5
BrCL2781	A05	6,290,904	GAAACGGAGGCAAAGGATGTAG	ACAATCTGCAGAGAGAGGCAAA	ACAGTGAAAATCATCAC	40	1
BrCL3392	A05	6,646,717	GAGTAAAGCCGAGCTTGTTCGT	GATCCTCAGCTTTTGGTGGAAT	ACCACACGTTGTTCGTT	50	0.5

Brsp6054	A05	7,293,761	AAGCCTAAGGCGAAGGAGAG	AGCCCCCTTCATAGACGATT	AAGAGGGCTTCGACGA	50	0.5
Brsp3771	A05	16,757,651	ACGCTAAGAAGTTGGGAGGAGA	GAGCATTCTCTTGTTCGGTCAT	CATACCCGAAAAAGCTT	50	1
Brsp5694	A05	16,611,050	AAGCTCGAGAAGCGATGTGT	TGGAAATGTGAGAAGGTCAGC	GAGACATCTGGCCTTG	50	1
BrCL1200	A05	21,215,000	CCCTTCCTCAGAGTTGGTTTTG	GATGATGTCTTCGCCGATGTTA	TCACCAAAGGAGGAGTC	45	0.5
Brsp6494	A05	21,702,815	TGCTTCATGCAAGCCTTAGAAC	TGTTGAGAGCAGCAGCATTACA	AATCAAAGTTATTCTCA	40	1
Brsp3252	A05	21,732,542	TCCTCTGGCTCCATTAGGTC	GTGTCCCCATTCCTGGTTAC	AAACCCCAAGGGAGAA	50	0.5
Brsp1497	A05	22,617,537	TGTTTGGGAGCTGAAGGAAG	CACACCAAATCTTTCGAACC	TGTGTGATGACGGTAT	50	1
Brsp3387	A06	654,009	GGTAAGAAGGCGACAGCTTTTC	GCTGGAGTGACAACTGACTGAA	AATGTGCTTTGCGCTGA	50	0.5
Brsp1005	A06	1,415,597	AACCAATGGCTTCCACACTTCT	AACCCTGAAAAGCTCAGTCACC	AGATCAGCAGTTCCTCG	50	1
Brsp3701	A06	5,212,848	AGAATGCCTAGGGTCAGATTCG	GTTGGAAGGCAACAAAATGG	CACTTCTTACGTTGATT	40	0.5
Brsp5804	A06	6,064,748	CAGTTTCAGGGAGGAGATGG	CCTGACACACTCAGCACTCAA	GTGTGATTAGTGCTGTTG	50	1
BrCL1078	A06	8,283,418	AGACCAATGCTTTCAACCGTCT	CCCTTTCCAACAAGCTAACGAC	ATTTTTCTCTTCTTTTT	35	0.5
BrCL3221	A06	12,312,536	TGCAGTTTCATGGAGCATTC	CCTCTCCAGAAATCCAAGGAG	CTTCCGCAGCACAAA	40	1
BrCL4007	A06	16,718,051	TCCTGCAAACTCCCATACTCAA	ATGGCTGCTCTAAAAGGCAAAG	TTCAGTGCGGCTTCCAC	50	0.5
Brsp1832	A06	17,726,236	ACTGAGGCGACTTTTGATGG	CGACATCACTCAGCAAGGTG	TCGCGATATGGCAAC	40	1
BrCL1332	A06	20,472,945	CACAGGTTTGGTCGCAGAACTA	GGAGAAGAGGTTGTTGGAGGAA	CAAAAAAGTTAAGCATA	35	0.5
BrCL1448	A06	25,045,969	AGCTCAAGCTCCTTGCTCAT	GGATGGGGAAAGACGATAATG	CAAATTTCTGAACCTG	50	1
BrCL1574	A07	8,391,967	GGACACATTTCACAACCACCAC	TCTCGTCAACGGTAGTTTTTCC	GTTTTTTTGTTTGTTTT	35	0.5
Brsp4232	A07	17,073,809	AAATCAGGGGGTGCTCTAATCC	GGGTGCAAAAGAGCCAATGT	ACGGTTCTCTTGGAGAA	50	1
Brsp4632	A07	18,003,380	GATCATGTCCTGGCATATTGGA	GGATCAGTCATTCCACGAACAA	GCTGAGGAGGAGAAGAA	45	0.5
Brsp1183	A07	18,772,574	TAAAGGTGTGATCCCAATGCAC	AAACGGTATGACCAACTCAGGA	TACTTGGGACTCATTGA	50	1
Brsp3860	A07	23,902,326	GGTCCCTCAGATTCCACAAACT	GCGCCGTGATTCACAATATAAG	TACTACTTCTACTTTTA	40	1
BrCL0752	A07	23,977,082	TTGTGGAAGTGCTTGTGTAGCA	CAAGGGGATTATTTCACGCAAC	TCATGAGAGCCGTACGG	50	0.5
BrCL3972	A08	6,732,161	CGCTATAGCTTGCGGTTACACA	TTTACACAACACGGCAAGAAGC	CTTCTCGCAGCAATTAT	45	0.5
BrCL0971	A08	12,510,970	CAAGTCCTCGGTTCTTCATTG	TCACTCATGTCCCTATTCATGG	ACTACTAACTTGGCATG	40	0.5
Brsp4152	A08	14,670,847	GAAGCAACTACAAAGCGTGGTG	ACTAAACGACACCGAGACCGTA	GTGATTTTAAATTAATT	30	0.5
Brsp6444	A09	1,319,826	CAATAGTCAACGACTCGACGTG	GAATACGAAACAGAGGGGGAGA	CCAAGCCAGTACAATGT	50	0.5
Brsp3917	A09	9,756,377	GGGCCTAACGTTCAGTGGAATA	AAGCCACCAACACATGTACGTT	AAGCTCGAGGTCCCTAG	50	0.5
BrCL0833	A09	11,570,510	AGGCTTGTGCCCTTGTATGT	TCACTGTGGACCTAGACGTTGT	TATTACATATGTTTTTT	35	0.5
Brsp7378	A09	19,094,110	GAGGCAATACACATGGGACAAG	AAGCTGCACAATAACAGGGTGA	AACATTAAGCTATTATTATT	40	1
Brsp6511	A09	27,767,282	TACCAAGGCCACAAAGAAGACA	CCACACACCATCGTGACTGTAA	CATTGAACAACGTTAAG	40	0.5
Brsp4255	A09	31,402,919	TTGACATAAGATCCGCAGAAGG	CGAACATGCTCCACAGTCTTTC	ATCTTCTTCCACGGCGAT	50	0.5

Brsp1004	A09	38,322,895	CGATTCAACATGGGAGTCTCTT	GAGATCCACATCACATGCTTCA	CACTGACGATTCTCCGGCGA	60	1
Brsp4458	A10	147,404	GGAACTCCAATCCCCACATA	CAGCCATAGCAAACATCACC	AAGATCATTGGAACATCT	50	1
Brsp4132	A10	3,434,120	AAGTGGCCGTATGGGATACA	GGTCCACTTTCTTTCCATGC	TGCAAGTGGTTGCAGT	50	0.5
Brsp3699	A10	6,942,410	ATAAAGCTGACCAGATGGGAGA	GTACATGGAAAGCATGCAACAG	TGAGTGACCTGAGAGTT	50	1
Brsp4013	A10	7,201,877	CGATGTTCTCGCCTTTGATT	TCCACTGGGTCAAGATCTCC	AGTGTCCGTGTTTAGTGAC	50	0.5
Brsp3316	A10	8,541,717	AGGAAAACAAGCTGCTCTGC	TTTCCAGCTTTTGACCCATC	AGTTAGCAGCATATAGAAG	50	0.5
Brsp1994	A10	11,003,559	CAGCGTAGGATGGTGAGAAAT	AACTTAGTACCAGCGGCTCATT	GACTGCGATTCTGCAGA	50	0.5
Brsp4309	A10	16,702,302	CGATCGCTTCTTCTCCTCTG	TGGGTAGACGTGTAAAGTCGAA	CAATAGTTGGAATTAACCA	50	0.5

Supplemental Table 2. Sequences of primers and oligonucleotide probes of *Brassica oleracea*-specific chromosome markers and conditions of hybridization and washing

Marker names	Chromosomes		Primer sequences (5' → 3')		Probe sequences	Hybridization conditions	
	No.	Position (bp)	Forward	Reverse		Temperature	SSC concentration
Bosp5432	C01	3,342,299	TCAGGCTTCGTCGAGTACATTC	AGCTCTCATAGCAACACCATCG	TTAAACGTTTCCGTGGC	40	0.5
Bosp7041	C01	3,611,889	CGTACGAGAATGCACACCAC	AAGGCCCAGAACACTGAAGA	TAGCTCTCTCTCACCGG	40	0.5
Bosp2308	C01	5,694,282	GAGACTGCATCTGGATTTGGTG	TTCACAGGAAGAAACCATGACC	GACATAGCCTCCGGAGG	40	0.5
Bosp7330	C01	7,043,931	CAATTTGGAGTCTGGGAAGC	CATGCTTTTCTTTGCCCTTC	AGTCCGAGCGTCATAG	40	0.5
Bosp0801	C01	10,748,339	AAGTCGAGGAGCTGTTGAGC	AACTTCGCCTCGAGTTCTTG	ACAAGAGTCTGCTTGA	40	0.5
Bosp8226	C01	11,129,359	TCCCATCCTTCACTCTCCAC	CAATCCGAAGTCTGTGCAAC	CGCTGCTTCTCTAGTTTTTCT	40	0.5
Bosp4205	C01	11,830,170	GCAAGGAAATTTTGCTGACC	CAGCTTGGTGAAAGGGAAAG	TCCTTGCCGTCAATTTTC	40	0.5
Bosp6247	C01	16,868,415	ACCAATACGATCCGACAAGC	TTCGACTCACCACGTTAGACC	GGTATGAGATGTCTTATGATGATG	40	0.5
Bosp6060	C01	20,424,788	CGGTTGCTTTCGCACTATCT	TGGCCTTTCTTAAACCGTTG	TTTTGGAGATGCAAATAT	40	0.5
Bosp4653	C01	21,447,577	GTTGGCCGTGACCTCATACT	CACCTCAAGAAGCGAGAAGC	TGTGGCGCATAAACACC	40	0.5
Bosp5533	C01	36,583,336	CAGCAGGTTAACTGCCATGA	AAACGTGAAAGGTCCATTGC	TGAGCGCTAAAGAGCT	40	0.5
Bosp6550	C01	37,150,157	TCTTTCCCTTTCTCCAGCTTC	GCCTTGTAGGGTGAGCTTTG	TCATCATCGACATGTAG	40	0.5
Bosp2645	C02	1,210,286	TACGGATCGGGTCAAATAAACC	CAAGATGGGACTCCTCACAAGA	GGAAGAGGCCATGAGAG	40	0.5
Bosp6200	C02	3,466,517	CTCCAAGAAAGCCTCTGGTG	GAAGCATACGACTTGCGTGA	GAACTTCGTTCTTCATT	40	0.5
Bosp7843	C02	3,604,467	CACCGGAGATAAAGCCAAGA	TTAGGGCTTCCTCTCACACC	AAGAAGAAACAGATGTC	40	0.5
Bosp3410	C02	5,922,944	TGGAGAGCTACGACATCACG	GTAACGGCGGATTCTTCAAA	CGGCCATGACTTTTGG	40	0.5
Bosp5545	C02	6,314,639	CGCAGTCTTTGGCTTTTGAT	ACGGCTCAAGTTGGTCAATC	GAACCGGGAGAGTTAGTTACTC	40	0.5
Bosp5979	C02	7,682,902	CTAAACGGGACCAAACCTGA	CATTAGTCCAAGGGGAAGCA	TTGCTCTTGGGATCGACT	40	0.5
Bosp7758	C02	31,292,824	AGCTTCATCTGCTCCTCCAG	TTAGACCGCAGCTTCTCGAT	TTCCACAATCTTGACCCTC	40	0.5
Bosp8667	C02	38,155,690	CCTTCTCTTTTTCTTCTTCTTTGAG	GTTGGTTGGTGTCAGCAAGA	GAGTGAAAAGATGAACT	40	0.5
Bosp5310	C03	696,026	GGCTGTCAAATGTGTGGTTG	GCGGATCATAATGCGAATCT	TGACTCGATAGAGGCATTG	40	0.5
Bosp8541	C03	4,342,209	GCGCAAATGGTTCCTTGTAT	ACCAGGTCACCGAGAATGAC	AAGAACTTCAGAAGCA	40	0.5
Bosp6883	C03	7,015,584	CAGCAAAAACGAGGATGACC	GGAACTATGCATTACCCATTCC	GGAACAAGAGAGGAAG	40	0.5
Bosp2567	C03	7,583,377	GCTGTGTGGTGAGGTTGTTG	CTCGGCTGAGGATGAAGAAG	AACTCATCGTAATGTACTCTTCTG	40	0.5
Bosp0756	C03	20,793,336	TTTGCTAGCAGATGCCTTCG	AGTATGACGAGCCACCATCC	TAGCCTTAGCAGAGGAGGA	40	0.5
Bosp8467	C03	33,103,812	CAAACCGGTTCTGTGAAATCTG	CACGCCTCAGAATAGCAATCAA	CTGCTTAAGGATAACCT	40	0.5
Bosp1823	C03	37,001,281	ACACCCGTGTTCTTTCAAGG	CCATATTTTGCGTTGACTCG	TGAGCCAACCTCGTGTAT	40	0.5
Bosp3783	C03	40,369,573	CAAGAAGTTCCCAGCCTCTG	GAAGAGCAGATCCCAGCAAG	CTTACGCTTAGCAAAAGCTG	40	0.5
Bosp2635	C04	3,724,738	TGGACGAACAGAACATTCCA	TTGGCCCCAATACAGTCTTC	GAAGAAGTCTCAAGTGCCTG	40	0.5

Bosp6965	C04	12,714,913	CCGCTTCTAAAATTCCTCTCCA	CGGAATCAATCTTGTTGCTACG	TGGCACTCGTTACTAAA	40	0.5
Bosp3226	C04	15,952,932	GTTCCTTCTCAAAGCCATCG	TTGAAGTGAGCATGGAGACG	AGAACAAACCTACGCTCCA	40	0.5
Bosp6419	C04	21,803,824	CTCCGAATCAGACTCCGAAC	GCCAAACACCTCTTCAGCTC	GCTGTTTCAGACGAATG	40	0.5
Bosp6364	C04	22,309,364	CCAGGATGTTCAGCATCACA	GGCTAAACCGAAAACTGCTG	GAGGAAGAAAATCACCA	40	0.5
Bosp5842	C04	24,328,303	CAGATTGAATGGGAGCTGGT	CCGGAGAGATCACAGCTTTC	ATTCCGGCGACGAAA	40	0.5
Bosp7046	C04	26,940,580	CCATCCGACATGACTGTGTC	CATCCTGCTGAACCTGATCC	TTGTGCGTGTTTCCACAT	40	0.5
Bosp3689	C04	33,499,932	AACCTCCACAAAAACCTCATCC	AGGAGCATCATCAGGGGAAT	ACCACGAAGCTTCGAAA	40	0.5
Bosp5355	C04	37,821,096	CACCGTGCATTGGCTACATA	CACTGACTCCACATGCCTTG	AGCTTAACAGTATGGCCCG	40	0.5
Bosp6009	C04	37,893,386	TGTGAGCAAGGTTACCGTCTTG	TTACCATGGCTTCCTCATCTTG	AAGAGTCCATGTTGTTG	40	0.5
Bosp6233	C05	1,737,090	CGCCCATATTGATTCTTGCT	CACATGCACGAGGAAGAGAG	GCATGAGTTTTCTAACGGGA	40	0.5
Bosp2575	C05	5,156,969	CTGAAGAACACCCCTCAAGC	TCCCCTGTTTTCTCAAATGC	TGACATCTTTCTCCTCA	40	0.5
Bosp2892	C05	7,825,799	CGAGGAAATCCTCAAGGTGA	CTTCGTCTGTTCCGGTTTTG	AATTCCTCGGACTGTTGAG	40	0.5
Bosp3929	C05	8,039,790	TTTGTTGCTCCAGATGTTGC	ATCCTTTGCAACAGCAAACC	TTGAAGATTCCGGGAC	40	0.5
Bosp4282	C05	15,618,745	ACTAAACCCTGGTGGTGTTTCC	CATCAGATCAGCCATCATGACA	ACCATAATCACAATCTA	40	0.5
Bosp6775	C05	23,132,359	TGCCTTCAGGGAGAAGAAGA	TGCTGGTCTCTCATTGTTGC	CAAAAGACACAAACCTAAACAATC	40	0.5
Bosp6346	C05	26,113,899	TGTAGTCGCAGAACCACAGG	CAAAGCCCAAAGCAAAGAAC	CTCTGGGTCATACATG	40	0.5
Bosp3171	C05	27,551,220	AAGATCCGAATGGGGTTAGG	TGCTCGACATGCTTTTAGGA	AACGCAACTGCTACAG	40	0.5
Bosp3543	C05	29,572,456	ACCCATTCTCATCAGCCTTG	GAAAAAGGACTCCGCAACTG	TCACTGGTAGCTGGAGGAG	40	0.5
Bosp1448	C06	24,283,654	GCCTTTAAACCACCGAACAA	TTCGAGTGTGTTGGAAGCAG	GTTTAAGCAAATTTCTGAACCT	40	0.5
Bosp0674	C06	31,946,888	CTGCCCTCTCATACCGAAAC	ACCCCTTGTTGTGTTCAACC	GAACCTACTACTGCTTCCGG	40	0.5
Bosp2426	C06	38,192,348	TCCCAAGTACTGTCGTCGAA	ACTCCTTTGAGACCGTGTGG	TGACACATCTCATGAAGTTCTGT	40	0.5
Bosp8113	C06	41,348,170	GGAAGCAGGGAAGGCTCTAT	TGGGCTCATAGGACTCCATC	ATTGGGGCTAAAGAGCTATG	40	0.5
Bosp3614	C06	46,404,754	AACAGGCGTATGGAAACCAG	ACCAGGCTTGAATTTGGAGA	CAGGTACCAGCATCATCG	40	0.5
Bosp2436	C06	48,303,007	TCTAACCCTTCCACCACCTG	GTAACCTTTCCATGGCGAAC	CCAATCTGTTTTTACGGTG	40	0.5
Bosp3876	C07	2,884,637	CGCACAAGGAGGGAGATACTTT	CGGCTTTCCAATGTAACCTCTT	GAACATTGGATGTGGGA	40	0.5
Bosp5593	C07	9,138,166	TCCATCTCTTCACCCACTTCTG	TGTCGATTCCGACATGGTTATC	GACGCCGGTCGGATGAG	40	0.5
Bosp6332	C07	9,265,675	GCGTTGGTCTACCGAAACAT	AGCTTCCTCCACACGAACAG	CTATGGCGATTCCGTTTT	40	0.5
Bosp1812	C07	13,342,112	GGACAGGAAAAGAAGCGTTG	GCCTAGTGCATTGTGACACG	GGATGGTGGACATCGATC	40	0.5
Bosp4441	C07	17,605,121	TTTGGGACACGAGGTCTGTT	TGCATACAAGCTGCCAAAAC	GAAATATCGGTGTCACT	40	0.5
Bosp5823	C07	18,176,696	TTGCCCTAACTCCAATGCTC	ACAAAAACCATCACGGCTTC	CTAGACAACGCGACAGC	40	0.5
Bosp3138	C07	21,651,292	CTCCGGTTTGCTTCTCTTTG	TTGACTGTCGCGGATATGAA	TATGATTAATTGATTGA	40	0.5
Bosp2693	C04	24,787,789	GTTCCTTCTCAAAGCCATCG	TTGAAGTGAGCATGGAGACG	AGAACAAACCTACGCTCCA	40	0.5

Bosp3433	C08	939,459	ATCCAAACGGCGTTAAACTG	ACACCGGAGAAGAAGCTTGA	CGATTGGAAGTGAAAGAAGC	40	0.5
Bosp4193	C08	9,083,323	GAAATTGGCCGGTTCATAGA	TCGTGTAACCTCACCCATGA	GTGTAGTGATTGTGGACTCACG	40	0.5
Bosp6590	C08	19,953,674	TATGTCGAGGGGAAGAACCA	TCTCCACACCATCATGGAAA	ATTTAGTTAGAGAAATC	40	0.5
Bosp2405	C08	27,339,382	TCACTGCCACTACTTGCAAAGC	AGCGAGTGCATCAGAACGTTTA	ACTGCTTTTATCAAAGG	40	0.5
Bosp2556	C08	28,779,601	CACACTCGACCCAATGTACG	ACGAGGGTACCCGTAAATCC	TGGTCCAAGTGGGAA	40	0.5
Bosp5993	C08	30,497,379	GGTTGCAAGGTGGCTATCAG	GCAAACTCTGCGGGTTTAAG	GGGCATTACACTCCTTACA	40	0.5
Bosp3581	C08	31,186,397	TCTCTGGCTACCTCGAATGG	TACACATTCCCCACCTTGTG	TGAAACATAGATTTGAC	40	0.5
Bosp8754	C08	31,595,079	CATGTCCATTGGTTGTTGGA	GGCCGTACAGTTTCAAATGC	CCGACAACATTTGGTT	40	0.5
Bosp3592	C08	33,556,132	GCTGATCAACTTCCATCTCCAA	GATATGGGTGATGGTTGGGTTT	ACCAGCCCTTGTCTGAA	40	0.5
Bosp7415	C08	35,444,655	TGAAGTTGAGCAATGGCTTG	CCGGGCAGTTATTCCTAACA	CGCCTTATGAGCTCACTGC	40	0.5
Bosp5573	C08	38,549,917	ATGTGCATGGACAATCGCTTAG	CATTCCTTTGAGAGGGAGGCTA	TCACCTATGGTATTTTG	40	0.5
Bosp6306	C08	41,408,261	GACCCAAACCAATCATCACC	AGATGGAATCGGATGAGCTG	CTTCCCAACAACATTTGACC	40	0.5
						40	0.5
Bosp2526	C09	1,410,010	ATGCTTGTGGACGAGAGAGG	CTCCAAAAGTCCTCCCAACA	GAGCTTAGCGGGTTATATGA	40	0.5
Bosp6327	C09	2,628,561	AACAGCCAACTTCATCTTCGTG	TCGAGCATATGACGAGCTTCTT	CGGAGAGTACAACAAGC	40	0.5
Bosp3197	C09	3,161,935	CTGCAAGCCTTTGAAGAACC	GCAGCAGATGACACAAAGGA	GTGGCTTCTTTGCTTCATTA	40	0.5
Bosp5708	C09	10,422,371	GCGCTTCGAATGAATCTCTCTT	AGCTGAATCACTTGCAGCTCCT	GATGATGAGAAAGAAGA	40	0.5
Bosp5899	C09	18,213,246	AACGTTGGTCCTCACGTTTC	CACCAAACTCAGGCCTCTTC	CTCAACTTCACTACGAAGCA	40	0.5
Bosp1789	C09	24,992,369	TTCTTGGGCCATCCATAGAG	AACGACACCATCCTCTGACC	GAAGCTGCCAATAGTAATAAGC	40	0.5
Bosp5066	C09	34,062,175	CATGCGGGCTATTTCAGAAT	AAGCCATGAGATGGATGGAG	CAACACAACTGTTACTCTGTCTTT	40	0.5