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ORIGINAL PAPER

Assigning *Brassica* microsatellite markers to the nine C-genome chromosomes using *Brassica rapa* var. *trilocularis–B. oleracea* var. *alboglabra* monosomic alien addition lines

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Abstract *Brassica rapa* var. *trilocularis–B. oleracea* var. *alboglabra* monosomic alien addition lines (MAALs) were used to assign simple sequence repeat (SSR) markers to the nine C-genome chromosomes. A total of 64 SSR markers specific to single C-chromosomes were identified. The number of specific markers for each chromosome varied from two (C3) to ten (C4, C7 and C9), where the designation of the chromosomes was according to Cheng et al. (Genome 38:313–319, 1995). Seventeen additional SSRs, which were duplicated on 2–5 C-chromosomes, were also identified. Using the SSR markers assigned to the previously developed eight MAALs and recently obtained aneuploid plants, a new *Brassica rapa–B. oleracea* var. *alboglabra* MAAL carrying the alien chromosome C7 was identified and developed. The application of reported genetically mapped SSR markers on the nine MAALs contributed to the determination of the correspondence between numerical C-genome cytological (Cheng et al. in Genome 38:313–319, 1995) and linkage group designations. This correspondence facilitates the integration of C-genome genetic information that has been generated based on the two designation systems and accordingly increases our knowledge about each chromosome. The present study is a significant contribution to genetic linkage analysis of SSR markers and important agronomic traits in *B. oleracea* and to the potential use of the MAALs in plant breeding.

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Introduction

The genus *Brassica* is composed of diploid and allopolyploid species. *Brassica rapa* ($2n = 2x = 20$, AA), *B. nigra* ($2n = 2x = 16$, BB) and *B. oleracea* ($2n = 2x = 18$, CC) are diploid species, whereas *B. napus* ($2n = 4x = 38$, AACC), *B. carinata* ($2n = 4x = 34$, BBCC) and *B. juncea* ($2n = 4x = 36$, AABB) are allotetraploid species; each generated from two of the three diploid species through natural hybridization and polyploidization process (UN 1935). *B. napus* is an amphidiploid species that originated from the hybridization between the diploid species *B. rapa* and *B. oleracea*, as confirmed by the identification of distinct linkage groups representing the A- and C-genomes (Parkin et al. 1995).

Within the last three decades, sets of *Brassica rapa–B. oleracea* monosomic alien addition lines (MAALs), which contain the entire diploid complement of *B. rapa* as a background genome and one of the nine chromosomes of *B. oleracea* (AA + 1 C-chromosome, $2n = 21$), have been generated and characterized (e.g. Quiros et al. 1987; Chen

et al. 1988; McGrath and Quiros 1990; McGrath et al. 1990; Hu and Quiros 1991; Chen et al. 1992, 1997a; Cheng et al. 1994a; Heneen and Jørgensen 2001). One of these sets of MAALs was generated using a pair of parental lines, *Brassica rapa* var. *trilocularis* (yellow sarson, K-151) and *B. oleracea* var. *alboglabra* (No. 4003). The development of this set of MAALs involves backcrossing of the resynthesized *B. napus* (AACC) to *B. rapa* (AA) to produce sesquidiploids (AAC), selfing or backcrossing of the sesquidiploids to the AA parent and the production of a progeny of aneuploids (AA + 1–9 C-chromosomes) and parental AA plants. The analyses of the aneuploids and their progenies have resulted in the detection and development of the MAALs that carry the different C-chromosomes. The different MAALs have been identified by detecting the alien chromosome through cytogenetic studies (Chen et al. 1992, 1997a, b; Cheng et al. 1994a, b, 1995; Jørgensen et al. 1996; Heneen and Jørgensen 2001; Hasterok et al. 2005), by genome and chromosome-specific markers that distinguish the various alien chromosomes (e.g. Jørgensen et al. 1996; Chen et al. 1997a; Heneen and Jørgensen 2001) and/or by the unique morphological features of plants bearing specific alien chromosomes (Heneen et al. 2012).

MAALs have various applications in plant genetic analysis and breeding by facilitating the genetic and cytological characterization of alien chromosomes (e.g. Hosaka et al. 1990; This et al. 1990; Chen et al. 1992). These include identification of gene loci and marker linkage groups and their assignments to specific chromosomes, together with determination of chromosome homoeology within and among the genomes involved. MAALs and substitution lines have also been proven to be useful in transferring genes between species to introduce new traits and/or to increase genetic variation in existing traits. For example, Banga (1988) successfully substituted a B-genome chromosome in *B. juncea* with its C-genome homoeologue from *B. napus*, which led to significant variations in the erucic acid content and bolting habit in *B. juncea*.

Brassica rapa–B. oleracea var. *alboglabra* MAALs have been used for various genetic and phylogenetic studies, such as intergenomic homoeology among specific chromosome arms between the A- and C-genomes, intergenomic introgression in the progenies of the addition lines, and the occurrence of interspecific chromosomal substitutions (e.g. Quiros et al. 1987; McGrath et al. 1990; Chen et al. 1992; Jørgensen et al. 1996; Chen et al. 1997a, b, 2007; Heneen et al. 2012). The advantages of using these MAALs for the characterization of the *B. oleracea* genome include genetic analyses of specific traits in the addition lines generated from parental genotypes known to have combinations of desirable traits. This may also facilitate the transfer of desirable traits from the alien *B. oleracea* chromosome to the *B. rapa* genome through introgression and/or the development of stable disomic alien addition lines ($2n$ = AA + 2 C-chromosomes = 22). The MAALs were previously used in the identification of specific chromosomes carrying genes controlling important agronomic traits such as erucic acid content and seed colour, as well as flower colour in *B. oleracea* var. *alboglabra* (Chen and Heneen 1992; Chen et al. 1992, 1997b; Cheng et al. 1994a, 1995; Heneen et al. 2012). In addition, they can be used to locate genes regulating other important traits, such as disease resistance, oil content and oil quality. Chen et al. (1992), through the use of these MAALs, showed that three distinct loci, which control the biosynthesis of erucic acid, white flower colour and the faster migrating band of leucine aminopeptidase are located on the same chromosome of the *B. oleracea* genome.

Characterisation of the different addition lines facilitates the identification of C-genome chromosome-specific markers. Such markers are very useful for marker-assisted selection that accelerates plant breeding, especially for introgression of traits into *B. rapa*. Chen et al. (1997b) reported 19 RAPD markers specific to an alien chromosome of C-genome through the analysis of *Brassica rapa* var. *trilocularis–B. oleracea* var. *alboglabra* MAALs, of which one marker was inferred to be located close to the seed colour gene.

Simple sequence repeat (SSR) markers, also called microsatellites, have been widely used for various genetic analyses including genetic diversity, linkage analyses and gene tagging. SSRs are among the markers of choice for tagging genes (e.g. Padmaja et al. 2005; Zhao et al. 2006) mainly because of their amenability to high-throughput analysis, high polymorphism, abundance and codominant inheritance (e.g. Gupta and Varshney 2000; Suwabe et al. 2002; Lowe et al. 2004; Cheng et al. 2009). The number of publicly available brassica SSRs including those derived from *B. oleracea, B. rapa* and *B. napus* is rapidly increasing (e.g. Suwabe et al. 2002; Lowe et al. 2004; Batley et al. 2007; Iniguez-Luy et al. 2008; Cheng et al. 2009; Parida et al. 2010; Gao et al. 2011; Ge et al. 2011; Wang et al. 2011). Such markers have been valuable in constructing and integrating genetic linkage maps (e.g. Padmaja et al. 2005; Gao et al. 2007; Cheng et al. 2009; Iniguez-Luy et al. 2009; Basunanda et al. 2010; Ge et al. 2011). In the present study, we aimed to (1) assign previously developed SSR markers to the nine *Brassica* C-genome chromosomes; (2) develop a new MAAL carrying the "missing" C-genome chromosome with the help of SSR markers; and (3) confirm the assignment of the nine cytological chromosomes with their corresponding linkage groups.

Materials and methods

Plant material

DNA samples from four different groups of *Brassica* plants were used. The first and second groups comprised ten individual plants of *B. rapa* var. *trilocularis* ($2n = 20$, AA) and ten individual plants of *B. oleracea* var. *alboglabra* ($2n = 18$, CC), respectively. The third group comprised eight previously identified *Brassica rapa* var. *trilocularis–B. oleracea* var. *alboglabra* MAALs ($2n = 21$, AA + 1 C-chromosome), each of which was represented by a minimum of 20 individual plants. The MAALs that carry one of the following C-genome chromosomes (C-chromosomes): C1, C2, C3, C4, C5, C6/7, C8, C9, were developed previously (Cheng et al. 1995; Chen et al. 1997a, b; Heneen and Jørgensen 2001; Heneen and Brismar 2001). Based on the available information the full set of C-chromosomes in the MAALs could not be unambiguously assigned to known linkage groups; consequently, the cytological numerical designation system of Cheng et al. (1995) was used to describe each C-chromosome in this study. The designation system of Cheng et al. (1995) was based on centromeric position and size of the chromosomes, and thus is different from the system applied by Armstrong et al. (1998) and Howell et al. (2002) that was based solely on chromosome size. C3 had a deleted arm in the available MAAL, and so was referred to as C3d. A sister line to the C4-carrier MAAL had a C4 with a small deletion in the short arm, and was thus designated C4d. One C-chromosome was referred to as C6/7, as it was not clear if the C-chromosome in this MAAL was C6 or C7 when this line was developed. However, this chromosome was later determined to be C6 (Heneen et al. 2012).

The fourth group comprised a large number of generated aneuploid plants that were believed to carry 1–9 C-genome chromosomes in addition to the full complement of 10 pairs of A-chromosomes. This group was used to identify aneuploid plants carrying the missing C-chromosome and to develop a new MAAL carrying this chromosome. The C-chromosome that was not part of the previously developed MAALs was referred to as "missing chromosome" until it was later determined to be the C7 chromosome based on the results from this study and the work of Heneen et al. (2012). Seeds of all nine MAALs and the parental lines have been delivered to the gene bank NordGen (http://www.nordgen.org) in Alnarp, Sweden, and are available for genetic and breeding studies. A description of the material and means of propagation for the different MAALs will be supplied by the gene bank on request.

DNA extraction

Seeds from the aforementioned four groups of plants were planted in a greenhouse and young leaf tissue was sampled for DNA extraction at about 2 weeks of age after germination. Individually sampled leaf tissue was placed in 2 ml Eppendorf microcentrifuge tubes and immediately frozen in liquid nitrogen and stored at −80 °C until DNA extraction. After the frozen samples were milled using a Retsch MM400 shaker (Haan, Germany), DNA was extracted using a modified CTAB procedure, as described in Bekele et al. (2007). DNA quality and concentration was measured using a Nanodrop® ND-1000 spectrophotometer (Saveen Werner, Sweden).

SSR-PCR and electrophoresis

This study was based on publicly available *Brassica* di, tri, tetra and penta repeat motif SSRs that were developed based on conventional SSR enriching procedure or genomic shotgun sequences. Initially, more than 180 primer-pairs previously reported to have amplified SSR loci in *B. olearcea* and/or in *B. napus* were screened for their amplification of only target loci and for the reproducibility of the loci amplified, using DNA samples from 10 *B. oleracea* var. *alboglabra* individual plants. Those primer-pairs that failed to amplify the target loci or amplified multiple loci were excluded. The remaining primer-pairs were screened for their specificity to the C-genome using DNA samples from 10 *B. oleracea* var. *alboglabra* and 10 *B. rapa* var. *trilocularis* individual plants. For simplicity, in the following text, SSR markers amplified in *B. oleracea* var. *alboglabra* but not in *B. rapa* var. *trilocularis* are referred to as C-genome specific SSR markers.

PCR was carried out in a total volume of 25 µl containing 25 ng genomic DNA, 0.3 µM of each primer, 0.3 mM of each dNTP, 1 U Taq DNA polymerase (Saveen Werner AB, Sweden) and 1× reaction buffer (20 mM Tris–HCl pH 8.55, 16 mM $(NH_4)SO_4$, 0.01 % Tween®20 and 2 mM $MgCl_2$). Reactions lacking DNA were included as negative controls, whereas reactions containing DNA from parental A- and C-genomes were included as positive controls during DNA amplification. The reactions were performed using the GeneAMP PCR system 9700 thermocycler (Applied Biosystems Inc, USA) using the following temperature profiles: initial denaturation at 95 °C for 3 min, followed by 38 cycles of 30 s denaturation at 94 °C, 30 s annealing at optimized annealing temperature (T_a) for each primer-pair and 45 s primer extension at 72 °C; followed by 20 min final extension at 72 °C. The annealing temperature (T_a) for each primer-pair was 3–6 °C below their melting temperature (T_m).

The amplified product was analysed on 1.5 % (w/v) agarose gels containing ethidium bromide after adding 5 µl of 6 × DNA loading dye and electrophoresed in 1× TAE buffer (0.04 M Tris–Acetate, 0.002 M EDTA) for 2 h at a constant voltage of 90 V. A 50-bp DNA ladder (GeneRulerTM, Fermentas Life Sciences) was used as a molecular size marker. After electrophoresis, the gel was photographed using a Saveen Werner AB UV camera equipped with a Sony Black and White Monitor SSM930CE and Sony Video graphic printer UP-895CE.

Some of the primer-pairs (BRAS068, BRAS003, BRAS019, Na10-B08, CB10010, CB10139 and CB10288) were also tested under different conditions while we were assessing markers for different C-chromosomes simultaneously in two laboratories. Approximately 1 cm^2 of leaf tissue was collected and frozen at −80 °C for 1 h. Samples were disrupted using a TissueLyser bead mill (Qiagen, UK) and DNA extracted following the protocol described in Edwards et al. (1991). The DNA was rehydrated with 100 µl of sterile water and quality assessed as before. The PCR was carried out using a Mastercycler Gradient thermocycler (Eppendorf, Germany) in a 10-µl reaction volume using HotStarTAQ (Qiagen, UK), following the manufacturers' instructions. The PCR was carried out with the following temperature profile; initial denaturation at 95 °C for 15 min, followed by 40 cycles of 15 s denaturation, 30 s annealing at 55 °C, and extension at 72 °C for 30 s and a final extension of 10 min at 72 °C. The DNA was then electrophoresed on a 2.0 % agarose gel at 6–7 V/cm for approximately 2 h. A gel image was recorded using a Gel Doc 2000 and associated software (Bio-Rad). These primer-pairs performed well and produced the same results under both conditions, suggesting their robustness.

Identification of C-chromosome-specific SSR markers

Those primer-pairs that amplified C-genome-specific SSR loci and/or alleles were applied to the eight previously developed *Brassica rapa* var. *trilocularis*–*B. oleracea* var. *alboglabra* MAALs in order to identify C-chromosome-specific SSR markers. Primer-pairs that amplified both A-genome and C-genome SSRs were used only when the size of the alleles of the two genomes was unambiguously different. After the analysis of the eight MAALs, those SSRs that were amplified only in one of the eight MAALs were considered as potentially C-chromosome-specific and were selected for further analysis.

Developing a MAAL carrying the missing chromosome

SSR markers that were amplified in parental *B. oleracea* var. *alboglabra* but absent in all eight available MAALs and parental *B. rapa* var. *trilocularis* were considered as candidate markers specific to the missing chromosome and were used for the analysis of aneuploid plants. Aneuploids that were positive for these markers were regarded as potential carriers of the missing chromosome and selected for further analysis. These aneuploid plants were tested for the presence of other C-chromosomes using the SSR markers specific to each of the eight MAALs. Plants with one or few C-chromosomes were targeted to develop the MAAL carrying the missing C-chromosome. Cytogenetic analysis of promising lines was carried out in order to confirm the presence of the missing chromosome and to determine whether it was C6 or C7. This was followed by final determination of SSR markers specific only to each of the nine C-chromosomes.

Determining the correspondence between numerical C-genome cytological and linkage group designations

The internationally agreed numerical designation system for the *Brassica* genome chromosomes is based on molecular genetic linkage groups (Parkin et al. 2005; http://www.brassica.info/resource/maps/lg-assignments.php; Wang et al. 2011). The linkage groups of the *B. oleracea* genome (C-genome) are numbered and orientated so as to match the corresponding linkage groups of *B. napus* as follows: O (*oleracea*)1 = N (*napus*)11 = C1; O2 = N12 = C2, O3 = N13 = C3…and O9 = N19 = C9. Some SSRs that were demonstrated to be specific only to one of the nine C-chromosomes in the present study had previously been mapped to specific linkage groups in *B. napus* and/or *B. oleracea* (e.g. Padmaja et al. 2005; Gao et al. 2007; Cheng et al. 2009; Iniguez-Luy et al. 2009; Basunanda et al. 2010; Ge et al. 2011). These SSRs were used to determine the correspondence between the cytological numerical designation system of the C-chromosomes in the MAALs (Cheng et al. 1995) with the C-genome linkage groups. In the following sections, the designation of C-chromosomes is according to Cheng et al. (1995) unless preceded by prefix "LG-" to refer to linkage group numerical designation.

Results

The screening of more than 180 *Brassica* SSR primers-pairs led to the selection of 151 primer-pairs that amplified single band SSRs in *B. oleracea* var. *alboglabra*. These included 77 "FITO" SSRs developed by Iniguez-Luy et al. (2008), 26 "BnGMS" SSRs developed by Cheng et al. (2009), 32 "BRAS" and "CB" SSRs developed by Celera AgGen *Brassica* Consortium and reported in Piquemal et al. (2005), and 16 "Ol" and "Na" SSRs developed by Lowe et al. (2004). Sixty-three of these SSRs were also

readily amplified in A-genome (see Online Resource 1), of which 21 were "FITO" SSRs that were reported to be amplified only in the C-genome by Iniguez-Luy et al. (2008). The remaining 88 SSRs were C-genome specific, of which 81 were those listed in Tables 1 and 2.

Developing a MAAL carrying the missing chromosome

The analysis of *Brassica rapa* var. *trilocularis–B. oleracea* var. *alboglabra* MAALs and their parental lines using C-genome-specific SSR primer-pairs resulted in the identification of 15 SSR markers that were absent in all eight previously developed MAALS. These markers were considered as potentially specific to the missing chromosome and were used to analyse a large number of aneuploid plants that carry different numbers of C-chromosomes and the complete diploid set of A-chromosomes. Aneuploid plants that were positive for most of these markers were selected as potential carriers of the final (missing) chromosome. The analysis of these plants using SSR markers that were specific to each of the eight characterized chromosomes revealed that most of these plants carried more than three C-chromosomes. However, few aneuploid plants carried three or less C-chromosomes. Only, one of these plants (*aneuploid-40*) carried a single C-chromosome. Out of the 15 markers, ten markers were unambiguously amplified in *aneuploid-40* but absent in all the eight previously developed MAALs and thus were considered as specific to the missing chromosome. These markers are those marked "+" under C7 (LG-2) in Table 1. The cytogenetic analysis conducted on *aneuploid-40* and its progenies, and also on certain monosomic plants among progenies of an aneuploid with $2n = 23$, confirmed the presence of only one C-chromosome that could be designated C7 after comparing it with C6/7 which consequently is now designated C6 (Heneen et al. 2012). Accordingly, chromosome C6/7 and the missing chromosome will be referred to as C6 and C7, respectively, in the following sections. Plants carrying the complete diploid set of A-genome and one C7 chromosome are considered the final *Brassica rapa* var. *trilocularis–B. oleracea* var. *alboglabra* MAAL, and referred to as C7 MAAL.

C-chromosome-specific SSR markers

The development of the C7 MAAL completes the set of *Brassica rapa* var. *trilocularis–B. oleracea* var. *alboglabra* MAALs which are now available for both genetic and breeding studies. The application of the SSR primer-pairs to these MAALs and parental *B. rapa* var. *trilocularis* and *B. oleracea* var. *alboglabra* lines led to the development of markers specific only to single C-chromosomes (Table 1; Fig. 1). Of the 64 SSRs identified as C-chromosome-specific in the present study, 40, 9, 6, 3, 3 and 3 were "FITO", "BnGMS", "CB" "BRAS", "Ol" and "Na" SSRs, respectively. The highest number of C-chromosome-specific markers (10) was recorded in chromosomes C4, C7 and C9, whereas only two SSR markers (FITO-504 and Na10-B08) were specific to chromosome C3d. The ten C7-specific markers were among the 15 markers that were absent in all eight C-chromosomes in the previously developed MAALs. The remaining five SSRs (BnGMS349, CB10132, CB1028, FITO-066 and FITO-515) were not amplified in C7 and thus specific to none of the nine MAALs suggesting possible chromosomal rearrangements/deletions during the development of the MAALs.

Duplicated C-genome-specific SSRs

Seventeen SSR markers present on more than one C-chromosome (duplicated SSRs) were also identified (Table 2; Fig. 1). The number of duplicated SSRs that each C-chromosome shared with other C-chromosomes was 4, 7, 5, 9, 4, 4, 6, 7 and 7, in the order of C1–C9. Ten of the 17 SSRs were duplicated only on two C-chromosomes. FITO-574 and BnGMS302 were amplified in three C-chromosomes, whereas FITO-380 and FITO-457b were distributed across four C-chromosomes (Table 2). FITO-086, FITO-457a, FITO-466 and FITO-467 had a minimum of five copies that were distributed across five of the nine C-chromosomes.

Chromosome C1 shared none of its SSRs with C2, C6 and C7 but shared two SSRs with C4, C5, C8 and C9 (Tables 2, 3). Similarly, no shared SSRs were revealed between C2 and C9, between C5 and C6, and between C6 and C7 (Table 3). Chromosome C2 shared five SSRs with C7 and similarly C4 and C9 shared five SSRs, suggesting the presence of a significant level of partial homoeologies between C2 and C7 and between C4 and C9. Of the five SSRs that C3d shared with other C-chromosomes, four were shared with C8 and only one with C7. Chromosome C5 shared four SSRs with other C-chromosomes, of which three were shared with C8 and C9. Similarly, C6 shared three of its four shared SSRs with C8 (Tables 2, 3).

The correspondence between the cytological and linkage group numerical designations of C-genome chromosomes

Some SSRs that had previously been mapped to specific linkage groups in *B. napus* and/or *B. oleracea* were demonstrated to be specific only to one of the nine C-chromosomes in the present study. The specificity of these SSRs to different C-genome chromosomes and linkage groups served as a basis for the establishment of the following correspondence between the cytological numerical

Table 1 *Brassica* C-genome SSRs specific only to one of the nine chromosomes/linkage groups

Locus	Genome		Chromosome									
	AA	CC	C1 LG-C9	C2 LG-C1	C3d[e] LG-C5	C4 LG-C3	C4d[f] LG-C3	C5 LG-C4	C6 LG-C6	C7 LG-C2	C8 LG-C7	C9 LG-C8
BnGMS185[a]	−	+	+	−	−	−	−	−	−	−	−	−
BnGMS634[a]	−	+	+	−	−	−	−	−	−	−	−	−
CB10288[b]	−	+	+	−	−	−	−	−	−	−	−	−
FITO-229[c]	−	+	+	−	−	−	−	−	−	−	−	−
FITO-231[c]	−	+	+	−	−	−	−	−	−	−	−	−
FITO-570[c]	−	+	+	−	−	−	−	−	−	−	−	−
Ol10-B01[d]	−	+	+	−	−	−	−	−	−	−	−	−
CB10277[b]	−	+	−	+	−	−	−	−	−	−	−	−
FITO-096[c]	−	+	−	+	−	−	−	−	−	−	−	−
FITO-318[c]	−	+	−	+	−	−	−	−	−	−	−	−
FITO-562[c]	−	+	−	+	−	−	−	−	−	−	−	−
FITO-504[c]	−	+	−	−	+	−	−	−	−	−	−	−
Na10-B08[d]	−	+	−	−	+	−	−	−	−	−	−	−
BRAS068[b]	−	+	−	−	−	+	?	−	−	−	−	−
FITO-094[c]	−	+	−	−	−	+	−	−	−	−	−	−
FITO-243[c]	−	+	−	−	−	+	+	−	−	−	−	−
FITO-306[c]	−	+	−	−	−	+	−	−	−	−	−	−
FITO-451[c]	−	+	−	−	−	+	+	−	−	−	−	−
FITO-459[c]	−	+	−	−	−	+	+	−	−	−	−	−
FITO-463[c]	−	+	−	−	−	+	+	−	−	−	−	−
FITO-505[c]	−	+	−	−	−	+	+	−	−	−	−	−
FITO-553[c]	−	+	−	−	−	+	−	−	−	−	−	−
Na12-B09[d]	−	+	−	−	−	+	+	−	−	−	−	−
BnGMS408[a]	−	+	−	−	−	−	−	+	−	−	−	−
BnGMS490[a]	−	+	−	−	−	−	−	+	−	−	−	−
BRAS003[b]	−	+	−	−	−	−	−	+	−	−	−	−
FITO-304[c]	−	+	−	−	−	−	−	+	−	−	−	−
FITO-336[c]	−	+	−	−	–	−	−	+	−	−	−	−
FITO-366[c]	−	+	−	−	−	−	−	+	−	−	−	−
FITO-454[c]	−	+	−	−	−	−	−	+	−	−	−	−
FITO-586[c]	−	+	−	−	−	−	−	+	−	−	−	−
FITO-067[c]	−	+	−	−	−	−	−	−	+	−	−	−
FITO-106[c]	−	+	−	−	−	−	−	−	+	−	−	−
FITO-146[c]	−	+	−	−	−	−	−	−	+	−	−	−
FITO-201[c]	−	+	−	−	−	−	−	−	+	−	−	−
FITO-329[c]	−	+	−	−	−	−	−	−	+	−	−	−
CB10010[b]	−	+	−	−	−	−	−	−	+	−	−	−
BnGMS280[a]	−	+	−	−	−	−	−	−	−	+	−	−
BnGMS454[a]	−	+	−	−	−	−	−	−	−	+	−	−
CB10026[b]	−	+	−	−	−	−	−	−	−	+	−	−
FITO-130[c]	−	+	−	−	−	−	−	−	−	+	−	−
FITO-149[c]	−	+	−	−	−	−	−	−	−	+	−	−
FITO-194[c]	−	+	−	−	−	−	−	−	−	+	−	−
FITO-237[c]	−	+	−	−	−	−	−	−	−	+	−	−
FITO-421[c]	−	+	−	−	−	−	−	−	−	+	−	−
FITO-527[c]	−	+	−	−	−	−	−	−	−	+	−	−

Table 1 continued

Locus	Genome		Chromosome									
	AA	CC	C1 LG-C9	C2 LG-C1	C3d[e] LG-C5	C4 LG-C3	C4d[f] LG-C3	C5 LG-C4	C6 LG-C6	C7 LG-C2	C8 LG-C7	C9 LG-C8
FITO-556[c]	−	+	−	−	−	−	−	−	−	+	−	−
FITO-303[c]	−	+	−	−	−	−	−	−	−	−	+	−
FITO-472[c]	−	+	−	−	−	−	−	−	−	−	+	−
FITO-497[c]	−	+	−	−	−	−	−	−	−	−	+	−
FITO-564[c]	−	+	−	−	−	−	−	−	−	−	+	−
Na12-F03[d]	−	+	−	−	−	−	−	−	−	−	+	−
Ol10-H04[d]	−	+	−	−	−	−	−	−	−	−	+	−
BRAS019[b]	−	+	−	−	−	−	−	−	−	1	+	−
BnGMS336[a]	−	+	−	−	−	−	−	−	−	−	−	+
BnGMS439[a]	−	+	−	−	−	−	−	−	−	−	−	+
BnGMS509[a]	−	+	−	−	−	−	−	−	−	−	−	+
CB10139[b]	−	+	−	−	−	−	−	−	−	−	−	+
CB10179[b]	−	+	−	−	−	−	−	−	−	−	−	+
FITO-024[c]	−	+	−	−	−	−	−	−	−	−	−	+
FITO-252[c]	−	+	−	−	−	−	−	−	−	−	−	+
FITO-439[c]	−	+	−	−	−	−	−	−	−	−	−	+
FITO-543[c]	−	+	−	−	−	−	−	−	−	−	−	+
Ol10-H07[d]	−	+	−	−	−	−	−	−	−	−	−	+

+ marker present, − marker absent, ? not analysed

[a] Cheng et al. 2009

[b] Piquemal et al. 2005

[c] Iniguez-Luy et al. 2008

[d] Lowe et al. 2004

[e] C3d has deleted arm

[f] C4d has small deletion in the short arm

designation system of the C-chromosomes in the MAALs (Cheng et al. 1995) with the C-genome linkage groups: C1, C2, C3d, C4, C5, C6, C7, C8 and C9 correspond to LG-C9, LG-C1, LG-C5, LG-C3, LG-C4, LG-C6, LG-C2, LG-C7 and LG-C8, in that order (Tables 1, 2). In most cases, this correspondence was also supported by the work of Heneen et al. (2012).

The number of SSR markers suggesting this correspondence varied among the C-chromosomes, as discussed in the following section. The specificity of BnGMS185 and BnGMS634 was the evidence for the correspondence between C1 and LG-C9. The correspondence between C2 and LG-C1 was mainly based on the specificity of CB10277. However, BnGMS271, BnGMS301 and CB10258 also support this correspondence (see below). SSR marker Na10-B08 suggested the correspondence between C3 and LG-5, whereas BRAS068, FITO-306 and FITO-505 suggested the correspondence between C4 and LG-C3. The correspondence between C5 and LG-C4 was based on BRAS003, BnGMS408 and BnGMS490 and that between C6 and LG-C6 was based on FITO-067 and CB10010. The correspondence between C7 and LG-C2, between C8 and LG-C7 and between C9 and LG-C8 was supported by at least four SSR markers: (1) BnGMS280, BnGMS454, CB10026 and FITO-237; (2) BRAS019, Na12-F03, Ol10-H04, FITO-472 and FITO-497; (3) BnGMS336, BnGMS439, BnGMS509, CB10139 and CB10179, in that order, suggested the correspondence.

Discussion

A large number of *Brassica* SSRs has been developed in recent years (e.g. Suwabe et al. 2002; Lowe et al. 2004; Batley et al. 2007; Iniguez-Luy et al. 2008; Cheng et al. 2009; Parida et al. 2010; Gao et al. 2011; Ge et al. 2011; Wang et al. 2011). However, in most cases, data on polymorphism and copy number of these SSRs are not available, although this information is very important for population genetic studies and genetic linkage and QTL mapping. Genetic linkage analysis requires mapping populations and a large number of polymorphic molecular

Table 2 *Brassica* C-genome SSRs specific to more than one C-chromosomes/linkage groups

Locus	Genome		Chromosome									
	AA	CC	C1 LG-C9	C2 LG-C1	C3d[d] LG-C5	C4 LG-C3	C4d[e] LG-C3	C5 LG-C4	C6 LG-C6	C7 LG-C2	C8 LG-C7	C9 LG-C8
FITO-491[c]	−	+	+	−	−	+	+	−	−	−	−	−
CB10344[b]	−	+	+	−	−	−	−	−	−	−	+	−
BnGMS271[a]	−	+	−	+	−	−	−	−	−	+	−	−
BnGMS301[a]	−	+	−	+	−	−	−	−	−	+	−	−
CB10258[b]	−	+	−	+	−	−	−	−	−	+	−	−
FITO-147[c]	−	+	−	+	−	−	−	−	−	+	−	−
FITO-404[c]	−	+	−	−	−	+	+	−	−	−	+	−
FITO-223[c]	−	+	−	−	−	+	+	−	−	−	−	+
FITO-008[c]	−	+	−	−	−	+	+	−	−	−	−	+
FITO-550[c]	−	+	−	−	−	−	−	−	+	−	+	−
FITO-574[c]	−	+	−	−	−	+	+	−	+	−	−	+
BnGMS302[a]	−	+	−	−	−	+	−	−	−	+	−	+
FITO-457b[c]	−	+	+	−	+	−	−	+	−	−	−	+
FITO-380[c]	−	+	−	−	+	−	−	+	−	−	+	+
FITO-086[c]	−	+	+	−	−	+	+	+	−	−	+	+
FITO-466[c]	−	+	−	+	+	+	+	−	+	−	+	−
FITO-467[c]	−	+	−	+	+	+	+	−	+	−	+	−
FITO-457a[c]	−	+	−	+	+	−	−	+	−	+	+	−

FITO-457 has two loci (a and b)

+ marker present, − marker absent

[a] Cheng et al. (2009)

[b] Piquemal et al. (2005)

[c] Iniguez-Luy et al. (2008)

[d] C3d has deleted arm

[e] C4d has small deletion in the short arm

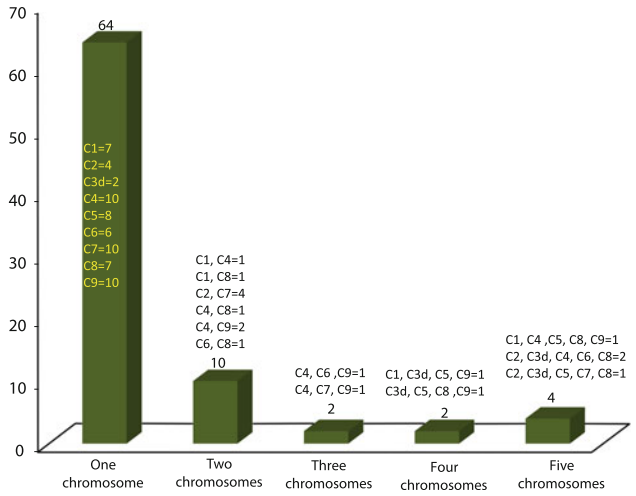

Fig. 1 Number of C-genome SSRs specific to different number of C-chromosomes

markers. The use of MAALs to develop molecular markers specific to different chromosomes allows selection of markers for linkage analysis and mapping, and thus facilitates the development of molecular markers for marker-assisted selection. The C-chromosome-specific SSR markers developed in the present study are useful resources that facilitate the development of markers for traits of interest.

Seven C-chromosomes (C1–C7) were suggested to influence the seed colour in *B. oleracea* var. *alboglabra* (Heneen et al. 2012). Two (C1 and C4) of these chromosomes carry major genes that control pigmentation of the entire seed coat. The C4 chromosome was also known to carry genes for flower colour and erucic acid content (Chen et al. 1992; Cheng et al. 1994a, 1995; Jørgensen et al. 1996). The SSRs located on C1 and C4 in the present study are useful resources for the linkage analysis of major seed colour genes, whereas C4 SSRs should be tested additionally for their linkage to erucic acid content in *B. oleracea*. After developing C-chromosome-specific RAPD markers, Chen et al. (1997b) reported that one of the 19 markers specific to C1 was closely linked to the seed colour gene. The C-chromosome-specific markers developed in the present study are potentially useful to develop more

Table 3 The number of SSRs shared among each pair of C-chromosomes/linkage groups

		C1 LG-C9	C2 LG-C1	C3d LG-C5	C4 LG-C3	C5 LG-C4	C6 LG-C6	C7 LG-C2	C8 LG-C7
C2	LG-C1	0							
C3d	LG-C5	1	3						
C4	LG-C3	2	2	2					
C5	LG-C4	2	1	3	1				
C6	LG-C6	0	2	2	3	0			
C7	LG-C2	0	5	1	1	1	0		
C8	LG-C7	2	3	4	4	3	3	1	
C9	LG-C8	2	0	2	5	3	1	1	2

markers linked to seed colour genes and other important traits, such as disease and pest resistance.

The gene for white flower colour in *B. oleracea* var. *alboglabra* is located on C4 (Chen et al. 1992; Cheng et al. 1994a, 1995; Jørgensen et al. 1996; Ramsay et al. 1996) and the white colour is dominant over yellow. While the C4 MAAL carrying intact C4 chromosome produces white flowers, the MAAL carrying C4d with a small deletion in the short arm (Heneen et al. 2012) produces yellow flowers suggesting that the gene for white flower colour is located on the deleted segment. Similarly, the MAALs with intact C4 chromosome appeared to show relatively more vigorous growth than those with the C4d chromosome suggesting the possibility that genes contributing to vigour are located on the deleted segment of the C4 chromosome. Three C4-specific SSR markers (FITO-094, FITO-306 and FITO-553) were amplified only in MAAL carrying the intact C4 chromosome. Apparently, the deleted segment carried these three markers. These markers might be linked to the gene for white flower colour and/or to genes that contribute to plant size and should be analysed for their linkage to these traits. The C3d chromosome has the least number of specific SSR markers (2) as compared to other C-chromosomes in the MAALs, which is partly related to the loss of one arm. However, it is interesting to note that it has more SSR markers (5) shared with other C-chromosomes than the number of markers that each of the intact C1, C5, and C6 chromosomes shared with other C-chromosomes.

The assignment of molecular markers such as SSRs to a particular C-chromosome or linkage group and analysis of marker duplication, without recourse to mapping populations, are among the advantages of using the *Brassica rapa–B. oleracea* MAALs for the characterization of the *B. oleracea* genome. Duplicated SSRs have been used as a tool for investigation of genetic duplication (e.g. David et al. 2003; Antunes et al. 2006; Zhang and Rosenberg 2007) due to their high variability. In the present study, duplicated SSRs represent about 20 % of C-genome-specific SSRs, suggesting a duplication of a significant fraction of the genome. Previous studies have also shown a high frequency of duplicated chromosomal segments in *B. rapa*, *B. oleracea* and *B. napus* (McGrath et al. 1990; Slocum et al. 1990; Song et al. 1991; Kianian and Qmros 1992; Kowalski et al. 1994; Parkin et al. 2005; Schranz et al. 2006, The *Brassica rapa* Genome Sequencing Project Consortium 2011; Wang et al. 2011). Considering the presence of a significant level of null alleles in the *Brassica* SSRs (e.g. Uzunova and Ecke 1999; Bond et al. 2004; Wang et al. 2011) the proportion of duplicated SSR loci in *B. oleracea* may be higher than the 20 % obtained in this study. The number of alleles has also been shown to correlate positively with the copy number of SSRs in plant genomes (Gao et al. 2009). Due to the rapid changes that occur in microsatellite copy numbers over time, duplicated SSRs may be more polymorphic than non-duplicated ones. Thus, duplicated SSRs revealed in the present study need to be considered for characterization of *B. oleracea* genetic resources, as the occurrence of three or more distinct alleles per SSR is possible.

Unlike the overwhelming majority of the C-genome-specific SSRs, two (FITO-326 and FITO-397, data not shown) were amplified in all nine *Brassica* var. *trilocularis–B. oleracea* var. *alboglabra* MAALs. It is less likely that these SSRs were distributed on all nine C-chromosomes. Rather, the result suggests the introgression of these markers into the A-genome background during the development of MAALs, most likely at the resynthesized *B. napus* (AACC) and/or sesquidiploids (AAC) stages. This is likely, as the two species show a high level of chromosomal homoeologies along their genomes and a close evolutionary relationship (e.g. Sharpe et al. 1995; Chen et al. 1997a; Szadkowski et al. 2010). Intergenomic introgression and chromosomal substitution between the genomes have been previously reported (e.g. McGrath et al. 1990; Chen et al. 1992, 1997a, 2007); Sharpe et al. 1995; Jørgensen et al. 1996.

Chromosome-specific markers and the correspondence between the cytological and linkage group numerical designations of C-genome chromosomes

The previously mapped SSRs to C-genome linkage groups that were specific to a particular C-chromosome in the

present study enabled unambiguous assignment of cytological (Cheng et al. 1995) and linkage group (Parkin et al. 2005) numerical designation approaches in *B. oleracea*. These markers and lines provide resources that now facilitate the assignments previously outlined by Howell et al. (2002).

Two SSR markers (BnGMS185 and BnGMS634) that were mapped to LG-C9 by Cheng et al. (2009) were specific to C1 in the present study. The correspondence between C1 and LG-C9 was also supported by a cytogenetic study conducted based on multiple target FISH (Heneen et al. 2012). However, the other two mapped C1-specific SSRs (CB10288 and Ol10-B01) did not support the correspondence, as CB10288 was mapped to LG-4 (Piquemal et al. 2005) and Ol10-B01 was mapped to LG-C7 (Hasan et al. 2008) and LG-C4 (Zhang et al. 2011). Considering the presence of significant levels of duplicated chromosomal segments (McGrath et al. 1990; Slocum et al. 1990; Song et al. 1991; Kianian and Qmros 1992; Kowalski et al. 1994) and SSR null alleles (e.g. Uzunova and Ecke 1999; Bond et al. 2004; Wang et al. 2011) in the *Brassica* genomes, it is likely that CB10288 was duplicated on LG-C9 and LG-C4 but having a null allele on LG-4 in our study, and a null/monomorphic allele on LG-C9 in the mapping population used by Piquemal et al. (2005). The case of SSR Ol10-B01 may also be similar, as the plant materials used in Hasan et al. (2008), Zhang et al. (2011) and the present study are different.

One of the C2-specific SSRs (CB10277) and three SSRs that were specific to both C2 and C7 (BnGMS271, BnGMS301 and CB10258) were mapped to LG-C1 (Piquemal et al. 2005; Cheng et al. 2009). On the other hand, four C7-specific SSRs (BnGMS280, BnGMS454, CB10026 and FITO-237) were mapped to LG-C2 (see Piquemal et al. 2005; Cheng et al. 2009; Iniguez-Luy et al. 2009), which is strong evidence supporting the correspondence of C7 and LG-C2. Given the fact that the correspondence between C7 and LG-C2 was strongly supported (four SSR markers), the specificity of CB10277, BnGMS271, BnGMS301 and CB10258 strongly suggests that C2 corresponds to LG-C1.

The SSR marker Na10-B08, which was specific to C3d in the present study, was previously mapped to LG-C5 (http://www.brassica.info/cgi-bin/cmap/feature?feature_aid=4161) suggesting the correspondence between C3 and LG-C5. This correspondence was also supported by a cytological study using multiple target FISH (Heneen et al. 2012). Similarly, SSR markers that were mapped to LG-C3, BRAS068 (Cheng et al. 2009) and FITO-306 and FITO-505 (Iniguez-Luy et al. 2008), were specific to C4 in the present study. The correspondence was in line with the evidence from a FISH-based study (Heneen et al. 2012). Three C5-specific SSRs (BRAS003, BnGMS408 and BnGMS490) were mapped to LG-C4 (Cheng et al. 2009), which is in line with the results from the FISH-based study (Heneen et al. 2012) suggesting the correspondence between C5 and LG-C4. The present study showed that FITO-067, FITO-146 and CB10010 are specific to C6. FITO-067 (Iniguez-Luy et al. 2008) and CB10010 (Piquemal et al. 2005) were mapped to LG-C6. FITO-146 was mapped to LG-C6 by Cheng et al. (2009) and to LG-C1, LG-C6 and LG-C8 by Iniguez-Luy et al. (2008). The results strongly suggest the correspondence of C6 and LG-C6, which is in line with the FISH-based study by Heneen et al. (2012). The restriction of FITO-146 only to C6 in the present study, and probably in the work of Cheng et al. (2009), suggests rearrangements/deletions of the chromosomal regions carrying this SSR on LG-C1 and LG-C8. Such events were previously suggested in *Brassica rapa* var. *trilocularis*–*B. oleracea* var. *alboglabra* MAALs (Chen et al. 1997a) and in *B. napus* (Wang et al. 2011).

Five SSR markers that were specific to C8 in the present study were previously mapped to LG-C7 (Piquemal et al. 2005, BRAS019; Lowe et al. 2004, Na12-F03 and Ol10-H04; Iniguez-Luy et al. 2009, FITO-472 and FITO-497). Similarly, five C9-specific SSRs (BnGMS336, BnGMS439, BnGMS509, CB10139 and CB10179) were mapped to LG-C8 (Piquemal et al. 2005; Cheng et al. 2009). These SSRs in combination with the evidence from the FISH-based study (Heneen et al. 2012) strongly suggest that C8 corresponds to LG-C7 and C9 corresponds to LG-C8. Overall, based on the evidence from the present study and the work of Heneen et al. (2012) C1, C2, C3d, C4, C5, C6, C7, C8 and C9 correspond to LG-C9, LG-C1, LG-C5, LG-C3, LG-C4, LG-C6, LG-C2, LG-C7 and LG-C8, in that order. The correspondence helps to integrate genetic information generated based on the two approaches and accordingly increase our knowledge of each C-chromosome. The integration will contribute to a wide range of research that includes providing complementary information to the physical maps of the species and location of genes in relation to features of chromosomal organization (Howell et al. 2002).

The C-chromosome-specific SSR markers developed in the present study have a direct application for the differentiation and definition of C-chromosome carriers from euploid *B. rapa* plants in the progeny generations of the MAALs. The markers also help to monitor the introgression of segments of the alien chromosome into the A-genome and for the identification of stable disomic alien addition lines, and thus have a significant contribution to the improvement of *B. rapa* through the transfer of desirable genes from the C-genome. The set of lines will be of particular value for the study of interspecific heterosis in the complex *Brassica* crop genomes.

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