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ORIGINAL PAPER

Development of a core set of single-locus SSR markers for allotetraploid rapeseed (*Brassica napus* L.)

Haitao Li · Muhammad Younas · Xiaofeng Wang · Xuemin Li · Lin Chen · Bo Zhao · Xun Chen · Jinsong Xu · Fan Hou · Baohua Hong · Gang Liu · Hongyang Zhao · Xueli Wu · Hongzhi Du · Jiangsheng Wu · Kede Liu

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Abstract *Brassica napus* (AACC) is a recent allotetraploid species evolved through hybridization between two diploids, *B. rapa* (AA) and *B. oleracea* (CC). Due to extensive genome duplication and homoeology within and between the A and C genomes of *B. napus*, most SSR markers display multiple fragments or loci, which limit their application in genetics and breeding studies of this economically important crop. In this study, we collected 3,890 SSR markers from previous studies and also developed 5,968 SSR markers from genomic sequences of *B. rapa*, *B. oleracea* and *B. napus*. Of these, 2,701 markers that produced single amplicons were putative single-locus markers in the *B. napus* genome. Finally, a set of 230 high-quality single-locus SSR markers were established and assigned to the 19 linkage groups of *B. napus* using a segregating population with 154 DH individuals. A subset of 78 selected single-locus SSR markers was proved to be highly stable and could successfully discriminate each of the 45 inbred lines and hybrids. In addition, most of the 230 SSR markers showed the single-locus nature in at least one of the *Brassica* species of the U's triangle besides *B. napus*. These results indicated that this set of single-locus SSR markers has a wide range of coverage with excellent stability and would be useful for gene tagging, sequence scaffold assignment, comparative mapping, diversity analysis, variety identification and association mapping in *Brassica* species.

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H. Li · M. Younas · X. Wang · X. Li · L. Chen · B. Zhao · X. Chen · J. Xu · F. Hou · B. Hong · G. Liu · H. Zhao · X. Wu · H. Du · J. Wu · K. Liu (✉)
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China
e-mail: kdliu@mail.hzau.edu.cn

H. Li · M. Younas · X. Wang · X. Li · L. Chen · B. Zhao · X. Chen · J. Xu · F. Hou · B. Hong · G. Liu · H. Zhao · X. Wu · H. Du · J. Wu · K. Liu
Key Laboratory of Rapeseed Genetic Improvement, Ministry of Agriculture, Wuhan 430070, China

Introduction

Plants of the genus *Brassica* comprise an exceptionally diverse group of crops grown for edible oil, vegetables, condiment mustards and forages. The cytogenetic and evolutionary relationships among the major oilseed and vegetable species of the genus *Brassica* are commonly depicted as the U's triangle (U N 1935). In the U's triangle, *B. rapa* (AA, $2n = 20$), *B. nigra* (BB, $2n = 16$), and *B. oleracea* (CC, $2n = 18$) are three diploids, and *B. carinata* (BBCC, $2n = 34$), *B. juncea* (AABB, $2n = 36$) and *B. napus* (AACC, $2n = 38$) are three allotetraploids each formed through interspecific hybridization among the three progenitor diploid species.

Comparative mapping using restriction fragment length polymorphism (RFLP) markers derived from the *A. thaliana* genome indicated that there is extensive co-linearity between the *Brassica* and *A. thaliana* genomes (Parkin et al. 2005). Most markers are presented as a single copy in the *A. thaliana* genome but as multiple copies, on average three copies, in the *Brassica* genomes (Cavell et al. 1998; Lagercrantz et al. 1996; Sadowski et al. 1996). Subsequent comparative sequencing of chromosomal segments among *B. rapa*, *B. oleracea* and *B. napus* confirmed the hypothesis that the genomes of *B. rapa* and *B. oleracea* had been

triplicated relative to *A. thaliana* (Cheung et al. 2009; Town et al. 2006). Comparison of orthologous A and C genome segments of *B. rapa*, *B. oleracea* and *B. napus* has indicated that the majority of chromosomal segments could be detected in 6–8 copies in *B. napus* genome (Parkin et al. 2005). Microsatellite markers or simple sequence repeats (SSRs) are tandem DNA repeats from 1 to 6 bp that are found throughout the coding and non-coding regions of eukaryotic genomes. In *B. napus*, most SSR markers display two or more loci due to the high level of homoeology between the A and C genomes (Cheng et al. 2009; Li et al. 2011; Ling et al. 2007; Piquemal et al. 2005). The multi-locus nature of most SSR markers makes it hard to integrate previously constructed genetic linkage maps and compare genes/QTLs detected using different genetic populations. In gene mapping and marker-assisted selection (MAS), it is necessary to distinguish which locus of the multi-locus marker linked to the gene of interest.

Multiplexed fluorescent PCR is a technique to significantly improve the throughput of SSR genotyping (Guichoux et al. 2011). Nevertheless, the multi-locus nature of SSR markers in *B. napus* limits the number of multiplexed markers and increases genotyping errors due to reciprocal overlapping fragments. Similarly, multi-locus SSR markers bring many problems for population genetic studies. The polymorphic alleles cannot be assigned precisely to specific genomic loci in diversity analyses (Chen et al. 2008). As a consequence, the measurement parameters for diversity including number of alleles, allele frequency and polymorphism information content (PIC) cannot be estimated correctly. It is widely accepted that single-locus SSR markers are ideal for association analysis (Comadran et al. 2009; Jin et al. 2010; Stich et al. 2005). Contrastingly, SSR markers with multiple loci usually bring ambiguous genotyping in *B. napus* and make it unsuitable for understanding the population structure and linkage disequilibrium (LD).

Single-locus markers have advantages over multi-locus markers in genetic studies and breeding programs since they would remove all of the abovementioned experimental problems and technical defects. Currently, more than 10,000 SSR markers are available in the *Brassica* community (Cheng et al. 2009; Choi et al. 2007; Iniguez-Luy et al. 2008; Kaur et al. 2009; Kim et al. 2009; Li et al. 2011; Parida et al. 2010; Wang et al. 2011a; Xu et al. 2010). However, most of these publically available SSR markers detect multiple loci in a panel of materials for polymorphism screening. Only a small proportion of SSR markers were alleged single-locus in *B. napus* genetic maps, and some of these were found to be accompanied by redundant monomorphic amplicons (Kaur et al. 2009; Lowe et al. 2004; Piquemal et al. 2005). The mapped loci of these SSR markers might not be polymorphic in other studies, whereas the monomorphic amplicons may be polymorphic. Hence, these alleged single-locus SSR markers can only be called population specific single-locus. Although these markers have been used widely in *B. napus*, a set of universal single-locus SSR markers are preferred for *B. napus* genetic research and breeding.

To overcome the difficulties of multi-locus markers and deficiency of genuine single-locus markers, it is necessary to develop a number of genuine universal single-locus SSR markers in *B. napus*. In this study, we aimed to (1) develop a set of high-quality single-locus SSR markers,(2) assign the single-locus SSR markers to a *B. napus* linkage map, (3) validate a subset of the single-locus markers across a panel of inbred and hybrid rapeseed lines and (4) test the universality of the single-locus markers across the other *Brassica* "U's triangle" species.

Materials and methods

Mapping population and DNA isolation

Six *B. napus* inbred lines (S1, S2, M201, M202, No2127 and ZY821) were used to screen polymorphism of microsatellite markers. These lines had previously been used as the parents of three different mapping populations. A total of 154 double-haploid (DH) lines (named as BnaNZDH hereafter) derived from the cross between 'No2127' and 'ZY821' were used to assign all the single-locus SSR markers to a *B. napus* genetic linkage map (Xiao et al. 2007). Of these DH lines, the first 88 lines had been previously used for construction of linkage maps (Cheng et al. 2009; Li et al. 2011; Wang et al. 2011a). In addition, to test the universality of the single-locus SSR markers, two accessions of inbred or DH lines with diverse genetic background and geographical distribution were selected from each of *B. rapa*, *B. oleracea, B. nigra*, *B. juncea*, *B. napus* and *B. carinata* (Table 1). Fresh leaves were collected from the inbred and DH lines for DNA isolation. DNA samples were diluted to approximate 25 ng/μl and used as template for PCR amplification.

Polymorphism screening and genetic localization

We collected 3,890 *B. napus* SSR markers from literature, including 627 BnGMS markers, 1,398 BoGMS markers, 1,000 BnEMS markers and 865 BrGMS markers from previous studies by our group (Cheng et al. 2009; Li et al. 2011; Wang et al. 2011a; Xu et al. 2010). Additionally, we developed 3,493 SSR markers from *B. rapa* BACs, 2,150 from *B. oleracea* whole genome shotgun sequences and 325 from *B. napus* BAC-end sequences (Table 2). These newly developed markers were also designated as BrGMS,

Table 1 List of *Brassica* species used for evaluation of universality of single-locus SSR markers

Accession	Genome	Species	Origin
Kenshin	AA	*B. rapa*	Korea
Yellow Sarson	AA	*B. rapa*	India
Chi jie lan	CC	*B. oleracea*	China
A12	CC	*B. oleracea*	Europe
B. nigra (L.) Koch cv Giebra	BB	*B. nigra*	Europe
N	BB	*B. nigra*	Europe
G37	AACC	*B. napus*	Australia
G38	AACC	*B. napus*	Canada
10H002-1	AABB	*B. juncea*	Hubei (China)
10H003-1	AABB	*B. juncea*	Shanxi (China)
CGN03995	BBCC	*B. carinata*	Europe
CGN03943	BBCC	*B. carinata*	Europe

BoGMS and BnGMS, respectively, in the way reported previously (Cheng et al. 2009; Li et al. 2011; Xu et al. 2010). Primer pairs of these markers were used to amplify the genomic DNA of the six rapeseed inbred lines for polymorphism screening. Primer design and synthesis, PCR amplification, products separation and polyacrylamide gel staining were performed as previously described (Cheng et al. 2009).

SSR markers that only produce a single amplicon in each of the six inbred lines and showed polymorphisms between ZY821 and No2127, the two parents of the BnaNZDH population, were used for population survey. Genotypes for each individual were scored as described previously (Xu et al. 2010) and those markers that segregated in the DH population in a bi-allele pattern were thought to be single-locus. Genetic linkage map was constructed using JoinMap3.0 (Van Ooijen and Voorrips 2001) with the following mapping parameters. The order of loci was determined using recombination frequency <0.4 and minimum logarithm of odds (LOD) scores of 2.0. A "ripple" was performed after adding each locus and the threshold for removal of loci with respect to jumps in goodness-fit was set to 5. Lastly, the markers were assigned to linkage groups using logarithm of odds (LOD) of 4.0–10.0. All genetic distances were expressed in centi-Morgan converted from recombination value using the Kosambi function (Kosambi 1944). In order to assign the single-locus SSR markers to specific linkage groups (LGs), 112 single-locus SSR markers were selected from previously published linkage maps and used as anchor markers, which included 87 markers from linkage maps constructed using the same BnaNZDH population (Cheng et al. 2009; Li et al. 2011; Wang et al. 2011a; Xu et al. 2010) and 25 SSR markers from other linkage maps (Choi et al. 2007; Kim et al. 2009; Lowe et al. 2004; Piquemal et al. 2005; Suwabe et al. 2008).

Validation of single-locus markers in rapeseed accessions

To confirm that the identified single-locus markers are stable in different rapeseed varieties and test the marker usage in DNA fingerprinting and diversity analysis, 41 markers that amplified only in *B. rapa* or *B. oleracea* and 37 markers that were present in both *B. rapa* and *B. oleracea* (see "Results") were selected to genotype 45 rapeseed inbred lines and 45 hybrid cultivars separately (Supplemental Table 2). These inbred lines, which included 28 from China, 6 from Europe, 4 from Canada, 5 from Australia, and 2 from Japan, were selected from four diverse subgroups representing a collection of 192 inbred lines from all over the world (Xiao et al. 2012). They had been selfed for more than ten generations and are considered to be homozygous for most regions of the genome. The hybrids were true F_1 generation bred by research institutes from nine provinces or cities. The observed heterozygosity (H_o), polymorphism information content (PIC)

Table 2 Amplification patterns of SSR markers collected from different sources

Marker type	Sources	Markers	Amplified[e]	Single amplicon (%)	Two amplicons (%)	≥ Three amplicons (%)
BnGMS[a]	*B. napus* GSSs	952	884	307 (34.7)	296 (33.5)	281 (31.8)
BnEMS[b]	*B. napus* ESTs	1,000	776	198 (25.5)	353 (45.5)	225 (29.0)
BrGMS[c]	*B. rapa* BACs	4,358	3,558	1,114 (31.3)	1,327 (37.3)	1,117 (31.4)
BoGMS[d]	*B. oleracea* GSSs	3,548	2,791	1,082 (38.8)	903 (32.4)	806 (28.8)
Total		9,858	8,009	2,701 (33.8)	2,879 (35.9)	2,429 (30.3)

[a] The marker includes 627 from Cheng et al. (2009) and 325 developed newly in this study

[b] The markers includes 1,000 from Wang et al. (2011a)

[c] The marker includes 865 from Xu et al. (2010) and 3,493 developed newly in this study

[d] The marker includes 1,398 from Li et al. (2011) and 2,150 developed newly in this study

[e] The amplified markers with clear main band

value and pair-wise genetic distances between accessions were calculated using Powermarker version V3.51 package (Liu and Muse 2005). The H_o is the proportion of observed heterozygous individuals in the population. At a single locus it is estimated as:

$$H_O = 1 - \sum_{u=1}^{n} p_{uu}$$

where p_{uu} is the frequency of individual with homozygous allele u, and n is the number of alleles. The PIC value was estimated using the standard equation (Botstein et al. 1980):

$$\mathrm{PIC} = 1 - \sum_{i=1}^{n} p_i^2 - 2\left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2\right]$$

where p_i is the frequency of the ith allele, and n is the number of alleles. The neighbor-joining (N-J) tree based on the Nei's distance using MEGA 4.0 was employed to display genetic relationship and reciprocal difference in the 45 inbred lines and 45 hybrids (Tamura et al. 2007).

Evaluation of single-locus markers across relative *Brassica* species

In order to detect the universality of the single-locus markers in the cultivated *Brassica* species, all the confirmed single-locus SSR markers in this study were used to amplify the genomic DNA of two accessions from each of the six species in the U's triangle. Band patterns of all markers in every species were visually observed on the denaturing polyacrylamide gels. Those markers that displayed a single amplicon in both accessions of a species were identified as putative single-locus, and ones that displayed two or more amplicons in at least one accession of a species were identified as putative multi-locus. Meanwhile, those markers without any positive PCR amplifications in both two accessions of a species were considered as null-locus.

Results

Screening for single-locus SSR markers

All 9,858 SSR markers, including 4,358 BrGMS markers, 3,548 BoGMS markers, 952 BnGMS markers and 1,000 BnEMS markers, were used to amplify the genomic DNA from six inbred lines to screen for polymorphisms. Of these SSR markers, 8,009 produced clear fragments, of which, 2,701 (33.8 %) displayed single amplicons, 2,879 (35.9 %) displayed two amplicons, and 2,429 (30.3 %) displayed three or more amplicons (Table 2).

Of each marker type, 1,082 (38.8 %) BoGMS, 307 (34.7 %) BnGMS and 1,114 (31.3 %) BrGMS markers detected single amplicons, where the BnEMS markers had the lowest level of single amplicons (25.5 %). For the markers with two amplicons, the rate of BnEMS (45.5 %) was significantly higher than BrGMS (37.3 %), BnGMS (33.5 %) and BoGMS (32.4 %) ($P < 0.05$). This observation might suggest that the coding regions are more conserved than intergenic and intron regions. Of the 2,701 putative single-locus SSR markers, 1,087 showed polymorphism between No2127 and ZY821, the two parents of the BnaNZDH population.

Assignment of the single-locus SSR markers to linkage map

A core set of clear and prominent putative single-locus SSR markers covering the whole genome of *B. napus* were sorted out from the 1,087 polymorphic markers with the following criteria: (1) high-quality amplification excluding strong stutter bands and background products; and (2) good resolution with appropriate spacings between adjacent alleles. Based on these criteria, a total of 283 high-quality putative single-locus SSR markers with single amplicon were selected, which included 25 single-locus SSR markers from other maps as anchors (Lowe et al. 2004; Piquemal et al. 2005; Suwabe et al. 2008).

To test whether these markers are truly inherited in a single-locus mode and assign them to *B. napus* linkage groups, the 283 selected markers were subjected to survey the BnaNZDH population. Of these 283 markers, 241 displaying bi-allele pattern were identified to be true single-locus and the remaining 42 were regarded as dominant markers with two loci from homoeologous chromosome regions. Finally, 230 high-quality single-locus markers were assigned to the BnaNZDH linkage map (Fig. 1; Supplemental Table S1), and 11 single-locus markers could not be assigned to any linkage group. To fill the gaps on linkage groups, 146 polymorphic single-locus SSR markers with one or two redundant monomorphic amplicons were selected to genotype the DH individuals and assigned to linkage groups (Fig. 1). The 230 single-locus markers were distributed randomly all over the genome, with 122 and 108 markers on the A and C chromosomes, respectively. The number of single-locus markers ranged from 3 on A4 and C1 to 31 on A3. The remaining 16 linkage groups had 6–26 single-locus markers (Fig. 1; Supplemental Table S1).

A total of 81 and 70 single-locus BrGMS and BoGMS markers were, respectively, assigned to the linkage map. Of the 81 BrGMS markers, 78 (96.3 %) were assigned to the A genome. Of the 70 BoGMS markers, 69 (98.6 %) were assigned to the C genome of *B. napus*. The BnGMS and BnEMS markers were evenly distributed in the A and C

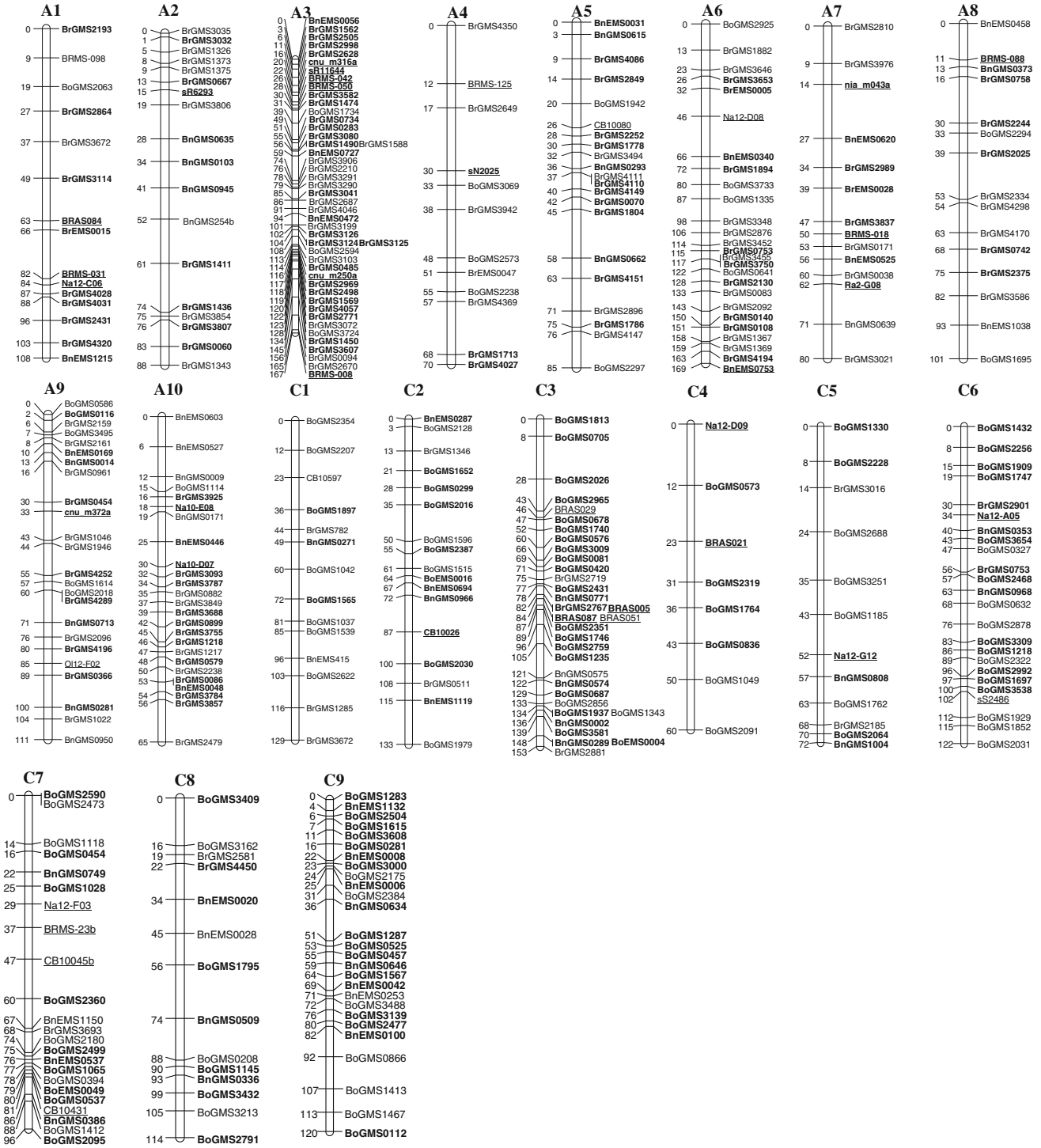

Fig. 1 Distribution of single-locus SSR markers on the genetic linkage map constructed using the BnaNZDH population derived from No2127 and ZY821. The single-locus SSR markers are highlighted in *boldface*. The SSR markers selected from previous maps published by other research groups are *underlined* (Lowe et al. 2004; Piquemal et al. 2005; Suwabe et al. 2008)

genomes, respectively. This result suggests that the single-locus BrGMS and BoGMS markers conservatively amplify loci from their source genome (Lowe et al. 2004; Suwabe et al. 2008). However, one BoGMS (BoMGS0116) marker was mapped in the A genome and 3 BrGMS (BrGMS2767, BrGMS2901and BrGMS4450) markers were mapped in the C genome. Further investigation indicated that these four markers, BoMGS0116, BrGMS2767, BrGMS2901 and BrGMS4450, could successfully amplify in both *B. rapa* (A genome) and *B. oleracea* (C genome) genomes

(Supplemental Table S2), suggesting that the 'misassignment' of these markers might be caused by deletion or mutation that occurred at the target primer binding sites in the corresponding cognate genome in *B. napus*.

Stability and utility of single-locus SSR markers in *B. napus*

The SSR markers displaying a single amplicon in the above-mentioned six *B. napus* varieties may not show a single-locus pattern in more diverse lines. To confirm if these single-locus markers are applicable in a diverse set of rapeseed germplasm, we selected 78 single-locus SSRs across the A and C genomes to genotype 45 *B. napus* inbred lines representing a diverse germplasm from all over the world (Xiao et al. 2012). Each SSR marker is expected to have a single allele in an inbred line. All markers detected a unique locus in these inbred lines as observed in the six inbred lines used for polymorphism screening and in above BnaNZDH mapping population. Allele frequency at each marker locus in the population was calculated (Supplemental Table S3). With this information, the observed heterozygosity (H_o) value at each locus was employed to display the percentage of heterozygous individuals in the inbred lines (Table 3; Supplemental Table S3). Theoretically, the H_o value should be close to 0 in inbred lines. In our collection, the H_o value of 36 loci was 0, which means that all inbred lines are homozygous at these loci. Seventeen markers or loci each detected one heterozygous line in the inbred lines and had a H_o value of 0.02 at each locus. Sixteen markers each detected 2–4 heterozygous lines and had a H_o value ranging from 0.04 to 0.10. The remaining nine markers each detected 5–10 heterozygous lines and had a H_o value ranging from 0.11 to 0.23. Taken together, the average H_o value was 0.04, which is very close to 0 and consistent with the genome characteristics of inbred lines. This phenomenon suggested that these markers could display single allele in most *B. napus* varieties except for a very few heterozygous genotypes, which revealed the single-locus SSR markers are universal in the rapeseed pool.

Furthermore, it is expected that the H_o value in hybrids would be higher than 0. We further validated the stability of the 78 single-locus SSR markers in 45 hybrid cultivars with the H_o value (Table 3; Supplemental Table S3). The H_o values of 26 markers ranged from 0.51 to 0.91 (21–39 heterozygous individuals at each locus). Forty-three markers had a H_o value between 0.11 and 0.49 (5–22 heterozygous individuals at each locus). Nine markers had a H_o value ranging from 0 to 0.02, which might be due to their much lower polymorphism. In total, the average heterozygosity value was 0.41 (Table 3), which is significantly higher than that in the inbred lines (0.04). These results confirmed these SSR markers were universal single-locus across a wide range of inbred lines and hybrid cultivars in *B. napus*, and thus can be used for various aspects of genetic studies in rapeseed gene pools.

The effectiveness of the single-locus markers was also evaluated in the 45 inbred lines and 45 hybrids by computing parameters for genetic diversity. The average PIC value in the inbred lines was 0.41 (varying from 0.04 to 0.74), which was slightly higher than that in the hybrids (0.37), ranging from 0 to 0.83 (Table 3). With this information, the genetic distance between every two rapeseed inbred lines and hybrids were calculated using the Powermaker software. Accordingly, the average pairwise genetic distance in inbred lines was 0.68, which was significantly higher than that of 0.36 in hybrids. This difference is consistent with their geographical origins, where the inbred lines were collected from all over the world but all of the hybrids were cultivars in China, which suggested that the markers have good ability for diversity analysis in rapeseed varieties. A total of 252 alleles were detected in inbred lines. Two to nine alleles were detected per single-locus marker, with an average of 3.23, which was almost the same as the average number of alleles in hybrids (3.01), ranging from 1 to 8 (Table 3). An N-J tree was generated using allelic data of the 78 SSRs. All 45 inbred lines and 45 hybrids were readily discriminated from one another (Supplemental Fig. 1).

Universality of single-locus markers to the relative species

To investigate whether the single-locus SSR markers also produce single amplicons in other species of the U's triangle, we amplified the genomic DNA from *B. rapa*, *B. oleracea*, *B. nigra*, *B. juncea* and *B. carinata* with the 230 mapped single-locus SSR markers by using *B. napus* as control (Table 1). Detailed amplification information is provided in Table 4 and Supplemental Table S4 and the distribution of markers that display single amplicon in each species is highlighted in Fig. 2. Of the 230 SSR markers, 118 (51.3 %), 125 (54.4 %), 85 (37.0 %), 134 (58.3 %) and 121 (52.6 %) markers produced a single fragment in *B. rapa*, *B. oleracea*, *B. nigra*, *B. juncea* and *B. carinata*, respectively. Another 77 (33.5 %), 47 (20.4 %), 41 (17.8 %), 36 (15.6 %), and 41 (17.8 %) markers produced two fragments in *B. rapa*, *B. oleracea*, *B. nigra*, *B. juncea* and *B. carinata*, respectively, and 20 (8.7 %), 24 (10.4 %), 25 (10.9 %), 19 (8.3 %) and 23 (10 %) amplified three or more fragments in *B. rapa*, *B. oleracea*, *B. nigra*, *B. juncea* and *B. carinata*, respectively. The remaining 15 (6.5 %), 34 (14.8 %), 79 (34.3 %), 41 (17.8 %), and 45 (19.6 %) markers did not have any amplification in *B. rapa*, *B. oleracea*, *B. nigra*, *B. juncea* and *B. carinata*, respectively.

Table 3 Genetic information in 45 inbred lines and 45 hybrids revealed by 78 SSR markers

Marker	LG_location (cM)	Inbred lines				Hybrids			
		No. of observation[a]	H_o[b]	Alleles	PIC[c]	No. of observation	H_o	Alleles	PIC
BRAS084	A1_63.454	44	0.00	2	0.32	45	0.20	3	0.40
BrGMS4028	A1_86.595	45	0.04	7	0.62	45	0.27	7	0.60
BrGMS4031	A1_88.15	45	0.02	5	0.65	45	0.27	5	0.70
BrGMS0667	A2_13.375	45	0.02	2	0.29	45	0.27	2	0.20
sR6293	A2_15.353	44	0.16	5	0.66	45	0.53	4	0.70
BnGMS0635	A2_28.183	42	0.00	3	0.56	45	0.49	4	0.40
BnGMS0945	A2_40.568	42	0.05	3	0.30	45	0.44	3	0.40
BrGMS1436	A2_74.153	45	0.02	2	0.37	44	0.52	4	0.39
BrGMS0060	A2_83.034	44	0.05	3	0.40	43	0.14	3	0.13
BnEMS0056	A3_0	45	0.11	6	0.71	45	0.51	4	0.46
BrGMS1474	A3_31.119	45	0.04	3	0.45	43	0.30	3	0.47
BrGMS3124	A3_103.537	44	0.00	2	0.35	45	0.42	2	0.37
cnu_m250a	A3_115.833	40	0.00	7	0.72	42	0.36	7	0.70
BrGMS1569	A3_118.722	44	0.02	4	0.55	45	0.49	4	0.55
BrGMS1450	A3_134.466	44	0.02	3	0.39	45	0.76	3	0.38
BrGMS1713	A4_68.431	45	0.09	5	0.60	43	0.58	4	0.61
BrGMS2849	A5_14.237	41	0.00	3	0.31	40	0.40	3	0.34
BrGMS2252	A5_27.532	44	0.00	3	0.25	42	0.45	3	0.36
BrGMS4110	A5_36.957	45	0.16	6	0.44	42	0.64	6	0.57
BrGMS4149	A5_39.718	44	0.07	4	0.56	41	0.49	4	0.61
BrGMS4151	A5_63.289	45	0.00	4	0.62	43	0.67	4	0.59
BrEMS0005	A6_31.583	44	0.00	4	0.49	44	0.41	3	0.49
BrGMS1894	A6_72.022	41	0.07	3	0.38	44	0.69	3	0.35
BrGMS2130	A6_127.889	45	0.00	3	0.46	45	0.37	3	0.49
BrGMS0108	A6_150.986	37	0.00	4	0.41	43	0.00	3	0.33
BrGMS4194	A6_162.767	45	0.00	3	0.12	45	0.48	1	0.00
BnEMS0753	A6_168.946	45	0.00	3	0.38	44	0.34	2	0.36
BnEMS0620	A7_26.69	44	0.02	4	0.60	44	0.55	3	0.55
BrGMS3837	A7_46.998	45	0.02	3	0.56	38	0.74	3	0.58
BRMS-018	A7_50.499	45	0.09	9	0.74	45	0.58	8	0.83
BnGMS0373	A8_13.19	43	0.02	2	0.27	45	0.29	2	0.24
BrGMS0454	A9_30.343	45	0.00	5	0.69	42	0.55	4	0.53
cnu_m372a	A9_33.433	45	0.07	6	0.68	39	0.77	6	0.75
BrGMS4252	A9_55.124	42	0.02	3	0.47	40	0.23	3	0.28
BnGMS0713	A9_70.936	45	0.04	4	0.61	40	0.55	4	0.50
BrGMS0366	A9_89.347	45	0.02	2	0.34	43	0.19	3	0.32
BrGMS0899	A10_41.782	42	0.00	3	0.46	41	0.51	3	0.45
BrGMS1218	A10_46.339	45	0.00	2	0.15	44	0.00	1	0.00
BrGMS0579	A10_47.95	44	0.05	3	0.50	44	0.57	3	0.45
BnEMS0048	A10_52.978	45	0.02	4	0.43	45	0.64	4	0.54
BoGMS1897	C1_35.982	45	0.02	2	0.35	44	0.44	2	0.36
BoGMS1565	C1_72.051	45	0.04	2	0.34	44	0.48	2	0.35
BoGMS1652	C2_20.711	45	0.00	2	0.37	45	0.41	2	0.29
CB10026	C2_86.685	44	0.00	2	0.30	43	0.58	2	0.37
BoGMS2030	C2_100.354	45	0.00	2	0.12	43	0.02	2	0.02
BoGMS1813	C3_0	44	0.11	2	0.36	45	0.38	2	0.34
BoGMS0678	C3_47.053	42	0.10	4	0.51	42	0.48	4	0.38

Table 3 continued

Marker	LG_location (cM)	Inbred lines				Hybrids			
		No. of observation[a]	H_o^b	Alleles	PIC[c]	No. of observation	H_o	Alleles	PIC
BoGMS1740	C3_52.439	41	0.07	4	0.50	43	0.35	3	0.38
BRAS087	C3_83.742	42	0.00	3	0.57	43	0.91	2	0.37
BoGMS1746	C3_88.693	44	0.00	2	0.08	45	0.02	2	0.02
BnGMS0002	C3_135.961	42	0.02	4	0.32	44	0.57	3	0.41
Na12-D09	C4_0	45	0.00	6	0.73	39	0.46	6	0.68
BoGMS0573	C4_11.949	42	0.00	2	0.37	42	0.12	3	0.46
BRAS021	C4_23.483	45	0.20	2	0.36	44	0.43	2	0.30
BoGMS1764	C4_35.874	44	0.00	2	0.36	45	0.36	2	0.31
BoGMS0836	C4_42.616	45	0.02	5	0.48	45	0.80	5	0.61
BnGMS0808	C5_57.181	45	0.00	2	0.12	43	0.02	2	0.02
BoGMS2256	C6_8.082	45	0.00	2	0.04	43	0.16	2	0.14
BoGMS1909	C6_14.642	43	0.12	4	0.57	43	0.58	3	0.49
BoGMS1747	C6_19.102	45	0.00	3	0.48	45	0.38	2	0.36
BnGMS0968	C6_63.2	45	0.00	2	0.30	45	0.11	2	0.10
BoGMS3309	C6_82.71	45	0.00	2	0.12	44	0.02	2	0.02
BoGMS1218	C6_86.415	44	0.18	4	0.23	39	0.46	2	0.29
BoGMS2992	C6_96.29	45	0.00	2	0.29	43	0.02	2	0.02
BoGMS1697	C6_97.171	44	0.05	3	0.57	45	0.56	3	0.39
BrGMS0753	C6_115.309	40	0.00	3	0.40	41	0.24	2	0.37
BoGMS2499	C7_74.688	45	0.00	2	0.36	44	0.80	2	0.37
BoGMS2095	C7_96.37	45	0.02	2	0.32	45	0.47	2	0.32
BnEMS0020	C8_34.225	45	0.00	2	0.37	45	0.58	2	0.37
BoGMS1795	C8_56.398	41	0.15	3	0.36	43	0.40	3	0.37
BoGMS1145	C8_89.613	45	0.00	3	0.40	45	0.49	2	0.34
BoGMS3432	C8_99.281	44	0.02	2	0.31	43	0.37	2	0.26
BoGMS1283	C9_0	45	0.07	2	0.24	45	0.22	2	0.18
BoGMS3608	C9_10.69	45	0.02	2	0.31	44	0.25	2	0.31
BoGMS1287	C9_50.978	45	0.00	2	0.12	43	0.02	2	0.02
BoGMS1567	C9_64.415	45	0.00	2	0.27	45	0.49	2	0.33
BoGMS3139	C9_75.793	43	0.23	3	0.43	39	0.56	2	0.37
BoGMS2477	C9_79.929	39	0.00	2	0.13	35	0.00	2	0.05
Average			0.04	3.23	0.41		0.41	3.01	0.37

[a] The No. of observation for a marker is defined as the number of cultivars with nonmissing genotypes observed in the population

[b] Observed heterozygosity

[c] Polymorphism information content

In total, 213 markers (92.6 %) could amplify single amplicons in at least one of the species. Of these, 21 markers amplified single amplicon in all of the five species, 32 markers amplified a single fragment in all three diploid species, and 65 markers could amplify single amplicon in both *B. juncea* and *B. carinata* (Fig. 2; Supplemental Table S4). In summary, these SSR markers producing single amplicon in each species could be identified as putative single-locus.

After observing the single-locus pattern of the 230 SSR markers in *B. napus* (AACC), we tested their amplification behavior in *B. rapa* (AA) and *B. oleracea* (CC). Of these, 181 markers (78.7 %) successfully amplified in both progenitor genomes A and C, indicating that most of the single-locus SSR markers are not genome specific. We also observed that 34 (14.8 %) markers mapped in the A genome of *B. napus* were unique to *B. rapa* (AA). Furthermore, 27 out of these 34 markers were also unique to *B. juncea* (AABB). While 15 markers (6.5 %) mapped in C genome of *B. napus* were unique to *B. oleracea* (CC) and 13 out of these 15 markers were also unique to *B. carinata* (BBCC) (Supplemental Table S4).

Discussion

SSR markers are useful tools for gene/QTL mapping, marker-assisted selection and diversity analysis. In simple diploid species like rice and barley, most SSR markers are single-locus and thus usually amplify a maximum of two alleles per marker in diverse varieties and segregating populations such as F_2 and BC_1. Unfortunately, in allotetraploid *B. napus*, the use of SSR markers is generally not as straightforward as in diploids because most SSR markers are multi-locus and amplify multiple alleles from homoeologous loci. Therefore, assigning alleles to distinct loci is a great challenge in *B. napus*. In the present study, we developed a set of 230 high-quality single-locus SSRs from a total of 9,858 SSR markers. These single-locus SSR markers were firstly selected based on their amplification fashion in six rapeseed inbred lines. Then these markers were confirmed to be genuine single-locus using a DH mapping population with 154 lines. The major advantage of DH populations is that every individual is homozygous for the two alleles of a single-locus SSR marker. Furthermore, a diverse set of 45 inbred lines and 45 hybrids was selected to validate the single-locus nature of 78 representative SSR markers. These SSR markers presented either in both *B. rapa* and *B. oleracea* genomes or only in *B. rapa* or *B. oleracea* genome (Supplemental Table S4) and distributed across each chromosome of *B. napus* genome. All the 78 markers detected at most two alleles in this diverse set of rapeseed varieties. Of these, about half (36) had a H_o value of 0 in the diverse set of inbred lines, indicating that at least 50 % of these markers were unambiguous single-locus. By combining amplification of six rapeseed varieties, segregation analysis in a segregating DH populations and validation with a diverse set of inbred lines and hybrids, we believe that most of the 230 SSR markers, if not all, were confirmed to be true single-locus in *B. napus*. It is worth noting that nine out of 78 markers (11.5 %) detected a H_o value higher than 0.10 in the inbred lines, which might be caused by pollen contamination in selfing. However, we could not exclude the possibility that some of the markers are not true single-locus, and suggest researchers further test these markers in a more diverse germplasm in their own studies.

Table 4 Amplification of the 230 SSR markers in other *Brassica* species of the U's triangle except *B. napus*

Species	Single amplicon (%)	Two amplicons (%)	≥ Three amplicons (%)	No product (%)
B. rapa	118 (51.3)	77 (33.5)	20 (8.7)	15 (6.5)
B. oleracea	125 (54.3)	47 (20.4)	24 (10.4)	34 (14.8)
B. nigra	85 (37.0)	41 (17.8)	25 (10.9)	79 (34.3)
B. juncea	134 (58.3)	36 (15.6)	19 (8.3)	41 (17.8)
B. carinata	121 (52.6)	41 (17.8)	23 (10.0)	45 (19.6)

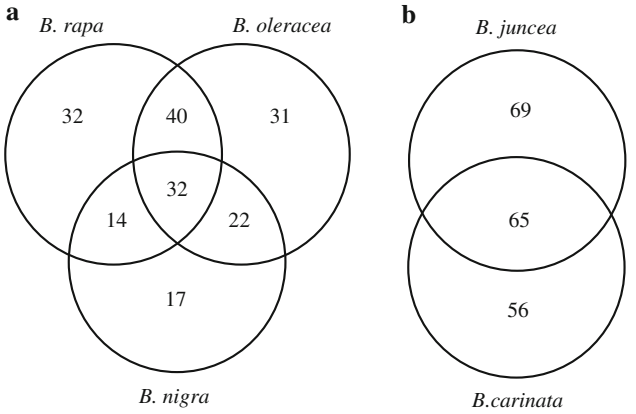

Fig. 2 Venn diagram displaying amplification patterns of the 230 single-locus SSR markers in **a** *B. rapa*, *B. oleracea*, and *B. nigra*; **b** *B. juncea* and *B. carinata*. The numbers in each circle represent the number of SSR markers that displayed single amplicon in corresponding species

Genetic diversity analysis and cultivar fingerprinting or identification are important aspects in germplasm collection and evaluation and in protection of a commercial variety (Chen et al. 2008). Ideal markers for diversity analysis and DNA fingerprinting should have the following features: (1) high-quality single-locus markers with reasonable polymorphism; (2) easily amplified and highly reproducible; (3) thoroughly distributed across the whole genome and (4) appropriate spacing between adjacent alleles (Cipriani et al. 2008; Ghislain et al. 2004; Varshney et al. 2008; Wang et al. 2011b). Core sets of SSR markers had been developed for diversity analysis and cultivar discrimination in plants such as potato, barley, maize and grape (Cipriani et al. 2008; Ghislain et al. 2004; Varshney et al. 2008; Wang et al. 2011b). In this study, the 230 single-locus SSR markers satisfied the above strict criteria, and thus will be very useful in rapeseed cultivar identification, diversity analysis and subsequent association mapping.

Cross-species amplification would bring similar advantages to species without single-locus SSR markers. When analyzed in other *Brassica* species in the U's triangle, these single-locus SSR markers demonstrated a high degree of universality in the three diploids and other two allotetraploids. Therefore, these markers would be also helpful for more accurate comparative studies of genome changes between species. Although the single-locus SSR markers were developed from the A or C genome, 85 markers could amplify a single fragment and 66 amplified two or more fragments in *B. nigra* (B genome). This result indicates that there is high homology between the B and A genomes, and between the B and C genomes. Meanwhile there were also 79 markers that could not amplify in *B. nigra*, which was

much more than the number of non-amplified markers in *B. rapa* (15 markers) and *B. oleracea* (34 markers), indicating that the relationship between the A and C genomes is much closer than that between the B and A or C genomes as revealed by molecular phylogenetic and cytogenetic analyses (Warwick and Black 1991; Xiao et al. 2010). It is worth noting that 27 markers only had amplification in the A genome-containing species (*B. rapa*, *B. napus* and *B. juncea*), suggesting that these markers might be A genome specific. In addition, 13 markers only had amplification in the C genome-containing species (*B. oleracea*, *B. napus* and *B. carinata*), suggesting that these markers might be C genome specific. These genome-specific markers would be useful to map and characterize the corresponding genome introgression at different generations in cross-species hybridization (Navabi et al. 2011).

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