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Establishment and application of molecular ID in the main inbred lines of Chinese cabbage

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Genet. Mol. Res. 16 (1): gmr16019144
Received September 2, 2016
Accepted December 12, 2016
Published March 8, 2017
DOI http://dx.doi.org/10.4238/gmr16019144

ABSTRACT. Chinese cabbage is an important cruciferous vegetable in China. The differences in the morphology and other characteristics of the different varieties of Chinese cabbage are generally caused by their different genes. Using the simple sequence repeat (SSR) DNA molecular markers is an effective way to identify different genotypes. The identification of a genetic relationship is a key point in the breeding process, and it plays an important role in guiding parent selection and breeding of high-yield varieties. Moreover, the establishment of genomic fingerprints is significant for plant variety protection. Three to five SSR sites were selected from each of the 10 Chinese cabbage chromosomes on the basis of the abundance of SSR loci on them. According to the differences in the SSR polymorphic bands, a genomic fingerprint comprising 36 different loci was established in the 20 main inbred lines of Chinese cabbage, and this fingerprint was converted digitally into a molecular ID with 36 numbers based on the 36 SSR sites. The utility of this core set SSRs was demonstrated in 20 main inbred lines of Chinese cabbage, which could be placed into six clusters that were

largely consistent with previous classification based on morphology data. Moreover, the molecular ID of an F1 hybrid can be deduced from its parents molecular IDs, and its purity can be determined by selecting one or two SSR loci from the 36 different loci.

Key words: *Brassica rapa*; Inbred line; SSR; Molecular ID; Fingerprint

INTRODUCTION

Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*) is an important cruciferous vegetable. Numerous varieties of Chinese cabbage are available in the Chinese market. However, many of them are highly similar, and good varieties are not much. Therefore, an accurate identification of the varieties of this vegetable is important for establishing a reference for the breeding of new varieties as well as for protecting the germplasm resources and rights of the breeders. Three main identification methods are applicable today: morphologic, biochemical marker, and DNA molecular marker identification (Mei and Lu, 2005). Morphologic identification involves the use of agronomic characters as standards, and thus, is time consuming and costly. Moreover, its accuracy is easily influenced by numerous factors, such as the environment, seasons, and experiences of evaluators, thereby preventing this method from meeting the required speed in variety renovation. The biochemical marker identification method essentially exhibits the same characteristics and drawbacks. However, DNA molecular marker identification is a fast and accurate method, which has been widely applied in the establishment of fingerprint databases, identification of germplasm resources, assessment of genetic distances of inbred lines, and identification of purity. As a type of molecular markers, the simple sequence repeat (SSR) markers have been frequently used to establish the fingerprint databases and identify the purity of *Zea mays* L. (Zhou and Chen, 2005), *Oryza sativa* L. (Dai et al., 2011), *Gossypium hirsutum* (Xue et al., 2010), *Brassica oleracea* var. *capitata* (Chen et al., 2011), *Musa* spp. (Wang et al., 2009), *Cucumis melo* L. (Wang et al., 2010), *Nicotiana tabacum* L. (Xu et al., 2011), *Brassica rapa* var. *chinensis* (Shen et al., 2016), *Cucumis sativus* (Hu et al., 2011), and *Citrus limon* (Lei et al., 2009). This is because of their simple operation, high polymorphism, codominance, good repeatability and stability, and abundance with random distribution in genomes (Zhou, 2005).

To date, many SSR molecular markers have been developed and analyzed in Chinese cabbage, but they are not evenly distributed on the 10 chromosomes of Chinese cabbage (Yu et al., 2003; Yang et al., 2013; Zhang et al., 2013; Kawamura et al., 2015). The present study aims to use 36 SSR markers that are evenly distributed on the 10 chromosomes of Chinese cabbage to screen its 20 highly inbred lines, and establish an inbred line ID. Through the codominant factor of SSR markers, we could identify the purity of hybrids and validate the accuracy and reliability of our results in combination with morphologic identification. Thus, we provided an accurate and effective method to identify the purity and authenticity of hybrids.

MATERIAL AND METHODS

Plant materials

Twenty inbred lines of Chinese cabbage (Table 1) developed in Northeast Agricultural

University were used to establish the molecular ID database. The F1 hybrid resulting from the cross between A204-1-1 and A8-1 was used to validate the accuracy and reliability of the molecular ID, whereas two hybrid combinations (A204-1-1 × A8-1 and A204-1-1 × A66-2-1) were used to identify the purity of hybrids. Seeds were obtained after mixed pollination.

Table 1. Molecular IDs of the 20 inbred lines of Chinese cabbage.

Number	Material	Molecular ID
1	A18-2	13113343111221513121433531202221 1131
2	A66-2-1	11211221242221513131212521311321 1231
3	A8-1	33223263211221513211313101321312131
4	A28-3	21113343131121113122132520222432 3111
5	A109	31111381241212632211111331323310121
6	C56	23122252112231611121112410212423222 3
7	A113-1	31111331241221513231111311232324242 1
8	C30	21112342132121633111132210322421 0111
9	A101	21132131111221512121421131321331112 1
10	A31-1	33113342112121513212132531132134213 3
11	A71-1-1	21122241141111632111111310232421212 1
12	A209	31122352241211613232122331332433221 2
13	A17-1	22122333132221312121131320332434253 3
14	A161	33113253142121322222131330212133223 1
15	C34-1	23211263111221312121112532322241131
16	A117	32211373131231212211111431132411223 1
17	A200	21121352221211613111112431322132213 3
18	A105-3-1	32113371131141313212111530232411213 1
19	A204-1-1	21123313132111531111131431322421013 1
20	A17-4	11113223131122411121311531212431132 1

The seeds were planted in 8 x 8 cm pots, which contained nutrient-rich soil and were placed in an illuminated incubator. The young leaves, whose lengths were less than 1.5 cm, were harvested during the 3-4-leaf stage, recorded, and frozen in liquid nitrogen for DNA isolation.

Genomic DNA extraction

The genomic DNA was extracted from the stored young leaves using the NuClean PlantGen DNA Kit (Beijing ComWin Biotech Co., Ltd., Beijing, China), and then analyzed via 0.6% agarose gel electrophoresis.

SSR primer screening

We selected 130 already published primers for screening the core primers. Thirty-six core primers were identified; these primers should have more than two polymorphic bands for establishing the molecular ID of inbred line (Table 2) (Suwabe et al., 2002, 2003; Lowe et al., 2004; Ge et al., 2005; Piquemal et al., 2005; Yu et al., 2009; Pino Del Carpio et al., 2011; Gao et al., 2012; Zhang et al., 2013; Sui et al., 2014). The primers were synthesized by Boshi Biological Technology Co., Ltd. in Harbin, China.

SSR amplification

Polymerase chain reaction (PCR) was performed in a 25-μL reaction volume comprising 12.5 μL 2X Taq MasterMix (Beijing ComWin Biotech Co., Ltd.), 8.5 μL ddH_2O,

2 μL primer pairs, and 2 μL template DNA (approximately 50 ng/μL). The amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at proper temperature for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

Table 2. Detailed information of 36 simple sequence repeat (SSR) markers.

Number	Marker	Primer sequence	Tm (°C)	References
1	P193	TCAAGATCCTCCACTTCT ACGGACTGTATAGCGACT	51	Zhang et al., 2013
2	BC22	CAAACTCCCCCAGATCCCCAAACC CGAGCGCGAACATGAGGAAGAGAA	60	Ge et al., 2005
3	NA14-D07	GCATAACGTCAGCGTCAAAC CTGCGGGACACATAACTTTG	53	Lowe et al., 2004; Piquemal et al., 2005
4	Na14-H11	GGATGTTTTCACAGACCCTG CTTTGCAGGTATGAACACGC	53	Lowe et al., 2004; Piquemal et al., 2005
5	FT-SSR-P1	GACGACAGCTTCGAAAGAGA TGAGCATTGTTTTGGTGATG	50	Gao et al., 2012
6	SAG12-SSR-P1b	CCATTGGAGCTTGTAAGCAAT GCTCCAAGGGGTAGAACTCC	53	Gao et al., 2012
7	BRMS-026	CCTATCCTCGGACTAATCAGAA GTGCTTGATGAGTTTCACATTG	52	Suwabe et al., 2002
8	CX272537	GCCAGAGATAGTGTGAGAGC AAGTTGTAAGGCTGAGATGC	57	Yu et al., 2009
9	BC7	TCGGCGATGGCTCTTTTCACC AAGGATCGTTCAGCGGAGAGTAT	56	Ge et al., 2005
10	BRMS-129	TGAGGTTAGACATGGCGCTGCTTG TTTGATCATTGTGGTCGCGAGTTC	57	Zhang et al., 2013
11	BRMS-042	GGATCAGTTATCTGCACCACAA TCGGAATTGGATAAGAATTCAA	50	Suwabe et al., 2002
12	Na12-A01	GCATGCTCTTGATGAACGAA GCTTCAACCTCTCAATCGCT	53	Lowe et al., 2004; Piquemal et al., 2005
13	BRU05099	TCTCCTCCCCGAATCTAC AAACGTTATTCTCATCAACGA	50	Zhang et al., 2013
14	Ol11-H02	TCTTCAGGGTTTCCAACGAC AGGCTCCTTCATTTGATCCC	54	Lowe et al., 2004; Piquemal et al., 2005
15	BRMS-034	GATCAAATAACGAACGGAGAGA GAGCCAAGAAAGGACCTAAGAT	53	Suwabe et al., 2002; Zhang et al., 2013
16	CX272976	CAACTCTAACGAACGAGAGC CATGTATTCTGCGTGAAGTG	57	Sui et al., 2014
17	BC25	GCGGCAAAAACGGGCTCATTA CTCTCCGGCTTCTTCCTCACCTCT	56	Ge et al., 2005
18	P193	TCAAGATCCTCCACTTCT ACGGACTGTATAGCGACT	51	Zhang et al., 2013
19	ENA19	AAGTTACCAAGGAGAGGACAG AAAGGGACGCTACAAGTCA	52	Sui et al., 2014
20	BRU00503	GTGCTTTAATGGAAGGAACTT TCACGAAGAAGATCGATTAGA	50	Zhang et al., 2013
21	EJU5	GGCACGTACATGGAGGATTC TGTTGGTCGAGCTGTTTCAG	54	Yu et al., 2009; Sui et al., 2014
22	ENA6	CTCGTCTTCTTCACCTACAAC CTGACATCTTTCTCACCCAC	53	Yu et al., 2009; Sui et al., 2014
23	BRMS296	GATCCTAATGTTGCTGAGAAAGAGG TATATGAAACCGATGAAGCTCCTTT	54	Zhang et al., 2013
24	Ra2-A01	TTCAAAGGATAAGGGCATCG TCTTCTTCTTTTGTTGTCTTCCG	52	Lowe et al., 2004
25	BRMS-036	GGTCCATTCCTTTTTGCATCTG CATGGCAAGGGGTAACAAACAT	54	Suwabe et al., 2002
26	BRMS-088	TATCGGTACTGATTCGCTCTTCAAC ATCGGTTGTTATTTGAGAGCAGATT	53	Suwabe et al., 2003
27	BR319	TCTATGATCATGGCTTCCTC TCTCCGGTGTAGAGTTTGTT	52	Pino Del Carpio et al., 2011
28	BRMS-006	TGGTGGCTTGAGATTAGTTC ACTCGAAGCCTAATGAAAAG	50	Suwabe et al., 2002
29	MR013	CGCTACTTCCGCTGATACTTT TCAGAATCGCGACTGTAGTCT	54	Piquemal et al., 2005
30	MR172	GGGTTTTAAGCCTTGTCTAAG AAACCAAAATGCATAGACTGA	49	Piquemal et al., 2005
31	BRMS-051	GGCCAAGCCACTACTGCTCAGA GCGGAGAGTGAGGGAGTTATGG	60	Suwabe et al., 2002; Zhang et al., 2013
32	Ni4-D09	AAAGGACAAAGAGGAAGGGC TTGAAATCAAATGAGAGTGACG	52	Lowe et al., 2004; Piquemal et al., 2005
33	Na10-G08	TTTCTTTTAACCTGATGTTTTGG TCACTGTGTTTACTTGCGCC	49.5	Lowe et al., 2004; Piquemal et al., 2005
34	Na12-H04	TTTATCGTCTTTCCCCTCCC ACAAGGAACTAGAGAGAGAGAG	54	Lowe et al., 2004; Piquemal et al., 2005
35	BRU02939	CCAAGATGATGCAGATACATT TGATCTTATATGCCCTTTTGA	49	Zhang et al., 2013
36	ENA18	TTAAAATGAAACCCACCCGA TGTTGGGCAACATCCATTTA	50	Sui et al., 2014

Electrophoresis analysis

PCR products were analyzed via 8% non-denaturing polyacrylamide gel electrophoresis at 35 V/cm for 3 h, using 1X TBE (Tris-Borate-EDTA) buffer. Subsequently, the polyacrylamide gels were silver stained for 15 min according to the method of Bassam et al. (1991). The banding pattern was also recorded.

Data analysis

Each polymorphic band was observed as an allele. When a band was present on a certain position of the polyacrylamide gel, it was marked as "1"; otherwise, it was marked as "0". After marking all bands, the data were analyzed using the numerical taxonomy and multivariate analysis system (NTSYS-pc) version 2.10 software (Song et al., 2012). The sequential, agglomerative, hierarchic, and non-overlapping methods, as well as the unweighted pair-group method with arithmetic average (UPGMA) program, were used to perform cluster analysis. The genetic relationship of the inbred lines was subsequently obtained.

Digitization of polymorphic bands and establishment of molecular ID

The establishment of a molecular ID was based on the differences in polymorphic bands (Lu et al., 2014). Each band was digitized to a specific number. On the basis of the 36 differential polymorphic bands, a molecular ID comprising 36 numbers for each inbred line was established.

Deduction and identification of the molecular ID of F1 hybrid

The molecular ID of the hybrid combination A204-1-1 x A8-1 was deduced according to the polymorphism of 36 SSR sites present in the inbred lines A204-1-1 and A8-1. Theoretically, the molecular ID of their F1 hybrid is composed of 72 numbers. Subsequently, the DNA of the F1 generation was extracted to perform an electrophoresis using 36 primers. The bands were recorded and compared with the theoretical bands.

Purity identification of F1 hybrid

One hundred hybrid seeds of the hybrid combinations A204-1-1 x A8-1 and A204-1-1 x A66-2-1 were selected randomly, and planted in nutrient-rich soil. DNA was extracted from the seedlings at a 3-4-leaf stage. A pair of specific primers was selected for PCR amplification, and then analyzed through electrophoresis. The bands were recorded and compared with the theoretical bands.

RESULTS

Screening and analysis of SSR primers

Using the genomic DNA of 20 inbred lines of Chinese cabbage as template for PCR, 130 pairs of SSR primers were screened. Most of the primers were effective in amplification.

After repeated verification, 36 polymorphic bands were selected; these bands were stable and clearly polymorphic. They were evenly distributed on the 10 Chinese cabbage chromosomes, each of which contained three to five SSR sites (Figure 1). Each primer can produce two to eight polymorphic bands (Table 3 and Figure 2). For example, the primers BRMS-129 and Ni4-D09 produce four polymorphic bands (Figure 2a and b). These four polymorphic fragments were sorted according to the amplified fragment length in the ascending (from small to large) order, and subsequently marked as 1, 2, 3, and 4 (Figure 3) for establishing the molecular ID.

Table 3. Basic information of 36 simple sequence repeat (SSR) primer pairs.

Number	Primer	Chromosome	Molecular weight (bp)	Number of polymorphic bands
1	P193	1	370-380	3
2	BC22	1	210-225	3
3	NA14-D07	1	130-135	2
4	Na14-H11	2	108-120	3
5	FT-SSR-P1	2	250-265	3
6	SAG12-SSR-P1b	2	180-220	3
7	BRMS-026	2	120-180	8
8	CX272537	3	115-123	3
9	BC7	3	215-235	2
10	BRMS-129	3	180-250	4
11	BRMS-042	3	125-145	2
12	Na12-A01	3	115-120	2
13	BRU05099	4	270-310	4
14	Ol11-H02	4	180-190	2
15	BRMS-034	5	125-145	6
16	CX272976	5	240-245	3
17	BC25	5	185-195	3
18	P193	6	370-380	2
19	ENA1	6	115-125	3
20	BRU00503	6	115-120	2
21	EJU	6	150-180	4
22	ENA6	7	110-120	3
23	BRMS296	7	150-162	3
24	Ra2-A01	7	100-130	5
25	BRMS036	7	115-120	3
26	BRMS-088	8	0-230	2
27	BR319	8	445-460	3
28	BRMS-006	8	145-180	4
29	MR013	9	160-165	2
30	MR172	9	220-240	4
31	BRMS-051	9	245-280	3
32	Ni4-D09	9	165-220	4
33	Na10-G08	10	145-260	3
34	Na12-H04	10	65-100	5
35	BRU02939	10	145-160	3
36	ENA18	10	100-110	3

Digitization of polymorphic bands and establishment of molecular ID

All bands were recorded for their quantity and amplified fragment length, and then arranged in order according to their amplified fragment length. These bands vary in number from 1 to N, where N denotes the total number of bands (Figure 2). Each inbred line could be represented as a molecular ID with 36 numbers based on the 36 SSR sites. For example, the molecular ID of material A18-2 is 131133431112215131214335312022211131. The first "1" represents a band in A18-2, and indicates that the first primer P193 can amplify the first band; the remaining 35 numbers were digitized in the same mode.

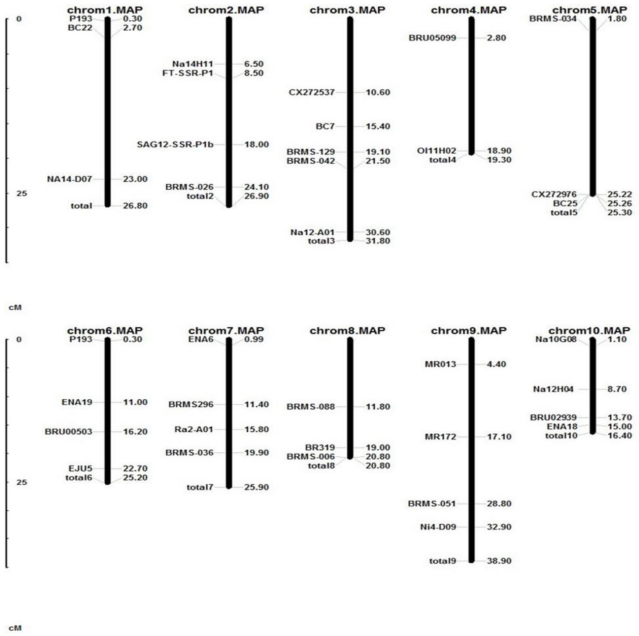

Figure 1. Distribution of 36 simple sequence repeat (SSR) sites on the 10 chromosomes in Chinese cabbage. The primers are mentioned on the left side of chromosomes, and their corresponding positions on the right side of the chromosomes.

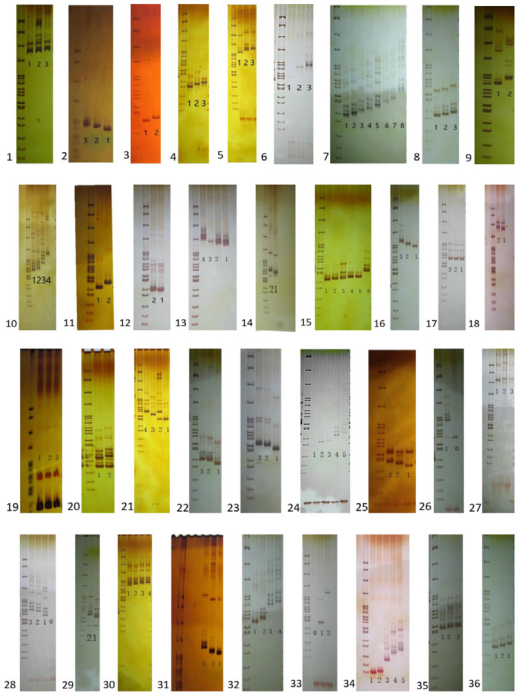

Figure 2. Polymorphic bands of 36 simple sequence repeat (SSR) loci.

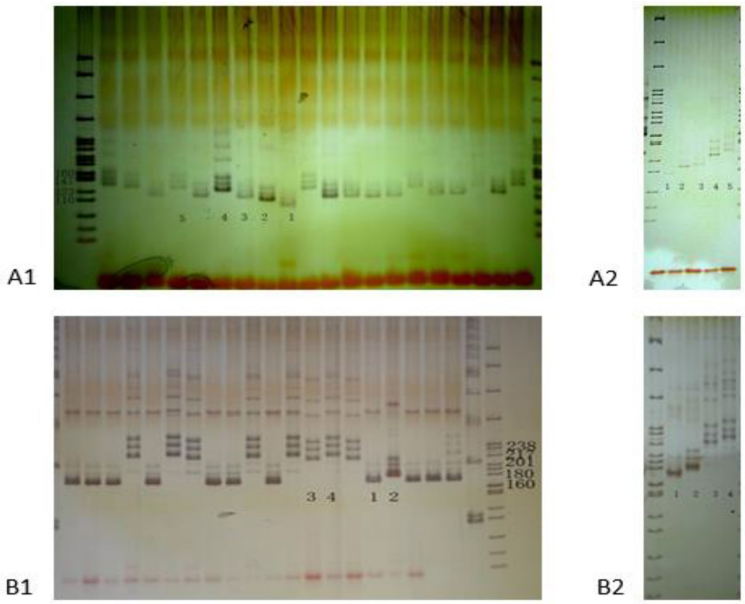

Figure 3. Polymorphism of some primers in 20 inbred lines of Chinese cabbage. **A1.** Amplified bands of Ra2-A01 in 20 inbred lines. **A2**. *Lanes 1-5* = 5 polymorphic bands corresponding to A1. **B1.** Amplified bands of Ni4-D09 in 20 inbred lines. **B2.** *Lanes 1-4* = 4 polymorphic bands corresponding to B1.

Genetic distance and relationship analysis of inbred lines

The data of all bands were encoded in the statistical analysis software NTSYS 2.10 to perform cluster analysis using the UPGMA method. Subsequently, 20 cluster figures corresponding to 20 inbred lines were obtained. These 20 inbred lines can be divided into six categories on the basis of their genetic similarity coefficient of 0.47. For example, the Chinese cabbage varieties A117, A113-1, A105-3, and A109 are all straight-head types; therefore, they belong to the same category in the cluster figure (Figure 4).

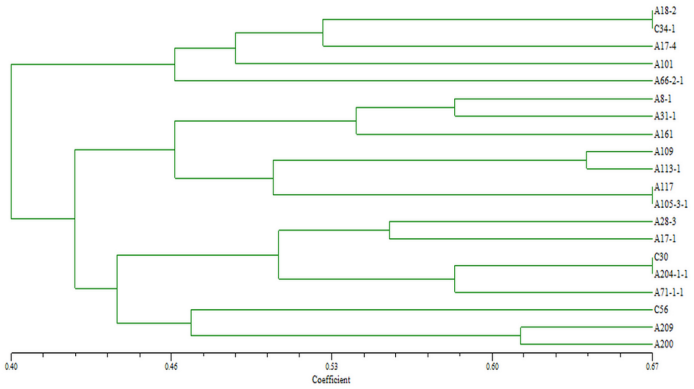

Figure 4. Cluster figure of the 20 inbred lines of Chinese cabbage based on the simple sequence repeat (SSR) molecular markers.

Deduction and verification of the molecular ID of F1 hybrid

The molecular ID established in this study can be used for protecting the variety rights of inbred lines and hybrids. We can obtain a molecular ID composed of 72 numbers, which is a combination of the parents' 36 numbers. In this experiment, the hybrid combination A204-1-1 x A8-1 was selected to deduce and verify the molecular ID of its F1 hybrid. In Table 1, the molecular IDs of the parents A204-1-1 and A8-1 are shown as 21123313213211153111113 14313224210131 and 33223263211221513211131310132131 2131, respectively. The SSR markers are codominant; hence, we can deduce the molecular ID of the hybrid combination A204-1-1 × A8-1 as 23 13 12 22 33 32 16 33 12 31 21 12 12 11 55 31 13 12 11 11 11 33 11 43 31 10 31 23 22 41 23 11 02 11 33 11. The electrophoresis results were consistent with our prediction, thus proving that our deduction was correct (Figure 5).

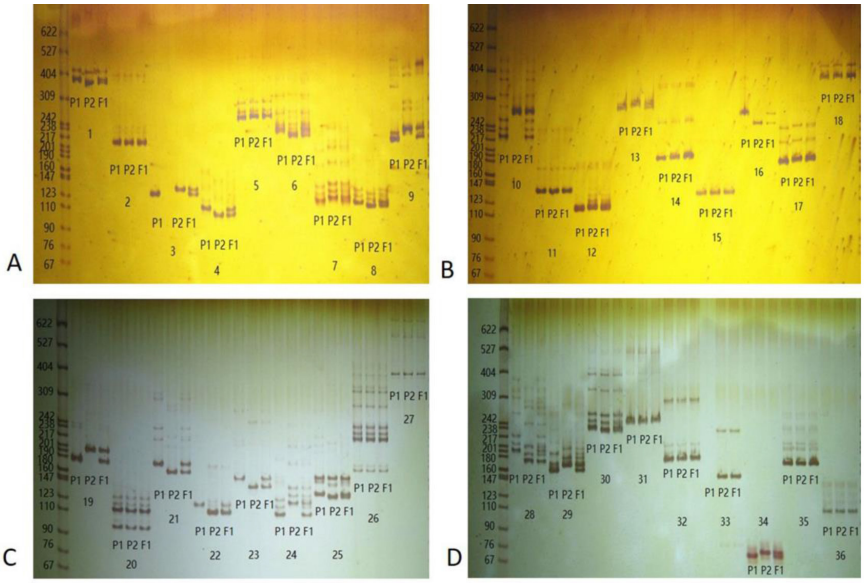

Figure 5. PCR-product bands obtained for the F1 hybrids of A204-1-1 (P1) x A8-1(P2) using 36 core primer pairs. **A.** 1-9 primers. **B.** 10-18 primers. **C.** 19-27 primers. **D.** 28-36 primers. P1 and P2 refer to parents 1 and 2, respectively.

Purity identification of F1 hybrid

To verify the feasibility of using molecular IDs in identifying the purity of hybrid combinations, the parental complementary band analysis was performed. We select one primer pairs from Table 1 which show polymorphism between parents to test the purity of the hybrid combinations A204-1-1 x A8-1 and A204-1-1 x A66-2-1. The BRMS-026 primer was selected to identify the hybrid combination A204-1-1 x A8-1. The parents, A204-1-1 and A8-1, produced the bands of 120 and 148 bp, respectively. Among 100 hybrid bands, 78 were complementary bands, 20 were identical to parent A204-1, and 2 were identical to parent A8-1 (Figure 6A). Therefore, the purity of the hybrid was 78.0%, which was consistent with value obtained in the field investigation (77.2%).

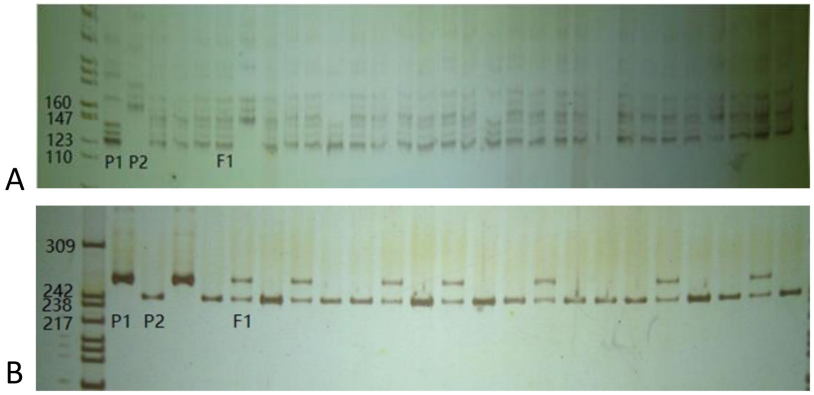

Figure 6. Purity identification of hybrid. **A.** A204-1-1(P1) x A8-1(P2). **B.** A204-1-1(P1) x A66-2-1(P2). If the two bands belonging to the parents P1 and P2 coexist in one gel track like F1 hybrid, then the sample is a hybrid generation, otherwise it is only a self-bred generation, which was not hybridized successfully.

The CX272976 primer was selected to identify the hybrid combination A204-1-1 x A66-2-1. The parents, A204-1-1 and A66-2-1, produced the bands of 240 and 245 bp, respectively. Among 100 hybrid bands, 40 were complementary bands, 60 were identical to parent A66-2-1, and none was identical to parent A204-1 (Figure 6B). Hence, the purity of the hybrid was 40.0%, which was consistent with the value obtained in the field investigation (41.3%). Therefore, using molecular IDs for identifying the purity of hybrid species is a reliable method.

DISCUSSION

The use of molecular markers has grown in recent years. Although single nucleotide polymorphism (SNP) markers are widely used, the SSR markers are still recognized as one of the ideal molecular markers because of their low cost, abundant polymorphism, and stable and good reproducibility. In our study, the SSR markers were used to establish a molecular ID database for 20 important inbred lines of Chinese cabbage. Unlike other studies, the present work used the inbred lines and digitized the obvious polymorphic bands. The molecular identification of inbred lines exhibits considerable application value in protecting the variety rights of inbred lines and F1 hybrids. A database based on the molecular IDs of the main inbred lines in Chinese cabbage can be established. This database can be expanded subsequently, and the data and query information can be retrieved at any time. Although the materials used in our study were relatively few in number, 20 inbred lines can yield a large number of hybrid combinations. Hence, the identification method presents a great application value in the future.

Core primers are the key to establishing a fingerprint database (Li et al., 2005). The primers we selected were distributed evenly and accurately on the chromosomes. A total of 36 core primers were selected, and the 20 inbred lines of Chinese cabbage were divided into six categories. The four straight-head type of inbred lines were classified into one group. These results indicated that the importance of the selection of polymorphic primers, which are evenly distributed on the chromosomes.

To validate the application of molecular IDs in the purity identification of inbred lines, we tested F1 hybrids using 36 core primers. The 72-number molecular ID was consistent with

our prediction, indicating that the SSR markers are codominant and stable. The molecular ID of a hybrid can be deduced from the molecular ID of two inbred lines. A database could be established on the basis of the molecular ID of a hybrid in Chinese cabbage. The data and query information about the F1 hybrids or other varieties can be retrieved from this database. Furthermore, the molecular ID can be used to protect the variety rights of inbred lines and hybrid combinations.

In purity identification, we selected the core primers to identify the two F1 hybrids of the inbred lines of Chinese cabbage, and compared them with the results of field investigation; the results showed a good agreement. Thus, purity identification using one to two SSR sites from the 36 SSR sites was found to be accurate and feasible when we obtained the parents' molecular IDs. Hence, establishing the molecular ID of hybrids and inbred lines is considerably valuable and presents broad application prospects.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Foundation for Key Technologies R&D Program (#2014BAD01B0802) of the Ministry of Science and Technology of the People's Republic of China.

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