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RESEARCH ARTICLE

Microsatellite-based analysis of genetic diversity in 91 commercial *Brassica oleracea* L. cultivars belonging to six varietal groups

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Abstract *Brassica oleracea* L. includes various types of important vegetables that show extremely diverse phenotypes. To elucidate the genetic diversity and relationships among commercial cultivars derived by different companies throughout the world, we characterized the diversity and genetic structure of 91 commercial *B. oleracea* cultivars belonging to six varietal groups, including cabbage, broccoli, cauliflower, kohlrabi, kale and kai-lan. We used 69 polymorphic microsatellite markers showing a total of 359 alleles with an average number of 5.20 alleles per locus. Polymorphism information content (PIC) values ranged from 0.06 to 0.73, with an average of 0.40. Among the six varietal groups, kohlrabi cultivars exhibited the highest heterozygosity level, whereas kale cultivars showed the lowest. Based on genetic similarity values, an UPGMA clustering dendrogram and a two-dimensional scale diagram (PCoA) were generated to analyze genetic diversity. The cultivars were clearly separated into six different clusters with a tendency to cluster into varietal groups. Model-based structure analysis revealed six genetic groups, in which cabbage cultivars were divided into two subgroups that were differentiated by their head shape, whereas cauliflower and kai-lan cultivars clustered together into a single group. Furthermore, we identified 18 SSR markers showing 27 unique alleles specific to only one cultivar that can be used to discriminate 22 cultivars from the others. Our phylogenetic and population structure analysis presents new insights into the genetic structure and relationships among 91 *B. oleracea* cultivars and provides valuable information for breeding of *B. oleracea* species. In addition, we demonstrate the utility of SSR markers as

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a powerful tool for discriminating between the cultivars. The SSR markers described herein will also be helpful for Distinctness, Uniformity and Stability (DUS) test of new cultivars.

Keywords *Brassica oleracea* L. · Genetic diversity · Heterozygosity · Microsatellite markers · Population structure

Introduction

Brassica oleracea L. (CC, 2n = 18) is a member of the *Brassicaceae* family with a wide center of origin in the Mediterranean Basin. The primitive ancestors of modern *B. oleracea* were cultivated and selected for several millennia (Quiros and Farnham 2011), resulting in diverse phenotypes in several vegetable crops that serve as important sources of dietary fiber, vitamin C and anticancer compounds (Fahey and Talalay 1995).

Brassica oleracea includes many subspecies, which show remarkable morphological diversity with regard to inflorescences, leaves, stems, roots, and terminal or apical buds (Paterson et al. 2001). These diverse cultivated forms consist of 14 taxonomic groups or varieties that are classified based on their crop type, including cabbage (*B. oleracea* L. var. *capitata* L.), savoy cabbage (*B. oleracea* L. var. *sabauda* L.), cauliflower (*B. oleracea* L. var. *botrytis* L.), broccoli (*B. oleracea* L. var. *italica* Plenck), Brussels sprout (*B. oleracea* L. var. *gemmifera* DC.), kale (*B. oleracea* L. var. *acephala* DC.), thousand headed kale (*B. oleracea* L. var. *ramosa* DC.), scotch kale (*B. oleracea* L. convar. *acephala* (DC.) Alef. var. *sabellica* L.), marrow stem kale (*B. oleracea* L. convar. *acephala* (DC.) Alef. var. *medullosa* L.), palm kale (*B. oleracea* L. convar. *acephala* (DC.) Alef. var. *palmifolia* L.), collard (*B. oleracea* L. var. *viridis* L.), kohlrabi (*B. oleracea* L. var. *gongylodes* L.), Portuguese Tronchuda cabbage (*B. oleracea* L. var. *costata* DC.) and kai-lan (*B. oleracea* L. var. *alboglabra* (L. H. Bailey) Musil) (Diederichsen 2001). Common cabbage, cauliflower, and broccoli are the most commonly grown vegetables in this species (Quiros and Farnham 2011). The extreme morphological divergence among cultivated *B. oleracea* subspecies has resulted from selection for different characteristics during domestication (Purugganan et al. 2000). Moreover, this morphological diversity in *Brassica* species may be linked to genomic changes associated with polyploidization and following diploidization (Kianian and Quiros 1992; Lukens et al. 2004).

Genetic diversity studies can provide potential genetic resources by elucidating genetic information and relationships between different populations for crop improvement and facilitating the identification of diverse parents to cross in hybrid combinations in order to maximize the expression of heterosis (Nienhuis and Sills 1992; Smith et al. 1990). Cost-effective and reliable method to identify cultivars is desirable in order to differentiate the increasing numbers of new cultivars and eliminate duplicates from germplasm collections (Louarn et al. 2007). An effective method for cultivar identification such as fingerprinting is essential for distinctness, uniformity and stability (DUS) testing of new cultivars and for protection of intellectual property of new cultivars (Lu et al. 2009).

Crop germplasm diversity can be exploited by numerous techniques such as analyses of morphological traits, total seed protein, isozymes, cytological and biochemical characteristics and various types of molecular markers. Of those techniques, molecular markers can serve as powerful and reliable tools for discerning variations and for studying genetic diversity and evolutionary relationships (Gepts 1993). Furthermore, molecular markers are not affected by physiology or the environment; they have been widely used in cultivar identification and seed purity testing (Lu et al. 2009).

Recently, genetic diversity and relationships among and within *Brassica* species have been examined using various molecular markers, such as random amplified polymorphic DNA (RAPD) (Chuang et al. 2004; Shengwu et al. 2003), restriction fragment length polymorphism (RFLP) (Santos et al. 1994; Song et al. 1988; Song et al. 1990), sequence-related amplified polymorphism (SRAP) (Riaz et al. 2001), amplification fragment length polymorphism (AFLP) (van Hintum et al. 2007), inter-simple sequence repeats (ISSRs) (Lu et al. 2009) and simple sequence repeats (SSRs) (Hasan et al. 2005; Louarn et al. 2007; Tonguc and Griffiths 2004). In comparison with other molecular markers, microsatellite markers, also called simple sequence repeats (SSRs), are the most informative molecular markers due to their reliability and abundant multi-allelic forms (Formisano et al. 2012; Powel et al. 1996). They are well distributed throughout the genomes of most eukaryotic species and are

known to be highly variable. Therefore, information from SSR analysis has been widely used to detect polymorphism of nuclear genomes among species (Jarne and Lagoda 1996; Moxon and Wills 1999).

Previously, phylogenetic analysis of 18 *B. oleracea* cultivars as representatives of 13 varietal groups was performed using RFLP markers, and they were classified into three groups. Group one consisted of thousand headed kale and kai-lan, and the second group contained cabbage, collard, kohlrabi and Portuguese Tronchuda cabbage, whereas group three was composed of broccoli, marrow stem kale, palm kale and Brussels sprout (Song et al. 1988). Another study of nine cultivated and 13 wild type *B. oleracea* using RFLP markers showed that cabbage, Portuguese Tronchuda cabbage and kai-lan were closely related, while broccoli and cauliflower were clustered together. Kohlrabi and collard were also found in the same cluster, whereas thousand headed kale seemed to be a distinct varietal type (Song et al. 1990).

Seed companies have contributed to the rising number of F_1 hybrid cultivars of *Brassica* species. The use of F_1 hybrid cultivars is preferred due to hybrid vigor, uniformity, disease resistance, stress tolerance and good horticultural traits including earliness and long shelf-life. Genetic diversity based on microsatellite markers for 54 *B. oleracea* F_1 hybrid cultivars belonging to three varietal groups, cabbage, cauliflower and broccoli, from eight seed companies, revealed that cabbage cultivars clustered in two separate groups, while cauliflower and broccoli cultivars clustered less regularly (Tonguc and Griffiths 2004). A more recent analysis identified four major groups using 59 *B. oleracea* F_1 hybrid cultivars belonging to five varietal groups, broccoli, Brussels sprout, cabbage, savoy cabbage and cauliflower, derived from 13 seed suppliers. The first group contained all ten cauliflower cultivars; group two was a cluster of red cabbage cultivars, except one, with one white cabbage cultivar; the third group comprised all six savoy cabbages, six white cabbages, one each Brussels sprout and red cabbage, while group four consisted of all broccoli cultivars, five white cabbages and nine Brussels sprout cultivars (Louarn et al. 2007).

In the present study, we analyzed genetic diversity and phylogenetic relationships and determined the population structure of 91 commercial *B. oleracea* cultivars belonging to six varietal groups: cabbage (*B. oleracea* var. *capitata*), broccoli (*B. oleracea* var. *italica* Plenck), cauliflower (*B. oleracea* var. *botrytis*), kohlrabi (*B. oleracea* var. *gongylodes*), kale (*B. oleracea* var. *acephala*) and kai-lan (*B. oleracea* var. *alboglabra*), derived from 24 seed companies worldwide. We identified 69 valuable cross-subspecies transferrable SSR markers by screening 148 SSR markers. These markers will be valuable for genetic study, DUS testing and seed purity testing of the increasing numbers of commercial F_1 hybrids and further selection of parental lines in breeding programs.

Materials and methods

Plant materials and DNA extraction

Ninety-one commercial *B. oleracea* cultivars including 49 cabbage (*B. oleracea* var. *capitata*), 22 broccoli (*B. oleracea* var. *italica* Plenck), five cauliflower (*B. oleracea* var. *botrytis*), nine kohlrabi (*B. oleracea* var. *gongylodes*), three kale (*B. oleracea* var. *acephala*) and three kai-lan (*B. oleracea* var. *alboglabra*) cultivars (Table 1) were used for analysis of genetic diversity and phylogenetic relationships using SSR markers. Eighty-five out of 91 cultivars were F_1 hybrids, whereas six cultivars were inbred lines. All materials used in this study were purchased from or kindly provided by seed companies.

Total genomic DNA was extracted from homogenized young leaf tissue, which derived from one individual plant of each cultivar, according to the modified cetyltrimethylammonium bromide (CTAB) method (Allen et al. 2006). The quality and quantity of the extracted DNA were estimated with a NanoDrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). The final concentration of each DNA sample was adjusted to 10 ng/μl for PCR analysis.

SSR analysis

A total of 148 SSR markers were tested to detect polymorphism among 91 *B. oleracea* cultivars. Of those, 104 primer pairs were derived from previous studies: 61 from the public database (Lowe et al. 2004; Piquemal et al. 2005) (see http://ukcrop.net/perl/ace/search/BrassicaDB), three from Louarn et al. (2007), six prefixed "PBCGSSR" from Burgess et al. (2006), four prefixed "BRMS" from Suwabe et al. (2002),

Table 1 Characteristics of the 91 *B. oleracea* L. cultivars used in this study and their proportion of heterozygosity

No	Cultivar #	Varietal group	Cultivar name	Origin	Country	Proportion of heterozygosity (%)	Main phenotypic characteristics[z, y]
1	C 1	Cabbage	8398	IVF, CAAS	China	27.54	EM, RH, LBt, CrT
2	C 2	Cabbage	Zhong gan 21	IVF, CAAS	China	30.43	EM, RH, LBt, CrT
3	C 22	Cabbage	Golden Acre	India	India	30.43	EM, RH, LBt, CrT
4	C 30	Cabbage	Xi wang	Sakata	Japan	28.99	EM, RH, LBt, CrT
5	C 31	Cabbage	Zennith	Seminis	Korea	24.64	EM, RH, LBt, CrT
6	C 33	Cabbage	Green Express	Sakata	Japan	33.33	EM, RH, LBt, CrT
7	C 37	Cabbage	Head Start	Seminis	U.S.A.	28.99	EM, RH, LBt, CrT
8	C 51	Cabbage	Charmant	Sakata	Japan	40.58	EM, RH, LBt, CrT
9	C 53	Cabbage	Kranti	Mahyco	India	39.13	EM, RH, LBt, CrT
10	C 54	Cabbage	GC 60	Golden Seed	India	40.58	EM, RH, LBt, CrT
11	C 70	Cabbage	Goody ball-65	Golden Seed	India	34.78	EM, RH, LBt, CrT, CT
12	C 87	Cabbage	Rinda	Seminis	Netherlands	47.83	EM, RH, LBt, CrT
13	C 102	Cabbage	Green Challenger	Seminis	Korea	46.38	EM, RH, EBt, HT
14	C 111	Cabbage	Saint	Seminis	Korea	40.58	EM, RH, LBt, HT
15	C 157	Cabbage	Blue Vantage	Sakata	Japan	37.68	MM, RH, LBt, HT, CT
16	C 158	Cabbage	Vantage Point	Sakata	Japan	27.54	MM, RH, LBt, HT, CT
17	C 159	Cabbage	Royal Vantage	Sakata	Japan	40.58	MM, RH, LBt, HT, CT
18	C 160	Cabbage	Rareball	Kaneko	Japan	43.48	EM, RH, MBt, HT
19	C 162	Cabbage	Lucky ball	Kaneko	Japan	44.93	EM, RH, MBt, HT
20	C 163	Cabbage	Wonder ball	Seminis	Korea	30.43	EM, RH, MBt, HT, DR
21	C 171	Cabbage	Gloria F1	Ohlsens Enke	Denmark	37.68	EM, RH, MBt, HT, DR
22	C 172	Cabbage	Pruktor F1	Ohlsens Enke	Denmark	37.68	EM, FH, MBt, HT, DR
23	C 174	Cabbage	KY-Cross	Takii	Japan	31.88	EM, FH, EBt, HT
24	C 176	Cabbage	Grand KK	Takii	Japan	37.68	EM, FH, EBt, HT, DR
25	C 177	Cabbage	Tropic Sun Plus	Seminis	Korea	40.58	EM, FH, EBt, HT, DR
26	C 181	Cabbage	Hayadori	Kobayashi	Japan	36.23	EM, FH, EBt, HT
27	C 185	Cabbage	New Star Cross	Tokida	Japan	37.68	EM, FH, EBt, HT
28	C 202	Cabbage	Grand 11	Chia Tai	Thailand	43.48	EM, FH, MBt, HT, DR
29	C 209	Cabbage	Green Nova	Takii	Japan	42.03	MM, FH, MBt, HT, DR
30	C 217	Cabbage	Ogane	Takii	Japan	40.58	MM, FH, MBt, HT, DR
31	C 220	Cabbage	Han Chun No. 4	Jing Tian Seed	Japan	36.23	MM, FH, LBt, CT
32	C 221	Cabbage	Han Kwang	Asahi	Japan	37.68	MM, FH, LBt, CT
33	C 222	Cabbage	Green Coronet	Takii	Japan	40.58	MM, FH, LBt, CT

Table 1 continued

No	Cultivar #	Varietal group	Cultivar name	Origin	Country	Proportion of heterozygosity (%)	Main phenotypic characteristics[z, y]
34	C 223	Cabbage	Super Coronet	Takii	Japan	42.03	MM, FH, LBt, CT
35	C 226	Cabbage	Han Chun No. 5	Jing Tian Seed	Japan	42.03	MM, FH, LBt, CT
36	C 244	Cabbage	YR Hogeol	Takii	Japan	42.03	MM, FH, LBt, CT
37	C 253	Cabbage	Primero	Bejo	Netherlands	18.84	RCb, EM, MBt, CT
38	C 254	Cabbage	Red Sun	Seminis	Korea	33.33	RCb, MM, MBt, CT
39	C 257	Cabbage	Kai Bi	Beijing Tang Yuan Seed	China	31.88	RCb, EM, MBt, CT
40	C 258	Cabbage	Danish Ballhead	OSC Seed	Canada	30.43	LM, RH, LBt, FHA, CT
41	C 259	Cabbage	Tekila	Syngenta	Switzerland	40.58	LM, RH, LBt, FHA, CT
42	C 260	Cabbage	Quisor	Syngenta	Switzerland	44.93	LM, RH, LBt, FHA, CT
43	C 261	Cabbage	Jewelry 068	Jewelry	China-imported from Europe	47.83	LM, RH, LBt, FHA, CT
44	C 268	Cabbage	Beltis	Seminis	Netherlands	39.13	LM, RH, LBt, FHA, CT
45	C 273	Cabbage	Quartz	Seminis	Korea	44.93	LM, RH, LBt, FHA, CT
46	C 277	Cabbage	Megaton	Bejo	Netherlands	49.28	LM, RH, LBt, FHA, CT
47	C 278	Cabbage	Jewelry 1698	Jewelry	China-imported from Europe	37.68	LM, RH, LBt, FHA, CT
48	C 279	Cabbage	Tobia	Seminis	Netherlands	46.38	LM, RH, LBt, FHA, CT
49	C 295	Cabbage	Atria	Seminis	Netherlands	39.13	LM, RH, LBt, FHA, CT
50	B 2008	Broccoli	Yuan you qing hua cai	Tokita	Japan	30.43	EM, DH, MB, HT
51	B 2013	Broccoli	Yu huang	Hongkong Seed	Japan	37.68	MM, DH, MB, CT
52	B 2014	Broccoli	Youshou	Sakata	Japan	36.23	EM, DH, FB, HT
53	B 2056	Broccoli	Heart Land	Sakata	Japan	39.13	MM, DH, AF, MB, CT
54	B 2060	Broccoli	Subaru	Brolead	Japan	33.33	EM, DH, FB, HT
55	B 2061	Broccoli	Fighter	Brolead	Japan	28.99	EM, DH, AF, FB, HT
56	B 2065	Broccoli	KB-052	Mikado-Kyowa	Japan	34.78	EM, FH, BB, HT
57	B 2070	Broccoli	Green Majic	Sakata	Japan	28.99	EM, DH, FB, HT
58	B 2071	Broccoli	Tradition	Seminis	U.S.A.	31.88	EM, DH, FB, HT
59	B 2073	Broccoli	Montop	Syngenta	Switzerland	30.43	EM, DH, FB, HT
60	B 2085	Broccoli	Green Belt	Sakata	Japan	26.09	MM, DH, MB, CT
61	B 2097	Broccoli	Grace	Bejo	Netherlands	37.68	MM, DH, MB, CT
62	B 2098	Broccoli	Super Grace	Bejo	Netherlands	40.58	MM, DH, MB, CT
63	B 2134	Broccoli	Castle	Takii	Japan	30.43	EM, FH, BB, HT
64	B 2135	Broccoli	Anfree-747	Takii	Japan	28.99	EM, FH, AF, BB, HT
65	B 2138	Broccoli	Marathon	Sakata	Japan	30.43	LM, HDH, FB, CT

Table 1 continued

No	Cultivar #	Varietal group	Cultivar name	Origin	Country	Proportion of heterozygosity (%)	Main phenotypic characteristics[z, y]
66	B 2139	Broccoli	BI-15(Monaco)	Syngenta	Switzerland	26.09	LM, HDH, FB, CT
67	B 2140	Broccoli	Heritage	Seminis	U.S.A.	27.54	LM, HDH, FB, CT
68	B 2145	Broccoli	Ironman	Seminis	Netherlands	31.88	LM, HDH, FB, CT
69	B 2193	Broccoli	Aosima	Sakata	Japan	40.58	LM, HDH, AF, FB, CT
70	B 2198	Broccoli	Green Dome	Takii	Japan	28.99	LM, DH, AF, FB, CT
71	B 2205	Broccoli	Endevour	Takii	Japan	27.54	LM, DH, AF, FB, CT
72	B 2266	Cauliflower	Snow Dream	Takii	Japan	20.29	MM, WC, HDH, GCv, CT
73	B 2267	Cauliflower	White Dream	Takii	Japan	26.09	MM, WC, HDH, GCv, CT
74	B 2268	Cauliflower	Snow March	Takii	Japan	26.09	MM, WC, HDH, GCv, CT
75	B 2270	Cauliflower	Violet Dream	Takii	Japan	10.14	EM, VC, EBt
76	B 2271	Cauliflower	Orange Dream	Takii	Japan	30.43	MM, OC, HDH
77	K 3001	Kohlrabi	Korist	Bejo	Netherlands	40.58	EM, RH, MSC, HT, DR
78	K 3008	Kohlrabi	Express Forcer	Takii	Japan	40.58	EM, FH, PGC, HT
79	K 3038	Kohlrabi	White Rookie	Numhems Korea	Korea	47.83	EM, FH, GC, HT
80	K 3039	Kohlrabi	Winner	Takii	Japan	50.72	MM, FH, PGC, HT
81	K 3044	Kohlrabi	UFO	Seminis	Korea	46.38	EM, FH, GC, HT
82	K 3048	Kohlrabi	Worldcol	Joeun Seed	Korea	52.17	EM, FH, GC, HT, DR, FHA
83	K 3065	Kohlrabi	Kolibri	Bejo	Netherlands	44.93	EM, FH, RC, HT
84	K 3066	Kohlrabi	Purple King	Joeun Seed	Korea	26.09	EM, FH, RC, HT, LB
85	K 3083	Kohlrabi	Dongchuan	Konmyeong Noksaeng Chaejo Seed	China	15.94	LM, FH, GC, HB, EBt
86	K 3598	Kale	Este	Sakata	Japan	8.70	Vg, BGC
87	K 3600	Kale	Kale	Joeun Seed	Korea	8.70	Vg, LBt, GC, HT
88	K 3601	Kale	Joeun kale	Joeun Seed	Korea	7.25	Vg, dGC, HT, DR
89	K 3603	Kai-lan	Chi Huajianye	Guangzhou seed company	China	11.59	EBt, HT
90	K 3608	Kai-lan	Khanabai	Chia Tai	Thailand	15.94	EBt, HT
91	K 3611	Kai-lan	Si Ji Da You	China local	China	11.59	EBt, HT

[z] *EM* early maturity, *MM* medium maturity, *LM* late maturity, *RH* round head shape, *FH* flat head shape, *DH* domed head shape, *HDH* high domed head shape, *RCb* red cabbage, *EBt* early bolting type, *MBt* medium bolting type, *LBt* late bolting type, *CrT* cracking tolerance, *CT* cold tolerance, *HT* heat tolerance, *DR* disease resistance, *FHA* very long field holding ability, *MB* medium bead size, *FB* fine bead size, *BB* big bead size, *AF* anthocyanin-free, *WC* white curd color, *VC* violet curd color, *OC* orange curd color, *GCv* good coverage, *MSC* milky skin color, *PGC* pale green color, *GC* green color, *RC* red color, *LB* less fiber, *HB* high fiber, *Vg* vigorous, *BGC* bluish green leaf color, *dGC* deep green color

[y] The phenotypic characteristics are based on description of each cultivars from the seed company and observation of plants growing in research farm of Joeun Seed Company

Table 2 Description of polymorphic EST-SSR markers developed in this study and their functional annotation by TBLASTX

Marker name	SSR motif	Forward primer	Reverse primer	Product size (bp)	Best hits	Arabidopsis Gene ID	E-value
BoESSR003	(GA)8	TGTTGTCGGAGACAGAGACG	TCTCGGAGAGAAGCAACCTC	160–180	Cellulose synthase A catalytic subunit 5 (UDP-forming)	830847	0
BoESSR012	(TTC)7	CTTCCTCTTCGCCTTCTTGA	TTGGGTAGAAACATGCCACA	382–390	Hypothetical protein	834043	7E−62
BoESSR020	(TTTC)5	TCTCCGGTGGGTATTGTCTC	TCGTTGGATGTTCCGTATGA	170–190	ACT domain-containing protein 3	844035	2E−144
BoESSR029	(GGA)6	ATTCGATCTCTCGCGTCACT	GACATGCTTGATCAGGTTCG	150–155	Hypothetical protein	842439	7E−46
BoESSR030	(CAG)10	GTGTGAATGGTGGACAGTCG	TGCTGAGATTGACTCCGTTG	230–290	Protein TIME FOR COFFEE	821807	3E−95
BoESSR031	(CAT)6	GGGATTATCACCGGAGGTTT	AGTTGCATCTCCACCTGCTT	290–295	Hypothetical protein	827540	8E−32
BoESSR037	(CAT)13	GAACAGGAAAAGGACCACCA	TCCTCAGATGAAGGGTCCAG	330–350	Heavy metal transport/ detoxification domain-containing protein	832028	1E−09
BoESSR040	(CAT)7	TCTTCTTCCACGTTCCCTTC	TGAGGTTTTTGCTTGGGAAC	250–280	Hypothetical protein	820719	1E−88
BoESSR049	(ATG)8	TGGAGGTTGATGAGGTAGCC	CATCTTCATTCCTAGCGCAGA	290–300	Transducin/WD40 domain-containing protein	827625	3E−34
BoESSR073	(TGG)7	GGACTGCCAAAAGACTGAGC	ACTCGCACAGGAACCAAAAT	220–260	Winged-helix DNA-binding transcription factor family protein	829743	1E−22
BoESSR074	(GGAGAA)4	CGGATAAAGGGCACATGAGT	TTTTGAATCTCAGCGACCAA	214–220	Hypothetical protein	841250	2E−26
BoESSR077	(GAA)6	GCTGACGAAGGAGATCAAGG	TTCTCCCTCTCCGACTTCAA	270–300	BCL-2-associated athanogene 7	836360	1E−144
BoESSR106	(T)12//(ATC)5	TTCGTTCGGGCTTGTTAGTC	GACAGTAGAGCCAATCCTCAA	200–230	Serine/threonine-protein phosphatase BSL1	828097	2E−93
BoESSR110	(CTT)6	TTGGCTTCTTCTTCCTCTGC	TAGGACGTCTGTCTGGCTCA	280–550	Putative nucleolar protein 5–2	819668	9E−18

11 prefixed "BnGMS" from Cheng et al. (2009), two prefixed "nga" from Bell and Ecker (1994), two prefixed "CNU" from Choi et al. (2007), one (CALSSR) from Smith and King (2000), and 14 prefixed "sN", "sR", "sO" or "sA" from Agriculture and Agri-Food Canada (http://brassica.agr.gc.ca/index_e.shtml). Those previously reported markers were selected randomly from nine linkage groups of *B. oleracea* maps (Supplementary Table 1). The remaining 44 primer pairs were developed in this study based on EST sequences. Of which, the ESTs containing polymorphic SSR primers were blasted against *Arabidopsis thaliana* (L.) Heynh. database using the TBLASTX algorithm (http://www.ncbi.nlm.nih.gov/Blast). The best hits of ESTs were assigned at expected value $<10^{-6}$ (Table 2).

PCR reactions were carried out in a total volume of 10 µl containing 10 ng DNA template, 1× PCR reaction buffer (Inclone Biotech), 0.2 mM each dNTP (Inclone Biotech), 0.2 µM each primer and 1 unit *Taq* DNA polymerase (Inclone Biotech). Amplifications were performed under the following conditions: initial denaturation at 94 °C for 4 min, and then 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55–60 °C, 30 s extension at 72 °C, and 10 min at 72 °C for final extension. PCR-amplified products were separated by 6 % non-denaturing polyacrylamide gel electrophoresis using 1× TBE buffer. The gels were stained with ethidium bromide for 20 min and DNA bands were visualized under UV light using the gel documentation system.

Data analysis

The polymorphic bands of each SSR marker were scored as binary characters for their presence (1) or absence (0) in the 91 cultivars and the resulting data were analyzed using NTSYS-PC version 2.1 (Rohlf 2000). Genetic similarity between cultivars was calculated based on the simple matching coefficient using the SIMQUAL subprogram of NTSYS-PC. Cluster analysis was performed using the unweighted pair group arithmetic mean method (UPGMA) in the SAHN subprogram of NTSYS-PC. Principal coordinate analysis (PCoA) based on the genetic similarity matrix was performed using DCENTER and EIGEN algorithm of the NTSYS-PC software package.

The number of alleles (N_A), rare alleles (R_A), major allele frequency (M_{AF}), gene diversity (GD), expected heterozygosity (H_e) and polymorphic information content (PIC) values were calculated using PowerMarker version 3.25 (Liu and Muse 2005). Rare allele refers to alleles with frequencies of less than 5 % among the 91 cultivars and major allele frequency (M_{AF}) was defined as the allele with the highest frequency.

Population structure analysis was performed with STRUCTURE version 2.3 using genotype data consisting of unlinked markers (Pritchard et al. 2000). Individuals in the sample were assigned to populations (genetic groups), or jointly to two or more populations if their genotypes indicated that they were admixed. The loci within populations are assumed to be at Hardy–Weinberg equilibrium and linkage equilibrium. The optimum number of populations (K) was selected by testing $K = 1$ to $K = 8$ using five independent runs of 10,000 burn-in period length at fixed iterations of 10,000 with a model allowing for admixture and correlated allele frequencies (Falush et al. 2003). In order to determine the best K, the log likelihood of each K, Ln P(D) or L(K) was calculated, of which the average of Ln P(D) slightly increased up to $K = 6$ and began to plateau at $K = 7$ and $K = 8$ (Supplementary Fig. 1). Therefore we could not get the obvious indication of which K value presented the best fit for the data and the groupings was examined based on six varietal groups of *B. oleracea*. Thus $K = 6$ was used to determine inferred ancestries of the 91 *B. oleracea* commercial cultivars.

Results

SSR markers and allele diversity

Out of 148 SSR markers, 78 markers generated reproducible, clear, distinct and polymorphic amplification products at one or more loci. Meanwhile, the other 70 were not valuable: 38 showed no polymorphism and the remaining 32 produced unclear bands. Of the 78 reproducible and polymorphic markers, nine were excluded from further analysis because they showed a large proportion of missing data among accessions (>5 %). Hence, a total of 69 polymorphic markers were used for the statistical analysis using PowerMarker (Table 3).

The polymorphic loci showed unique fingerprints providing a total of 359 alleles for all 91 cultivars. The number of alleles per locus ranged from two to 14, with

Table 3 Characteristics of the 69 polymorphic SSR loci across 91 *B. oleracea* L. cultivars

Locus	Number of alleles	Number of rare alleles[a]	Size range (bp)	Frequency of major alleles[b] (%)	Gene diversity	Observed heterozygosity (H_e)	PIC[c]
BoESSR003	5	4	160–180	40	0.26	0.11	0.24
BoESSR012	2	–	382–390	38	0.21	0.23	0.19
PBCGSSRBo2	6	3	180–205	15	0.67	0.18	0.59
BoREM1b	4	2	170–210	38	0.23	0.24	0.22
BoKAH45TR	6	3	170–200	15	0.58	0.36	0.49
BoESSR020	3	1	170–190	34	0.27	0.32	0.24
BoESSR029	3	–	150–155	20	0.49	0.31	0.37
BoESSR031	3	–	290–295	21	0.47	0.62	0.42
BoESSR030	4	–	230–290	19	0.53	0.52	0.46
sR12387	8	5	280–300	18	0.59	0.51	0.54
BoDCTD1	11	7	150–180	22	0.60	0.39	0.56
sN11670	4	2	150–200	28	0.40	0.39	0.33
PBCGSSRBo33	3	–	120–150	23	0.46	0.39	0.35
PBCGSSRBo22	6	3	260–270	30	0.39	0.33	0.36
BoESSR040	4	2	250–280	33	0.31	0.30	0.27
BoESSR037	4	2	330–350	40	0.24	0.15	0.22
BoESSR049	5	3	290–300	40	0.25	0.12	0.23
sR5795	3	2	200–230	46	0.10	0.07	0.10
CB10064	13	10	140–180	16	0.68	0.59	0.65
PBCGSSRBo34	6	2	195–230	22	0.60	0.25	0.53
sR12384	2	–	280–310	39	0.19	0.21	0.17
BoESSR073	7	5	220–260	19	0.56	0.49	0.49
BoESSR074	3	–	214–220	20	0.50	0.41	0.37
BnGMS51	3	1	230–270	36	0.31	0.20	0.26
BoESSR077	5	2	270–300	26	0.49	0.19	0.39
BRMS-006	2	1	150–155	47	0.06	0.07	0.06
BRMS-034	3	–	140–160	21	0.50	0.19	0.37
CB10267	3	1	120–150	27	0.40	0.54	0.32
CB10005	4	3	250–270	44	0.14	0.08	0.13
CB10172	2	–	210–230	34	0.26	0.31	0.23
BRAS039	4	2	200–240	35	0.31	0.22	0.27
CB10632	3	–	170–180	32	0.38	0.20	0.31
CB10130	2	–	240–295	40	0.18	0.20	0.16
BRAS112	6	3	240–280	34	0.48	0.19	0.43
Na10D11	5	2	170–205	19	0.64	0.23	0.56
Ol10-D02	11	8	140–210	22	0.62	0.56	0.54
Na10F06	5	2	100–150	20	0.51	0.22	0.39
MR133.1	3	1	240–250	37	0.36	0.03	0.30
CB10427	5	1	150–180	15	0.57	0.40	0.48
CB10288	5	2	200–220	31	0.48	0.18	0.42
Ol10-F08	4	2	160–200	38	0.29	0.13	0.26
MR049	9	6	170–290	20	0.64	0.22	0.59
Ol13G05	4	2	130–160	32	0.51	0.30	0.45

Table 3 continued

Locus	Number of alleles	Number of rare alleles[a]	Size range (bp)	Frequency of major alleles[b] (%)	Gene diversity	Observed heterozygosity (H_e)	PIC[c]
CB10109	2	–	250–290	34	0.27	0.32	0.23
Ol11H09	10	8	150–230	23	0.59	0.19	0.51
sORF73	14	10	130–200	12	0.73	0.54	0.69
BoESSR106	4	3	200–230	41	0.21	0.14	0.20
sNRH63	8	5	90–160	24	0.54	0.34	0.49
Na10-D07	2	1	150–200	47	0.13	0.00	0.12
CB10629	4	2	100–150	23	0.46	0.46	0.37
CB10258	7	3	180–200	24	0.61	0.30	0.56
CB10028	14	13	120–190	32	0.48	0.24	0.47
CB10014	5	1	200–220	25	0.56	0.24	0.50
nga111	9	6	120–160	21	0.64	0.55	0.59
CB10611	8	6	160–180	35	0.42	0.11	0.38
Na12-B11	4	–	150–160	23	0.55	0.24	0.45
BoESSR110	2	1	280–550	47	0.50	0.94	0.37
BnGMS539	4	–	180–200	32	0.60	0.76	0.53
BnGMS326	4	1	270–290	24	0.61	0.74	0.53
Na10-H03	3	1	100–120	32	0.32	0.32	0.27
CB10229	4	2	270–295	38	0.61	0.97	0.54
CNU400	4	1	260–290	21	0.74	0.84	0.70
Ol10-C05	7	2	100–160	18	0.70	0.59	0.66
CALSSR	10	5	140–200	18	0.77	0.93	0.73
CB10435	8	6	140–170	25	0.51	0.36	0.45
BnGMS160	8	3	280–380	20	0.62	0.48	0.58
Na12-A02	2	–	180–190	40	0.31	0.00	0.27
BnGMS83	6	4	200–240	26	0.59	0.13	0.52
MR216	3	1	170–200	35	0.30	0.23	0.25
Mean	5.20	3.33	–	28.75	0.45	0.34	0.40

[a] Rare alleles are defined as alleles with a frequency less than 5 %

[b] Major allele is defined as the allele with the highest frequency

[c] Polymorphic information content

an average of 5.20 alleles across the 69 loci (Table 3). Of those, nine loci, i.e. BoESSR012, sR12384, BRMS-006, CB10172, CB10130, CB10109, Na10-D07, BoESSR110, and Na12-A02, exhibited only two alleles among the 91 cultivars, while two loci, sORF73 and CB10028, showed 14 different alleles. Gene diversity (GD) ranged from 0.06 to 0.77 with an average of 0.45. The PIC values ranged from 0.06 to 0.73 with an average of 0.40. Among the SSRs, CALSSR showed the highest value for both PIC (0.73) and gene diversity (0.77), and BRMS-006 had the lowest gene diversity and PIC value (0.06).

The frequency of the major allele at each locus ranged from 12 % (sORF73) to 47 % (BRMS-006, Na10-D07 and BoESSR110). On average, 28.75 % of the 91 cultivars shared a common major allele at any given locus. The number of rare alleles, which were defined as those alleles with a frequency of less than 5 %, varied from one to 13 alleles per locus. Marker CB10028 exhibited the highest number of rare alleles. Rare alleles were identified at 54 loci, with an average of 3.33 per locus (Table 3). Of the 54 SSRs showing rare alleles, 18 produced 27 unique alleles, each of which was found in only one specific cultivar and was

Table 4 Summary of cultivar-specific allele markers (CAMs)

Marker	No. of alleles	Unique alleles	Varietal type	Representative cultivar
BoESSR073	7	a/c	Cabbage	Tropic Sun Plus
CB10267	3	b/b	Cabbage	Wonder ball
Na10F06	5	a/d	Cabbage	Han Kwang
		b/c	Cabbage	Han Chun No. 5
CB10611	8	a/d	Cabbage	Han Chun No. 5
sNRH63	8	b/f	Cabbage	Jewelry 1698
		a/g	Cabbage	Megaton
CALSSR	10	a/d	Cabbage	Gloria F1
CB10435	8	a/c	Cabbage	Zennith
nga111	9	c/f	Cabbage	Red Sun
		a/c	Broccoli	KB-052
MR049	9	d/e	Broccoli	Fighter
		e/f	Broccoli	Tradition
CB10064	13	c/f	Broccoli	Montop
		a/b	Kale	Este
BnGMS83	6	a/a	Kale	Joeun kale
BoDCTD1	11	e/e	Kale	Joeun kale
BoESSR077	5	a/a	Kale	Este
		d/d	Kai-lan	K3608 Thailand
sORF73	14	f/f	Kai-lan	K3603 China
		b/e	Kohlrabi	White Rookie
BoREM 1b	4	b/b	Kohlrabi	Kolibri
Ol10-D02	11	a/f	Kohlrabi	Kolibri
BRAS039	4	b/b	Kohlrabi	Purple King
BRAS112	6	a/a	Kohlrabi	White Rookie
		a/b	Kohlrabi	UFO
		b/b	Cauliflower	Snow Dream

designated as a cultivar-specific allele marker (CAM) (Table 4). Among these 18 SSR markers, BRAS112 detected CAMs for three different cultivars ('White Rookie', 'UFO' and 'Snow Dream'), seven SSR markers including Na10F06, sNRH63, nga111, MR049, CB10064, BoESSR077 and sORF73 detected two CAMs, and the remaining 10 SSR markers detected one CAM. Ten CAMs were found for cabbage cultivars, 4 CAMs were present in broccoli cultivars, 4 CAMs were in kale cultivars, 6 CAMs were in kohlrabi cultivars, 2 CAMs were in kai-lan cultivars, and 1 CAM was in cauliflower. A total of 22 cultivars including nine cabbage, four each kohlrabi and broccoli, two each kale and kai-lan, and one cauliflower cultivar could be identified by these 18 cultivar-specific allele markers.

Except for two loci (Na10-D07 and Na12-A02), all loci used in this study could identify heterozygous individuals across the 91 *B. oleracea* cultivars. The proportion of heterozygous cultivars (H_e) ranged from 0.03 at MR133.1 to 0.97 at CB10229, with an average of 0.34 (Table 3).

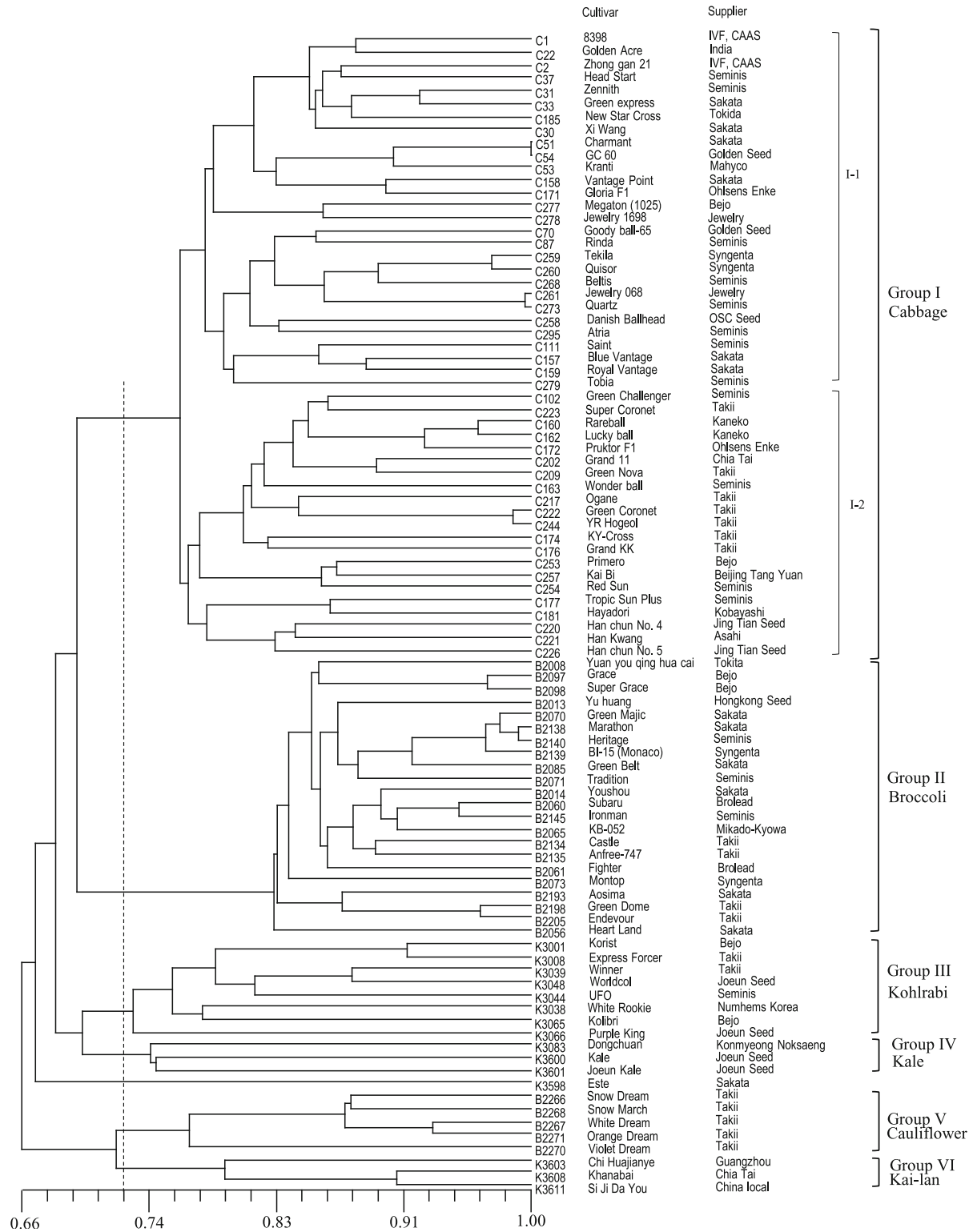
Genetic diversity and phylogenetic relationships among 91 cultivars

Phylogenetic analysis using 69 SSR markers clearly elucidated the relationships among the 91 cultivars and revealed that all cultivars tended to cluster within their own varietal groups (Fig. 1). Using a similarity coefficient of 72 % as the threshold level for UPGMA clustering, all the cultivars were classified into six major groups, which coincided with the six varietal groups except for one kale cultivar 'Este' bred by the Sakata seed company that did not belong to any group and one kohlrabi cultivar 'Dongchuan' bred by the Konmyeong Noksaeng seed company, which grouped with kale cultivars. The first group (group I) was a population of cabbage cultivars that was further divided into two sub-groups. Group II consisted of a set of 22 broccoli cultivars; group III held eight kohlrabi cultivars; group IV contained two kale cultivars along with the kohlrabi cultivar 'Dongchuan'; group V consisted of five cauliflower cultivars, and group VI comprised three kai-lan cultivars. The groupings identified by PCoA were also similar to those identified by the UPGMA cluster analysis (Supplementary Fig. 2).

Overall, 89 (97.8 %) cultivars could be differentiated from each other using 69 microsatellite loci, while the other two cabbage cultivars ('Charmant' and 'GC 60') gave rise to identical results with those loci.

Cabbage (Group I)

Forty-nine cabbage cultivars formed a cluster (group I) that was further sub-divided into two sub-groups at a 77 % similarity coefficient. Sub-group I consisted of 28 cabbage cultivars that were dominated by round head shape with varying maturity, bolting type and head size characteristics. This sub-group also contained several cultivars displaying cracking tolerance, an important characteristic in cabbage that can confer good standing ability in the field. It is interesting to note that cultivars 'Charmant' and 'GC 60' showed identical phenotypic and molecular characteristics even though they came from two different seed


Cultivar
Supplier
C1 8398 IVF, CAAS
C22 Golden Acre India
C2 Zhong gan 21 IVF, CAAS
C37 Head Start Seminis
C31 Zennith Seminis
C33 Green express Sakata
C185 New Star Cross Tokida
C30 Xi Wang Sakata
C51 Charmant Sakata
C54 GC 60 Golden Seed
C53 Kranti Mahyco
C158 Vantage Point Sakata
C171 Gloria F1 Ohlsens Enke
C277 Megaton (1025) Bejo
C278 Jewelry 1698 Jewelry
C70 Goody ball-65 Golden Seed
C87 Rinda Seminis
C259 Tekila Syngenta
C260 Quisor Syngenta
C268 Beltis Seminis
C261 Jewelry 068 Jewelry
C273 Quartz Seminis
C258 Danish Ballhead OSC Seed
C295 Atria Seminis
C111 Saint Seminis
C157 Blue Vantage Sakata
C159 Royal Vantage Sakata
C279 Tobia Seminis
C102 Green Challenger Seminis
C223 Super Coronet Takii
C160 Rareball Kaneko
C162 Lucky ball Kaneko
C172 Pruktor F1 Ohlsens Enke
C202 Grand 11 Chia Tai
C209 Green Nova Takii
C163 Wonder ball Seminis
C217 Ogane Takii
C222 Green Coronet Takii
C244 YR Hogeol Takii
C174 KY-Cross Takii
C176 Grand KK Takii
C253 Primero Bejo
C257 Kai Bi Beijing Tang Yuan
C254 Red Sun Seminis
C177 Tropic Sun Plus Seminis
C181 Hayadori Kobayashi
C220 Han chun No. 4 Jing Tian Seed
C221 Han Kwang Asahi
C226 Han chun No. 5 Jing Tian Seed
B2008 Yuan you qing hua cai Tokita
B2097 Grace Bejo
B2098 Super Grace Bejo
B2013 Yu huang Hongkong Seed
B2070 Green Majic Sakata
B2138 Marathon Sakata
B2140 Heritage Seminis
B2139 BI-15 (Monaco) Syngenta
B2085 Green Belt Sakata
B2071 Tradition Seminis
B2014 Youshou Sakata
B2060 Subaru Brolead
B2145 Ironman Seminis
B2065 KB-052 Mikado-Kyowa
B2134 Castle Takii
B2135 Anfree-747 Takii
B2061 Fighter Brolead
B2073 Montop Syngenta
B2193 Aosima Sakata
B2198 Green Dome Takii
B2205 Endevour Takii
B2056 Heart Land Sakata
K3001 Korist Bejo
K3008 Express Forcer Takii
K3039 Winner Takii
K3048 Worldcol Joeun Seed
K3044 UFO Seminis
K3038 White Rookie Numhems Korea
K3065 Kolibri Bejo
K3066 Purple King Joeun Seed
K3083 Dongchuan Konmyeong Noksaeng
K3600 Kale Joeun Seed
K3601 Joeun Kale Joeun Seed
K3598 Este Sakata
B2266 Snow Dream Takii
B2268 Snow March Takii
B2267 White Dream Takii
B2271 Orange Dream Takii
B2270 Violet Dream Takii
K3603 Chi Huajianye Guangzhou
K3608 Khanabai Chia Tai
K3611 Si Ji Da You China local
I-1
I-2
Group I
Cabbage
Group II
Broccoli
Group III
Kohlrabi
Group IV
Kale
Group V
Cauliflower
Group VI
Kai-lan
0.66
0.74
0.83
0.91
1.00

◀ **Fig. 1** UPGMA cluster dendrogram showing the genetic relationships among 91 commercial *B. oleracea* L. cultivars based on 69 microsatellite loci. Each cultivar is identified by cultivar number, name and seed supplier

companies, in Japan and India, respectively. Similar results were found between cultivars 'Jewelry 068' and 'Quartz', which showed a 99.5 % similarity coefficient, even though they were from different breeding companies, Jewelry (China) and Seminis (Korea), respectively.

Sub-group II of cabbage comprised 21 cultivars, which also displayed various types of maturity, head size and bolting. However, the majority of cultivars in this sub-group (14 cultivars) had a flat head shape, which differentiated them from sub-group I. Three red cabbage cultivars, 'Primero', 'Red Sun' and 'Kai Bi', were closely clustered in this sub-group. Among the other members in this sub-group, two cultivars, 'Green Coronet' and 'YR Hogeol', derived from the same seed company (Takii) showed the highest similarity (98 %). This is likely due to the use of parental lines with similar genetic backgrounds for breeding of the two cultivars.

Broccoli (Group II)

All 22 broccoli cultivars were separated at a genetic similarity of 83 % and obviously placed in group II. The members of this group had various types of head shape, bead size and maturity, and some of these cultivars were also referred to as being anthocyanin-free. A medium-maturity cultivar 'Heart Land' was quite distinct in the clustering compared to other members in this group. Meanwhile, cultivars 'Marathon' and 'Heritage' showed about a 99 % similarity coefficient even though they were from different seed suppliers, Sakata and Seminis, respectively.

Kohlrabi and Kale (Groups III and IV)

Kohlrabi and kale cultivars were the most closely related varietal groups that had diverse genetic backgrounds even though the major cultivars were separated into group III for kohlrabi and group IV for kale. Eight out of nine kohlrabi cultivars clustered together in group III, while the other, 'Dongchuan', was clustered into group IV with the kale cultivars. 'Dongchuan' was the most distinct compared to the other kohlrabi cultivars. Although this cultivar had a flat head shape and green color, other characteristics, such as high fiber, early bolting type and late maturity, were relatively different, consistent with this cultivar having a different genetic background.

The majority of the kohlrabi cultivars in group III had a flattish head shape, green color and early to medium bolting type. Meanwhile, 'Korist' had a milky skin color and the cultivars 'Kolibri' and 'Purple King' had red skin. However, their genetic diversity did not correspond to skin color differences. 'Purple King' was separated from others at about a 72.5 % similarity coefficient, which might be related to its phenotype of low fiber because the other cultivars did not display this characteristic.

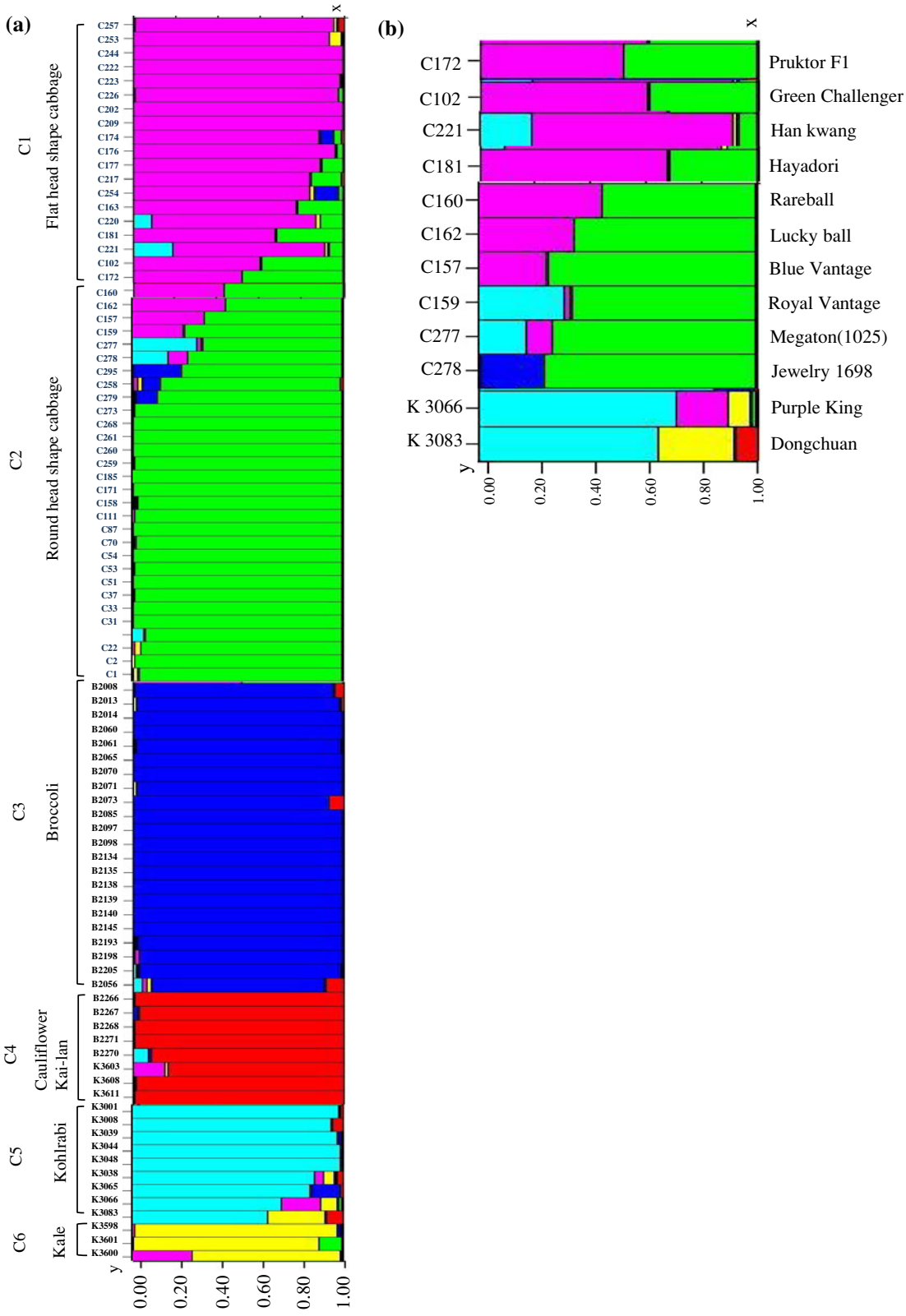
Kale cultivars were more diverse than the other cultivars. In particular, 'Este', which had bluish green leaves, did not belong to any group. Meanwhile, the two other cultivars, 'Kale K 3600' and 'Joeun kale', which had green leaves and heat tolerance, were clustered into the same group with the kohlrabi cultivar 'Dongchuan' (group IV) at a similarity coefficient value of 74 %.

Cauliflower and Kai-lan (Groups V and VI)

Cauliflower and kai-lan were grouped independently as groups V and VI, respectively. However, they showed a close relationship to each other. Five cauliflower cultivars from the Takii seed company showed relatively low diversity. Among them, 'Violet Dream' was separated from others at 77 % genetic similarity. That coincided with the major phenotype differences between the cultivars: 'Violet Dream' exhibited early maturity, early bolting and violet curd color, whereas the other cauliflower cultivars showed medium maturity, high-domed shape and white or orange curd colors. Three kai-lan cultivars, two from China and one from Thailand, showed similar genetic diversity based on molecular genetic analysis.

Population structure analysis

Population structure and inferred ancestry based on analysis using the STRUCTURE program revealed that the 91 cultivars belonged to six genetic groups (C1–C6) ($K = 6$) (Fig. 2a). Two groups, C1 and C2, corresponded to the cabbage subgroups I and II that


(a)
C1
Flat head shape cabbage
C257
C253
C244
C222
C223
C226
C202
C209
C174
C176
C177
C217
C254
C163
C220
C181
C221
C102
C172
C2
Round head shape cabbage
C160
C162
C157
C159
C277
C278
C295
C258
C279
C273
C268
C261
C260
C259
C185
C171
C158
C111
C87
C70
C54
C53
C51
C37
C33
C31
C22
C2
C1
C3
Broccoli
B2008
B2013
B2014
B2060
B2061
B2065
B2070
B2071
B2073
B2085
B2097
B2098
B2134
B2135
B2138
B2139
B2140
B2145
B2193
B2198
B2205
B2056
C4
Cauliflower
Kai-lan
B2266
B2267
B2268
B2271
B2270
K3603
K3608
K3611
C5
Kohlrabi
K3001
K3008
K3039
K3044
K3048
K3038
K3065
K3066
K3083
C6
Kale
K3598
K3601
K3600
y
0.00
0.20
0.40
0.60
0.80
1.00
x
(b)
C172
C102
C221
C181
C160
C162
C157
C159
C277
C278
K 3066
K 3083
Pruktor F1
Green Challenger
Han kwang
Hayadori
Rareball
Lucky ball
Blue Vantage
Royal Vantage
Megaton(1025)
Jewelry 1698
Purple King
Dongchuan
y
0.00
0.20
0.40
0.60
0.80
1.00
x

◀ **Fig. 2** Population structure analyses of the 91 *B. oleracea* L. cultivars. Analysis was carried out using STRUCTURE software with *K* set at 6. **a** Inferred ancestries of the 91 *B. oleracea* cultivars based on six genetic groups. Each group is represented by a different color. 79 cultivars shared over 75 % ancestry with one of the genetic groups. **b** Twelve *B. oleracea* cultivars that showed admixture (sharing less than 75 % ancestry)

were identified in the UPGMA cluster analysis (Fig. 1). The other four groups corresponded to three varietal groups, broccoli (C3), kohlrabi (C5) and kale (C6), and the merging of two varietal groups, cauliflower and kai-lan, into group C4. Each *B. oleracea* varietal group was also examined for membership in the six genetic groups described above. The proportion of membership is the average of inferred ancestry value in each varietal group. Broccoli, cauliflower and kai-lan had a proportion of membership greater than 90 % in the C3 and C4, whereas those of kohlrabi and kale were more than 85 % in the C5 and C6. Cabbage cultivars were divided into two groups with proportions of membership about 37 and 58 % for cabbage C1 and C2, respectively (Table 5).

The C1 group included 19 cabbage cultivars, of which ten shared more than 90 % ancestry and other five had 78–88 % shared ancestry. The remaining four cultivars were admixed. The C2 group was composed of 30 cabbage cultivars, of which 21 showed more than 90 % shared ancestry and three cultivars ranged from 77 to 88 %, while the other six cultivars were of mixed ancestry. The 22 broccoli cultivars clustered in group C3 had more than 90 % shared ancestry, except cultivar 'Heart Land' which had the lowest shared ancestry at 81 %. The C4 group, a cluster of cauliflower and kai-lan cultivars, revealed more than 90 % shared ancestry with the exception of the kai-lan cultivar 'K 3603'. The C5 group included nine kohlrabi cultivars; five of them had more than 90 % shared ancestry and two other cultivars ranged from 84 to 86 %, whereas the remaining two cultivars showed mixed ancestry. The C6 group consisted of three kale cultivars with varying levels of shared ancestry. Although cultivar 'Este' was not designated into any group based on the UPGMA analysis (Fig. 1), its level of shared ancestry was the highest (>95 %) compared to the other two kale cultivars, 'Joeun kale' (>85 %)

Table 5 Proportion of membership for each varietal group in each of the six clusters

Given population	Inferred cluster						# of individuals
	C1	C2	C3	C4	C5	C6	
Cabbage	0.371	0.586	0.014	0.003	0.019	0.007	49
Broccoli	0.004	0.002	0.972	0.013	0.005	0.003	22
Cauliflower	0.002	0.001	0.007	0.972	0.017	0.002	5
Kohlrabi	0.023	0.005	0.019	0.021	0.882	0.05	9
Kale	0.073	0.032	0.005	0.005	0.007	0.878	3
Kai-lan	0.055	0.001	0.002	0.932	0.004	0.007	3

Table 6 Genetic differentiation among six varietal groups of *B. oleracea* L. cultivars

Varietal group	No. of cultivars tested	Mean no. alleles/ locus	Major allele frequency	Mean genetic diversity	Mean heterozygosity	Mean PIC value
Cabbage	49	3.81	0.32	0.39	0.38	0.34
Broccoli	22	2.42	0.37	0.28	0.32	0.25
Cauliflower	5	1.80	0.41	0.26	0.23	0.22
Kohlrabi	9	2.81	0.33	0.41	0.41	0.35
Kale	3	1.77	0.39	0.33	0.08	0.27
Kai-lan	3	1.46	0.43	0.22	0.13	0.18
Total	91	14.07	2.25	1.89	1.55	1.61
Average		2.35	0.38	0.32	0.26	0.27

and 'kale K 3600' (>75 %) (Fig. 2a, Supplementary Table 2).

Genetic diversity among members in each of the six varietal groups

Among the six varietal groups, kohlrabi had the highest genetic diversity (0.41), while kai-lan exhibited the lowest (0.22) (Table 6). The mean number of alleles per locus among each of six varietal groups ranged from 1.46 to 3.81 with an overall mean of 2.35. The cabbage cultivars demonstrated the highest number of alleles (3.81), and kai-lan cultivars had the lowest number of alleles (1.46). The mean of the major allele frequency within varietal groups varied from 0.32 in cabbage to 0.43 in kai-lan, with an overall mean of 0.38. These low values for genetic diversity and number of alleles in kai-lan might be due to the small number of cultivars used in the analysis.

Variation in heterozygosity

Since the majority of the cultivars used in the present study were F_1 hybrid cultivars, we were interested to know their proportion of heterozygosity at the 69 SSR loci (Table 1). The level of heterozygosity among 49 cabbage cultivars ranged from 18.8 to 49.3 %. Of which, the highest level of heterozygosity was detected in cultivar 'Megaton', while the lowest was in cultivar 'Primero'. The cultivars 'Super Grace' and 'Aosima' demonstrated the highest degree of heterozygosity (40.58 %) in broccoli but cultivars 'Green Belt' and 'BI-15 (Monaco)' showed the lowest (26.09 %). Of the five cauliflower cultivars, 'Orange Dream' had the highest level of heterozygosity (30.43 %) and cultivar 'Violet Dream' showed the lowest (10.14 %).

Interestingly, kohlrabi cultivars showed the highest mean heterozygosity (41 %) compared to the other varietal groups. Among nine kohlrabi cultivars, 'Worldcol' had 52.17 % heterozygosity, while cultivar 'Dongchuan' had 15.94 %. In contrast to kohlrabi cultivars, kale cultivars exhibited the lowest mean heterozygosity (8 %) among the six varietal groups. The highest degree of heterozygosity in kale cultivars was 8.70 %, which were represented by cultivars 'Este' and 'K 3600'. Meanwhile, the highest heterozygosity level in kai-lan cultivars was 15.94 % which shown by cultivar 'K 3608' from Thailand.

Discussion

Transferability and diversity of SSR markers

Microsatellite markers are widely known to have high transferability from the focal species in which they were identified to other subspecies or even to other related genera. In *Brassica*, there are reports of transferability of microsatellite markers among species of the genus (Lowe et al. 2004; Marquez-Lema et al. 2010; Plieske and Struss 2001). In this study, 148 microsatellite markers derived from several *Brassica* species and *Arabidopsis thaliana* were used to determine genetic diversity and relationships of *B. oleracea*. Of those markers, 69 (46.62 %) showed perfect transferability to each varietal group examined herein and were appropriate for assessing the genetic diversity of a wide range of *B. oleracea* subspecies. We found that the remaining 79 markers (53.38 %) were not suitable for this purpose because they produced monomorphic or non-specific bands or did not allow successful amplification. Among the 69 reproducible and polymorphic markers, 54 (78.3 %) were derived from the *B. oleracea* genome and 11 (15.9 %), 3 (4.3 %), and 1 (1.4 %) were derived from *B. napus*, *B. rapa*, and *A. thaliana,* respectively.

The number of alleles per SSR locus ranged from 2 to 14, with an average of 5.23, which is significantly higher than those of the previous reports in which 2 to 8 alleles per locus with an average of 4.46 (Tonguc and Griffiths 2004) and 2-9 alleles per locus with an average of 4.27 (Louarn et al. 2007) were found. In addition, the finding of many rare alleles reveals a unique source of genetic diversity within *B. oleracea* varietal groups. On the other hand, we also identified 18 SSR markers producing 27 cultivar-specific allele markers (CAM) that can differentiate 22 cultivars from the others (Table 4). These markers provide an effective means for cultivar identification among the rising number of commercial cultivars and will be useful for cultivar protection and DUS testing.

Although a relatively low PIC value was found in this study (0.40, compared to above 0.5 in previous studies (Louarn et al. 2007; Tonguc and Griffiths 2004)), the diversity of SSR markers here proved to be a reliable tool for cultivar discrimination and identification. We could discriminate all the cultivars except two using the 69 SSR markers, which will also be helpful for DUS testing in relation to the release of

new cultivars (Louarn et al. 2007). Even though our SSR markers had high discrimination power, we could not differentiate two cultivars, 'Charmant' and 'GC 60', from Japan and India, respectively. We presume that they might have been sold with different cultivar names in different countries but originate from the same cultivar. This result is in agreement with previous reports, which showed that several varieties with different names might be genetically identical (Jain et al. 2004). We also found that many cultivars in the same clade originated from different seed suppliers, in agreement with Lu et al. (2009), who found that cultivars with different origins can be clustered together in the same group, and Belaj et al. (2003), who reported that breeding materials were often shared by a variety of institutions or used as common elite lines under different names.

Phylogenetic relationships between varietal groups according to UPGMA and population structure analyses

The genetic similarity-based analysis of the 91 cultivars demonstrated a clear classification into six major groups with a tendency to cluster within varietal groups (Fig. 1), except for one kale cultivar 'Este' and one kohlrabi cultivar 'Dongchuan'. This finding provides more clarity than earlier studies, which could not clearly separate several varietal groups (Louarn et al. 2007; Song et al. 1988; Song et al. 1990; Tonguc and Griffiths 2004). The results regarding phylogenetic relationships are consistent with the expectation that each varietal group would be classified separately within its group, considering that each varietal group remained genetically distinct after selection for several millennia (Quiros and Farnham 2011).

Population structure analysis also showed that the 91 cultivars could be divided into six groups, with strong similarity to those found by UPGMA dendrogram (Fig. 1). The main difference was that the population structure analysis divided cabbage cultivars into two different groups: cabbages with flattish head shape were positioned in group I (C1), whereas round head-shape cabbages were in group II (C2). In addition, cauliflower and kai-lan cultivars were placed into the same group (C4).

In a previous study, cabbage landraces in China did not show any association between the molecular classification based on AFLP data and head type (Kang et al. 2011). However, in our UPGMA and population structure analyses, cabbage cultivars formed two distinct groups that coincided with the classification based on head shape, suggesting that the head shape of cabbage is genetically more distinct compared to other agronomic traits, such as maturity, head size and bolting type. This result also may signify that a gene responsible for the head shape of cabbage is associated with SSR markers used in the present study.

Although the UPGMA dendrogram clearly classified most commercial cultivars into varietal groups, the population structure analysis placed cauliflower and kai-lan into the same group. Kai-lan, also known as Chinese broccoli, has vestigial flower heads similar to those of broccoli. Meanwhile, cauliflower is characterized by its undifferentiated inflorescences, called curd, resembling those in broccoli. Based on their characteristics, cauliflower and kai-lan have similar traits that are related to broccoli cultivars. Thus, even though cauliflower and kai-lan are different varietal groups, the similarity of their flower heads could be related to their presence together in the same group.

When inferred ancestry was computed, 79 out of 91 *B. oleracea* cultivars had more than 75 % of their shared ancestry derived from one of the six groups (Fig. 2a, Supplementary Table 2). The remaining 12 cultivars were identified as admixtures having 52–73 % shared ancestry with a major group (Fig. 2b, Supplementary Table 2). The low level of admixture types found among the *B. oleracea* cultivars may be a result of breeding programs that mainly focus on developing new cultivars within the same varietal group. Therefore, the gene flow occurred only within each varietal group. Overall, our population structure analysis provides new insight into the genetic structure and relationships among six varietal groups of *B. oleracea*, which has previously been unclear.

Allele diversity and heterozygosity

Genetic variability within varietal groups was relatively high, with an average of 0.32 and 2.35 for overall gene diversity and alleles per locus, respectively. Among the six varietal groups, kohlrabi cultivars showed the highest gene diversity (0.41), followed by cabbage cultivars (0.39). Conversely, cabbage cultivars had an average of 3.81 alleles per locus, higher than the average number of alleles (2.81)

in kohlrabi cultivars (Table 6). A previous study reported that the gene diversity in cabbage, broccoli and cauliflower were 0.59, 0.58 and 0.56, respectively (Louarn et al. 2007), which is higher than found in our study. Meanwhile, a recent study of genetic diversity in kale landraces, cultivars and wild populations in Europe reported a total gene diversity of 0.32 (Christensen et al. 2010), which are similar to our findings. The variation in gene diversity and allele numbers per locus among cultivars in each varietal group represents how wide and diverse the genetic resources that were used for breeding programs. In this study, kohlrabi and cabbage cultivars showed the highest gene diversity and allele numbers per locus, respectively, indicating that relatively diverse wild resources were included in the development of desirable cultivars in these two varietal groups.

Among the six varietal groups, kohlrabi cultivars exhibited the highest heterozygosity value (0.41), followed by cabbage cultivars (0.38). Meanwhile, kale and kai-lan cultivars showed lower heterozygosity levels (0.08 and 0.13, respectively) than the other varietal groups, indicating that cultivars in these two groups may not be F_1 hybrids, but rather inbred lines. The higher values of heterozygosity among cultivars of kohlrabi and cabbage coincided with their higher values for gene diversity and allele numbers per locus. In addition, we can conclude that most breeders have a good F_1 seed production system using self-incompatibility or male sterility for these two varietal groups. Because heterozygosity plays an important role in performance of the F_1 hybrid (Syed and Chen 2005), it is important to know the heterozygosity level of each F_1 hybrid cultivar. One cabbage cultivar 'Megaton', two broccoli cultivars 'Super Grace' and 'Aosima', and one kohlrabi cultivar 'Worldcol' were F_1 hybrid cultivars and showed the highest heterozygosity levels (over 40 %). Among cauliflower cultivars, 'Orange Dream' showed the highest heterozygosity (30.43 %). It will be interesting to explore whether the higher heterozygosity levels we found here do indeed correspond to superior agronomic performance. In addition, the identified F_1 hybrid cultivars containing high heterozygosity are good candidate breeding and genetic materials because they have higher allele diversity.

With regard to molecular markers, we found that marker CB10229 has the highest contribution in detecting heterozygous individuals across 91 cultivars. A total of 97 % cultivars were identified as heterozygous. This finding suggests this locus as a potential marker for predicting hybrid performance or heterosis in hybrid materials considering the strong correlation between molecular marker heterozygosity and hybrid performance or heterosis (Zhang et al. 1996). By contrast, marker MR133.1 identified only 3 % heterozygous cultivars across 91 cultivars. This result is interesting because that marker detected most cultivars as homozygous, even though the majority of cultivars used in this study were F_1 hybrids. This suggests that the MR133.1 locus remained highly conserved across *B. oleracea* germplasms.

Summary

The information regarding genetic diversity, relationships, heterozygosity levels and population structure among 91 commercial *B. oleracea* cultivars presented here is important for future breeding programs and will facilitate the utilization of those cultivars for crop improvement. This study also demonstrates the usefulness of a set 69 microsatellite markers as a potential tool for assessing genetic diversity, detecting heterozygous individuals, differentiating and identifying cultivars, DUS testing, and F_1 seed purity testing in breeding programs.

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