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ANALYSIS OF GENETIC DIVERSITY AMONG RAPESEED CULTIVARS AND BREEDING LINES BY SRAP AND SSR MOLECULAR MARKERS

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Abstract

The knowledge of genetic diversity is very important for developing new rapeseed (*Brassica napus* L.) cultivars. The genetic diversity among 77 rapeseed accessions, including 22 varieties and 55 advanced breeding lines were analyzed by 47 sequence-related amplified polymorphism (SRAP) and 56 simple sequence repeat (SSR) primers. A total of 270 SRAP and 194 SSR polymorphic fragments were detected with an average of 5.74 and 3.46 for SRAP and SSR primer, respectively. The cluster analysis grouped the 77 accessions into five major clusters. Cluster I contained spring and winter type varieties from Czech Republic and semi-winter varieties and their respective breeding lines from China. The 16 elite breeding lines discovered in Cluster II, III, IV and V indicated higher genetic distance than accessions in Cluster I. The principal component analysis and structure analysis exhibited similar results to the cluster analysis. Analysis of molecular variance revealed that genetic diversity of the selected breeding lines was comparable to the rapeseed varieties, and variation among varieties and lines was significant. The diverse and unique group of 16 elite breeding lines detected in this study can be utilized in the future breeding program as a source for development of commercial varieties with more desirable characters.

Key words: *Brassica napus* L., Genetic diversity; Molecular markers; SRAP; SSR.

Introduction

Rapeseed (*Brassica napus* L.; AACC, 2n=38) is the second most economically important oilseed crop in the world and the first important oilseed crop in China (Wang & Yin, 2014). China, Canada, and European countries are the leading rapeseed producing countries in the world (Li *et al*., 2012). In China rapeseed quality improvement breeding programme was initiated in the late 1970s after the introduction of Oro, Tower and other single-low or double-low *B. napus* cultivars from Europe, Canada and Australia (Liu, 2000). Using these canola varieties as parental lines, Chinese breeders have successively developed a series of new varieties adapted to local conditions. *B. napus* has become the most important oilseed crop in China (Liu, 2000).

Genetic diversity information is very important for developing new varieties of Brassica (Li *et al*., 2012). The importance of such information has been demonstrated in several genetic diversity studies of rapeseed. On the basis of ecotypes, Diers & Osborn (1994) and Becker *et al*. (1995), classified rapeseed into spring type, winter type and semi-winter type groups. Hybridization between winter, semi-winter, and spring rapeseed is an important approach to broaden the genetic base of these three types of rapeseed germplasm (Qian *et al*., 2009; Kebede *et al*., 2010). European rapeseed (Hu *et al*., 2007) and US rapeseed (Li *et al*., 2012) were discovered as important germplasm resource for enriching the genetic background of Chinese rapeseed. Chen *et al*. (2008) reported higher genetic diversity and genetic distinctiveness of Chinese germplasm, compared with Australian germplasm.

Various molecular markers have been successfully used to study genetic diversity in Brassica, such as restriction fragment length polymorphism (RFLP) (Diers & Osborn, 1994), random amplified polymorphic DNA (RAPD) (Hu *et al*., 2003; Yu *et al*., 2005; Li *et al*., 2012), amplified fragment length polymorphism (AFLP) (Jiang *et al*., 2007; Qi *et al*., 2008), simple sequence repeat (SSR) (Hasan *et al*., 2006; Wang *et al*., 2009; Turi *et al*., 2012; Younas *et al*., 2012), sequence-related amplified polymorphism (SRAP) (Li & Quiros, 2001; Li *et al*., 2011; Ahmad *et al*., 2014), and single-nucleotide polymorphisms (SNPs) (Wang *et al*., 2014). SRAP is a PCR-based molecular marker system primarily developed for mapping and gene tagging in Brassica species (Li & Quiros, 2001). Ahmad *et al*. (2014) reported desirable genetic diversity between the maintainer and restorer inbred lines in *B. napus*. SSR markers are simple, reliable, easily detected, genome specific, highly polymorphic and commonly used in genomic applications (Li *et al*., 2013). Cunmin *et al*. (2012) reported that SSR analysis is very effective for the assessment of genetic diversity among *B. napus* inbred lines.

The main objectives of this study were to (i) compare the genetic variation between *B. napus* varieties and their respective breeding lines on the basis of SRAP and SSR markers data, (ii) identify desirable breeding lines for future breeding program. This study will be useful for plant breeders and geneticist in understanding the genetic structure of *B. napus* in identification of desirable lines for developing new varieties with superior characters in future.

Materials and Methods

Plant samples: In this study, a set of 77 accessions of *B. napus* (22 varieties and 55 breeding lines) were analyzed for their genetic diversity (Table 1). Out of 22 tested

varieties 19 belong to different rapeseed growing provinces of China with 11 from Hubei, five from Shaanxi, one each from Henan, Jiangsu and Zhejiang provinces, and three exotic varieties were from Czech Republic procured from the Crop Research Institute, Prague. All breeding lines used in this study were developed by Rapeseed Research Centre of Northwest A&F University, Yangling, Shaanxi, PR China, by using hybridization and/or backcrossing followed by pedigree selection. All these accessions were planted in the experimental station of Northwest A&F University at Yangling, Shaanxi, PR China on 20 September 2013.

DNA extraction: Young leaves from 15 plants of each accession were randomly collected from plants grown in the field and ground in liquid nitrogen. Genomic DNA was extracted following the cetytrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1990). All DNA samples were tested on 0.8% agarose gel and DNA concentration calculated by using Epoch Microplate Spectrophotometer (Epoch, USA) and the final dilution was done before use at 50 ng/µl.

Sequence-related amplified polymorphism analysis: Forty seven primer combinations of SRAP were screened from a collection of 350 primer pairs (Table 2) because of their superior polymorphism shown in previous work of our laboratory. These 47 selected SRAP primer combinations were synthesized by BioAsia (Shanghai, China). PCR were performed in a PCR bar containing 2 µl of 50 ng/µl DNA template; 0.3 µl of each reverse and forward primer 10 µmol/L; 5 µl PCR Mix containing 1 x PCR buffer, 500 µM dNTP each, 20 mM Tris-HCL (pH 8.3), 100 mM KCL, 3 mM $MgCl_2$; Golden DNA Polymerase; 2.5 U/µl (Tiangen Biotech Co., Ltd, Beijing, China) and 3 µl ddH_2O were used to complement the reaction. The PCR amplification program was performed in C1000 Thermal Cycler (BioRad Co. Ltd., USA) following PCR cycle steps: 1 min for pre-degeneration at 95°C; 1 min for degeneration at 94°C, 1 min for annealing at 35°C and 1 min for extension at 72°C for 5 cycles; 1 min for degeneration at 94°C , 1 min for annealing at 50°C and 1 min for extension at 72°C for 35 cycles; and finally 7 min incubation at 72°C. Polymorphism at each locus was assessed by electrophoresis of PCR products on 8% polyacrylamide (w/v) gel in 1 x Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) and visualized by silver staining.

Simple sequence repeat analysis: SSR analysis was performed by PCR with 56 pairs of SSR primers (Table 3). These primers were selected for good polymorphism and easy to score quality. The PCR amplification program was also performed in C1000 Thermal Cycler following PCR cycle steps: 2 min for pre-degeneration at 94°C; 1 min for degeneration at 94°C, 0.5 min for annealing at 60°C and 0.75 min for extension at 72°C for 10 cycles; 1 min for degeneration at 94°C, 0.5 min for annealing at 55°C and 0.75 min extension at 72°C for 30 cycles; and finally 5 min incubation at 72°C. Polymorphism at each locus was assessed by electrophoresis of PCR products on 8% polyacrylamide (w/v) gel in 1 x TBE solution and visualized by silver staining.

Data collection and analysis: In the SRAP and SSR molecular analysis only strong, reproducible and clearly distinguished polymorphic fragments among all accessions were used in the data analysis. The profile data produced by SRAP and SSR markers were scored manually for each fragment in every accession for each primer pair and recorded as 1 representing the presence of a fragment and 0 representing the absence of a fragment.

The following formula was used to calculate the polymorphic information content (PIC) per marker:

$$\text{PIC} = 1 - , \quad \sum_{j=1}^{n} P_{ij}^{\ 2}$$

where P_{ij} is the frequency of the *j*th allele of the *i*th marker locus and *n* is the total number of alleles. The data were analyzed using the qualitative routine to generate simple matching coefficients (SMCs), calculated as SMC = *a/(n-d)*, where *a* is the number of fragments in common between two accessions, *n* is the number of fragments in the matrix, and *d* is the number of fragments absent in both accessions (Sokal & Michener, 1958). Simple matching coefficients (SMCs) were used to construct a dendrogram by the unweighted pair-group method with arithmetic mean (UPGMA) and the sequential, hierarchical and nested clustering (SHAN) routine in the NTSYS program version 2.10 (Rohlf, 1998). The principal component analysis (PCA) was performed with the same program using the Decenter and Eigen procedures. The 0, 1 matrix of SRAP and SSR markers were used for population structure analysis by Structure version 2.3.4 (Pritchard *et al*., 2000). All markers were treated as dominant markers, so the recessive allele model was used. The allotetraploid rapeseed was treated as diploid and the admixture model was used in the analysis. Length of burn-in period before the start of data collection and number of Markov Chain Monte Carlo analysis repeats after burn-in were both set at 10,000. The number of groups (*K*) ranged from 1 to 10. For the analysis of molecular variance (AMOVA) analysis, all accessions were classified into two groups, variety and breeding line. The components of variance attributable to different varieties and breeding lines were estimated from the genetic distance matrix, as specified in the AMOVA procedure in ARLEQUIN version 3.1 (Schneider *et al*., 2000). A nonparametric permutation procedure with 3000 permutations was used to test the significance of variance components associated with the different possible levels of genetic structure in this study (Excoffier *et al*., 1992). The pairwise F_{st} values, a value of F statistic analogs computed from AMOVA, were used to compare genetic distances between any two groups.

Table 1. ***Brassica napus*** **L. accessions used in the present investigation.**

S. No.	Code	Name	Pedigree	Quality †	Oil content (%)	Type	Geographical region
1.	2D74		Zhongshuang No.9 × 07H-195	00	39.45	Semi-winter, line	
2.	2C203	Y6		00	41.35	Semi-winter, line	
3.	ZS07	Zhongshuang No.7	(Topas/84001)F1/821	00	39.86	Semi-winter, variety	Hubei, China
4.	2D028		Zhongshuang No.4 × Zhongshuang No.7	00	44.87	Semi-winter, line	
5.	CZ25	ZL-02-4	B12-153 × S5125 × Ledos	00	46.63	Spring, variety	Czech Republic
6.	2C183	Qinyou No.211		00	38.6	Semi-winter, line	
7.	2D10		Zayou No.1 × Zhongshuang No.5	00	41.7	Semi-winter, line	
8.	2C269	Ganza No.1R		0	37.95	Semi-winter, line	
9.	2C212	9722		00	39.21	Semi-winter, line	
10.	ZS10	Zhongshuang No.10	9126/(Zhongyou119 220 9248 9246 9245	00	42.19	Semi-winter, variety	Hubei, China
11.	D89	D89	Tower × Ning 7	00	35.06	Semi-winter, variety	Jiangsu, China
12.	2C198	D1560		00	39.02	Semi-winter, line	
13.	2D78		Zhongshuang No.9 × GYK2008	00	41.66	Semi-winter, line	
14.	2D56	CH10-9		00	43.07	Semi-winter, line	
15.	2D17		Jingyou No.1 × Zhongshuang No.4	00	42.17	Semi-winter, line	
16.	2D52	Chuyouza No.99		00	44.05	Semi-winter, line	
17.	ZS04	Zhongshuang No.4	Zhongyou 821/Zhongshuang No.2	00	45.91	Semi-winter, variety	Hubei, China
18.	2C209	2000-5		00	44.49	Semi-winter, line	
19.	ZS02	Zhongshuang No.2	Start × Ganyou No.5	00	40.43	Semi-winter, variety	Hubei, China
20.	2D11		C3 × zhongshuang No.9	00	41.09	Semi-winter, line	
21.	2C268	Qinyou 7P		00	44.37	Semi-winter, line	
22.	2D64		Zhongshuang No.7 × KS2185	00	41.91	Semi-winter, line	
23.	2D54	GYK2008		00	43.02	Semi-winter, line	
24.	2C178	Shaanyou No.8		00	35.57	Semi-winter, line	
25.	2C224	8C		00	42.53	Semi-winter, line	
26.	CZ02	Ozima repka sonata		00	44.23	Winter, variety	Czech Republic
27.	2D65		SH11 × Lisolde	00	40.18	Semi-winter, line	
28.	2C221	Zhongyou 821R		00	39.78	Semi-winter, line	
29.	ZS11	Zhongshuang No.11	(Zhongshuang No.9 × 2F10) × 26102	00	43.29	Semi-winter, variety	Hubei, China
30.	CZ38	ZL-Var-55		00	46.91	Winter, variety	Czech Republic
31.	2D37	T16		00	42.96	Semi-winter, line	
32.	2D43	Zayou No.86		00	44.7	Semi-winter, line	
33.	2D08	N310B		00	38.11	Semi-winter, line	
34.	2D77		Zhongshuang No.9 × Qinyou No.7	00	42.61	Semi-winter, line	
35.	C3	C3	Restorer C3	+	38.42	Semi-winter, variety	Shaanxi, China
36.	2C194	SH11		00	41.93	Semi-winter, line	
37.	2D32		Zhongyou 821 × SH11	00	39.63	Semi-winter, line	
38.	ZS09III	Zhongshuang No.9III	(Zhongyou821 × 84004 × Zhongshuang No.4	00	39.6	Semi-winter, variety	Hubei, China
39.	ZS09II	Zhongshuang No.9II	(Zhongyou821 × 84004 × Zhongshuang No.4	00	42.21	Semi-winter, variety	Hubei, China
40.	2D01		CZ44 × Zhongshuang No.7	00	42.33	Semi-winter, line	

Table 1. (Cont'd.).

S. No.	Code	Name	Pedigree	Quality †	Oil content (%)	Type	Geographical region
41.	2C195	SH11		00	40.62	Semi-winter, line	
42.	Shaan 2B	Shaan 2B		+	37.22	Semi-winter, variety	Shaanxi, China
43.	ZY17	Zheyou·No.17	Zheshuang No.6 × G166	00	42.33	Semi-winter, variety	Zhejiang, China
44.	2D51	H8117		00	42.74	Semi-winter, line	
45.	2C225	Z6C		00	43.62	Semi-winter, line	
46.	ZS05	Zhongshuang No.5	(84039 × 84001 × 821	00	42.99	Semi-winter, variety	Hubei, China
47.	2D13		Zhongshuang No.2 × Gan	00	39.77	Semi-winter, line	
48.	ZS06	Zhongshuang No.6	9328/ zhongyou119, 220, 9548, 9558	00	41.84	Semi-winter, variety	Hubei, China
49.	2D60	Mianza No.03-33		00	42.99	Semi-winter, line	
50.	Qinyou3	Qinyou No.3	SE8 × Midas	0	34.69	Semi-winter, variety	Shaanxi, China
51.	2C272	Qinyou 10C		00	40.67	Semi-winter, line	
52.	2D79		Zhongshuang No.9 × Shaanyou No.17	00	43.66	Semi-winter, line	
53.	2D50	Zayou No.105		00	43.44	Semi-winter, line	
54.	2D18	Fuyou No.668		00	43.84	Semi-winter, line	
55.	2D20	Ganza No.5		00	42.43	Semi-winter, line	
56.	2D75		Zhongshuang No.9 × T16	00	40.77	Semi-winter, line	
57.	Zaofeng1	Zaofeng No.1		0	39.72	Semi-winter, variety	Shaanxi, China
58.	2D44	CH1012		00	42.27	Semi-winter, line	
59.	2D19	Liangyou No.586		00	42.94	Semi-winter, line	
60.	2D67		Zhongshuang No.9 × 07H-195	00	42.08	Semi-winter, line	
61.	2D16		Zhongshuang No.9 × Gan	00	46.45	Semi-winter, line	
62.	2C226	D1526		00	40.79	Semi-winter, line	
63.	ZS04-2	Zhongshuang No.4	Zhongyou 821/Zhongshuang No.2	00	33.74	Semi-winter, variety	Hubei, China
64.	2C243	Qinyou 7P		00	41.4	Semi-winter, line	
65.	2D21		Zhongshuang No.4 × Jingyou No.1	00	39.77	Semi-winter, line	
66.	Zaofeng5	Zaofeng No.5		+	38.32	Semi-winter, variety	Shaanxi, China
67.	2D80	Qingza No.1		00	45.61	Semi-winter, line	
68.	2D46	SY06-18		00	42.62	Semi-winter, line	
69.	2D69		Zhongshuang No.9 × CH10-9	00	41.42	Semi-winter, line	
70.	220	220	(7818×Marrnoo) × Qva	00	37.08	Semi-winter	Henan, China
71.	2D34	ZY1007		00	44.66	Semi-winter	
72.	2D58	ZY0913		00	42.02	Semi-winter	
73.	ZS09I	Zhongshuang No.9I	(Zhongyou821, 84004 × Zhongshuang No.4	00	39.39	Semi-winter	Hubei, China
74.	2D22		Jingyou No.1 × SH11	00	38.77	Semi-winter	
75.	2D02	Sheng No.11		00	45.44	Semi-winter	
76.	2D73		Zhongshuang No.9 × Qinyou No.7	00	41.16	Semi-winter	
77.	2D24		Qinyou 7R × Zhongyou 821H	00	39.12	Semi-winter	

† "0" low erucic acid, "00" low erucic acid and low glucosinolates, "+" high erucic acid and glucosinolates.

Table 2. The SRAP combination primers used in the present investigation, amplified alleles, polymorphic alleles, polymorphism and PIC values.

S. No.	SRAP primers	Forward primer (5'-3')	Reserve primer (5'-3')	Amplified alleles	Polymorphic alleles	Polymorphism (%)	PIC Value
1.	Em1 + Me7	GACTGCGTACGAATTATT	TGAGTCCAAACCGGTAA	9	6	66.67	0.77
2.	Em1 + Me12	GACTGCGTACGAATTATT	TGAGTCCAAACCGGACG	10	8	80.00	0.84
3.	Em1 + Me18	GACTGCGTACGAATTATT	TGAGTCCAAACCGGAAG	7	6	85.71	0.75
4.	Em10 + Me25	GACTGCGTACGAATTCAC	CAAATGTGAACCGGATA	15	8	53.33	0.84
5.	Em10 + Me31	GACTGCGTACGAATTCAC	CAGGACTAAACCGGATA	11	7	63.64	0.79
6.	Em10 + Me17	GACTGCGTACGAATTCAC	TGAGTCCAAACCGGAGA	7	4	57.14	0.66
7.	Em10 + Me9	GACTGCGTACGAATTCAC	TGAGTCCAAACCGGAAT	10	8	80.00	0.84
8.	Em10 + Me16	GACTGCGTACGAATTCAC	TGAGTCCAAACCGGAAC	9	5	55.56	0.68
9.	Em12 + Me17	GACTGCGTACGAATTCAT	TGAGTCCAAACCGGAGA	8	6	75.00	0.68
10.	Em12 + Me18	GACTGCGTACGAATTCAT	TGAGTCCAAACCGGAAG	8	4	50.00	0.549
11.	Em12 + Me20	GACTGCGTACGAATTCAT	TGGGGACAACCCGGCTT	7	4	57.14	0.67
12.	Em12 + Me22	GACTGCGTACGAATTCAT	GGTGAACGCTCCGGAAG	7	4	57.14	0.63
13.	Em12 + Me24	GACTGCGTACGAATTCAT	GAGCGTCGAACCGGATG	10	5	50.00	0.74
14.	Em12 + Me17	GACTGCGTACGAATTCAT	TGAGTCCAAACCGGAGA	12	7	58.33	0.83
15.	Em13 + Me24	GACTGCGTACGAATTCTA	GAGCGTCGAACCGGATG	11	5	45.45	0.68
16.	Em13 + Me30	GACTGCGTACGAATTCTA	GACCAGTAAACCGGATG	9	5	55.56	0.75
17.	Em14 + Me20	GACTGCGTACGAATTCTC	TGGGGACAACCCGGCTT	7	4	57.14	0.69
18.	Em14 + Me22	GACTGCGTACGAATTCTC	GGTGAACGCTCCGGAAG	8	4	50.00	0.68
19.	Em14 + Me31	GACTGCGTACGAATTCTC	CAGGACTAAACCGGATA	13	6	46.15	0.79
20.	Em14 + Me32	GACTGCGTACGAATTCTC	ATCAGTCGGACCGGATT	13	7	53.85	0.81
21.	Em15 + Me23	TGAGTCCAAACCGGAAA	AGCGAGCAAGCCGGTGG	14	9	64.29	0.86
22.	Em15 + Me2	TGAGTCCAAACCGGAAA	TGAGTCCAAACCGGAGC	7	6	85.71	0.75
23.	Em15 + Me5	TGAGTCCAAACCGGAAA	TGAGTCCAAACCGGGAT	10	6	60.00	0.77
24.	Em15 + Me8	TGAGTCCAAACCGGAAA	TGAGTCCAAACCGGTGC	8	5	62.50	0.75
25.	Em15 + Me13	TGAGTCCAAACCGGAAA	TGAGTCCAAACCGGACT	10	8	80.00	0.79

Table 2. (Cont'd.).

S. No.	SRAP primers	Forward primer (5'-3')	Reserve primer (5'-3')	Amplified alleles	Polymorphic alleles	Polymorphism (%)	PIC Value
26.	Em16 + Me2	GACTGCGTACGAATTGAT	TGAGTCCAAACCGGAGC	8	5	62.50	0.69
27.	Em16 + Me3	GACTGCGTACGAATTGAT	TGAGTCCAAACCGGATG	12	5	41.67	0.70
28.	Em16 + Me7	GACTGCGTACGAATTGAT	TGAGTCCAAACCGGTAA	10	6	60.00	0.76
29.	Em16 + Me8	GACTGCGTACGAATTGAT	TGAGTCCAAACCGGTGC	7	3	42.86	0.53
30.	Em17 + Me24	GACTGCGTACGAATTGTC	GAGCGTCGAACCGGATG	15	12	80.00	0.84
31.	Em19 + Me4	TGTGGTCCGCAAATTTAG	TGAGTCCAAACCGGACA	10	5	50.00	0.75
32.	Em19 + Me12	TGTGGTCCGCAAATTTAG	TGAGTCCAAACCGGACG	7	4	57.14	0.65
33.	Em19 + Me8	TGTGGTCCGCAAATTTAG	TGAGTCCAAACCGGTGC	10	7	70.00	0.81
34.	Em4 + Me1	GACTGCGTACGAATTTGA	TGAGTCCAAACCGGATA	12	7	58.33	0.79
35.	Em4 + Me2	GACTGCGTACGAATTTGA	TGAGTCCAAACCGGAGC	5	4	80.00	0.66
36.	Em4 + Me7	GACTGCGTACGAATTTGA	TGAGTCCAAACCGGTAA	9	5	55.56	0.72
37.	Em4 + Me8	GACTGCGTACGAATTTGA	TGAGTCCAAACCGGTGC	6	3	50.00	0.49
38.	Em5 + Me19	GACTGCGTACGAATTAAC	TTCAGGGTGGCCGGATG	8	3	37.50	0.56
39.	Em5 + Me21	GACTGCGTACGAATTAAC	CTGGCGAACTCCGGATG	8	4	50.00	0.64
40.	Em5 + Me22	GACTGCGTACGAATTAAC	GGTGAACGCTCCGGAAG	10	4	40.00	0.68
41.	Em5 + Me23	GACTGCGTACGAATTAAC	AGCGAGCAAGCCGGTGG	8	4	50.00	0.70
42.	Em5 + Me24	GACTGCGTACGAATTAAC	GAGCGTCGAACCGGATG	10	5	50.00	0.72
43.	Em5 + Me27	GACTGCGTACGAATTAAC	GTACATAGAACCGGAGT	9	5	55.56	0.72
44.	Em5 + Me30	GACTGCGTACGAATTAAC	GACCAGTAAACCGGATG	13	8	61.54	0.82
45.	Em5 + Me23	GACTGCGTACGAATTAAC	AGCGAGCAAGCCGGTGG	14	10	71.43	0.87
46.	Em9 + Me9	GACTGCGTACGAATTCAA	TGAGTCCAAACCGGAAT	10	7	70.00	0.82
47.	Em9 + Me18	GACTGCGTACGAATTCAA	TGAGTCCAAACCGGAAG	13	7	53.85	0.83
			Total	454	270		34.51
			Average	9.65	5.744		0.73

Table 3. List of SSR primers used in the present investigation, amplified alleles, polymorphic alleles, polymorphism and PIC values.

S. No.	SRAP primers	Forward primer (5'-3')	Reserve primer (5'-3')	Amplified alleles	Polymorphic alleles	Polymorphism (%)	PIC Value
1.	BRAS011	TGGGACGTAGTCAGTCAACAA	CCAAGTGCGAGAAGAGGAAG	7	4	57.14	0.65
2.	BRAS014A	CCCATTGACAACTCTTCTCTT	CTGTGTTCGCCCATTATG	3	2	66.67	0.36
3.	BRAS072A	GCCATCTACACATTTATCCC	CACTAACCTTCTTGCTACCGT	11	2	18.18	0.37
4.	BRAS078F	ATTGGGTTCTGACCTTTTCTC	CTTTTCCTCATCGCTACCAC	5	3	60.00	0.49
5.	BRAS084	ATTGGGTTCTGACCTTTTCTC	TTTTCCTTCATCGCTACCAC	6	3	50.00	0.51
6.	CB10026	TCGTTCTGACCTGTCGTTAT	GGAAATGGCTGCTCATGTT	6	3	50.00	0.56
7.	CB10028	CTGCACATTTGAAATTGGTC	AAATCAACGCTTACCCACT	7	5	71.43	0.73
8.	CB10092B	TTGATCCGAAATTCTCTGG	AGGCAAGCAATAGATAAAGG	7	3	42.86	0.54
9.	CB10143	CATGGGAGGCTGTCTAAA	TTGCACCCATACGTTTTC	4	2	50.00	0.35
10.	CB10369	CATTCACAGGACCAGAGC	CAAAGCCAAGACAACCAT	12	3	25.00	0.57
11.	CB10427A	TCCCAACAAAAGAGTCCA	CAGCGAACCGAGTCTAAA	6	3	50.00	0.51
12.	CB10545	CTCGCAATAGTCGCAGAT	TGCCCTACTGTCTCCTCA	8	5	62.50	0.63
13.	CB10587	TTGTGTTTTGCCTTCTGA	TTTGCGCACAAACAATAA	4	4	100.00	0.64
14.	Na12-E02	TTGAAGTAGTTGGAGTAATTGGA	CAGCAGCCACAACCTTACG	6	4	66.67	0.64
15.	Ol10-B06	GCTTTGGCTCGTGTAAATGG	CATCTTTGGTTGTGTATGGTTAGG	6	2	33.33	0.21
16.	Ol10-D03A	GCCAAAGACCTCAAAGATGG	AAGCCACGTGAAGAAAGTCC	9	4	44.44	0.66
17.	Ol11-B05	TCGCGACGTTGTTTTGTTC	ACCATCTTCCTCGACCCTG	7	3	42.86	0.41
18.	Ol12-F08A	TGATAGGATGCGTGAATCAG	AGAATGTAGATCAGTTACGG	4	3	75.00	0.39
19.	Ol13-G05	GTGTGCAGGAAACGATGTTC	GGGAGTTTGAAGAGAAAGCG	3	3	100.00	0.47
20.	A77096A	TAGGACACGTGACAAAACTTCAT	TATCGATGGTATCAAAGAATGGA	3	3	100.00	0.49
21.	BRAS038	TGAGCTGTTACCACTTTCT	AAAACACTTATTTCTTTCCTG	6	3	50.00	0.35
22.	BRMS-007	AAATTGTTTCTCTTCCCCAT	GTGTTAGGGAGCTGGAGAAT	8	3	37.50	0.53
23.	BRMS-042	GGATCAGTTATCTGCACCACAA	TCGGAATTGGATAAGAATTCAA	5	3	60.00	0.39
24.	CB10027	CGGCTTGTAAACCTTG	GACTCGAAAATCACTAACAC	6	3	50.00	0.45
25.	CB10031	ACAAGTCCTTCACGAGAGCCT	CAAGAGATTGTGTCTGTAGCG	2	1	50.00	Monomorphic loci
26.	CB10081	GGCTTTAGCACTGTGATCCT	TTGGGAGAGAAAACTATACG	2	1	50.00	Monomorphic loci
27.	CB10097	ACTTCGGTGGTTCTATTTCT	CGACGGTTAATCAAGTTTCT	4	3	75.00	0.41
28.	CB10099A	CTTCCCCTTTCATCGAACT	TAGAAGCATTTGGAAACGCA	7	4	57.14	0.65
29.	CB10364	GAGACGATGCAAAGATCG	TGCAGACACATTCGAACA	6	3	50.00	0.53

Table 3. (Cont'd.).

S. No.	SRAP primers	Forward primer (5'-3')	Reserve primer (5'-3')	Amplified alleles	Polymorphic alleles	Polymorphism (%)	PIC Value
30.	CB10528	ATGCTTTCTTTGCACGAG	ACCAGACTGATGGTGTGC	5	2	40.00	0.34
31.	CB10569A	TCTCCATCATCTTCGTTCTC	GTGGAGTTGGAGCAACAG	6	1	16.67	Monomorphic loci
32.	CB10597	AAGCGCGCATAACTACAC	AACACTGCTCCTTTCCCT	2	2	100.00	0.375
33.	CB10628	CCGTGAGAAGTCATTTGG	AATCATTTTTCGATGACAGAA	5	5	100.00	0.74
34.	Na10-B01	CAAGTGTCTGCTAGGTGGGG	TCGATCGAAGAAACCAGACC	7	6	85.71	0.78
35.	Na10-B08	AGAGAAAAACACTTCCCGCC	GTGAGCTTTGCGAAACACG	14	7	50.00	0.77
36.	Na10-E02	TCGCGCATGTAATCAAAATC	TGTGACGCATCCGATCATAC	8	2	25.00	0.37
37.	Na10-C06	TGGATGAAAGCATCAACGAG	ATCAATCAACACAAGCTGCG	2	2	100.00	0.37
38.	Na12-C08	GCAAACGATTTGTTTACCCG	CGTGTAGGGTGATCTAGATGGG	3	1	33.33	Monomorphic loci
39.	Na12-G05	CCGATCATACCTTTTACTCTAGCC	GATGTTCCTCTCGGTGATGC	13	8	61.54	0.76
40.	Na14-G02	TTCCCTTTATTGAGCAAGCTG	TCCCGGTCGCTAAGATATTG	8	3	37.50	0.55
41.	Ni2-C12B	ACATTCTTGGATCTTGATTCG	AAAGGTCAAGTCCTTCCTTCG	10	5	50.00	0.38
42.	Ni2-F11A	AAAGGGTTTCAATTTCACGC	GGGAAACATACTCACCACGC	7	4	57.14	0.64
43.	Ol10-C05	GGCTACAAAATGTTTGATAAGCTCT	ACCTGAAAGAGAGGCTACACAT	4	3	75.00	0.57
44.	Ol10-G06	GACAAGTTCCCTTGTAATGGC	TGTAATCATCACACATTTTGGG	2	2	100.00	0.24
45.	Ol11-G11	GTTGCGGCGAAACAGAGAAG	GAGTAGGCGATCAAACCGAG	11	9	81.82	0.77
46.	Ol12-F11A	AAGGACTCATCGTGCAATCC	GTGTCAGTGGCTACAGAGAC	11	8	72.73	0.74
47.	Ra1-F03	AACTCGCTTTTACCGTCGTC	CAAGACGTGGAGCTGAAGTG	8	2	25.00	0.37
48.	Ra2-A01	TTCAAAGGATAAGGGCATCG	TCTTCTTCTTTTGTTGTCTTCCG	4	2	50.00	0.33
49.	Ra2-D04A	TGGATTCTCTTTACACACGCC	CAAACCAAAATGTGTGAAGCC	6	1	16.67	Monomorphic loci
50.	Ra2-E04	ACACACAACAAACAGCTCGC	AACATCAAACCTCTCGACGG	6	3	50.00	0.51
51.	Ra3-H09	GTGGTAACGACGGTCCATTC	ACCACGACGAAGACTCATCC	14	11	78.57	0.85
52.	sN11722	CGATCTGAGCGTTGTTGCTA	GCGCGACTCAAAGAAGAAGT	5	3	60.00	0.52
53.	sN2834	CGTGCTTGTTCTTCACTCCA	TCTTTGCGGCTGTGTTCTAA	9	4	44.44	0.16
54.	sR12156	TTCTTGGTGTGTCCTGCAAA	GAGCTATGGCAATCCTCCTG	12	6	50.00	0.71
55.	sR12387	GGGTCTGGGTTTTTCTGTGA	GATTGGGCCGTGTAATATCG	8	2	25.00	0.35
56.	sR7223	AGGACCCGACTTTCCTTGTT	ACCAAACTCGGCGTACAAAT	5	2	40.00	0.35
				365	194		26.17
			Average	6.51	3.46		0.51

Results

Marker polymorphism: On the basis of high level of polymorphism and display of strong banding patterns revealed in our initial screening, 47 SRAP primer and 56 SSR primer combinations (Tables 2 and 3) were used for analysis of genetic diversity among 77 accessions including 22 varieties and 55 breeding lines of *B. napus* (Table 1). SRAP and SSR primers produced sufficient polymorphism in the tested 77 accessions, which was used for calculating similarity coefficient. A total of 270 and 194 polymorphic fragments were detected by SRAP and SSR markers, respectively (Table 4). The fragment size ranged from 200 to 2000 bp for SRAP markers and 100 to 600 bp for SSR markers. The average numbers of polymorphic fragments detected by SRAP primer combinations were 5.74, whereas those of SSR were 3.46. Among 47 SRAP primer combinations, Em17 + Me24, Em5 + Me23 and Em15 + Me23 proved highly effective in generating maximum number of polymorphic bands 12, 10, and 9, respectively (Table 2). In case of 56 SSR primers highest number of polymorphic bands 11 and 9 were revealed by primers Ra3-H09 and Ol11-G11 (Table 3). The percentage of polymorphic bands ranged from 37.50% to 85.71% and 25% to 100% for SRAP and SSR primers, respectively.

Polymorphism information content (PIC) was calculated for each of the 47 SRAP primer combinations and 56 SSR primers to quantify allelic diversity at each locus. The PIC values of the selected SRAP primer combinations ranged from 0.49 for Em4 + Me8 to 0.87 for Em5 + Me23 with an average PIC value 0.73 per locus (Table 2). The PIC values for SSR markers widely varied among loci and ranged from 0.16 for primer sN2834 to 0.85 for Ra3-H09 with an average PIC value 0.51 per locus, whereas, five primers were monomorphic (Table 3). For SRAP all primer combinations PIC value above 0.50 (50%) with maximum genetic diversity of 87%, whereas for SSR 29 primers PIC value above 0.50 (50%) with maximum genetic diversity of 85% was observed for these genotypes which indicates high genetic diversity in the studied plant materials.

Cluster analysis: The molecular marker data of 464 SRAP and SSR markers were used to calculate genetic similarity coefficient for all accessions and a dendogram was constructed from the similarity coefficient data with the UPGMA method (Fig. 1). Genetic similarity estimates calculated among 77 accessions varied from 0.50 to 0.77 for all accessions except for two breeding lines numbered 13 and 23 (2D78 and 2D54). Two accessions, numbered 21 and 29 (breeding line 2C268 and variety ZS11) were found the most divergent accessions and appeared in a separate cluster than other *B. napus* accessions at a similarity coefficient value of 0.53. The similarity coefficient among the rest of the 75 *B. napus* accessions ranged from 0.56 to 0.77. The Cluster analysis placed all 77 accessions of *B. napus* into five major clusters. Among these five clusters, Cluster I was found the largest cluster which contained 58 accessions (19 varieties and 39 breeding lines) including one spring type, two winter type and 16 semi-winter type varieties, and 39 breeding lines. This cluster was further divided into three sub-clusters (I-a, I-b, and I-c). Sub-cluster I-a included two varieties, one spring type exotic variety (CZ25) of Czech Republic and one semi-winter type variety (ZS10) of Hubei province of China and three breeding lines (2D74, 2C203, and 2C183) from Shaanxi province of China. Sub-cluster I-b included two exotic winter type rapeseed varieties (CZ02 and CZ38) of Czech Republic and 11 semi-winter type varieties (ZS07, ZS09II, D89, ZS06, ZS04, ZS02, C3, Shaan 2B, ZS05, ZS09III, and ZY17) from Hubei, Jiangsu, Shaanxi, and Zhejiang provinces of China along with their 21 respective breeding lines (2D028, 2D08, 2C178, 2C198, 2D78, 2D54, 2D64, 2D56, 2D17, 2D65, 2C224, 2D11, 2C221, 2D37, 2D52, 2D43, 2D77, 2C225, 2C194, 2C195, and 2D01) from Shaanxi province of China. Sub-cluster I-c included four semi-winter type varieties (Qinyou3, Zaofeng1, Zaofeng5, and ZS04-2), three from Shaanxi and one from Hubei province of China, and 15 breeding lines (2D51, 2D13, 2C272, 2D20, 2D60, 2D19, 2D50, 2D75, 2C243, 2D18, 2D16, 2D21, 2C226, 2D79, and 2C212). The Cluster II contained only three semi-winter type breeding lines (2D10, 2D32, and 2D67) with different pedigree. The Cluster III consisted 10 semi-winter type accessions including two varieties (220 and ZS09I) from Henan and Hubei province of China and eight breeding lines (2D80, 2D34, 2D58, 2D02, 2D73, 2D24, 2D46, and 2D69). The Cluster IV included four breeding lines (2C269, 2D44, 2C209, and 2D22). The Cluster V included two elite accessions ZS11 semi-winter type variety of Hubei province and one breeding line 2C268 from Shaanxi province of China.

Principal component analysis: The principal component analysis result was similar to the cluster analysis (Fig. 2). The first two principal components accounted for 5.19% and 4.19% of the total variation, respectively. The PCA diagram revealed that all breeding lines assembled with their respective varieties having similar genetic background which gives an indication of genetic distance among the varieties and breeding lines, and furthermore, all breeding lines scattered more widely than the tested varieties.

Table 4. Summary of amplification results of SRAP and SSR.

Marker type	SRAP	SSR
Polymorphic bands	270	194
Range of fragment size	200 – 2000 bp	100 – 600 bp
Average No. of polymorphic bands/primer	5.74	3.46
Range of polymorphic bands	3 – 12	1 – 11
Maximum % of Polymorphic bands	85.71%	100%
Minimum % of Polymorphic bands	37.5%	25%
Range of PIC	0.49 – 0.87	0.16 – 0.85

SRAP, sequence-related amplified polymorphism; SSR, simple sequence repeat; PIC, polymorphic information content

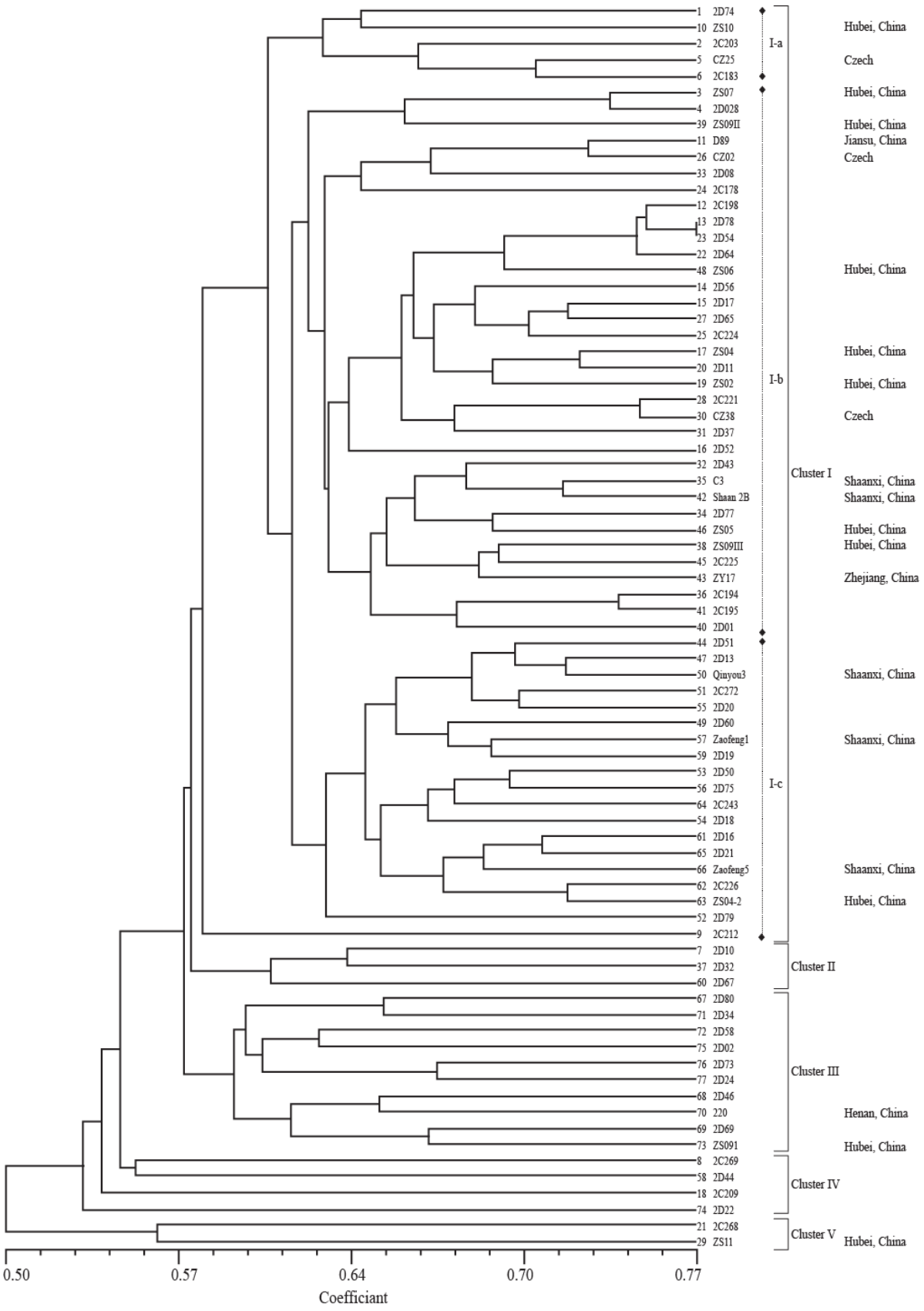

Fig. 1. Clustering of 77 rapeseed accessions by unweighted pair-group arithmetic mean method with Dice index.

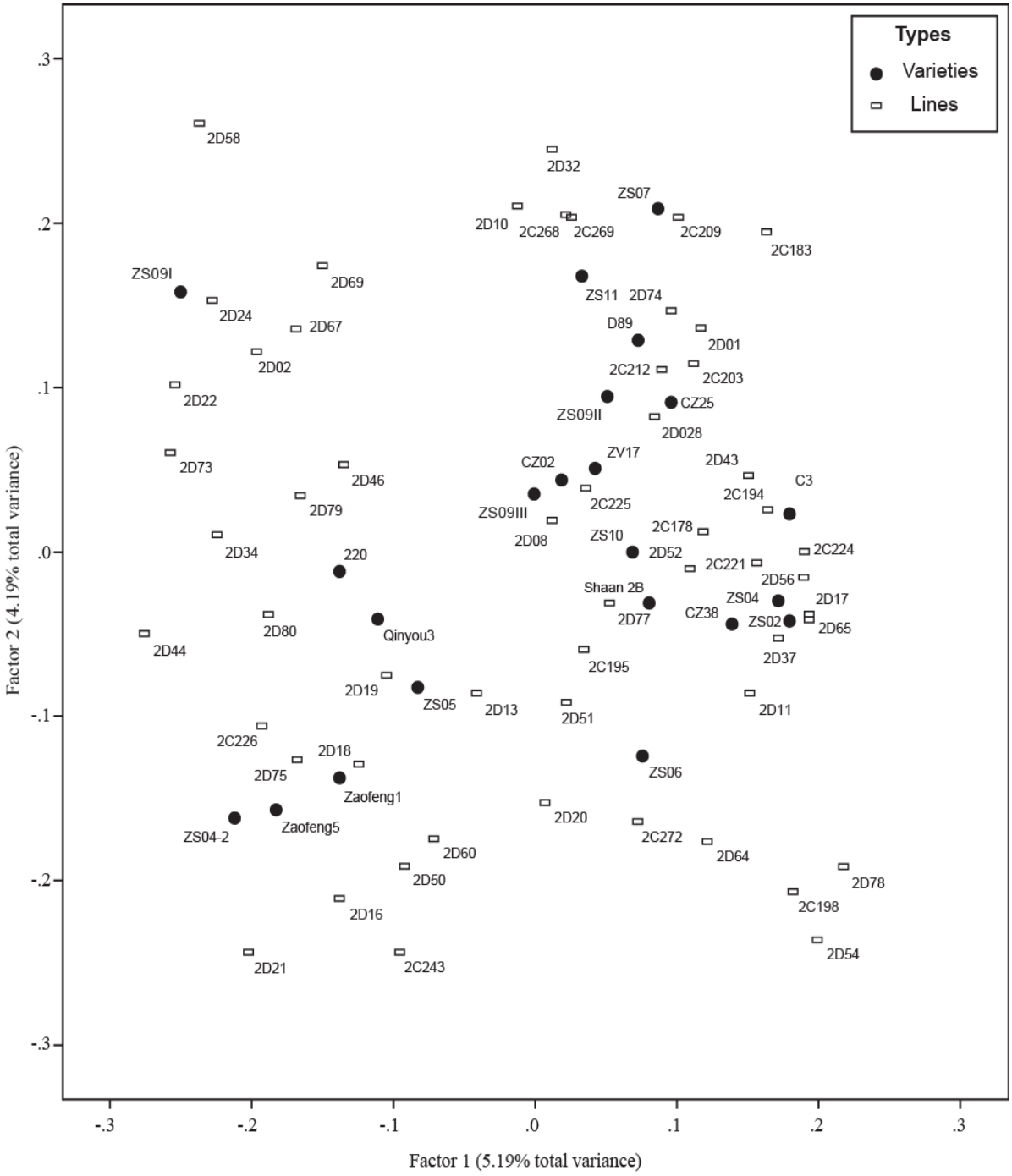

Fig. 2. Biplot of the first two major principal components extracted from SRAP and SSR data.

Table 5. Analysis of molecular variance of 77 rapeseed accessions.

Source of variation	Df	Sum of squares	Variance components	Percentage of variation
Among populations	1	179.15	3.07	3.60
Within populations	75	6177.21	82.36*	96.40
Total	76	6356.36	85.44	

‡Fixation Index F_{st} (a value of *F* statistic analogs): 0.03604; Significance tests (1023 permutations)

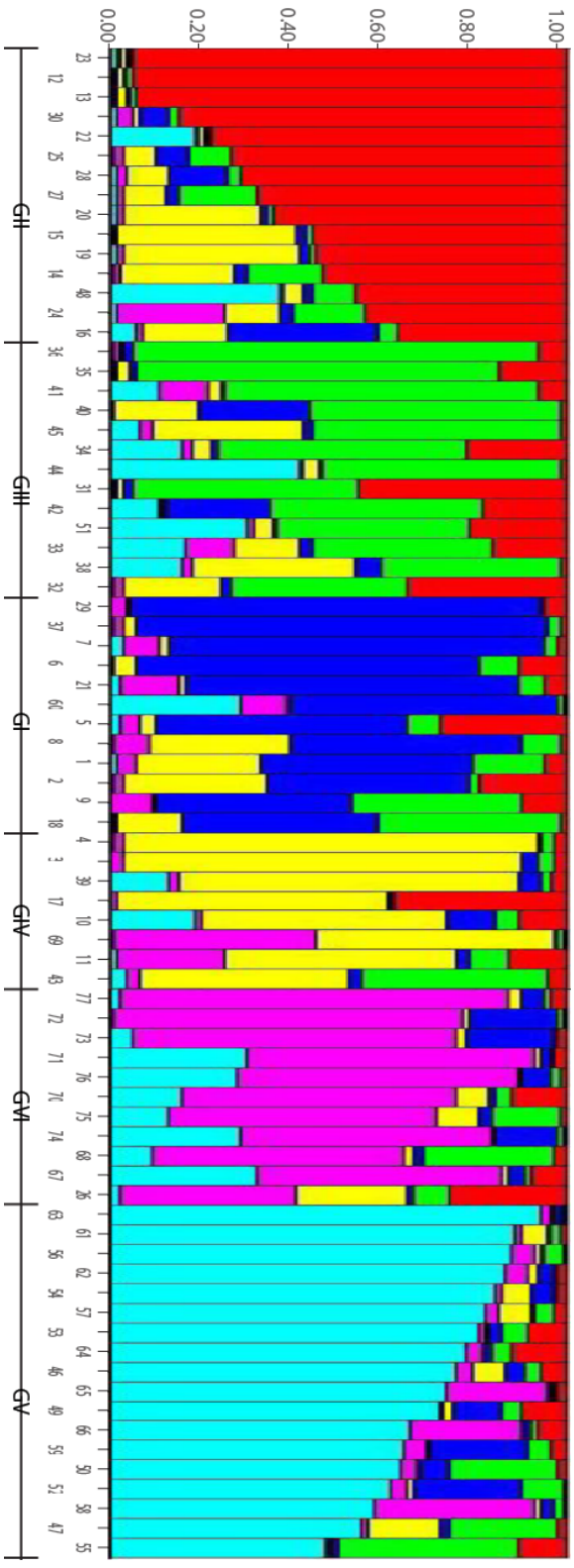

Fig. 3. Population structure of the tested rapeseed accessions suggested by structure analysis (K = 6). Six colors represent six inferred groups (GI-GVI). Each bar represents each accession. The estimated genetic fraction of each accession of each inferred group was indicated in different colors. The numbers under each bar is the same accession numbers in Table 1.

Population structure analysis: The break point of the estimated Ln probability of data [Ln P (D)] was obtained when K = 6, suggesting the presence of six groups, GI-GVI (Fig. 3). Group I included 12 accessions, two varieties and ten breeding lines, a group of five accessions in Cluster I, three accessions in Cluster II and two accessions in each Cluster IV and Cluster V, respectively in the cluster analysis. Group II included 15 accessions, three varieties and 12 breeding lines in Cluster I; Group III included 13 accessions, three varieties and 10 breeding lines in Cluster I; Group IV included eight accessions, seven varieties and one breeding lines, out of these eight accessions seven revealed in Cluster I and one in Cluster III; Group V included 18 accession, five varieties and 13 breeding lines, 17 accessions revealed in Cluster I and one in Cluster IV; Group VI included 11 accessions, three varieties and eight breeding lines, one accessions in Cluster I, nine in Cluster III and one in Cluster IV. Generally, structure analysis result corresponds with the clustering result that all breeding lines assembled with their respective varieties having similar genetic background.

Analysis of molecular variance: All 77 accessions were classified into two groups, varieties and breeding lines, on the basis of genetic background for analysis of molecular variance (AMOVA). The results indicated that 3.60% of the variance was due to differences among populations and 96.40% difference was within populations (Table 5). The pairwise F_{st} value of varieties and lines was 0.036, which is significant. This indicated that variation within populations was high, whereas variation among population was very low but significant.

Discussion

Recently a number of studies on genetic diversity in *B. napus* have been conducted throughout the world using different molecular markers (Hu *et al*., 2003; Hasan *et al*., 2006; Wang *et al*., 2009; Wu *et al*., 2009; Li *et al*., 2012; Turi *et al*., 2012; Ahmad *et al*., 2014; Laila *et al*., 2014). However, the ultimate purpose of assessment of the genetic diversity in available *B. napus* genetic resources is to effectively use them in the breeding programs. We have developed a set of rapeseed breeding lines by using hybridization and/or backcrossing followed by pedigree selection method. In this study, a set of 55 elite breeding lines were selected to compare their genetic diversity with 22 representative varieties, 19 from China and 3 Czech by employing SRAP and SSR molecular markers.

The SRAP and SSR molecular markers were quite useful and revealed considerable genetic diversity among 77 accessions of *B. napus*. The 47 SRAP primer combinations amplified a total of 270 polymorphic alleles with an average number of 5.74 polymorphic alleles ranging from 3 to 12 polymorphic alleles per locus, which is less than the SRAP marker of 8 alleles per locus as observed by Ahmad *et al*. (2014) in rapeseed. Whereas, 56 SSR primer combinations amplified a total of 194 polymorphic alleles with an average number of 3.46

polymorphic alleles per locus ranging from 1 to 11 polymorphic alleles per locus, which is more than 1.7, 2.7, 2.66, and 2.69 observed in genetic diversity studies in rapeseed by previous researchers (Hasan *et al*., 2006; Zhou *et al*., 2006; Turi *et al*., 2012; Laila *et al*., 2014), respectively. This suggested the existence of considerable genetic variation among the tested rapeseed accessions.

The cluster analysis separated all 77 *B. napus* accessions into five major groups. Cluster I contained a large group of 58 accessions including 19 varieties and 39 breeding lines into three sub-clusters. The spring type variety CZ25 from Czech Republic and semi-winter variety Zhongshuang No.10 from Hubei and three breeding lines (2D74, 2C203, and 2C183) from Shaanxi province of China clustered together in Cluster I-a (Fig. 1). Cluster I-b contained 34 accessions, two winter type CZ02 and CZ38 varieties from Czech Republic, seven semi-winter type varieties from Hubei, one each from Jiansu and Zhejiang, and two from Shaanxi province of China together with 21 breeding lines. Most of semi-winter type varieties (ZS07, ZS09II, ZS06, ZS04, ZS02, ZS05, and ZS09III) from Hubei province of China along with their seven respective breeding lines (2D028, 2D78, 2D64, 2D17, 2D11, 2D77, and 2D01) of Shaanxi province of China clustered closely with winter type CZ02 and CZ38 varieties from Czech Republic in cluster I-b because these accessions have Australian, European and Canadian winter and spring type germplasm in their genetic background, for example, accessions ZS07, ZS06, and ZS05 possess Topas, Marrano spring type varieties, and ZS09II, ZS04, ZS02, and ZS09III possess Start winter type variety, respectively in their ancestry (Table 1) The genetic diversity within this cluster was very narrow because majority of the accessions were closely related by pedigree and derived from common germplasm with some specific characteristics. Breeding lines 2D028 and 2D17, 2D64 and 2D01, 2D78, 2D11, and 2D77 contain Zhongshuang No.4, Zhongshuang No.7, and Zhongshuang No.9 varieties, respectively in their pedigree. This has made the genetic background of these accessions very narrow and molecular markers have grouped them into a single large cluster. It is probably because since initiation of rapeseed quality improvement in China during last five decades lot of spring and winter type germplasm from Australia, Europe and Canada have been extensively used in Chinese rapeseed breeding programmes (Liu, 2000; Ren *et al*., 2008). Cluster I-c included 19 semi-winter type accessions, one variety (ZS04-2) from Hubei province, and three varieties (Qinyou No.3, Zaofeng1, and Zaofeng5) and their fifteen respective breeding lines from Shaanxi province. All accessions except 2D13, 2D75, 2D16, 2D21, ZS04-2, and 2D79 have spring type germplasm in their genetic background. This sub-cluster exhibited enough genetic diversity than accessions revealed in Cluster I-b.

Cluster II consisted three elite semi-winter breeding lines with different pedigree. Cluster III contained two semi-winter type varieties 220 from Henan and ZS09I from Hubei and eight elite breeding lines (2D80, 2D34, 2D58, 2D02, 2D73, 2D24, 2D46, and 2D69) from Shaanxi province of China. Cluster IV included four semi-winter elite breeding lines (2C269, 2D44, 2C209, and 2D22) and Cluster V consisted one ZS11 semi-winter variety from Hubei and Qinyou 7P elite breeding line from Shaanxi province of China. The 16 elite breeding lines in Cluster II, III, IV and V indicated higher genetic distance than accessions appeared in Cluster I, and they will be valuable resource for the future rapeseed breeding program. The principal component analysis and structure analysis exhibited similar results to the cluster analysis. AMOVA results indicated that 3.60% of the variance was due to differences among populations and 96.40% difference was within populations (Table 5). This indicated that variation within populations was high, whereas variation among populations was very low but significant.

Conclusions

The present study demonstrated that SRAP and SSR molecular markers contributed well in exploration of the genetic diversity among *B. napus* accessions. The integrative results of cluster analysis, PCA analysis, structure analysis, and AMOVA analysis revealed the genetic diversity of the selected breeding lines evaluated was comparable to the *B. napus* varieties, and variation between variety and line groups was very low but significant. The diverse and unique group of 16 elite breeding lines detected in this study can be utilized in the future breeding program as a source for development of commercial varieties with more desirable characters.

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