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THE ASSESSMENT OF GENETIC DIVERSITY BETWEEN AND WITHIN *BRASSICA* SPECIES AND THEIR WILD RELATIVE (*ERUCA SATIVA*) USING SSR MARKERS

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Abstract

Microsatellites markers were tested for their ability to distinguish genomic distribution of the *Brassica* species of the U Triangle and *E. sativa*. The objectives of the present study were to investigate the genetic diversity of six *Brassica* species from U-Triangle (representing three genomes, A, B, C) and one from genus *Eruca* and to identify promising sources of genetic variation for breeding purposes. A total of 54 SSR markers were analyzed in order to detect variation between and within the selected genomes. Three primer pairs depicted the greatest genetic diversity showing 97% polymorphism between *Brassica* and *Eruca* genomes (2.55 alleles per locus). Polymorphic Information Content (PIC) values ranged from 0.40 (SSR primer Na14-DO7) to 0.79 (NA10-G09). For comparison within *Brassica* genomes and *Eruca*, all the genomes were grouped in three modules i.e., ABE, ACE and BCE (Fig. 1). The tetraploid originating from their parental diploids along-with *Eruca* was considered in the same module. For the estimation of relatedness within and among genomes, dice coefficients were computed as a measure of genetic similarity matrix. On the basis of genetic distances, dendrogram was constructed through cluster analysis. Two major clusters at coefficient of similarity level (0.47) were observed. One cluster comprised of all *Brassica* genomes and their accessions, while another consisting of all accessions of *Eruca* genome. The cluster containing *Brassica* genomes was further subdivided into four sub-groups that contained diploid and tetraploid species in a way that tetraploid species were grouped in between their diploid parental species with varying genetic distances. Present findings confirmed the validity of SSR markers in genomic studies.

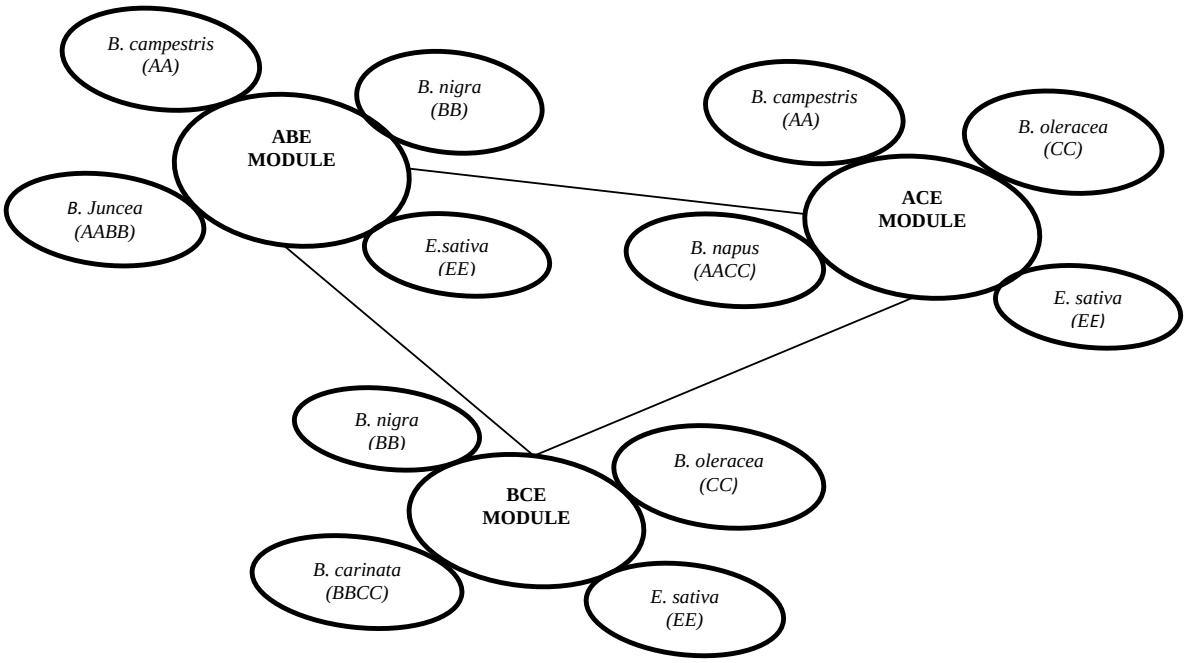

Fig. 1. Diagrammatic representation of modules with their respective genomes and species.

Introduction

The family Brassicaceae (Cruciferae) comprises around 3000 species in 360 genera, organized into 13 tribes. The family's major centers of diversity are Southwestern and Central Asia and the Mediterranean region. The most economically important species in the genus Brassica includes six species that are *B.rapa, B. nigra, B. oleracea, B. napus, B. juncea, B. carinata* (Price et al., 1994) and are grown world widely for a variety of uses. Some of them are important sources of edible and industrial oils, vegetables, condiments, fodder and forage etc. Three of the species, *B.rapa, B. nigra* and *B. oleracea* are diploids and the other three species, *B.*

juncea, B.napus and *B. carinata* are amphidiploids. In family Cruciferae, Brassica is highly diverse group of crop plants with great economic value. Unfortunately, wide genetic diversity for improved crop productivity is largely unexplored in the wild relatives of Brassica crops and there is no clear cut association of genetic and taxonomic classification within Brassicaceae (Gomez-Campo and Prakash, 1999, Warwick et al., 2000). The distribution of genetic diversity within and among plant populations is a function of gene flow between them (Fayyaz *et al*., 2014; Ali *et al*., 2013). Estimation of genetic variation and their distribution within and among populations of species gives bases of genetic diversity (Fayyaz *et al*., 2014; Ahmad *et al*., 2014; Dumolin-Lapegene *et al*., 1997; Rabbani *et al*., 2010; Zeb *et al*., 2011). In addition to biochemical and morphological characterization (Azam *et al*., 2013), the advancement in scientific research leads to the development of molecular markers for exploration of polymorphism present in the DNA of different plants (Ahmad *et al*., 2013; Shinwari *et al*., 2013). The principle, methodology and application of these molecular markers depend on the objectives of study and available resources (Ahmad *et al*., 2014; Semagn *et al*., 2006). Although many different molecular markers have been tested in Brassica (Zada *et al*., 2013a) but among these Simple Sequence Repeats (SSRs) or microsatellites are the choice of most scientists (Ahmad *et al*., 2013) as they can detect polymorphisms even between closely related lines, require low amount of DNA for amplification, can be exchanged between laboratories and are highly transferable between populations (Gupta *et al*., 1994). SSRs are useful tool for the characterization of most of the crops including Brassica species (SzewcMcFadden *et al.*, 1996; Saal *et al.,* 2001, Lowe *et al.,* 2004). SSRs are genome specific as well as trait specific, hence their use depends upon the objective of the researcher. In the present study, SSRs were used as scientific tools for the estimation of genetic diversity between and within Brassica species and their wild relative *Eruca sativa* and also for the estimation of phylogenic relationship in Brassicaceae family.

Materials and Methods

Plant materials: Plant material comprised of six *Brassica* species forming U-triangle and one from the genome *Eruca*. For the estimation of genetic diversity within *Brassica* and *Eruca* genomes, four accessions were selected from each genome belonging to different geographical regions.

DNA extraction: DNA extraction from individual seedlings (germinated in small buckets), was carried out at Plant Genetic Resources Institute (PGRI), National Agricultural Research Centre (NARC), Islamabad Pakistan. The CTAB protocols (Doyle & Doyle, 1990) were used with some modifications. After extraction, DNA was treated with RNase enzyme to remove the free RNA. For this purpose, 2µl of RNase was added to the sample and was incubated at 40°C for 1 hr.

Polymerase chain reaction (PCR): The SSR markers used are given in Table 1. The PCR was carried out in final volume of 15µl for each sample (having 1µl of DNA sample, 9µl of ddH2O, 2.2µl 10xPCR buffer, 0.3µl dNTPs, 0.75µl of reverse and forward primers (Gene Link, USA) and 0.15µl Taq polymerase). After PCR, the amplification of alleles was checked with corresponding set of primers on 2.5% agarose gel. After completion of electrophoresis, gel was visualized under UV light by gel documentation system. Clear glowing bands within the range of DNA ladder were considered as amplified alleles and photographs were taken.

Data analysis: For analysis, the bands were scored using bi-variate (0, 1) cluster analysis. Cluster analysis was performed using NTSys PC version 2.1 (Applied Biosystems Inc, USA). Similarity coefficients were generated using absence-presence pattern for pair wise comparison of species for both similar and dissimilar bands. Estimates for genetic similarity (F) for subjected *Brassica* species and *E. sativa* were calculated by the DICE algorithm according to Nei & Li (1979).

Results

Genetic diversity between *Brassica* and *Eruca* genomes: A total of 81 alleles were amplified in all *Brassica* and *Eruca* genomes by using initially screened 32 SSR primer pairs in which five primer pairs (BRMS-37, PBCJU-8, BN8-3B1, NA10-B10, and NI3-G05) gave single allelic polymorphism, while rest of 27 primer pairs gave polymorphic bands ranging from 2-5 in all *Brassica* species along with *Eruca sativa* (Figs. 2-5). Overall ratio of polymorphism to total allele amplification was found as 2.55. PIC value (Polymorphic Information Content) was calculated according to the given formula (Tonguc & Griffith, 2004).

$$PIC = 1 - \Sigma (pi)^2$$

PIC value ranged from 0.40 to 0.79 for selected set of primers. Among these primers, the highest PIC value was generated by the primer pair NA10-G09, while the lowest PIC value was observed for primer set Na14-D07.

Genetic diversity within *Brassica* genomes and *E. sativa:* For the estimation of genetic diversity within *Brassica* and *Eruca* genomes, all the genomes were grouped into three modules in a way that the tetraploid genome originating from diploid genomes along-with *Eruca* were considered as one module (Fig. 1). Description of each module is given below:

ABE module: The responsive 32 polymorphic primer pairs amplified 76 alleles in ABE module (*B. campestris*, *B. juncea*, *B. nigra* and *E. sativa).* Among these 76 alleles, 74 were polymorphic. On average, 2.38 polymorphic alleles were detected which showed high polymorphic ability of these markers and relationship within *Brassica* and *Eruca* genome. PIC value for these species fell in the range of

0.38 by primer pair BRMS-27 and 0.77 by a primer pair BRMS-01 which showed greater tendency of polymorphism in species from ABE module.

ACE module: In case of this module (*B. campestris, B. napus*, *B. oleracea* and *E. sativa*), 31 primer pairs amplified a total of 76 alleles in which 73 were polymorphic. Average allelic amplification was 2.45 in which 2.35 were polymorphic. PIC value calculated for the polymorphic efficiency of selected primer pairs in these species ranged from 0.26 by primer pair BRMS-19 which showed its less ability of detecting the polymorphic alleles in studied species, while the highest PIC value (0.79) was given by a primer pair NA10-G09.

BCE module: This Module included (*B. nigra*, *B. carinata*, *B. oleracea* and *E. sativa*). In these species, 32 primer pairs amplified a total of 74 alleles in which 71 were polymorphic. On average, 2.29 alleles were polymorphic out of a total of 2.38. PIC value for these species ranged from 0.38 by a primer pair 0112-E03 to 0.79 by a primer pair NA10-G09.

Table 1. SSR markers with their reported band size (bp), observed band size (bp), total amplified alleles, polymorphic alleles, total loci and PIC value in all *Brassica* species and *E. sativa*.

S #	SSR Marker	Reported band size (bp)	Observed band size (bp)	TA	PA	TL	PIC value
1.	BRMS-19	220	250-500	2	2	23	0.47
2.	PBCE-B040	90-150	50-200	4	3	71	0.71
3.	RA2-E12	189	150-200	3	3	33	0.60
4.	BRMS-08	145	50-108	3	3	46	0.56
5.	NA14-D07	150-175	50-100	2	2	25	0.40
6.	NI2-B01	256	150-200	3	3	33	0.63
7.	OII2-Eo3	110-250	112-200	2	2	19	0.41
8.	BRMS-oo1	139	50-136	5	4	88	0.74
9.	Ni2-C12	50-200	100-110	3	3	27	0.62
10.	Ra2-E03	225-275	229-245	2	2	35	0.44
11.	RA3-H10	141	50-150	2	2	25	0.48
12.	Pbcju11	206-236	201-225	3	3	38	0.63
13.	PBCGSSRB07	157-250	162-238	4	4	58	0.68
14.	Ra2-D04	50-250	50-200	2	2	35	0.65
15.	Na12-A07	20-170	50-100	2	2	30	0.48
16.	NA10-GO9	300-755	250-800	5	5	84	0.79
17.	NA10-D11	205-550	200-600	4	4	53	0.75
18.	NA10-B08	100-175	125-160	2	2	27	0.50
19.	NA10-Eo2	120-200	134-190	3	3	28	0.66
20.	NI2-BO3	100-250	150-200	2	2	20	0.42
21.	NA12-AO8	150-300	50-200	3	3	48	0.65
22.	BRMS-27	205	100-500	2	2	32	0.48
23.	Pbc-NA3	150-550	200-500	2	2	25	0.50
24.	RA2-G09	141	50-150	3	3	31	0.53
25.	PBC-NA8	200-425	275-400	4	4	51	0.74
26.	PBCE-SRJU7	188-224	200-210	2	2	32	0.50
27.	Na12-Co7	200-250	50-200	3	3	40	0.44
			Single allelic polymorphic markers:				
28.	BRMS-37	154	135	1	1	17	-
29.	PBCJU-8	203-226	200-250	1	1	16	-
30.	BN8-3B1	194	50-200	1	1	11	-
31.	NI3-G05	100-200	110-145	1	1	16	
	Total	-	-	**81**	**79**	**1117**	
	Average			**2.61**	**2.55**	**36**	
			Monomorphic marker:				
32.	Na10-B10	-	-	-	-	-	

T_A= Total alleles, P_A = Polymorphic alleles, T_L = Total loci, bp = Base pairs

Genetic similarity matrix and cluster analysis: For the assessment of genetic relatedness between and within *Brassica* genomes and their relation with *Eruca*, dice coefficient values were computed using the binary data from the scoring of common primers in all genomes. On average, the values of dice coefficients fell in the range of 0.30 showing more distant groups to 0.94 showing closely related species. On basis of these genetic distances, cluster analysis was performed. The dendrogram obtained from cluster analysis revealed that the major cluster was divided into two sub-clusters (Fig. 6). First cluster comprised of all accessions of *Brassica* species including diploids and tetraploid, while the second cluster comprised of all accessions of the wild relative *E. sativa*. In 1st cluster, there was distribution of genomes in a way that tetraploid that originated from its diploid parental genomes, was placed in between of parental genomes with varying pattern of divergence towards any of the parent.

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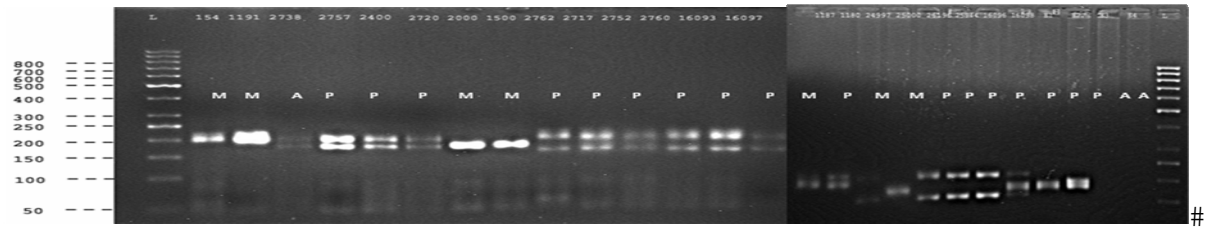

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Fig. 2. SSRs banding patterns in *Brassica* species and *E. sativa* generated by microsatellite primer Na12-Co7.

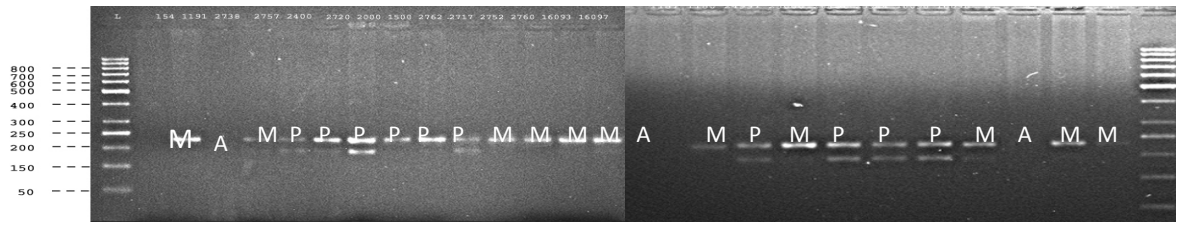

Fig. 3. SSRs banding patterns in *Brassica* species and *E. sativa* generated by a microsatellite primer PBCE-SRJU.

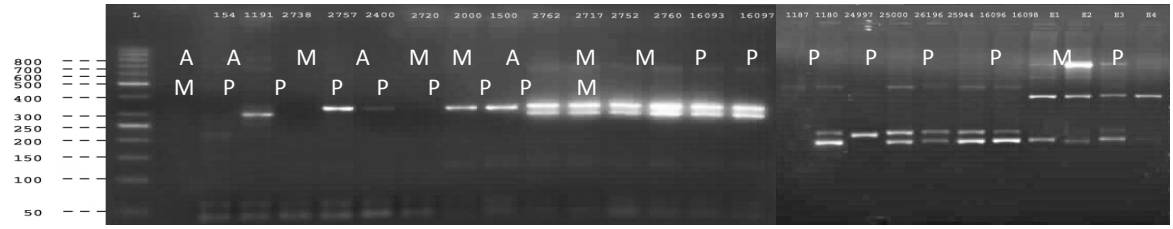

Fig. 4. SSRs banding patterns in *Brassica* species and *E. sativa* generated by a microsatellite primer PBC-NA8.

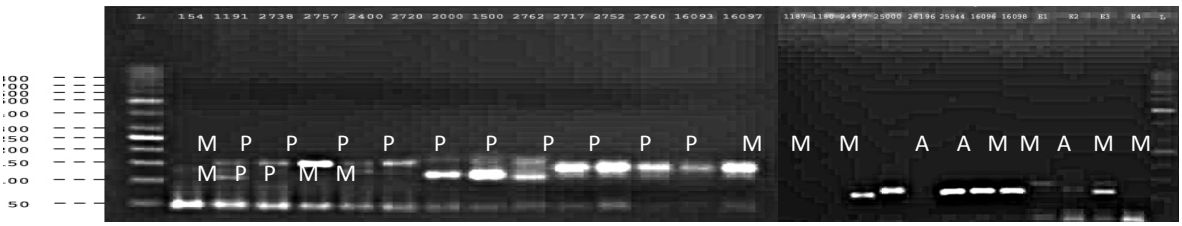

Fig. 5. SSRs banding patterns in *Brassica* species and *E. sativa* generated by a microsatellite primer Ra2-G09.
Lane represents: L. Ladder or marker of 50bp (Top left); *B. nigra* (N-154, N-1191) diploid parent (BB genome); *B.juncea* (J-2738, J-2757, J-2400, J-2720) tetraploid hybrid (AABB genome); *B. campestris* (C-2000, C-1500) diploid parent (AA genome); *B.napus* (N-2762, N-2717, N-2752, N-2760) tetraploid hybrid (AACC genome); *B.oleracea* (O-16093, O-16097) diploid parent (CC genome); *B. nigra* (N-1187, N-1180) diploid parent (BB genome); *B. carinata* (Ca-24997, Ca-2500, Ca-26196, Ca-25944) tetraploid (BBCC genome); *B.oleracea* (O-16096, O-16098) diploid parent (CC genome); *E. sativa* (E1-15, E2-7, E3-7, E4-15) (Top right).

Discussion

Overall, from 31 set of SSR markers, a total of 81 alleles were detected with varying banding patterns. This result was in agreement of Lowe *et al*., (2001) who studied 12 SSR primers derived from *Brassica* species of U-triangle and all these primers were polymorphic. In the present study, allele size ranged from 90 to 300bp in these 31 SSR markers. Polymorphic bands in *Brassica* species

were also observed by other researchers (Ahmad *et al*., 2013 & 2014; Cansian & Echeverrigarary, 2000; Geraci *et al*., 2001; Cartea *et al*., 2005; Tekelewold & Becker, 2006) in brassica crops. Maximum numbers of alleles were amplified by primer pair BRMS-001 and NA10-G09, which showed that these primers can be used efficiently in *Brassica* species as well in *E. sativa* for their ability for polymorphism detection. The results were supported by the findings of Suwabe *et al*., (2002). The highest value for PIC (0.79) was attained by a primer pair Na10-G09, while the minimum PIC value (0.40) was obtained by a primer pair Na14-D07. Same results for this primer set were also observed by Hassan *et al*., (2004). In studying all three genomes of *Brassica* and their comparison with *Eruca sativa*, it was observed that less primer sets amplified polymorphic bands in B, C genome (25 primer pairs) with 448 loci having 95% polymorphic alleles as compared to A, B genome (28 primer pairs) with 454 loci having 97% polymorphic alleles and AACC genome (27 primer pairs) with 525 loci having 96% polymorphic alleles. Results showed that maximum loci were detected in AACC genome but high polymorphism was observed in AABB genome with 454 loci. These results are in agreement with Poulsen *et al*., (1994), who stated that there is an uneven distribution of SSR within *Brassica* species. In this study, this relationship was proved with the utilization of molecular tools which was in agreement with that of Demeke *et al*., (1992), Lagercrantz & Lydiate (1996) and Snowdon *et al*., (1997). However, two accessions of *B. nigra* fell into separate cluster than their amphidiploids and diploid species. The possible reason may be collection from diverse origins. These results were in agreement with that of Warwick *et al*., (1992). The genetic similarity between these two accessions was 0.79 which meant that they were more close to each other, while the genetic difference was more between these two accessions with other two accessions of *B. nigra* having range from 0.46 to 0.59, which means that despite of being same species, they exhibited diversity as they belong to different origins. These results were supported by Zhou *et al*., (2005) who stated that the similar morphotypes form different origins are different due to their separate breeding and domestication. *Eruca sativa* fell into a separate cluster due to its different genus from rest of the *Brassica* species. However on the basis of dice coefficients, it was estimated that the minimum genetic distance of 0.61 was observed between accession E1 from Saudi Arabia and two accessions of *B. juncea* 2720 and 2400 from Pakistan as compared to other species of Brassica. The maximum genetic distance (0.30) was observed between E4 from Pakistan and one accession of *B. carinata* i.e. 26196 from Germany. The possible reason for this relation may be that *E. sativa* is evolved from Mediterranean and Asiatic origins and also the two accessions of *B. juncea* belonged to the Asiatic origins, while the accession of *B. carinata* was from non-Asiatic origins. These results were in line with the findings of Zeven & de Wet (1992). Description of origins is not enough for proving the relatedness of *E. sativa* with *B. juncea*. Form above findings, it was observed that *Eruca sativa* showed maximum genetic distance with *B. carinata* although they were evolved from same Asiatic regions. So the valid reason is the contribution of AA genome parent in *B. juncea*. However, *B. carinata* didn't show any resemblance at any level of study with *E. sativa*. The reason is that it contains BBCC genome. All these findings postulate that there are some conserved sequences between *B. juncea* and *E. sativa*, which are the reason for their more relatedness as compared to other *Brassica* species. These findings were also in correspondence to the previous findings of Westman & Kresovoch (1998) who proposed the relatedness of *B. juncea* and *B. carinata* with wild relative of Brassicaceae family which included *Sinapsis*, *Arabidopsis*, *Raphanus* and *Diplotaxis*. Our studies for comparison of wild relative with *Brassica* species were coinciding with previous researches (Harberd & McArthur, 1980; Warwick & Black, 1993; Prakash *et al*., 1999; Warwick *et al*., 2000; Snowdon *et al*., 2007).

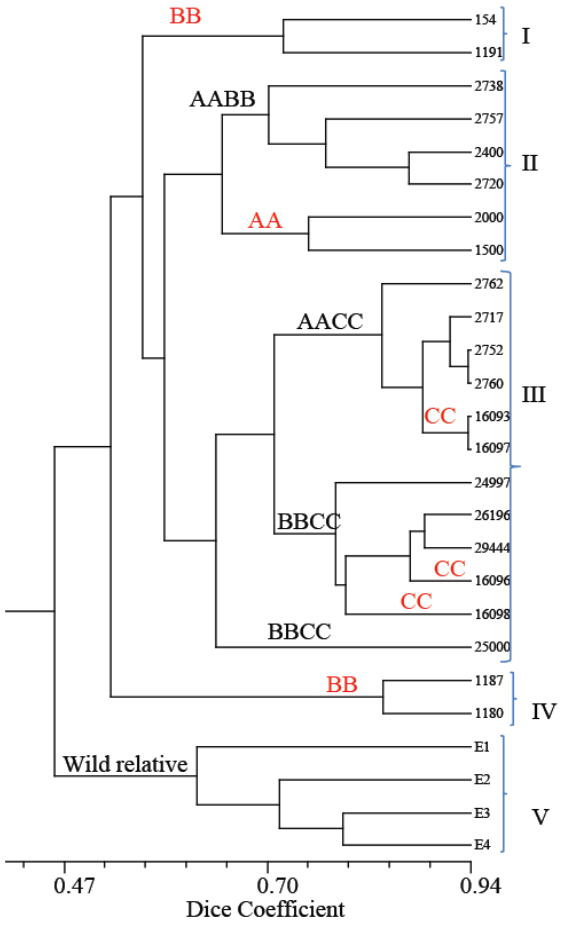

Fig. 6. Dendrogram representing the genetic relationship of *Brassica* species from U-triangle and their wild relative *E. sativa*.

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