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GENETIC DIVERSITY IN THE LOCALLY COLLECTED *BRASSICA* SPECIES OF PAKISTAN BASED ON MICROSATELLITE MARKERS

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Abstract

Genetic diversity among 120 different accessions of *Brassica* species were characterized with the help of SSR markers. These species include *Brassica rapa, B. juncea* and *B. napus*. 39 SSR primers were used and they produced 162 scorable bands in which 105 were polymorphic. The average rate of polymorphic loci was 46%, which indicates high genetic diversity among the accessions. The UPGMA cluster analysis revealed two main clusters and nine sub-clusters. From the gene pool of the collected Brassicas considerable variation was observed among the *B. rapa* accessions, which clearly differentiate the *B. rapa* of northern part of the country from the *B. rapa* of rest of the country. Groupings also reflected geographical similarities and suggested misidentification of certain accessions in the germplasm collection. Based on our study, SSR analysis proved to be a useful tool in assessing the genetic diversity of leafy Brassica germplasm.

Introduction

Brassica is a highly diverse genus of plants belonging to the family Brassicaceae (or Cruciferae). It contains species that are of great economic importance since most species are the source of edible oil, forage, ornamental and vegetable in the world. Almost all parts of a Brassica plant have been developed to be edible including roots, stems, buds, leaves, flowers and seeds (AVRDC, 2000). Brassica crops consist of three primary species viz., *Brassica rapa* (Chinese cabbage, n = 10), *B. oleracea* (Kale, n = 9), and *B. nigra* (Koch, n = 8) and three amphidiploids, *B. carinata* (n = 2x = 17), *B. juncea* (n = 2x = 18) and *B. napus* (n = 2x = 19) (Ren *et al*., 1995). Three amphidiploids arose from crossing and paleopolyploidization among the primary species. Although most Brassica crops originated from Western Europe and the Mediterranean, East Asia is the major secondary center of diversity for leafy Brassica. Because of the allogamous breeding system in Brassicas, morphological and botanical variability in the many subspecies and cultivar groups of *B. rapa* and *B. juncea* has increased (Li, 1981; Lee, 1982; Opena *et al.*, 1988). Genetic diversity is very important to a successful crop improvement. It helps in the protection of our food supply by broadening the range of genes available to meet agricultural production challenges (Pervaiz *et al*., 2010). The evaluation of genetic diversity that presents in germplasm collections promotes the efficient use of genetic variation in establishing a breeding program (Paterson, 1991; Rabbani *et al*., 2010).

There have been studies on identifying genetic relationships of Brassica species using different genetic markers. Ren *et al*., (1995) used RAPD markers to assess the diversity of Brassicas. An *et al*., (2000) studied the genetic relationships among Brassica species based on RAPD markers used to provide information of the fitness of selected parents for crossing and the improvement of breeds of Brassica species. Furthermore, Zhao *et al*., (2005) inferred genetic relationships within *B. rapa* using AFLP fingerprints.

Microsatellites or SSRs (simple sequence repeats) are tandem repeat sequences having less than six base pairs. They are very polymorphic due to the high mutation rate affecting the number of repeat units. They are also very abundant and randomly distributed in the genome. Polymorphisms of SSR can be easily detected on high-resolution gels (Gianfranceschi, 1998). SSRs are advantageous over other DNA-based markers because they are co-dominant, evenly distributed in the genome, and allow the identification of many alleles at a single locus. It also requires only a small amount of DNA for PCR (polymerase chain reaction) analysis. SSR markers have been developed and characterized for the efficient use of genetic studies of Brassica species (Sadia *et al*., 2010). More recently, Louarn *et al*., (2007) used database derived SSR markers for cultivar differentiation in *B. oleracea*.

Therefore, an effort was made to characterize the collected Brassica species applying SSR markers to assess the relationships among economically important leafy Brassica species including *B. rapa, B. napus* and *B. juncea.* Since the genetic diversity of Brassica species of the country is not well intact, by identifying the promising genotypes can be used in future breeding projects.

Materials and Methods

Plant materials: The accessions included in study were 28 rapeseed (*B. napus*), 41 Indian mustard (*B. juncea*), 51 turnip rapeseed (*B. rapa* syn. *B. campestris*) and three commercially released varieties as a check (Table 1). These accessions were collected from different geographical locations of Pakistan.

Microsatellite marker analysis: Seeds of the selected accessions were grown in pots. DNA was extracted from young leaves of 3-week-old seedlings by using a standard protocol (Doyle & Doyle, 1990). After the extraction of DNA, the purity and concentration of the obtained DNA was calculated using NanoDrop ND-2000 Spectrophotometer (Wilmington, USA). Before using for microsatellite study, all DNA samples were diluted to a working concentration of 200ng/µl with TE. Bulk genomic DNA samples were made from 5 individual seedlings of each accession for studying SSR analysis.

Table 1. List of *Brassica* species selected for SSR study.

No.	Accession	Location	No.	Accession	Location	No.	Accession	Location
	Brassica napus		40.	24949	Vehari	80.	25051	Bunner
1.	Shiralee	Check	41.	24933	Bahawalpur	81.	25023	Sialkot
2.	24854	Narowal	42.	24934	Lodhran	82.	25038	Chakwal
3.	24852	Okara	43.	24911	Islamabad	83.	25039	Kalar Kahar
4.	24882	Dara	44.	24952	Chakwal	84.	25055	Bannu
5.	24873	Swabi	45.	24920	Faisalabad	85.	25058	Abakhel
6.	24872	Rawalpindi	46.	24955	Pannu Akil	86.	25059	Shahbazkhel
7.	24881	Gilgit	47.	24930	D.G.Khan	87.	25060	Paharkhel
8.	24842	Islamabad	48.	24984	Mardan	88.	25062	Hassanabdal
9.	24887	Haripur West	49.	24923	Rajanpur	89.	25066	Sheikhan
10.	24874	Nowshera	50.	24971	Lasbela	90.	25046	Batgram
11.	24897	Batgram	51.	24954	Faisalabad	91.	25047	Balakot
12.	24875	Charat	52.	24982	Mattani	92.	25048	Karak
13.	24867	Mianwali	53.	24966	Kohat	93.	25029	Peshawar
14.	24864	Bannu	54.	24936	Lodhran	94.	25069	Miandam
15.	24907	Nowshera	55.	24912	Islamabad	95.	25056	Mangla
16.	24901	Lakki Marwat	56.	24959	Ghizer	96.	25034	Attock
17.	24903	Charsada	57.	24960	Swat	97.	25064	Haji Shah
18.	24843	Islamabad	58.	24972	Haripur	98.	25030	Hazara
19.	24908	Bunair	59.	24975	Khuzdar	99.	25031	Haripur
20.	24888	Swat	60.	24980	Naseerabad	100.	25032	Haripur
21.	24906	Risalpur	61.	24964	Mansehra	101.	25012	Naseerabad
22.	24876	Chamkani	62.	24967	Hangu	102.	25036	Multan
23.	24905	Akora Khattack	63.	24981	Ziarat	103.	25037	Mianwali
24.	24896	Wazirabad	64.	24935	Rawalakot	104.	25024	Hunza
25.	24891	Shabqadar	65.	24974	Pakpattan	105.	25071	Malam Jabba
26.	24866	Khairabad	66.	24978	Hunza	106.	25079	Juglote
27.	24861	Kurram Agency	67.	24977	Chilas	107.	25084	Sundas
28.	24860	Mahandri	68.	24979	Hunza	108.	25028	Swat
	Brassica juncea		69.	24991	Bannu	109.	25022	Hungu
29.	BARD-1	Check		***Brassica rapa***		110.	25075	Chunda
30.	24958	Bunair	70.	BSA	Check	111.	25021	Mastung
31.	24983	Takhtbhai	71.	25027	Skardu	112.	25054	Nawarkhel
32.	24970	Pabbi	72.	25035	Charsada	113.	25076	Hashupi
33.	24939	Pashin	73.	25085	Hunza	114.	25077	Parhi
34.	24963	Mansehra	74.	25068	Mardan	115.	25078	Sultanabad
35.	24941	D.I.Khan	75.	25018	Batkhela	116.	25081	Chilas
36.	24942	Sheikhupura	76.	25045	Haripur	117.	25080	Sundas
37.	24932	Mianwali	77.	25033	Jassian	118.	25074	Kota
38.	24927	Rahim Yar Khan	78.	25049	Nigger	119.	25067	Rustam
39.	24916	Islamabad	79.	25050	Bunair	120.	25083	Sherqila

A total of 55 SSR primers were selected for the genetic diversity analysis on the basis of various published articles on oilseed Brassica diversity (Lowe *et al*., 2002; Tommasini *et al*., 2003; Low *et al*., 2004). Of these, 10 primers produced monomorphic bands and 6 produced unsatisfied results, therefore they were not included in the further study. Microsatellite primer pairs used in the study were obtained from Hokkaido Science System (Sapporo, Hokkaido, Japan). SSR analysis was performed following the protocol of Hasan *et al*., (2006) with minor modifications. PCR amplification reactions were carried out in a total volume of 20µl containing 1x PCR buffer (10mM Tris HCl pH 8.3, 50mM KCl), 2.0mM $MgCl_2$, 200µM each of dNTPs, 0.5µM of each forward and reverse primer, 1 unit Taq DNA polymerase (Fermentas Life Sciences) and 20ng template DNA. The reaction was carried out in MyGene Series Peltier Thermal Cycler (UniEquip GmbH, Munich, Germany). Thermal cycler was programmed to 1 cycle of 5 min at 94°C as an initial hot start and strand separation step. This was followed by 35 cycles of 1 min at 94°C for denaturation, 1 min for annealing temperature depending on the marker used (55°C-65°C) and 2 min at 72° C for primer elongation. Finally, 1 cycle of 7 min at 72°C was used for final extension. Amplified products were stored at -20°C until further use. The reproducibility of the amplification products was checked twice for each primer.

Electrophoresis of amplified products: After amplification, a 10µl aliquot of the amplified SSR samples was combined with 3µl of a loading buffer (0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanole and 5ml of glycerol) and was analyzed directly on 3% (w/v) Gene Choice High Resolution agarose (CLP, USA) gels in 1xTBE buffer (10mM Tris-Borate, 1mM EDTA) containing 0.5µg per ml of Ethidium bromide. A 20bp DNA ladder

(Fermentas Life Sciences) was used as a size marker to compare the molecular weights of amplified products. After electrophoresis, the gels were documented using an UVI Doc. Gel Documentation System (JICA, JAPAN).

Allele scoring and data analysis: Electrophoresis of PCR products on agarose gel produced many bands. The size of these bands was compared with the DNA ladder (size marker). The data was entered into a binary matrix as a discrete variable based on presence and absence of bands. Those presented bands were marked as 1 and the absented bands were scored as 0. The most informative primers were selected based on the extent of polymorphism. The polymorphic information content (PIC) value of a marker was calculated according to Anderson *et al*., (1993). Mean allele numbers, PIC values, and genetic similarities were calculated on the basis of different Brassica accessions. Pair-wise comparisons of the genotypes based on the proportion of unique and shared amplification products (alleles) were used to measure the genetic similarity by Dice coefficients using PAST program (Hammer *et al*., 2001; Pervaiz *et al*., 2010). Genetic similarities (F) between all pairs of the accessions were calculated according to Nei & Li (1979). A dendrogram was constructed using pair-group method to get genetic relationships among accessions.

Results and Discussion

After studying the presence of diverse bands, a total 164 bands were produced by using 39 primers and among these 105 were polymorphic (Fig. A, B and C). On the basis of polymorphic average, a total of 2.7 polymorphic bands were generated by each primer. Among the studied primers, 22 primers were identified as the best primers for Brassica genetic diversity as they produced the rich polymorphisms. Of these, the highest polymorphic rates were produced by the primer Ra2-E07, which produced 5 polymorphic bands, whereas the least number of polymorphic bands (1) were generated by the primers Ol10-A05 and Na10-B10. The percentage for polymorphism ranged from 19% to 100% with an average of 46%. Our findings were supported by the earlier reports of Chen *et al*., (2000); Agrama & Tuinstra (2003); Osborn & Lukensd (2003); and Stephanie *et al*., (2009) who also reported the highly polymorphic rate of SSR markers. The results obtained from this study are very important for the future scientists who want to use different microsatellite primers for the genetic study of the local accessions or germplasm. In this study, the primers Na12-C03, Ra2-D04 and Ra2-E11 are the most recommended for such purposes because they were able to demonstrate the highest polymorphism.

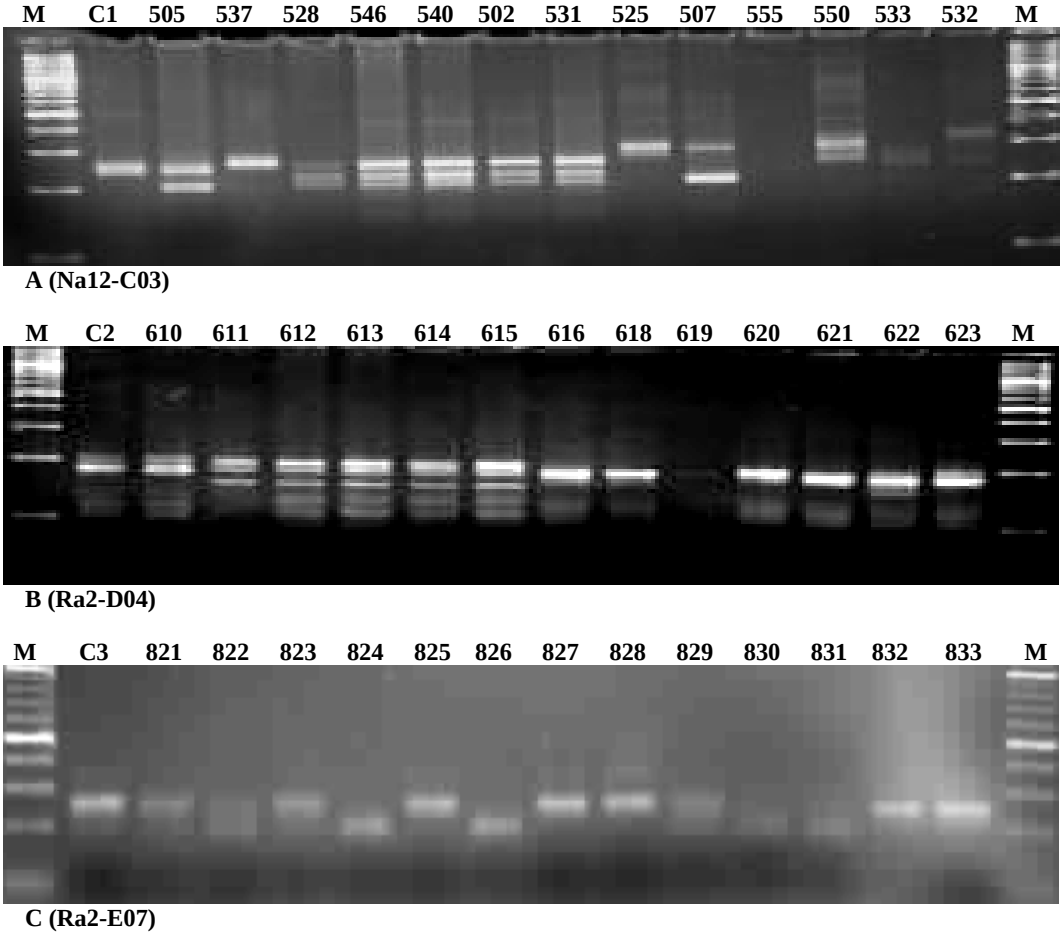

Fig. 1. SSR banding pattern of oilseed *Brassica* accessions generated by primer pair Na12-C03 (A), Ra2-D04 (B) and Ra2-E07 (C) in *B. napus*, *B. juncea* and *B. rapa* landraces, respectively. The lanes represent M-molecular size marker and numbers as given in Table 1.

The banding pattern obtained after running the PCR product on agarose gel showed that the bands produced by the *B. rapa* species were different from the other Brassica species and also showed high level of polymorphism. The possible reason for that high genetic diversity in *B. rapa* accessions may be the local originations, because most of the *B. rapa* originated from the subcontinent and therefore there are a large number of that species. Our findings were further supported by the past findings of Ren *et al*., (1995) who also obtained large variations in *B. rapa* species. Similar results were reported by Chyi *et al*., (1992). From the present study it was also observed that some accessions of the *B. juncea* showed same banding pattern and those were that *B. juncea* which were obtained from the north of the country as *B. juncea* originated from India and north of the Pakistan also adjacent to India. Therefore those accessions had some relations with the *B. rapa* species. Data on the basis of both morphological and biochemical level observed earlier in the present study of these accessions also showed similarity with *B. rapa* accessions. Therefore further study of these accessions should be done to further clarify the identifications of these accessions.

The level of polymorphism among the 120 accessions was evaluated by calculating PIC values for each of the 39 SSR primers. The PIC values varied widely among loci and ranged from 0.17 (Ni4-D09) to 0.75 (Ra2-E07) with an average of 0.461 per locus (Table 2).

Table 2. Details of SSR markers used, indicating *Brassica* motif type, number of alleles detected, allele size range and polymorphism information content (PIC).

Marker/primer	Motif type	Total alleles	Polymorphic alleles	Size range	Difference	PIC value
Na10-B08	di GA/CT	4	3	145-100	45	0.33
Na10-B10	tri GGC/GGT	3	1	130-80	50	0.56
Na10-C06	di GA/CT	4	2	270-220	50	0.56
Na10-D03	di GT/CA	5	3	155-120	35	0.37
Na10-D09	di GT/CA	4	2	240-180	60	0.41
Na10-D11	di GA/CT	3	2	180-135	50	0.52
Na10-E02	di GA/CT	4	3	135-115	20	0.27
Na10-F06	tri GGC/CCG	4	2	120-70	50	0.39
Na10-G08	di GA/CT	3	2	285-200	85	0.33
Na10-G10	di GA/CT	4	3	220-160	60	0.42
Na12-A02	di GA/CT	4	3	290-240	50	0.50
Na12-A07	di GT/CA	5	3	155-125	30	0.36
Na12-C07	di GA/CT	3	3	180-120	60	0.31
Na12-C08	di GA/CT	4	3	320-220	100	0.49
Na12-D04	di GT/CA	4	2	290-230	60	0.51
Na12-E02	tri TTG/AAC	4	2	110-65	45	0.52
Na12-F03	di GA/CT	5	3	300-240	60	0.49
Na14-C12	di GA/CT	4	2	240-180	60	0.39
Na14-D07	tri GGC/CCG	4	2	140-90	50	0.47
Ni2-B01	dis. di GT/CA	4	3	250-200	50	0.51
Ni2-B02	tri GGC/CCG	3	2	85-45	30	0.45
Ni2-B03	di GA/CT	4	3	135-100	35	0.34
Ni2-F02	di GA/CT	4	2	250-160	90	0.45
Ni4-A03	di GA/CT	4	3	225-190	35	0.50
Ni4-D09	di GA/CT	4	3	200-140	60	0.17
Ol9-A03	di GA/CT	5	3	160-130	30	0.59
Ol10-A05	di GA/CT	3	1	210-140	70	0.37
Ol10-B01	di GA/CT	4	3	180-150	30	0.54
Ol11-H02	tri AAT/AAG	4	3	180-140	40	0.55
Ra2-A11	di GA/CT	6	3	300-220	80	0.52
Ra2-D04	di GT/CA	4	3	150-105	45	0.45
Ra2-E03	di GA/CT	3	2	280-225	55	0.39
Ra2-E07	di GA/CT	8	5	150-120	30	0.75
Ra2-E11	di GA/CT	4	3	185-120	65	0.45
Ra2-E12	di GA/CT	5	2	190-150	40	0.42
Ra2-F11	di GA/CT	5	3	240-160	80	0.44
Ra2-G09	diGA/CT	4	3	140-80	60	0.37
Ra2-H06	di GT/CA	4	3	200-110	90	0.56
Ra3-H10	di GA/CT	3	3	120-80	40	0.54
Total		**164**	**105**			**17.98**
Average		**4.21**	**2.69**			**0.46**

A similarity matrix based on the proportion of shared SSR fragments was used to establish the level of relatedness between the various collected Brassica accessions. Pair-wise estimates of similarity ranged from 0.11 to 0.67 and the average similarity among all 120 accessions was 0.41. Accessions 825 and 826 had the highest similarity index of 69%. This was followed by 68% similarity between two accessions 820 and 821. The lowest similarity (11%) was observed between accession Shiralee and 833. These results were further strengthened by the earlier findings of Das *et al*., (1999); and Cansian & Echeverrigaray (2000) who observed more or less similar range of genetic dissimilarities in Brassica lines.

On the basis of cluster study, the total accessions were distributed into two main clusters. These two main clusters were further sub-divided into nine sub-groups. Among these nine sub-groups, six fell in main cluster one and the remaining three sub-groups were part of main cluster two (Fig. 2 and Table 3). In the main cluster one the six sub-groups mainly comprised of the *B. napus* and *B. juncea* and also some accessions of *B. rapa*. These findings strengthened our earlier morphological and biochemical results which showed that there was less variations among the accessions of *B. napus* and *B. juncea* and also some kind of similarity among *B. rapa* accessions. These accessions of *B. rapa* were collected from the same geographic region. While cluster two mainly comprised of *B. rapa*. These were accessions of *B. rapa* which were mostly collected from the northern belt of the country. These findings strengthened our earlier assumptions that the flora and fana of the northern part of the country still intact. It is evident from the cluster analysis that the *B. rapa* accessions of the northern part of the country are quite different from the other part of the country. This confirms that geographic affinity would contribute to the similarity between accessions (Ren *et al*., 1995).

Table 3. Main cluster, sub-groups among the main clusters and total number of accessions in each cluster.

Main cluster	Sub-group	Total accessions
Cluster-I	G1	14
	G2	10
	G3	16
	G4	13
	G5	16
	G6	14
Cluster-II	G7	14
	G8	09
	G9	14

As discussed above, accessions belonging to different subspecies but were collected from the same region were more similar to each other than to accessions of the same species but of different origin. Zhao *et al*., (2005) made the same observation as they studied different morphotypes of *B. rapa* using AFLP markers. Their studies suggested an independent origin in both sites of collection and/or a long and separate domestication and breeding history in both regions.

Conclusions

Several conclusions were obtained from this molecular study: (1) Genetic diversity was found among the studied Brassica accessions. High ratio of genetic diversity was obtained in *B. rapa* accessions than in *B. napus* and *B. juncea* accessions. The possible reason for high genetic variation in *B. rapa* is the local adaptability and existence. According to literatures, *B. rapa* originated from the Himalayan regions of subcontinent due to that reason large number of local *B. rapa* still found in that part of Pakistan. The reason for low genetic diversity in other Brassica species is their genetic erosion as well as using the same genotype again and again in our breeding programmes as a result the genetic background of these genotypes become narrow. (2) The germplasm collected from Northern areas of the country had clear cut variations as compared to other part of the country. The possible reason for that variation is that flora and fauna found in that part of the country is still intact and have full of genetic variations. So it is recommended that if we explore the flora and fauna of Northern areas of the country that will be used in the future breeding programme. In this way one can save one's precious existing varieties from genetic erosion and deterioration and at the same time can increase our agricultural productivity. (3) Also the accessions collected from some part of Baluchistan showed some kind of variations. But in general the accessions collected from other geographical origin of the country present close relationship between each other and have low genetic diversity. (4) SSR markers proved to be useful in the germplasm characterization of Brassica species in future studies.

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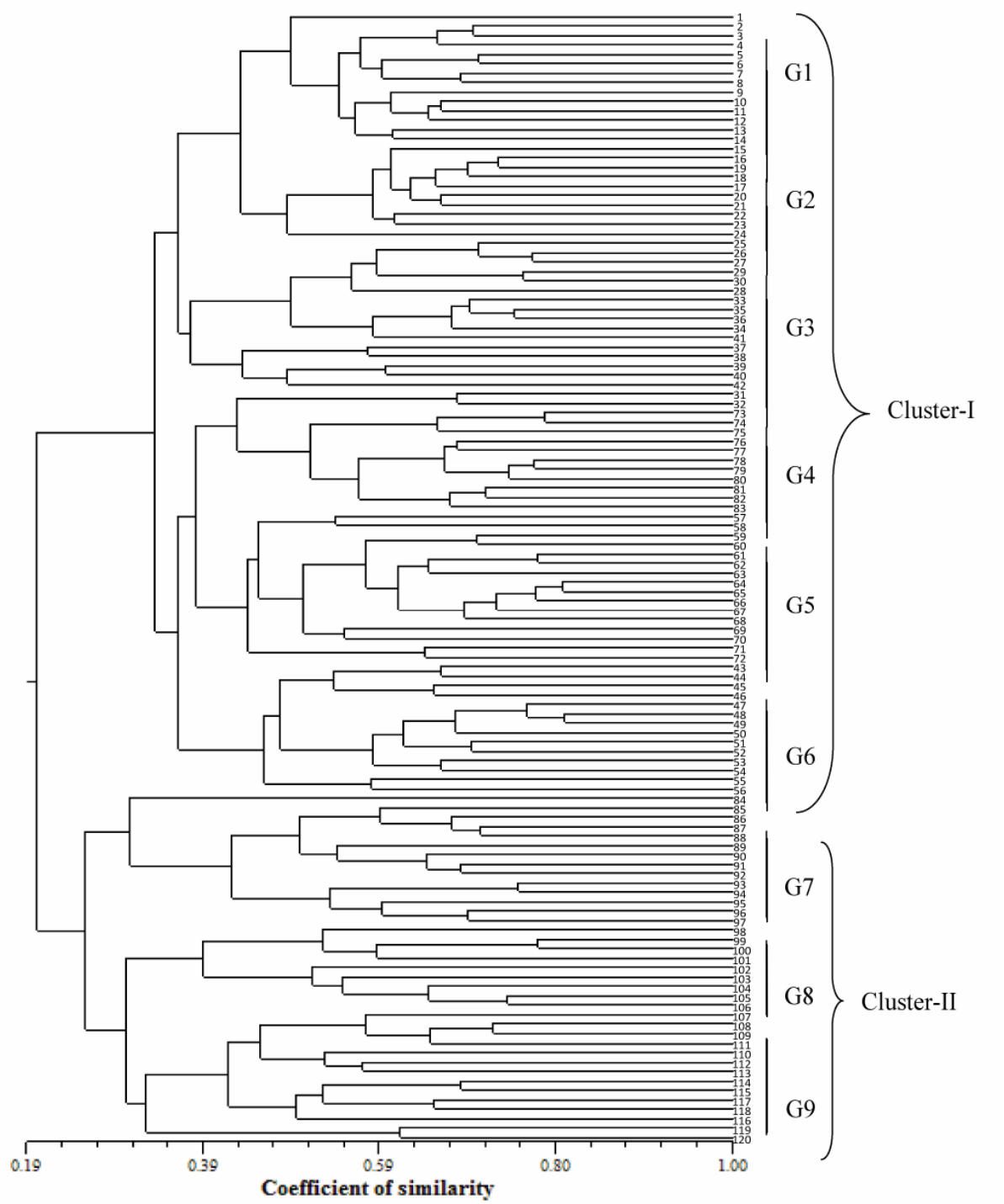

Fig. 2. UPGMA cluster analysis showing the diversity and relationship among 120 *Brassica* accessions based on 164 alleles generated by 39 SSR markers.

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