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Genetic Diversity of *Brassica juncea* L. Accessions Using Isozyme and RAPD Markers

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Abstract: The genetic variability within species and the distribution of genetic diversity among populations are crucial for improvement of crop species. The main objective of this study was to assess genetic diversity on the basis of isozymes and RAPDs in 15 mustard (*Brassica juncea* L.) accessions. Using nine isozymes, twenty-two loci with 51 alleles were detected. 64% of the detected loci were polymorphic in at least one accession. The average number of alleles per polymorphic locus was 2.14 and effective number of alleles per locus was 1.54. Two accessions PI280637 and PI470241 exhibited one unique allele. A total of 54 polymorphic amplicons were obtained using five RAPD primers. Genetic distances among accessions based on isozyme and RAPD markers were calculated. RAPDs showed higher genetic distance average (0.614) than that (0.449) obtained with isozyme markers. This implies higher levels of genetic diversity and differentiation among accessions using RAPDs than isozyme markers. Based on the genetic distances of isozymes and RAPDs, dendrograms were constructed. Both dendrograms based on RAPDs and isozymes exhibited three main phylogenetic groups. However the accessions of each main cluster group were not identical. The RAPDs-based dendrogram showed a kind of notable geographic origin compared to isozymes-based dendrogram.

Key words: Isozymes · RAPD · *Brassica juncea* L. · Genetic distance

INTRODUCTION

Brassica. juncea L. Czern. and Cross. species is one of the six most agriculturally and economically important crop species of genus *Brassica* (*B. rapa* (AA), *B. nigra* (BB), *B. oleraceae* (CC), *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC)). The mustard (*B. juncea* (L.) Czern. and Cross.) is widely cultivated in Asia and Europe [1]. It is oilseed, salad, condiment, green manure or fodder crop. It is principally self-pollinated, although cross-pollination (7.5%-30%) occurs in nature [2]. *Brassica juncea* (2n = 36, AABB) was first naturally developed in the Middle East by cross between its putative ancestral parents species *B. rapa* (2n = 20, AA) and *B. nigra* (2n = 16, BB). This gave rise to current *Brassica juncea* L. that appeared in the region of Eastern India, Caucasus and China, which are the major centers of diversification [3]. The *B. juncea* L. has a number of potential advantages over *B. napus* including enhanced seedling vigour, blackleg resistance and shatter resistance. Moreover it has tolerance to drought and high temperature stresses [4, 5]. Therefore, *B. juncea* L. can serve as a most important source of genes, which are rare in other oilseed *Brassica*s. The information related to the extent and nature of genetic diversity within a crop species is crucial for characterizing individual accessions and cultivars as well as in the selection of parents for hybridization and eventually for future improvement of this crop [2].

Various types of markers have been used for studying the genetic variability including morphological traits, total seed proteins, isozymes and DNA molecular markers [2]. DNA-based molecular markers are neutral. They are informative and superior over traditional types of markers such as morphological traits and biochemical markers [6-8]. Isozyme makers have been used widely to describe the genetic structure of populations [9]. Isozymes reflect variability in protein coding sequences and thus are selectively restrained in ways that noncoding regions are not represented [10]. RAPD (random amplified polymorphic DNA) markers [11-13] have been increasingly employed for population studies. They are simple compared to other DNA-based markers like RFLPs which is labor intensive, time consuming and expensive. As well as, RAPD markers need no prior sequence knowledge [13]. Therefore, they facilitate studies of large numbers of loci and are expected to provide a far more random sample of genomic DNA than do isozymes. However, RAPDs also have significant limitations compared to isozymes. Most important is that

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the large majority of RAPD loci provide biallelic, dominant markers. This limits RAPDs, especially in studying diploid tissues and may bias some population genetic parameters when compared with codominant, multiallelic markers such as isozymes [14]. Studies on conifers [15, 16] have suggested that inferring RAPD allele frequencies from diploid tissues may result in overestimates of population differentiation when compared with isozyme markers.

In the genus *Brassica*, isozymes and proteins have been employed to characterize various genotypes [17], to demonstrate interspecific variability [18-20] and to evaluate phylogenetic relationships among different taxa [21]. RAPDs have been applied to develop genome specific markers [22], to find out genetic relationships between species [23-25] and to evaluate genetic diversity among germplasm [2, 26, 27]. They have also been used for cultivar identification [28-31].

The objectives of the study were to assess the genetic variability among *Brassica juncea* L. accessions as well as to determine the genetic relationships using isozyme and RAPD markers.

MATERIALS AND METHODS

Plant Material: Fifteen *Brassica juncea* L genotypes were kindly provided by U.S. National Plant Germplasm System (NPGS, USDA-ARS). The material included four cultivars, nine landraces collected from different localities and two wild accessions. The origins of the different accessions, the seed bank codes as well as local plant names are shown in Table 1.

Isozyme Analysis: Crude protein extraction was done by grinding, on ice seven-day old seedlings in 0.1 M Tris-HCl buffer pH 7.5 containing 1% β-mercaptoethanol, 0.001 M EDTA, 0.01 M $MgCl_2 6H_2O$, 0.01 KCl and 4% PVP 40,000 [32]. The homogenate was centrifuge at 4°C at 15,000 ×g for 20 min. The supernatant with soluble proteins was stored at -20°C until use. Electrophoretic separation of the extracts was carried out on 10% native PAGE following the procedure described by Laemmli [33] and gels were stained for nine different isozyme systems: Acid phosphatase (ACP, E.C.3.1.3.2), Alkaline phosphatase (ALP, E.C.3.1.3.1), Aspartate amino-transferase (AAT, E.C.2.6.1.1), α-esterase (α-EST, E.C.3.1.1.1) and β-esterase (β-EST, E.C.3.1.1.1), Glutamate dehydrogenase (GDH, E.C.1.4.1.3), Malate dehydrogenase (MDH, E.C.1.1.1.37), Peroxidase (PER, E.C.1.11.1.7) and Polyphenol oxidase (PPO, E.C. 1.14.18.1) using appropriate staining procedures. The gels of ACP, AAT, α-EST, β-EST, GDH, MDH, PER enzymes were stained according to the protocols described by Soltis *et al.* [32] while gels for ALP and PPO were stained following Murphy *et al.* [34] and Sato and Hasegawa [35], respectively.

Table 1: List of *Brassica juncea* L. accessions used in this study

No.	Accession code	Accession type	Country	Local plant name
1	Ames725	Cultivar	USA	Oriental Mustard
2	PI175602	Landrace	Turkey, Kayseri	-
3	PI208734	Landrace	Cuba	-
4	PI249555	Landrace	Thailand	-
5	PI280637	Landrace	Ethiopia	CARINATA
6	PI379103	Landrace	Yugoslavia	CANAKLIJSKI ELE
7	PI426178	Landrace	Afghanistan	K-1072
8	PI432367	Landrace	Bangladesh	BAU-M/18
9	PI470241	Landrace	Indonesia	Sawi
10	PI531267	Cultivar	China	GAI-CAI-TAI
11	PI531271	Cultivar	Hungary	VITTASSO
12	PI633089	Landrace	Russian Federation	Stepnyanka
13	PI633109	Wild	Germany	CR 78/90
14	PI633113	Cultivar	Italy, Calabria	BRA 1209/87
15	PI633115	Wild	Mongolia	96S-114

DNA Isolation and RAPD Analysis: Total genomic DNA was individually extracted from young leaves of ten days old seedlings using Qiagen DNeasy Plant Mini Kit (QIAGEN, Germany). RAPD analysis was performed according to William *et al.* [11] with some modifications. Amplification reactions were performed in a volume of 50 µl. Reaction mixtures contained 1× PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 10 *p*mol of 10-mer primer, 3 units Taq DNA polymerase and 50 ng template DNA. Arbitrary primers, OPA-01, OPA-02, OPA-03, OPA-04 and OPB-01 (Operon Tech., Alameda, CA, USA), were used for PCR amplification. The PCR amplification was performed in Biometra thermal cycler (Biometra GmbH, Germany) for 40 cycles after initial denaturation at 94°C for 3 min. Each cycle consisted of 94°C for 1 min, 36°C for 1 min and 72°C for 90 sec and a final extension at 72°C for 7 min. The RAPD amplicons were separated on 1.5% agarose gels in 1x TAE buffer for 2 h at 110 V and then visualized with UV light after staining with ethidium bromide. 50 bp DNA ladder (Promega, Madison, USA) was used as a size marker.

Data Analysis: Based on isozyme allele frequencies, percentage of polymorphic loci (*P*), mean number of alleles per polymorphic locus (*Ap*), mean number of alleles per locus (*A*), effective number of alleles per locus

(A_e), observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated. Percent of polymorphic loci at accession level (P) was calculated by dividing the number of loci polymorphic within an accession by the total number of loci analyzed. The Ap (mean number of alleles per polymorphic locus) values were calculated based on A (mean number of alleles per locus). Population genetics software package POPGENE [36] was used to calculate A_e, H_o and H_e. Nei's genetic identity values [37, 38] were generated for each pair-wise comparison of populations using GENESTAT2 [39]. A tree illustrating the genetic distances among accessions, using unweighed pair-group method with arithmetic average (UPGMA) and based on Nei's genetic distance (40), was generated utilizing NTSYS version 2.1 [41].

The RAPD bands were scored as 0 (absence) or 1 (presence). The genetic distance among accessions was calculated using POPGENE software [36] according to Nei [37]. The obtained genetic distance matrix was analyzed by unweighed pair-group method with arithmetic average (UPGMA) cluster analysis, to generate a dendrogram by the program NTSYS version 2.1 [41]. The goodness of fit of the cluster analysis, for both of isozyme and RAPD data, was determined by calculating the cophenetic value matrix from the tree matrix to perform the Mantel test [42] to determine the agreement between the two matrices.

RESULTS

Isozyme Diversity: A total of 22 isozyme loci with 51 alleles were detected by means of nine enzyme systems in the 15 accessions of *Brassica juncea* L (Fig.1). Fourteen of the 22 loci (64%) were polymorphic in at least one accession. The only locus detected for alkaline phosphatase was monomorphic. The percentage of polymorphic loci (P) ranged from 23% in PI432367 [8] and PI531271 [11] to 50% in PI249555 [4] and PI633109 [13] with an average of 36.6% of all the accessions (Table 2). The average number of alleles per locus was 1.31. However, the average of number of alleles per polymorphic locus was 2.14. They ranged from 2.00 in five accessions, Ames725 [1], PI379103 [6], PI426178 [7], PI531267 [10] and PI633115 [15] to 2.43 in accession PI470241 [9]. The effective number of alleles per locus ranged from 1.36 in PI531271 [11] to 1.79 in PI633115 [15] with an average of 1.54. The average of the observed heterozygosity (H_o) was 0.542. The expected heterozygosity (H_e) value ranged from 0.182 in PI531271 [11] to 0.393 in PI633115 [15]. Two accessions, PI280637 [5] and PI470241 [9] exhibited one unique allele each that was not fond in any other accessions (Fig. 1 B and C). This unique allele was found in β-estrase locus 2 and polyphenol oxisade locus 2 (Fig. 1 B and C).

Table 2: Isozyme variation of 15 *B. juncea* L. accessionsbased on 9 enzyme systems

No.	Accession	P	A	Ap	A_e	H_o	H_e
1	Ames725	32	1.18	2.00	1.42	0.424	0.212
2	PI175602	36	1.27	2.13	1.53	0.531	0.267
3	PI208734	36	1.36	2.25	1.53	0.531	0.267
4	PI249555	50	1.41	2.10	1.69	0.689	0.344
5	PI280637	27	1.23	2.33	1.41	0.406	0.203
6	PI379103	36	1.27	2.00	1.56	0.559	0.279
7	PI426178	45	1.41	2.00	1.58	0.583	0.292
8	PI432367	23	1.14	2.20	1.48	0.485	0.242
9	PI470241	32	1.45	2.43	1.50	0.500	0.250
10	PI531267	32	1.32	2.00	1.39	0.395	0.197
11	PI531271	23	1.18	2.40	1.36	0.361	0.182
12	PI633089	41	1.41	2.11	1.58	0.583	0.292
13	PI633109	50	1.41	2.10	1.70	0.697	0.349
14	PI633113	41	1.36	2.11	1.60	0.600	0.300
15	PI633115	45	1.23	2.00	1.79	0.786	0.393
	Mean	36.6	1.31	2.14	1.54	0.542	0.271
	Std.Dev	8.72	0.10	0.14	0.12	0.12	0.06

The values of genetic identity between pairs of accessions, ranged between 0.992 and 0.410. The highest value was 0.992 for accessions Ames725 [1] and PI531267 [10] and the lowest value 0.410 was for PI432367 [8] and PI531271 [11]. The average genetic identity was 0.701. The lowest genetic distance value (0.007) was between accessions Ames725 [1] and PI531267 [10] and the highest genetic distance value (0.891) was between accessions PI 432367 [8] and PI531271 [11]. The average genetic distance was 0.449. The estimated genetic diversity parameters, over all 22 loci in the 15 populations, indicated that the mean value of total genetic diversity for the species (H_T) was 0.424. The mean value of Nei's relative genetic differentiation (G_{ST}) was 0.453. The other two important genetic diversity parameters were the genetic diversity among populations (D_{ST}) and genetic diversity within populations (H_S). Their values were 0.192 and 0.231, respectively.

Cophenetic correlation, i.e., the correlation between the cophenetic matrix and the matrix based on Nei's genetic distance, was r = 0.91 indicating a very good fit to the dendrogram derived from the cluster analysis. The dendrogram of genetic distance indicated that Ames725 [1] and PI531267 [10] were alike and that PI280637 [5], PI432367 [8] and PI633113 [14] were considerably different from other 12 accessions. The two accessions, PI432367 [8] and PI633113 [14], grouped separately from the rest of the accessions. Actually, at genetic distance of 0.50, the

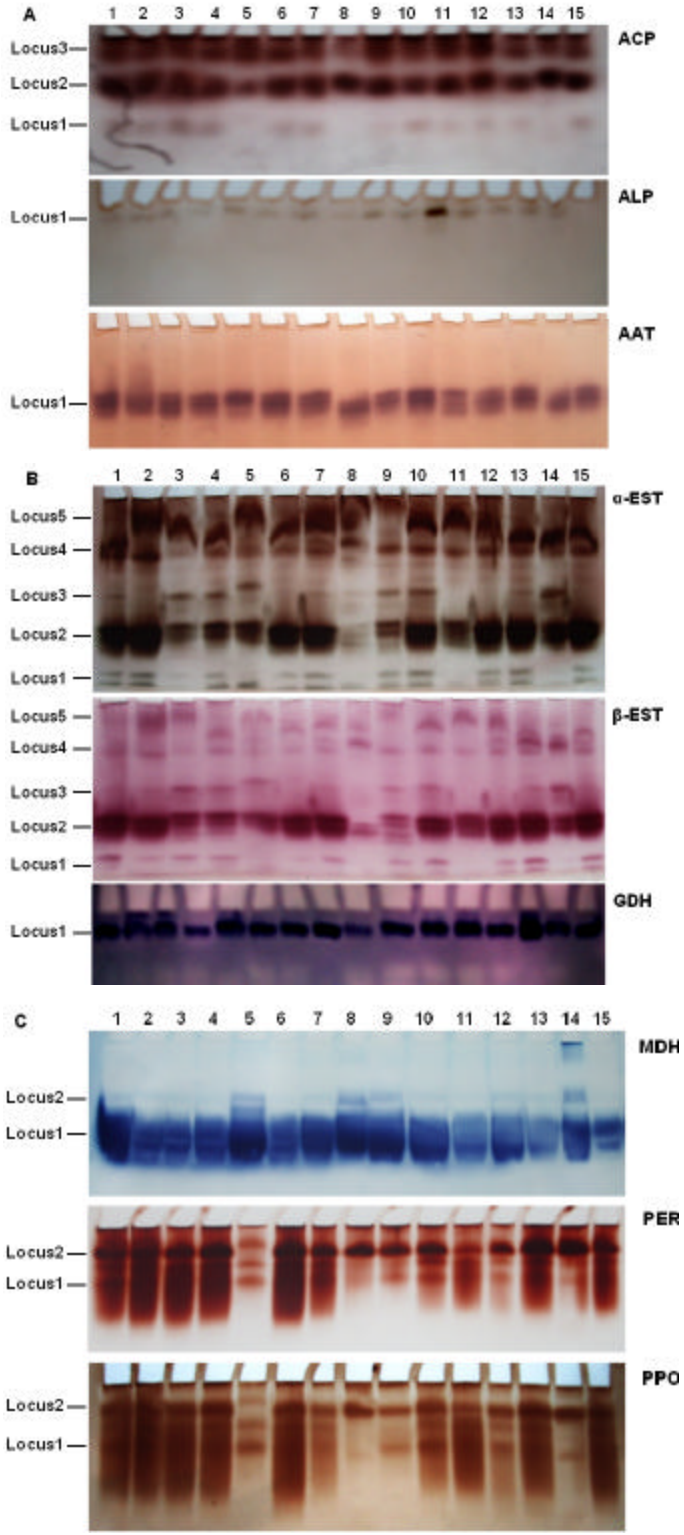

Fig. 1: PAGE zymograms of Acid phosphatase: ACP, Alkaline phosphatase: ALP, Aspartate amino-transferase: AAT (**A**), α-esterase: α-EST, ß-esterase: ß-EST, Glutamate dehydrogenase: GDH (**B**), Malate dehydrogenase: MDH, Peroxidase: PER and Polyphenol oxidase: PPO (**C**) isozyme patterns of 15 *Brassica juncea* L. accessions

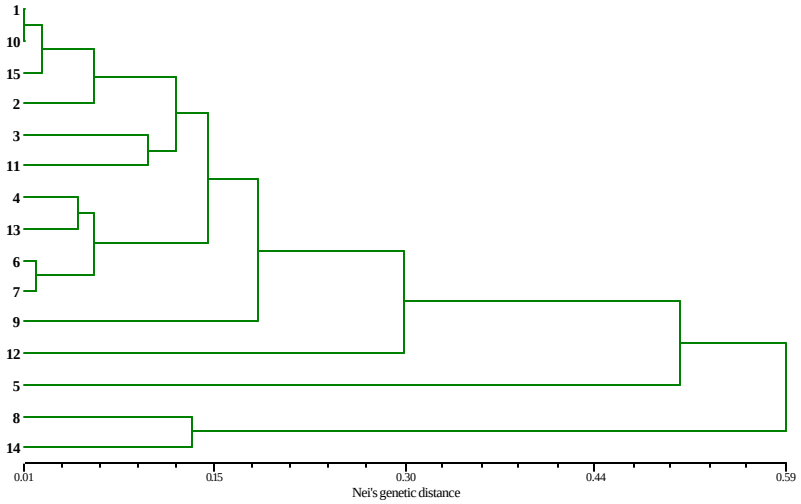

Fig. 2: Dendrogram of UPGMA clustering of 15 *Brassica juncea* L. accessions based on Nei's genetic distance using 22 isozyme loci

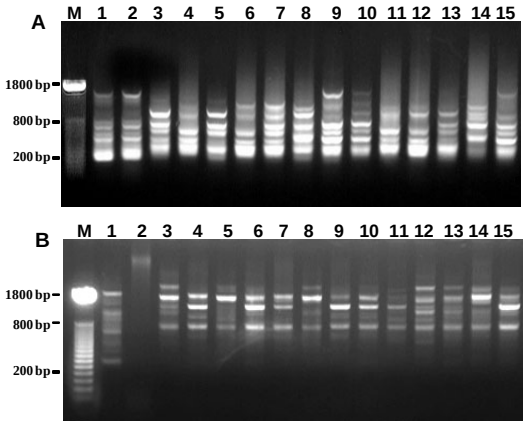

Fig. 3: RAPD profiles of 15 *Brassica juncea* L. accessions generated by OPA-01 (A) and OPA-04. M: is 50 bp DNA ladder

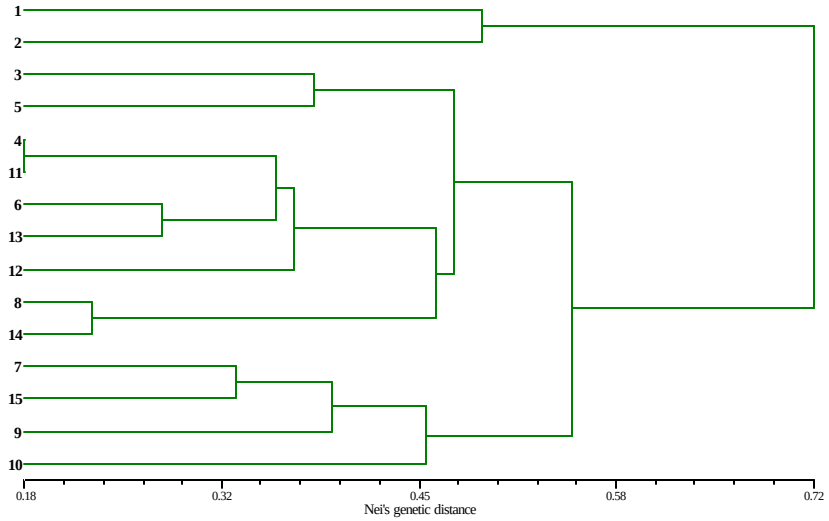

Fig. 4: Dendrogram of UPGMA clustering of 15 *Brassica juncea* L. accessions based on Nie's genetic distance using 54 RAPD markers

Table 3: Nucleotide sequences of five selected primers, number of amplified RAPD bands and number of shared bands by all accessions in 15 *Brassica juncea* L. accessions

Primer	Sequence (5' to 3')	No. of amplified RAPD bands	No. of shared RAPD bands by all accessions
OPA-01	CAGGCCCTTC	11	0
OPA-02	TGCCGAGCTG	11	0
OPA-03	AGTCAGCCAC	12	0
OPA-04	AATCGGGCTG	10	1*
OPB-01	GTTTCGCTCC	10	1*
Total		54	2

* not present in accessions Ames725 (1) and PI175602 (2)

dendrogram indicated three phylogenetic groups (clusters), one containing 12 accessions and the second cluster containing one accession, PI280637 [5]. The third cluster contained two accessions PI432367 [8] and PI633113 [14] (Fig. 2).

RAPD Diversity: A total of fifty four RAPD bands were found in all fifteen accessions used in this study (Table 3). Except for accessions Ames725 [1] and PI175602 [2], one band was observed in all accessions. The amplified RAPD-PCR products ranged from 200 bp to 3500 bp. As an example of PCR amplification with RAPD primers OPA-1 and OPA-04 is shown in Figure 3 A and B. The calculated genetic distances ranged from 0.182 to 1.045. The smallest genetic distance was between accessions PI249555 [4] and PI531271 [11]. The highest genetic distance was between accessions Ames725 [1] and PI208734 [3]. The average genetic distance based on the RAPD data was 0.614.

Based on the genetic distance matrix of the RAPD data, UPGMA clustering used to generate a dendrogram (Fig. 4). The dendrogram showed that the fifteen accessions clustered, at the genetic distance of 0.54, into three main phylogenetic groups (clusters). Two accessions Ames725 [1] and PI175602 [2] represented the first cluster. The second main cluster contained nine accessions PI208734 [3], PI280637 [5], PI249555 [4], PI531271 [11], PI379103 [6], PI633109 [13], PI633089 [12], PI432367 [8] and PI633113 [14]. The second main cluster was subdivided into three subclusters, the first contained two accessions PI208734 [3] and PI280637 [5]. The second subcluster represented by five accessions PI249555 [4], PI531271 [11], PI379103 [6], PI633109 [13] and PI633089 [12]. The remained two accessions PI432367 [8] and PI633113 [14] comprised the third subcluster. The other four accessions PI426178 [7], PI470241 [9], PI531267 [10] and PI633115 [15] represented the third main cluster. Cophenetic correlation test, used to measure the goodness of fit of RAPD cluster analysis, showed r = 0.80 which indicated a good fit of the generated dendrogram.

DISCUSSION

The results of the present study clearly revealed that the fifteen *Brassica juncea* accessions were highly differentiated and the amounts of diversification among accessions (intrapopulation diversity) were high. Moreover, RAPD markers showed higher levels of diversity among population (accessions) than isozyme markers.

The mean value of expected heterozygosity (H_e = 0.271) was high compared to the accepted mean value for all plant species, H_e = 0.141 [43]. Meanwhile it was corresponding to the value recorded for *Brassica*, 0.244 [21]. The number of polymorphic alleles per locus (Ap = 2.14) was higher than that recorded for all plant species, Ap = 1.69 [43]. On the other hand, the percent of polymorphic loci (P = 36.6%) was in concordant with the value (P = 36.8%); reported with Soltis and Soltis [43] and the value reported for *Brassica macrocarpa*, 36.36% [21]. Hence, it is proposing the need of conservation programs in order to preserve the existing diversity. The lower H_e value (0.182) of PI531271 compared to the other populations suggested that the PI 531271 (a cultivar from Hungary) may be a more recently established population [44]. The genetic identity ranged from 0.992 and 0.410 which indicated wider range of genetic identity of the *B. juncea* L.

In the present study, Nei's genetic differentiation (G_{ST}), obtained for the fifteen studied accessions of *B. juncea* L. was = 0.453. This value was higher than the mean genetic heterogeneity G_{ST} = 0.118 recorded by Loveless and Hamrick [45]. However, this result is in accordance with the results found by Làzaro and Aguinagalde [21]. They found high G_{ST} = 0.330 for *Brassica* spp. The high G_{ST} showed that 45% of the total genetic diversity observed was interpopulational while 55% was intrapopulational. The high genetic differentiation among the 15 populations could be due to those populations were from very different distant geographic regions.

Comparison of isozyme and RAPD results is elaborated due to the biallelic and dominant nature of RAPD markers. Therefore, the genetic diversity parameters (P, He, H_o and G_{ST}) could not be calculated for RAPDs. RAPD 0 or 1 data matrix were converted into genetic distance matrix based on Nei's [37]. The RAPDs mean value (0.614) of genetic distance among accessions

was higher than that of isozymes (0.449). This indicated high amount of genetic diversity shown by RAPD markers which could be explained by high rate of detectable mutations as well as low selectivity at RAPD compared to isozyme loci. In addition, RAPDs are assumed to be amplified from noncoding sequence regions and the noncoding sequences generally have higher rates of nucleotide substitutions than the coding ones [46, 47]. Moreover, as reported by William *et al.* [11], RAPD markers are sometimes amplified from regions of repetitive DNA.

The RAPDs-based dendrogram showed relatively higher Nei's [37] genetic distances among accessions than those observed with isozymes. The highest value between populations using RAPDs was 1.024 compared to 0.891 using isozymes. Therefore, it is clear that RAPDs detected higher levels of polymorphism than isoyzmes. Although, this result is in correspondence with Làzaro and Aguinagalde [25], they detected higher genetic distances (1.825) among taxa.

In this study, phylogenetic relationships using RAPD markers indicated that RAPDs are able to geographically distinguish the accessions. Meanwhile, isoyzymes cluster analysis demonstrated mixed-up *Brassica juncea* accessions. Only two accessions PI432367 [8] and PI633113 [14] were similarly grouped together using both RAPDs and isozymes. In contrast to what was found in this study, no correlation between RAPD patterns and the geographic origin of *Brassica juncea* L. accessions from Pakistan [2]. In another study, Ren *et al.* [24] found no association between the similarity and the geographic origin of Chinese vegetable *Brassicas* analyzed with RAPD markers.

In conclusion, higher levels of the average polymorphism have been detected by RAPD loci compared to isozyme loci. This information of genetic diversity in cultivars and their wild relatives, may assist in breeding improvement of crops with the help of the available genetic resources.

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