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Full Length Research Paper

Study of total seed storage protein in indigenous *Brassica* species based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

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Genetic diversity was studied in 234 accessions of locally collected *Brassica* species for total seed protein content through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). These accessions were collected from different locations of Pakistan. After the study of these accessions on SDS-PAGE, 28 reproducible bands were used for cluster analysis and with the help of these bands, genetic diversity were estimated. Out of 28, four major bands were observed. Dendogram was constructed and the accessions were divided into two main groups comprising 11 clusters. The results obtained from these clusters showed minimum genetic diversity in these accessions on SDS-PAGE level. Due to low genetic diversity on SDS-PAGE level, it is suggested that two dimensional (2D)-electrophoresis can be used for protein study.

Key words: Genetic variation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), protein electrophoresis, cluster analysis, brassica.

INTRODUCTION

An immense deal of research has been inattentive on oil crops of various plants, especially members of the mustard family (*Brassicaceae* or *Cruciferae*) such as species of *Brassica*. Widespread reviews did not show much attention on seeds of various plants with respect to their oil extracts. Food with high amount of protein and high percentage of seed oil also increase food value and its shelf life (Munazza et al., 2009). Similarly, plants having high food values also contain certain amount of important drugs. Among the oilseed crop, oilseed rape (*Brassica* and related species, *Brassicaceae*) is now the second largest oilseed crop in the world providing 13% of the world supply. On a large scale, the world demand mainly depends on two species, *Brassica napus* L. and *Brassica rapa* L. The oil and protein percentage in the seed of these species are 40% or more and 30 to 35%,Respectively (Nasar et al., 2006). In a common practice, genetic improvement is easy in those species/crops which have immense genetic diversity and the information regarding these hidden genetic resources is easily available. Research on *Brassica* germplasm could increase the edible oil production and its nutritional benefits; therefore, collection of these higher value genetic resources and the estimation of genetic assortment within and between landraces should have priority for varietal improvement. Further, it is necessary to develop better methods of characterization and evaluation of germ-plasm, in order to improve strategies for conservation and collection of germplasm and increase the utilization of plant genetic resources. As a result, electrophoresis of seed storage protein is a method used to explore genetic difference and classify plant varieties. As we know, seed protein is not perceptive pattern is very to environmental

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Table 1. Presence and absence of protein subunits in SDS-PAGE analysis of Indian mustard *B. juncea*.

Protein zone	Protein band	Number of genotype	
		Presence	Absence
A	1	6	83
	2	85	4
	3	89	0
	4	89	0
	5	86	3
	6	89	0
B	7	89	0
	8	89	0
	9	89	0
	10	89	0
	11	89	0
	12	5	84
	13	89	0
	14	8	81
	15	89	0
	16	89	0
	17	78	11
	18	81	8
C	19	89	0
	20	85	4
	21	79	10
	22	88	1
	23	89	0
	24	86	3
D	25	89	0
	26	89	0

changes; and as a result, its banding steady, in that it advocated for cultivars identification in crop. Similarly, it has also been used for the identification of disputed cultivars during a patent process. Seed storage protein is also used for the comparison of cultivated species with its wild relatives. Although we are using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for different purposes, its application and attentity is still limited. The objective of the present study is to check the genetic variation in these indigenous collected *Brassica* species for seed storage protein with the help of SDS-PAGE.

MATERIALS AND METHODS

A total of 234 accession *Brassica* species of *Brassica* species (*B. napus, Brassica juncea, Brassica campestris and Brassica olaracea*) were used for the total protein content with the help of SDS-PAGE. The studied accessions were collected across the country. For protein content, electrophoresis seeds were grinded to fine powder with the help of mortar and pestle. Being an oilseed crop, we added sample buffer (400 µl) to a 0.02 g of fine seed flour as extraction liquid and bromophenol blue (BPB) as tracking dye to follow the movement of protein in the gel. The active ingredients used for the extraction of protein buffer contained 0.5 M Tris-HCl (pH 8.0), 0.2% SDS, 5 M urea and 1% 2-mercaptoethanol. In order to know the movement of protein in the gel, a dye in the form of bromophenol blue was added to the extraction buffer. When all these chemicals are tightly put together than the solution needs to be purified and homogenated, we mixed the samples thoroughly by vortexing and centrifugation at 15,000 rpm for 5 min at room temperature (RT). After centrifuging samples, the crude proteins were recovered as clear supernatant on the top of the tube. Then the supernatant were transferred into new 1.5 ml eppendorf tubes and were stored at -20°C until gel electrophoresis.

Staining and destaining

After completion of electrophoresis, the gels were stained with 0.2% (w/v) coomassie brilliant blue (CBB) R250 dissolved in a solution containing 10% (v/v) acetic acid, 40% (v/v) methanol and water in the ratio of 10:40:60 (v/v) for an hour. When the staining procedure was completed, the gels were then destained by washing these with a solution containing 5% (v/v) acetic acid, 20% (v/v) methanol and water in the ratio of 5:20:75 (v/v), so that the blue color of the coomassie brilliant blue (CBB) disappears and the electrophoresis bands on the gels were clearly visible. After destaining, gels were dried, using Gel Drying Processor for about two hours.

Data analysis

After the process of staining and destaining of the gel, similarity index was used for the presence or absence of polypeptide bands and possible pairs of protein types were calculated (Tables 1 to 3). Since the objective of the study is to investigate genetic diversity in the collected species, we did not take intensity of the bands into consideration, but only focused on the presence or absence of bands. Presence of the band was scored 1, while 0 for absence of the band. Data compiled after the presence and absence of the bands were subjected to binary data matrix. Based on the results of electrophoretic band spectra, similarity index (S) was calculated for all possible pairs of protein type electrophoregrams by the following formula (Sneath and Sokal, 1973):

$$S = W/(A + B - W)$$

Where, W = Number of bands of common mobility; A = number of bands in protein type 'A'; B = number of bands in protein type 'B'.

After the generation of similarity matrix, the generated similarity matrix was then converted into a dissimilarity matrix (dissimilarity = 1 - similarity) and used to construct dendrogram by unweighted pair-group method with arithmetic averages (Sneath and Sokal, 1973). All the analyses were carried out using statistical package NTSYS-pc, version 2.1 (Applied Biostatistics Inc., USA).

RESULTS AND DISCUSSION

Bulk seed samples were used for total protein comparisons. The banding pattern of some of the total seed protein presented in Figures 1, 2 and 3 showed close relationship among these studied accessions, while the difference in banding pattern showed the range of geographic differences. For *B. napus,* a total number of 28 bands were detected; and among these, four were detected as major bands (Figure 1). There were also observed

Table 2. Presence and absence of protein subunits in SDS-PAGE analysis of rape (*B. napus*).

Protein zone	Protein band	Number of genotype	
		Presence	Absence
A	1	3	67
	2	10	60
	3	8	62
	4	67	3
B	5	62	8
	6	70	0
	7	63	7
	8	68	2
	9	70	0
	10	2	68
	11	70	0
	12	70	0
	13	62	8
	14	70	0
C	15	70	0
	16	65	5
	17	70	0
	18	66	4
	19	70	0
	20	70	0
	21	67	3
	22	61	9
	23	70	0
	24	70	0
	25	66	4
	26	68	2
D	27	70	0
	28	1	69

differences for minor bands, but we only consider the variation in major bands and the reason behind this was the reproducibility and consistency in these bands. The ratio of the polymorphic banding was 60%. However, dendrogram was constructed on the basis of these polymorphic bands.

After the study of the banding patterns in these accessions, four zones were observed (A to D) showing variations. As a result, Zone-A was having protein weight from 116 - 66 kDa. A total of four bands were observed in Zone-A, and among these, two were polymorphic. The protein detected in Zone-A had comparatively, light stained bands. However, the protein in Zone-B ranged from 45 to 35 kDa. Protein bands detected in this region were 10, among which five were polymorphic. Zone- B comprised both light and dark stained bands, while Zone-C ranged from 35 to 25 kDa. Protein bands produced in this region were 12 and polymorphic were seven.

Consequently, the region showed both light and dark stained bands. Zone-D consists of two bands with one y being polymorphic. This region consists of a dark region and the protein banding pattern in this region was from 18 to 14. The study's finding falls in the same category with the early finding of Rabbani et al. (2001) who also reported the same banding region in mustard accessions from Pakistan. Similarly Kour and Singh (2004) obtained the same results.

Table 3. Presence and absence of protein sub units in SDS-PAGE analysis of rapa (*B. campestris*).

Protein zone	Protein band	Number of genotype	
		Presence	Absence
A	1	84	1
	2	85	0
	3	83	2
	4	82	3
B	5	84	1
	6	80	5
	7	81	4
	8	82	3
	9	81	4
	10	84	1
	11	1	84
C	12	84	1
	13	3	82
	14	79	6
	15	2	82
	16	81	3
	17	84	1
	18	82	2
D	19	85	0
	20	85	0

In *B. campestris,* a total of 20 bands were detected and divided into four zones. Zone-A comprised three, among which polymorphic was one of them. Majority of the bands in this region were light stained and the size of this zone lies in between 116 and 66 kba. Zone-B consists of eight bands and three bands were polymorphic. The banding size falls in the range of 66 to 45 kba, whereas the bands lying in this region were light stained. Zone-C comprised seven bands and polymorphic bands in this region were four. This region consists of both light and dark stained. The range of bands in this region was 35 to 25. Zone-D comprised two bands and one is polymorphic among these bands. The colors of band in this region were dark; however, the protein sizes in this region were 25 to 18 kba.

For Indian mustard *B. juncea*, a total of 26 bands were detected. These bands were divided into four main zones. Zone-A consists of six bands and among these, four were polymorphic. The banding range in this zone was 116 - 66 kba and their bands were light stained. Zone-B comprised 12 bands; and among these, seven

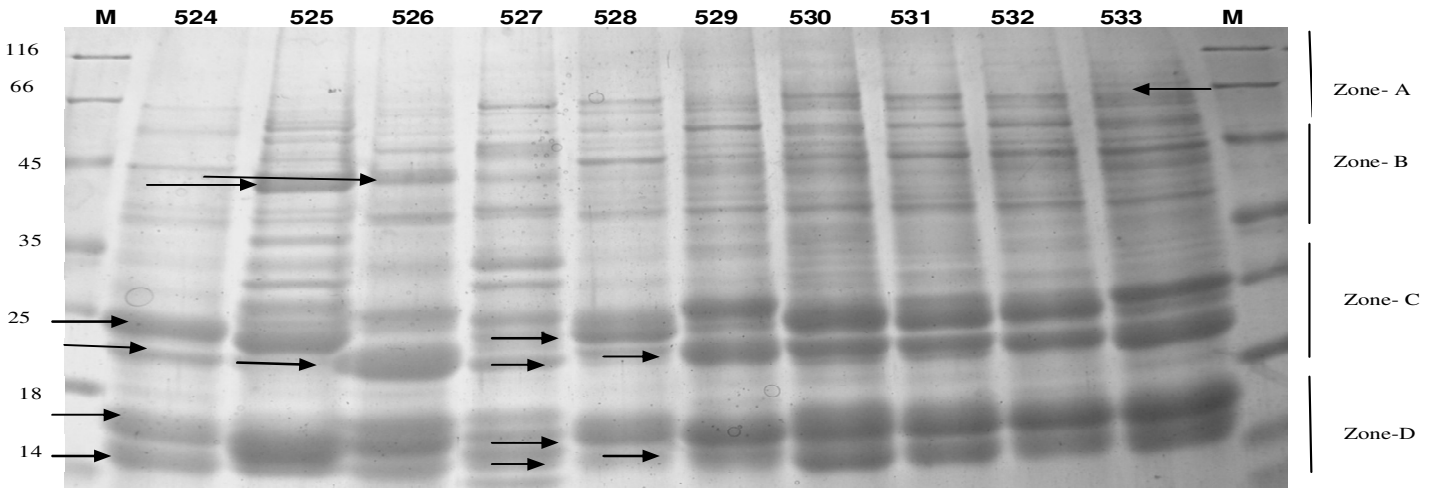

Figure 1. Electrophoretic banding pattern generated by SDS-PAGE of seed storage proteins of *B. napus* accessions. M = Markers, while 511 - 520 = accession numbers given in appendix 1.

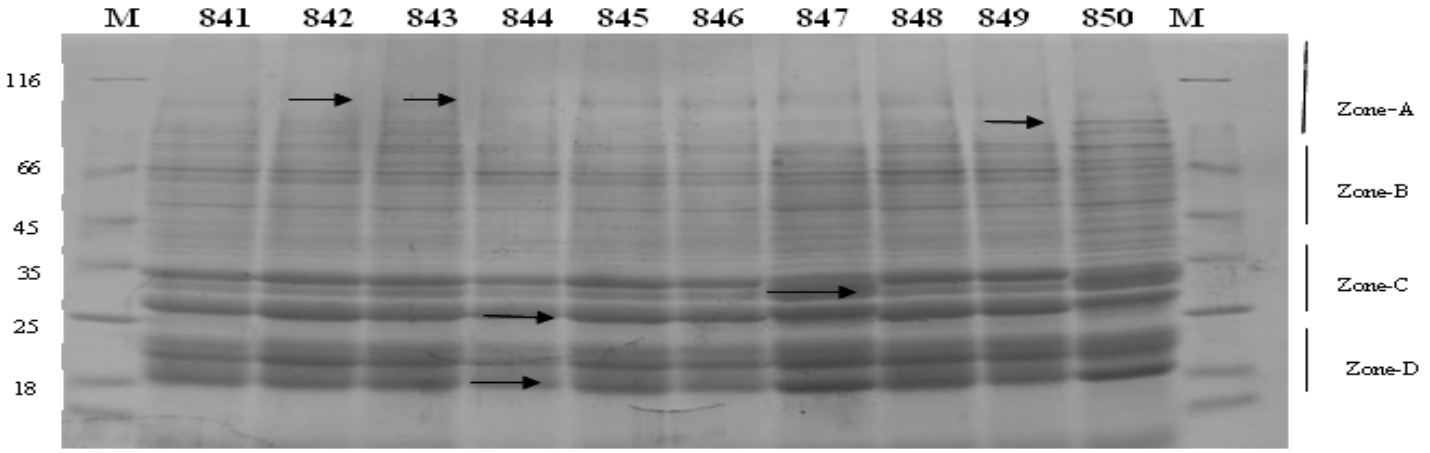

Figure 2. Electrophoretic banding pattern generated by SDS-PAGE of seed storage proteins of *B. napus* accessions. M = markers, while 511 - 520 = accession numbers given in appendix 1.

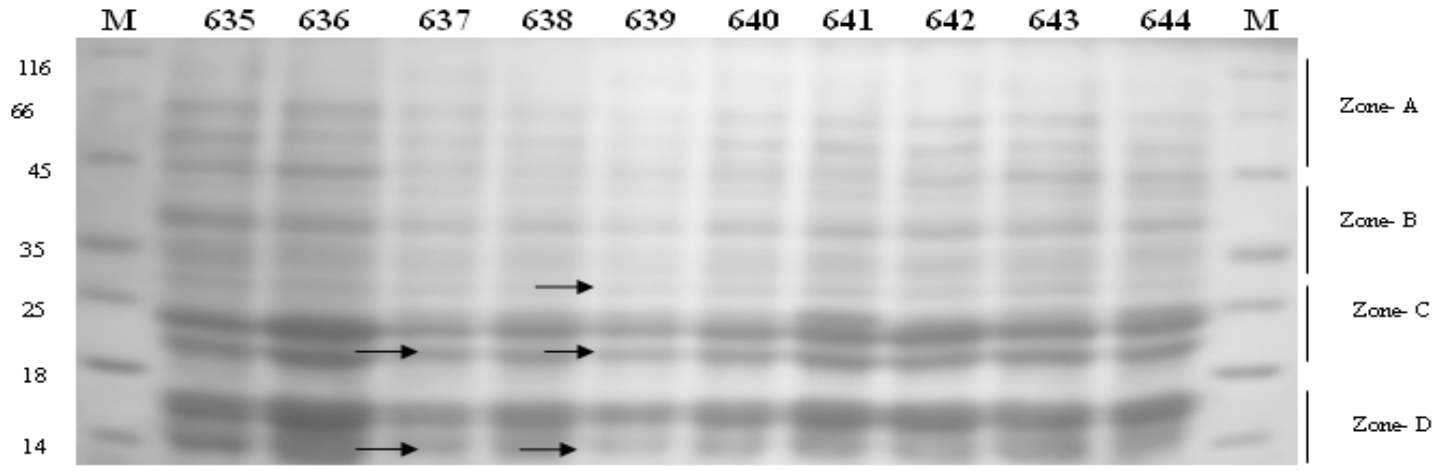

Figure 3. Electrophoretic banding pattern generated by SDS-PAGE of seed storage proteins of Indian mustard accessions. Lanes M = marker; 635 - 644 = accession numbers given in appendix 1.

Table 4. Dice coefficients between different protein types based on SDS-PAGE.

	A(63)	B(4)	525	526	C(2)	D(62)	E(2)	F(2)	G(2)	H(8)	I(2(	J(3)	K(30)	L(30)	M(2)	N(5)	O(6)	P(3)	Q(8)	R(3)
A(63)	1																			
B(4)	0.98	1																		
525	0.95	0.93	1																	
526	0.93	0.91	0.95	1																
C(2)	0.93	0.91	0.95	0.93	1															
D(62)	0.95	0.95	0.92	0.91	0.93	1														
E(2)	0.95	0.95	0.92	0.92	0.93	0.89	1													
F(2)	09.1	0.95	0.92	0.93	0.92	0.93	0.92	1												
G(2)	0.94	0.95	0.92	0.91	0.93	0.77	0.88	0.87	1											
H(8)	0.93	0.95	0.92	0.94	0.91	0.79	0.78	0.91	0.88	1										
I(2)	0.97	0.95	0.92	0.95	0.93	0.83	0.92	0.82	0.92	0.81	1									
J(3)	0.93	0.95	0.92	0.91	0.93	0.82	0.79	0.88	0.71	0.79	0.88	1								
K(30)	0.95	0.95	0.92	0.98	0.88	0.74	0.83	0.96	0.98	0.94	0.94	0.81	1							
L(30)	0.81	0.83	0.87	0.81	0.85	0.82	0.82	0.82	0.82	0.82	0.82	0.82	0.82	1						
M(2)	0.89	0.91	0.91	0.88	0.88	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.84	1					
N(5)	0.89	0.87	0.87	0.85	0.85	0.89	0.89	0.89	0.89	0.89	0.89	0.89	0.89	0.82	0.88	1				
O(6)	0.85	0.83	0.87	0.85	0.85	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.76	0.84	0.88	1			
P(3)	0.96	0.95	0.92	0.88	0.91	0.76	0.99	0.91	0.82	0.74	0.72	0.81	0.77	0.82	0.86	0.89	0.86	1		
Q(8)	0.62	0.59	0.59	0.56	0.51	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.64	0.59	0.49	0.63	0.63	0.64	1	
R(3)	0.59	0.56	0.56	0.52	0.48	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.55	0.45	0.63	0.61	0.61	0.97	1

were polymorphic, having a size of 45 - 35 kba. However, their bands were light stained. Zone- C produced six bands, in which 3 were polymorphic and having a band size of 25 to 18 kba. Bands in this region were both light and dark stained. Zone-D has two bands and one of them is polymorphic. The bands' size in this region was 14 kba and bands were dark stained.

Among the 234 studied brassica accessions, it was noticed that there were close association among these accessions apart from a few accessions. These accessions were found in *B. napus*, *B. juncea* and *B. campestris*. The different accessions, 511, 512, 513, 514, 515, 520, 535, 550, 555 and 560 for *B. napus*; 635, 640, 644, 651, 660, 665, 668 and 670 for *B. juncea* and 805, 808, 811, 818, 820, 828, 840, 844, 850, 853 and 860 for *B. campestris*, were different.

Genetic similarity matrix and cluster analysis

After calculating protein banding patterns, Nei and Li's similarity indices were calculated among the observed protein types. Based on similarity estimates, the ranges among these species were from 45 to 99%. The highest similarity estimates were recorded in P(3) and E(2), followed by 98% in B(2) and A(63) and 97% in I(2) and A(63), while the lowest similarity estimates (45%) were recorded in R(3) and M(2), followed by 49% in Q(8) and M(2) and 51% in Q(8) and C(2) (Table 4) .

After the construction of dendrogram of the total seed proteins, the dendrogram of total seed proteins dependent on dissimilarity matrix using unweighted pair group method with arithmetic

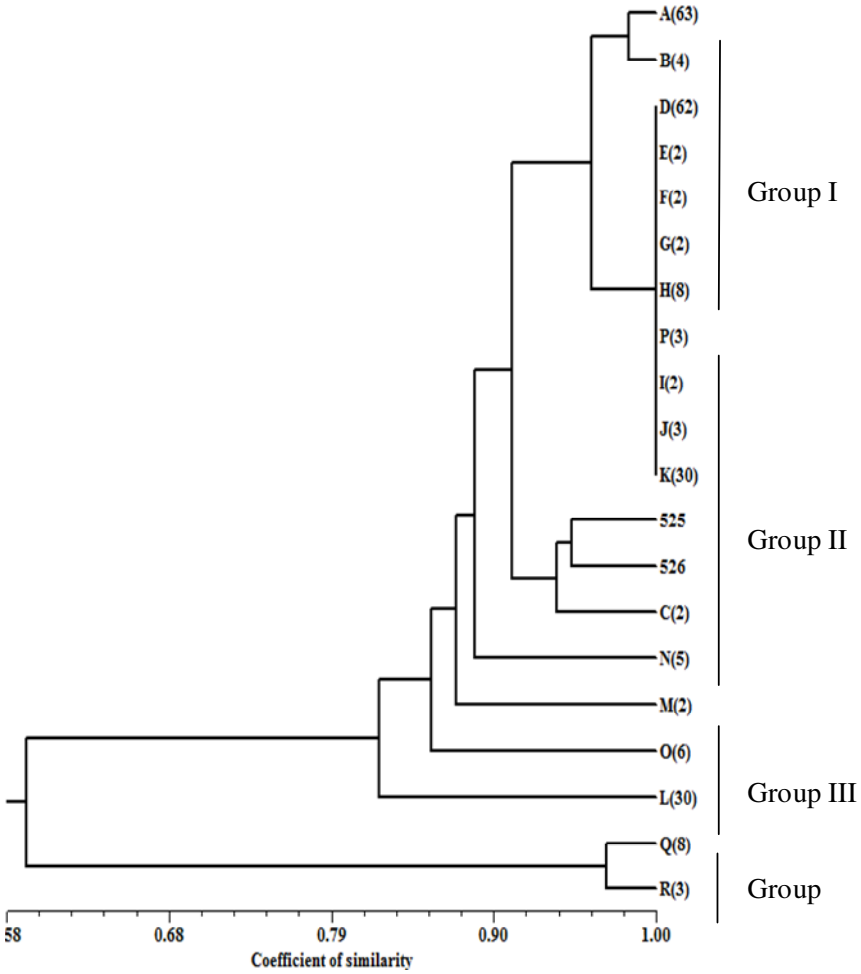

Figure 4. Dendrogram showing the relationship between different protein types in indigenous brassica species based on SDS-PAGE of total seed proteins.

averages (UPGMA) divided the protein into four main groups, I, II, III and IV (Figure 4). Group I comprised 143 accessions, group II has 47 accessions, group III has 38 and group IV has 11 accessions. To study the generic and specific level, poly-acrylamide gel electrophoresis provides the best basic tool (Ladizinsky and Hymowitz, 1979) and proved to be a good asset for classical taxonomic classi-fication. However, protein kinds and their differences between diverse crop species may help in the untimely detection of species at seed level and to dig up the record of transparency of genetic resources (Rahman and Hirata, 2004).

From the verdict of the present study, the genetic diversity in the different *Brassica* species collected was pragmatically inadequate on SDS-PAGE level; but on the other hand, there is clear cut variation between accessions collected from Northern areas and the accessions of the rest of the country. The possible reason for these variations is that the flora and fauna in these areas are still not explored by the scientific community; while in the rest of the country, these have been explored in time past. Therefore, same is the case with *Brassica*. If we crossed these accessions with the rest of the accessions, then there will be a much larger genetic diversity, because the genetic diversity of our local accessions is less and the possible answer for that is the narrow genetic background of these local accessions. Similarly, Rabbani et al. (2001) also observed narrow genetics in Indian mustard having different geographic origin. Earlier researchers focused on SDS-PAGE for species inequity (Vaughan and Denford, 1968; Yadava et al., 1979). After evaluating various *Brassica* species of different varieties of the same species, the study concluded that 21.2% of polymorphism was recorded in a variety of *B. rapa,* followed by 6.3% in *B. napus* and 3.2 and 3.2% in *B. juncea* (Rahman and Hirata, 2004). Rahman et al. (2004) recorded 18.8% polymorphism in 32 genotypes of *B. rapa* collected from Bangladesh, Japan and China. Similarly, Munazza et al. (2009) evaluated 30 accessions of different *Brassica* species for genetic diversity of the total seed protein, though SDS-PAGE found no genetic diversity among these genotypes on protein level. Raymond et al. (1991)

also found that the cluster pattern for sunflower genotypes showed variation having no relation with its locality. Anupreet and Singh (2008) also observed genetic diversity in oilseed brassica genotypes. Similarly, Sihag et al. (2004) also investigated that there is no direct relation between genetic diversity and geographic distribution. Alipour et al. (2002) evaluated the experiment on seed protein level and observed 30 protein bands among these 5 that were among the studied accessions.

From the cluster analysis, it is clear that there is less variation in local accessions. Eleven clusters and 4 groups were constructed after the analysis of these accessions on a qualitative level. The study's finding is further strengthened by the early report of Ghafoor et al. (2003), which also concluded the same results. Similarly, Ghafoor et al. (2002) concluded that SDS-PAGE will be the best tool in the case of interspecific variation rather than intraspecific variation.

The study's result is further strengthened by the early findings of Javaid et al. (2004) who also reported minimum genetic diversity in groundnut for SDS-PAGE and suggested two dimensional (2-D) electrophoresis. The accessions, having alike banding patterns, are suggested to be studied in the future with the help of 2-D electrophoresis and DNA markers. This may be due to the fact that these differences have some important hidden qualities and we can preserve these precious materials in genebank for use in breeding program (Celis and Bravo, 1984; Beckstrom-Sternberg, 1989).

By studying these local accessions, it is concluded that these materials are full of genetic diversity and we can explore this diversity by using modern and advance techniques including 2-D electrophoresis and molecular markers techniques.

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APPENDIX 1

List of the collected *Brassica* accessions from different locations of Pakistan.

Brassica juncea							
Acc. No	**Location**	**Acc. No**	**Location**	**Acc. No**	**Location**	**Acc. No**	**Location**
600	Islamsbad	622	D.G.Khan	644	Islamabad	666	Lasbella
601	Islamsbad	623	Islamsbad	645	Chakwal	667	Haripur
602	Islamsbad	624	Mianwali	646	Faisalabad	668	Mansehra
603	Islamsbad	625	Bhawalpur	647	Faisalabad	669	Pakpattan
604	Islamsbad	626	Lodhran	648	panu akil	670	Khuzdar
605	Islamsbad	627	Rawalkot	649	USA	671	Naseerabad
606	Islamsbad	628	Charsada	650	Bunner	672	Khairabad
607	Sarghoda	629	USA	651	Bunner	763	Chilas
608	Kohat	630	Islamabad	652	Ghizer	674	Hunza
609	Chakwal	631	Pashin	653	Swat	675	Hunza
610	D.M.Jamali	632	Multan	654	Bhawalpur	676	Naseerabad
611	Gilgit	633	D.i.Khan	655	Swabi	677	Ziarat
612	Faisalabad	634	Shiekhupura	656	Mansehara	678	Mattani
613	Islamsbad	635	Shangla	657	Mansehara	679	Takhtbhai
614	Mianwali	636	swabi	658	Batkhawala	680	Maradan
615	Rajanpur	637	Hangu	659	Mattani		
616	Rahim Yar Khan	638	Attock	660	Kohat		
617	Fateh jang	639	Pindi gaph	661	Hangu		
618	Rawalkot	640	Bhakkar	662	Jhelum		
619	Rahim Yar Khan	641	Multan	663	Sari e norung		
620	Islamsbad	642	Vehari	664	Pabbi		
621	Islamsbad	643	Islamabad	665	Naseer abad		

Brassica napus							
Acc. No	**Location**	**Acc. No**	**Location**	**Acc. No**	**Location**	**Acc. No**	**Location**
500	Islamsbad	522	Hassan Abdal	544	Naseer abad	566	Rawalpindi
501	Islamsbad	523	Bannu	545	Jaglot	567	Karak
502	Rawalakot	524	Karak	546	Haripur North	568	Akora Khattack
503	Sibi	525	Khairabad	547	Haripur West	569	Rsialpur
504	Rahim Yar Khan	526	Mianwali	548	Swat	570	Nowshera
505	Rajan pur	527	Mazafar abad	549	Islamabad		
506	D.G. Khan	528	Thank	550	Kasur	*Brassica caranita*	
507	Mianwali	529	Ganche 03	551	Shab qadar	700	Unknown
508	Rajanpur	530	Ganche 02	552	bunner	701	Unknown
509	Pakpathan	531	Diamer 01	553	Kaghan	702	Unknown
510	Okara	532	Rawalpindi	554	Jarid	703	Peshawar
511	Kasur	533	Swabi	555	karak	704	Islamabad
512	Narowal	534	Nowshera	556	Pir Sabak	705	Khairabad
513	tarnul	535	Charat	557	Wazir abad	706	Sheikhupura
514	Rajanpur	536	Chamkani	558	Batgram	707	Mangla
515	Balakot	537	Peshawar	559	Nigger	708	Unknown
516	Lodhran	538	Rawalpindi	560	Bannue 01	709	Unknown
517	Taxila	539	Giligit	561	Laki 08	710	Unknown
518	Kaghan	540	Hunza	562	Laki 09		
519	Mahandri	541	Giligit	563	Laki 10		

APPENDIX 1 Cont.

520	Kurram Agency	542	Dara	564	peshawar		
521	D.G. Khan	543	Shangla	565	Charsada		

Brassica campestris							
Acc. No	**Location**	**Acc. No**	**Location**	**Acc. No**	**Location**	**Acc. No**	**Location**
800	Lasbilla	821	Attock	842	Laki 01	863	Rustam
801	Thall	822	Charsada	843	Laki 02	864	Maradn
802	Islamsbad	823	Multan	844	Laki 04	865	Miandam
803	Mardan	824	Mianwali	845	Nawarhkel	866	D.I.Khan
804	Batkhalla	825	Chakwal	846	Bannue	867	Malam jabba
805	Mendi bhauddin	826	Khalar khar	847	Bannue	868	Malakhand
806	Rawalpindi	827	Gujrat	848	Abakhel	869	Charsada
807	Shiekhupura	828	Faisalabad	849	Abakhel	870	kota
808	Mastug	829	Sangarh	850	Swabi	871	Chunda
809	Hngue	830	Ghazi	851	Manglah 01	872	Hashupi
810	Sialkot	831	Kamra	852	Manglah 02	873	Parhi
811	Hunza	832	Hattian	853	Abdullahkhel	874	Sultana bad
812	Rajanpur	833	Mansehara	854	Abdullahkhel	875	Juglot
813	D.I.Khan	834	Haripur	855	Shahbazkhel	876	Sundas
814	Skardu	835	Batgram	856	Peharkhel 01	877	Chilas
815	Swat	836	Balakot	857	Peharkhel 02	878	Nagar
816	Peshawar	837	Karak 03	858	Hassan abdal	879	Sherqila
817	Hazara	838	Nigger	859	mula mansoor	880	Sundas
818	Haripur	839	Bunner 01	860	Haji shah	881	Hunza
819	Haripur	840	Bunner 02	861	Karimabad (22)		
820	Jassian	841	Bannue 01	862	Sheikhan		