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PHENOTYPIC, BIOCHEMICAL AND MOLECULAR DIVERSITY AND DIVERGENCE IN *BRASSICA* ACCESSIONS REPRESENTING DIFFERENT SPECIES

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Abstract

Genetic improvement in *Brassica* is crucial for the production of sufficient edible oil, which requires exploitation of diverse genetic resources. The present study assessed divergence and diversity of 24 *Brassica* accessions belonging to different species through molecular genotyping, biochemical characterization and field testing. Considering yield contributing parameters, number of siliquas main raceme^{-1} ranged from 39.3 (local genotype) to 48.7 siliqas (IBGE-2); number of primary branches ranged from 3.3 (wester-209) to 6.0 (p118 and Altex 232); number of siliquas plant^{-1} ranged from 173 (Torch-237) to 409 (IBGE-4); seeds siliqua^{-1} ranged from 13 (IBGE-4) to 21 (p-118); yield ranged from 560 g (Mun-1) to 1462 g (IBGE-4). Variability was also observed for oil content (%), which ranged from 47 (Zahoor-15 and IBGE-1) to 53 (Mun-1 and Crusher-212), while the protein content was overall in higher content, with the highest value of 24% (Zahoor-15). Molecular genotyping with a set of 16 polymorphic loci revealed the maximum number of loci (5) for OPC-05, followed by OPC-09 and OPB-04 (4). The maximum gene diversity (0.497) was recorded for loci OPB-04-L3, while the minimum (0.080) was for locus OPC-05-L5. The neighbor-joining tree showed that accessions representing *B. juncea* were dispersed among various genotypes. In network analysis, two clusters were identified. Cluster I consisted of *B. compestres* while cluster II comprised of *B. juncea* with similarity to other *B. napus* crossed genotypes. The divergence and diversity observed in the present study should be useful for genetic improvement.

Key words: Brassica, Genotypic diversity, Phenotyping, Molecular markers, Pakistan.

Introduction

Brassica belongs to the family *Brassica*ceae, which is also called crucifers or oleiferous family. The family *Brassica*ceae consists of 350 genera and more than 3500 species. Six species of the genus *Brassica* are widely used around the world as oilseeds (Cheng *et al*., 2014), among these three are considered as parental species of oilseed *Brassica* which are diploid. These include *B. campestris* or *B. Rapa* AA – 2n=2x=20, *B. nigra* BB - 2n=2x=16 and *B. oleracea* CC - 2n=2x=18. These three ancestral species gave rise to three other amphidiploid or tetrapolid species through inter specific breeding which are *B. juncea* AABB - 2n=4x=36 (Indian mustard), *B. napus* AACC - 2n=4x=38 (rapeseed) and *B. carinata* BBCC - 2n=4x=34 (Ethiopian mustard). Sometime they are called allotetraploid because these consist of two different diploid genomes (Mason & Wendel, 2020).

Brassica family has a high diversity for broad range of phenotypic characters (Christopher *et al*., 2005). *Brassica napus*, *B. juncea*, *B. rapa*, and *B. carinata*, are commonly cultivated and utilized as commercial oilseeds. *B. napus* is often referred to as canola because it possesses low levels of erucic acid and glucosinolates. However, canola-type varieties of *B. rapa,* and *B. juncea* are also available. These varieties were initially developed in Canada, and due to their health benefits, canola varieties are now popular worldwide (Cardoza & Stewart, 2004). Following soybean oil and palm oil, Brassica ranks as the third most important oil-producing crop globally, owing to its adaptability to diverse climatic conditions and cropping systems.

The broad adaptability of rapeseed and thus their cultivation at diverse agroecological zones, including the subsistence rainfed agricultural production systems (Iqbal *et al*., 2014; Ali & Hodson, 2017), plays a key role in oil production (Ton *et al*., 2020). World largest producers of *Brassica* are China, India, Europe and Canada followed by South America, Australia and United States. *Brassica* production was increased significantly in the recent past (Oram *et al*., 2005). During growing session (2017-18) overall cultivated area in Pakistan was 198 thousand hectares with an average yield of 180.7 thousand tons and per area yields of 812 kg/ha (Anon., 2017-18). This yield is much lower than many other *Brassica* growing countries, mainly due to lack of improved varieties adapted to local conditions and resistant to biotic and abiotic stresses. In *Brassica* genetic advance of is the most important component of oilseed breeding programs throughout the world.

Genetic advance in any crop is dependent on the level of diversity and divergence in that particular crop, which serve as the source for genetic improvement. Genetic improvement of *Brassica*, like other crop improvement programs, involves characterization of local materials, which include collection, molecular characterization and field evaluation of the germplasm (Rashid *et al*., 2016). In crop improvements programs, assessment of germplasm diversity and genetic linkage help us to improve our selection efficiency, while assessment of genetic divergence help to identify parental combinations for crossing. Genetic diversity could be obtained from local germplasm accessions, exotic introduced material, segregating population and advanced lines with the use of subsequent selection methods. Indigenous accessions and traditional varieties may carry

some special characters like good quality and flavor along with resistance to biotic and abiotic stresses, compared to introduce materials, however, introduced material could provide high yielding traits (Williams *et al*., 1991). For improvement of quality and quantity of *Brassica* different species the presence of sufficient genetic variability is of utmost importance, which needs to be determined through various available methods.

Characterization of *Brassica* could be achieved through various methods such as molecular, biochemical and field based morphological data (Mohammadi & Prasanna, 2003). Various markers, including DNA, morphological, biochemical, cytological, and genetic markers, have been utilized to assess the presence and extent of genetic diversity (Chesnokov *et al*., 2020). Most of the genetic markers are considered as the most suitable as they are more reliable, efficient and precise in differentiation of likely genotypes. Association mapping and QTL studies may highlight agronomic traits by using DNA markers, which are the powerful tools in modern plant breeding (Hu *et al*., 2006).

Different molecular markers have evolved along with classical breeding tools, to evaluate the genetic diversity and conduct molecular characterization (Iqbal *et al*., 2023a; Iqbal *et al*., 2023b; Iqbal *et al*., 2020). Random amplified polymorphic DNA (RAPD) markers are the easiest and less time consuming, among various molecular markers, which could easily be adopted with least cost and complications (Jan *et al*., 2020). Similarly, simple sequence repeat/microsatellites have also been widely utilized to understand genetic diversity, divergence and temporal variation (Ali *et al*., 2016; Ali *et al*., 2014b; Brar *et al*., 2018). In *Brassica* RAPD markers are extensively used for calculating genetic diversity (Javed *et al*., 2021). In most of the crop plants, RAPD technique is used which is suitable for finding genetic variability, especially when limited resources are available (Naz *et al*., 2019). They are simple, efficient, easy and do not need sequence information (William *et al*., 1991). Such information based on RAPD can provide preliminary information on diversity and divergence, which can be taken into account for further studies with sequencing and genomics tools (Rashid *et al*., 2016; Naz *et al*., 2019). This information could also be coupled with phenotypic data from the field to assess the relationship among various genetic clusters in terms of their phenotypic variability.

Finally, being an oilseed crop, the biochemical characteristics of Brassica germplasm should also be evaluated to identify the candidate lines for further cultivation (Kopsell, 2007; Ali *et al*., 2014a). The overall production is linked with both seed yield and oil content (Oram *et al*., 2005). Considering the biochemical characteristics, both oil content/quality and the protein content/quality are important, as both, oil are used for the human consumption and the remaining cakes are used for livestock.

Considering the importance of characterization of *Brassica* accessions in crop improvement, this study was devised based on assessment of diversity and divergence in the *Brassica* germplasm from various species in relation with the advanced breeding lines/candidate varieties of Institute of Biotechnology and Genetical Engineering (IBGE), University of Peshawar. The objectives of the study were: i). to assess the field-based diversity in *Brassica* accessions for agronomic and morphologic parameters. ii). to genetically characterize *Brassica* accessions using molecular markers to identify the relationship of these lines with the advanced breeding lines and candidate varieties of IBGE. iii). to assess the level of diversity and divergence among accessions from various species and assess its correlation with the phenotypic traits.

Material and Method

The present study aimed to assess the level of diversity and divergence in Brassica accessions from various species through phenotypic, molecular, and biochemical characterization, in comparison to the advanced lines/ candidate varieties at IBGE. The tested *Brassica* genotypes were evaluated for phenotypic variability under field conditions at the IBGE research plots, Agriculture Research Farm, University of Agriculture Peshawar, Pakistan, while the molecular work was conducted at the Genomics and Bioinformatics division.

Selection of germplasm and field testing: A set of 24 *Brassica* lines were selected for the study, which represented three *Brassica* species and four inter-specific cross progenies. *B. napus* was represented by 16 lines, *B. juncea* by three lines and *B. compestris* by a single line (Table 1). These lines were selected with the idea to represent important *Brassica* species along with Zahoor-15, as a local check. The experiment was carried out in randomized complete block design with three replications. Each plot/genotype consisted of three rows, and row to row space was maintained 60 cm, with row length of four meter.

Morphological parameters: Phenotypic variability was assessed through taking data on morphological characteristics, which included days to 100% flowering, plant height (cm), number of primary branches, main-raceme length (cm), siliquas plant^{-1} and siliquas main raceme^{-1}, siliqua length (cm), seeds siliqua^{-1}, beak length and siliqua angle.

Molecular genotyping: DNA was extracted from fresh young leaves of the *Brassica* accessions through Cethyl Tri methyl Ammonium Bromide (CTAB) method (Ali *et al*., 2017). The DNA was stored for further genotyping at -20°C. After DNA extraction DNA quantification was done through Nano drop by measuring their purity and concentration. The quality of the extracted DNA was checked through gel electrophoresis.

PCR amplification and gel electrophoresis: Amplification of the extracted DNA was done with RAPD and SSR primers (Table 2) by making polymerase chain reaction (PCR) and scored on the agarose gel. Each genotype underwent PCR using 10 µL solutions, comprising 5 µL master mix, 2.8 µL distilled or PCR water, 1 µL primer, 0.2 µL Taq DNA, and 1 µL DNA sample in PCR tubes. Conditions were optimized for amplification of various primers. The products were run on 1% and 2% agarose gel, visualized with ethidium bromide under UV light and photographs of the bands were captured on gel.

Table 1. Details on *Brassica* accessions selected for assessment of diversity and divergence at the phenotypic and molecular levels.

S. No.	Code	Species	Genotype	Source code	Source
1.	SABr-1	*Brassica compestres*	P-118	-	PBG
2.	SABr-2	*Brassica juncea*	L638	-	PBG
3.	SABr-3	*Brassica juncea*	Altex-237	E.9	IBGE
4.	SABr-4	*Brassica juncea*	Raya-Anmol	E.10	IBGE
5.	SABr-5	*Brassica napus*	Wester-209	E.1	IBGE
6.	SABr-6	*Brassica napus*	Ganyou-211	E.2	IBGE
7.	SABr-7	*Brassica napus*	Rainbow-214	E.3	IBGE
8.	SABr-8	*Brassica napus*	Oscar-213	E.4	IBGE
9.	SABr-9	*Brassica napus*	Vangard-210	E.5	IBGE
10.	SABr-10	*Brassica napus*	Crusher-212	E.6	IBGE
11.	SABr-11	*Brassica napus*	Torch-233	E.7	IBGE
12.	SABr-12	*Brassica napus*	Legend-248	E.8	IBGE
13.	SABr-13	*Brassica napus*	Zahoor-15	-	PBG
14.	SABr-14	*Brassica napus*	Dunkled	E.11	IBGE
15.	SABr-15	*Brassica napus*	BNWC8YS	E.12	IBGE
16.	SABr-17	*B.napus* × *B.napus*	IBGE-1	WesterxSumner	IBGE
17.	SABr-18	*B. juncea* × *B.napus*	IBGE-2	Raya-AnmolxArtic	IBGE
18.	SABr-19	*B.napus*×*B. Compestres*	IBGE-4	CON-IIIXT-16	IBGE
19.	SABr-20	*B.napus*×*B.carinata*	IBGE-3	MolukoXTP571	IBGE
20.	SABr-21	*Brassica napus*	Mun-1	-	IBGE
21.	SABr-22	*Brassica napus*	Mun-2	-	IBGE
22.	SABr-23	*Brassica napus*	Mun-3	-	IBGE
23.	SABr-24	*Brassica napus*	Mun-4	-	IBGE
24.	SABr-25	*Brassica napus*	Local line	-	PBG

Table 2. RAPD and SSR primers, along with their sequences and annealing temperatures, utilized for genotyping of *Brassica* accessions.

S. No.	Primer	Primer name	Primer sequence	Annealing temp.
1.	RAPD	OPB-04	GGACTGGAGT	37 ℃
2.	RAPD	OPC-05	GATGACCGCC	39 ℃
3.	RAPD	OPC-09	CTCACCGTCC	37 ℃
4.	SSR	Pr5-FIT0262	F:TCATAATAGCACAAGTCATAGTC R:GAATCAAACCCAACACCTC	65 ℃
5.	SSR	CB10504	F:GGTGCCCAACTGTTGAA R:CATTGGCATAGGAACAGG	47 ℃
6.	SSR	BRMS-018	F:TCCCACGCCTTCTAGCCTTC R:ACCGGAGCTTTTGTTGCC	56 ℃
7.	SSR	Na10-B08	F:AGAGAAAAACACTTCCCGCC R:GTGAGCTTTGCGAAACAGC	52 ℃

Table 3. Mean squared variation (MSE), least significant differences (LSD) and coefficient of variation (CV) for various parameters studied in 24 *Brassica* accessions.

Parameters	Mean square values	least significant difference	coefficient of variation
Days to flowering	0.74**	2.8	1.2
Plant height	406.6667**	66.82273	12.53951
Siliqua length	0.8143264**	2.990227	16.47927
Seeds siliqua	10.18889	-	18.61777
Main raceme length	78.33333*	29.32772	14.10456
Siliqua main raceme-^{1}	46.77778	22.66338	15.20812
Siliqua plants^{-1}	6668.365*	-	30.77913
Beak length	4.416667	6.963896	23.5692
Siliqua angle	180.4167**	44.50853	17.84644
Seed yield	72971.76*	-	28.3753

** Highly significant (p<0.001)

Data analyses: Data were compiled in MS Excel sheets for both fields (phenotypic and molecular data) and conclusions were drawn on diversity and divergence. The morphological and yield related data were analyzed using analyses of variance (ANOVA) technique appropriate for RCB Design using R statistical software. Association between parameters was assessed through correlation and regression using MS Excel and R software. Cluster analyses based on various parameters were carried out in R statistical software. Molecular data was analyzed with POPPR package of R software to assess diversity and construct phylogenetic tree, while considering the overall variability for molecular markers (Ali *et al*., 2014a, b). Clustering for molecular data was done, while considering either RAPD and SSR markers or both.

Results

Significant variability was observed for the studied parameters except for seed siliqua^{-1} (fruit), fruit main receme^{-1} and beak length (Table 3). The variability was further confirmed through molecular markers, which revealed divergence among different genotypes.

Variability at the level of Species: Substantial variability was observed in the representative genotypes of various species (Table 4). Overall mean for days to flowering were 72 days ranged from 71 days to 72. The maximum 72 days were recorded to attain flowering in *B. compestres* and *B. juncea* while the minimum 71 days were recorded in *B. napus* crossed species.

Overall mean value for plant height were 160.8 cm, with range from 152.2 to 194.5 cm. The maximum plant height (194.5 cm) was recorded for *B. napus* crossed progeny while lower value (152.2 cm) was observed for *Brassica napus* genotypes. The maximum siliqua length (6.5 cm) was recorded for *B. napus* crossed progeny while (3.5 cm) for *B. juncea*. Overall mean of raceme length was 62.7 cm, with range from 53.4 to 75 cm. The maximum raceme length (75 cm) was recorded for *B. napus* cross while the minimum (53.4 cm) for *B. compestres*. Overall mean for beak length of *Brassica* species was 8.9 mm, with range from 6.2 to 10 mm. The maximum beak length value (10 mm) was calculated for *B. napus* cross while the minimum beak length value (6.2 mm) was recorded for *B. juncea.* Overall mean for siliqua angle was 75.3. Highest value of siliqua angle was calculated for *B. napus* crossed progeny while lowest siliqua angle was observed for *B. juncea* species.

The maximum siliquas main raceme^{-1} (47.4) was calculated for *B. napus* crossed progeny while the minimum siliquas main raceme^{-1} (42) was observed for *B. compestres* (Fig. 1). Data on number of primary branches ranged from 4.3 to 5.3 branches. The maximum no. of primary branches 5.3 were calculated for *B. juncea* and *B. napus* crossed progeny while the minimum number of primary branches 4.3 was calculated for *B. napus*. Data for number of siliquas plant^{-1} ranged from 239.9 to 370.6 siliquas. The maximum siliquas plant^{-1} (370.6) was calculated for *B. napus* crossed progeny species while the minimum siliquas plant^{-1} (239.9) was calculated for *B. napus.* The maximum number of seeds siliqua^{-1} (18.6) was observed for *B. napus* crossed species whereas the minimum number of seeds siliqua^{-1} (15.3) was calculated for *B. juncea* and *B. napus* species. The maximum number of seed yield (703.6) was recorded for *B. napus* crossed while the minimum number of seed yield (1411.2 g) was recorded for *B. juncia.*

Variability at the level of individual genotypes: Significant differences were observed for days to flowering, which ranged from 70 to 73 days (Table 5). The maximum days to flowering (73 days) was observed for genotype p-118 followed by Altex-237, Ganyou-211, crusher-212 and Dunkled whereas the minimum days to flowering (70 days) was reported for genotype IBGE-3 and IBGE-4 respectively. The candidate varieties IBGE-2 and IBGE-1 had days to flowering value of 71 days. A total of nine *Brassica* accessions including Mun-2, Mun-3 and Mun-4, had a day to flowering value of 72 days.

Among *Brassica* lines, plant height ranged from 136 cm to 204 cm (Table 5). The maximum plant height (204 cm) was recorded for genotype IBGE-4 whereas genotype Rainbow-214 exhibited the minimum plant height (136 cm). The siliqua length data ranged from 3.5 to 6.5 cm. Overall mean for siliqua length recorded was 5.48 cm. Siliqua length ranged from 3.44 to 6.75 cm. The maximum siliqua length (6.75 cm) was recorded for genotype IBGE-3 while the minimum siliqua length (3.44 cm) was observed for genotype Altex-237. Main raceme length ranged from 52.3 to 78.9 cm. The maximum raceme length (78.9 cm) was measured for genotype IBGE-4 whereas the minimum raceme length (52.3) was recorded for genotype p-118. The data for fruit beak length of genotypes ranged from 4.33 to 11 mm. The highest value for beak length (11 mm) was recorded for genotype IBGE-3, whereas the lowest value (4.33 mm) was observed for genotype Altex-237. The maximum siliqua angle (85.7) was recorded for genotype p-118 while the minimum siliqua angle (42.3) was measured for genotype Mun-1.

Among the yield contributing traits the maximum siliquas main raceme^{-1} (48.7) was calculated for genotype IBGE-2 whereas the minimum siliquas main raceme^{-1} (39.3) for genotype local line (Table 5). The maximum number of primary branches (6) was recorded for genotype p-118, Altex-232 and IBGE-3 while the minimum no of primary branches (3.33) was counted for genotype Wester-209. The maximum number of siliquas plant^{-1} (409 siliquas) was observed for genotype IBGE-4 while minimum number of siliquas plant^{-1} (173 siliquas) was recorded for genotype Torch-237. The maximum number of seeds siliqua^{-1} (21) was recorded for genotype p-118 while the minimum number of seeds siliqua^{-1} (13) was recorded for genotype IBGE-4. The highest value for seed yield (1462 g) was calculated for genotype IBGE-4, while the minimum number of seed yield (560 g) was observed for genotype Mun-1.

As for as biochemical analysis is concerned, the maximum oil content (53%) was recorded for genotype Mun-1 and Crusher-212 while the minimum (47%) for genotype Zahoor-15 and IBGE-1. Overall mean for protein content (21%) was recorded where the maximum value for protein content (24%) was recorded for genotype Zahoor-15 and the minimum (19%) was observed for genotype L638, Crusher-212, Dunkled, Mun-1 and Oscar-213.

Table 4. Mean values for various morphological and yield parameters as observed in different species of *Brassica* germplasm tested during 2018-19.

Parameter	*B. compestres*	*B. juncea*	*B. napus*	*B. napus crossed* progeny	Overall mean
Days to flowering	72.7	72.7	71.9	70.8	71.8
Plant height	163.1	160.0	152.2	194.5	160.8
Siliqua length	4.6	3.5	5.6	6.5	5.5
Main raceme length	53.4	60.4	61.1	75.0	62.7
No of primary branches	5.2	5.3	4.3	5.3	4.6
Beak length	7.0	6.2	9.2	10.0	8.9
Siliqua angle	63.3	46.7	78.6	82.0	75.3

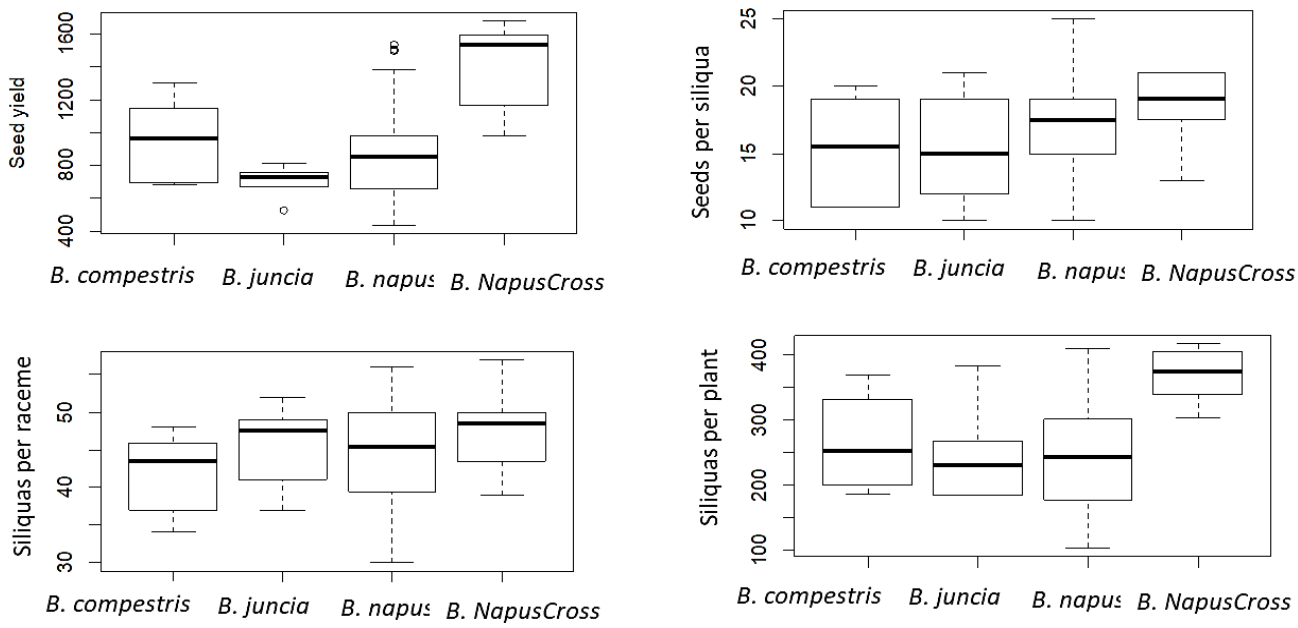

Fig. 1. Variability among various germplasm for yield and yield contributing parameters of different *Brassica* species of tested during 2018-19.

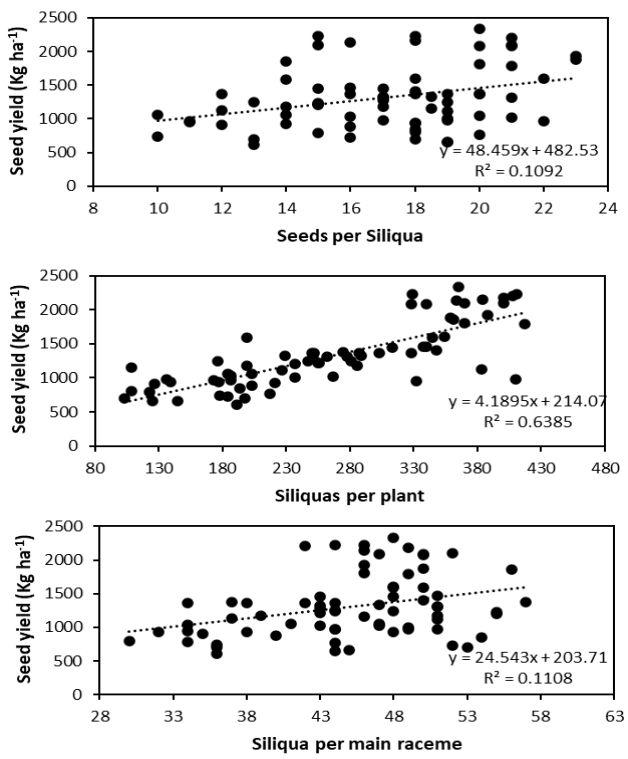

Fig. 2. Association of seed yield (g per plot) with yield parameters, as assessed in *Brassica* germplasm representing various species, tested during 2018-19.

***Correlation of yield with phenotypic parameters*:** The correlation of seed yield was assessed with parameters like seeds siliqua^{-1}, siliquas plant^{-1}, siliquas main raceme^{-1} (Fig. 2). Among the yield parameters, there was a strong correlation between seed yield, seeds siliqua^{-1}, siliquas plant^{-1} and siliquas main raceme^{-1}. Association of seed yield with seeds siliqua^{-1} was positive, showing an increasing trend. Genotypes with higher value of seeds siliqua^{-1} had higher seed yield. Similarly, genotypes having higher number of siliquas plant^{-1} and siliquas main raceme^{-1} had higher seed yield (g per plot). Further studies with intensive consideration of this relation may be useful to clearly elucidate these relationships.

Molecular genotyping of *Brassica* accessions: The collected *Brassica* accessions were genotyped with seven molecular markers, including both RAPD and microsatellite markers (Table 6). Total numbers of loci obtained were 16, with an average of 2.7 loci per primer, which showed different diversity and divergence level. During genotyping of 24 *Brassica* accession, all the markers were highly polymorphic. The RAPD markers amplified more loci, compared to the microsatellite markers. The maximum number of loci (5) was recorded for OPC-05, followed by OPC-09 and OPB-04 (4). The microsatellite markers (CB10504, BRMS-018 and Na10-B08) produced as single locus was considered present or absent.

Table 5. Variability in morphological traits, yield traits and biochemical traits assessed in *Brassica* accessions from different species tested during 2018-19.

Species	Genotype	Morphological traits						Yield traits					Biochemical traits		
		Days to flowering	Plant height	Siliqua length	Main raceme length	Beak length	Siliqua angle	Primary branches	Siliquas plant^{-1}	Siliquas main raceme^{-1}	Seeds pod^{-1}	Seed yield (g)	Oil %	Proteins %	Moisture %
B. compestres	L638	72	169	5.37	54.5	7.33	84.3	4.33	281	43.7	17.7	1145	52	19	6.2
	P-118	73	157	3.87	52.3	6.67	42.3	6.00	249	40.3	13.0	777	48	23	6.7
B. juncea	Altex-237	73	165	3.44	55.3	4.33	45.3	6.00	263	45.7	14.0	668	49	22	6.1
	Raya-Anmol	72	155	3.48	65.6	8.00	48.0	4.67	229	45.7	16.7	739	51	20	5.9
B. napus	BNWC8YS	72	155	5.62	62.3	10.00	81.3	3.67	216	47.3	19.0	820	49	22	6.4
	Crusher-212	73	159	5.96	62.5	9.00	82.7	4.33	271	44.3	20.7	990	53	19	6.8
	Dunkled	73	147	5.79	59.9	8.67	74.3	4.67	272	48.3	15.3	1027	52	19	5.8
	Ganyou-211	73	151	5.83	65.0	7.00	81.3	4.67	233	43.7	16.7	870	49	22	6.4
	Legend-248	72	141	6.01	61.3	8.53	74.3	4.00	255	45.0	17.0	1051	50	22	6.4
	Local	72	151	5.15	62.2	10.00	83.3	4.00	252	39.3	15.0	899	50	20	5.9
	Mun-1	71	167	6.27	65.4	10.93	85.7	4.67	219	44.0	18.7	560	53	19	6.7
	Mun-2	72	148	5.08	56.7	10.67	66.3	5.00	239	45.0	17.7	928	51	20	6.0
	Mun-3	72	144	4.50	67.4	7.33	83.3	3.67	248	44.0	16.8	1037	49	23	6.4
	Mun-4	72	167	6.18	63.4	10.00	80.7	4.67	351	47.0	19.7	1141	51	21	6.1
	Oscar-213	71	154	5.19	61.3	10.33	72.7	5.00	271	43.0	16.0	982	52	19	5.8
	Rinbow-214	72	136	4.86	54.3	9.33	80.0	4.67	229	43.7	14.0	746	49	22	6.6
	Torch-233	71	151	5.91	61.5	9.00	71.0	4.67	173	50.0	18.5	799	50	20	6.2
	Vangard-210	71	154	5.54	65.9	9.00	82.0	3.67	192	41.0	15.3	564	50	21	6.1
	Wester-209	71	153	6.06	52.4	9.00	76.0	3.33	191	40.7	19.0	675	51	21	6.7
	Zahoor-15	72	158	5.39	55.9	8.77	83.3	3.67	226	48.0	16.3	768	47	24	6.8
B. napus Cross	IBGE-1	71	179	6.38	73.6	9.00	78.0	4.33	347	47.3	17.7	1431	47	23	7.4
	IBGE-2	71	199	6.49	70.5	9.60	81.9	5.33	342	48.7	19.7	1346	50	21	6.6
	IBGE-3	70	197	6.75	77.0	11.00	84.0	6.00	384	46.0	16.0	1406	49	22	6.5
	IBGE-4	70	204	6.33	78.9	10.33	84.0	5.33	409	47.7	21.0	1462	51	20	5.9
Overall mean		**72**	**161**	**5.48**	**62.7**	**8.91**	**75.3**	**4.60**	**264**	**45.0**	**17.1**	**951**	**50**	**21**	**6.3**

Table 6. Diversity parameters for molecular markers amplified in the *Brassica* germplasm.

Molecular marker loci	Gene diversity	Simpsons diversity index	Evenness
OPC-09-L1	0.469	0.489	0.941
OPC-09-L2	0.486	0.507	0.973
OPC-09-L4	0.330	0.344	0.737
OPC-09-L5	0.219	0.228	0.612
OPC-05-L1	0.278	0.290	0.676
OPC-05-L2	0.153	0.159	0.543
OPC-05-L3	0.153	0.159	0.543
OPC-05-L4	0.330	0.344	0.737
OPC-05-L5	0.080	0.083	0.459
OPB-04-L1	0.469	0.489	0.941
OPB-04-L2	0.469	0.489	0.941
OPB-04-L3	0.497	0.518	0.993
OPB-04-L4	0.413	0.431	0.850
CB10504	0.153	0.159	0.543
BRMS-018	0.153	0.159	0.543
Na10-B08	0.330	0.344	0.737

The RAPD markers had more diversity than microsatellite markers (Table 4). The maximum gene diversity (0.497) was recorded for loci OPB-04-L3, while the minimum gene diversity (0.080) was observed for locus OPC-05-L5. The maximum diversity index (0.518) was recorded for loci OPB-04-L3 while the minimum diversity index (0.083) was observed for locus OPC-05-L5. The maximum Evenness index (0.993) was observed for OPB-04-L3, while minimum Evenness index (0.459) was recorded for locus OPC-05-L5.

The finding of maximum number of multilocus genotypes under panmixia and there plotting against the total number of loci re-sampled confirmed the suitability of markers to detect the genetic diversity and divergence in *Brassica* accessions (Fig. 3A). The molecular markers were able to detect all of multilocus genotypes (MLGs) which were recorded at loci number 10, which was increased by increasing g number of markers. The (Fig 3B) showed linkage among various studied loci, showing lack of strong association between loci. The r^2 was calculated using pairwise linkage disequilibrium analysis (Fig 3B).

Clustering of *Brassica* accessions based on various set of markers: Clustering revealed different grouping when RAPD, SSR or both RAPD and SSR loci were considered altogether (Fig. 4). The dendrogram obtained from RAPD data clearly separated the studied genotypes into two main groups having five sub clusters as represented in Fig 4A. The *Brassica* accessions Local, L638 and IBGE-1 were placed in the first main cluster while the second clustered contained the rest of *Brassica* genotypes. The second main cluster was sub divided into three sub cluster.

The dendrogram generated with SSR markers also divided the inbred lines in to two main clusters (Fig. 4B). The first main cluster consisted of two sub-group having five genotypes such as Mun-1, Mun-2 etc. while the rest of genotypes were placed into second main clustered having three subgroups having maximum genetic similarity in between them compared to first groups genotypes.

Based on overall polymorphism, both RAPD and SSR primers showed an overall diversity and grouping pattern for the tested *Brassica* accessions (Fig. 4C). The clustering based on all loci divided the genotypes into two main clusters. The first cluster comprised of two genotypes (Local and L638). The second cluster was subdivided into four sub clusters having rest of *Brassica* genotypes and was genetically related and similar. On the basis molecular data, noticeable position differences were detected of all genotypes except for the genotypes Local which separated into separate groups in both methods of molecular analysis which indicated that this genotype was highly divergent than other studied genotypes. Considering these clustering results, all subsequent analyses were conducted based on all the molecular loci together, to capture the full extent of the studied genetic diversity.

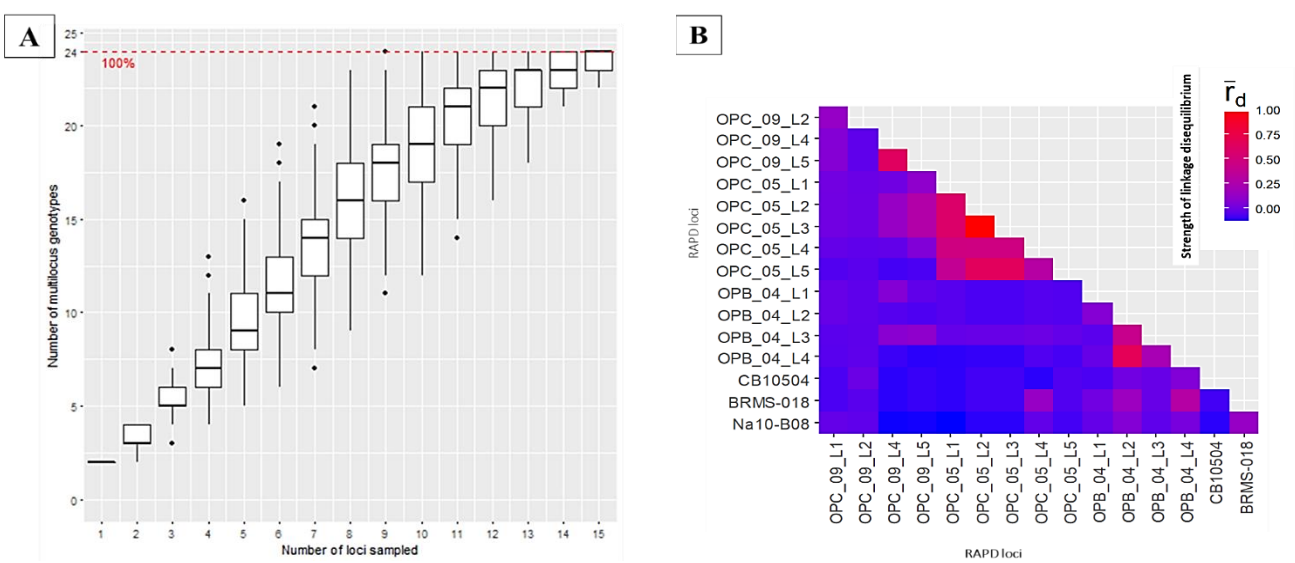

Fig. 3. Plotting the multilocus genotypes as detected against the number of loci resampled (A) and the linkage across loci (B) amplified in *Brassica* germplasm.

Table 7. Diversity parameters estimated in various *Brassica* species germplasm based on molecular markers.

***Brassica* species**	**Sample size**	**MLGs detected**	**Gene diversity**	**Genotypic diversity**
B. compestres	2	2	0.500	0.500
B. juncea	2	2	0.188	0.500
B. napus	16	16	0.315	0.938
B. napus crossed progeny	4	4	0.271	0.750
Overall germplasm	**24**	**24**	**0.325**	**0.958**

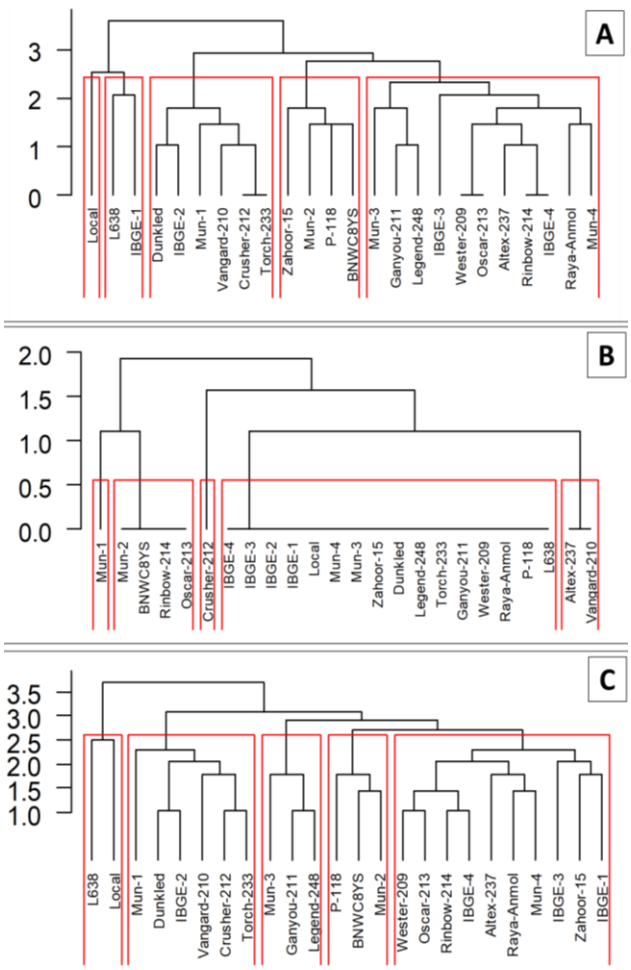

Fig. 4. Clustering of *Brassica* accessions from different species based on polymorphism in RAPD loci (A), SSR loci (B) and overall polymorphism in both RAPD and SSR loci (C).

Molecular diversity and divergence in different *Brassica* species: High diversity was calculated for all *Brassica* species (Table 7). Every genotype represented a distinct multilocus genotypes (MLGs) i.e., 24 MLGs were observed out of 24 tested genotypes. The genotype diversity ranged from 0.500 to 0.938 with an average of 0.958, while maximum gene diversity was detected in *B. compestres* and minimum was detected in *B. juncea* with an overall diversity of 0.325.

The principal component analysis identified a pattern of *Brassica* genotypes. The first component contributed (27.01%) while second components contributed (17.19%) with an overall component contribution was 44.2% (Fig 5A). The coordinate analysis showed that no clear groups were identified and all the genotypes were dispersed across all genotypes and was overlap with one another (Fig. 5B). The neighbor-joining tree showed that accessions representing *B. juncea* were dispersed among various genotypes and showed similarity with other accessions. In network analysis, two clusters were identified. The cluster I consisted of *B. compestres* while cluster II comprised with *B. juncea* with similarity to other *B. napus* crossed genotypes (Fig. 5C&D).

Discussion

Field based phenotyping and molecular genotyping revealed an overall high diversity in *Brassica* accessions from different species, along with substantial divergence based on morphological, molecular and biochemical analysis. The observed diversity would be useful for genetic improvement of *Brassica* crop, which stands 3rd among the oilseed crop. *Brassica* and were sown on about 23 million hectares area due to which the worlds oilseed production was over 36 million tones (Anon., 2004). Genetic improvement is an important strategy to increase the crop yield as per unit area productivity in Pakistan is much lower (Nasim *et al*., 2013). Genetic improvement is thus of utmost important to increase yield and oil content of *Brassica* in Pakistan (Hassan *et al*., 2008), which could not be achieved without assessment of phenotypic and molecular diversity of the crop.

Agronomic and morphological based characteristics are important determents to identify best genotypes in field trials which is also useful in further biochemical and molecular studies (Jan *et al*., 2016). Therefore, morphological screening of different genotypes is quite important for plant breeders (Iqbal *et al*., 2017). The Present study was conducted to screen *Brassica* genotypes for morphological characters such as days to 50% germination**,** days to 100% flowering, plant height (cm) and number of primary branches. Significant variation was reported among the tested genotypes on the basis of agro-morphological evaluation. The highest value for plant height, siliqua length and main raceme length were recorded 204 cm, 6.75 cm and 78.9 cm respectively. Our results are the confirmation of Zada *et al*., (2013) they also detected significant variation in both quantitative and qualitative characters among diverse group of *B. carinata.* They reported maximum variation for main qualitative characters i.e. main raceme length, leaf length, plant height, day to flowering 50% and day to flowering 100%. Jan *et al*., (2017) also detected significant variation in flower initiation, day to flowering 50%, leaf length and width, plant height and siliqua length.

Variability was observed in earliness among different varieties of *Brassica.* The differences among varieties in days to emergence and days to flowering were to cold climatic condition of the area as discussed in (Nasim *et al*., 2013). Further studies should be focused on genetic basis of earliness, which is important for efficient breeding programs (Iftikhar *et al*., 2024; Mohammadi & Prasanna, 2003; Iftikhar *et al*., 2021).

Variability was observed for morphological parameters, including primary branches and plant height. Primary branches play a key role in seed yield production as they bear secondary branches and fruits and hence increase number of fruits per plant. Plant height is also an important character of *Brassica* genotypes, though the potential risk of lodging needs to be considered. Plant height determines the growth of plant (Rameeh, 2016). Fruit length is also an important character of *Brassica* as the fruit is long; they contain more seeds which help in yield. The current results of significant variability for morphological parameters were similar to the previous finding (Hassan *et al*., 2008; Rameeh, 2011; Rameeh, 2016).

The seed yield is the ultimate objective along with the oil content, which is directly linked with the yield parameters. In this study, significant variability was observed for yield related traits. The variability was significant for siliquas main raceme^{-1}, siliquas plant^{-1} and seed yield, significance could be attributed to the genetic variation among these lines along with the effect of environment Khan *et al*., (2013). To understand the existence of variability at genetic level, we genotyped the tested lines with molecular markers.

Biochemical analyses of the tested lines revealed substantial variability for oil content, protein content and moisture contents of different *Brassica* accessions. The oil content (%) was in overall high percentage with a range of 47 (Zahoor-15) and IBGE-1to 53 (Mun-1 and Crusher-212). The protein content (%) was also high in the tested lines with the highest in Zahoor-15 (24%), reflecting an over elite biochemical attributes of these tested *Brassica* accessions, to be exploited for *Brassica* breeding. *Brassica* oil content and its quality remains a major objective in *Brassica* germplasm improvement programmers (Kopsell, 2007; Ali *et al*., 2014a), as the overall yield is based on the seed yield and oil content. During improvement in oil quality, breeders were also working to improve the quality of protein in oil. The residue left after oil extraction from *Brassica*, used as a fodder for livestock due to high content of protein in residue. The highest protein contents were reported in genotype Zahoor-15 while the lowest protein contents were detected in genotype L638 followed by Crusher-212, Dunkled, Oscar-213 and Mun-1. Similar results were detected by Sayal & Khan (2020). They reported an overall protein content of 22% with maximum value of 27.86% and minimum was 18.31% in some lines of *Brassica*. As a result of the above discussion, several genotypes having several favorable characters including both qualitative and quantitative characters such as high oil and protein contents could be identified. Also, further fatty acid profiles of most diverse genotypes should be studied to identify low erucic acid.

Along with morphological and biochemical traits-based variability, our finding showed significant variability among all the tested lines at the genetic level as assumed through molecular genotyping. Molecular genotyping of 24 *Brassica* lines with 7 molecular markers (OPB-04, OPC-05, OPC-09, pr5-FIT0262, BRMS-018, CB10504 and Na10-B08) resulted in amplification of a total number of 16 loci, which showed different level of variation. Among the tested markers, RAPD were the most polymorphic, as revealed by previous study of Shahin *et al*., (2015), which conducted molecular characterization of *Brassica* genotypes using RAPD markers. These markers could be used for DNA based fingerprinting, particularly in research environment with limited resources.

Identification of the *Brassica* accessions using different RAPD markers is clear evidence of the high discrimination capacity of these markers (Naz *et al*., 2019), although with the limitation of reproducibility and advantage of low cost. Genotyping with our RAPD markers showed high genetic diversity present among the tested genotypes and had proved to be a powerful tool for germplasm evaluation. Normally RAPD is used due to simplicity and its high polymorphic level (Belaj *et al*., 2004; Shahin *et al*., 2015). The studied markers were useful for DNA fingerprinting as every accession represented a distinct multilocus genotypes (MLGs) i.e., 24 MLGs were observed out of 24 tested genotypes. These results however must be further explored with molecular sequencing and other useful markers considering the fast-growing next generation sequencing technology.

As two different set of markers were used i.e., three RAPD loci and four SSR loci, cluster analyses was done using various sets of molecular data for the 24 different *Brassica* accessions. Previously, different kinds of markers have been used for finding genetic diversity (Naz *et al*., 2019; Ali *et al*., 2014b). Clustering revealed different grouping when RAPD, SSR or both RAPD and SSR loci were considered altogether, with more elaborated clustering achieved when both set of markers were used altogether. Considering these clustering results, subsequent diversity and divergence analyses were done considering all the molecular loci together, to capture all of the studied genetic diversity.

Interestingly, genotyping of *Brassica* lines from different species revealed an overall high diversity in the studied *Brassica* accessions. Each single individual represented a distinct multi-locus genotype. This high level of polymorphism agreed with the result of previous studies carried out in *Brassica* genotypes with RAPD markers (Shahin *et al*., 2015). This high diversity could be exploited in future breeding for making crosses, while considering both phenotypic data and level of diversity.

Analyses of genetic divergence across various species by the network analysis, neighbor joining tree and principal coordinate analysis, showed the overall divergence among different *Brassica* genotypes. Mostly *B. napus* and *B. napus* crossed genotypes were near to each other while other was most divergent from one and other. Divergence of accessions representing different species was expected, though some overlap was observed, which could be possible due to limited crosses among accessions from different species (Belaj *et al*., 2004).

Thus, the results of this study, suggesting an overall high diversity and divergence across the species, should be useful for identifying potential parents, while considering their yield potential, earliness and morphological diversity. The results also revealed superiority and distinctiveness of candidate *Brassica* lines from other accessions.

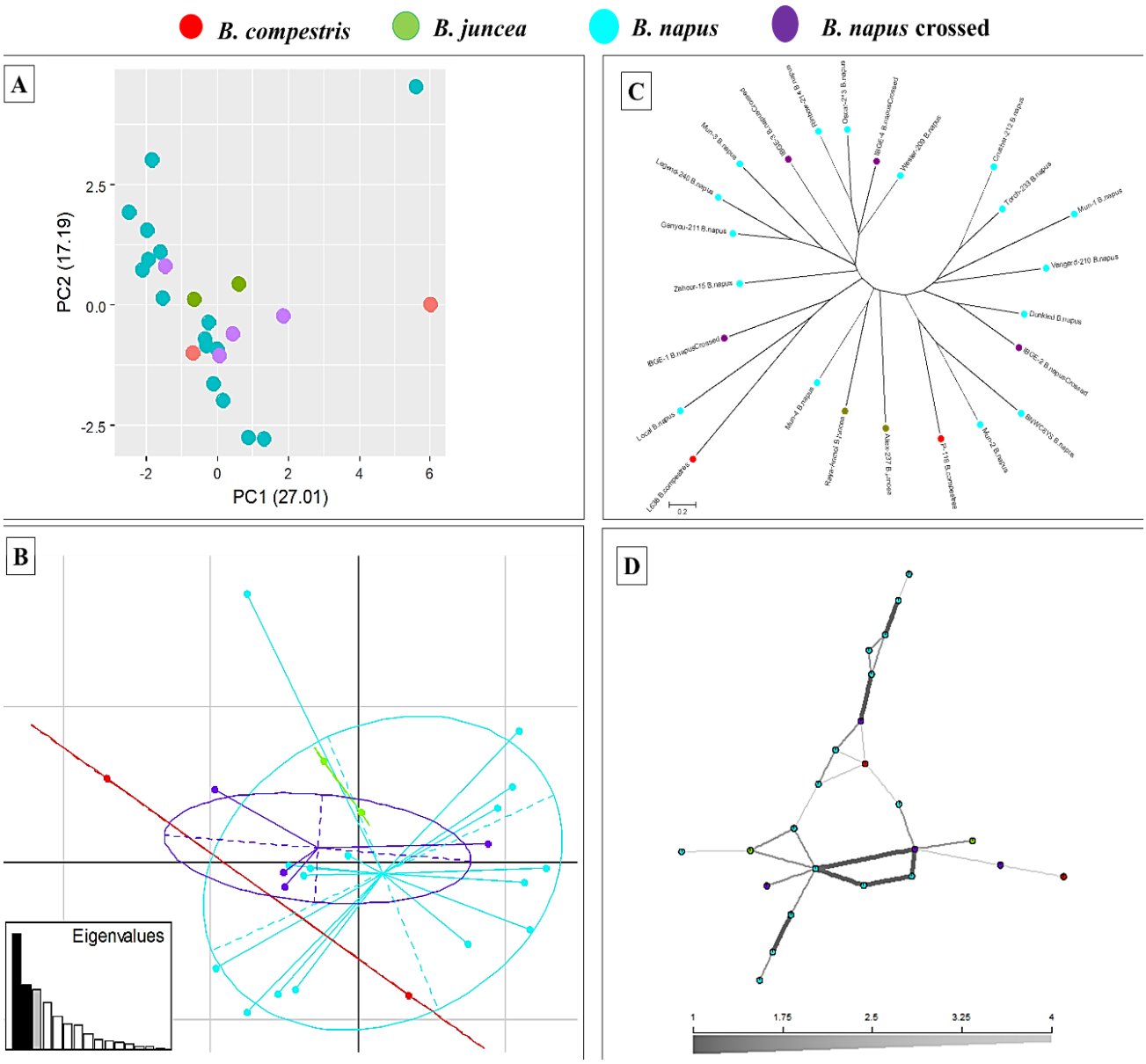

Fig. 5. Principal component analyses (A) and Principal co-ordinate analyses (B) based on RAPD data of *Brassica* genotypes; Phylogenetic tree (C) and Network based analyses (D) based on molecular data of *Brassica* genotypes.

Conclusion

The present study conducted on diversity and divergence of different *Brassica* accessions showed a high diversity in *Brassica* genotypes, which could be used in further *Brassica* cultivation and improvement. Genotype IBGE-4 had the maximum seed yield (1462 g), while the lines Mun-1 and Crusher-212 had the maximum oil content (53%), and Zahoor-15 had the maximum protein content (24%), which could be recommended for further cultivation considering different criteria. The studied markers were useful for DNA fingerprinting as every accession represented distinct multilocus genotypes (MLGs). The maximum gene diversity was detected in *B. compestres* and the minimum was detected in *B. juncea* with an overall diversity of 0.325, respectively. The knowledge on diversity and divergence must be helpful for future breeding programs and development of *Brassica* cultivars.

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