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Assessment of Genetic Diversity in *Brassica rapa* Using SDS-PAGE and SSRs Markers

by

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My Work is Dedicated To The Only GOD ALLAH ALMIGHTY, To The Last Holy PROPHET HAZRAT MUHAMMAD S.A.W.W. (PBUH) And To My Family.

CERTIFICATE OF APPROVAL

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(Wajeeh Ul Hassan)

Abstract

Brassica rapa is among the significant oilseed crops, that has been grown worldwide for thousands of years related to variety of purposes. The present work was designed to study genetic diversity among 30 *B. rapa* genotypes. Investigation of *B. rapa* seed storage protein diversity is helpful for the enhancement and effective usage of this crop. In this study diverse *B. rapa* genotypes were examined for electrophoratic total seed storage protein variation. A highly efficient SDS-PAGE protocol was optimized for diversity analysis. A low to moderate to high level of genetic variance was observed in the 30 tested genotypes of *B. rapa*. Maximum of 13 bands were recorded and all of them (100%) were polymorphic. The highest similarity value of 100% was observed between OkaBr-860/MulBr-803, DerBr-825/LayBr-820, SheBr-855/FaiBr-850, FaiBr-851/MuzBr-847 and MulBr-804/DerBr-823 genotypes. The lowest similarity 15% was recorded among genotypes NorBr-841/LhrBr-814.

The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to cluster 30 genotypes into seven major groups. Cluster I-VII consisted of 6, 12, 5, 2, 3, 1 and 1 genotypes, respectively. The group VI and VII has unique genotypes ChkBr-812 and LhrBr-814 that were distinct from other genotypes. The genotypes were further visualized via 2D and 3D methods. The 2D analysis indicated 4 diverse genotypes that were LhrBr-814, ChkBr-812, LayBr-818, and DerBr-822 while the 3D indicated genotypes were LayBr-818, ChkBr-812 and LayBr-819. These results will be valuable as a core analysis into the characterization of *B. rapa* genotypes.

In the Molecular analysis, 30 different *B. rapa* genotypes were examined for genomic differences through 10 simple sequence repeats (SSRs) marker. The primers were specific for *B. rapa* crop, majority of these primers amplified fragments ranged sizes from 100-400 bp. 100% polymorphism was observed for all genotypes through ten distinct microsatellites. However, seven primers (70%) detected only one allele while the other two primers (20%) (Ni2-F02, Ra2-A11) amplified two alleles each and only 1 primer (Na10-D09) generated 3 alleles. 14 alleles were detected

by 10 primers and all were highly polymorphic. The genetic similarity values ranged 0.0-100% was recorded between different *B. rapa* genotypes. The lowest similarity coefficient 0.0% was found between ChkBr-812/MulBr-803, ChkBr-812/OkaBr-860, ChkBr-812/ChkBr-811 and ChkBr-812/LayBr-919 genotypes etc. while the highest similarity value 100% was noted between DerBr-825/OkaBr-860, SadBr-831/OkaBr860 and FaiBr-851/DerBr-825 etc. Using the UPGMA similarity method, all genotypes from different origins were divided into five groups. Group I contained seven genotypes. Group II had 13 genotypes, making it the largest group. Group III had six genotypes, whereas Group IV had only three genotypes. However, group V was comprised of only one genotype i.e., ChkBr-812. Promising genotypes were identified using 2D and 3D analysis. In 2D analysis some diverse genotypes, such as ChkBr-812, FaiBr-853, MulBr-804 and FaiBr-850, were found. While 3D analysis revealed some rare genotypes too, including LhrBr-814, SheBr-855, MuzBr-845, and LayBr-820. For more research and evaluation these research could be used as a baseline.

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Abbreviations

2D	Two Dimensional
3D	Three Dimensional
APS	Ammonium Per Sulphate
Acetic acid	CH_3COOH
B. napus	*Brassica napus*
B. juncea	*Brassica juncea*
B. nigra	*Brassica nigra*
B. campestris	*Brassica campestris*
B. rapa	*Brassica rapa*
B. carinata	*Brassica carinata*
B. oleracea	*Brassica oleracea*
CBB	Coomassie Brilliant Blue
Distt. H_2O	Distilled Water
EST	Expressed Sequence Tags
FAO	Food and Agricultural Organization
ISSRs	Inter-simple Sequence Repeats
Methanol	CH_3OH
NARC	National Agricultural Research Centre
NTSYS-PC	Numerical Taxonomy System for Personnel Computer
PCR	Polymerase Chain Reaction
PGRI	Plant Genetic Resource Institute
PIC	Polymorphism Information Content
RFLP	Restriction Fragment Length Polymorphism
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

SSRs	Simple Sequence Repeats
SCAR	Special Sequence Characterized Amplified Region
Sol.	Solution
THAM	Tris Hydroxymethyl Amino Methane
UPGMA	Unweighted Pair Group Method with Arithmetic Averages
UN	United Nation
Vol.	Volume
WTO	World Trade Organization

Chapter 1

Introduction

1.1 Significance of *Brassica*

Brassica is the highly diverse genus of the plants belonging to family *Brassicaceae*. These species are source of forage, edible oil, vegetables and ornamental that has been a cause of accelerating economies of many developing countries. The *Brassicas* are rich in oil content, essential metabolites and even its oil extracted press cakes are nutrients supply for animals. The specie contains a variety of members that are utilized as vegetables for human diets, such as Chinese cabbage, Pak-choi etc [1]. It is among the plant groups recognized, worldwide and commercially, because of its diverse importance's for mankind. Broccolis, cabbage, cauliflower, kale, kohlrabi and brussel sprouts are other names of *Brassica* crops around the world. In China and India, newborn variety of rapeseed containing little erucic acid and glucosinolate is being now cultivated. The evaluation in this crop has led to an increase in industrial rapeseed demand. Because it has fewer glucosinolate, it is also beneficial for cattle and poultry. It has shown to be compatible to soybean meal and has the potential to be used in the development of high-value protein foods [2]. Its seeds contain 40% oil that is used as a human food i.e., a cooking medium, also in making pickles and many more businesses. Lightning lamps, oil-cakes, leather softening, and domestic animal feed are all made with the oils, and

the seeds are used as medication for digestive disorders, swelling, cardiovascular diseases as an anticancer and so forth [3]. Rapeseed oil is widely used as edible oil and in breeding programmes because of its low glucosinolate concentration. The name erucic acid was coined in 1974 by Canadian oil, which is employed in human nutrition [4]. Among the crop families, the *Brassicaceae* family is crucial for producing edible oil of high quality and high yield. *Brassica* crops such as canola, *Brassica oleracea*, and mustard are cultivated in Pakistan because of their high oil quality (44 to 46%). Its meal is made up of 38-40% protein and contains the essential amino acids for instance cystine, lysine, and methionine. After palm and soybean, *Brassica* oil seeds are the third most significant source of edible oil in the world, contributing almost 15% of edible vegetable oil (FAO, 2010). In Pakistan, all *Brassica* species are grown, but *B.napus*, *B.rapa*, *B. juncea* and *B.compesrtieset* are particularly more popular because of their high oil and protein content [5].

Plant breeder's major goal is to explore the genetic variability of distinct genotypes of vital crops and to identify the promising genotypes for efficient yield. The information about genetic resources and genetic diversity of crops could help in the improvement of crops quality, production and in breeding techniques. The diversity evaluation and investigation of *Brassica* species would be a reason of identification of novel genes that are viable and resistant to abiotic stress [6]. The genetic diversity estimation of plant germplasm plays an important role in the improvement of crop varieties for better production and other desirable traits such as disease protection, insect and pest resistance, early shedding, heat and cold tolerant. In the past, the harvesting, cultivation and yield of crop plants have increased and improved significantly because of using many morphological, biochemical and molecular methods. Botanical researchers have been studying genomic diversity for ten thousand years ago, resulted in the birth of hybridization, in order to increase the yield and quality by exploring the genes of interest [7]. Hence it is understood that for the successful crop improvement genetic diversity analysis is crucial. Genomic studies helped in the security of food chain by determining the genes that were proven to be a cure for many agricultural problems. The genetic diversity analysis also provided us with advanced and improved

breeding techniques that are a reason for modern known efficient genotypes [8]. Exploring a country's hidden and disregarded indigenous materials can help it thrive in socio-economic and scientific crisis. For future morpho-biochemical and molecular evaluations geographic information of selected germplasm is also critical. According to the WTO, every country's customizing policy should be to get the most out of the global market by expanding their existing genetic resources. To measure the genetic variability in plant species, morpho-physiological, molecular, and biochemical markers are being used [9]. In many crop species, now a day, plant breeders are utilizing molecular markers to study reproduction, speciation, and population spatial-temporal dynamics [10]. The *Brassicaceae* family is famous for economic significances among all plant families. *Brassica* vegetables are a dietary staple food in every part of the world with exception of tropical regions. According to the Food and Agricultural Organization of the UN, world commercial production of cauliflowers, broccoli, cabbages and other *Brassica* vegetables in 2013, was estimated above 93 million tons over an area of about 3.7 million hectares, with a 2013 farm gate value of some USD 31 billion (FAOSTAT, 2013).

1.2 Origin and Distribution of *Brassica*

The most prominent member of *Brassicaceae* family is *Brassica*, which is among one of the top 10 economically and strategically important crops with over 3,500 species and 350 genera [11]. *Brassica* mainly consist of three diploid wild members that are *B. nigra* (Kali sarson ,n = 8), *B. oleracea* (Kale, n = 9) and *B. rapa* (Chinese cabbage, n = 10) and three amphidiploids that is *B. napus* (n = 2x = 19), *B. carinata* (n = 2x = 17) and *B. juncea* (n = 2x = 18) [12]. Many parts of the world are thought to be the center of origin of *Brassica* but according to modern scientist's community Europe is most likely to be the origin of wild types of this specie. *Brassica* is currently growing in Ethiopia, Tanzania, Kenya, Zimbabwe, and Mali on the African continent [13]. Kazakhstan, China, Japan, Afghanistan, Iran, India, and Pakistan are among the Asian countries that grow it. It is frequently cultivated in southern Australia's medium and high rainfall

zones [14]. *Brassica* is cultivated throughout the United States, including in Idaho, Minnesota, North Dakota, Montana, Oklahoma, Argentina, and Central America [12].

1.3 Biochemical Based Diversity

The evaluation of genetic diversity among distinct *Brassica* species is important in the selection of elite genotypes. To identify novel genotypes among plant species/subspecies, various morpho-biochemical and physiological techniques are commonly used [15] [16]. Maximum polymorphism could be obtained using biochemical methods as the technique is unaffected by many environmental factors. It is frequently used to investigate the evolutionary relationships between several significant plant species or subspecies [17]. Genetic indicators such as total seed proteins, isozymes, and other biochemical markers, are modern methods to measure the genetic diversity. Genetic assessment is particularly important for *Brassica* species as biochemically investigated differences among species could plays a key role in novel and efficient genotypes identification. SDS-PAGE is a simple, competent, and rapid method for studying genetic diversity between species. The SDS-PAGE method provides information about polypeptide profiles that is considered as a source of genetic variation [15]. Researchers have examined protein-based variation among various *Brassica* species using the SDS-PAGE method for example the technique was used to examine protein profiles of the *Eruca sativa L.* [18]. SDS-PAGE is the method that is used to achieve high resolution analytical separation of protein mixtures. SDS-PAGE examination of vital *Brassica* genus is valuable to inspect genetic variability [19]. It is used for detection of different types of protein subunits [21]. The serious protein based categorization of *Brassica* species is mandatory to monitor varied genotypes. The SDS-PAGE process confers resourceful and rapid protein profiling of unusual crop varieties and it is harmless from every ecological cause [15]. However, using SDS-PAGE alone for protein characterization of diverse *Brassica* varieties is not enough [20].

1.4 Molecular Markers Based Diversity

Previous researches are still not very clear about the origin of *B. rapa*. The phylogenetic studies that have been using RFLP markers revealed that it may have originated in Europe, although the majority of its wild genotypes are found in Asia, from southern China to Japan. However, turnip rape was not seen in East Asia [22]. Based on SSRs marker analysis three separate *B. rapa* groups were found in South Asia, Northern Europe, and Southern-Eastern Europe while the other two subgroups were only present in South Asia. A proper genomic research of this crop is necessary as *B. rapa* is crucial source of oil and food [23]. Many scientists have studied the molecular marker based genetic variability of *B. rapa* that showed that these species are often found in Asian and European environments; with the Europe containing usually oil seed turnip rape. Similarly, the Asian varieties also included a range of vegetables belonging to this crop for example turnips, bok choy, tatsoi etc. It is crucial for future breeding programmes to understand the genetic diversity among distinct genotypes of *B. rapa* [24] [25]. The molecular markers can be used to compare species genetic differences. Also, those markers could be applied to detect chromosomal mutation that is caused by duplication, deletion, inversion, and insertion. Molecular markers have little effect on the phenotypes as these are only present in close vicinity to the genes in the genome. The molecular markers have a lot of advantages over standard phenotypic markers as that are permanent and detectable in all tissues at any stage of growth [26].

Moreover, numerous molecular techniques have been developed to evaluate genotypes in order to analyze genomic-based variation. SSRs are among these markers as they too are dispersed across the genome that is crucial for assessing genotype divergence. For *B. rapa*, various markers have been tested, but the SSRs marker proven to be better as compared to others due to its robustness, high polymorphism detection and co dominance expression. Subsequently SSRs marker are also used for other species of *Brassica* such as rapeseed, Indian mustard etc [27]. Simple sequence repeats (SSRs), also known as microsatellites, are DNA-based

markers found in eukaryotic organism's genomes that have 1-6 base pair tandem repeats in them. SSRs length variation is caused by high mutation rates ranging from 2-10 to 6-10 bp [28]. SSRs are simple, reproducible, affordable, and co-dominant markers. SSRs based analysis is used for detecting genomic variability in closely related organisms. SSRs markers are now frequently used to study wild populations and to conserve, use, and characterize crop variety. Molecular marker approaches have been developed and widely used in the study of genetic relationships among plants in recent years, including species identification, phylogenetic relationship reconstruction, and gene mapping [29]. Plant geneticists employ a variety of approaches to investigate genetic variation and relationships among plant assemblage members. Crop germplasm collection has engaged agricultural researchers and numerical taxonomists to categorize species and examine genetic relationships between and within species [30].

1.5 Research Gap

There are many areas of Punjab, Pakistan where *B. rapa* is still needed to be explored. Biochemical and molecular diversity can be used to find new genotypes. Scientists and researchers have concentrated on genetic link analysis of many species using molecular markers.

Here, at present, no comprehensive study is available to characterize *B. rapa* genotypes from Punjab area, therefore, the goal of this research is to assess the seed protein variability and genetic diversity of *B. rapa*.

1.6 Scope

Brassica is one of Pakistan's long-term hopes for increasing edible oil production. Promising genotypes discovered by selecting elite genotypes using biochemical and molecular techniques can be used in *Brassica* breeding programs. As a result, local

edible oil production can be improved and the need for importing edible oil will be reduced.

1.7 Aim and Objectives

1.7.1 Aim

- To evaluate *B. rapa* genotypes through biochemical and molecular markers.

1.7.2 Objectives

- To study the protein diversity among different collected genotype using SDS-PAGE.
- To study SSRs based variability among *B. rapa* genotypes collected from different sites of Punjab.
- To identify promising *B. rapa* genotypes.

Chapter 2

Review of Literature

2.1 SDS-PAGE Based Literatures

Ali et al. [31] investigated the genetic diversity of 136 genotypes of *B. napus L.* The biochemical technique of SDS-PAGE was used to characterize it. The study used germplasm from the Plant Genetic Resources Institute (PGRI), NARC, Islamabad, Pakistan, which included 135 accessions and one control cultivar. In this work, 12.25% PolyAcrylamide Gels were employed, and a total of 21 protein subunits were found among the genotypes using the Laemmli (1970) method. 16 (76.19%) of the 21 bands were polymorphic, while the remaining 5 (23.81%) were monomorphic. These 21 protein subunits were discovered with molecular weights ranging from 6 to 180 kDa.

The coefficients of similarity between these genotypes ranged from 0.83 to 0.98. The genotypes tested were classified into five primary clusters using UPGMA to create a dendrogram based on the dissimilarity matrix. In general, there were little genetic variations among the *B. napus* L. genotypes investigated. As a result, it is suggested that in the future, 2-D gel electrophoresis, along with other modern techniques, should be used to discover high levels of genetic diversity among these genotypes, as the SDS-PAGE technique alone is insufficient to fully explore the genetic diversity present in these genotypes. Shinwari et al. [18] used

SDS-PAGE to characterize 102 Pakistani genotypes of *Eruca sativa L.* to assess the genetic diversity present. They discovered 17 protein subunits in total, 11 of which were polymorphic and the remaining monomorphic. These protein subunits had molecular weights ranging from 15 to 220 kDa. The genetic closeness between these genotypes ranged from 60% to 100%. Based on the dissimilarity matrix and UPGMA, they recorded four broad groups. They noted four protein subunits in all genotypes, namely protein subunits 2, 7, 12, and 16, while protein subunit number six was not observed. Likewise, the protein subunit number ten was discovered in 82 genotypes but not in the others. SDS-PAGE revealed a low to medium amount of genetic diversity in these genotypes, and they concluded that in the future, contemporary molecular techniques such as 2-D gel electrophoresis should be utilized to detect significant genetic diversity among these genotypes.

Khurshid et al. [32] used SDS-PAGE and salt soluble protein to investigate genetic diversity across 30 *Brassica* genotypes. They discovered significant polymorphism in the studied genotypes. They separated their protein's profile into three distinct sections. Three highly polymorphic protein subunits were discovered in the first area. A total of nine protein sub-units were found in the second area, but only a few of them were polymorphic. In the last section of the gel, six protein subunits were discovered, two of which were polymorphic. A total of nine protein sub-units were found in the second area, but only a few of them were polymorphic. In the last section of the gel, six protein subunits were discovered, two of which were polymorphic. The study's dendrogram was created using UPGMA, and the total genotypes were divided into four major groups. The genotypes have a genetic closeness of 91%. In the dendrogram of salt soluble protein, they discovered four groups as well. They concluded that polymorphism reveals variations among *Brassica* species, and that the formation of groups was unaffected by geographic factors.

Zada et al. [33] used a biochemical technique called SDS-PAGE to explore the genetic diversity of 94 *Brassica carinata L.* (Ethiopian mustard) genotypes. They observed a total of 31 polypeptide subunits, fourteen of them were polymorphic and the remaining monomorphic, ranging in size from 8 to 180 kDa. 50% and 100%

genetic similarity was found to exist between the genotypes.Using UPGMA the studied dendrogram was classified into five major groups based on dissimilarity matrix. The genetic variations found among local genotypes were of a modest degree, whereas the genetic differences revealed between foreign genotypes were of a medium to high level. The authors also advised that 2-D gel electrophoresis and molecular markers be employed to analyze high genetic diversity because SDS-PAGE is ineffective in determining genetic variety in these genotypes.

Rabbani et al. [34] used SDS-PAGE to investigate the total seed protein pattern in oil seed mustard. In 52 genotypes, they identified eight different types of protein subunits. Type-I protein was recognized in 43 oil seed collections and genotypes, and type-VI protein was located in three vegetable genotypes, whereas the other six forms of protein were only found in a single genotype. The protein subunits were divided into four groups. The dendrogram was created using UPGMA and the grouping was done using Jaccard's similarity index. The SDS-PAGE result for seed protein revealed that this approach was not very effective in distinguishing genotypes that were closely related. However, they discovered that this method was sufficient to distinguish *B. juncea L.* from *B. campestris L.* and vegetable mustard from oil seed mustard.

Nasr et al. [35] used SDS-PAGE analysis of total protein to investigate 10 *B. napus L.* genotypes. During their research, they discovered many protein subunits. There were 19 protein subunits in genotypes one, four, and five, but only twelve protein subunits in genotypes six and seven. The study's dendrogram was separated into five primary categories. Genotypes 1, 10, 19, 8 and 3 were identified in the first group; in the second, 4 and 5 genotypes were present; genotypes 6 was found in third group; genotype 7 was discovered in fourth group; and the genotype 9 was present in the last group. They argued in support of utilizing protein profile trends in distinct plant germplasm samples using SDS-PAGE. Shahid et al. [36] on the basis of total seed storage proteins, 136 genotypes of rapeseeds (*B. napus L.*) were analyzed using the biochemical technique of SDS-PAGE. The study used germplasm from the PGRI, NARC, and Islamabad, Pakistan, which included 135 accessions and one control cultivar. In this investigation, 12.25% PolyAcrylamide

Gels were employed, and the genotypes were found to have a total of 21 protein subunits. 16 (76.19%) of the 21 bands were polymorphic, while the remaining 5 (23.81%) were monomorphic. The 21 protein subunits were discovered with molecular weights ranging from 6 to 180 kDa. These genotypes had similarity coefficients ranging from 0.83 to 0.98. By utilizing UPGMA to create a dendrogram based on dissimilarity matrix, the genotypes analyzed were grouped into five primary clusters. There was a minimal amount of genetic divergence identified overall. As a result, it is suggested that in the future, 2-D gel electrophoresis, along with other modern techniques, should be used to discover high levels of genetic diversity among these genotypes, as the SDS-PAGE technique alone is insufficient to fully explore the genetic diversity present in these genotypes.

Ali et al. [37] employed SDS-PAGE to examine and describe seed storage protein patterns in four pistachio cultivars (Akbari, Ahmad Aghaei, Fandoghi, and Kaleghouchi). The total protein content of pistachio seeds did not differ significantly across cultivars. A few protein bands were found to be up regulated on SDS PAGE, whereas others were shown to be down regulated. Protein markers for pistachio cultivars could be developed using the found protein patterns.

Asghar et al. [38] used SDS-PAGE to examine distinct protein banding patterns. They can be identified on the basis of these patterns. To determine the quantity of genetic diversity and the geographic distribution of that diversity, 29 accessions of *Cicer arietinum* (Chickpea) germplasm were analyzed for total seed protein profile. Protein banding pattern showed a significant variance that was geographically situated. Inter-specific variation was greater than intra-specific variation. Jiang et al. [39] designed a novel SDS PAGE procedure to see polypeptide bands clearly in the 1-30 KDa size range. This technique was created by adding and blending several gel compositions at varying quantities i.e acryl amide and bisacrylamide, urea, and glycerol. For clear resolution of micro mass proteins, a gel containing 10% glycerol and 4.2M urea was shown to be optimal. The visibility of tiny proteins was improved by loading a 5μl sample, staining for 1 hour, then de-staining for 2 hours. They were successful in developing efficient and highly reproducible technique. Khan et al. [40] used the SDSP-PAGE method to assess the biochemical

differences of *Vigna mungo* (black gramme) germplasm. The 33 accessions were collected from various locations throughout Pakistan. Protein banding patterns showed the most variance. According to Euclidean dissimilarity coefficient values, all genotypes split into three categories. Clusters 1, 2, and 3 each had 12 genotypes, 11 genotypes, and 10 genotypes, respectively. They discovered a wide range of polymorphic protein bands in seed samples. In comparison to other minor bands, bands 3 and 4 were practically universal in all genotypes. In all of the seed materials studied, however, they found more variability in minor bands than large bands. This gave researchers a powerful tool to assess the genetic diversity of this key agricultural species. Shinwari et al. [41] used SDS-PAGE to investigate seed storage protein variation among geographically different safflower germplasm. The polymorphic bands (60%) were greater than monomorphic (40%) bands. The coefficient of similarity was computed between different genotypes and ranged from 0.00 to 1.00. 16327 and 26752 genotypes were determined to be distinct from other genotypes with the highest dissimilarity score. On the basis of phylogenetic analysis, all of the accessions were divided into four groups.

There are 23, 75, 8, and 8 genotypes in Groups I, II, III, and IV, respectively. They proposed that these genotypes be investigated further using modern molecular and biochemical approaches. Sardar et al. [42] used SDS-PAGE to assess features related to seed proteins for glutelin diversity in Pakistani rice genetic resources. 475 accessions were obtained from three rice producing zones as well as other regions of the country. At 57kD pro-glutelin and 40kD glutelin acidic subunit bands 3 and 4, a lot of variance in glutelin portion of rice protein was present. The enriched glutelin variant at 57kD could be used to create better protein crops in terms of both quality and quantity.

Shuaib et al. [43] collected wheat grains from 13 distinct wheat kinds from Pakistan's various ecological zones. SDS-PAGE electrophoresis was used to examine the diversity of seed storage proteins. The presence or absence of each band was noted using electropherogram. The Jaccard's Similarity Index (JSI) was calculated using electrophoresis band spectra. The dendrogram for HMW and LMW gluten component was used to assess wheat genital diversity. It is suggested that

polypeptide profiles could be helpful in research on genomic diversity and organization of suitable cultivars, boosting the efficacy of wheat breeding programs, more likely in underdeveloped nations like Pakistan.

Iqbal et al. [44] did research to examine if there was a link between chickpea genotypes and blight disease reaction, as well as in vitro growth of *A. rabiei* on chickpea sap and seed protein pattern on SDS-PAGE. Seed proteins were tested using a slab type SDS-PAGE with a Polyacrylamide gel of 11.25% and a sample volume of 6μl. The majority of the genotypes were classified depending on illness reaction and in-vitro fungus growth, although there was no link among disease and SDS-PAGE. 6 of the 12 markers were polymorphic, and the gel was split into three sections. It was suggested that genotypes with comparable banding patterns be tested for genetic diversity using 2-D electrophoresis and DNA markers. Cluster analysis revealed a mixed grouping of susceptible and tolerant genotypes, indicating that SDS-PAGE could not be used to categorize chickpea for disease responses. 57 genotypes were found to have a low level of genetic variety, despite the fact that they came from multiple sources. Jan et al. [45] emphasize on the measurement of polypeptide based diversity among different *Brassica* sub-species. The variance in total seed protein between *B. rapabrown* subspecies was investigated in this study. SDS PAGE was used to examine the genotypes of twenty different brown kinds. Proteins of different sizes were identified. They obtained total 12 bands of which 10 were polymorphic and 2 were monomorphic. The molecular weight varies from 10 kDa to 180 kDa. The UPGMA method was used to analyze the data of variable proteins, all genotypes formed four cluster groups. These clusters contain 3, 6, and 10 genotypes. The similarity values were calculated across genotypes. The highest similarity (96%) was found between genotypes Br-607 and Br-560, as well as between Br-589 and Br-607, respectively. The brown sub-species of *B. rapa* displayed the most protein-based diversity, which might be used as a model to look for protein-based variation in other key plant sub-species. Jan et al. [46] investigated variations in total seed proteins in three ecotypes of *B. rapa*. SDS-PAGE was used to examine 20 distinct genotypes from all ecotypes, and their phylogenetic relationships were noted. Protein size polymorphism was examined,

and four primary groupings were identified based on molecular weights ranging from 10 kDa to 180 kDa. Large size proteins were in group A, small size proteins were in group D, and medium size proteins were in groups B and C. The UPGMA was used to evaluate total soluble seed protein changes, which aggregated all three ecotypes into four primary groups. One toria and one brown sarson genotype each were found in clusters I and III, respectively. When compared to other cluster groupings, these two displayed the most polymorphism. All three ecotypes were found in clusters III and IV. For all three kinds, similarity coefficient values ranging from 47 to 100% were recorded. Brown sarson and toria genotypes had the highest similarity coefficient value of 100%, while brown sarson and yellow sarson ecotypes had the lowest similarity indices of 47%. They identified significant protein-based differences in all three ecotypes of *B. rapa* for the first time. Their findings will be useful as a starting point for characterizing *B. rapa* ecotypes. Mukhlesar et al. [47] empirically analyze the genetic diversity in *Brassica* species collected from different countries around the globe using SDS-PAGE Analysis. This study is conducted to find the protein content and to identify the polymeric genetic markers for the evaluation of the genetic resources. In this study the leaf protein and seed storage protein of various *Brassica* species were analyzed. For performing taxonomy studies SDS-PAGE has proven to be a useful tool. As the protein types and their variation are unique among different species the information will help us to get the data about purity of genetic sources and to early identification of the species.

Jan et al. [48] investigated the possibility of updating a systematic SDS-PAGE procedure used for fast protein examination of *B. rapa*. The current study aimed to develop an efficient SDS-PAGE protocol for *B. rapa*. To examine their electrophoratic protein profiling diverse genotypes were used. By enhancing different parameters, an improved SDS-PAGE procedure for essential *B. rapa* was developed. Kakaei et al. [49] was used to examine the seed protein pattern of 12 *B. napus* cultivars using SDS-PAGE. Their findings revealed sufficient diversity in seed protein content among rapeseed cultivars. They discovered polymorphism based on the protein intensity between genotypes. The polymorphism was more

prevalent in proteins weighing 66-100 KDa, while it was absent in certain significant genotypes measuring 15-27 KDa. Their data can be used to achieve maximum heterosis by selecting the most distinct cultivars.

2.2 Molecular Markers Based Literatures

Ali et al. [50] used molecular markers to reveal the genetic diversity of *B. rapa* germplasm in KPK, Pakistan. Accessions of native *B. rapa* were composed from a range of areas in KPK, Pakistan. The most diverse genotypes among the obtained samples were identified using SSRs markers. SSRs markers revealed a surprising amount of heterogeneity among the genotypes studied. The presence and absence of bands were used to calculate the differences between genotypes.

Hobson et al. [51] discovered a large number of SSRs markers in the *Brassica* genome and the importance of a genome in breeding. The goal of this study was to expand SSRs markers from the publicly accessible *B. rapa* genome sequence and provide the actual location of these markers on the chromosomes for breeding and research purposes. SSRs and annotated elements from the *B. rapa* assembly annotation file were compared in terms of start and stop positions.

Thakur et al. [52] sheds light on the variation of SSR marker in *Brassica* species for the beginning and development of *Brassica* amphidiploids. SSR markers were used to untie genetic differences in three diploids and three amphidiploids *Brassica* species to better be aware of the basis and advancement of *Brassica* amphidiploids. This study's SSRs marker collection will be helpful for DNA fingerprinting of several *Brassica* cultivars, assessing genetic diversity in *Brassica* germplasm, genome mapping and linkage map creation, gene tagging, and other genomics-related studies in *Brassica* species. Plieske et al. [53] assessed the diversity of *Brassica* species based on microsatellite markers. A total of 121 SSRs markers were utilized, with *B. rapa* and *B. oleracea* accounting for 83% of amplified fragments and *B.nigra* accounting for just 30%. Other species contains 35% of fragments, including major *Eruca sativa* plants, were amplified. 198 polymorphic bands were formed using

61 SSRs markers across 32 rapeseed cultivars in another investigation. Except for *Eruca sativa*, all other species resulted in distinct band patterns. All *Brassica* species were divided into spring and winter types.

Wu et al. [54] examined genetic diversity in several *Brassica* species using SSRs markers. In 248 *B. napus* and 25 *B. oleracea* inbred lines, a total of 48 SSRs markers were checked. When comparing the A and C genomes, the A genome had the most genetic diversity. Semi-winter genotypes seemed to have the least variation in C genome diversity. In comparison to *B. napus* genotypes, *B. oleracea* genotypes contain highest diversity.

Celucia et al. [55] investigated the molecular maker based variations of three *B. rapa* subspecies. 54 SSRs markers were used to analyze the genotypes of *B. rapachinensis L.*, *B. rapa parachinensis*, and *B. oleracea alboglabra*, respectively. A total of 122 scoring fragments were found, with 77% of them being highly polymorphic. Among genotypes with a high degree of genomic variability, the mean total diversity was 71.07%. In contrast to the other *B. oleracea alboglabra* sub-species, both *B. rapa chinensis* and *B. rapa parachinensis* belonged to the same cluster group. According to their findings, *B. oleracea alboglabra* differs from the other two subspecies. They looked at the genetic diversity of three different sub-species of *B. rapa* that are regularly farmed in different parts of the world.

Yuzhen et al. [56] established a DNA fingerprint database sufficient for cotton cultivar verification by creating a set of perfect SSRs markers with a single copy in the cotton genome. They improved the detection efficiency and multiplatform compatibility of the PCR technology. Single-copy polymorphic SSR sites were found and produced as diploidization SSRs markers using the reference genome of upland cotton and 10 resequencing data of 48 basic cotton germplasm lines. Initial screening was done using denaturing PAGE, followed by secondary screening with fluorescence capillary electrophoresis. 210 lines from various sources were used to test and verify the final perfect SSRs markers in Chinese cotton regional trials.

Wen et al. [57] used *Jatropha curcas L.* because it has sparked a lot of interest around the world due to its potential as a new bio-diesel crop. However, knowledge

of this crop is very restricted, and little genetic research has been conducted. From 45 accessions of *J. curcas* they examined genetic relationship; we employed SSRs markers that could be transferred from *Manihot esculenta* (cassava).

Dini et al. [58] studied other species like *Eucalyptus* dunnii, the use of SSRs markers as seed orchard selection tactics has proven to be an effective tool for reducing inbreeding. The tight selection of reproductive populations in long-term breeding operations limits the number of genotypes participating in the final orchard, limiting genetic diversity and increasing the risk of depression in subsequent generations due to inbreeding. SSRs markers transferability between *Eucalyptus* species has been extensively researched. The goal of their study was to find highly polymorphic *E. globulus* SSRs markers to aid breeding operations.

Rauscher et al. [59] suggested that from the group of leafy vegetables, lettuce (*Lactuca sativa L.*) is the most common crop. Several types of molecular markers have been created for use in lettuce breeding and genetic research. However, there are just a few microsatellite-based markers that are publicly available. To produce 97 genomic SSRs markers, we used the enriched microsatellite libraries approach.

Xu et al. [60] analyzed that BAC libraries, BAC-end sequences, genetic and physical maps, and seed BAC sequences for *B. rapa* have all been created by the Multinational *B. rapa* Genome Sequencing Project (BrGSP). The quick transfer of these rich resources from *B. rapa* to *B. napus* will be facilitated by an integrated linkage map between the amphidiploid *B. napus* and diploid *B. rapa* (Oilseed rape, Canola).

Parthiban et al. [61] assessed the efficacy of primers, 59 sugarcane genetic stocks were used to test twenty-five primer pairs produced from SSRs and 25 expressed sequence tags (EST) SSRs. When compared to the PIC value obtained by the EST-SSR marker, the mean PIC of genomic SSRs was higher 0.62 to 0.72. The low amount of polymorphism in EST-SSR markers could be attributed to the fact that these markers are located in more conserved and expressed sequences than genomic sequences, which are dispersed throughout the genome. Dendrogram based on genomic SSRs and EST-SSR marker data revealed genotype grouping

disparities. Using genomic SSRs and EST-SSR, 59 sugarcane accessions were divided into 4 and 6 clusters, respectively.

Xu et al. [62] showed that markers with high polymorphism and co-dominance degrees, known as SSRs, are a valuable resource for genetic research. In the genome of the cabbage 'TO1000,' researchers discovered 64,546 perfect and 93,724 imperfect SSR motifs. They separated SSRs into different linkage groups depending on their overall length and repeat number. After that, they characterized cabbage genomes in terms of motif length, motif type classification, and SSRs level, and compared them to other cruciferous genomes. Moreover, a high number of 64,546 primer pairs were successfully identified, yielding 1,113 SSRs primers, with 916 (82.3%) demonstrating repetitive and stable amplification. Based on their findings, the genomic SSRs markers discovered in this study may aid cabbage research, laying the groundwork for future gene tagging and genetic linkage investigations such as marker-assisted selection, genetic mapping, and comparative genomic analysis.

Kumar et al. [63] studied that SSRs markers technology is continuously evolving as sequencing techniques improve and software to discover SSRs markers is developed. Comparative SSRs marker research targeting *Arabidopsis thaliana* and *Brassica* species that are from the same family helped in salt tolerance-related QTLs, the identification of "candidate genes," and the discovery of the origin of significant QTLs. Even though there have been some reports on utilizing molecular markers to improve salt tolerance in *Brassica* species, SSRs markers proved to be so helpful in improving salt tolerance in *Brassica* crops. SSRs marker-driven breeding procedures play a significant role in obtaining the finest harvests, and it has been explored, particularly for the gene flow of salt tolerance characteristics in crops. Shen et al. [64] used ISSRs and special SCAR markers to examine the molecular markers-based diversity among 11 non-heading Chinese cabbage genotypes. All of the examined genotypes had similarity coefficient values that ranged from 0.545 to 0.896%. Comparing the Fragrant Bok Choy genotype to other markers, the ISSR-840 primer amplified a particular segment. They came to the conclusion that such markers are suitable for effective morphological screening of non-heading Chinese genotypes. Iqbal et al. [65] showed significant genetic differences amongst

16 *B. rapa* genotypes by using 12 RAPD markers. In comparison, the 03 markers were shown to be very polymorphic. Maximum polymorphism was present in 38 (94.87%) of the 40 bands. OPB-04 and GLA-11 amplified the least, while the primer OPD-02 exhibited bands that were entirely polymorphic (92.31%). 12.67 polymorphic fragments and 13.33 scorable bands per primer were recorded. These genotypes were split into three main categories based on similarity coefficient values. There were unique genotypes for each group.

Gupta et al. [66] in their study evaluated the genetic diversity of Indian mustard genotypes using molecular markers. The genotypes of 23 *B. juncea* and 04 other *Brassica* species were screened using both RAPD and EST-SSR markers. With 15 RAPD primers, an average of 17 per primer, approximately 260 bands were computed. The 236 bands among these bands displayed high levels of polymorphism, with an average of 15.7 bands per primer. When comparing RAPD markers to EST-SSR markers, the level of polymorphism was at its highest from 86.6 to 91%.

Talebi et al. [67] revealed ISSRs markers based on diversity across several exotic *B. rapa* germplasm. 47 distinct genotypes were identified using a total of 12 of these markers. With the help of these primer sets, 99 distinct polymorphic bands could be detected, and the most genomic diversity was found in rare germplasm. An UPGMA method that divided all genotypes into three groups was used to build the genetic tree. Turnip kinds and particularly oily content made up group I. Types of oil, cabbage, and turnips were found in group II. Group III had greasy and cabbage-type items. Group III had all of the genotypes found in China and Korea. Their findings can be used to divide the European and Asian *B. rapa* genotypes into distinct groups. Takuno et al. [68] evaluated the phylogenetic relationships of a prominent cultivar of *B. rapa* by using AFLP markers. The genotypes that were evaluated come in a variety of morpho-types, including turnip, Chinese cabbage vegetable sarson, pot herb, and Pak-choi varieties. These markers were used to screen the 32 different genotypes that were obtained from the European and Asian regions of the world. A total of 452 amplified fragments, of which 392 were highly polymorphic, were obtained. Two further sub-groups were created from the *B. rapa* ecotypes. *B. rapa* species and *B. oleifera* were found in one group, while

East Asian genotypes predominated in other groupings. Additionally, they stated that compared to European varieties, Asian *B. rapa* genotypes are more primitive.

Sharma et al. [69] study SSRs markers and several agromorphological, physiological, and biochemical parameters to assess the genetic diversity in leafy mustard germplasm. They noted that 59 accessions varied significantly in both qualitative and quantitative agro-morphological features, indicating the accessions promising potential for use in breeding programmes and for human consumption. The 482 alleles produced by the 155 SSRs that were analyzed ranged in number from 1 to 8, with an average of 3.11 alleles per marker. 122 SSRs in total (78.70%) gave rise to polymorphic amplicons. Per SSR locus, the PIC value ranged from 0.32 to 0.77, with an average value of 0.44. All 59 accessions were classified into two primary groups using agro morphological characteristics and SSRs markers in the unweighted neighbor-joining-based dendrogram analysis.

Chapter 3

Materials and Methods

3.1 SDS-PAGE Based Characterization of *B. rapa* Germplasm

3.1.1 Plant Materials

The above experiment was carried out at Plant Genetic Resources Institute (PGRI), National Agriculture Research Centre (NARC) and Islamabad, Pakistan. The mature seeds of 30 genotypes of *B. rapa* were collected from different sites of Punjab, Pakistan including 1 check variety from NARC (Table 3.1) and are subjected to the SDS-PAGE for seed protein.

3.1.2 Protein Extraction

Fresh 10-15 seeds of *B. rapa* were delicately grind with mortar and pestle. Mashed supplies (0.02g) will be carried to every 1.5 ml Eppendorf tube with addition of 400 μl protein extraction buffers (0.5M Tris-HCl (pH 8.0), 0.2% SDS, 5M urea, 1% 2-mercaptoethanol, and bromophenol blue dye). The specimen was suitably mixed by vertex for 1-2 mints and stored overnight in refrigerator at -20^{o}C.

3.1.3 Electrophoresis Staining and Destaining

With a few minor modifications, the separation and stacking gels, Ammonium Per sulphate (APS), staining and destaining solutions were made in accordance with the SDS-PAGE protocol of Jan et al [48].

Chemicals of different concentrations were used for the preparation of separation and stacking gels (Tables 3.7 and 3.8). The sample stored in refrigerator was centrifuged at 12000 rpm for about 10 mints. 10 μL of each sample was loaded into each well along with a protein marker at 100 V by using an electrode buffer. The activity of proteins was judged continuously until reached at the base of the plates. After that, the gels was shifted into staining solutions and shaken for 4-5 hours.

The gels were cleaned twice with distilled water before being placed in a new destaining solution and shaken for 24 hours. To remove the surplus blue color from the autoclave tissue paper, it was placed on a gel. The ideal pH of all sorts of solutions is critical for protein mobility and separation. The movement and visibility of different sizes of protein are strongly affected by the low or very high pH of different solutions and protein extraction buffers.

3.1.4 Data Analysis

The band's pattern of all genotypes was recorded. The obvious bands were scored 1 and nonappearance of bands with 0. The Nei and Li method [71] was used to calculate the genetic similarity estimates. The UPGMA method was used to create the dendrogram [71].

NTSYS-pc, version 2.1, a piece of computational software, was used to check genetic diversity among genotypes. Principal Coordinates Analysis (PCoA) was used in the two-dimensional (2D) and three-dimensional (3D) studies for observing genotype dispersal using the NTSYS pc, version 2.1 [72] software package.

TABLE 3.1: Detail of *Brassica rapa* genotypes selected for SDS-PAGE analysis.

Sr. No.	**Acc. No**	**Source**
1.	MulBr803	Multan, Punjab.
2.	OkaBr860	Okara, Punjab.
3.	MulBr807	Multan, Punjab.
4.	LhrBr814	Lahore, Punjab.
5.	ChkBr811	Chakwal, Punjab.
6.	ChkBr812	Chakwal, Punjab.
7.	ChkBr810	Chakwal, Punjab.
8.	LhrBr815	Lahore, Punjab.
9.	LhrBr817	Lahore, Punjab.
10.	LayBr818	Layyah, Punjab.
11.	LayBr819	Layyah, Punjab.
12.	LayBr820	Layyah, Punjab.
13.	DerBr822	Dera Ghazi Khan, Punjab.
14.	DerBr823	Dera Ghazi Khan, Punjab.
15.	DerBr825	Dera Ghazi Khan, Punjab.
16.	SadBr831	Sadiqabad, Punjab.
17.	SadBr832	Sadiqabad, Punjab.
18.	SadBr834	Sadiqabad, Punjab.
19.	NorBr840	Narowal, Punjab.
20.	NorBr841	Narowal, Punjab.
21.	NorBr843	Narowal, Punjab.
22.	MuzBr847	Muzaffargarh, Punjab.
23.	MuzBr847	Muzaffargarh, Punjab.
24.	FaiBr850	Faisalabad, Punjab.
25.	FaiBr851	Faisalabad, Punjab.
26.	FaiBr853	Faisalabad, Punjab.
27.	SheBr855	Shakhupura, Punjab.
28.	MulBr804	Multan, Punjab.
29.	OkaBr862	Okara, Punjab.

TABLE 3.1: Detail of *Brassica rapa* genotypes selected for SDS-PAGE analysis.

Sr. No.	**Acc. No**	**Source**
30	Toria	NARC, Islamabad

TABLE 3.2: Composition of protein extraction buffer.

Ingredients	**Amounts**
Distt. H_2O	75ml
Tris(hydroxymethyl) aminomethane (THAM)	0.7g
HCL (conc)	Adjust to pH 8.0
SDS	0.4g
Urea	30g
2-Mercaptoethanol 1ml	1ml
Total vol.	100ml

TABLE 3.3: Composition of Sol. A

Ingredients	**Amounts**
Distt. H_2O	100ml
THAM	34g
SDS	0.8g
pH	8.0

Kept in freezer at -4^{o}C.

TABLE 3.4: Composition of Sol. B

Ingredients	**Amounts**
Distt. H_2O	100ml
THAM	7g
SDS	0.7g
pH	7.0

Kept in freezer at -4^{o}C

Table 3.5: Composition of Sol. C

Ingredients	**Amounts**
Acrylamide	31g
Bis (bis-acrylamide)	1g
Distt. H_2O	100ml

Kept in freezer at -4^{o}C.

Table 3.6: Composition of Ammonium Per Sulphate (APS)

Ingredients	**Amounts**
Ammonium Per Sulphate (APS)	0.2g
Distt. H_2O	1ml

Kept in freezer at -4^{o}C.

Table 3.7: Composition of Separation Gel

Ingredients	**Amounts**
Distt. H_2O	7.5ml
Sol. A	5ml
Sol. C	7.5ml
10% APS	230μl
TEMED	60μl

Kept in freezer at -4^{o}C.

Table 3.8: Composition of Stacking gel

Ingredients	**Amounts**
Distt. H_2O	6.0ml
Sol. B	5ml
Sol. C	2ml

Table 3.8: Composition of Stacking gel

Ingredients	**Amounts**
10% APS	100μl
TEMED	50μl

Kept in freezer at -4^{o}C.

Table 3.9: Composition of electrode buffer solution

Ingredients	**Amounts**
THAM	3.2g
Glycine	14g
SDS	1.3g
Distt. H_2O	1000ml

Kept at room temperature

Table 3.10: Composition of staining solution

Ingredients	**Amounts**
Distt. H_2O	470ml
Acetic acid	70ml
Methanol	460ml
CBB	2.10g

Kept at room temperature.

Table 3.11: Composition of destaining solution.

Ingredients	**Amounts**
Distt. H_2O	700ml
CH_3COOH	250ml
CH_3OH	50ml

Kept at room temperature.

3.2 Genetic Diversity of *Brassica rapa* Based on SSRs Marker

3.2.1 Experimental Material

The 30 distinct *B. rapa* genotypes were collected from several locations around the Punjab region, including one checked variety (Toria). The genetic diversity of these genotypes was examined using the 10 SSRs markers unique to this crop. The data of *B. rapa* genotypes and SSRs marker used are provided in Table 3.12 and 3.13.

3.2.2 Sample Preparation

In pots, the 4-5 fresh seeds of each genotype were planted. After two days inside, these pots received frequent watering. Following germination for two to three weeks, samples of fresh leaves were collected. For later usage, the leaf samples were kept in a refrigerator at -80^{o}C.

3.2.3 DNA Extraction

All the stock solutions were prepared prior to DNA extraction and the DNA was extracted using CTAB method [73]. The DNA was extracted as follows:

- Each genotype's three to four leaves were mashed in a mortar and pestle with the addition of a CTAB solution (700 μl) containing a tiny amount of mercaptoethanol (30 μl/1 ml CTAB solutions). After being suitably crushed, the samples were placed in a 1.5 ml Eppendorf tube.

- All samples were kept in the water bath for 40 minutes at 65^{o}C, rotating over four times every 5 mins.
- After the samples were cooled to room temperature, 600 μl of chloroform was added. The right amount of isoamyl alcohol (24:1) was added and stirred.
- Samples were centrifuged at 13000 rpm for 10 mins at 4^{o}C.
- The supernatant obtained was then transferred to new tubes for each sample i.e., 600 μl and the waste was discarded.
- Then for 2-3 times ice-chilled Isopropanol was added in amount 350 μl and was then placed in freezer at 4^{o}C for at least 30 mins.
- Centrifuged again for 10 minutes at 4^{o}C at 13000 rpm.
- From each tube the small white pellets were collected.
- After carefully removing the supernatant, 200 μl of ethanol at a 70% concentration were added to each tube.
- Then again centrifugation takes place at room temperature but for 8 minutes and at 13000rpm.
- On the sterilized filter paper, the tubes were left open at room temperature after the supernatant was carefully discarded.
- Unless the smell of ethanol finished entirely the pellet was dried on filter paper for about an hour.
- Then in every single tube 100 μl of fresh TE buffer was added and vertexed.
- Added each tube with 1 μl of RNase A (10 mg/ml) and incubated for 35 minutes at 40^{o}C in a water bath.
- Then at -20^{o}C in freezer all the extracted DNA samples were stored.
- Each DNA sample's quality and purity were examined using a Nano Drop ND-1000 Spectrophotometer at 260 and 280 nm.

- To obtain accurate PCR findings, all DNA samples were diluted to a working concentration of 20 mg/μl.

3.2.4 Specification of the Primers

To investigate molecular variability among *B. rapa* accessions, ten simple sequence repeats (SSRs) markers were used. In *Brassica* literature, both the monomorphic and polymorphic bands were produced by the desired primers.

3.2.5 Amplification of SSRs Markers in *B. rapa* Germplasm

The PCR conditions were optimized based on available data for each primer, with minor changes to the annealing temperature. Table 3.12 contains detailed information on the 20μl PCR reaction volume and PCR conditions. Agarose concentrations varied depending on the size of the primer.

To obtain clear PCR bands, 2 to 3% agarose gels were typically used. The high resolution agarose gel was prepared with a 1xTBE buffer (Tris-Borate = 10mM and EDTA = 1 mM) with the addition of 5μl ethidium bromide. The optimum condition for different PCR steps is given in Table 3.13. Tables of PCR rxn/mix & cycles 3.14 & 3.15.

3.2.6 Electrophoresis of Amplified Products

After PCR 4 μl of 6x loading dye was added to PCR tubes. In each well, the 7 μl PCR sample was placed. To verify the exact size of SSR markers, a DNA ladder of 50 and 100 bp was used. Under UVI Gel Doc Documentation System, the gels were seen, and the desired PCR product sizes were noted.

3.2.7 Allele Scoring and Data Analysis

For each sample, for each primer the banding pattern was recorded in the presence of a single, two, or multiple bands. Band presence was indicated by 1 and band absence by 0. All the data were recorded in MS-excel. For data analysis, only clear DNA bands were used. Each primer's total allele presence, total polymorphic allele count, and ideal annealing temperature were noted. Pair wise comparisons of *B. rapa* genotypes based on the presence or absence of alleles were used to compute genetic similarity (F) coefficients by the dice algorithm [70]. The values of the similarity coefficients were used to construct a phylogenetic tree based on UPGMA. NTSYS pc, version 2.1, was used for the analysis [72].

TABLE 3.12: List of *B. rapa* genotypes used for SSR analysis (n=30).

Sr. No.	**Genotypes**	**Source**
1	MulBr803	Multan, Punjab
2	OkaBr860	Okara, Punjab
3	MulBr807	Multan, Punjab
4	LhrBr814	Lahore, Punjab
5	ChkBr811	Chakwal, Punjab
6	ChkBr812	Chakwal, Punjab
7	ChkBr810	Chakwal, Punjab
8	LhrBr815	Lahore, Punjab
9	LhrBr817	Lahore, Punjab
10	LayBr818	Layyah, Punjab
11	LayBr819	Layyah, Punjab
12	LayBr820	Layyah, Punjab
13	DerBr822	Dera Ghazi Khan, Punjab
14	DerBr823	Dera Ghazi Khan, Punjab
15	DerBr825	Dera Ghazi Khan, Punjab
16	SadBr831	Sadiqabad, Punjab
17	SadBr832	Sadiqabad, Punjab
18	SadBr834	Sadiqabad, Punjab

TABLE 3.12: List of *B. rapa* genotypes used for SSR analysis (n=30).

Sr. No.	Genotypes	Source
19	NorBr840	Narowal, Punjab
20	NorBr841	Narowal, Punjab
21	NorBr843	Narowal, Punjab
22	MuzBr845	Muzaffargarh, Punjab
23	MuzBr847	Muzaffargarh, Punjab
24	FaiBr850	Faisalabad, Punjab
25	FaiBr851	Faisalabad, Punjab
26	FaiBr853	Faisalabad, Punjab
27	SheBr855	Shakhupura, Punjab
28	MulBr804	Multan, Punjab
29	OkaBr862	Okara, Punjab
30	Toria	NARC, Islamabad

TABLE 3.13: SSRs primers for diversity evaluation of *B. rapa* germplasm.

Sr No.	Primer	Forward primer (bp)	Reverse primer (bp)
1.	Na10-C06	TGGATGAAA GCATCAACG	ATCAATCAAC ACAAGCTGCG
2.	Na10-D09	AAGAACGTCA GATCCTCTGC	ACCACCACGG TAGTAGAGCG
3.	Na12-C07	ACTCAACCCC ACAAACCTG	AGTTCCCCGG ATCCGATTAG
4.	Na12-C08	GCAAACGATT TGTTTACCCG	CGTGTAGGGT GATCTAGATG GG
5.	Na14-D07	GCATAACGTC AGCGTCAAAC	CTGCGGGACA CATAACTTTG

TABLE 3.13: SSRs primers for diversity evaluation of *B. rapa* germplasm.

Sr No.	**Primer**	**Forward primer (bp)**	**Reverse primer (bp)**
6.	Ni2-F02	TGCAACGAAA AAGGATCAGC	TGCTAATTGAG CAATAGTGATT CC
7.	Ni4-D09	AAAGGACAAA GAGGAAGGGC	TTGAAATCAAA TGAGAGTGACG
8.	O109-A06	TGTGTGAAAG CTTGAAACAG	TAGGATTTTTT TGTTCACCG
9.	O110-F11	TTTGGAACGT CCGTAGAAGG	CAGCTGACTTC GAAAGGTCC
10.	Ra2-A11	GACCTATTTTA ATATGCTGTTT GA	ACCTCACCGGA GAGAAATCC

TABLE 3.14: Microsatellite PCR analysis (reaction mix)

Components	**Stock concetra -tion**	**Final concetra -tion**	**Vol/ Rxn**	**Samples**	**Total Vol.**
ddH_2O	-	-	10.7µl	x30	321µl
PCR Buffer minus MgCl2	10x	1 x	2.0µl	x30	60µl
dNTP Mixture	100 mM Each	2mM	2µl	x30	60µl

Table 3.14: Microsatellite PCR analysis (reaction mix)

Components	**Stock concetra -tion**	**Final concetra -tion**	**Vol/ Rxn**	**Samples**	**Total Vol.**
Forward Primer	20 pmoles/µl (20µM)	0.8 µM	0.8µl	x30	24µl
Reverse Primer	20 pmoles/µl (20µM)	0.8 µM	0.8µl	x30	24µl
Taq DNA Polymerase	5 Units/µl	1 unit/r x n	0.2µl	x30	6µl
Template DNA	20-50 ng/µl	20-50 ng/r x n	1.0µl	-	-
Total Volume	-	-	20.0µl	-	-

Table 3.15: PCR thermal cycler profile

Profile	**Temperature °C**	**Time**	**No. of Cycles**
Initial Denaturation	94 ^{o}C	5 minutes	1
Final Denaturation	94 ^{o}C	1 minute	
Annealing	55-60 ^{o}C	40 seconds	35
Initial Extension	72 ^{o}C	2 minutes	
Final Extension	72 ^{o}C	7 minutes	1

Chapter 4

Results and Discussions

4.1 Total Seed Proteins Based Variations of *B. rapa* through SDS-PAGE Method

Biochemical-based characterization is crucial for the genetic improvement of *Brassica* species. SDS-PAGE is a quick, easy, and affordable approach for defining various crop species. This approach is used to classify genotypes and examine intra-specific based changes among them.

Research on protein-based variation in several *B. rapa* species and subspecies is still in its infancy. Smaller molecules travel through gels more quickly than larger ones therefore it makes sense to divide protein subunits based on their molecular weight.

The molecular weight of known proteins is used to calculate the molecular weight of an unknown protein. The SDS-PAGE method, which provides information on polypeptide profiles, is thought to be a center of genetic variety. For the protein-based seed storage diversity among several plant species, SDS-PAGE has been useful. A few techniques were used in the past to characterize the various *B. rapa* accessions. Both quality and stability are crucial for an effective SDS-PAGE profiling.

4.1.1 Polymorphism in Protein Banding Patterns

In this study, 30 local *B. rapa* genotypes were included in the total seed storage-based variability analysis. All genotypes showed polymorphism and the majority of genotypes displayed distinctive banding patterns. The protein banding pattern is shown in Fig 4.1 (a-c). We recorded both the major and minor bands' pattern. Its sizes ranging from (∼10 kDa to ∼180 kDa) depend on the genotype being used. Maximum of 13 bands were recorded and all of them showed polymorphic banding patterns (100%). These polypeptide bands were divided to 4 main regions (A-D) on the basis of their molecular weight Fig 4.1 (a-c). High molecular weight proteins were present in A region that size ranged from ∼130 kDa to ∼180 kDa. In this region most of the bands were polymorphic. Second part (B) has molecular weight ranging from ∼100 kDa to ∼129 kDa. Medium size proteins were present in the third part (C) and these sizes varied from ∼40 kDa to ∼99 kDa. Small molecular weight proteins were present in the fourth part (∼10 kDa to ∼39 kDa).

4.1.2 Similarity Co-efficient of *Brassica rapa* Genotypes

The genetic diversity of *B. rapa* genotypes was evaluated using SDS-PAGE following the method of Nei and Li [73]. All investigated genotypes had similarity coefficient values that ranged from 15 to 100% across all genotypes (Table 4.1). The highest similarity value 100% was observed between OkaBr-860/MulBr-803, DerBr-825/LayBr-820, SheBr-855/FaiBr-850, FaiBr-851/MuzBr-847 and MulBr-804/DerBr823 accessions, shows that these genotypes share certain genetic similarities. The least similarity coefficient value 15% was noted between NorBr-841/LhrBr-814 followed by 16% in SadBr-831/LhrBr-814 and DerBr-822/LhrBr-814 then 20% between LayBr-818/LhrBr-814 and then 28% between SheBr-855 /LhrBr-814, LayBr-820/LhrBr814, DerBr-825/LhrBr-814 and FaiBr-850/LhrBr-814. Our research revealed that the similarity coefficient values across the genotypes of *B. rapa* differed. Our findings further supported the idea that protein-based variability varies depending on the genotype or subspecies of *B. rapa*.

4.1.3 Cluster Analysis Study of *B. rapa*

30 different genotypes of *B. rapa* were examined to study phylogenetic relationships among them using the UPGMA algorithm. Based on the genetic similarity between genotypes, all different types of germplasm were grouped into seven groups (Fig. 4.2; Table 4.2). Group I- VII consisted of 6, 12, 5, 2, 3, 1 and 1 genotypes respectively. Group I contained 6 genotypes that were MulBr-803, OkaBr-860, ChkBr-810, MulBr-807, LhrBr-817, ChkBr-811. Group II integrated the largest number of genotypes 12, indicating that these genotypes were more intra-specific as compared to others. The genotypes which were present in group II were LhrBr-815, FaiBr-853, NorBr-840, LayBr-820, DerBr-825, DerBr-823, MulBr-804, OkaBr-862, NorBr-841, FaiBr-850, SheBr-855 and SadBr-834 respectively. Group III consisted of 5 genotypes which means it was less similar genotypes than other 2 groups. The genotypes present in group III were MuzBr-847, FaiBr-851, SadBr-831, NorBr-843 and SadBr-832. Group IV contains less genotypes than other groups which were DerBR-822 and Toria. Group V also contains less genotypes which was LayBr-818, LayBr-819 and MuzBr-845 (Table 4.2). All groupings are extremely polymorphic.The genotypes ChkBr-812 and LhrBr-814, were unique to the group VI and VII only, and differed from other genotypes tested. Our research revealed that there is the most proteome diversity among the various *B. rapa* populations. In contrast to other diverging genotypes, the genotypes that are identical to one another occupied the same group. This indicates that there is a significant amount of seed protein-based variability among *B. rapa* subspecies. From all studied groups, two of them showed maximum variability which are accessions LhrBr-814 and ChkBr-812 (Fig. 4.2; Table 4.3).

4.1.4 PCoA Analysis of SDS-PAGE Data

A multivariate method called PCoA is used to segregate individuals based on their genetic distances. Thus, a DICE similarity coefficient matrix is used for a clear comprehension of genetic variety, all of the *B. rapa* genotypes were dispersed on

2D and 3D scatter plots. A distinct variation in any dimension is provided by the 2D and 3D study based on PCoA. The 2D and 3D studies enable the identification of distinct genotypes in a large population.

All of the genotypes in our study were divided into 4 main groups using a 2D dendrogram. Each group has unique genetics. However, certain distinct, diversified genotypes are outliers, such as LhrBr-814, ChkBr-812, LayBr-818, DerBr-822 etc., were also recorded.

These genotypes greatly differ from the other genotypes. Genotypes were seen in three feature dimensions from any angle. The 3D map revealed various rare genotypes, including LayBr-818, ChkBr-812 and LayBr-819 among others (Figures. 4.3, 4.4, 4.5 and 4.6).

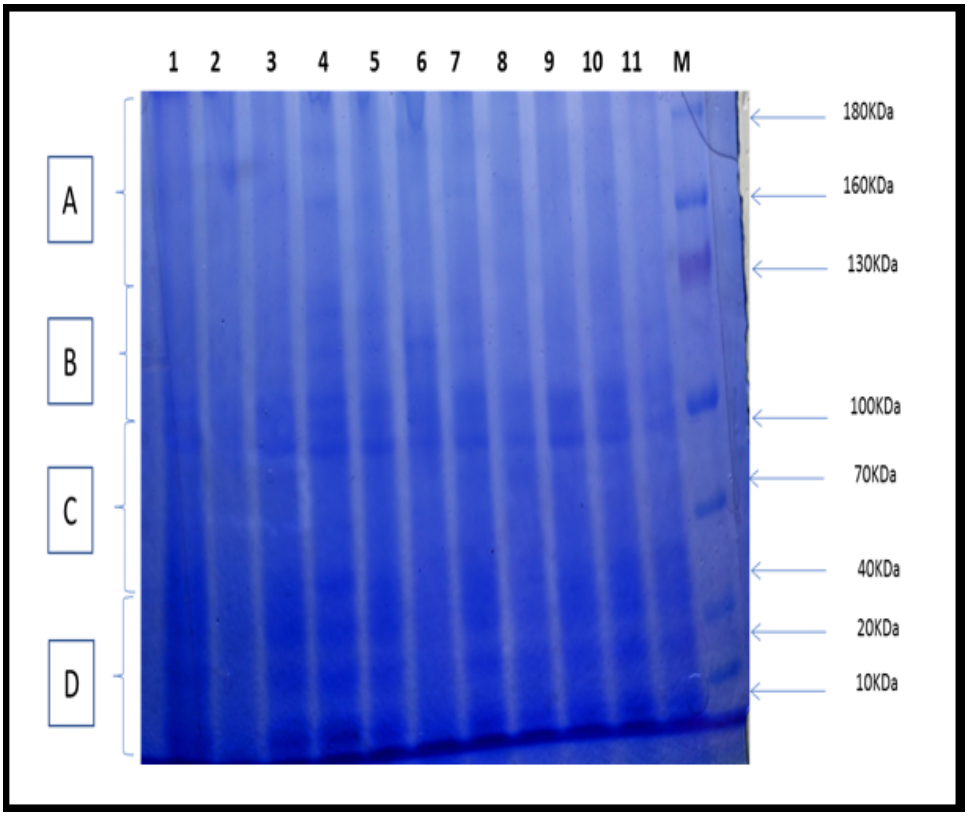

FIGURE 4.1: (A): Banding pattern of *B. rapa* genotypes generated through SDS-PAGE of total seed storage proteins 1-11 represent accessions MulBr-803, OkaBr-860, MulBr-807, LhrBr-814, ChkBr-811, ChkBr-812, ChkBr-810, LhrBr-815, LhrBr-817, LayBr-818, LayBr-819 and LayBr-820 respectively.

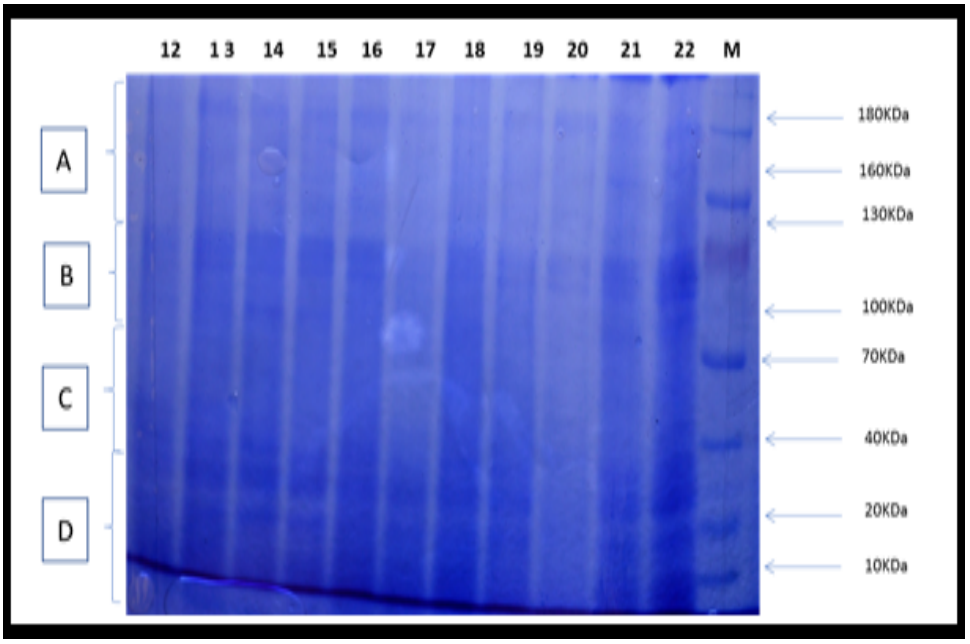

FIGURE 4.2: (B): Banding pattern of *B. rapa* genotypes generated through SDS-PAGE of total seed storage proteins 12-22 represent accessions DerBr-822, DerBr-823, DerBr-825, SadBr-831, SadBr-832, SadBr-834, NorBr-840, NorBr-841, NorBr-843, MuzBr-845 and MuzBr-847 respectively.

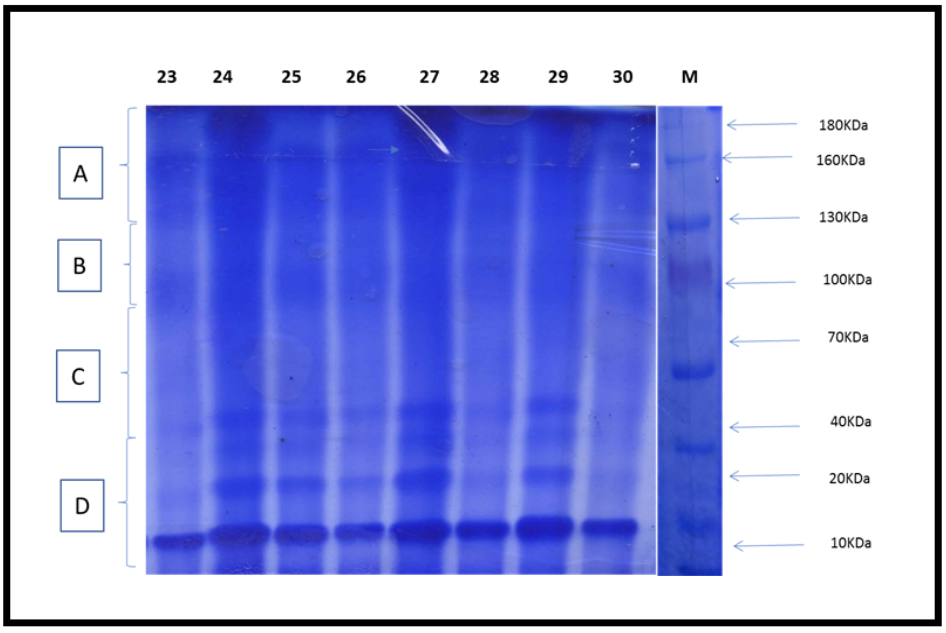

FIGURE 4.3: (C): Banding pattern of *B. rapa* genotypes generated through SDS-PAGE of total seed storage proteins 23-30 represent accessions FaiBr-850, FaiBr-851, FaiBr-853, SheBr-855, MulBr-804, OkaBr-862 and Toria, respectively.

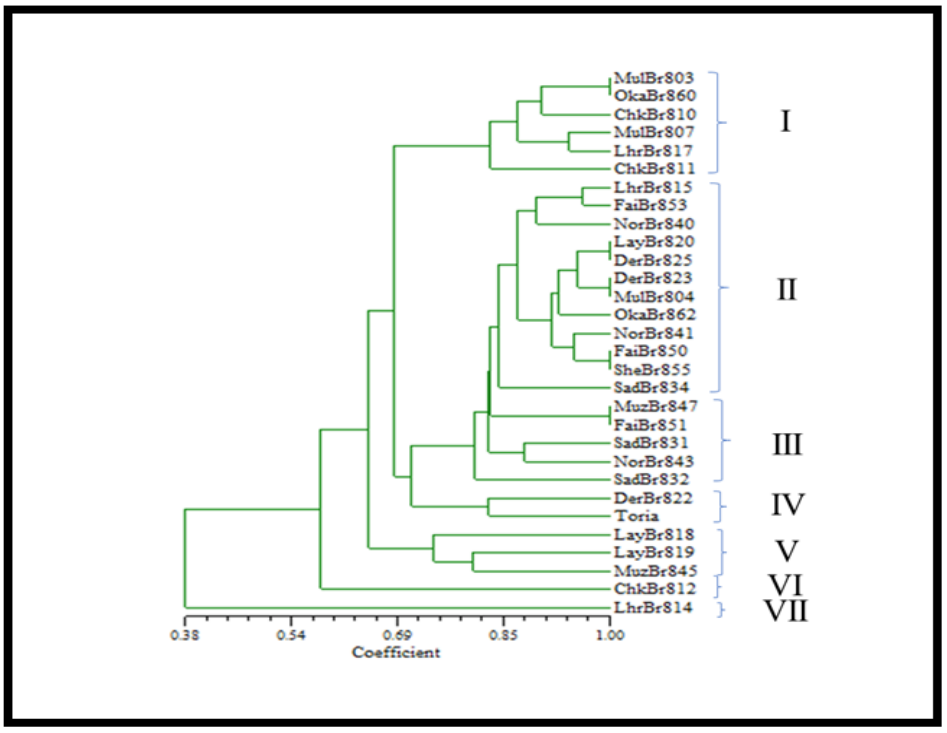

FIGURE 4.4: SDS-PAGE based diversity in 30 genotypes of *B. rapa* using cluster analysis.

TABLE 4.1: Clusters of 30 *Brassica rapa* genotypes based on SDS-PAGE method.

Cluster	**No. of genotypes**	**Genotypes**
I	6	MulBr-803, OkaBr-860, ChkBr-810
		MulBr-807, LhrBr-817 and ChkBr-811
II	12	LhrBr-815, FaiBr-853, NorBr-840,
		LayBr-820, DerBr-825, DerBr-823,
		MulBr-804, OkaBr-862, NorBr-841
		FaiBr-850, SheBr-855 and SadBr-834
III	5	MuzBr-847, FaiBr-851, SadBr-831
		NorBr-843 and SadBr-832
IV	2	DerBR-822 and Toria
V	3	LayBr-818, LayBr-819 and MuzBr-845
VI	1	ChkBr-812
VII	1	LhrBr-814

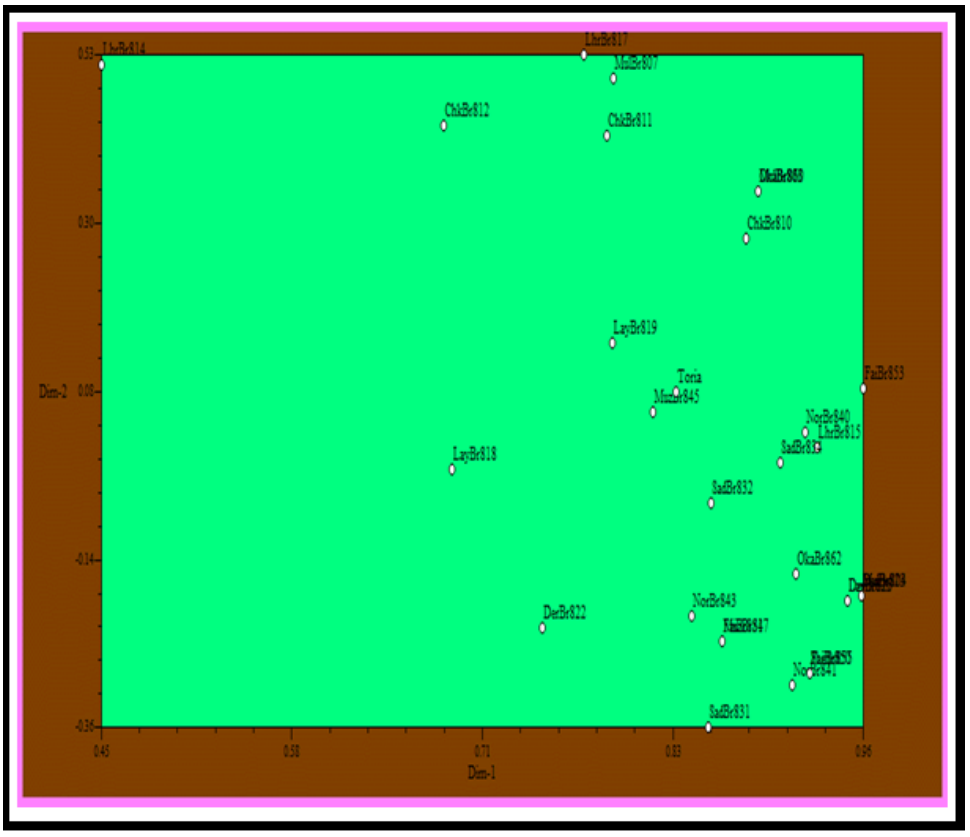

FIGURE 4.5: 2D analysis of 30 genotypes of *B. rapa* based on SDS-PAGE.

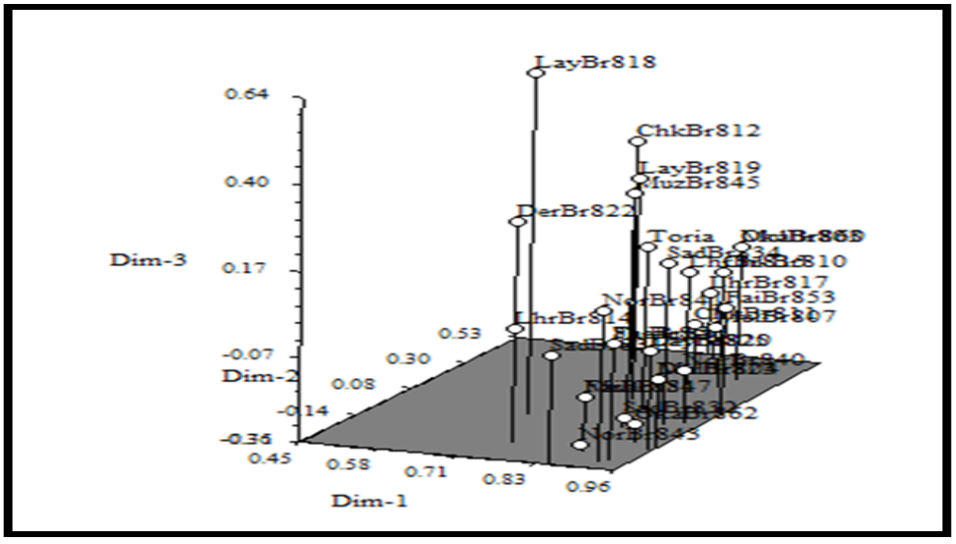

FIGURE 4.6: 3D visualization and vector analysis of 30 genotypes of *B. rapa* based on SDS-PAGE.

Table 4.2: Dice coefficient of similarity among 30 *B. rapa* genotypes on the basis of SDS-PAGE analysis.

Acc#	**MulBr803**	**OkaBr860**	**MulBr807**	**LhrBr814**	**ChkBr811**	**ChkBr812**	**ChkBr810**	**LhrBr815**
MulBr803	100.00%							
OkaBr860	100.00%	100.00%						
MulBr807	84.21%	84.21%	100.00%					
LhrBr814	42.86%	42.86%	61.54%	100.00%				
ChkBr811	84.21%	84.21%	88.89%	61.54%	100.00%			
ChkBr812	70.59%	70.59%	62.50%	54.55%	62.50%	100.00%		
ChkBr810	90.00%	90.00%	84.21%	42.86%	73.68%	70.59%	100.00%	
LhrBr815	81.82%	81.82%	76.19%	37.50%	76.19%	63.16%	81.82%	100.00%
LhrBr817	88.89%	88.89%	94.12%	50.00%	82.35%	66.67%	88.89%	70.00%
LayBr818	62.50%	62.50%	40.00%	20.00%	40.00%	61.54%	62.50%	66.67%
LayBr819	82.35%	82.35%	62.50%	36.36%	62.50%	57.14%	70.59%	73.68%
LayBr820	80.00%	80.00%	63.16%	28.57%	63.16%	58.82%	80.00%	81.82%
DerBr822	55.56%	55.56%	47.06%	16.67%	47.06%	53.33%	55.56%	80.00%
DerBr823	76.19%	76.19%	70.00%	40.00%	70.00%	55.56%	76.19%	86.96%
DerBr825	80.00%	80.00%	63.16%	28.57%	63.16%	58.82%	80.00%	81.82%
SadBr831	66.67%	66.67%	47.06%	16.67%	58.82%	40.00%	66.67%	80.00%

TABLE 4.2: Dice coefficient of similarity among 30 *B. rapa* genotypes on the basis of SDS-PAGE analysis.

Acc#	**MulBr803**	**OkaBr860**	**MulBr807**	**LhrBr814**	**ChkBr811**	**ChkBr812**	**ChkBr810**	**LhrBr815**
SadBr832	77.78%	77.78%	70.59%	33.33%	58.82%	40.00%	77.78%	70.00%
SadBr834	80.00%	80.00%	63.16%	42.86%	63.16%	70.59%	80.00%	81.82%
NorBr840	85.71%	85.71%	80.00%	40.00%	80.00%	55.56%	76.19%	86.96%
NorBr841	73.68%	73.68%	55.56%	15.38%	55.56%	50.00%	73.68%	85.71%
NorBr843	66.67%	66.67%	58.82%	33.33%	58.82%	40.00%	77.78%	70.00%
MuzBr845	77.78%	77.78%	58.82%	33.33%	58.82%	66.67%	77.78%	80.00%
MuzBr847	63.16%	63.16%	55.56%	46.15%	66.67%	50.00%	63.16%	76.19%
FaiBr850	70.00%	70.00%	63.16%	28.57%	63.16%	47.06%	70.00%	90.91%
FaiBr851	63.16%	63.16%	55.56%	46.15%	66.67%	50.00%	63.16%	76.19%
FaiBr853	86.96%	86.96%	81.82%	47.06%	81.82%	70.00%	86.96%	96.00%
SheBr855	70.00%	70.00%	63.16%	28.57%	63.16%	47.06%	70.00%	90.91%
MulBr804	76.19%	76.19%	70.00%	40.00%	70.00%	55.56%	76.19%	86.96%
OkaBr862	70.00%	70.00%	73.68%	42.86%	63.16%	47.06%	80.00%	81.82%
Toria	73.68%	73.68%	66.67%	46.15%	66.67%	75.00%	73.68%	76.19%

TABLE 4.3: Dice coefficient of similarity among 30 *B. rapa* genotypes on the basis of SDS-PAGE analysis.

Acc#	**LhrBr817**	**LayBr818**	**LayBr819**	**LayBr820**	**DerBr822**	**DerBr823**	**DerBr825**	**SadBr831**
LhrBr817	100.00%							
LayBr818	42.86%	100.00%						
LayBr820	66.67%	62.50%	70.59%	100.00%				
DerBr822	37.50%	71.43%	53.33%	66.67%	100.00%			
DerBr825	66.67%	62.50%	70.59%	100.00%	66.67%	95.24%	100.00%	
SadBr831	50.00%	57.14%	66.67%	88.89%	62.50%	84.21%	88.89%	100.00%
SadBr834	66.67%	75.00%	70.59%	90.00%	66.67%	85.71%	90.00%	77.78%
NorBr840	73.68%	47.06%	66.67%	85.71%	73.68%	90.91%	85.71%	73.68%
NorBr843	62.50%	42.86%	53.33%	88.89%	50.00%	84.21%	88.89%	87.50%
MuzBr847	47.06%	53.33%	62.50%	84.21%	70.59%	90.00%	84.21%	82.35%
FaiBr850	55.56%	62.50%	70.59%	90.00%	77.78%	95.24%	90.00%	88.89%
FaiBr853	76.19%	63.16%	70.00%	86.96%	76.19%	91.67%	86.96%	76.19%
SheBr855	55.56%	62.50%	70.59%	90.00%	77.78%	95.24%	90.00%	88.89%
MulBr804	63.16%	58.82%	66.67%	95.24%	73.68%	100.00%	95.24%	84.21%
OkaBr862	66.67%	50.00%	58.82%	90.00%	66.67%	95.24%	90.00%	77.78%
Toria	58.82%	66.67%	62.50%	73.68%	82.35%	80.00%	73.68%	58.82%

Table 4.4: Dice coefficient of similarity among 30 *B. rapa* genotypes on the basis of SDS-PAGE analysis.

Acc#	**SadBr832**	**SadBr834**	**NorBr840**	**NorBr841**	**NorBr843**	**MuzBr845**	**MuzBr847**	**FaiBr850**
SadBr832	100.00%							
SadBr834	77.78%	100.00%						
NorBr840	84.21%	76.19%	100.00%					
NorBr841	82.35%	84.21%	80.00%	100.00%				
NorBr843	87.50%	77.78%	73.68%	82.35%	100.00%			
MuzBr845	62.50%	77.78%	63.16%	82.35%	62.50%	100.00%		
MuzBr847	70.59%	73.68%	80.00%	77.78%	82.35%	58.82%	100.00%	
FaiBr850	77.78%	80.00%	85.71%	94.74%	77.78%	77.78%	84.21%	100.00%
FaiBr851	70.59%	73.68%	80.00%	77.78%	82.35%	58.82%	100.00%	84.21%
FaiBr853	76.19%	86.96%	91.67%	81.82%	76.19%	76.19%	81.82%	86.96%
SheBr855	77.78%	80.00%	85.71%	94.74%	77.78%	77.78%	84.21%	100.00%
MulBr804	84.21%	85.71%	90.91%	90.00%	84.21%	73.68%	90.00%	95.24%
OkaBr862	88.89%	80.00%	85.71%	84.21%	88.89%	66.67%	84.21%	90.00%
Toria	58.82%	73.68%	80.00%	66.67%	58.82%	58.82%	77.78%	73.68%

TABLE 4.5: Dice coefficient of similarity among 30 *B. rapa* genotypes on the basis of SDS-PAGE analysis.

Acc#	**FaiBr 851**	**FaiBr 853**	**SheBr 855**	**MulBr 804**	**OkaBr 862**	**Toria**
FaiBr851	100.00%					
FaiBr853	81.82%	100.00%				
SheBr855	84.21%	86.96%	100.00%			
MulBr804	90.00%	91.67%	95.24%	100.00%		
OkaBr862	84.21%	86.96%	90.00%	95.24%	100.00%	
Toria	77.78%	81.82%	73.68%	80.00%	73.68%	100.00%

4.2 Microsatellite (SSR) Based Inter-specific Variability Among *B. rapa* Genotypes

For SSR-based molecular analysis, a core collection of 30 diverse *B. rapa* genotypes was chosen. Ten microsatellite markers were used, with the majority exhibiting polymorphic banding patterns.

4.2.1 Inter-specific Variations Among *B. rapa* Genotypes

The genomic variability of 30 *B. rapa* genotypes was screened using 10 SSR markers. The desired amplified fragments were seen when these marker sizes were compared to their known sizes.

The range of alleles per locus was between one and two. Polymorphic banding patterns were observed in all primers (Fig. 4.8, 4.9 and 4.10, 4.11). The majority of these primers amplified fragments ranging from 100 to 400bp.

In all tested *B. rapa* genotypes, 7 primers (70%) detected only one allele. The other two primers (20%) (Ni2-F02, Ra2-A11) each amplified two alleles (Table 4.7). Only one primer (10%) (Na10-D09) amplified three allele.

4.2.2 Genetic Similarity and Cluster Analysis

The genetic similarity values between different *B. rapa* genotypes ranged from 0.0 to 100%. The genotypes with the lowest similarity coefficient 0.0 % were found between ChkBr-812/MulBr-803, ChkBr-812/OkaBr-860, ChkBr-812/ChkBr-811 and ChkBr-812/LayBr-919 etc. While the highest similarity value 100% was noted between DerBr-825/OkaBr-860, SadBr-831/OkaBr860, SadBr-831/DerBr825 and FaiBr-851/DerBr-825 etc. It is shown that genotypes exhibit large levels of genetic diversity. Using the UPGMA similarity method, all genotypes from various origins were classified into five major groups.

The clusters I to V had 7, 13, 6, 3 and 1 genotypes, respectively. There are 7 genotypes in group I i.e MulBr-803, FaiBr-850, FaiBr-853, FaiBr-851, LayBr-819, MuzBr-845 and MuzBr-847. With 13 genotypes, group II is the largest group containing OkaBr-860, DerBr-825, SadBr-831, OkaBr-862, SadBr-832, LayBr-818, LhrBr-815, MulBr-807, Toria, ChkBr-810, NorBr-841, NorBr-843 and DerBr-822. Group III comprises 6 genotypes i.e LhrBr-814, LhrBr-817, DerBr-823, SadBr-834, NorBr-840 and MulBr-804; however group IV only has 3 genotypes which are much smaller than other four groups containing genotypes ChkBr-811, SheBr-855 and LayBr-820 (Table 4.6).

ChkBr-812 is the sole genotype found in group V. It is the most varied genotype. Overall highly diverse groups are recorded (Fig 4.7; Table 4.6) Our findings revealed a high level of inter-specific similarity between genotypes however, a high degree of genetic variation among all genotypes was also observed. These findings can serve as a foundation for future genomic variability studies of important *B. rapa* and other *Brassica* subspecies.

4.2.3 PCoA Analysis

Modern techniques of 2D and 3D analysis are being used to investigate the most diverse genotypes in order to better understand genetic diversity. Principal Coordinate Analysis is the name given to the entire method (PCoA). It is a statistical tool that transforms data based on distance between items into a map-based visualization. It aids in the understanding of items that are closely related to one another and allows for the identification of groups or clusters. In our PCoA analysis results, all genotypes were classified into five major groups in a 2D dendrogram. Some diverse genotypes, such as ChkBr-812, FaiBr-853, MulBr-804 and FaiBr-850 were found. These genotypes differ greatly from the rest of the genotypes (Fig.4.12). The 3D diagram allowed genotypes to be visualized from any angle. The 3D plot also revealed some rare genotypes, including LhrBr-814, SheBr-855, MuzBr-845, and LayBr-820 (Fig. 4.13).

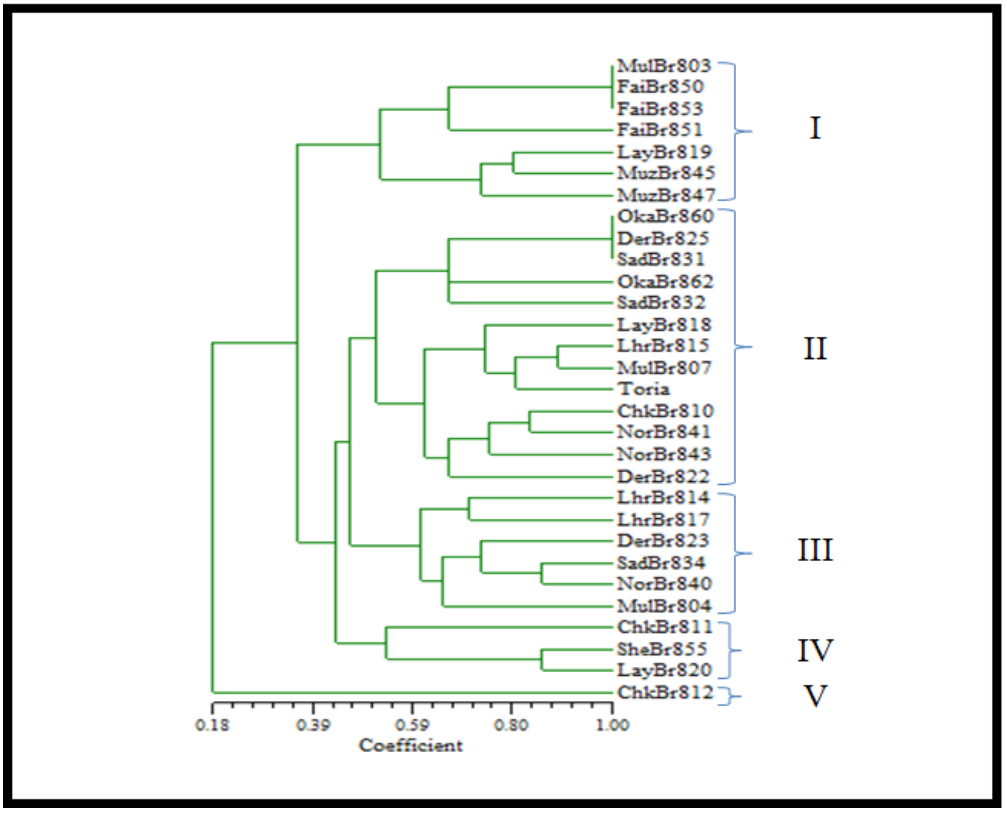

FIGURE 4.7: SSRs marker-based variability among 30 genotypes of *B. rapa* using cluster analysis.

TABLE 4.6: Grouping of 30 genotypes of *B. rapa* through cluster analysis.

Cluster	**No. of genotypes**	**Genotypes**	**Origin**
I	7	MulBr-803, FaiBr-850, FaiBr-853, FaiBr-851, LayBr-819, MuzBr- 845 & MuzBr-847.	Multan, Faisalabad, Layyah, & Muzaffargarh.
II	13	OkaBr-860, DerBr-825, SadBr-831, OkaBr-862, SadBr-832, LayBr-818, LhrBr-815, MulBr-807, Toria, ChkBr-810, NorBr-841, NorBr-843 & DerBr-822.	Okara, D. G. Khan, Sadiqabad, Layyah, Lahore, Multan, Chakwal & Narowal
III	6	LhrBr-814, LhrBr-817, DerBr-823, SadBr-834, NorBr-840 & MulBr-804.	Lahore, D.G. Khan, Sadiqabad, Narowal, & Multan.

TABLE 4.6: Grouping of 30 genotypes of *B. rapa* through cluster analysis.

Cluster	**No. of genotypes**	**Genotypes**	**Origin**
IV	3	ChkBr-811, SheBr-855 & LayBr-820.	Chakwal, Sheikhpura & Lyyah.
V	1	ChkBr-812.	Chakwal.

TABLE 4.7: Details of SSRs markers used including total generated alleles, polymorphic alleles, % polymorphism and size of amplified alleles.

Primers	**Total amplified alleles**	**Polymorphic alleles**	**% Poly morphism**	**Size range (bp)**	**Melting tempera-ture °C**
Na10-C06	1	1	100	200	59.8
Na10-D09	3	3	100	110-290 310	58.5
Na12-C07	1	1	100	450-500	58.4
Na12-C08	1	1	100	210	60.3
Na14-D07	1	1	100	110	55
Ni2-F02	2	2	100	400-200 210	58
Ni4-D09	1	1	100	200	55

TABLE 4.7: Details of SSRs markers used including total generated alleles, polymorphic alleles, % polymorphism and size of amplified alleles.

Primers	**Total amplified alleles**	**Polymorphic alleles**	**% Poly morphism**	**Size range (bp)**	**Melting tempera-ture °C**
O109-A06	1	1	100	200	59
O110-F11	1	1	100	180-200	55
Ra2-A11	2	2	100	290-310	57

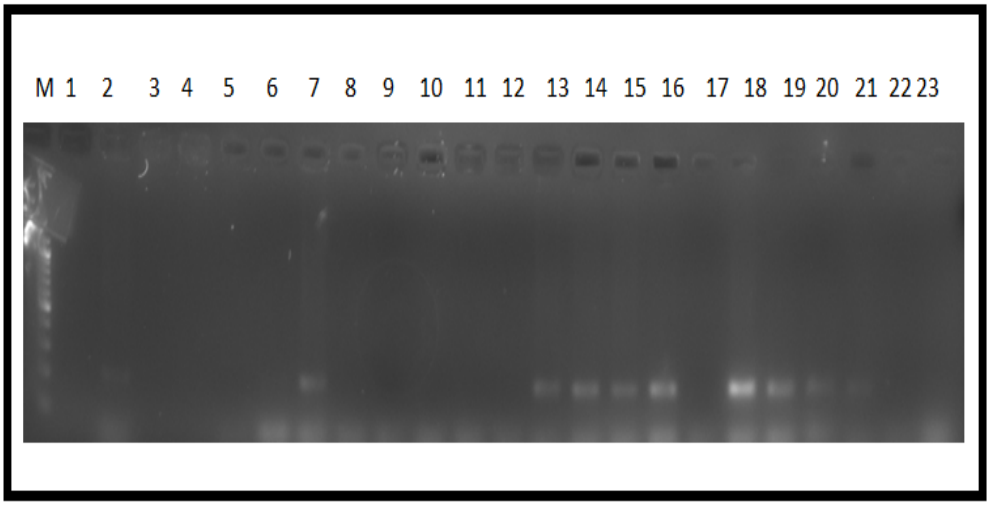

FIGURE 4.8: (a). SSRs marker banding patterns of 23 genotypes of *B. rapa* by SSRs primer Na10-C06, 1-23. M = 100 bp molecular marker, MulBr-803, OkaBr-860, MulBr-807, LhrBr-814, ChkBr-811, ChkBr-812, ChkBr-810, LhrBr-815, LhrBr-817, LayBr-818, LayBr-819, LayBr-820, DerBr-822, DerBr-823, DerBr-825, SadBr-831, SadBr-832, SadBr-834, NorBr-840, NorBr-841, NorBr-843, MuzBr-845 and MuzBr-847 respectively.

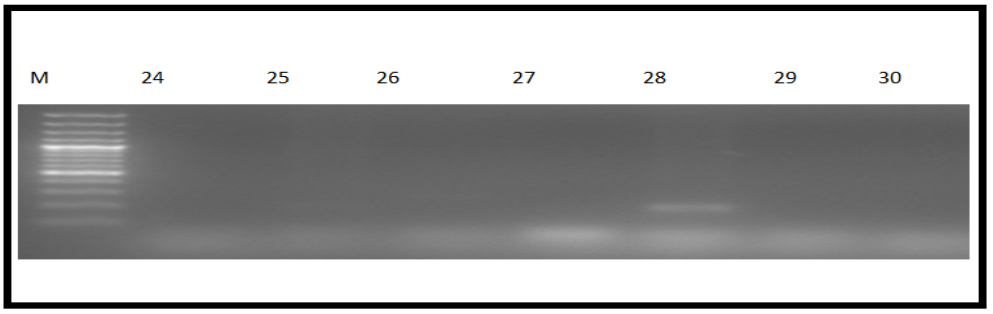

FIGURE 4.9: (b). SSR banding patterns of 8 genotypes of *B. rapa* by SSR primer Na10-C06, 24-30. M = 100 bp molecular marker, FaiBr-850, FaiBr-851, FaiBr-853, SheBr-855, MulBr-804, OkaBr-862 and Toria, respectively.

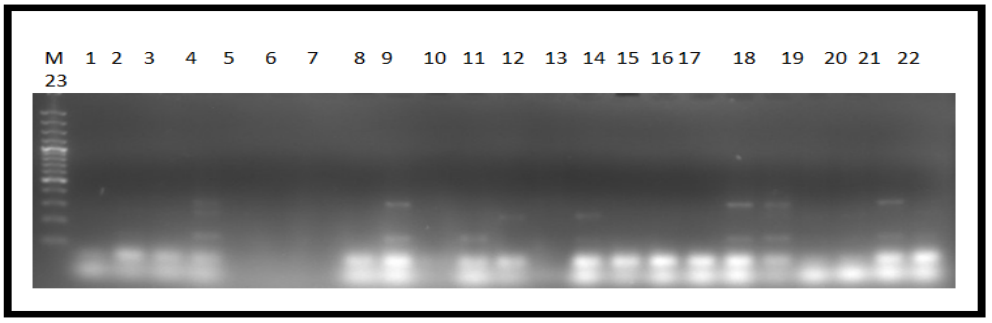

FIGURE 4.10: (a). SSRs banding patterns of 23 genotypes of *B. rapa* by SSR primer Na10-D09, 1-23. M = 100 bp molecular marker, MulBr-803, OkaBr-860, MulBr-807, LhrBr-814, ChkBr-811, ChkBr-812, ChkBr-810, LhrBr-815, LhrBr-817, LayBr-818, LayBr-819, LayBr-820, DerBr-822, DerBr-823, DerBr-825, SadBr-831, SadBr-832, SadBr-834, NorBr-840, NorBr-841, NorBr-843, MuzBr-845 and MuzBr-847 respectively.

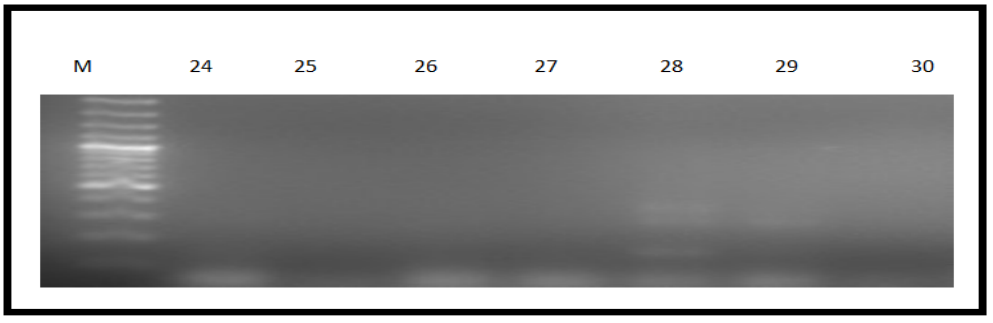

FIGURE 4.11: (b). SSRs banding patterns of 8 genotypes of *B. rapa* by SSR primer Na10-D09, 24-30. M = 100 bp molecular marker, FaiBr-850, FaiBr-851, FaiBr-853, SheBr-855, MulBr-804, OkaBr-862 and Toria, respectively.

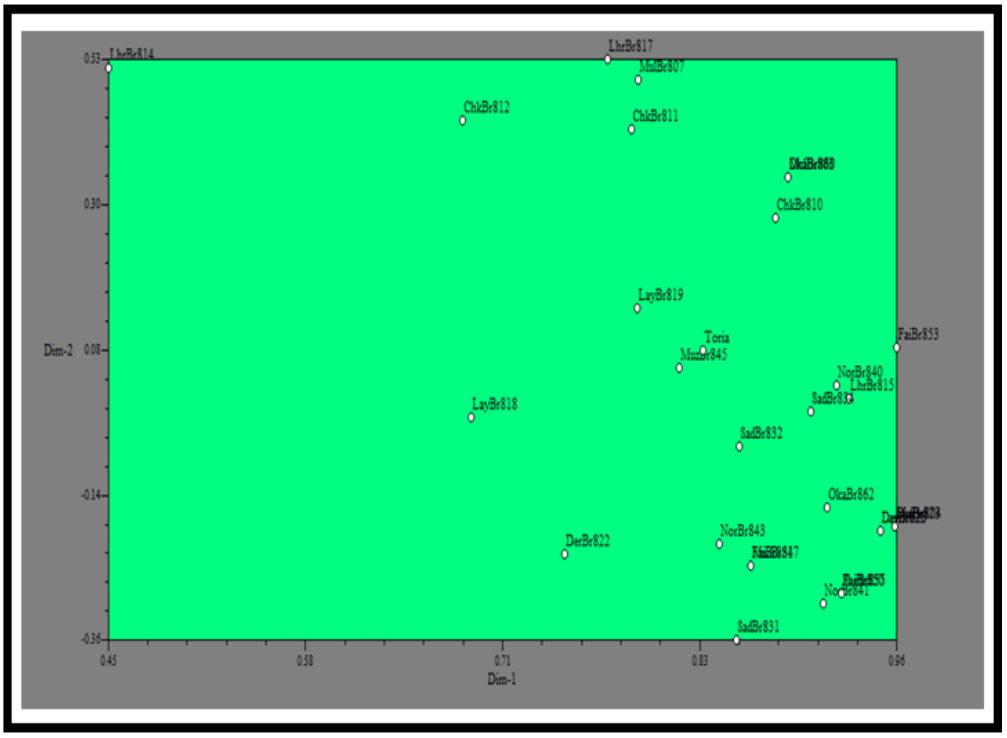

FIGURE 4.12: Two-dimensional (2D) diversity analysis of 30 genotypes of *B. rapa* using SSRs primer.

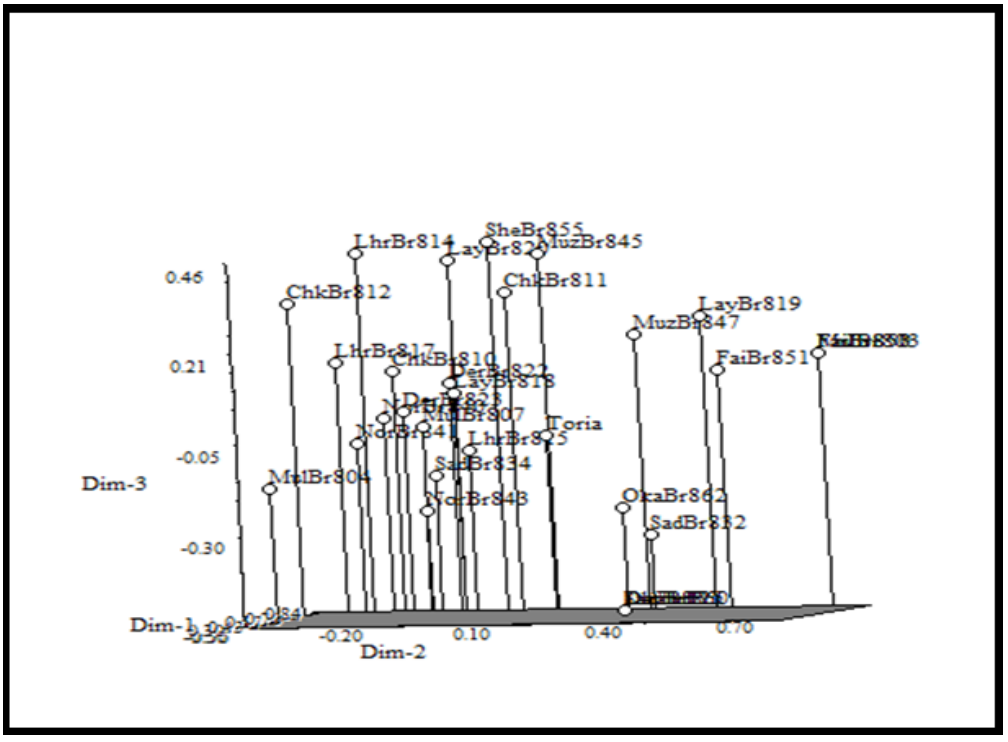

FIGURE 4.13: Three-dimensional (3D) analysis of 30 *B. rapa* genotypes evaluated via SSRs.

TABLE 4.8: Dice similarity coefficient among 30 *B. rapa* genotypes on the basis of SSRs.

Acc#	**MulBr803**	**OkaBr860**	**LayBr818**	**LhrBr814**	**ChkBr811**	**SheBr855**	**ChkBr810**	**LhrBr815**
MulBr803	100.00%							
OkaBr860	50.00%	100.00%						
LayBr818	33.33%	50.00%	100.00%					
LhrBr814	22.22%	18.18%	61.54%	100.00%				
ChkBr811	40.00%	28.57%	66.67%	66.67%	100.00%			
SheBr855	50.00%	33.33%	50.00%	54.55%	57.14%	100.00%		
ChkBr810	33.33%	50.00%	40.00%	46.15%	44.44%	75.00%	100.00%	
LhrBr815	40.00%	57.14%	66.67%	33.33%	25.00%	57.14%	66.67%	100.00%
LhrBr817	20.00%	33.33%	57.14%	70.59%	46.15%	50.00%	57.14%	61.54%
MulBr807	33.33%	50.00%	80.00%	46.15%	44.44%	50.00%	60.00%	88.89%
LayBr819	66.67%	40.00%	28.57%	40.00%	33.33%	40.00%	28.57%	33.33%
LayBr820	40.00%	28.57%	44.44%	66.67%	50.00%	85.71%	66.67%	50.00%
DerBr822	33.33%	50.00%	60.00%	46.15%	44.44%	50.00%	60.00%	44.44%
DerBr823	22.22%	54.55%	46.15%	62.50%	33.33%	54.55%	61.54%	50.00%
DerBr825	50.00%	100.00%	50.00%	18.18%	28.57%	33.33%	50.00%	57.14%
SadBr831	50.00%	100.00%	50.00%	18.18%	28.57%	33.33%	50.00%	57.14%
SadBr832	50.00%	66.67%	50.00%	18.18%	28.57%	33.33%	25.00%	57.14%

Table 4.8: Dice similarity coefficient among 30 *B. rapa* genotypes on the basis of SSRs.

Acc#	**MulBr803**	**OkaBr860**	**LayBr818**	**LhrBr814**	**ChkBr811**	**SheBr855**	**ChkBr810**	**LhrBr815**
SadBr834	25.00%	60.00%	33.33%	53.33%	36.36%	40.00%	50.00%	36.36%
NorBr840	25.00%	60.00%	50.00%	66.67%	36.36%	60.00%	66.67%	54.55%
NorBr841	25.00%	60.00%	66.67%	53.33%	54.55%	60.00%	83.33%	72.73%
NorBr843	28.57%	66.67%	54.55%	28.57%	20.00%	44.44%	72.73%	80.00%
MuzBr845	50.00%	33.33%	50.00%	54.55%	28.57%	66.67%	50.00%	57.14%
MuzBr847	50.00%	33.33%	25.00%	36.36%	28.57%	33.33%	25.00%	28.57%
FaiBr850	100.00%	50.00%	33.33%	22.22%	40.00%	50.00%	33.33%	40.00%
FaiBr851	66.67%	40.00%	28.57%	20.00%	33.33%	40.00%	28.57%	33.33%
FaiBr853	100.00%	50.00%	33.33%	22.22%	40.00%	50.00%	33.33%	40.00%
ChkBr812	0.00%	0.00%	33.33%	22.22%	0.00%	50.00%	33.33%	40.00%
MulBr804	0.00%	36.36%	30.77%	50.00%	0.00%	18.18%	46.15%	50.00%
OkaBr862	50.00%	66.67%	50.00%	36.36%	28.57%	33.33%	25.00%	57.14%
Toria	50.00%	66.67%	75.00%	36.36%	28.57%	66.67%	50.00%	85.71%

Table 4.9: Dice similarity coefficient among 30 *B. rapa* genotypes on the basis of SSRs.

Acc#	**LhrBr817**	**MulBr807**	**LayBr819**	**LayBr820**	**DerBr822**	**DerBr823**	**DerBr825**	**SadBr831**
LhrBr817	100.00%							
MulBr807	71.43%	100.00%						
LayBr819	36.36%	28.57%	100.00%					
LayBr820	46.15%	44.44%	33.33%	100.00%				
DerBr822	42.86%	60.00%	28.57%	44.44%	100.00%			
DerBr825	33.33%	50.00%	40.00%	28.57%	50.00%	54.55%	100.00%	
SadBr831	33.33%	50.00%	40.00%	28.57%	50.00%	54.55%	100.00%	100.00%
SadBr834	62.50%	33.33%	44.44%	36.36%	33.33%	66.67%	60.00%	60.00%
NorBr840	75.00%	50.00%	44.44%	54.55%	50.00%	80.00%	60.00%	60.00%
NorBr843	53.33%	72.73%	25.00%	40.00%	72.73%	57.14%	66.67%	66.67%
MuzBr845	50.00%	50.00%	80.00%	57.14%	50.00%	54.55%	33.33%	33.33%
MuzBr847	33.33%	25.00%	80.00%	28.57%	50.00%	36.36%	33.33%	33.33%
FaiBr850	20.00%	33.33%	66.67%	40.00%	33.33%	22.22%	50.00%	50.00%
FaiBr853	20.00%	33.33%	66.67%	40.00%	33.33%	22.22%	50.00%	50.00%
OkaBr862	33.33%	50.00%	40.00%	57.14%	25.00%	54.55%	66.67%	66.67%
Toria	50.00%	75.00%	40.00%	57.14%	50.00%	54.55%	66.67%	66.67%

TABLE 4.10: Dice similarity coefficient among 30 *B. rapa* genotypes on the basis of SSRs.

Acc#	**SadBr832**	**SadBr834**	**NorBr840**	**NorBr841**	**NorBr843**	**MuzBr845**	**MuzBr847**	**FaiBr850**
SadBr832	100.00%							
SadBr834	40.00%	100.00%						
NorBr840	40.00%	85.71%	100.00%					
NorBr841	40.00%	57.14%	71.43%	100.00%				
NorBr843	44.44%	46.15%	61.54%	76.92%	100.00%			
MuzBr845	33.33%	40.00%	60.00%	40.00%	44.44%	100.00%		
MuzBr847	33.33%	40.00%	40.00%	20.00%	44.44%	66.67%	100.00%	
FaiBr850	50.00%	25.00%	25.00%	25.00%	28.57%	50.00%	50.00%	100.00%
FaiBr851	40.00%	22.22%	22.22%	22.22%	25.00%	40.00%	40.00%	66.67%
FaiBr853	50.00%	25.00%	25.00%	25.00%	28.57%	50.00%	50.00%	100.00%
ChkBr812	0.00%	0.00%	25.00%	25.00%	28.57%	50.00%	0.00%	0.00%
MulBr804	18.18%	66.67%	66.67%	53.33%	57.14%	36.36%	18.18%	0.00%
OkaBr862	66.67%	40.00%	40.00%	40.00%	44.44%	33.33%	33.33%	50.00%
Toria	66.67%	40.00%	60.00%	60.00%	66.67%	66.67%	33.33%	50.00%

TABLE 4.11: Dice similarity coefficient among 30 *B. rapa* genotypes on the basis of SSRs.

Acc#	**FaiBr 851**	**FaiBr 853**	**ChkBr 812**	**MulBr 804**	**OkaBr 862**	**Toria**
FaiBr851	100.00%					
FaiBr853	66.67%	100.00%				
ChkBr812	0.00%	0.00%	100.00%			
MulBr804	0.00%	0.00%	22.22%	100.00%		
OkaBr862	40.00%	50.00%	0.00%	36.36%	100.00%	
Toria	40.00%	50.00%	50.00%	36.36%	66.67%	100.00%

4.3 Discussion

The use of protein storage is utilized for the classification of various varieties and to assess genetic variations in different crops. It is particularly helpful to differentiate between various plant species and subspecies based on total seed storage diversity. Better protein-based diversity of varied plant populations is made possible by its critical analysis [73], [74]. There are numerous biochemical techniques used to study genetic diversity. One of the most important techniques for differentiating *Brassica* species and subspecies on the basis of protein size is the SDS-PAGE method, which also provides a clear protein profile of significant varied genotypes. This method was used in the current study to assess the genotypes of *B. rapa*, and the highest genetic diversity between genotypes was found. The SDS-PAGE method, which is cheaper and faster than other molecular methods, offers the most protein-based genotype diversity [73], [74], [44], [75], [47]. Numerous biochemical and molecular techniques are used to investigate the genetic diversity of various crop species and subspecies [76], [77]. Through the use of the SDS-PAGE method, the genetic divergence between the genotypes of *B. rapa* was identified. There were 13 bands altogether, and they were all polymorphic. All genotypes displayed varying protein band sizes, demonstrating that seed total storage protein

varies depending on the plant subspecies. Zada et al. [33] gives information about 31 diverse polypeptide sub units in *B. carinata* genotypes. Shinwari et al. [18] recorded maximum polymorphic and very less monomorphic proteins in *Eruca sativa* genotypes. Turi et al. [78] used similar methods and reported four different proteins in many *Brassica* species. The maximum polymorphism was noticed in soybean genotypes by using SDS-PAGE and RAPD markers [79]. Ahmad et al. [80] also used this technique to examine polypeptide based polymorphism in Pakistani cultivar *Hyppophaerhamnoides L.* ssp Turkestanica. The protein bands pattern depends upon the kind of genotype used [17], [81]. The changes in examined genotypes, the amount of gel used, and the choice of protein sub-units during data scoring may account for the differences in the number of protein sub-units between the previous and present findings. All 30 genotypes of *B. rapa* were calculated for the genetic similarity coefficient. Among different genotypes the similarity value ranges from 15% to 100% (Table 4.2). The thirty genotypes were divided into seven main groups by the phylogenetic tree (Fig 4.4 Table 4.1). Our results are not similar with the findings of Shinwari et al. [84] who noticed 60% to 100% similarity for *Eruca sativa* species. This deviation is because of the different plant species. Mukhlesur et al. [47] also used similar method to classify different cultivars of *Brassica* into different clustered groups.

The 2D and 3D representations of genotype grouping allows us to see clearly from various angles. However, the 3D structure gives us more details and information than 2D structure. In the current study, both types provide us information about diverse genotypes. Our findings are consistent with the conclusion of Gupta et al. [82] who used PCoA analysis to achieve maximal polypeptide based variability in 45 genotypes of chickpea. Their work showed FLIP-90-160 has a distinct relation based on 3D study. Through the use of principal coordinate analysis (PCoA), Mottaghi et al. [83] discovered that Iranian Achillea species had the greatest and clearest protein-based divergence. They claim that the first three PCoA categories contributed more than 83% variations. In this study 10 SSR primers were used for 30 diverse collected genotypes from Punjab province. Different marker sets showed different fingerprints in these genotypes. Polymorphic banding patterns

were recorded in all genotypes. Our findings correlate with reports of Chen et al. [84] and Agrama and Tuinstra [85]. Their findings revealed the most SSR-based diversity across the several *Brassica* species. A considerable level of chromosomal variation among the *Brassicaceae* family is revealed using microsatellite markers [53]. Ma et al. [86] conducted a similar study to investigate genomic divergence in 20 non heading Chinese cabbages which were collected from China and Japan. The maximum amount of polymorphism was noted in the amplified allele. Havlickova et al. [87] measured maximum genomic variability in 94 winter rapeseed Czech genotypes using SSRs and AFLP markers. 53 to 100% similarity was observed among genotypes. They also discovered several new and diverse elite genotypes. In addition, Indian mustard (*B. juncea*) and other *Brassica* species showed substantial levels of RAPD and SSR-based genomic diversity by Gupta et al. [66]. Abbas et al. [88] used 10 different RAPD and SSRs markers each and recorded 458 and 258 alleles. Highly polymorphic bands with sizes ranging from 250 to 2000bp were seen. SSRs markers had fragment sizes that were significantly smaller than RAPD markers.

In the current study, genotype similarity coefficient values were determined, and all genotypes displayed a range of genetic variability from low to high (0 to 100%). The results of our study differed with those of Ofori et al. [89] who found 83% genetic variation among various *B. rapa* winter genotypes. Within species, interspecific genetic variability was only found at a relatively low level (17%). With the exception of only three genotypes, they reported maximal polymorphic alleles using 15 SSR markers. Our results differ from those of Shen et al. [64], who found that eleven non-heading Chinese cabbage genotypes had similarity coefficient values ranging from 54 to 89% using microsatellite markers.

The examined genotypes were sorted into five groups using cluster analysis. (Fig. 4.7)/ (Table 4.6). The first group had seven genotypes from the Multan, Faisalabad, Layyah, and Muzaffargarh regions. Group two consisted of 13 genotypes, the majority of which were from Okara, D.G. Khan, Sadiqabad, Layyah, Lahore, Multan, Chakwal, and Narowal. The third group contained 6 genotypes from Lahore, D.G. Khan, Sadiqabad, Narowal and Multan. The fourth group contained

3 genotypes from Chakwal, Sheikupura and Layyah. Fifth group is the smallest group and only contains 1 genotype from Chakwal. Similar findings were obtained by Das et al. [90] who noted a similar range of genetic dissimilarities in *B. campestris* and cabbage genotypes. Framarzpour et al. [91] studied 25 cultivars of *B. napus* that were grown in greenhouse conditions via 12 pairs of SRAP primers. They detected 96 polymorphic bands with 33% of genetic similarity. They use PCoA analysis to analyze the diverse genotypes of *B. napus* more vividly. The 2D and 3D analysis estimated about 83.66% of variation that was the indication of inappropriate distribution of SRAP markers on the genome of *B. napus*. Their PCoA analysis indicated a high level of genetic diversity among genotypes. Singh et al. [92] used 48 SSRs markers which generated 114 alleles with average value of 2.38 alleles per primer and the genetic similarity of 50%. They performed 2D and 3D analysis for 16 *B. Juncea* genotypes to distinguish elite genotypes from others. Their PCoA analysis confirms the places and clustering of genotypes that was in line with the findings of structure analysis. Their results differentiated the resistant and susceptible genotypes. Takahashi et al. [93] analyzed 24 cultivars of *B. rapa*, *B. juncea*, *B. napus* and *B. oleracea* using RAPD and ISSR markers through individual and bulked based approaches. They examined 2 genotypes for each cultivar of *Brassica* and in total they scored 305 and 422 bands for each approach and recorded 100% polymorphism. According to PCoA the inter-specific variation was less than the intra-specific variation. The results of their PCoA analysis were similar with the findings of UPGMA analysis.

Chapter 5

Conclusions and Recommendations

The diverse *B. rapa* genotypes were collected from different areas of Punjab, Pakistan and was characterized for seed storage protein profiling. The similarity coefficient values ranged from 15 to 100% across all genotypes. The highest similarity was observed between OkaBr-860/MulBr-803, DerBR-825/LayBr-820 etc., while the lowest similarity 15% was noted between NorBr-841/LhrBr-814. The polymorphic data was further visualized via PCoA that generated 2D and 3D plots of genotypes. Through modern 2D and 3D techniques some unique genotypes were recorded. Our results established that biochemical diversity of *B. napus* genotypes could be utilized for exploration of genetic diversity. However, we recommend applying more sophisticated methods, like as 2-D gel electrophoresis and molecular markers, to examine the evolutionary basis of divergence in the *B. rapa* germplasm. The genotypes LhrBr-814, ChkBr-812, LayBr-818, DerBr-822, LayBr-818, ChkBr-812 and LayBr-819 were founded unique and are recommended for further studies.

Simple sequence repeats (SSRs) markers were used for molecular characterization to identify some rare alleles in various *B. rapa* accessions. The genetic similarity values ranged from 0.0 to 100%. All the tested genotypes showed 100% polymorphism. A total of 10 primers were used out of which 7 primers detected only one

allele. The other two primers amplified two alleles while only one primer amplified 3 alleles. After this study we found some unique genotypes like ChkBr-812, FaiBr-853, MulBr-804, FaiBr-850, LhrBr-814, SheBr-855, MuzBr-845 and LayBr-820 and these novel genotypes are highly recommended for further studies. We should use modern tools for further studies like Genome Wide Association Study (GWAS) along with SSRs to analyze genetic diversity and to identify genotypes of *B. rapa*.

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