CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

# Assessment of Genetic Diversity in *Brassica rapa* Genotypes of Azad Jammu and Kashmir by using SDS-PAGE and SSRs Markers

by

Uzma Banaras

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
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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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**(Uzma Banaras)**

# *Abstract*

*Brassica rapa* is commonly grown all over the world because it is a very important crop and is used for multiple purposes. It is an important oilseed crop. The current research was designed to study biochemical and molecular-based variations among *B. rapa* genotypes collected from diverse areas of Azad Jammu and Kashmir and to identify promising genotypes among studied genotypes. For the improvement and utilization of the *B. rapa* crop, seed storage protein-based variability is very important. In this research 30 genotypes of *B. rapa* were used for the study of total seed storage protein-based variation through the SDSPAGE method. Tweleve Protein bands were noted and these bands showed 100% polymorphism for all the tested genotypes. These polypeptide bands varied in size within the range of 10-180 kDa. The maximum similarity coefficient of 95% was noted among BSA/PooBr152 genotypes the lowest similarity of 0% was noted among genotypes PooBr147/ MuzBr115, PooBr149/MuzBr115, PooBr152/ MuzBr115, MirBr158/ MuzBr115, and MirBr160 /MuzBr115. Unweighted Pair Group Method with Arithmetic Mean (UPGMA), was used to study the total soluble seed protein based -variations and classified all tested genotypes into 8 groups. The group I, II, III, IV, V, VI, VII and, VIII contained 8, 1, 5, 3, 5, 2, 5 and, 1 genotype respectively. Group II and VIII had unique genotypes and these genotypes were BagBr137 and NeeBr125 different from all other genotypes. The 2D and 3D analyses were used to further analyze these genotypes from different dimensions. The unique genotype of 2D analysis was BagBr137, BagBr130, NeeBr125, and MirBr158. and 3D analysis of unique genotypes was BagBr137 and NeeBr125. There were total 10 Simple Sequence Repeats (SSRs) markers that were used to identify genomic variability among 30 Brassica rapa genotypes. The size of the fragment that were obtained from the primer ranged from 100-500bp. In the banding pattern, 100% polymorphism was recorded. Eight primers (80%) detected only one allele. One primer (10%) (Na10-A02) amplified 3 alleles and one primer (10%) name (BRMS-001) amplified 2 alleles in all *B. rapa* genotypes. A total of 13 alleles were obtained by using 10 primers and they were all highly polymorphic. Genetic similarity value among *B. rapa* genotypes range of 0-94% was noted. The highest

similarity value (94%) was noted between MirBr156/PooBr150 genotypes followed by 89% among following genotypes KotBr166/NeeBR120, MirBr160/NeeBr122, BagBr130/NeeBr122. The lowest similarity coefficient value 0% was noted among genotypes MuzBr118/HatBr103, NeeBr122/ HatBr103 and PooBr147/ HatBr103 followed by 14% between genotypes BhiBr168/HatBr110 and 17% between BhiBr170 /MirBr158. 0n the basis of the UPGMA similarity method all 30 genotypes of B. rapa were classified into seven groups. Group I was the largest group among all the groups and consisted of 9 genotypes. Groups II and III contained six genotypes each. Group IV had three genotypes and groups VI and VII had 2, 2 genotypes. Between these groups, VII comprised promising genotypes PooBr147 and BhiBr170 and these genotypes were variant and highly diverged from all other genotypes. 2D and 3D structures provided a more prominent picture of the promising genotype. In 2D analysis, some unique genotypes, such as PooBr147, KotBr165, PooBR155, and PooBr150 were found. While 3D analysis revealed some promising genotypes too, including HatBr103, PooBr147, HavBr143, and BagBr137.Results showed that maximum polymorphic diversity was present among different genotypes of *B. rapa* and provide a baseline for the future study of *B. rapa* genotype in Azad Jammu and Kashmir.

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# Abbreviations

| | |
|---|---|
| **APS:** | Ammonium per sulphate |
| ***B. rapa*:** | *Brassica rapa* |
| ***B. napus*:** | *Brassica napus* |
| ***B. nigra*:** | *Brassica nigra* |
| ***B. Oleracea*:** | *Brassica Oleracea* |
| ***B. juncea*:** | *Brassica juncea* |
| ***B. carintes*:** | *Brassica carintes* |
| **Bp:** | Base pair |
| **CBB:** | Coomassie brilliant blue |
| **CTAB:** | Cetyl trimethyl ammonium bromide |
| **KDa:** | Kilo Dalton |
| **NTSYS:** | Numerical taxonomy and multivariate analysis system |
| **PCoA:** | Principal coordinate system |
| **PCR:** | Polymerase chain reaction |
| **SDS:** | Sodium dodecyle sulphate |
| **SSRs:** | Simple sequence repeats |
| **SDS-PAGE:** | Sodium dodecylsulphate polyacrylamide gel electrophoresis |
| **UPGMA** | Unweighted pair group method with arithmetic averages |
| **2D** | Two dimensional |
| **3D** | Three dimensional |

# Chapter 1

# Introduction

## 1.1 Background

Genetic diversity of plant genotypes plays vital role for the production of enhanced varieties of crops for better production and other attractive traits and due to the use of these valuable properties, the production and quality of crop plant backup tools have been improved manifold in the last few years. Since the early days of agriculture (about 10,000 years ago), many plant and animal breeders have sought genetic diversity to achieve desired yields and quality by carrying genes for desired traits and avoiding others [1].

In the early days, some parameters like agronomic and phonotypical parameters were used to assess genetic diversity and different important achievements were achieved but there were some misconceptions about using these parameters alone as some traits are similar to those of different loci. Identifying the specific origins of different phenotypic traits was difficult because many are allele-controlled. Numerous characteristics are influenced by environmental factors and cannot be differentiated from characteristics that are inherited.

Therefore, the only and necessary approach is to reliably evaluate different traits for better and efficient use of plant genotypes were achieve better and better crop yields [2]. The *Brassicaceae* and *Brassica* discussed below.

## 1.2 *Brassicaceae*

Brassicaceae includes 350 genera and 3500 species. This family has broad agronomic characteristics and is one of the 10 most economically beneficial plant families [3]. It consists of very essential crop species that are economically very important for example used as vegetable, industrial, cooking oil, and condiments. However, in plant biology, important species is *Arabidopsis thaliana*, the model organism of flowering plant and has a complete sequenced genome (The Arabidopsis Genome Initiative, 2000) used in every field of experimental biology and provides a better understanding of every aspect of plant biology [4]. The Brassicaceae family is very important in agriculture because of the genus Brassica, which is a valuable source of oil seeds and also has a wide range of vegetable and grain crops.

## 1.3 *Brassica*

Generally, six unique species of Brassica are cultivated. Out of these six, three are diploid, *B. nigra* (2n = 16, genome BB), *B. oleracea* (2n = 18, genome CC), and *B. rapa* (2n = 20, AA) though the remaining three amphidiploid derivatives *Brassica carinata* (2n = 34, BBCC), *Brassica juncea* (2n = 36, AABB), and *Brassica napus* (2n = 38, AACC) [5]. These six *Brassica* crop species have a genomic relationship (called the triangle of U) nuclear DNA content, genome-specific markers used, sequence analysis, and pairing of chromosomes are shown in the figure (1.1).

In one group Diplotaxis ecocide's (n=7) give both textitB. rapa and *B. olercea* because nuclear DNA sequence data and chloroplast restriction site data confirmed that they have a separate evolutionary pathway. In the second group B. nigra and S. arvensis (n=9) are assigned [6].

Genus *Brassica* has species that are very beneficial economically in the world. For example turnips, cauliflower, broccoli cabbage, weeds, and various mustards are utilized for edible oil, scavenging, and decorative and vegetable purposes. Nearly complete *Brassica* plant, which includes non-reproductive parts such as root, stem,

buds, flowers, and seeds, used for edible purposes. The three important *Brassica* crops species are *Brassica nigra*, *Brassica oleracea* and *Brassica rapa*.

Amphidiploid species are *Brassica juncea, Brassica carinata,* and *Brassica napus* [7]. The five most significant rapeseed and mustard species grown in Pakistan are *Eruca sativa, Brassica napus, Brassica carinata, Brassica campestris,* and *Brassica juncea*. Between these *B. napus L.* is a very important species because its seed contains 40 to 45% oil content, 3.5% fats, and 0.35% phosphorous. *B. napus* traits have a very limited genetic diversity but *B. rapa* and *B. oleracea* have incredible differences within their types and origin [8].

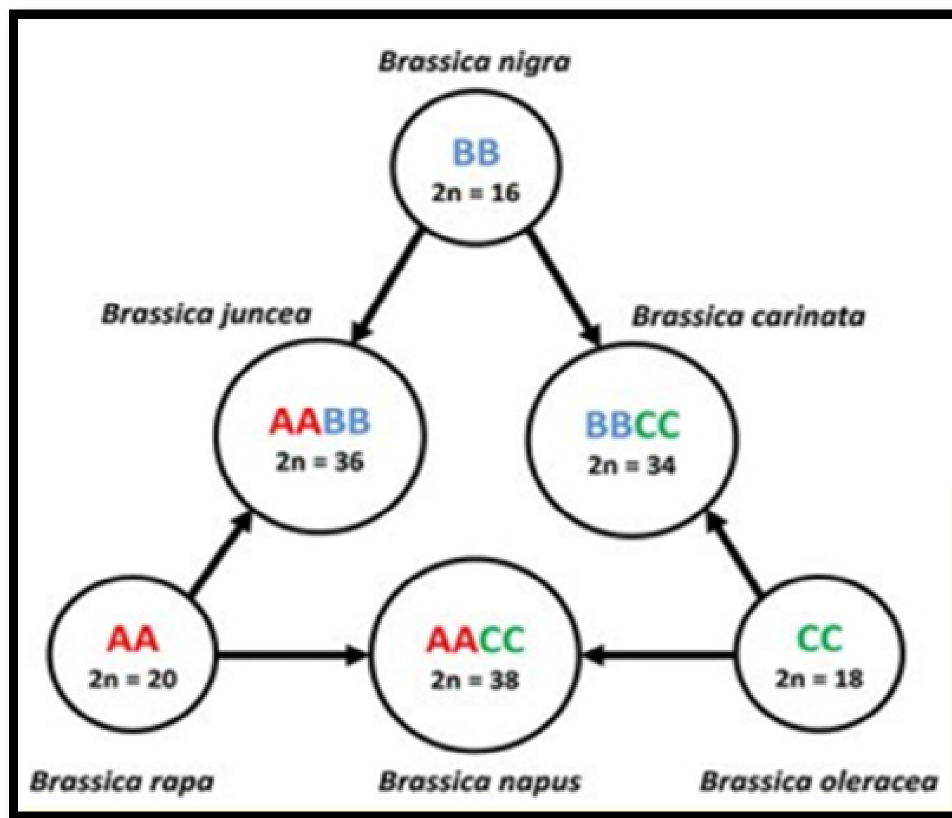



FIGURE 1.1: This diagram shows the genomic relationship between six *Brassica* crop species (called the triangle of U) [6].

## 1.4 *Brassica rapa*

*B. rapa* formerly known as *Brassica compasteris* is native to the region of the Himalayas. *Brassica* species have diversity in structure and genetic varieties and

can be cultivated in different climatic regions [9]. *Brassica* is supposed to have originated in several locations throughout the globe. Modern scientists believe that the wild varieties of this species are most likely to have originated in Europe. On the African continent, *Brassica* is currently growing in Ethiopia, Tanzania, Kenya, Zimbabwe, and Mali. Among the Asian nations that cultivate it are Kazakhstan, China, Japan, Afghanistan, Iran, India, and Pakistan. In the regions of southern Australia with medium and high rainfall, it is frequently grown. All around the United States, especially in Idaho, Minnesota, North Dakota, Montana, Oklahoma, Argentina, and Central America, Brassica is grown for food [10].

*B. rapa* grows under temperate conditions (Rabi season) in Kashmir valley and a few high altitudes areas of Jammu province. This crop is a famous and only consumable oil seed crop in northwestern India Himalayan state of Jammu and Kashmir [11]. Oil seed crops are quite versatile in the conventional farming system; that's why they are grown all over the country. Important seed crops include mustard and rapeseed.

*Brassica rapa* is the only crop in the rapeseed mustard group that works well as an oilseed. *Brassica rapa* the main Rabi crops in the Kashmir valley. Rapeseed mustard is very susceptible to weather, as shown by its inconsistent response to various sowing dates [12].

## 1.5 Importance of *Brassica rapa*

*Brassica* is the name of the extremely diverse genus of plants in the *Brassicaceae* family. These species are a source of feed, edible oil, vegetables, and ornamentals. The *Brassica* are abundant in vital metabolites, oil, and even their oil-extracted press cakes, which are a source of nutrients for animals. Chinese cabbage, Pak-choi, and other species members are among those used as vegetables in human diets [13]. Many important vegetables and oil crops are the part of *B. rapa* species. Due to paleohexaploid ancestry and its close relation with *Arabidopsis thaliana* and some other *Brassica* species, genomic studies like polyploidy can be easily studied using

this model species. So its genome is of high importance due to its usage in both basic and applied research on the side of cultivated *Brassica* species. With a wide diversity of species that can be utilized as a variety of oilseed, vegetable, and fodder crops, the *Brassica* are one of the most significant oilseeds for agronomy. The high oil content of the seeds, which can range from 17 to 40% in wild relatives, is the key distinguishing characteristic of *Brassica* species [14]. Triacylglycerols (TAG), the primary component of seed oil, not only have a significant impact on edible oil but may also be used to produce biodiesel or biofuel for industrial purposes Since Kennedy initially suggested the TAG biosynthesis pathway in 1961, the production of glycerol has come to be recognized as a distinctive feature of lipid biochemistry the TAG biosynthesis, metabolic, and degradation pathways in *Arabidopsis* have been thoroughly investigated and the genes encoding the majority of the TAG metabolism-related enzymes have been identified and cloned [15].

Rapeseed oil is widely utilized as edible oil and in breeding programs because of its low glucosinolate concentration. Erucic acid was given its name by Canadian oil, which is used in human nutrition, in 1974 [16]. Hepatoprotection and antihepatotoxicity are two additional significant biological properties of *B. rapa L.* It was claimed that turnip root ethanolic extract had antidiabetic effects on all animals with oxidized diabetes. Turnips have a wide range of biological processes that have been verified, many of which can be categorized as antioxidant potential. According to additional other research, the ethanol extracted from the roots contains substances that can prevent cancer, such as those that can reduce power and prevent lipid peroxidation as well as those that can remove free radicals, nitrites, and lipid peroxidation [17].

Genus *Brassica* has species that are very beneficial economically in the world. For example Turnip, cauliflower, broccoli cabbage, weeds, and various mustards are utilized for edible oil, scavenge, decorative and vegetable purposes. Nearly complete *Brassica* plant which includes non-reproductive parts such as root, stem, buds, flowers, and seeds used for edible purposes [18]. Many lethal diseases are controlled and treated through medicinal plants all around the world. Leaves of the mustard plant contain many secondary metabolites that have the potential

for antioxidant activities. For the protection against chromosomal damage and oxidative stress, leaves of *B. rapa* and *B. compestris* are used. *Brassinosteroids* are highly distributed in both pollen and seed of *B. campestris* and *B. napus* and their concentration changes with plant tissues used Brassinosteroids play important role in controlling both prostate and breast cancers [19].

Different parts of *B. rapa* have strong anticancer and antioxidant activities. In recent studies, its metabolites indicate strong anti-cancerous potential against ovary, colon, bladder, lung, prostate, breast, and many other cancer types. The leaf and roots of different vegetable sub-species are used against different types of cancer cell lines and showed strong inhibitory activities against cancer [20]. *B. rapa* is the most commonly used species in the treatment of many kinds of cancer. Species of *B. rapa* like Pak Choi, Chinese, Cabbage and *selvistric rapa*, and many more vegetative parts not only have anti-cancerous property but also behaves as an anti-oxidant. Its plant parts like roots, leaves, fruits, and seeds are a major source of anti-cancerous or antioxidant chemicals. A lot of cancer cell lines are treated by these chemicals. Following (Figure 1.2) shows the anticancer activity of *B. rapa* against different types of cancer (In-vivo and In-vitro) [21].

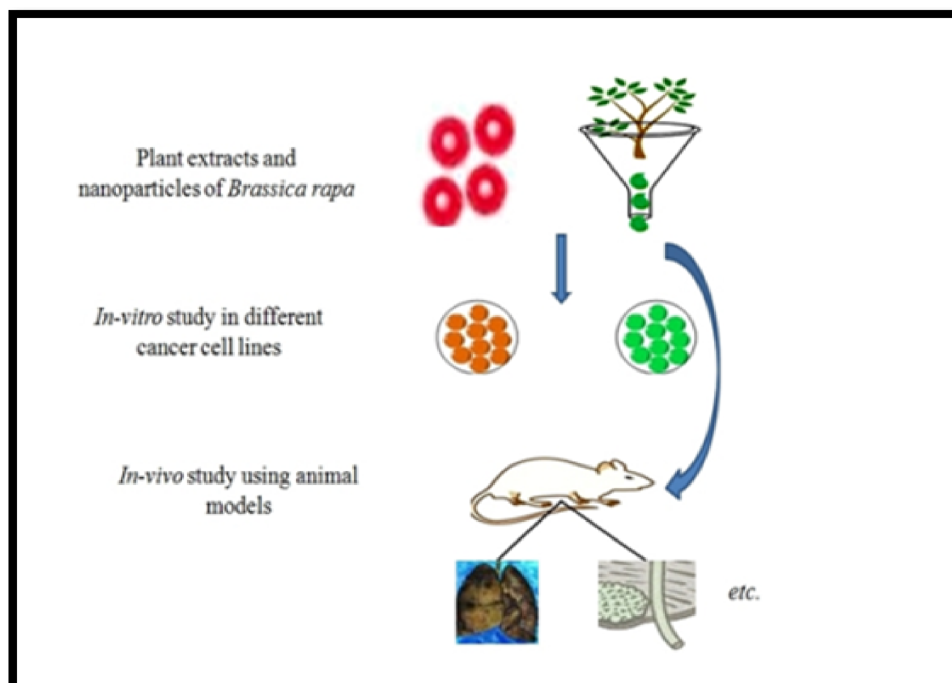



FIGURE 1.2: Anticancer activity of *B. rapa* against different types of cancer (In-vivo and In-vitro) [21].

Among all plant families, the Brassicaceae are famous for their economic importance. Except for tropical regions, *Brassica* vegetables are a staple aliment in every region of the world. The Food and Agricultural Organization (FAO) of the UN estimates that approximately 93 million tons of cauliflower, broccoli, cabbage, and other *Brassica* vegetables were produced commercially worldwide in 2013, on an area of roughly 3.7 million hectares, with a farm gate value of about USD 31 billion [22].

## 1.6 Biochemical Based Diversity

It is essential to assess the genetic diversity among distinct *Brassica* species in order to choose elite genotypes. To identify novel genotypes among plant species and subspecies, numerous physiological and morpho-biochemical techniques were often used [23]. To find out the genetic variations and genotypes collections, different new methodologies are now in consideration that identifies the genetic diversity in desirable traits. These may include morphological characterization and biochemical markers evaluation at the protein level SDS-PAGE. Many workers also have discussed the electrophoretic evidence in plant systematics. In some plant species like Leguminosae, based on electrophoresis many studies have been carried out. Electrophoretic patterns of total seed proteins predicted through PAGE with SDS have been used to solve the taxonomic and evolutionary problems of some plant species [24]. The study on seed storage proteins helps to identify and characterize the genetic diversity, their wild type, and cultivars. They also determine phylogenetic relationships [25]. If the data of the genetic source and genetic variety are accessible, the Genetic development of vegetation can be greater. Exploration of SDS-PAGE evaluation of important *Brassica* species is beneficial to analyze genetic variants and also categorizing plant diversity. SDS-PAGE techniques are used to analyze the genetic divergence between numerous plant species. One of the most efficient, quick, straight, and precise techniques is this one. Seeds are treated to SDS-PAGE analysis, which provides exact polypeptide profile data that is unaffected by any environmental factors. Examine the evolutionary and taxonomic

relationships between crop species and subspecies using this method. SDS-PAGE is an extremely effective method for examining the polypeptide diversity in several Brassica species [26]. SDS-PAGE protein analysis reveals the proteins in many species of plants. Identification of seed protein by electrophoresis indicates whether the protein profile of the seed is stable or not. Secondly, experimental conditions do not affect seed protein profile. SDS-PAGE is a cost-effective procedure, and easy to carry out. So many workers use this to find out the genetic variations or diversity in plant species [27].

## 1.7 Molecular Markers Based Diversity

To find out the unique genes and evaluate the traits of native gathered genotypes we used molecular markers, DNA based markers ended up being a valuable device for the assessment and usage of vital traits because of their reliable and outstanding outcomes [28]. For marker-assisted selection, the efficacy of resistant genotypes is suggested to make a combination with a molecular marker. Due to increasing difficulty in field evaluation and screening, biochemical markers are more in demand in recent years by crop genetics for assessing genetic diversity [29]. Molecular markers are now commonly used to track loci and genome regions in many crop-breeding programmers because molecular markers form a tight connection with a large range of agronomic and disease-resistant traits [30]. Among the variety of applications in plant genetics and breeding, different molecular markers are used. The most favorable of them are microsatellites or simple sequence repeats. They are selected because of their multi-allelic property, codominance, high abundance, reproducibility, and extensive genome coverage [31]. Microsatellite DNA or SSRs are commonly used in marker technology. These markers are termed as a tandem array of a short nucleotide sequence of small bases length and produce polymorphism based on the differences in DNA repeat units at a given locus. SSRs are generally used for genome mapping, evolutionary and population studies in plant and animal kingdoms, and also fingerprinting [32]. SSRs are located in repetitive regions of DNA so the random selection from genomic DNA can result in a

distorted map where the markers are clustered around the pericentric heterochromatin. Screening for SSRs and around the coding region required that a set of markers in the coding region must be recognized first. And complete genome coverage must be obtained. For entire genome coverage, it is required to complement the complete genome coverage of SSRs with CAPs assay. In the SSRs technique, primers are used that are complementary to the conserved regions that flank a microsatellite. By using agarose gel electrophoresis especially standard agarose or sequencing gels are used to detect length polymorphism [33].

## 1.8 Research Gap

SSRs are highly polymorphic and show simple repeated sequences. *B. rapa* is a very important crop economically. Many researches was carried out on biochemical and molecular characteristics in Pakistan and worldwide but in Kashmir very limited reports are available about the biochemical and molecular markers based diversity of the huge *Brassica* species population. The biochemical and molecular, 2D and 3D study of *Brassica* (*B. rapa* genotypes) species has not been reported yet for Azad Jammu and Kashmir. The SSRs marker and SDS-PAGE-based characterization of the present local genotypes of Kashmir have not been reported yet. There is a need to explore the *B. rapa* genotype from the region of Azad Jammu and Kashmir. Therefore, the goal of this research is to analyze the seed protein variability and genetic diversity of *B. rapa*.

## 1.9 Scope

In Pakistan *Brassica* is the one of the major source for long term production of edible oil. In Brassica breeding programs biochemical and molecular techniques was used to determine promising genotype through selecting elite genotype. In conclusion production of local edible oil can be increased and it is necessary to reduce the import of edible oil.

## 1.10 Aim and Objectives

**Aim**

- The Aim of present study is to analyze *B. rapa* genotypes through biochemical and molecular markers

**Objectives**

- To study the diversity among collected genotypes through SDS-PAGE
- To study SSRs based variability among *B. rapa* genotypes collected from different area of Azad Jammu and Kashmir
- To find out the promising genotype of *B. rapa*

# Chapter 2

# Review of Literature

## 2.1 SDS-PAGE Based Literatures

Many techniques including seed protein electrophoresis, isozymes, and different forms of molecular markers are now utilized for analyzing variability and relationships within genotypes. Seed proteins can give stable, easily used, and direct gene products with little environmental influence. This ability makes them good molecular markers. SDS-PAGE is globally utilized to analyze seed protein and to sort out inter and intra-specific genetic diversity studies, accession characterization, phylogenetic relationship, and identification. Seeds from different regions of the world can be collected and information can be provided by using variations in seed protein [34]. SDS-PAGE is a convenient method for identifying a genetic assortment of crops in a brief period. It can be commonly used for various reasons, for example, classification of germplasm, the study of biosystematics, certified varieties, identifying the association of phylogeny in different varieties, and estimating exact data in markers like RAPD[35].

For the estimation of crop genetic diversity, different techniques are available such as morphological, biomolecular, and molecular (DNA) markers. For the investigation of plant genetic diversity and classification of plant germplasm used electrophoresis of total seed storage protein. In Pakistan, the different genotypes of

oil seed *Brassica mustard* expose high diversity by electrophoretic seed protein analysis. As compared to the agro-morphological methods SDS-PAGE methods are more effective, simple, and expensive that's why plant breeders and scientists used the SDS-PAGE technique[36].

*Zada et al.* [37] study the genetic diversity of the 94 *Brassica carinata* (Ethiopian mustard) genotypes SDS-PAGE. It was reported that there were 31 reproducible protein subunits, 14 out of them were polymorphic, and reaming was monomorphic lies within the range from 8 kDa to 180 kDa. It was observed that there were 50 to 100% genetic similarities among the genotypes. The study dendrogram was grouped into five main classes based on matrix dissimilarity with the help of the UPGMA. Low levels of genetic differences were reported within local genotypes and medium to high levels of genetic differentiation were reported in foreign genotypes. It was suggested that only SDS-PAGE was not sufficient to investigate the genetic diversity of these genotypes, so further study is required by using the high genetic diversity 2-D gel electrophoresis and molecular markers. *Jan et al.* [38] investigate the variation of seed protein among brown *B. rapa* sub-species. SDS-PAGE method was used to analyze the 20 different types of brown genotypes. It was observed that protein sizes were small, medium, and large. It was reported that there were 12 total bands out of which 10 (83.33%) were highly polymorphic and 2(15.38%) bands were monomorphic. The protein was divided into various categories according to its size and polymorphism based on its molecular weight, which ranges from 10 kDa to 180 kDa. The UPGMA method was used to investigate the variable protein data, this method classified all genotypes into four major cluster groups. Each cluster has a different number of genotypes such as Cluster I, II, III, and IV have 3, 3, 6, and 10 genotypes respectively. Similarity coefficients (40 to 96%) between several genotypes were calculated. Br-607, Br560, and the highest similarity coefficient (96%) between Br-589 and Br-607 were reported for genotypes.

*Shinwari et al.* [39] used the SDS-PAGE technique to investigate the genetic diversity among 102 genotypes of *Eura Sativa*. It was observed that there were 17 total protein sub-units, 6 out of these are monomorphic and 11 were polymorphic.

The protein sub-unit ranged from 15 to 220 kDa in molecular weight. About 60100% of genetic similarities were reported among these genotypes. The study of the dendrogram was placed into 4 main groups based on the dissimilarity matrix by using UPGMA. All of the accessions were divided into four groups based on phylogenetic analysis. Groups I, II, III, and IV each include 23, 75, 8, and 8 genotypes. SDS-PAGE technique was used and reported that low to medium level of genetic diversity of genotypes and it was suggested that the use of modern molecular techniques like 2-D gel electrophoresis for a high level of genetic diversity among these genotypes.

*Jiang et al.* [40] used unique SDS PAGE technique to recorded clear polypeptide bands in the 1-30 KDa size range. This method was developed by adding and combining various gel compositions, such as urea, glycerol, acrylamide, and bisacrylamide, in variable amounts. A gel with 10% glycerol and 4.2M urea was shown to be ideal for the clear resolution of micro mass proteins. By loading a 5µl sample, staining for 1 hour, and then destaining for 2 hours, the visibility of minute proteins was increased. They were successful in developing a procedure that is very effective and repeatable.

*Akber et al.* [41] reported that SDS-PAGE was used for the characterization of one hundred and five genotypes of sesame named *Sesame Indicum L.* According to this study, fourteen (70%) of the twenty protein subunits were polymorphic, and the other eight were monomorphic. These protein subunits range from 13.5 to 100 kDa in terms of molecular weight. Three main clusters can be seen in the dendrogram of the study, which was based on UPGMA. Eighty-nine cultivars are part of cluster I, fourteen are part of cluster II, and two are part of cluster III. From 50% to 100% made up the similarity coefficient. Based on these findings, it was concluded that SDS-PAGE is not as significant to find genetic diversity up to a high level in a for mentioned genotypes because the range of genetic diversity varies from low to medium level. It was therefore recommended that other techniques, such as 2-D gel electrophoresis and modern DNA tools, be used to find genetic diversity up to a high level in mentioned genotypes. *Sadia et al.* [42] used SDS-PAGE to report the total protein profile in the seed of almost thirty genotypes of different

species of Brassica such as *B. napus L., B. juncea L., B. rapa L.,* and *B. carinata L.* They stated that protein subunits in every genotype were present from twenty-nine to thirty-one. The dendrogram of the study was divided into three clusters of genotypes. They found 15 cultivars in the first cluster, 13 in the second cluster and 3 in the third cluster. A significant level of genetic diversity of the genotypes of mustard-which was gathered from Pakistan, was found. They discovered a lower amount of interspecific genetic diversity in the mentioned *Brassica* genotypes.

*Turi et al.* [43] found the total protein patterns of 234 different Brassica genotypes were determined using SDS-PAGE. *Brassica napus L.* had twenty-eight protein subunits, fifteen of which were regarded as polymorphic and the remaining twelve as monomorphic. There was a total of twenty protein subunits in *Brassica campestris L.*; eleven of them were polymorphic, while the remaining twelve were monomorphic. Twenty-six protein subunits altogether, of which fifteen were polymorphic and the rest were monomorphic, were present, just as in *Brassica juncia L.*

The study's dendrogram demonstrated how the total number of genotypes was divided into two major groups, each of which included eleven subgroups. They generalized that the level of genetic diversity was low in studied genotypes, so they considered that SDS-PAGE only found the low level of genetic diversity estimation in these genotypes, in result 2D gel electrophoresis techniques should be used for the study of protein level of the mentioned genotypes.

*Noor Saleem et al.* [44] reported the characterization of genetic diversity of one hundred local Indian mustard named *Brassica juncea L.*, for total protein profile in seed through SDS-PAGE. The 12.25% polyacrylamide gels generalized the total protein in seeds, so a total of twenty-one bands were produced on the criteria of molecular weight varied from 6 to approximately 180kDa.

Out of twenty-one bands, seventeen were polymorphic and the rest of them were monomorphic resulting in a high level of variability present in genotypes. Similarity index varied from 0.62 to 1.0. UPGMA was used to produce a dendrogram, in results all hundred accessions were divided into five main clusters. The medium

level of genetic diversity in the protein profile of the seed was inferred through grouping pattern, although it is recommended that 2D gel electrophoresis and other modern molecular techniques should be used to search for genetic diversity up to the highest level, due to limited significance of SDS-PAGE for genetic diversity found in seed protein profiling of mentioned accessions.

*Khurshid et al.* [45] used salt soluble protein and SDS-PAGE to analyze the genetic diversity of 30 *Brassica* genotypes. They discovered significant polymorphism in the studied genotypes. They divided the profile of their protein into three different parts. In the first region, three highly polymorphic protein subunits were found. The second region had a total of nine protein sub-units; however, only a few of them were polymorphic. Six protein subunits were found in the last region of the gel, including two polymorphic. The second region had a total of nine protein sub-units, however, only a few of them were polymorphic. The entire genotypes were sorted into four main groups and a dendrogram was produced for the study using UPGMA. A 91% genetic similarity exists between the genotypes. They also found four groups in the dendrogram of salt-soluble protein. They concluded that polymorphism indicates differences among Brassica species and that the formation of groups was unaffected by geographic factors.

*Khakaei et al.* [46] investigate 16 genotypes of *Brassica napus L.* were characterized using the SDS- PAGE technique. In order to study the differences in the protein subunit profiles of plants cultivated under two distinct environments, researchers extracted protein from the leaves of the plants when they were fully flowering. According to research, the genotypes of Brassica napus L. that grow under normal conditions have a mean genetic distance between them of 0.056 and 0.632, but those that grow under stress have genotype similarity between 0.0 and 0.5.Based on Jaccard's similarity coefficient during the study, both genotypes under normal and stress conditions were classified into 3 major groups by using cluster analysis. It was observed that the protein profile pattern was different for genotypes that were grown in normal conditions and different for those that grew in stress conditions. *Rabbani et al.* [47] used the SDS-PAGE technique to evaluate the seed protein pattern of the oil seed of mustard. In 52 genotypes, they observed 8 different

types of protein sub-units. While just one genotype revealed the other six forms of protein, the type-I protein was found in 43 oil seed genotypes and the type-IV protein in 3 genotypes. Four groups of protein subunits were developed. In group I, the protein subunits were weakly stained, whereas in group II, eight protein subunits were seen, and in group III, very wide protein subunits with molecular weights ranging from 24.5 to 19.5 were seen, while in group IV the bands observed were 13kDa. UPGMA was used to make a dendrogram and Jaccard's similarity index was used for grouping. The result of seed protein by using SDS-PAGE indicated that this technique is not strongly able to differentiate the genotypes that are closely associated. But it was useful to differentiate *B. juncea L.* from *B. campestris L.* oilseed mustard from vegetables.

*Akbar et al.* [48] used Polyacrylamide Gel Electrophoresis as a biochemical marker to study thirteen different *Brassica* leaf storage protein variations. There were discovered 19 different protein bands in all, with a high polymorphism of 89.47%. The location, molecular weight, and staining strength of the bands may be used as fingerprints for variant identification. UPGMA was used to construct a dendrogram that showed two main clusters for all the *Brassica* variations that were examined.

*Muklesur et al.* [49] evaluated 85 *Brassica* genotypes such as *B. carinata L. B. napus L. B. rapa L. B. oleracea L. B. juncea L.* and hexaploid *Brassica* obtained from various countries including China, Bangladesh, Denmark, and Japan. For seed protein, SDS-PAGE was used and identified that 34 to 35 protein subunits in seed among them 10 (31.4%) were polymorphic and the rest was monomorphic. Among the genotypes highest polymorphism was indicated in B. rapa L. which was 21.2%, about 6.3% observed in *B. napus L.* and 3.2% observed in polymorphism *B. juncea L.* Uppermost indexes were reported for hexaploid Brassica by analyzing of seed protein and on the second place were the amphidiploids species such as *B. juncea L. B. carinata L.* and *B. napus L.* and on the third place were the diploid species including *B. oleracea L.* and *B. rapa L.* were observed. *Jan et al.* [50] studied variations in total seed proteins in three ecotypes of *B. rapa* 20 various genotypes from all ecotypes were examined using SDS-PAGE, and their

phylogenetic relationships were identified. Based on molecular weights varying from 10 kDa to 180 kDa, four basic categories of protein size polymorphism were discovered. Small proteins belonged in group D, medium proteins in groups B and C, while large proteins were in group A. Total soluble seed protein variations were analyzed using the UPGMA, which divided the three ecotypes into four major groups. Clusters I and III, respectively, included one each of the brown sarson and one toria genotype. These two cluster groupings showed the largest polymorphism in comparison to other cluster groupings. Clusters III and IV contained all three ecotypes. Similarity coefficient measurements for all three types ranged from 47 to 100%. Toria and brown sarson genotypes shared a 100% similarity coefficient, while brown sarson and yellow sarson ecotypes shared a 47% similarity index. For the first time, they identified significant protein-based variations in all three *B. rapa* ecotypes. Their research will be helpful as a foundation for describing *B. rapa* ecotypes.

*Siddiqui et al.* [51] evaluate the characteristics of seed proteins for glutelin diversity in Pakistani rice genetic resources, through SDS-PAGE. From three rice-producing regions as well as other parts of the country, 475 accessions were collected. At 57kD pro-glutelin and 40kD glutelin acidic subunit bands 3 and 4, a lot of variance in the glutelin portion of rice protein was present. Better protein harvests may be produced using the enriched glutelin version at 57kD, both in terms of quality and quantity.

According to *Shahid et al.* [52] 136 genotypes of rapeseeds (*B. napus L.*) were examined using the biochemical method of SDS-PAGE based on total seed storage proteins. One control cultivar and 135 accessions of germplasm from the PGRI, NARC, and Islamabad, Pakistan, were used in the study. In this study, 12.25% Polyacrylamide Gels were used, and it was discovered that each genotype had a total of 21 protein subunits. The 5 (23.81%) of the 21 bands were monomorphic, and remaning16 (76.19%) that were polymorphic. The molecular weights of the 21 protein subunits, which range from 6 to 180 kDa, were identified. The similarity coefficients for these genotypes ranged from 0.83 to 0.98. The investigated genotypes were divided into five major groups using UPGMA to produce

a dendrogram based on a dissimilarity matrix. Overall, just a little percentage of genetic difference was found. The SDS-PAGE technique alone is insufficient to properly investigate the genetic diversity contained in these genotypes; hence it is recommended that in the future, 2-D gel electrophoresis, together with other modern techniques, should be used to find high levels of genetic diversity among these genotypes.

## 2.2 Molecular Markers Based Literatures

The era of genomics began with the development of genetic tools such as DNA based molecular markers, which have been widely used in different fields such as genetic engineering, taxonomy, physiology, and embryology etc. Molecular markers are defined as a specific part of DNA that is representative of differences in genome analysis. To calculate genetic diversity, scientists used molecular markers, often known as genetic markers. These molecular markers can be used to assess genetic variation in various animals. They can recognize variations caused by chromosomal duplication, dilatation, inversion, and insertion. These markers are only found in traits that are controlled by genes, so they cannot affect the phenotypes of the traits [53].

Plant breeding and plant systematics utilize molecular markers as effective instruments for genetic study. SSRs markers are crucial among these markers. They have been applied to various agricultural evaluations. SSRs markers were developed for two different purposes, one for the nuclear genome and the other for chloroplasts [54]. Chloroplast SSRs (cpSSRs) exhibit a high level of intraspecific variation and are employed for evolutionary investigations.

Nuclear SSRs (nuSSRs) are utilised to characterise nuclear polymorphism and differentiate genetic variants. SSRs was used to investigate inter- and intraspecific genetic relationships in *Brassica* and closely related species [55]. It was reported that the SSRs markers were used to investigate the purity of hybrids between salt-resistant and susceptible genotypes. Breeding by the early selection of the

genotypes than the phenotypic screening was assisted by the microsatellite markers. It was studied that SSRs were used in determining the genetic diversity and population structure in 104 genotypes of cucumber through 23 SSRs primers. The information gathered through this research will be helpful for the identification of genotypes, molecular breeding, and further improvement of the crop SSRs markers associated with yield traits were used to characterize the collection of 96 common beans. A high level of genetic diversity observed could be used for genetic improvement of common bean targeting yield attributing traits [56].

SSRs or microsatellites are found everywhere in the eukaryotic genome. These makers are DNA-based. Microsatellites have 1-6 base pair tandem repeats. Because the mutation rate is considerable and ranges from 2-10 to 6-10bp, variations are also found in the length of SSRs [57]. These markers are very informative and polymorphic when combined with other molecular markers. If information about primers is available, these markers are inexpensive, straightforward, reproducible, and co-dominant. SSRs are used to identify genetic variation in organisms that are genetically related. These markers are used to analyze the genetic composition of natural populations regularly. SSRs are also employed to characterize, use, and conserve crop variety [58]. The entire list of nuclear SSRs in the Brassicaceae family is accessible. These markers are very informative and polymorphic when combined with other molecular markers. If information about primers is available, these markers are inexpensive, straightforward, reproducible, and co-dominant. SSRs are used to identify genetic variation in organisms that are genetically related. These markers are used to analyze the genetic composition of natural populations regularly. SSRs are also employed to characterize, use, and conserve crop variety.

The entire list of nuclear SSRs in the *Brassicaceae* family is accessible[59]. Through the use of SSRs markers, *El-Esawi et al.* [60] evaluated 118 B. oleracea genotypes. There were found to be 47 distinct sizes of alleles. High levels of heterozygosity (0.699) were observed among accessions. A total 72.9% intra-specific variability and 27.1% inter-specific genetic differentiation was discovered. The genotypes of Brussels sprouts and kale were further separated by the dendrogram. They came

to the conclusion that spring cabbage genotypes exhibit the greatest degree of diversity.

*Thakur et al.* [61] explains how the SSRs marker variation in *Brassica* species affected the emergence and growth of *Brassica amphidiploids*. To better understand the origins and development of *Brassica amphidiploids*, SSRs markers were used to unravel genetic differences in three diploid and three amphidiploid *Brassica* species. The SSRs marker collection from this study will be useful for gene tagging, genome mapping, assessing genetic diversity in *Brassica* germplasm, DNA fingerprinting of various *Brassica* cultivars, and other genomics-related studies in *Brassica* species.

*Iqbal et al.* [62] investigate significant genetic differences between 16 *B. rapa* genotypes Using 12 markers. In comparison, the three were shown to be extremely polymorphic. Out of 40 bands, 38 (94.87%) displayed the highest levels of polymorphism. While OPB-04 and GLA-11 amplified the least (92.31%), the primer OPD-02 exhibited polymorphic bands with a purity of 100%. 12.67 polymorphic fragments and 13.33 bands per primer were recorded. These genotypes are split into three main categories based on similarity coefficient values. TheCelucia2009oxre were unique genotypes for each group.

*Celucia et al.* [63] examined the molecular maker-based variations of three of *B. rapa* subspecies. In order to examine the genotypes of *B. rapa Parachinensis, B. oleracea alboglabra*, and *B. rapachinensis L.*, 54 SSRs markers were utilized. There were 122 scoring fragments discovered, and 77% of them exhibited high polymorphism. The mean total diversity among genotypes with a high level of genomic variability 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. Their findings suggested that *B. oleracea alboglabra* different from the other subspecies. They examined the genetic diversity of three distinct *B. rapa* subspecies, that are routinely grown across the world.

*Tahira et al.* [64] using (RAPD) markers, thirty *B. juncea* genotypes were examined for genetic differences. With an average of 8.6 alleles amplified per primer,

approximately 104 loci were found. Bands with maximum polymorphism were detected. With sizes ranging from 300 bp to 3kb, several banding patterns were seen. The three RAPD primers, A-5, A-19, and B-05, each produced a maximal fragment with a distinct 3 kb size. Overall, all genotypes showed 84.5% similarity. When compared to other evaluated genotypes, the RBJ-97001 genotypes displayed the highest level of variety (29%). All three genotypes were divided into major groups using the phylogenetic analysis method. Based on similarities among the groups, these groups were further split into two small subgroups. They also suggested this research be used to create brand-new canola cultivars.

*Parthiban et al.* [65] investigate 25 primer pairs made from SSRs and 25 SSRs from expressed sequence tags (EST), 59 sugarcane genetic populations were used. The mean PIC of genomic SSRs was higher, ranging from 0.62 to 0.72, when compared to the PIC value produced by the EST-SSRs marker. The fact that EST-SSRs markers are positioned in more conserved and expressed sequences than genomic sequences, which are scattered throughout the genome, may be responsible for the low level of variability in these markers. Genotype grouping differences were revealed by a dendrogram created using genomic SSRs and EST-SSRs marker data. 59 sugarcane accessions were sorted into 4 and 6 clusters, respectively, using genomic SSRs and EST-SSRs.

*Ali et al.* [66] characterized the total of 96 indigenous *B. rapa* accessions obtained from different places in Khyber Pakhtunkhwa, Pakistan. From the collected samples, the SSRs markers identify the most diverse genotypes. To evaluate the genetic variability among collected genotypes, 26 different SSRs primers were used. Literature was used for the selection of the primers based on their previous results and these primers indicated a total of 135 bands. Among them, 75 showed polymorphic, having an average of 55.5% polymorphic loci, and indicated the broader genetic variation of the collected samples of genotypes. UPGMA was used to classify all genotypes into 3 major groups. Three clusters were present in group one, while two clusters were present in groups two and three. This study indicated that SSRs markers were an efficient tool for the genetic variability of other local and exotic *B. rapa* genotypes.

# Chapter 3

# Materials and Methods

## 3.1 Research Methodology Flow Chart

To find out the promising genotype and study the protein profile diversity of *B. rapa* used two types of methods.

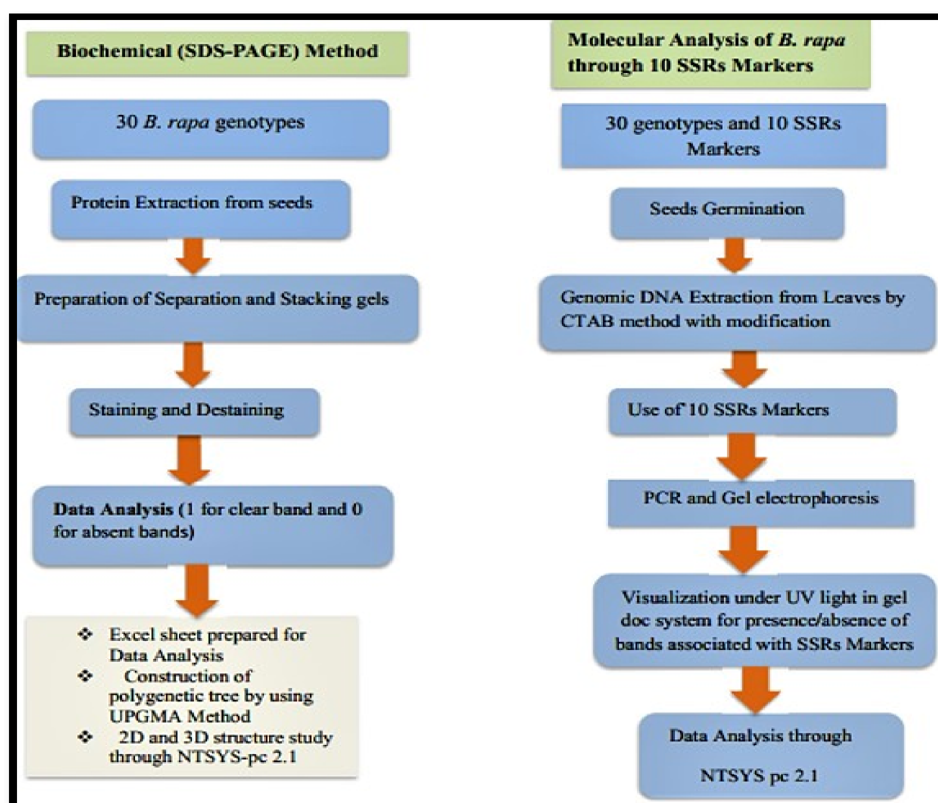



FIGURE 3.1: Overview of methodology.

## 3.2 SDS-PAGE Based Characterization of *B. rapa* Genotype

### 3.2.1 Plant Material Collection of Seeds

The fresh and mature seeds of *B. rapa* genotypes were collected from different districts of Azad Jammu and Kashmir for SDS-PAGE analysis with the help of native peoples, family members, and friends including one check variety from NARC. These 30 genotypes of *B. rapa* were used in SDS-PAGE for seed protein at PGRI, NARC, and Islamabad, Pakistan. All these genotypes with their accession numbers are listed in (Table 3.1).

Table 3.1: List of diverse genotypes of *Brassica rapa* selected for SDS-PAGE analysis (n=30)

| Serial Number | Accession Number | Origin |
|---|---|---|
| 1 | HatBr103 | Hattian Bala,Jhelum vally,AJK |
| 2 | HatBr107 | Hattian Bala,Jhelum vally,AJK |
| 3 | HatBr110 | Hattian Bala,Jhelum vally,AJK |
| 4 | MuzBr113 | Muzaffarabad ,AJK |
| 5 | MuzBr115 | Muzaffarabad ,AJK |
| 6 | MuzBr118 | Muzaffarabad ,AJK |
| 7 | NeeBr120 | Neelum,AJK |
| 8 | NeeBr122 | Neelum,AJK |
| 9 | NeeBr125 | Neelum,AJK |
| 10 | BagBr130 | Bagh ,AJK |
| 11 | BagBr132 | Bagh ,AJK |
| 12 | BagBr135 | Bagh ,AJK |
| 13 | BagBr137 | Bagh ,AJK |
| 14 | HavBr140 | Havli ,AJK |
| 15 | HavBr141 | Havli ,AJK |
| 16 | HavBr143 | Havli ,AJK |

Table 3.1: List of diverse genotypes of *Brassica rapa* selected for SDS-PAGE analysis (n=30)

| Serial Number | Accession Number | Origin |
|---|---|---|
| 17 | PooBr147 | Poonch ,AJK |
| 18 | PooBr149 | Poonch ,AJK |
| 19 | PooBr150 | Poonch ,AJK |
| 20 | PooBr152 | Poonch ,AJK |
| 21 | PooBr155 | Poonch ,AJK |
| 22 | MirBr158 | Mirpur ,AJK |
| 23 | MirBr160 | Mirpur ,AJK |
| 24 | MirBr162 | Mirpur ,AJK |
| 25 | KotBr163 | Kotli ,AJK |
| 26 | KotBr165 | Kotli ,AJK |
| 27 | KotBr166 | Kotli ,AJK |
| 28 | BhiBr168 | Bhimber, AJK |
| 29 | BhiBr170 | Bhimber ,AJK |
| 30 | BSA | NARC, Islamabad, PAK |

### 3.2.2 Protein Extraction from Seeds

*B. rapa* fresh 10-15 seeds were finely ground in a mortar and pestle. Every 1.5 ml Eppendorf tube with mashed supplies (0.02g) was received 400µl protein extraction buffers (0.5M Tris-HCl (pH 8.0), 0.2% SDS, 5M urea, 1% 2-mercaptoethanol, and bromophenol blue dye) is shown in (Table 3.2).

Table 3.2: Protein extraction buffer composition.

| Ingredients | Amounts |
|---|---|
| Distt. $H_2O$ | 75ml |
| Tris(hydroxymethyl) aminomethane (THAM) | 0.7g |
| HCl (conc) | Adjust to pH 8.0 |

TABLE 3.2: Protein extraction buffer composition.

| **Ingredients** | **Amounts** |
|---|---|
| SDS | 0.4g |
| Urea | 30g |
| 2-Mercaptoethanol 1ml | 1ml |
| Total vol. | 100ml |

The sample was properly blended by vertex for a few minutes and kept overnight in the refrigerator at -200C.

### 3.2.3 Preparation of Separation Gel and Stacking Gel

The Ammonium Per sulphate (APS), separation and stacking gel were prepared line with the SDS-PAGE technique of *Jan et al.* [67] with a few minor changes. To make separation and stacking gels, various chemical concentrations were used (Table 3.3 to 3.8).

TABLE 3.3: Sol. A composition.

| **Ingredients** | **Amounts** |
|---|---|
| Distt. $H_2O$ | 100ml |
| Tris (hydroxtmethyl) aminomethane | 34g |
| SDS | 0.8g |
| pH | 8.0 |

Kept in freezer at -4$^o$C.

TABLE 3.4: Sol. B Composition.

| **Ingredients** | **Amounts** |
|---|---|
| Distt. $H_2O$ | 100ml |
| Tris(hydroxtmethyl) aminomethane | 7g |
| SDS | 0.7g |

Table 3.4: Sol. B Composition.

| Ingredients | Amounts |
|---|---|
| pH | 7.0 |

Kept in freezer at -4$^o$C.

Table 3.5: Sol. C Composition.

| 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 |
|---|---|
| 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 | 60 µl |

Kept in freezer at -4$^o$C.

TABLE 3.8: Composition of stacking gel.

| **Ingredients** | **Amounts** |
| --- | --- |
| Distt. $H_2O$ | 6.0ml |
| Sol. B | 3ml |
| Sol. C | 2ml |
| 10% APS | 100 µl |
| TEMED | 50 µl |

Kept in freezer at -4$^o$C.

The sample that had been kept in the refrigerator underwent a ten-minute centrifugation at 12000 rpm. A protein marker and 10 L of each sample were added to each well at a voltage of 100 V using an electrode buffer (Table 3.9). Protein activity was assessed constantly until it reached the plates' base.

TABLE 3.9: Composition of electrode buffer solution.

| **Ingredients** | **Amounts** |
| --- | --- |
| Distit. $H_2O$ | 1000ml |
| Tris(hydroxtmethyl) aminomethane | 3.2g |
| SDS (Sodium dodecyl sulphate) | 1.3g |
| Glycine | 14g |

Keep at room temperature

### 3.2.4 Staining and Destaining

The gels were next put in a staining solution (Table 3.10) and kept for two to three hours. The gels were then put into a new de-staining solution (Table 3.11) and shaken for 24 hours after being cleaned twice with distilled water. The autoclaved tissue paper was placed on a gel to remove the excess blue color. All genotypes' band patterns were observed. The movement and visibility of various sizes of

protein are significantly affected by the low or excessively high pH of various solutions and protein extraction buffers.

Table 3.10: Composition of staining solution.

| **Ingredients** | **Amounts** |
|---|---|
| Distt. $H_2O$ | 470ml |
| Acetic acid ($CH_3OH$) | 70ml |
| Methanol ($CH_3COOH$) | 460ml |
| CBB & R250 | 2.10g |

Keep at room temperature

Table 3.11: Composition of destaining solution.

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

Keep at room temperature

### 3.2.5 Data Analysis

The banding patterns were observed and each band was scored according to the scoring system. The scoring system of 1/0 was used to give scores to the bands. The presence of the band was indicated with 1 score and the absence of bands indicate 0. The genetic coefficients were measured by following the equations of *Nei and Li* [68]. The dendrogram was constructed using the UPGMA method [69]. NTSYS-pc, version 2.1 computational software was used to check genetic diversity among genotypes. Principal Coordinates analysis (PCoA) was used to study the two-dimensional (2D) and three-dimensional (3D) studies for observing genotype dispersion through NTSYS pc, version 2.1 software and friendly [70] .

## 3.3 Genetic Diversity of *B. rapa* Based on SSRs Marker

### 3.3.1 Experimental Material

From different locations of Azad Jammu and Kashmir, almost 30 distinct *Brassica rapa* genotypes were collected. Among these genotypes, BSA was a check genotype. The detail of *Brassica rapa* seed genotypes and SSRs marker used are provided in Tables 3.12 and 3.13.

TABLE 3.12: List of diverse genotypes of *B. rapa* selected for SSRs analysis (n=30)

| Serial Number | Accession Number | Source |
|---|---|---|
| 1 | HatBr103 | Hattian Bala,Jhelum vally,AJK |
| 2 | HatBr107 | Hattian Bala,Jhelum vally,AJK |
| 3 | HatBr110 | Hattian Bala,Jhelum vally,AJK |
| 4 | MuzBr113 | Muzaffarabad ,AJK |
| 5 | MuzBr115 | Muzaffarabad ,AJK |
| 6 | MuzBr118 | Muzaffarabad ,AJK |
| 7 | NeeBr120 | Neelum,AJK |
| 8 | NeeBr122 | Neelum,AJK |
| 9 | NeeBr125 | Neelum,AJK |
| 10 | BagBr130 | Bagh ,AJK |
| 11 | BagBr132 | Bagh ,AJK |
| 12 | BagBr135 | Bagh ,AJK |
| 13 | BagBr137 | Bagh ,AJK |
| 14 | HavBr140 | Havli ,AJK |
| 15 | HavBr141 | Havli ,AJK |
| 16 | HavBr143 | Havli ,AJK |
| 17 | PooBr147 | Poonch ,AJK |
| 18 | PooBr149 | Poonch ,AJK |

Table 3.12: List of diverse genotypes of *B. rapa* selected for SSRs analysis (n=30)

| Serial Number | Accession Number | Source |
|---|---|---|
| 19 | PooBr150 | Poonch ,AJK |
| 20 | PooBr152 | Poonch ,AJK |
| 21 | PooBr155 | Poonch ,AJK |
| 22 | MirBr158 | Mirpur ,AJK |
| 23 | MirBr160 | Mirpur ,AJK |
| 24 | MirBr162 | Mirpur ,AJK |
| 25 | KotBr163 | Kotli ,AJK |
| 26 | KotBr165 | Kotli ,AJK |
| 27 | KotBr166 | Kotli ,AJK |
| 28 | BhiBr168 | Bhimber ,AJK |
| 29 | BhiBr170 | Bhimber ,AJK |
| 30 | BSA | NARC, Islamabad. |

SSRs/microsatellite primers for genotype diversity analysis of *Brassica rapa.*

Table 3.13: SSRs/microsatellite primers for genotype diversity analysis of *Brassica rapa.*

| Sr No | Primers | Forward Primer (bp) | Reverse Primer (bp) |
|---|---|---|---|
| 1 | BRMS-001 | GGTGGCTCTA ATTCCTCTGA | ACTT TCTCTCACCA ACCCC |
| 2 | BRMS-007 | AAATTGTTT CTCTTCCCC AT | GTGT TAGG GAGC TGGAG AAT |

TABLE 3.13: SSRs/microsatellite primers for genotype diversity analysis of *Brassica rapa*.

| Sr No | Primers | Forward Primer (bp) | Reverse Primer (bp) |
|---|---|---|---|
| 3 | Na10-D09 | AAGAACGT CAAGATCCT CTGC | ACCA CCAC GGTA GTAGA GCG |
| 4 | Na12-A02 | AGCCTTGT TGCTTTTCA ACG | AGTG AATC GATG ATCTC GCC |
| 5 | Na14-C12 | CACATTT TGGTTCA ATTCGG | TACG ACGC TGGT TTCGA TTC |
| 6 | Ni2-A06 | TGGCTC CTTTATG GTCTTGC | AACC ATGTA GCTTT GGACGC |
| 7 | Ni2-A11 | AACAAA CAAGAG TCGAAT ACGG | AATG CCCT CTAA CTGAGCCC |
| 8 | Ni2-B03 | ACTTCT TGCCCT CCTCACC | AAAT ACTCACTGCA ATACCCAGG |

TABLE 3.13: SSRs/microsatellite primers for genotype diversity analysis of *Brassica rapa*.

| **Sr No** | **Primers** | **Forward Primer (bp)** | **Reverse Primer (bp)** |
|---|---|---|---|
| 9 | Ni4-A03 | ACACAG AAACAT CAAACA TACC | GGAC CGGTT TTATT TGTTC G |
| 10 | Ni4-H03 | GATGAA CAGCAAC AGCTTGG | CAAA ATGT CGTT TGTT AGTC TTGG |

### 3.3.2 Sample Preparation

Each genotype's 4-5 fresh seeds were planted in pots. For two days inside, these pots received frequent watering. After germination for two to three weeks, fresh leaf samples were taken. The leaf samples were kept in a refrigerator at -80°C for later use.

### 3.3.3 DNA Extraction

Before DNA extraction, all stock solutions were prepared, and the CTAB technique was used to extract the DNA [71]. This is how the DNA was extracted:

- Three to four leaves of each genotype were mashed in mortar and pestle, with the addition of CTAB solution (700 µl) also containing a little volume

of mercaptoethanol (30 µl/1 ml CTAB solution). After proper crushing, the samples were placed in a 1.5ml Eppendorf tube.

- All the samples were kept in water bath at about 65°C for 40 min. samples were rotated about four times after five minutes.
- The sample was cooled to room temperature. Then 600 µl 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°C.
- 600µl of clear supernatant was collected and transferred to the other tube while the waste was discarded.
- The amount of 350µl of Isopropanol was added for 2-3 times and was placed in the freezer at 4°C for at least 30 mins.
- Centrifuged again for 10 minutes at 4°C at 13000 rpm.
- From each tube the small white pellets were collected.
- Supernatant was removed and 200 µl ethanol of 70
- 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.
- Then in every single tube 100 µl of fresh TE buffer was added and vortexed.
- Added each tube with 1 µl of RNase A (10 mg/ml) and incubated for 35 minutes at 40°C in a water bath.
- Then at -20°C in freezer all the extracted DNA samples were stored.
- A NanoDrop ND-1000 Spectrophotometer was used to check the quality and purity of each DNA sample at 260 and 280 nm.
- All DNA samples were diluted to a working concentration of 20 mg/l to achieve accurate PCR results.

### 3.3.4 Specification of the Primers

To find out the molecular variability among *B. rapa* accessions, Ten simple sequence repeats (SSRs) markers were used. The literature analysis of Brassica spp. The desired primers produce monomorphic and polymorphic bands.

### 3.3.5 Amplification of SSRs Markers in *B. rapa* Genotypes

Based on the data that was supplied for each primer, the PCR conditions were optimized with a few minor changes to the annealing temperature. Details on the 20µl PCR reaction volume and PCR conditions was provided in Appandix 5.1. The concentration of agarose changed according to the size of the primer. To obtain clear PCR bands, 2 to 3 % agarose gels were typically used. With the addition of 5µl of ethidium bromide, a 1xTBE buffer containing Tris-Borate (10 mM) and EDTA (1 mM) was used to generate the high-resolution agarose gel. In Appandix 5.2 the ideal conditions for several PCR stages are listed.

### 3.3.6 Electrophoresis of Amplified Products

After PCR 4 µl of 6x loading dye was added to PCR tubes. The 7 µl PCR sample was put into each well. A DNA ladder of 50 and 100 bp was used to confirm the precise size of SSRs markers. The gels were seen using UVI Gel Doc Documentation System, and the desired PCR product sizes were recorded.

### 3.3.7 Allele Scoring and Data Analysis

It was noted whether there were one, two, or more bands present for each sample depending on the primer used. 1 indicated band presence, whereas 0 indicated band absence. MS Excel was used to record all of the data.

Only clear DNA bands were used for data analysis. The optimal annealing temperature and the overall polymorphic allele count for each primer were recorded.

The dice method was used to construct genetic similarity coefficients based on pair-wise comparisons of *B. rapa* genotypes based on the presence or absence of alleles [68]. A phylogenetic tree based on UPGMA was constructed using the values of the similarity coefficients. The analysis was performed using NTSYS pc, version 2.1 [70].

# Chapter 4

# Results and Discussions

## 4.1 *B. rapa* Genotypes Show Total Seed Proteins Based Variations through SDS-PAGE Method

Biochemical based characterization is essential for the genetic improvement of *Brassica* species. For the characterization of *B. rapa* SDS-PAGE method was used. It is very efficient, inexpensive, quick, and safe. To study the variations among genotypes and their classification this method was used. This approach divides protein subunits based on molecular weight since smaller molecules move more quickly than larger ones in the gel is used and to calculate the molecular weight of unidentified proteins, by using the molecular weight of known protein. The SDS-PAGE technique, which provides information on polypeptide profiles, is considered to be a center of genetic diversity.

### 4.1.1 Polymorphism in Protein Banding Patterns

In the current study, 30 genotypes of *B. rapa* were investigated for diversity in total seed storage protein. These genotypes were all polymorphic to one another,

and distinctive banding patterns were observed. Total banding pattern of protein profiles is shown in Figure 4.1 (a- c).

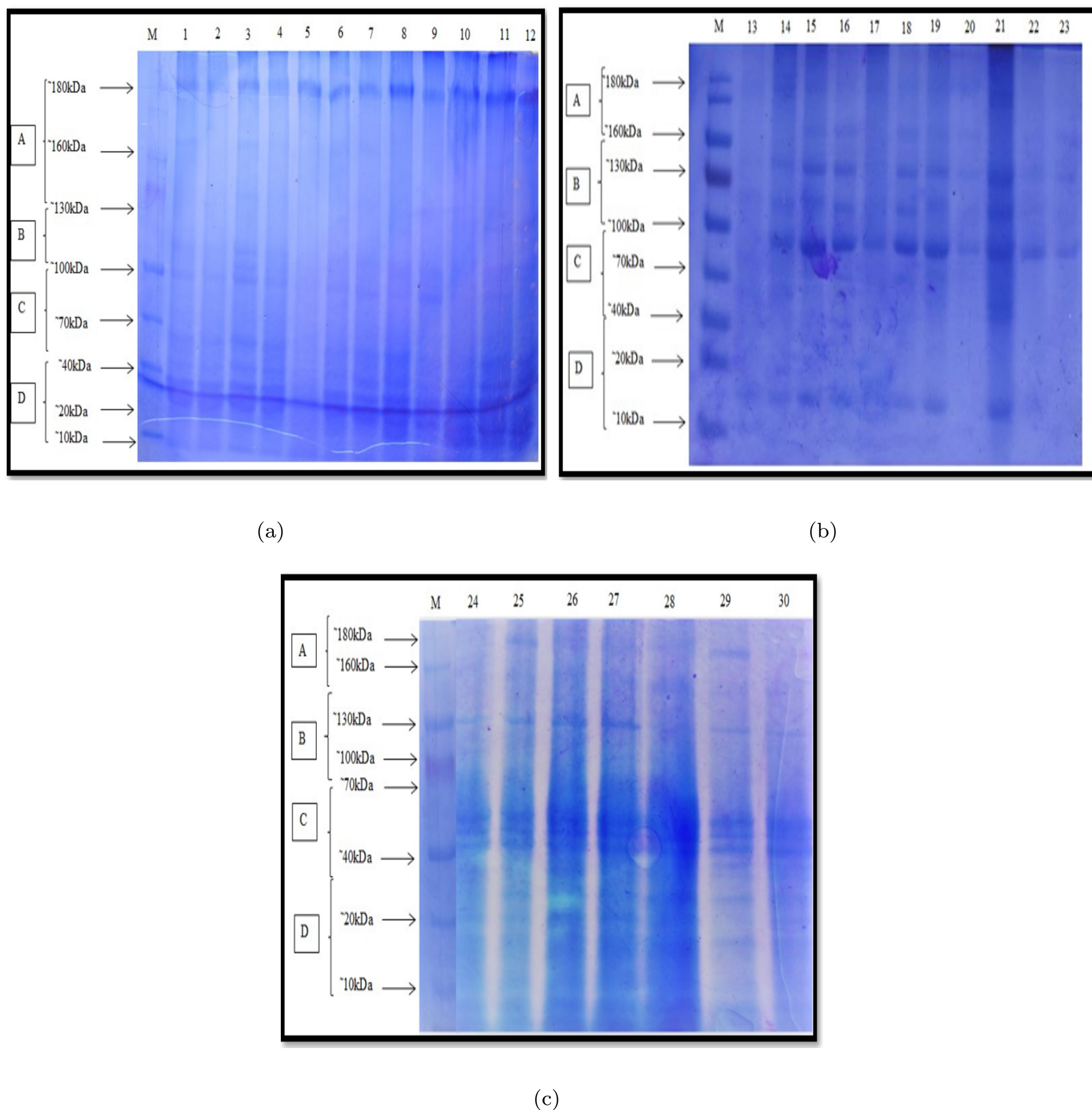



FIGURE 4.1: (a) SDS-PAGE of total seed storage proteins reveals the genotype of *Brassica rapa* electrophoretic banding patterns. M stands for molecular size marker ∼10kDa to ∼180kDa, while numbers from 1-12 represent genotypes HatBr103, HatBr107, HatBr110, MuzBr113, MuzBr115, MuzBr118, NeeBr120, NeeBr122, NeeBr125, BagBr130, BagBr132 and BagBr135.
(b) Electrophoretic banding patterns via SDS-PAGE of total seed storage proteins of *B. rapa* genotype. Molecular size of marker is ∼10kDa to ∼180kDa and represented by M, while numbers from 13-23 represent genotypes BagBr137, HavBr140, HavBr141, HavBr143, PooBr147, PooBr149, PooBr150, PooBr152, PooBr155, MirBr158 and MirBr160.
(c) Electrophoretic banding patterns via SDS-PAGE of total seed storage proteins of *B. rapa* genotype. The molecular size of a marker is ∼10kDa to ∼180kDa represented by M, while numbers from 24-30 represent genotypes MirBr162, KotBr163, KotBr165, KotBr166, BhiBr168, BhiBr170 and BSA.

We noted major and minor bands patterns depending on the genotype being utilized; its size range from ∼10kDa to ∼180kDa. The maximum number of bands reported was 12, and 100% of them displayed polymorphic banding patterns. Based on their molecular weight, these polypeptide bands were divided into 4 main regions (A–D) in Figure 4.1 (a-c).

In part A high molecular weight proteins with sizes ranging from ∼130kDa to ∼180kDa. The majority of the bands in this area were polymorphic. Second part (B) has molecular weight ranging from ∼100kDa to ∼131kDa. The third section (C) contained proteins that ranged in size from ∼38kDa to ∼99kDa. The fourth section contained proteins with small molecular weights ∼10kDa to ∼39kDa.

### 4.1.2 Similarity Co-Efficient of *Brassica rapa* Genotypes

SDS-PAGE was used to assess the genetic diversity of the several genotypes of *B. rapa* in line with Nei and Li's methodology [68]. In all these genotypes similarity coefficient value was measured and the genetic similarity values among genotypes ranged from 0 to 95% (Table 4.1 to 4.4). Highest genetic similarities values are 95% between the genotypes.

- BSA/PooBr152 followed by 94% among NeeBr120/HatBr107, KotBr163/BagBr135 and PooBr155/PooBr150.
- On the other hand, lowest similarity value of 0% was observed among genotypes PooBr147/ MuzBr115, PooBr149/ MuzBr115, PooBr152/MuzBr115, MirBr158/MuzBr115, and MirBr160/MuzBr115 etc. (Table 4.1 to 4.4).
- The similarities among the other genotypes ranged from low to high, depending on the genotype. Our findings demonstrated that all genotypes of *B. rapa* had different similarity coefficient values.
- Our results provide additional evidence that protein-based variability varies between *B. rapa* genotypes.

TABLE 4.1: Genetic similarities among 30 genotypes of *B.rapa*.

| **Acc** | **HatBr103** | **HatBr107** | **HatBr110** | **MuzBr113** | **MuzBr115** | **MuzBr118** | **NeeBr120** | **NeeBr122** |
|---|---|---|---|---|---|---|---|---|
| HatBr103 | 1 | | | | | | | |
| HatBr107 | 0.82 | 1 | | | | | | |
| HatBr110 | 0.9 | 0.84 | 1 | | | | | |
| MuzBr113 | 0.82 | 0.88 | 0.84 | 1 | | | | |
| MuzBr115 | 0.71 | 0.77 | 0.63 | 0.62 | 1 | | | |
| MuzBr118 | 0.82 | 0.75 | 0.84 | 0.75 | 0.62 | 1 | | |
| NeeBr120 | 0.78 | 0.94 | 0.8 | 0.82 | 0.71 | 0.71 | 1 | |
| NeeBr122 | 0.82 | 0.75 | 0.74 | 0.75 | 0.77 | 0.75 | 0.82 | 1 |
| NeeBr125 | 0.67 | 0.71 | 0.71 | 0.71 | 0.73 | 0.71 | 0.67 | 0.71 |
| BagBr130 | 0.57 | 0.62 | 0.5 | 0.62 | 0.8 | 0.46 | 0.71 | 0.77 |
| BagBr132 | 0.82 | 0.75 | 0.74 | 0.88 | 0.62 | 0.75 | 0.82 | 0.88 |
| BagBr135 | 0.59 | 0.63 | 0.74 | 0.63 | 0.46 | 0.5 | 0.71 | 0.63 |
| BagBr137 | 0.46 | 0.33 | 0.53 | 0.5 | 0.44 | 0.67 | 0.31 | 0.5 |
| HavBr140 | 0.53 | 0.43 | 0.71 | 0.57 | 0.18 | 0.57 | 0.4 | 0.29 |

Table 4.1: Genetic similarities among 30 genotypes of *B.rapa*.

| **Acc** | **HatBr103** | **HatBr107** | **HatBr110** | **MuzBr113** | **MuzBr115** | **MuzBr118** | **NeeBr120** | **NeeBr122** |
|---|---|---|---|---|---|---|---|---|
| HavBr141 | 0.59 | 0.5 | 0.74 | 0.63 | 0.15 | 0.63 | 0.59 | 0.5 |
| HavBr143 | 0.71 | 0.63 | 0.84 | 0.75 | 0.31 | 0.75 | 0.59 | 0.5 |
| PooBr147 | 0.46 | 0.5 | 0.53 | 0.67 | 0 | 0.5 | 0.46 | 0.33 |
| PooBr149 | 0.5 | 0.4 | 0.67 | 0.53 | 0 | 0.53 | 0.5 | 0.4 |
| PooBr150 | 0.59 | 0.5 | 0.74 | 0.63 | 0.15 | 0.5 | 0.59 | 0.5 |
| PooBr152 | 0.43 | 0.31 | 0.63 | 0.46 | 0 | 0.46 | 0.29 | 0.15 |
| PooBr155 | 0.67 | 0.59 | 0.8 | 0.71 | 0.29 | 0.59 | 0.67 | 0.59 |
| MirBr158 | 0.31 | 0.17 | 0.53 | 0.33 | 0 | 0.33 | 0.15 | 0.17 |
| MirBr160 | 0.53 | 0.43 | 0.71 | 0.57 | 0 | 0.57 | 0.4 | 0.29 |
| MirBr162 | 0.71 | 0.63 | 0.74 | 0.75 | 0.46 | 0.5 | 0.71 | 0.63 |
| KotBr163 | 0.67 | 0.71 | 0.8 | 0.71 | 0.43 | 0.59 | 0.78 | 0.59 |
| KotBr165 | 0.67 | 0.59 | 0.8 | 0.59 | 0.43 | 0.59 | 0.67 | 0.59 |
| KotBr166 | 0.67 | 0.59 | 0.8 | 0.59 | 0.29 | 0.59 | 0.67 | 0.59 |
| BhiBr168 | 0.53 | 0.57 | 0.59 | 0.71 | 0.18 | 0.43 | 0.67 | 0.57 |
| BhiBr170 | 0.78 | 0.71 | 0.9 | 0.82 | 0.43 | 0.71 | 0.67 | 0.59 |
| BSA | 0.74 | 0.67 | 0.86 | 0.78 | 0.4 | 0.67 | 0.74 | 0.67 |

TABLE 4.2: Genetic similarities among 30 genotypes of *B.rapa*.

| **Acc** | **NeeBr125** | **BagBr130** | **BagBr132** | **BagBr135** | **BagBr137** | **HavBr140** | **HavBr141** | **HavBr143** |
|---|---|---|---|---|---|---|---|---|
| NeeBr125 | 1 | | | | | | | |
| BagBr130 | 0.55 | 1 | | | | | | |
| BagBr132 | 0.57 | 0.77 | 1 | | | | | |
| BagBr135 | 0.43 | 0.62 | 0.63 | 1 | | | | |
| BagBr137 | 0.6 | 0.44 | 0.5 | 0.33 | 1 | | | |
| HavBr140 | 0.5 | 0.18 | 0.43 | 0.43 | 0.6 | 1 | | |
| HavBr141 | 0.43 | 0.31 | 0.63 | 0.75 | 0.33 | 0.71 | 1 | |
| HavBr143 | 0.57 | 0.31 | 0.63 | 0.63 | 0.67 | 0.86 | 0.75 | 1 |
| PooBr147 | 0.4 | 0 | 0.5 | 0.33 | 0.25 | 0.6 | 0.67 | 0.67 |

Table 4.2: Genetic similarities among 30 genotypes of *B.rapa*.

| **Acc** | **NeeBr125** | **BagBr130** | **BagBr132** | **BagBr135** | **BagBr137** | **HavBr140** | **HavBr141** | **HavBr143** |
|---|---|---|---|---|---|---|---|---|
| PooBr149 | 0.31 | 0.17 | 0.53 | 0.67 | 0.36 | 0.77 | 0.93 | 0.8 |
| PooBr150 | 0.29 | 0.31 | 0.63 | 0.75 | 0.33 | 0.71 | 0.88 | 0.75 |
| PooBr152 | 0.36 | 0 | 0.31 | 0.46 | 0.44 | 0.91 | 0.77 | 0.77 |
| PooBr155 | 0.4 | 0.43 | 0.71 | 0.82 | 0.46 | 0.67 | 0.82 | 0.82 |
| MirBr158 | 0.4 | 0 | 0.17 | 0.5 | 0.5 | 0.8 | 0.67 | 0.67 |
| MirBr160 | 0.33 | 0 | 0.43 | 0.57 | 0.4 | 0.83 | 0.86 | 0.86 |
| MirBr162 | 0.57 | 0.62 | 0.75 | 0.63 | 0.5 | 0.71 | 0.63 | 0.75 |
| KotBr163 | 0.4 | 0.57 | 0.71 | 0.94 | 0.31 | 0.53 | 0.82 | 0.71 |
| KotBr165 | 0.53 | 0.43 | 0.59 | 0.71 | 0.46 | 0.67 | 0.71 | 0.71 |
| KotBr166 | 0.4 | 0.29 | 0.59 | 0.71 | 0.31 | 0.67 | 0.82 | 0.71 |
| BhiBr168 | 0.33 | 0.36 | 0.71 | 0.57 | 0.2 | 0.5 | 0.71 | 0.57 |
| BhiBr170 | 0.53 | 0.43 | 0.71 | 0.82 | 0.46 | 0.67 | 0.82 | 0.82 |
| BSA | 0.5 | 0.53 | 0.78 | 0.78 | 0.57 | 0.75 | 0.78 | 0.89 |

Table 4.3: Genetic similarities among 30 genotypes of *B.rapa*.

| **Acc** | **Poo Br 147** | **Poo Br 149** | **Poo Br 150** | **Poo Br 152** | **Poo Br 155** | **Mir Br 158** | **Mir Br 160** | **Mir Br 162** |
|---|---|---|---|---|---|---|---|---|
| Poo Br147 | 1 | | | | | | | |
| Poo Br149 | 0.73 | 1 | | | | | | |
| Poo Br150 | 0.67 | 0.93 | 1 | | | | | |
| Poo Br152 | 0.67 | 0.83 | 0.77 | 1 | | | | |
| Poo Br155 | 0.62 | 0.88 | 0.94 | 0.71 | 1 | | | |
| Mir Br158 | 0.5 | 0.73 | 0.67 | 0.89 | 0.62 | 1 | | |
| Mir Br160 | 0.8 | 0.92 | 0.86 | 0.91 | 0.8 | 0.8 | 1 | |
| Mir Br162 | 0.5 | 0.67 | 0.75 | 0.62 | 0.82 | 0.5 | 0.57 | 1 |
| Kot Br163 | 0.46 | 0.75 | 0.82 | 0.57 | 0.89 | 0.46 | 0.67 | 0.71 |
| Kot Br165 | 0.46 | 0.75 | 0.82 | 0.71 | 0.89 | 0.62 | 0.67 | 0.82 |
| Kot Br166 | 0.62 | 0.88 | 0.94 | 0.71 | 0.89 | 0.62 | 0.8 | 0.71 |
| Bhi Br168 | 0.8 | 0.77 | 0.86 | 0.55 | 0.8 | 0.4 | 0.67 | 0.71 |
| Bhi Br170 | 0.62 | 0.75 | 0.82 | 0.71 | 0.89 | 0.62 | 0.8 | 0.71 |
| BSA | 0.57 | 0.82 | 0.89 | 0.67 | 0.95 | 0.57 | 0.75 | 0.89 |

TABLE 4.4: Genetic similarities among 30 genotypes of *B.rapa*.

| **Acc** | **KotBr 163** | **KotBr 165** | **KotBr 166** | **BhiBr 168** | **BhiBr 170** | **BSA** |
|---|---|---|---|---|---|---|
| KotBr163 | 1 | | | | | |
| KotBr165 | 0.78 | 1 | | | | |
| KotBr166 | 0.78 | 0.89 | 1 | | | |
| BhiBr168 | 0.67 | 0.67 | 0.8 | 1 | | |
| BhiBr170 | 0.89 | 0.78 | 0.78 | 0.67 | 1 | |
| BSA | 0.84 | 0.84 | 0.84 | 0.75 | 0.84 | 1 |

### 4.1.3 Cluster Analysis Study of *B. rapa*

UPGMA algorithm was used to study the phylogenetic relationship among B. rapa genotypes. Based on genetic similarities phylogenetic tree was constructed among all 30 genotypes and classified into 8 groups (Figure 4.2, Table 4.5). The group I, II, III, IV, V, VI, VII, and VIII contained 8, 1,5,3,5, 2, 5, and 1 genotype, respectively. All groupings are highly polymorphic.

TABLE 4.5: Cluster of 30 genotype of *B. rapa* through cluster analysis based on SDS-PAGE method.

| **Cluster** | **No.of Genotypes** | **Genotypes** |
|---|---|---|
| I | 8 | HatBr103, HatBr110, MuzBr118, HatBr107, NeeBr122, MuzBr113, BagBr132, NeeBr122 |
| II | 1 | NeeBr125 |

TABLE 4.5: Cluster of 30 genotype of *B. rapa* through cluster analysis based on SDS-PAGE method.

| **Cluster** | **No.of Genotypes** | **Genotypes** |
|---|---|---|
| III | 5 | MuzBr115, BagBr130, BagBr135, KotBr163, BhiBr170 |
| IV | 3 | HavBr141, PooBr149, MirBr160 |
| V | 5 | PooBr150, KotBr166, PooBr155, BSA, KotBr165 |
| VI | 2 | HavBr143 MirBr162 |
| VII | 5 | HavBr140, PooBr152, MirBr158, PooBr147, BhiBr168 |
| VIII | 1 | BagBr137 |

Our results show that maximum polymorphic diversity is present among the different populations of B. rapa. Those genotypes that show similarity with each other exist in the same group as compared to other different genotypes. Among *B. rapa* genotypes high level of seed protein-based diversity was observed. Of all clusters, cluster I is the largest one and consists of eight genotypes of *B. rapa* i.e., HatBr103, HatBr110, MuzBr118, HatBr107, NeeBR120, MuzBr113, BagBr132,

and NeeBr122. Clusters II and VIII show maximum variability among all studied clusters and these clusters consist of only one genotype each. These genotypes BagBr137 and NeeBr125 are novel genotypes and both are highly diverged as compared to all other genotypes.

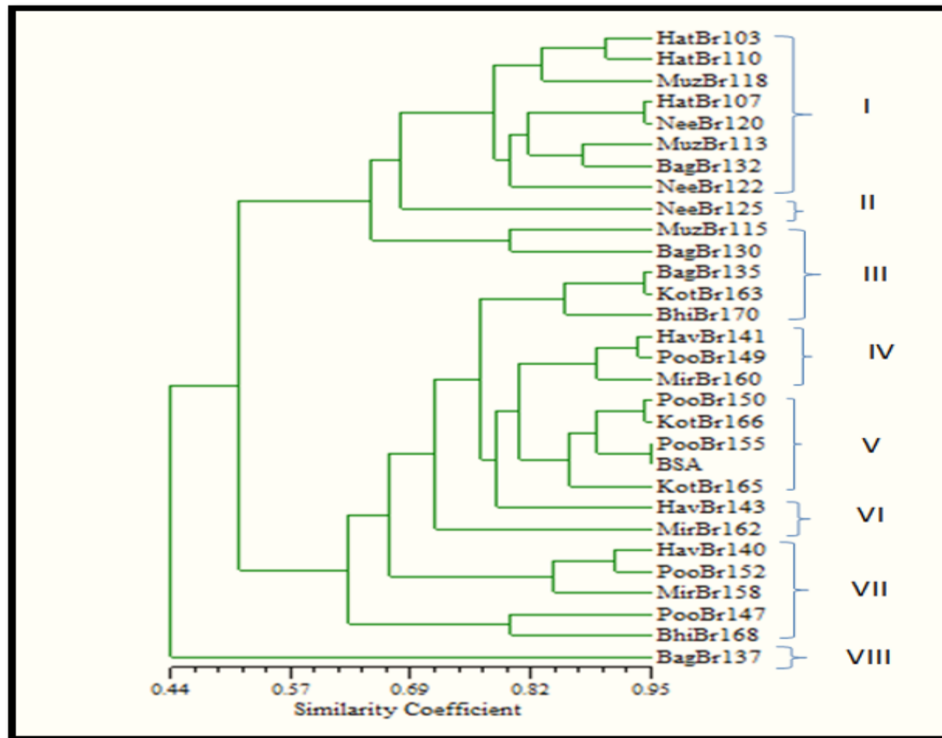



FIGURE 4.2: The phylogenetic tree represented the phylogenetic relationship between 30 elite genotypes of *B. rapa* through cluster analysis based on SDS-PAGE.

### 4.1.4 PCoA Analysis of SDS-PAGE Data

Individuals are divided into groups according to their genetic distances using a multivariate technique called PCoA. For a clear understanding of genetic variation, a DICE similarity coefficient matrix is used, and all of the *Brassica rapa* genotypes were scattered on 2D and 3D scatter plots. A distinct variation in any dimension is provided by the 2D and 3D study based on PCoA. To differentiate the novel genotypes among all the population of B. rapa 2D and 3D diagram was used. Using a 2D dendrogram, all of the genotypes in our research were separated into 4 major groups. Each group has unique genetics. However, there are a few unique,

diverse genotypes that are outliers such as BagBr137, BagBr130, NeeBr125 and MirBr158.These genotypes highly diverge from all other studied genotypes (Figure 4.3).

FIGURE 4.3: Representation of 30 promising genotype of *B. rapa* genotypes in 2D structure on the basis phylogenetic tree and SDS-PAGE banding patterns.

3D diagram provided a more clear picture from any direction. Some unique and diverse genotypes were recorded among all other genotypes in the 3D model these are BagBr137 and NeeBr125 (Figure 4.4).

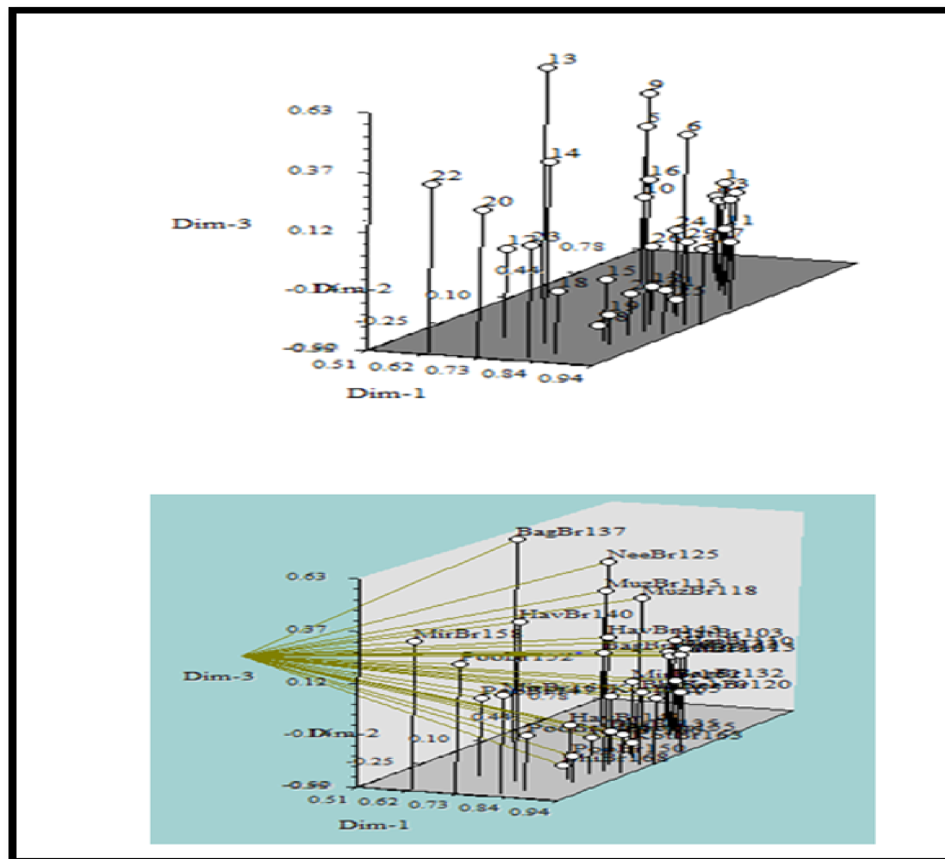



FIGURE 4.4: Representation of 30 promising genotypes of *B. rapa* genotypes in 3D structure and vector diagram based on the phylogenetic tree and SDS-PAGE banding patterns.

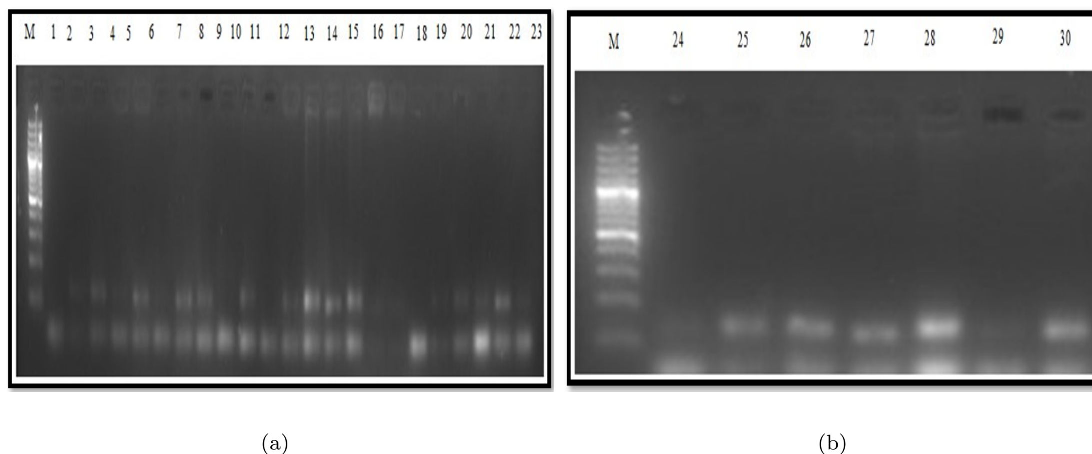
## 4.2 Microsatellite Markers (SSRs) Based Inter-specific Variability among *B. rapa* Genotypes

For SSRs-based molecular analysis, 30 diverse *B. rapa* genotypes were selected. Ten microsatellite markers were used and these markers showed the most diverse banding patterns.

### 4.2.1 Inter-Specific Variations among *B. rapa* Genotypes

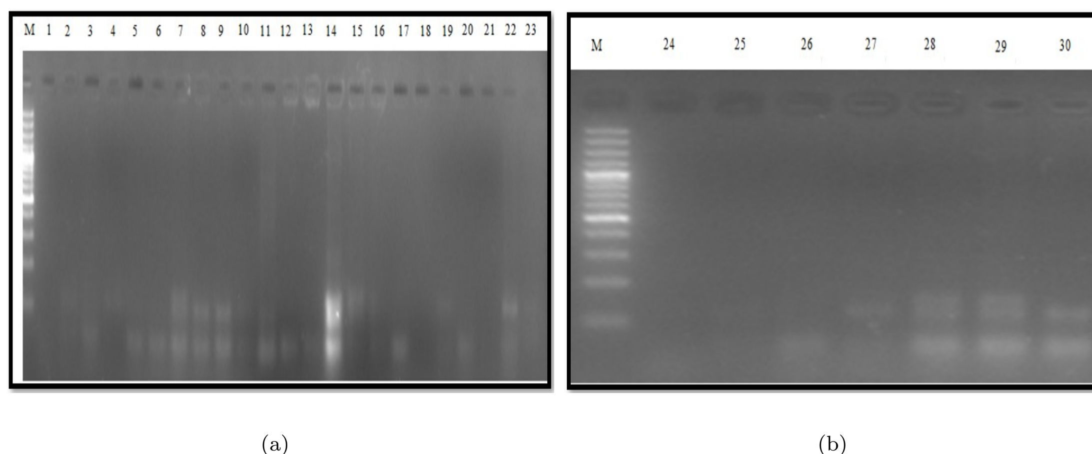
Using 10 SSRs markers, the genomic variability of 30 *B. rapa* genotypes was studied. These marker sizes were compared to their known sizes, and the desired amplified fragments showed variability per locus ranged from 1-3. A polymorphic

banding pattern was observed in all primers (Figure 4.5( a and b) and Figure 4.6 (a & b)).




(a) (b)

FIGURE 4.5: (a) *B. rapa* genotypes SSRs banding patterns were generated by the SSRs primer Na12-A02, 1–23.M = 100 bp of molecular marker, HatBr103, HatBr107, HatBr110, MuzBr113, MuzBr115 ,MuzBr118, NeeBr120, NeeBr122, NeeBr125, BagBr130, BagBr132, BagBr135, BagBr137, HavBr140, HavBr141, HavBr143, PooBr147, PooBr149, PooBr150, PooBr152, PooBr155, MirBr158 and MirBr160. (b) *Brassica rapa* genotypes SSRs banding patterns were generated by the SSRs primer Na12-A02, 24-30. M = 100 bp of molecular marker, MirBr162, KotBr163, KotBr165, KotBr166, BhiBr168, BhiBr170 and BSA.




(a) (b)

FIGURE 4.6: (a) *Brassica rapa* genotypes SSRs banding patterns were generated by the SSRs primer BRMS-007, 1-23. M = 100 bp of molecular marker, HatBr103, HatBr107, HatBr110, MuzBr113, MuzBr115, MuzBr118, NeeBr120, NeeBr122, NeeBr125, BagBr130, BagBr132, BagBr135, BagBr137, HavBr140, HavBr141, HavBr143, PooBr147, PooBr149, PooBr150, PooBr152, PooBr155, MirBr158 and MirBr160.
(b) *Brassica rapa* genotypes SSRs banding patterns were generated by the SSRs primer BRMS-007, 24-30. Lanes represent. M = 100 bp of molecular marker, MirBr162, KotBr163, KotBr165, KotBr166, BhiBr168, BhiBr170 and BSA.

The size of the fragment that are obtained from the primer ranged from 100-500bp. Out of ten primers eight primers (80%) detected only 1 allele, one primer (10%) (Na10-A02) amplified 3 alleles and one primer (10%) (BRMS-001) amplified 2 alleles in all *B. rapa* genotypes Table 4.6.

Table 4.6: Details of SSRs markers used including total generated alleles, polymorphic alleles, % polymorphism and size of amplified alleles.

| **Primer Name** | **Total Amplified Alleles (a)** | **Poly-morphic Allele (b)** | **Percentage (%) Polymor-phism** | **Size Range (bp)** | **Melting tm (°C)** |
|---|---|---|---|---|---|
| BRMS -001 | 2 | 2 | 100 | 110, 190 | 54.5 |
| BRMS -007 | 1 | 1 | 100 | 190 | 57.3 |
| Na10 -D09 | 1 | 1 | 100 | 110 | 58.5 |
| Na12 -A02 | 3 | 3 | 100 | 100, 190, 400 | 55 |
| Na14 -C12 | 1 | 1 | 100 | 200 | 60 |
| Ni2 -A06 | 1 | 1 | 100 | 280 | 55 |
| Ni2 -A11 | 1 | 1 | 100 | 280 | 57 |
| Ni2 -B03 | 1 | 1 | 100 | 490 | 56 |
| Ni4 -A03 | 1 | 1 | 100 | 200 | 54.2 |
| Ni4 -H03 | 1 | 1 | 100 | 110 | 59 |

### 4.2.2 Genetic Similarity of *B. rapa* Genotypes

Among various *B. rapa* genotypes genetic similarity value range of 0-94% was noted (Table 4.7 to 4.10). The highest similarity value 94% was noted between MirBr156/ PooBr150 genotypes followed by 89% among following genotypes KotBr166/ NeeBR 120, MirBr160/ NeeBr122, BagBr130/ NeeBr122. The least similarity coefficient value 0% was noted among genotypes MuzBr118/ HatBr103, NeeBr122/ HatBr103, PooBr147/ HatBr103, PooBr147/ HatBr107 and KotBr166/ HatBr103 etc. followed by 14% among genotypes BhiBr168/HatBr110 and 17% between BhiBr170/ MirBr158. It is shown that genotypes exhibit large levels of genetic diversity.

Table 4.7: Dice similarity coefficient among 30 *B. rapa* genotypes on the basis of SSRs.

| **Acc** | **Hat Br103** | **Hat Br107** | **Hat Br110** | **Muz Br113** | **Muz Br115** | **Muz Br118** | **Nee Br120** | **Nee Br122** |
|---|---|---|---|---|---|---|---|---|
| Hat Br103 | 1 | | | | | | | |
| Hat Br107 | 0.57 | 1 | | | | | | |
| Hat Br110 | 0.44 | 0.67 | 1 | | | | | |
| Muz Br113 | 0.36 | 0.71 | 0.63 | 1 | | | | |
| Muz Br115 | 0.33 | 0.67 | 0.73 | 0.62 | 1 | | | |
| Muz Br118 | 0 | 0.29 | 0.22 | 0.18 | 0.33 | 1 | | |
| Nee Br120 | 0 | 0.6 | 0.33 | 0.43 | 0.44 | 0.57 | 1 | |
| Nee Br122 | 0.57 | 0.8 | 0.5 | 0.57 | 0.44 | 0.29 | 0.6 | 1 |

Table 4.7: Dice similarity coefficient among 30 *B. rapa* genotypes on the basis of SSRs.

| **Acc** | **Hat Br103** | **Hat Br107** | **Hat Br110** | **Muz Br113** | **Muz Br115** | **Muz Br118** | **Nee Br120** | **Nee Br122** |
|---|---|---|---|---|---|---|---|---|
| Nee Br125 | 0.22 | 0.5 | 0.43 | 0.75 | 0.36 | 0.44 | 0.5 | 0.5 |
| Bag Br130 | 0.33 | 0.67 | 0.36 | 0.46 | 0.25 | 0.33 | 0.67 | 0.89 |
| Bag Br132 | 0.29 | 0.6 | 0.33 | 0.57 | 0.22 | 0.57 | 0.6 | 0.6 |
| Bag Br135 | 0.25 | 0.55 | 0.46 | 0.53 | 0.4 | 0.5 | 0.55 | 0.55 |
| Bag Br137 | 0.33 | 0.44 | 0.36 | 0.31 | 0.25 | 0.33 | 0.44 | 0.67 |
| Hav Br140 | 0.18 | 0.57 | 0.38 | 0.67 | 0.31 | 0.36 | 0.71 | 0.57 |
| Hav Br141 | 0.25 | 0.55 | 0.46 | 0.4 | 0.4 | 0.5 | 0.73 | 0.55 |
| Hav Br143 | 0.5 | 0.29 | 0.22 | 0.18 | 0 | 0.5 | 0.29 | 0.57 |
| Poo Br147 | 0 | 0 | 0.22 | 0.18 | 0.33 | 0 | 0 | 0 |
| Poo Br149 | 0.5 | 0.36 | 0.31 | 0.53 | 0.2 | 0.25 | 0.18 | 0.55 |
| Poo Br150 | 0.36 | 0.71 | 0.5 | 0.78 | 0.46 | 0.36 | 0.57 | 0.71 |
| Poo Br152 | 0.33 | 0.67 | 0.55 | 0.46 | 0.75 | 0.33 | 0.44 | 0.44 |
| Poo Br155 | 0.5 | 0.73 | 0.62 | 0.53 | 0.6 | 0.5 | 0.55 | 0.73 |

TABLE 4.7: Dice similarity coefficient among 30 *B. rapa* genotypes on the basis of SSRs.

| **Acc** | **Hat Br103** | **Hat Br107** | **Hat Br110** | **Muz Br113** | **Muz Br115** | **Muz Br118** | **Nee Br120** | **Nee Br122** |
|---|---|---|---|---|---|---|---|---|
| Mir Br158 | 0.2 | 0.62 | 0.4 | 0.71 | 0.33 | 0.4 | 0.62 | 0.62 |
| Mir Br160 | 0.67 | 0.67 | 0.55 | 0.46 | 0.5 | 0.33 | 0.44 | 0.89 |
| Mir Br162 | 0.33 | 0.44 | 0.36 | 0.31 | 0.5 | 0.67 | 0.44 | 0.44 |
| Kot Br163 | 0.33 | 0.67 | 0.55 | 0.46 | 0.75 | 0.67 | 0.67 | 0.67 |
| Kot Br165 | 0.4 | 0.75 | 0.6 | 0.5 | 0.86 | 0.4 | 0.5 | 0.5 |
| Kot Br166 | 0 | 0.67 | 0.36 | 0.46 | 0.5 | 0.67 | 0.89 | 0.67 |
| Bhi Br168 | 0 | 0.33 | 0.14 | 0.63 | 0.18 | 0.22 | 0.67 | 0.5 |
| Bhi Br170 | 0.33 | 0.44 | 0.18 | 0.46 | 0.25 | 0 | 0.44 | 0.44 |
| BSA | 0.29 | 0.6 | 0.5 | 0.43 | 0.44 | 0.29 | 0.6 | 0.8 |

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

| **Acc** | **Nee Br125** | **Bag Br130** | **Bag Br132** | **Bag Br135** | **Bag Br137** | **Hav Br140** | **Hav Br141** | **Hav Br143** |
|---|---|---|---|---|---|---|---|---|
| Nee Br125 | 1 | | | | | | | |
| Bag Br130 | 0.55 | 1 | | | | | | |
| Bag Br132 | 0.83 | 0.67 | 1 | | | | | |

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

| **Acc** | **Nee Br125** | **Bag Br130** | **Bag Br132** | **Bag Br135** | **Bag Br137** | **Hav Br140** | **Hav Br141** | **Hav Br143** |
|---|---|---|---|---|---|---|---|---|
| Bag Br135 | 0.46 | 0.6 | 0.55 | 1 | | | | |
| Bag Br137 | 0.36 | 0.75 | 0.44 | 0.8 | 1 | | | |
| Hav Br140 | 0.63 | 0.62 | 0.71 | 0.8 | 0.62 | 1 | | |
| Hav Br141 | 0.46 | 0.6 | 0.55 | 0.83 | 0.8 | 0.8 | 1 | |
| Hav Br143 | 0.44 | 0.67 | 0.57 | 0.5 | 0.67 | 0.36 | 0.5 | 1 |
| Poo Br147 | 0.22 | 0 | 0 | 0.25 | 0.33 | 0.18 | 0.25 | 0 |
| Poo Br149 | 0.62 | 0.4 | 0.55 | 0.5 | 0.6 | 0.53 | 0.5 | 0.5 |
| Poo Br150 | 0.75 | 0.62 | 0.71 | 0.67 | 0.62 | 0.78 | 0.67 | 0.36 |
| Poo Br152 | 0.18 | 0.25 | 0.22 | 0.6 | 0.5 | 0.46 | 0.6 | 0 |
| Poo Br155 | 0.46 | 0.6 | 0.55 | 0.83 | 0.8 | 0.67 | 0.83 | 0.5 |
| Mir Br158 | 0.8 | 0.67 | 0.77 | 0.71 | 0.67 | 0.82 | 0.71 | 0.4 |
| Mir Br160 | 0.36 | 0.75 | 0.44 | 0.6 | 0.75 | 0.46 | 0.6 | 0.67 |
| Mir162 | 0.36 | 0.25 | 0.44 | 0.6 | 0.5 | 0.46 | 0.6 | 0.33 |
| Kot Br163 | 0.36 | 0.5 | 0.44 | 0.6 | 0.5 | 0.46 | 0.6 | 0.33 |

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

| **Acc** | **Nee Br125** | **Bag Br130** | **Bag Br132** | **Bag Br135** | **Bag Br137** | **Hav Br140** | **Hav Br141** | **Hav Br143** |
|---|---|---|---|---|---|---|---|---|
| Kot Br165 | 0.2 | 0.29 | 0.25 | 0.44 | 0.29 | 0.33 | 0.44 | 0 |
| Kot Br166 | 0.55 | 0.75 | 0.67 | 0.6 | 0.5 | 0.62 | 0.6 | 0.33 |
| Bhi Br168 | 0.57 | 0.55 | 0.5 | 0.46 | 0.36 | 0.75 | 0.46 | 0.22 |
| Bhi Br170 | 0.18 | 0.25 | 0.22 | 0.2 | 0 | 0.46 | 0.2 | 0 |
| BSA | 0.33 | 0.67 | 0.4 | 0.36 | 0.44 | 0.43 | 0.36 | 0.29 |

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

| **Acc** | **Poo Br147** | **Poo Br149** | **Poo Br150** | **Poo Br152** | **Poo Br155** | **Mir Br158** | **Mir Br160** | **Mir Br162** |
|---|---|---|---|---|---|---|---|---|
| Poo Br147 | 1 | | | | | | | |
| Poo Br149 | 0.25 | 1 | | | | | | |
| Poo Br150 | 0.18 | 0.8 | 1 | | | | | |
| Poo Br152 | 0.33 | 0.4 | 0.62 | 1 | | | | |
| Poo Br155 | 0.25 | 0.67 | 0.8 | 0.8 | 1 | | | |
| Mir Br158 | 0.2 | 0.71 | 0.94 | 0.5 | 0.71 | 1 | | |
| Mir Br160 | 0 | 0.6 | 0.62 | 0.5 | 0.8 | 0.5 | 1 | |

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

| **Acc** | **Poo Br147** | **Poo Br149** | **Poo Br150** | **Poo Br152** | **Poo Br155** | **Mir Br158** | **Mir Br160** | **Mir Br162** |
|---|---|---|---|---|---|---|---|---|
| Mir Br162 | 0.33 | 0.6 | 0.62 | 0.75 | 0.8 | 0.5 | 0.5 | 1 |
| Kot Br163 | 0 | 0.4 | 0.62 | 0.75 | 0.8 | 0.5 | 0.75 | 0.75 |
| Kot Br165 | 0 | 0.22 | 0.5 | 0.86 | 0.67 | 0.36 | 0.57 | 0.57 |
| Kot Br166 | 0 | 0.2 | 0.62 | 0.5 | 0.6 | 0.67 | 0.5 | 0.5 |
| Bhi Br168 | 0 | 0.46 | 0.63 | 0.18 | 0.31 | 0.67 | 0.36 | 0.18 |
| Bhi Br170 | 0 | 0.2 | 0.31 | 0.25 | 0.2 | 0.17 | 0.25 | 0.25 |

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

| **Acc** | **Kot Br163** | **Kot Br165** | **Kot Br166** | **Bhi Br168** | **Bhi Br170** | **BSA** |
|---|---|---|---|---|---|---|
| Kot Br163 | 1 | | | | | |
| Kot Br165 | 0.86 | 1 | | | | |
| Kot Br166 | 0.75 | 0.57 | 1 | | | |
| Bhi Br168 | 0.36 | 0.2 | 0.55 | 1 | | |
| BhiBr170 | 0.25 | 0.29 | 0.25 | 0.55 | 1 | |
| BSA | 0.67 | 0.5 | 0.67 | 0.5 | 0.44 | 1 |

### 4.2.3 Cluster Analysis of *B. rapa* Genotypes through SSRs

Based on the UPGMA similarity method all 30 genotypes of B. rapa were classified into seven major groups (Figure 4.7, Table 4.11). The group I, II, III, IV, V, VI and VII contained 9, 6, 6, 3, 2, 2, and 2 genotypes, respectively.

TABLE 4.11: Cluster of 30 genotypes of *B. rapa* through cluster analysis

| Cluster | No. of Genotypes | Genotypes |
|---|---|---|
| I | 9 | HatBr103, HavBr143, HatBr107, MuzBr113, NeeBr125, BagBr132, HavBr140, PooBr150 MirBr158 |
| II | 6 | PooBr149, BhiBr168, NeeBr120, KotBr166, NeeBr122 BagBr130 |
| III | 6 | MirBr160, BSA, Bag Br135, HavBr141, PooBr155 BagBr137 |

Table 4.11: Cluster of 30 genotypes of *B. rapa* through cluster analysis

| **Cluster** | **No. of Genotypes** | **Genotypes** |
|---|---|---|
| IV | 3 | HatBr110, MuzBr115 KotBr165, |
| V | 2 | PooBr152, KotBr163. |
| VI | 2 | MuzBr118, MirBr162 |
| VII | 2 | BhiBr170, PooBR147 |

- Group I was the largest group among all the groups and consisted 9 genotypes of different origins i.e., HatBr103, HavBr143, HatBr107, MuzBr113, NeeBr125, BagBr132, HavBr140, PooBr150 and MirBr158.
- Group II comprises of 6 genotypes these genotypes are PooBr149, BhiBr168, NeeBr120, KotBr166, NeeBr120, and BagBr130.
- Group III also contains 6 genotypes and these are MirBr160, BSA, BagBr135, HavBr141, PooBr155, and BagBr137.
- Group IV only has 3 genotypes; these are HatBr110, MuzBr115, and KotBr165. Group V, VI, and VII have only 2 genotypes each which are much smaller than the other four groups containing genotypes
- Group V genotypes are PooBr152 and KotBr163.
- Groups VI genotypes are MuzBr118 and MirBr162 and group VII contains more diverged genotypes from rest of all groups, these are BhiBr170 and PooBr147.
- Our results showed that genotypes were highly inter-specifically similar, and also observed a high degree of genetic variation present among all genotypes.

These results can serve as a base for future genomic variability studies of significant *B. rapa* and other *Brassica* subspecies.

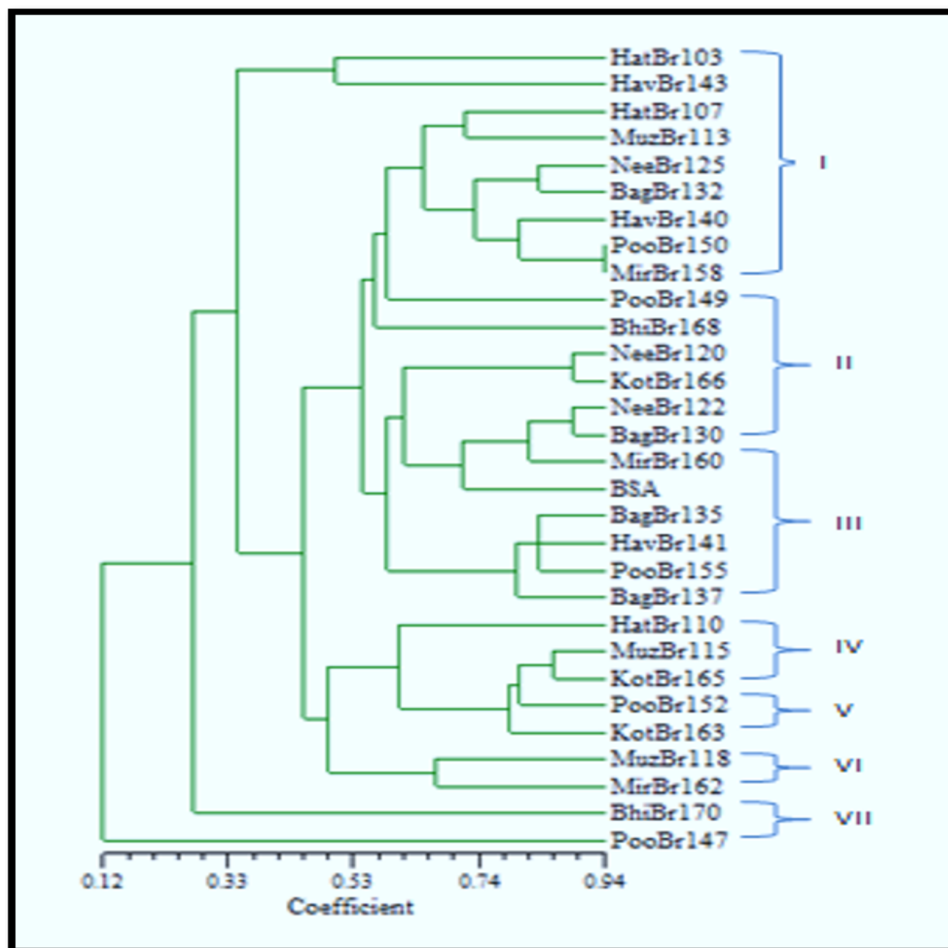



FIGURE 4.7: SSRs marker-based variability among 30 genotypes of *B. rapa* using cluster analysis.

### 4.2.4 PCoA Analysis

To better understand genetic variety, the most diverse genotypes are being investigated using contemporary 2D and 3D analysis methods. The overall technique is known as principal coordinate analysis (PCoA). It is a statistical tool that converts distance-based statistics from individual item data into a map-based display. In this study, 2D structures showed unique genotypes PooBr147, KotBr165, PooBr155, and PooBr150 etc. Among the above genotypes these genotypes are highly diverged Figure 4.8.

FIGURE 4.8: Representation of 30 promising genotypes of *B.rapa* through 2D structure based on SSRs Markers.

The 3D structure provides future clarification of genotypes from any direction and gives some promising genotypes such as HatBr103, PooBr147, HavBr143 and BagBr137 etc As shown in figure 4.9 below.

FIGURE 4.9: Representation of 30 promising genotypes of *B. rapa* through 3D structure from different directions based on the SSRs markers.

Vector diagram also provided clearer picture of promising genotypes from different dimensions. Figure 4.10 showed that promising genotypes are PooBr147, PooBr155, PooBr150 and KotBr165 etc.

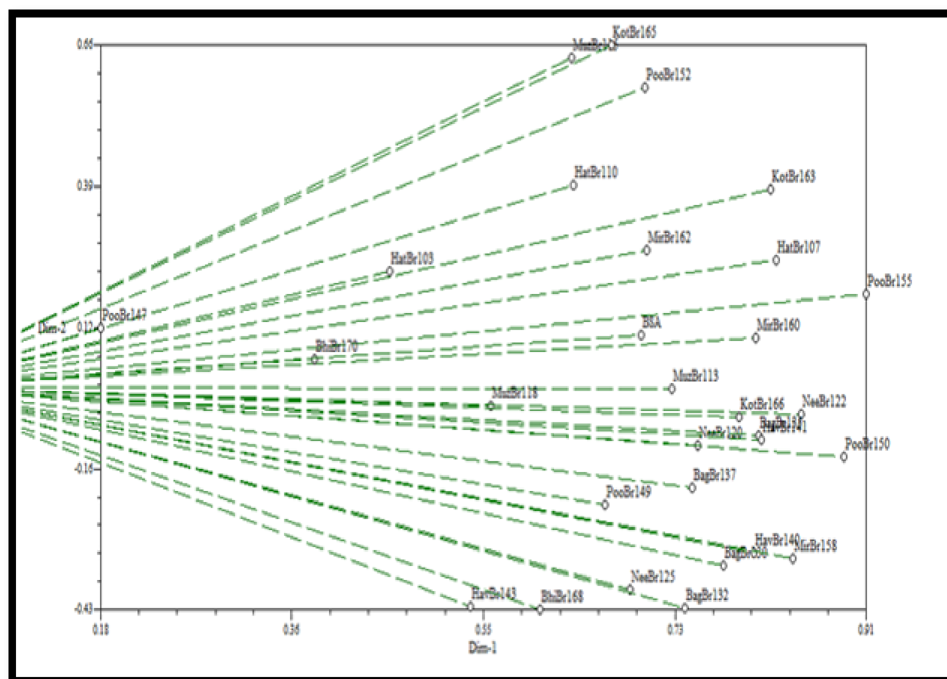



FIGURE 4.10: Representation of 30 promising genotypes of *B. rapa* through vector diagram based on the SSRs markers.

## 4.3 Discussion

The use of protein storage is utilized for the classification of various varieties and to assess genetic variations in different crops. Based on total seed storage diversity, it is particularly useful to distinguish between diverse plant species and subspecies. Its critical analysis provides improved protein-based diversity of diverse plant populations [72]. Various biochemical methods were used to investigate genetic diversity. The SDS-PAGE method, which provides a clear protein profile of significant variable genotypes, is one of the most essential method for distinguishing *Brassica* species and subspecies based on protein size. The genotypes of *B. rapa* were evaluated using this technique in the current investigation, and the highest genetic diversity between genotypes was found. The most protein-based genotype variety is provided by the SDS-PAGE method, which is inexpensive and quicker than other molecular techniques [73], [74]. For the study of genetic diversity among different crop species and subspecies different molecular and biochemical methods were used[75] to [77]. In the current study, 30 elite genotypes of *B. rapa* were used for SDS-PAGE-based characterization. Low to moderate and high levels of

variability were recorded among all tested genotypes. A total of 12 bands were recorded and 12 (100%) of these bands showed polymorphism to each other and no band was monomorphic. The sizes of these band patterns ranged from 10-180 kDa. The SDS-PAGE method provides excellent protein-based variability among diverse plant genotypes. *Shinwari et al.* [39] recorded maximum polymorphic and very less monomorphic proteins in *Eruca sativa* genotypes. *Zada et al.* [37] studied the genetic diversity of the 94 *Brassica carinata L.* (Ethiopian mustard) genotypes through SDS-PAGE. It was reported that there were 31 reproducible protein subunits, and majority showed that polymorphism lies within the range from 8 kDa to 180 kDa.

*Turi et al.* [78] used similar methods and reported four different proteins in many *Brassica* species. SDS-PAGE and RAPD markers were used to identify the genotypes of soybeans that showed the greatest polymorphism. *Kakaei and Kahrizi* [46] results show similarities with our findings. They used the SDS-PAGE method and characterized canola genotypes. Seventeen protein bands were recorded by using this method and majority of them were polymorphic. The results of *Turi et al.* [78] are not similar to our findings, which recorded a high level of genetic variability among 234 Brassica genotypes and found four diverse types of proteins. Among all tested genotypes a total of 28 major bands were observed including 60% polymorphic bands and others monomorphic.

Genetic similarity values among 30 genotypes were recorded. The maximum similarity value was 95% and the minimum was 0%. 8 groups were classified for all 30 genotypes through a genetic tree based on UPGMA. Our results are not similar with the findings of *Shinwari et al.* [39] who recorded low to high genetic similarity values between different Eruca sativa accessions and its similarity value ranged from 60-100%. *Zada et al.* [37] investigated *B. carinata* germplasm by using the SDS-PAGE method and observed 50 to 100% similarity values. In the genetic tree, genotypes were classified into five different groups, based on these similarity values. The various cluster groups were also recorded by *Nasr et al.* [79] for *B. napus* which showed deviation from our results. Similar classification methods

were used by *Mukhlesur et al.* [49] to group several *Brassica* cultivars into various clusters. To study the clear picture of all genotypes from various angles was used 2D and 3D structures. 3D structures provided more clarification than 2D structures. In the current study, some unique divergent genotypes were recorded by using these structures. Our results are in line with the finding of *Gupta et al.* [80] who obtained the most polypeptide-based variability in 45 genotypes of chickpea using PCoA analysis. Based on a 3D analysis, their results revealed a clear relationship between FLIP-90 and 160. Principal coordinate analysis (PCoA) is used. In this current study 10 SSRs primers were used for 30 *B. rapa* genotypes and gave positive results. All of the genotypes had unique fingerprints comprised of various marker sets. All recorded alleles showed polymorphism. Our findings are consistent with *Chen et al.* [81] who studied maximum SSRs-based variability among various Brassica species. The Brassicaceae family contains many diverse species and subspecies, and the microsatellite markers reveal a significant amount of chromosomal variation. *Gupta et al.* [82] recorded a high level of genomic variability in Indian mustard (B. juncea) and other Brassica species by using RAPD and SSRs markers. *Ma et al.* [83] investigated genomic divergence in 20 non-heading Chinese cabbages that were collected in China and Japan using a similar method. It was found that the amplified allele exhibited the most polymorphism.

*Havlickova et al.* [84] measured maximum genomic variability in 94 winter rapeseed Czech genotypes using SSRs and AFLP markers. *Abbas et al.* [85] recorded 458 and 258 alleles using different SSRs and RAPD markers. Highly polymorphic bands were observed and their size ranged from 250 to 2000bp. Size of Fragments of RAPD markers was higher than the SSRs markers. All genotypes in the current study showed a low to a high level of genetic diversity from all other studied genotypes when similarity coefficient values were determined among genotypes. Our results showed that close relations were recorded in those genotypes that have the same origin among all genotypes similarity and dissimilarity values varied (0%-94%).

Our results showed deviation from the finding of *Ofori et al.* [86] studied diverse *B. rapa* winter genotypes and recorded 83% genetic variability. They observed

maximum polymorphic alleles by using 15 SSRs markers. Our results are similar to *Wu et al.* [87] who reported the genomic variability of 2 essential Brassica species. In winter rapeseed genotypes low level of SSRsbased variability was observed. In *B. napus* species more variability was recorded as compared to the *B .oleraceae. Havlickova et al.* [84] reported about *B. napus* genotypes. He recorded 49.4 and 35.5% genetic distance values with 89 different SSRs and 1003 AFLP markers in between B. napus genotypes. Microsatellite markers were used by *Shen et al.* [88] to measure the 54 to 89% similarity coefficient values between eleven non-heading Chinese cabbage genotypes our results are not similar.

Based on the UPGMA similarity method all 30 genotypes of *B. rapa* were classified into seven groups and a phylogenetic tree was constructed. The group I, II, III, IV, V, VI, and VII contained 9, 6, 6, 3, 2, 2, and 2 genotypes, respectively. Group one was the largest group among all the groups and consisted of 9 genotypes of different origins. Group seven consists of promising genotypes PooBr147 and BhiBr170. Our findings indicate that among the many B. rapa genotypes, the maximum polymorphism variation was present. Our findings are similar to the findings of *Talebi et al.* [89] reported 3 major groups of 47 different exotic *B. rapa* genotypes Using 12 ISSR markers through the UPGMA. Similarly, *Yu et al.* [90] examined five different subspecies of significant *B. rapa* (Pak-choi). All 80 genotypes were classified into five primary groups using the UPGMA method. Smooth oval leafy types are present in group II. Group III consisted of typical Chinese rape, group IV had compact plant types, and sub-species are included in group *V. Ali et al.* [66] used UPGMA to classify all genotypes into 3 major groups. Three clusters were present in group one, while two clusters were present in groups two and three each. To study the clear genetic diversity of B. rapa genotypes, 2D and 3D structures were used. For this purpose, these structures were plotted based on the DICE similarity coefficient matrix.
These structures are very important because they provide clear variation in any dimension and find out the promising genotype. *Takahashi et al.* [91] examined 24 cultivars of *B. rapa B. Juncea, B. oleracea and B. napus* were examined, using individual and bulk-based methods with RAPD specific variation was smaller,

according to PCoA, than the intra-specific variation. The results of their PCoA analysis were similar with the findings of UPGMA analysis. *Singh et al.* [92] used 48 SSR markers to produce 114 alleles with a genetic similarity of 50% and an average value of 2.38 alleles per primer. They performed 2D and 3D analyses for 16 *B. Juncea* genotypes to distinguish elite genotypes from others. Their PCoA analysis confirmed the locations and genotype clusters that were consistent with the results of the STRUCTURE study. Their findings distinguished between the susceptible and resistant genotypes.

# Chapter 5

# Conclusions and Recommendations

The diverse *B. rapa* genotypes were characterized for seed storage protein profiling from different areas of Azad Jammu and Kashmir. Among various *B. rapa* genotypes genetic similarity value range of 0-94% was noted. Highest genetic similarities values are 95% between the genotypes BSA/PooBr152 while lowest similarity value of 0% was observed among genotypes PooBr147/ MuzBr115, PooBr149/ MuzBr115, PooBr152/ MuzBr115, MirBr158/ MuzBr115, MirBr160/ MuzBr115etc. Protein bands were observed and they showed high polymorphism. PCoA, which generated 2D and 3D genotype plots, was used to further examine the polymorphism data. Modern 2D and 3D techniques were used to record several unique genotypes. Our findings showed that *B. rapa* genotype biochemical diversity might be used to explore genetic diversity. However, we recommend using more advanced techniques to investigate the evolutionary origins of divergence in the B. rapa genotypes, such as 2-D gel electrophoresis and molecular markers. The genotypes BagBr137, BagBr130, NeeBr125, MirBr158, BagBr137 and NeeBr125 were found to be unique and are recommended for further studies. To identify several rare alleles in different simple sequence repeats (SSRs) markers were used for molecular analysis in *B. rapa* accession. The genetic similarity values ranged from 0.0 to 94%. All the tested genotypes showed 100% polymorphism. Out of

ten primers eight primers detected only 1 allele, one primer Na10-A02 amplified 3 alleles and one primer BRMS-001 amplified 2 alleles. After this study we found some unique genotypes PooBr147, KotBr165, PooBr155, PooBr150, HatBr103, HavBr143 and BagBr137 and these unique genotypes are highly recommended for further studies. Modern tools like GWAS (Genome Wide Association Study) along with SSRs, used to interpret the genetic diversity and identification of *B. rapa* genotypes.

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# An Appendix

## 5.1 A1

Table 5.1: Microsatellite/SSRs PCR analysis (reaction mix)

| Components | Stock Concentration | Final Concentration | Vol/ Rxn | | Samples | Total Vol. |
|---|---|---|---|---|---|---|
| $ddH_2O$ | - | - | 10.7 µl | x | 30 | 695.5 µl |
| PCR Buffer minus $MgCl_2$ | 10x | 1 x | 2.0 µl | x | 30 | 130 µl |
| $MgCl_2$ | 25 mM | 2.0 mM | 2.5 µl | x | 30 | 162.5 µl |
| dNTP Mixture | 100 mM Each | 2 mM | 2 µl | x | 30 | 130 µl |
| Forward Primer | 20 pmoles/µl (20µM) | 0.8 µM | 0.8 µl | x | 30 | 52 µl |
| Reverse Primer | 20 pmoles/µl (20µM) | 0.8 µM | 0.8 µl | x | 30 | 52 µl |

Table 5.1: Microsatellite/SSRs PCR analysis (reaction mix)

| Components | Stock Concentration | Final Concentration | Vol/ Rxn | | Samples | Total Vol. |
|---|---|---|---|---|---|---|
| Taq DNA Polymerase | 5 Units/µl | 1 unit/ rxn | 0.2 µl | x | 30 | 13 µl |
| Template DNA | 20-50 ng/µl | 20-50 ng/rxn | 1.0 µl | - | - | - |
| Total volume | | - | 20.0 µl | - | - | - |

## 5.2 A2

Table 5.2: PCR thermal cycler profile

| Profile | Temperature | Time | No. of cycles |
|---|---|---|---|
| Initial denaturation | 94°C | 5 Minutes | 1 |
| Final denaturation | 94°C | 1 | |
| Annealing | 55-60°C | 40 Sec | 35 |
| Initial extension | 72°C | 2Mins | |
| Final extension | 72°C | 7 Mins | 1 |