CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

To Study the Genetic Variability Among *Brassica napus* Germplasm from Punjab, Pakistan Using SDS-PAGE and Molecular Markers

by

Syed Muhammad Ouwn Haider Shamsi

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

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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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(Syed Muhammad Ouwn Haider Shamsi)

Abstract

Brassica napus L. (*B. napus*) is the third most preferable source of edible oil after soybean and palm oil, and also an emerging alternative of fossil fuel renown as green energy or biodiesel. The current study was designed to investigate the genetic diversity among 31 distinct genotypes of *Brassica napus*. The reliable, accurate and competent biochemical and molecular analysis were used for this purpose. Seed storage protein analysis through Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to analyze genomic variation and the efficiency of these 31 genotypes of *B. napus*. Seed storage protein-based variability is a useful tool. A highly efficient SDS-PAGE protocol was optimized for diversity analysis. A low to moderate to high level of genetic variance was observed in the 31 tested genotypes of *B. napus*. The polymorphic proteins analyzed were of molecular weight ranged 10-180 kDa and 14 polypeptide bands were observed in total. Among the 14 protein bands 13 (92.85%) were found polymorphic while 1 (7%) was monomorphic. The coefficient of similarity ranged 26% to 95.24% with maximum genetic similarity between OkaBn261 and SheBn253 genotypes while the least similarity value of 26% was estimated among SheBn250 and JamBn227. The results indicated low to high level of genetic similarity between *B. napus* genotypes. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) categorized all the diverse 31 genotypes into seven distinctive clusters. Cluster 1-7 contained 5, 10, 8, 1, 1, 4 and 2 genotypes, respectively. The cluster 4 and 5 contained one genotype each, JamBn225 and ChaBn249, respectively, that were found highly diverse. The modern 2D and 3D methods were also introduced to enhance visualization of genomic diversity of these genotypes from different angles in X-Y plane. 2D analysis indicated 6 diverse genotypes that were NorBn237, SheBn250, FaiBn201, JamBn227, MulBn240 and, MulBn241. However, 3D analysis revealed only 2 diverse genotypes that were MulBn240 and MulBn241. Although SDS-PAGE based variations were certified enough to enhance the knowledge in selection and identification of diverse genotypes but still to better understand genomic variance more clearly molecular analysis was used for these genotypes. Similarly, 10 diverse Simple Sequence Repeats (SSRs) primer

unique to these *B. napus* genotypes, studied from previous literature of *Brassicas*, were used to identify the genomic level diversity i.e., molecular analysis. Out of 10 SSRs used for 31 genotypes of *B. napus* a total of 12 alleles were generated. The amplified fragments were ranged from 100-480 bp in length and almost all the primers showed maximum polymorphic banding patterns. Out of 10 primers used 9 detected one allele each while the primer PBCESSRNA3 amplified 3 alleles. Almost, a low to high level of genetic similarity was observed among all the genotypes with values ranged from 0-100% however, maximum level (100%) of genetic similarity was among DerBn217 and DerBn215 only, that revealed the genotypes of same origin may share common ancestors. But all other genotypes showed low level or medium level similarity. Genetic similarity values of 0% was observed among ChaBn249 / FaiBn201, SheBn250 / MuzBn211 etc. The cluster analysis based on UPGMA divided all the genotypes into 5 diverse groups comprised of 6, 6, 9, 5 and 5 genotypes, respectively. The group 5 was highly diverse genotypes containing group. The 2D and 3D (Principal Coordinate Analysis) analysis further identified promising genotypes. The observations recorded with 2D analysis indicated 5 diverse genotypes LayBn222, MuzBn211, LhrBn258, OkaBn260 and LhrBn255 on the outskirts, while 3D analysis showed MulBn241, LayBn220 and SheBn250 genotypes. These results could be used as a baseline for future *Brassica napus* research, evaluation and breeding selection programs.

Contents

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Abbreviations

2D:- Two Dimensional

3D:- Three Dimensional

Acetic Acid:- CH_3COOH

AFLP :- Amplified Fragment Length Polymorphism

APS:- Ammonium Per Sulphate

B. campestris :- *Brassica campestris*

B. carinata:- *Brassica carinata*

B. juncea:- *Brassica juncea*

B. napus:- *Brassica napus*

B. nigra:- *Brassica nigra*

B. oleracea:- *Brassica oleracea*

B. rapa:- *Brassica rapa*

BPB:- Bromophenol Blue

CPC:- Canola Press Cakes

CBD :- Convention on Biological Diversity

CBB R250:- Coomassie Brilliant Blue R250

Distilled Water:- $d.H_2O$

FAO:- Food and Agriculture Organization

FAS:- Foreign Agricultural Services

IGF:- Insulin Growth Like Factors

ITPGR:- International Treaty on Plant Genetic Resource

Isopentyl Alcohol:- $C_5H_{12}O$

KPK:- Khyber Pakhtunkhwa

Kg ha- 1:- Kilogram Per Hectare

LDL-Cholesterol:- Low Density Lipid Cholesterol

Methanol:- CH_3OH

Mh:- Million Hectares

MT:- Million Tons

NARC :- National Agricultural Research Centre

NIRS:- Near Infrared Reflectance Spectroscopy

NTSys-PC:- Numerical Taxonomy System for Personnel Computer

PGRI:- Plant Genetic Resource Institute

PCR:- Polymerase Chain Reaction

PCoA :- Principal Coordinate Analysis

RAPD :- Random Amplified Polymorphic DNA

RCBD :- Randomized Complete Blocks Design

RFLP:- Restriction Fragment Length Polymorphism

Rf. :- Retardation Factor

SRAP:- Sequence Related Amplified Polymorphism

SSRs:- Simple Sequence Repeats

SNPs:- Single Nucleotide Polymorphism

SCLA:- Single Linkage Cluster Analysis

SDS-PAGE:- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sol. :- Solution

THAM:- Tris (hydroxymethyl) Amino Methane

USM:- Unani System of Medicine

UN:- United Nations

USD:- United States Dollar

USDA :- United States Department of Agriculture

UPGMA:- Unweighted Pair Group Method with Arithmetic Averages

Urea:- CH_4N_2O

Chapter 1

Introduction

1.1 Background

To improve the performances of plants, researchers are continuously struggling from the ancient times. Evolution in crop plants naturally or by human efforts is described by a term genetic diversity. Genomic variation in the population is actually the degree of differentiation between or within species. Either intra-specific or inter-specific, if had not possessed diversity among species, all the individuals would have been similar leaving no space for different traits to express restraining the plants to evolve. However, with the passage of time natural ways of evolution got depleted because of many reasons, for instance use of selected genotypes for breeding programs, improvement of only yield related factors and its components etc., that lead to huge amount of similarity among crop cultivars. Moreover, the concept of molecular studies that all the organisms even maternal twins are different from each other is the fundamental reason for modern day elucidation of genetic diversity among various crop plants [1].

Many novel techniques have been introduced in order to measure the diversity of a population. For example, development of biochemical markers i.e., use of protein markers to measure the genetic diversity via total seed storage proteins through SDS-PAGE analysis, also the use of DNA markers to estimate the more precise,

efficient and cost-effective molecular analysis of whole plant genome. Additionally, the recent methods of characterizing genomic data of a germplasm, the use of molecular markers to detect allelic variation in genes expressing target traits, number of nucleotides, halophyte diversity, expected heterozygosity and population structuring helped the modern-day researchers to investigate and conserve the genetic diversity of many plant species.

Also, the New Generation Sequencing (NGS) enabled the analysis of large quantities of molecular data via molecular marker analysis for example RAPD, AFLP, SNP's, SSRs etc. With the explosion of population, breeders now a days are enhancing breeders preferred traits and farmers preferred traits to improve and cope the requirement of surplus food [2]. In the past, till 1960's, developing countries were mainly focusing on the morphological based diversity assessment of plant crops only. Using morphological analysis techniques alone brought some significant benefits but with some worse situation i.e., prolonged activities resulted in loss of genetic diversity and extinction of primitive and adaptive genes.

Therefore, scientists felt the dire need of new and more advanced analytical methods based on biochemical and molecular analysis to further investigate the genomic diversity of plants along with old morphological methods [3]. Furthermore, the use of antique techniques was not only the reason of draggling but also the naturally occurring and man-made limitations contributed as a hurdle in the way of enhanced level research.

Human based global challenges of deforestation, land degradation, coastal urbanization and development and environmental stress, because of all of these activities the world required modern solutions. In the past times, naturally occurring Irish potato famine and Southern corn leaf blight epidemic in U.S.A of potato and corn, respectively, highlighted an immediate action and concern to focus on importance of plant genetic resources to spread the green revolution and assess the genetic diversity among crop plants to attain better genotypes for future and to feed the growing world population. The Food and Agriculture Organization supported the International Treaty on Plant Genetic Resource, and United Nation's supported

Convention on Biological Diversity CBD are international agreements that recognizes the important role of genetic diversity conservation [4].

1.2 *Brassicaceae* Family

Except Antarctica, the family *Brassicaceae* can be found all around the world, almost in all continents. This botanical family is an important source of good quality and high yields of edible oil, secondary metabolites with specific taste and as an experimental model for genetical studies. The family is rich source of glucosinolate, isothiocyanate, indoles, phenolic compounds, carotenoids, phytoalexins, terpenes etc [5]. However, phenolic compounds are rich in specie *Eruca sativa* Mill [6]. Depending upon the taxonomic, genomics, systematics, paleobotany and phylogenetic analysis the possible origin of *Brassicaceae* family is believed to be Europe, but now it is growing in almost all parts of the world. It has several other names regarding to the regions, but in English it is commonly known as rapeseed, oilseed rape, rape kale, Siberian kale, swede rape, canola, colza, and winter rape etc. Being an important source of human nutrition, cultivation of *Brassicaceae* has covered almost the entire globe. Africa, Australia, America, Canada and Asia have been cultivating *Brassicaceae* species from a long time. The main cultivators of *Brassicaceae* in Asia are China, Japan, India, Iran, Afghanistan and Pakistan [7]. The experimental model for genetic diversity study *Arabidopsis thaliana* belongs to this family. Also, the other *Brassica* species revolutionized our knowledge in every field of modern plant biology [8].

1.3 *Brassica*

Brassica is a valuable and economically important member of the family *Brassicaceae* comprising of 350 genera and 3500 species with 16% of world edible oil production [9]. After the soybean and palm oil, oilseed *Brassicas* are crucial source of edible oil and are produced in large quantities around the world [10].

Seven crops that are mostly preferred for abstraction of edible oil are Indian mustard or (*B. juncea*), Black mustard or (*B. nigra*), yellow sarson or (*B. campestris*), Taramira or (*Eruca sativa*), Gobhi sarson or (*B. napus*), Ethiopian mustard or (*B. carinata*) and *Brassica rapa* [11]. *B. napus* L. oil contains low quantity of glucosinolate and erucic acid and is the major edible oil in many countries like Australia, Japan and Canada [12]. *B. carinata* plays an important role in the production of biodiesel from vegetable oil for industrial use. It is a source of direct energetic usage and high value biochemical mining as comprises for a huge proportion of biomass [13]. Mostly, in Europe and Asia, morphotypes of *B. rapa* are cultivated as a source of vegetables, fodder, condiment and oil. The crops of *B. rapa* are used for extraction of oil are annual spring and biennial winter types. It includes 199 diverse accessions with East Asian ciaxin selected from Pak-choi with rapidly long and tender floral terms [14]. Another important crop to this family is *B. juncea* also known as Indian mustard commonly. Due to its low greenhouse gas emissions and higher tolerance to changing climate conditions it has gained a reputation of scientific model for future research projects.

It is an allotetraploid plant (AABB, 2n=36) probably originated on West and Central Asia through natural hybridization of *B. rapa* and *B. nigra*. China, Pakistan and India are the main cultivar of this oilseed specie. Concern to this crop raised when its use as a biodiesel was observed. It contains a high oil content fatty acids and erucic acid up to 40% and is a non-food crop, therefore, is free of limitations of food vs fuel debate [15].

Found along the narrow geographical range of Mediterranean, *Eruca sativa*, is an insect pollinated and self-compatible crop of *Brassica* [16]. According to Unani System of Medicine (USM) that deals with medicine from plants, *Eruca sativa* is of various therapeutic importance's as it contains erucic acid, oleic acid, linoleic acid, saturated fatty acids, flavonoids, phenolics etc. It serves as an herbal medicine for anti-ulcer, anti-bacterial, fertility, hyper-lipidemic, antioxidant, anti-hypertensive, anti-inflammatory, anti-edema, nephro-protective, anti-fungal, anti-diabetic and anti-cancer purposes [17]. Five major species of *Brassica* are mostly cultivated in Pakistan including *B. napus, B. juncea, B. carinata, B. compesrties* and, *Eruca*

sativa [18]. These species appear to be genetically and morphologically similar up to a point, but their breeding, morphological, and some other traits differ significantly. Depending upon their genomic and genetic relationship, a scheme known as U's triangle was developed to show the mutual relationship between these species [19] as shown in Figure 1.1.

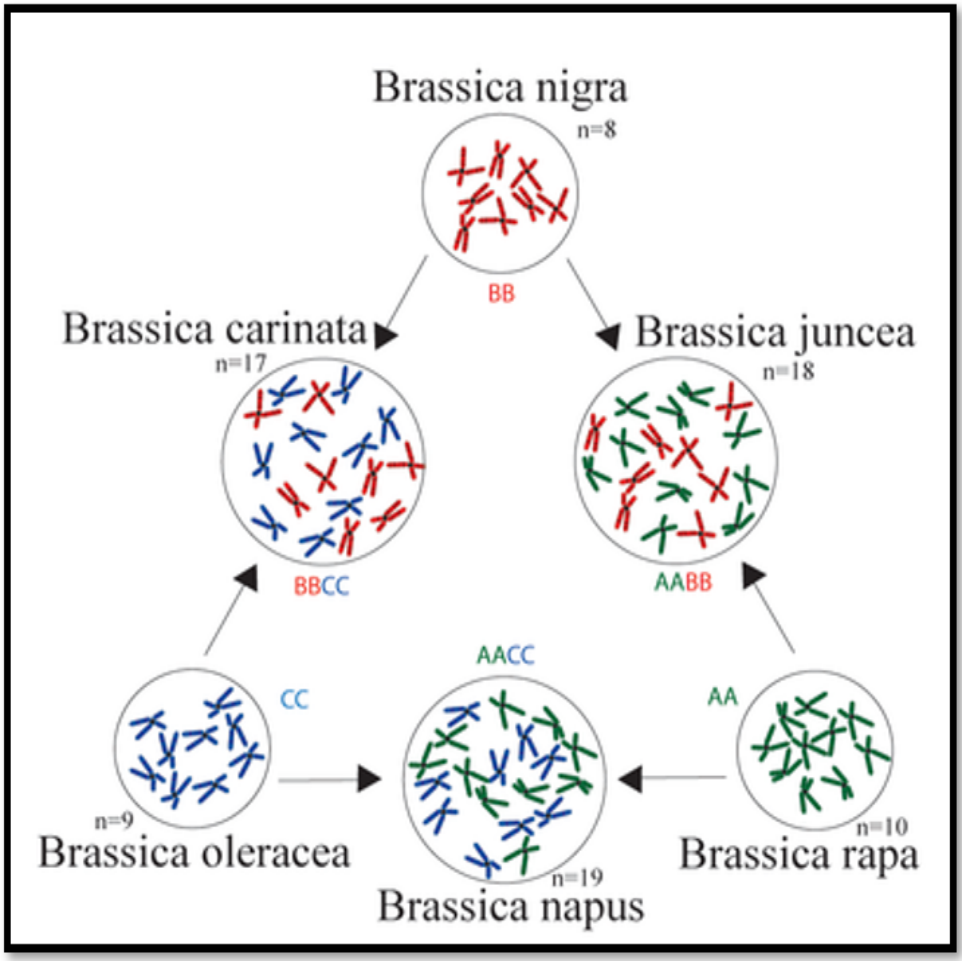

FIGURE 1.1: U's triangle indicating relationship among *Brassica* species [19].

1.4 *Brassica napus* L.

It is an allotetraploid with interspecific cross between diploid *B. rapa* (2n=20, AA) and *B. oleracea* (2n=18, CC) about 10,000 or 7000 years ago. It is thought and observed through many evolutionary analysis techniques that the possible origin of *B. napus* is either Europe or Asia, but it is still difficult to determine its

exact precise origin. Shahzadi *et al.* [20] determined the possible natural origin of wild types of *B. napus* is Europe and New Zealand. Studies showed that A. genome of *B. napus* has evolved from a European ancestor *B. rapa. Spp. rapa.* and C. genome from *B. montana* (2n=18) [[21], [22]]. Commonly, *B. napus* L. is known as rapeseed, oilseed rape and colza, however, the name 'Canola' was originated in Canada after is modified variant that constituted less erucic acid (less than 2%) and low glucosinolate quantity of almost 30 µmol/mg was developed [23]. It is an annual specie with winter, semi-winter and spring types that shows differences in their cold and drought tolerances i.e., variation in growing conditions. For example, winter types grow in high humidity and cool temperatures. It was initially recognized as a vegetable crop for instance leaf rape (*Brassica napus* var. *pabularia*) that grows fastly and attain early maturity [24]. These species require a pH ranged from 5.5-8.5 for optimal growth and 110-150 days to achieve full maturity. The seeds of it are mostly red-brown, dark-brown or black in color [7] Figure 1.2.

FIGURE 1.2: *Brassica napus* seeds color [7].

1.5 Production: Worldwide and in Pakistan

After soybean and palm oil the third most important and emerging source of vegetable oil in world is *B. napus* extracted oil. Rapeseed could be grown all year around in Zimbabwe mostly in its sowing periods from February-July and April-September [25]. It is a widely cultivated crop in many parts of the world. India has been cultivating it since 4000 B. C while China and Japan about 2000 years ago [26]. A total of 66 countries together produces almost 70 million tons (MT) of rapeseed, with Europe comprises the largest part i.e., 34 countries, 15 in Asia, 9 in America, 6 in Africa and 2 in Oceania [27]. In the year 2019/2020 the maximum rapeseed producing countries were Canada with 19 million tons occupying 1st position followed by China 13.1 million tons, India and European Union with 7.7 MT and 16.83 MT, respectively. Overall, 68.90 MT were produced in the year 2020/2021 [28] as shown in Figure 1.3.

According to Foreign Agricultural Services USDA, 2021, 66 MT of rapeseed were harvested globally in the year 2019/2020 and 39 MT of residues were produced after mechanical oil processing method [29].

Pakistan is an agricultural dependent economic country. The country like other developing nations is under the serious influence of drought, climate change, salinity etc. The major proportion of vegetable oil of country is imported that disturbs the country's economy. One can conclude that the nation is facing serious edible oil scarcity as local manufacture of edible oil from classic and modern oilseeds are barely capable to meet one fourth of country's demand. In Pakistan, oilseed crops are grown over an area of 0.23 million hectares (mh), yet the production per capita is 803 kg ha-1 [30]. In the year 2000-2001, the total necessity of oil to Pakistan was 1.9 MT of which 32% was fulfilled by local generation and 68% was traded in to meet the demand chain, at a cost of 788 million USD. The total area under cultivation in 2000 was 47.2 thousand hectares and annual production of rapeseed was 59 thousand tons [31]. The same figures were observed in year 2002-2004, with annual requirement of 1.95 MT of edible oil, provided 29% (0.606 MT) by local resources and 71% was imported at a cost of 800 million USD [32], [33].

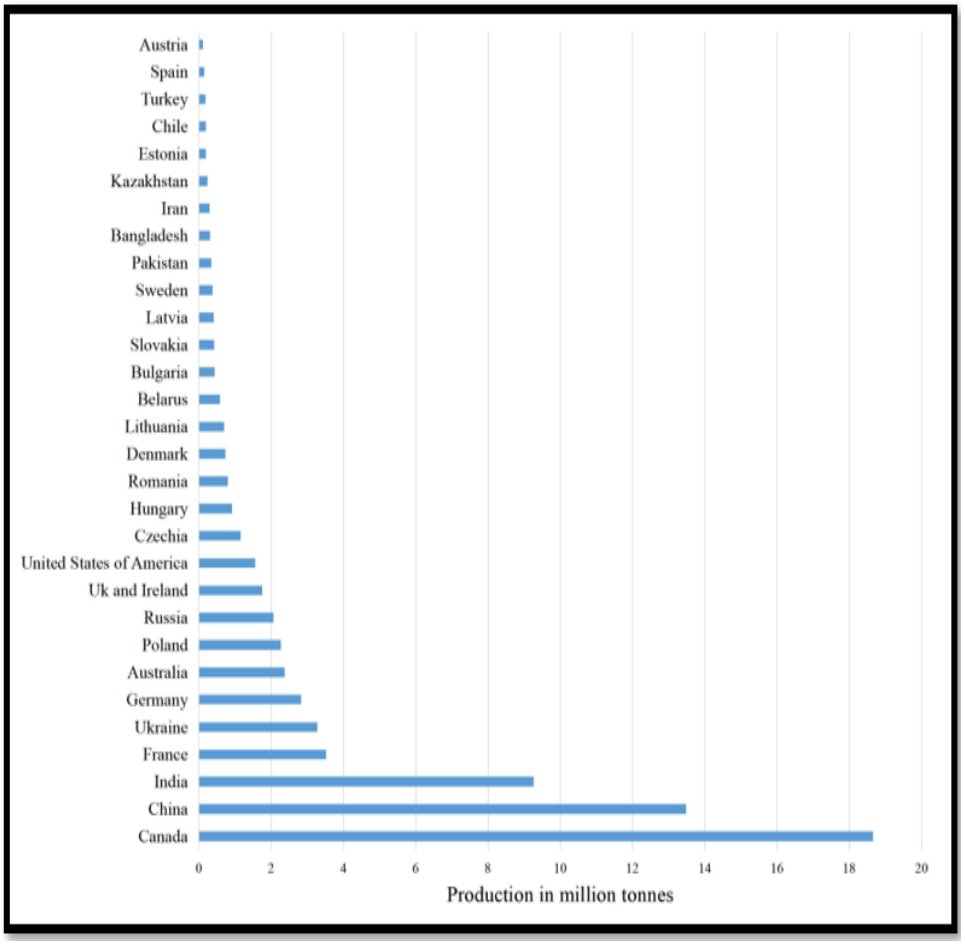

FIGURE 1.3: Worldwide production of rapeseed by countries in the year 2020/2021 [28].

Similarly, production of canola in years 2007-2010, were almost in line with the previous observations. The net production was 3 times less than the world production of 2180 kg ha-1 [34] and the requirement of the country was 2.764 in year 2010 with domestic production of only 0.857 MT and caused a heavy import of 84 billion USD [35]. In the year 2012-2013, total availability of rapeseed oil required was 2.9 MT in which 662 thousand tons was locally available and 1054.7 million USD was imported [36]. Traditional oilseed varieties grown in Pakistan in three provinces Punjab, KPK and Sindh are Mustard, Linseed, Sesame and Castor crops while non-traditional imported crops are Soybean, Safflower and Sunflower. The net demand of edible oil in year 2015-16 was 2.78 million tons with 0.83 million tons provided by country's local resources among which the share of rapeseed and mustard was 17%. The total area under cultivation for rapeseed in 2015-16 was 14,164 hectares. However, in 2017-18 index's a total of 1.98MT was imported

and 0. 446 MT was domestically produced over an area of 201 and 193 thousand hectares, respectively [37].

On the other hand, the demand and supply of oilseed crops in Pakistan is not in order as it should be, therefore, all the biotic and abiotic factors involved in its disturbance must be analyzed clearly. There are multiple issues that are causing chronic scarcity of edible oil production that should be met with immediate and accurate measures to capture the problem. For this purpose, it is the need of time to acquire upgraded array of *Brassicas* to fulfil the disparity between production and import [38]. Continuously worsening effects of climate change are among the main concerns that is adversely affecting the growth of *Brassicas* worldwide. Irregular rainfall, drought, salinity and many more fall under the this very issue [39]. Other factors include Insects attacks, nutrients deficiency to the crop, competition with other crops, non-availability of modern technology for harvesting and oil extraction of oilseed crops, flawed and corrupt marketing system etc. The insects like *Lipahis eyrsimi* Kalf. causes loss of yield about 10-90%, *Brevicoryne brassicae* L. causes damage to cabbage varieties i.e., Cabbage aphid, *Athalia proximia* Klug. or sawfly damages rapeseed varieties in mostly northern areas of Khyber Pakhtunkhwa and Sindh. Nutrients like nitrogen and phosphorous mechanism must be observed to estimate the right proportions of their application that is vital of increase in yield of selective crops. For example, proper and known increase in nitrogen quantity causes the yield increase of 40 to 160 kg ha-1 but not the seed oil content [40].

Rapeseed production, like other crops, frequently faces major challenges due to a variety of factors, including a decrease in labor hands and farmers as labor and agricultural input costs rise, resulting in lower outputs, a lack of agricultural mechanization, yield instability due to climate variability, and weak cultivars (shatter, biotic and abiotic factors). For example, stem rot disease, caused by *Sclerotinia sclerotium*, and clubroot disease, caused by *Plasmodiophora brassicae*, are two of the most damaging infections that weaken rapeseed crops worldwide. Stem rot and clubroot diseases have caused yield losses of 10-80% and 20-30% in China, respectively. Of some solutions few natural remedies are available for yield enhancement of oilseed crops. For instance, *Moringa* genus is the only member of

its family *Moringaceae* i.e., *Moringa olifera* L., its leaves consist of a substance Zeatin in it that is an important plant hormone as it acts as cytokinin. Also, the *Brassica* water extracts contain a substance *Brassinolide* in it that is a natural plant steroid that can be used to increase the yield of *Brassica* species. Furthermore, the use of allelochemicals and enzymes could help in increasing the yield, oil content, protein quality and weather tolerance in *Brassica* species [41].

1.6 Significance of *Brassica napus* L.

There are many reasons for its importance among which one is that these crops produce edible material for human and animals use [42].

1.6.1 Human Food Perspective

After soyabean and cottonseed the most renown source of vegetable oil is *Brassica*. Its demand of the world is rising day-by-day, as it is currently yielding 56.23 average million tons of oil all around the globe [43]. Its leaves and seeds are used as source of vegetable for human diet i.e., cooking, flavoring, pickle making as it contains 40% of oil. The high thermostability of Oleic acid in *B. napus* seeds makes it desirable for cooking also. It is also used for medical and industrial purposes [44]. Rapeseed oil contains a nutritive number of amino acids and 2 to 3 times high proportion of proteins then Wheat and Rice and is also a better emulsifier than Soybean [45], [46].

1.6.2 Animal Fodder

The residues of *B. napus L.* are rich source of proteins (35-40%), carbohydrates (30-35%), crude fibers (10-15%) and minerals (8-14%) [47]. Canola raw is used as livestock feeds as it contains essential nutrients of diet. European commission authorized Canola Press Cakes (CPC) i.e., oil extracted residues as a novel food

with reduced levels of phytates [48], [49]. The CPC constitutes of (35g/100g) of dietary fibers, 27g/100g of proteins or 81mg/100g of glutamic acid (amino acid), 20g/100g of fats and 5510mg/100g of sugars mostly, sucrose however, Malic acid, Citric acid and Phosphoric acid are present in 67, 17 and 12% ratio. The oil content of *B. napus* is ranged between 29.1%-50.1% and also contains in it Palmitic acid, Eicosenoic acid and Stearic acid but in relatively small quantities as compared to others present [50].

1.6.3 Health Perspective

It gained more importance because of high yielding, high oil content and good quality of its oil comprising 38-40% protein, high concentration of oleic acid (60%), appropriate quantity of linoleic acid (20%) and linolenic acid (10%) and less than 2% of erucic acid and also a 100g of canola oil provides 884 calories [51]. Observing this via health point of view high nutritional value compound linolenic acid helps in reducing the LDL-Cholesterol levels in body, improves insulin sensitivity and blood pressure. High concentration of Oleic acid also helps in lowering of cholesterol levels and act as anti-inflammatory controlling heart conditions. Erucic acid that is mostly higher or limited in many commercial plant oil is present in very minute quantity of 2% in "Double-zero" canola as it has many anti-nutritional properties [52].

It also contains low quantities of glucosinolate [53]. Phytic acid that makes complexes with proteins and minerals is its constituent, it reduces the ability of digestion i.e., glucosinolate (Progoitrin 4-hydroxy glucobrassicin, gluconapin) that affect functioning of thyroid. Although, glucosinolate gives potential benefits of reducing the risk of cancer and cardiovascular diseases but it should be used in reduced quantity in human diet that makes *B. napus* a risk-free crop desired [54]. This will important for the health perspective. Hydrolysates, proteins of rapeseed oil, could be used as inhibitors of angiotensin I, converting enzymes, as an antioxidant, anti-hypertensive agent, meat flavor production and in growth promotion of ovary cells [[55], [56], [57]].

1.6.4 Industrial Perspective

Erucic acid present in huge quantity among many *Brassica* species (old variants of *B. napus* L.) make it favorable for industrial use for instance as an adhesive, anti-corrosive material, anti-blocking agent in polyethylene films etc [52]. High seed oil and protein content with additional presence of unsaturated fatty acids has made it widely accepted as vegetable oil for human consumption and as a biofuel in Green energy industry [58]. Rapeseed oil was used as fuel about 20 years ago, for the first time, it was trans-esterified in the presence of a catalyst [59]. Former was then reduced with alcohol (methanol) [60]. Canola oil cold point is quite low i.e., 0oC and pour point is -15oC that made it suitable for biodiesel usage [61]. In Europe, 50-70% of biodiesel is from rapeseed oil [62]. It is preferred over fossil fuel because it reduces the greenhouse gas emissions up to 90% [63].

1.7 Diversity Analysis

Crop diseases cause yield losses; it is estimated that unprotected crops lose 16% of their yield due to diseases. The variety is required to combat numerous diseases, rapidly changing ecological situations, and essentially to meet the increasing human consumption challenges. There are different methods to assess the genetic diversity which have been used by scientists and researchers to help finding the promising crops and to combat diseases. These methods are morphological, biochemical and DNA based markers analysis [39].

1.7.1 Morphological Based Diversity

Features that are visible to naked eye such as seed shape, flower color, growth, seed weight, plant height, flower initiation, days to flowering formation, siliqua length etc. are some important morphological characteristics that are proved to be helpful in breeding programs. These markers allow us to determine the effect of environmental changes [36].

1.7.1.1 Biochemical Based Diversity

For better genetic improvement, vast genetic diversity and information must be available. Recent advancements in biochemical field have served this purpose well i.e., the characterization of seed storage proteins provided a helpful hand to successfully study the genetic diversity [64]. The SDS-PAGE is being used extensively to investigate, identify, and separate the seed storage proteins that are used to investigate evolutionary relations among several crop plants. It is now a widely used technique to characterize proteins [65]. It is the most accepted and appreciated technique as environmental factors have no effect on it. Many researchers found that polymorphism reveals differences between species that are unaffected by geographic conditions [66]. Therefore, in our current study, we have used SDS-PAGE to characterize variability among *Brassica napus* genotypes.

1.7.1.2 Molecular Based Diversity

A molecular marker is a specific location of DNA that is detected by specific primers that measure its presence and clearly recognize the nearby region's attributes. Rapidly evolving and continuously growing field of genomics provides alternative and advanced methods to study these crops breads at whole genome level and have certain advantages over other methods [67]. Now -a-days, to increase the genetic heterogeneity DNA markers are in use and are proving to be best assessing the genetic diversity and polymorphism among oilseed rape [9], [68]. Various DNA markers are in use for this purpose such as RFLP, RAPD, AFLP, SRAP, SSRs and SNP [69]. SSRs marker genotyping technology is preferred over others as it helps to identify the genetic diversity of rapeseed varieties and the genetic homogeneity of reproductive material, selection of parent for crosses and control the transfer of genetic material from parents to hybrids [9]. As these markers are persistent and detectable in every tissue at all growth rates, they have a number of advantages over traditional phenotypes. We can see variation in chromosomes through molecular markers, which result from duplication, deletion, inversion, and insertion [70].

1.8 Research Gap

Biochemical and molecular diversity can help in identification of highly diverse plant genotypes. For this purpose, investigators and experts have concentrated on genomic correlation analysis of various crop species, using SDS-PAGE and, molecular markers. SDS-PAGE has been a prominent method, used by many researchers, to study the protein profiles. Similarly, SSRs have become increasingly popular among molecular markers due to their high-level duplicability and ability to detect elevated levels of natural polymorphism, potency, vast genome dissemination, and genetic diversity. Yet, there is no comprehensive study available about *Brassica napus* genotypes of the Punjab, Pakistan regions, therefore, the goal of this research is to assess the seed protein variability and genetic diversity of Punjab belonged, *Brassica napus* varieties using SDS-PAGE and SSRs markers in order to find-out fast growing, environment favorable and economically beneficial and promising genotypes.

1.9 Scope

Brassica is one of Pakistan's future hopes to improve edible oil production. By selecting elite genotypes through biochemical and molecular techniques promising genotypes found can be further used in *Brassica* breeding programs. Consequently, the local production of edible oil can be improved, thus reducing the import burden of edible oil and ultimately saving valuable foreign exchange.

1.10 Aim and Objectives

Aim:

- To evaluate the biochemical and molecular marker (SSRs) based variability among *B. napus* genotypes.

Objectives

- To identify the differences in seed proteins of *B. napus* genotypes using SDS-PAGE.
- To assess the *B. napus* genotypes using SSRs markers.
- To identify promising genotypes of *B. napus*.

Chapter 2

Review of Literature

2.1 Seed Protein Based Previous Studies

Choudhary *et al.* [51] worked on 7 genotypes of *Brassica napus* to estimate protein-based variations. Using SDS-PAGE they found different banding patterns in the diverse genotypes that were collected from various geographic regions. A total of 10 bands were observed, with five of them being main bands. The polymorphic to monomorphic band ratio was nearly 50%. The bands were divided into three distinct regions (A, B and C). Those regions were made up of large, intermediate, and small-scale protein subunits. GSC101 and HNS0901 had the highest similarity coefficient.

While genotypes RSPN25 and RSPN29 had the lowest similarity estimates. All genotypes were divided into four cluster groups that differed significantly from one another. Polymorphic bands obtained through this research proved SDS-PAGE a feasible technology to distinguish different species of *Brassica napus* L. Their work showed close relationship among studied genotypes, and they deduced that the differences are because of geographical reasons but also found polymorphism in protein bands ranging 15-10 kDa. For breeders this characterization of seed proteins and selection of desirable genotypes is of great importance and may be used for crossing in breeding programs.

Sadia *et al.* [64] used SDS-PAGE to inspect the biochemical characterization of various *Brassica species.* Maximum polymorphism in protein band pattern was observed in four diverse *Brassica* species, including *Brassica rapa, Brassica carinata, Brassica napus,* and *Brassica Juncea.* A total of 31 bands were estimated, mostly being polymorphic. These bands were classified into thirteen different areas (A-M) based on their molecular weight. Different genotype groups showed intra- and inter-specific diversity. Group I contain 15 *Brassica juncea* and *Brassica carinata* genotypes, Group II contains 13 *Brassica napus* genotypes, and Group III contains 3 *Brassica rapa* genotypes. There was a significant difference between *Brassica* varieties. They established that protein-based variation differs depending on the species/sub-species.

Mukhlesur *et al.* [66] used Sodium dodecyl sulphate polyacrylamide gel electrophoresis to study protein profiles in seed and esterase, acid phosphate, and peroxidase in 32 genotypes of *Brassica rapa* from Bangladesh, Japan, and China. During their protein profile pattern analysis, they discovered thirty-one to thirty-two protein bands, of which 31.3 percent were polymorphic and the rest were monomorphic. Similarly, nine subunits were discovered for esterase, with 18.8 percent being polymorphic and the rest being monomorphic. However, no polymorphic bands were discovered for acid phosphate or peroxidase. The SDS-PAGE dendrogram was divided into five groups. This classification system clearly distinguished the yellow sarson, self-compatible genotypes from those that were self-incompatible and had brown seeds.

Abbas *et al.* [71] assessed *Brassica napus* and Indigenous *Brassica campestris* species through biochemical and molecular methods. While researching on parental lines along with five F_2S they found out, through (NIRS) Near Infrared Reflectance Spectroscopy, that parental lines contained more oil 45.85%, F_2S contain more protein 25.92% as compared with parents, but there was high glucosinolate and fatty acid in both. Using IGF (Insulin Growth like Factors) primers in molecular assessment they estimated high level of genetic dissimilarity among all genotypes. Dendrogram analysis allowed them to identify diverse genotypes that would be helpful in further breeding programs creating genetic variation in local germplasm.

Ahmad *et al.* [72] analyzed 12 genotypes of *Brassica* by biochemical method to identify the seed quality and high yielding genotypes. Those genotypes were evaluated for oil, protein, glucosinolate, moisture, oleic acid, linolenic acid and erucic acid. Results varied among all genotypes, for example, Oscar was best for its high oil content 52.10%, T-16-401 had the highest protein value of 25.12%, Rainbow had lowest glucosinolate content 67.35 µmg-1, Oscar was also highest in moisture content 7.09%, Linolenic acid was highest in Raya Anmol 12.8% and erucic acid was lowest in Crusher 36.44%. But the only genotype that found best for all the demands was Rainbow. Their results provided the future researchers with a genotype that could further be assessed through 2D and 3D analysis for better results and to help the country's economy.

Akbar *et al.* [73] characterized *Sesame* (*Sesamum indicum* L.) germplasm for total seed storage protein using SDS-PAGE for 105 accessions that were collected from different ecological regions of Pakistan. They used 12% polyacrylamide gel to separate proteins electrophoretically. 20 polypeptides bands were found in total among which 14 (70%) were polymorphic and 6 (30%) were monomorphic with proteins ranging from 13.5 to 100 kDa. 6 bands i.e., 7, 11, 12, 15, 16 and 18 were present in all genotypes.

Dendrogram based on dissimilarity matrix using UPGMA divided all the accessions in three groups A, B and C. As SDS-PAGE showed low to medium level of genetic variability, therefore, 2D gel electrophoresis, Molecular Analysis and a greater number of accessions were recommended for future genetic evaluation. To a fact, that no comprehensive study has been done on *Sesame* genotypes for total seed storage proteins, their research could support for classification, genetic evaluation and conservation of *Sesame*. Geetha *et al.* [74] studied Mustard genotypes by SDSPAGE to find the diversity of its seed proteins. A total of 9 varieties were studied and the total protein subunits were divided into 5 portions. In the first portion contained the protein subunits with an Rf value of 0.237, the molecular weight of the protein subunits found in these regions was 97.4kDa. In the second portion, the entrapped protein subunits had a molecular weight of 43 kDa with an Rf value between 0.314 and 0.382. In, the contained protein subunits of the third

part had a molecular weight of twenty-nine kDa and similarly 20.1 kD a protein subunits were found in the fourth portion with the highest Rf value of 0.477. In the last and fifth part of the contained gel protein subunit, the Rf value was 0.662. They concluded from their study that the diversity occurred mainly in portions I, II and V of the protein subunit gel and these portions showed the value of high and low molecular weight proteins. They found 3 common protein subunits for each variety, while protein subunits with an Rf value of 0.237 were only found in five genotypes, namely Maya, GM2, Varuna, PCR 7 and Pusa Bold, and found the protein subunit with an Rf value of 0.384 in all varieties except RN 393.

Javaid *et al.* [75] performed a study on 15 groundnut accessions from the five continents using slab-type gel electrophoresis with 11.25% polyacrylamide gel. They recorded 5 main bands and found that most of the accessions were similar but only 8 differed by one band. Due to the low genetic diversity of SDSPAGE, they also proposed 2D gel electrophoresis to separate different proteins. Their data indicated that SDSPAGE could be a good tool to study interspecific diversity and the phylogenetic or evolutionary relationship between different species, rather than intraspecific variation. Hybridization between accessions from two groups, i.e., one with all 5 bands and one with 4 missing bands, is proposed to study the inheritance and linkage of this band. This would help plan marker assisted breeding experiments in peanuts.

Chittora *et al.* [76] used SDS-PAGE to study seed protein polymorphism among three genotypes of *Abrus precatorius* with three different seed coat color i.e., White, Black and Red. They recorded 44 bands out of which 26 were common among all three genotypes and 18 (40.90%) were polymorphic. They analyzed data using UPGMA clustering analysis that black and white seed coat color were closer as compared to genotypes with red seed coat color. Those all have same polypeptide bands that were peculiar to them only. SDS-PAGE helped them to distinguish all 3 genotypes on basis of specific fragments. Ibrahim *et al.* [77] conducted comparative study of 53 genotypes of Indian Mustard (*Brassica juncea*) to evaluate extent of genetic variation using SDS-PAGE. They obtained 12 different types of bands. On the basis of banding pattern of all the genotypes seven were found

polymorphic i.e., 58% and five were monomorphic. Protein size base polymorphism revealed the range of protein bands based on their molecular weight ranging from 10 kDa to 180 kDa. Their similarity coefficient values were ranged from 17% to 100%. Their data showed that SDS plays an essential role in study of protein-based variation among different genotypes of plant species.

Jan *et al.* [78] studied the genetic variability in elite Guar (*Cyamopsis tetragonoloba*) using SDS-PAGE. They took 24 Guar germplasms and characterized them through SDS-PAGE. To study the variation at enhanced level they carried out 2D and 3D methods to visualize diverged genotypes from closest angle. They found significant variability in protein profile of all the genotypes. The polypeptide bands were ranging from 10-180 kDa. 100 % polymorphism was observed while the genetic similarity values were lowest i.e., 20% in accessions 31731 and 31764 and 100% in accessions 28952 and 31682. The innovative techniques of 2D and 3D identified 3 unique accesses (31764, 31731 and 31761), which would be useful for further improvements of this plant species.

Saleem *et al.* [79] assessed genetic diversity among 100 accessions of local mustard (*Brassica juncea*) to evaluate total seed storage proteins using SDS-PAGE. Those accessions were obtained from Gene bank of PGRI, NARC Islamabad, Pakistan. They used 12.25% polyacrylamide gel and obtained total of 21 bands ranged from 6 to approximately 180 kDa based on molecular weight. Out of 21 bands 17 were found polymorphic i.e., 80.95% while 4 (19.04%) were monomorphic that indicated high level of variability. Similarity index among accessions was 0.62 to 1.0. Dendrogram was constructed using UPGMA that distributed the accessions in 5 main clusters. This grouping system revealed low level of genetic variability, therefore, 2-D gel electrophoresis along with other molecular analysis techniques was recommended. Their work showed that SDS-PAGE alone is not sufficient to estimate genetic variability. Yousuf *et al.* [80] assessed the variation based on seed proteins between *B. campestris* varieties. The protein profile of hundred and fourteen *B. campestris* was monitored using the SDS-PAGE approach. A 15% polyacrylamide gel was observed to be optimal for the efficient resolution of polypeptide bands. They observed 16 bands, 75% of which were polymorphic

while others were monomorphic. Cluster analysis classified all genotypes into IV groups based on the similarity. Clusters I, II, III and IV contained 17, 28, 25 and 44 accessions, respectively. Their research revealed huge diversity at proteomic levels.

Rani *et al.* [81] investigated the changes in protein pattern of seedlings of Indian Mustard (*Brassica juncea*) after exposing those to high temperature stress (45 ± 0.5)0C. Two heat-tolerant genotypes and two thermo-prone genotypes were screened at 45 ± 0.50C with time of 50% seedling mortality and used SDS-PAGE to determine protein pattern. A main band of 53.12 kDa and minor band of 100, 89.12, 74.13, 46.76 and 38.9 kDa in heat-tolerant genotypes and protein bands of weight 25.79 and 30.7 kDa in thermo-prone genotypes were obtained via SDS. But the stress relief lead to disappearance of bands of the thermo-prone genotypes. This new protein could be the heat shock protein that can play an important role in inducing heat tolerance.

Olatunji *et al.* [82] studied genetic variation and affiliation among four Capsicum varieties via electrophoretically separating their leaf and seed protein. They separated protein using 12% polyacrylamide gel. Total of 38 polypeptide bands were found in seeds and 17 bands in leaves were obtained. Both in leave and seed samples variation existed, in numbers of bands and in intensity of bands. Coefficient of similarity showed high level of resemblance in seed protein bands ranging from 50 to 100% while there was a median level of similarity in leaf protein bands ranged from 16.7 to 83.3%. On the basis of SCLA (Single linkage cluster analysis) assembled dendrogram that revealed two main clusters. This report could help plant breeders for better development of pepper. Iqbal *et al.* [83] conducted research on 83 genotypes of maize of Pakistan and Japan origin using SDS-PAGE through vertical slab unit. They recorded 18 protein bands of which 7 (39%) were monomorphic and 11 (61%) were polymorphic with molecular weight ranged from 10-122 kDa. The Coefficient of similarity was relatively low ranged between 0.8-1.00. Dendrogram was constructed using UPGMA clustering method. Two main clusters were revealed; first contained 9 genotypes including Sahiwal-2002 while second cluster had 74 genotypes including Aaiti-2002 and Sadaf. But the overall

polymorphism to be found was relatively low therefore, in future breeding programs to determine genetic variation more reliable results are needed that could be attained using novel biochemical techniques.

Kakaei *et al.* [84] investigated 16 genotypes of *Brassica napus* via SDS-PAGE. 16 genotypes were studied in Randomized Complete Blocks Design (RCBD) with three replications under drought and non-drought stress conditions. Proteins in both conditions were extracted during the complete flowering stage. Using SDS-PAGE they separated proteins through Laemmle method in a 12.5% and 5% resolving and stacking gels, respectively. Mean genetic stress in normal condition was ranged from 0.056 to 0.632 while under drought condition it was 0.0 to 0.5. Cluster analysis placed genotypes in three main groups. SDS-PAGE showed that bands were different among all genotypes.

Turi *et al.* [85] analyzed local *Brassica* species via SDS-PAGE, that were collected from different areas of Pakistan. They discovered high degree of variation was observed among all the genotypes. Total 28 major bands were observed among which four major and 24 were minor bands. A total of 60% polymorphism was calculated and based on those distinct bands, a dendrogram was constructed that classified all genotypes into 11 clusters and found at least 18 degrees of polymorphism between different cluster groups. Although their result was affirmation of their work but still, they proposed the 2-D gel electrophoresis method for higher protein-based diversity in future experiments. Kakaei *et al.* [86] observed the seed protein pattern of 12 *Brassica napus* cultivars using SDS-PAGE. Their work demonstrated enough variation for seed protein content among the rapeseed cultivars. A total of 17 polypeptide, bands were recorded, most of them were polymorphic. Depending upon the protein intensity among the genotypes they found-out polymorphism. The polymorphism was higher in proteins weighing 66-100 kDa while in some major genotypes ranging 15-27 kDa polymorphism was not detectable. All the genotypes were clustered in three groups. Their data can be used for maximum heterosis achievements by choosing the cultivars that are most distant. Khan *et al.* [87] studied 37 accessions of *Brassica napus* belonged to Australia, Pakistan and China for detecting the genomic variation through biochemical markers i.e., using

SDS-PAGE. They used 12.25% of Polyacrylamide gel to separate the proteins. A total of 17 bands were recorded containing 9 minor and 8 major bands among which 10 were polymorphic. Bands ranging from 100-170 kDa were all polymorphic. Chinese accession contained minimum number of bands. Some novel bands ranging 170 kDa were also found. The results from their research depicted huge diversity among foreign accessions. On the basis of cluster analysis those genotypes were divided in four groups. Among Chinese and Australian genotypes there was a huge diversity as compared to Pakistani's. This work could help molecular biologists and plant breeders for improvement of *Brassica napus* crops, in future.

2.2 Molecular Markers Based Previous Studies

Klyachenko *et al.* [9] used SSRs to investigate the genomic assortment of rapeseed varieties for selection and identification of drought and salt tolerant varieties. Four (4) markers were used, and 41 alleles were detected i.e., 10.3 alleles per marker among which number of polymorphic loci were 24. The varieties differ at least one marker from each other indicating the possibility of using a set of markers for their identification. They found great diversity between winter and spring varieties. Differentiation of varieties according to their origin was also revealed. Their work showed that SSRs markers provided the efficient diverse data that could be used in in vitro selection for drought and salt tolerance. Li *et al.* [67] conducted research on 25 *Brassica napus* hybrids using AFLP and SSRs and detected above average level of heterozygosity. 9 AFLP and 11 SSRs were used for this purpose and generated 16 loci and 22 loci, respectively. Shannon's information index and inherent disparity attained by SSRs were superior to AFLP. Their data proved efficiency of SSRs over AFLP while AFLP are more suitable for DNA fingerprinting. This research deduced high level of genetic diversity among *Brassica napus* hybrids that could be used for favorable cultivation programs. El-Esawi *et al.* [88] assessed genetic diversity, population structure and relationship of 118 individuals from 25 accessions of *B. oleracea* in Ireland via molecular markers (SSRs). They observed 27 alleles that vary in size. An extraordinary level of variation (0.699)

among accessions was recorded that was higher than expected indicated by the negative values of fixation indices (F). Genetic diversity revealed 27.1% interspecific and 72.9% intra-specific variability. The cluster analysis further determined Kale and Brussels sprouts genotypes. Their data revealed that spring cabbages showed high degree of variation. Moreover, according to them, SSRs loci were found operative for variation analysis of *Brassica oleracea*.

Khazaei *et al.* [89] estimated the genomic differences of about 352 diverse lentil accessions of around 54 countries by using 1194 different polymorphic SNP markers. They classified all the genotypes into three main groups by using principal coordinate analysis, population analysis and cluster analysis. The three groups reflected origin, lineage and farming history of those genotypes. The names allotted to those diverse groups were Northern temperate, South Asia (sub-tropical savannah) and Mediterranean i.e., genotypes belonging to different geographical region were present among 3 groups. They found narrow diversity among South Asian and Canadian germplasms. Guo *et al.* [90] investigated the center of origin and center of diversity of 173 worldwide diverse *Brassica rapa* accessions by using a total of 51 SSRs markers. They recorded a total of 715 polymorphic bands. On the basis of structure analysis those polymorphic germplasms were clustered into 3 major groups. All the groups contained highly diverse genotypes from all continents. They found a unique wild type of genotype in group one (1) named *B. rapa var. sylvestris* and that group had the highest number of unique alleles. *Brassica rapa* ecotypes varied across all groups, including leafy, Rooty, and oilseed vegetable types. Their conclusion suggested that the old world i.e., group one Asian countries are the original center of origin of *Brassica rapa*.

Havlickova *et al.* [91] estimated the genetic variation among 94 *Brassica napus* genotypes of Czech Republic, Europe by employing different molecular markers i.e., SSRs, ISSR and AFLP. They evaluated total of 89 SSRs, 53 ISSR and 1003 AFLP screening markers that were varied on the scale of polymorphism found in each accession. The high genetic variation among accessions observed through polymorphic bands that was about 100%, 90.6% and 53.9% with SSRs, ISSR and AFLP markers, respectively. The lowest genetic distance values were found

through SSRs and AFLP (49.4% and 35.5%, respectively) while ISSR indicated highest value of 62.3%. Genetic analysis distributed the accessions in two groups that revealed a noticeable swing in breeding. Numerous new scout lines and one distinctive variety were tested.

Tahira *et al.* [92] employed RAPD markers to explore the genomic variation between thirty *Brassica juncea* genotypes. All primers generated a total of 104 alleles with an average of 8.6 alleles per primer with a polymorphic range of bands between 300bp to 3kb. Maximum polymorphic bands were recorded with the largest fragment of 3kb that was amplified by using three primers. Almost, among all the genotypes there observed a similarity of about 84.5%. With highest diversity of 29% the genotype RBJ-97001 was considered most diverse as compared to other tested genotypes. The Raya 49/2 and RBJ-97001 showed the maximum diversity with genetic similarity of 71%. Cluster analysis using UPGMA classified all thirty genotypes into two main groups. On the basis of resemblance within the groups the main groups were further categorized into two sub-groups. Their results could help in future for development of novel canola varieties. Celucia *et al.* [93] assessed three sub-species of *Brassica rapa* namely *Brassica rapa* chinensis, *Brassica rapa* parachinensis and *Brassica oleracea* alboglabra. They used 54 SSRs markers to identify variation among three sub-species. Among 122 scorable bands obtained by SSRs primers 77% were highly polymorphic. The average rate of polymorphism (71.08%) indicated the high genetic diversion among tested genotypes. Phylogenetic analysis clustered the *B. rapa* chinensis and *B. rapa* parachinensis in one group revealing that these two are closely related to each other while the *B. oleracea* alboglabra had separate cluster group. This work demonstrated that *B. oleracea* alboglabra is distinct from other two sub-species. Their results proved that SSRs is a useful tool in evaluating the variation among *Brassica* genotypes.

Abbas *et al.* [94] examined the SSRs, and RAPD based variation in 15 *Brassica* species. The augmented 25.8 and 45.8 DNA fragments with SSRs and Random Amplified Polymorphic DNA *Brassica* specific markers. Genetic similarity values recorded with RAPD were 26-89% and 5-61% with SSRs markers. Total scorable bands recorded with 10 RAPD markers were 458 and 258 DNA fragments with

SSRs with size ranged from 250-2000bp. Among 14 genotypes high level of dissimilarity was observed, and those genotypes were advised to be used for further oilseeds breeding program.

Ofori *et al.* [95] investigated the genetic diversity in thirty-two genotypes of *Brassica rapa* winter cultivars with sixteen SSRs markers by comparing those genotypes to 3-open pollinated cultivars from different breeding programs. Allele number ranged from 59-55 or mean allele number ranging 3.68-3.50 were observed. Similarly, Shannon's information index and expected heterozygosity supported the prior result. According to Molecular Variance analysis there was about 83% of intra-specific variation and 17% of inter-specific variation. Overall, high degree of diversity was found within the tested germplasms. Their work suggested that quality improvement of *Brassica* would not lead to any serious threats of loss in performance. Babayeva *et al.* [96] analyzed diversity among 39 genotypes of Lentil (*Lens culinaris Medik.*) from Central Asia and Caucasian through five SSRs markers. They recorded total of 33 alleles ranged from 3 to 8 per primer with genetic variation value of 0.66 for 33 loci. All the accessions were classified into six groups based on cluster analysis using UPGMA with similarity coefficient value of 0.5. The largest cluster was formed by the accessions from Tajikistan indicating high level of genetic diversity. Moreover, SSRs were found efficient for differentiating diversity among *Lentil* crops suggesting that this work could be used to introduce tropical germplasms in breeding programs.

Dikshit *et al.* [97] examined 86 accessions of three species of *Lens* through twelve genomic and thirty-one EST-SSRs markers to evaluate genetic diversity. The tested genotypes were diverse in nature and collected from various regions of India and Mediterranean. They recorded maximum polymorphism through genomic SSRs markers as compared to EST-SSR markers. Highest number of alleles were determined by GLLC. 598 with gene diversity index of 0.80. SSRs effectiveness proved when 43 SSRs detected most alleles *L. orientalis*. They found genetic similarity among tested genotypes via Nei's genetic distance i.e., *L. culinaris Subsp.* was found closer to its predecessor *L. culinaris Subsp. Orientalis*. Maximum level of polymorphism was found among species than among populations.

Bibi *et al.* [98] analyzed 30 stable mutants of Wheat along with parental lines to investigate polymorphism using SSRs marker. They recorded a total of 269 alleles, among which 75.46% were polymorphic. They found moderate level of genetic diversity with Nei's genetic diversity value of 0.165-0.479 and Shannon's index value of 0.23-0.672. Moreover, they also recorded the genetic relatedness within the population of 16.39% with gene flow value of 2.55. However, mutant SE4/12-1 indicated high level of dissimilarity while the mutant SG1/12-41 showed the least. Phylogenetic analysis using UPGMA categorized the genotypes into three main and nine minor groups. In a nutshell, their work indicated the efficiency, effectiveness and feasibility of SSRs markers that could be used as a future experimental tool for assessing genetic diversity.

Abbasov *et al.* [99] used 11 SSRs markers to evaluate the genetic diversity of 139 genotypes of diploid *Triticum* including diverse species namely *Triticum urartu, Triticum boeoticum* and *Triticum monococcum*. SSRs marker-based analysis detected a total of hundred and eleven alleles with ten alleles per locus. Among all the studied genotypes *Triticum Urartu* specie showed maximum level of genetic diversity and DNA fragments i.e., 81. However, regionally Turkish genotypes were found more diverse as compared to Georgian genotypes. Through Cluster analysis, they investigated that highest genetic similarity was between *Triticum boeoticum* and *Triticum monococcum* (0.84) while *Triticum Urartu* and *Triticum monococcum* showed lowest similarity (0.46). They also found some exotic genotypes and suggested that SSRs marker should be used to analyze diversity in einkorn wheat species.

Kumar *et al.* [100] analyzed genetic diversity and DNA fingerprinting through thirty-nine polymorphic SSRs marker in forty-one Indian origin and thirteen mysterious genotypes of Wheat. They generated 112 DNA fragments in whole i.e., 2.87 with each SSRs primer, however, lower level of genetic diversity was observed in total. Their similarity values were between 22.8% to 78.7%. A total of 54 genotypes were clustered into four groups using UPGMA based cluster analysis. Although group D contained the greatest number of genotypes (43) which was further classified into seven sub-groups. The exotic genotypes D-2, D-6 and D-7

indicated high level of similarity with genotypes of Indian origin. Their study demonstrated the potential of SSRs marker to analyze genetic diversity in wheat germplasms.

He *et al.* [101] genotyped 242 worldwide accessions of Alfalfa (*Medicago sativa* L.) using one hundred and two pairs of SSRs markers that were equally distributed on eight chromosomes of Alfalfa genome. They generated a total of 471 alleles with maximum level of polymorphism recorded in perennial wild population of Alfalfa, i.e., 89 markers showed diversity and only thirteen were homozygous. The Unweighted pair group method and Principal coordinates analysis divided 242 accessions into three groups. Molecular variation recorded within the population was 94.94% while among population it was 5.06%. Their results indicated high genetic variation among perennial Alfalfa population as compared to annual Alfalfa populations and maybe, therefore, there was a less gene flow among both of these populations. Tariq *et al.* [102] assessed 63 Rice (*Oryza sativa*) genotypes for salt tolerance through SSRs marker. Already tested genotypes for salt stress through conventional methods were further analyzed with molecular technology using 21 micro satellite markers. Maximum level of polymorphism (90%) was recorded. Furthermore, cluster analysis categorized the genotypes into three groups i.e., tolerant, moderate and sensitive however, phylogenetic analysis combined the tolerant and moderately tolerant genotypes into one group. Their data indicated the dire need of implementing the molecular technology for diversity analysis as proved by the effectiveness of SSRs marker analyzing salt stress. Moreover, this work could help the researchers in genetic breeding programs to introduce tolerant genes of tested genotypes to enhance the yield of rice varieties.

Liu *et al.* [103] analyzed genetic variation in 43 elite clones of Populus deltoides through six SSRs primer pairs. They recorded sixty-two alleles with 10 alleles amplified per primer. The genetic diversity values were between 84 to 94%. Among all the primer pairs, NJFUP-poly02 showed maximum level of polymorphism as it identified 22 out of 43 clones. The primers used in their research were proved to be reliable and accurate for differentiating Populus genotypes. Anyhow, phylogenetic analysis plotted using UPGMA clustered these clones into groups. However, few

clones showed greatest diversion from others in an ascending order. Moreover, they suggested that these clones could be more affected if introduced with gene introgression programs.

Ali *et al.* [104] analyzed the genetic diversity of *Brassica rapa* varieties gathered from separate sites of Khyber Pakhtunkhwa Pakistan. They used SSRs for assessment of genetic variation. Their results were more than excellent and showed that SSRs marker are preferable over other molecular markers for remarkable results of variation among the tested genotypes. The genotypes of Banu, Swat, Kohat and Haripur depicted substantial volume of deviation.

Chapter 3

Materials and Methods

3.1 SDS-PAGE Based Characterization:

3.1.1 Plant Material

The plant material contained 31 local genotypes of *Brassica napus* collected from various areas of Punjab, Pakistan. The detailed information of *B. napus* genotypes characterized through SDS-PAGE is given in Table 3.1.

3.1.2 Protein Extraction

Initially, to extract protein, 10-15 seeds of *Brassica napus* were crammed with the help of grinder and mortar up until fine powder was obtained. The following was steps involved in the protein extraction.

- After this, 0.02g of crushed material was collected in 1.5 ml of centrifugation tube and 400µl protein extraction buffer (0.5M Tris-HCl (pH 8.0).

- 0.2% Sodium dodecyl sulphate (SDS), 5M urea (CH_4N_2O), and 1% 2- mercaptoethanol) was added and appropriately mixed with small glass bar.

- Then, a dye (BPB) was added to protein extraction buffer as an indicator as shown in Table 3.2 to observe the movement of protein in the separation gel.
- The homogeneous mixture was then carefully mixed via vertexing for 1-2 minutes to ensure the extraction and was stored for electrophoresis at -20^{o}C.

3.1.3 Electrophoresis

Polyacrylamide gels are created when acrylamide and bis-acrylamide (N, N'- methylene bis-acrylamide) react, resulting in a highly cross-linked gel matrix. The gel acts as a sieve, allowing proteins to move in response to an electric field. Proteins have an overall positive or negative charge, which allows them to move towards the isoelectric point, where they have no net charge. It is possible to separate proteins based on size as they migrate towards the positive electrode by denaturing them and giving them a uniform negative charge. To run the process of electrophoresis, separation and stacking gels were prepared by properly mixing the chemicals provided in Tables 3.3 to 3.8 following the protocol of Jan *et al.* [105].

- The sample stored in refrigerator was centrifuged at 12000 rpm for about 10 minutes.
- The supernatant was collected after centrifugation an approximately 10µL of each sample was loaded into each well along with protein marker at 100 V.
- The protein motion through the gel was observed carefully and noted down continuously until it reached the bottom of the plates.
- The gels were then transferred to staining solutions and kept there for 2-3 hours on the shaker followed by twice of washing by distilled water.
- The staining procedure was followed by 1-2 days of destaining of the gels in destaining solution and were kept on shaker for 24 hours.

- Composition of staining, destaining and electrode buffer solution shown in Tables 3.9 to 3.11.
- Autoclaved tissue paper was used to eliminate the surplus blue color. Then, the banding pattern of the genotypes was observed.

3.1.4 Data Analysis

The banding patterns of all genotypes were observed. The scoring system of 1/0 was used to score the bands i.e., bands that are clear were scored 1 while absence of band were indicated through 0. The genomic similarity coefficients were assessed by method of Nei and Li [106].

Then the Dendrogram was constructed using UPGMA method [107]. To analyze the data, NTSYS-pc, version 2.1 (Applied Biostatistics Inc., USA) software were used. The two-dimensional (2D) and three-dimensional (3D) studies were carried out using PCoA for observing genotype dispersal by way of NTSYS pc, version 2.1 software [108].

TABLE 3.1: Detail of *Brassica napus* genotypes selected for SDS-PAGE analysis (n=31).

Sr. No.	**Acc. Code**	**Origin**
1.	FaiBn201	Faisalabad, Punjab, Pakistan.
2.	FaiBn203	Faisalabad, Punjab, Pakistan.
3.	FaiBn204	Faisalabad, Punjab, Pakistan.
4.	MuzBn207	Muzaffargarh, Punjab, Pakistan.
5.	MuzBn210	Muzaffargarh, Punjab, Pakistan.
6.	MuzBn211	Muzaffargarh, Punjab, Pakistan.
7.	DerBn215	Dera Ghazi Khan, Punjab, Pakistan.
8.	DerBn217	Dera Ghazi Khan, Punjab, Pakistan.
9.	LayBn220	Layyah, Punjab, Pakistan.
10.	LayBn222	Layyah, Punjab, Pakistan.
11.	JamBn225	Jampur, Punjab, Pakistan.

TABLE 3.1: Detail of *Brassica napus* genotypes selected for SDS-PAGE analysis (n=31).

Sr. No.	**Acc. Code**	**Origin**
12.	JamBn227	Jampur, Punjab, Pakistan.
13.	SadBn230	Sadiqabad, Punjab, Pakistan.
14.	SadBn231	Sadiqabad, Punjab, Pakistan.
15.	NorBn233	Narowal, Punjab, Pakistan.
16.	NorBn235	Narowal, Punjab, Pakistan.
17.	NorBn237	Narowal, Punjab, Pakistan.
18.	MulBn240	Multan, Punjab, Pakistan.
19.	MulBn241	Multan, Punjab, Pakistan.
20.	MulBn243	Multan, Punjab, Pakistan.
21.	ChaBn245	Chakwal, Punjab, Pakistan.
22.	ChaBn247	Chakwal, Punjab, Pakistan.
23.	ChaBn249	Chakwal, Punjab, Pakistan.
24.	SheBn250	Sheikhupura, Punjab, Pakistan.
25.	SheBn251	Sheikhupura, Punjab, Pakistan.
26.	SheBn253	Sheikhupura, Punjab, Pakistan.
27.	LhrBn255	Lahore, Punjab, Pakistan.
28.	LhrBn258	Lahore, Punjab, Pakistan.
29.	OkaBn260	Okara, Punjab, Pakistan.
30.	OkaBn261	Okara, Punjab, Pakistan.
31.	A. Canola	NARC, Islamabad, Pakistan.

TABLE 3.2: Composition of Extraction buffer.

Ingredients	**Concentration**
SDS	0.2g
CH_4N_2O	30.3g
d. H_2O	About 70 ml
HCl	Adjust pH to 8.0
2-Mercaptoethanol	1 ml

Table 3.2: Composition of Extraction buffer.

Ingredients	**Concentration**
Tris (hydroxymethyl) aminomethane (THAM)	0.6057 g
Total volume	100ml

A very minute amount of Bromophenol blue (BPB) was added after these ingredients then the solution was stored in the refrigerator.

Table 3.3: Composition of sol. A

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

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

Table 3.4: Composition of sol. B

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

Kept in freezer at -4^{o}C

Table 3.5: Composition of sol. C

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

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

Table 3.6: Composition of APS

Ingredients	**Amounts**
APS	0.2g
Distilled water	1ml

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

Table 3.7: Composition of separation gel

Ingredients	**Amounts**
d. 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**
Distilled water	6.0ml
Solution B	3ml
Solution C	2ml
10% APS	100 µl
TEMED	50 µl

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

Table 3.9: Composition of electrode buffer solution

Ingredients	**Amounts**
Distilled water	1000ml
Tris (hydroxtmethyl) aminomethane	3.2g
SDS (Sodium dodecyl sulphate)	1.3g
Glycine	14g

Kept at room temperature

Table 3.10: Composition of staining solution

Ingredients	**Amounts**
d. H_2O	470ml
CH_3COOH	70ml
CH_3OH	460ml
CBB R250	2.10g

Kept at room temperature.

Table 3.11: Composition of destaining solution.

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

Kept at room temperature.

3.2 *Brassica napus* Diversity Evaluation Based on SSRs Marker

3.2.1 Material

A 31 distinct genotypes of *Brassica napus* were collected from different locations around the Punjab, Pakistan. Among these 31, A. Canola was a check genotype. The genetic diversity of these genotypes was examined using the 10 SSRs markers unique to this crop. The thorough data of diverse *Brassica napus* genotypes and SSRs marker used is given in Tables 3.12- 3.13.

3.2.2 Sample Preparation

In little pots, the 4-5 fresh seeds of each genotype were planted with frequent watering after two days. After germination about two to three weeks, samples of fresh leaves were collected. The leaves were kept in a freezer at -80°C.

3.2.3 DNA Extraction

All the stock solutions were prepared prior to DNA extraction and the DNA was extracted via CTAB method Sika *et al.* [109]. The DNA extraction steps were as follows:

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

- All samples were kept in the water bath for 40 minutes at 65 °C, rotating over four times every five minutes.

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

- Each DNA sample's quality and purity was examined using a Nano Drop ND-1000 Spectrophotometer at 260 and 280 nm.
  To obtain accurate PCR findings, all DNA samples were diluted to a working concentration of 20 mg/µl.

3.2.4 Specification of the Primers

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

3.2.5 Amplification of SSRs Marker in *Brassica napus* Genotypes

The PCR conditions were optimized based on available data for each primer, with minor changes to the annealing temperature. The detailed information of the 20µl PCR reaction volume is in Table 3.13. Agarose concentrations varied depending on the size of the primer. To obtain clear PCR bands, 2 to 3 % agarose gels were typically used. The high-resolution agarose gel was prepared 1xTBE buffer (Tris-Borate = 10mM and EDTA = 1 mM) with the addition of 5µl ethidium bromide. The optimum condition for different PCR steps is given in Table 3.15.

3.2.6 Electrophoresis of Amplified Products

After PCR 4 µl of 6x loading dye was added to PCR tubes.

- In each well, the 7 µl PCR sample was placed.
- To verify the exact size of SSRs markers, a DNA ladder of 50 and 100 bp was used.
- Under UVI Gel Doc Documentation System, the gels were seen, and the desired PCR product sizes were noted. In that it will represented in the form of bands.

3.2.7 Allele Scoring and Data Analysis

For each sample, the amplified banding pattern for each primer was recorded in the presence of a single, two, or multiple bands. Band presence was indicated by 1 and band absence by 0. All the data was recorded in MS-excel. The intensity and clarity were also considered.

For data analysis, only clear DNA bands were used. For each primer, the overall presence of alleles, the total number of polymorphic alleles, and the optimal annealing temperature were recorded. The Pairwise comparisons of *B. napus* genotypes as a result of presence or absence of alleles present were used to calculate genetic similarity coefficients by the Dice algorithm Nei and Li [106].

The values of similarity coefficients were used to construct a genetic tree, 2D and 3D based on UPGMA, NTSYS pc, version 2.1, was used for this purpose and for 2D and 3D analysis [107] and [108].

TABLE 3.12: List of *B. napus* genotypes used for SSRs analysis (n=31)

Sr. No.	**Genotypes**	**Source**
1.	MulBn240	Multan, Punjab
2.	OkaBn260	Okara, Punjab
3.	MulBn241	Multan, Punjab
4.	SheBn250	Sheikhupura, Punjab
5.	ChkBn245	Chakwal, Punjab
6.	ChkBn247	Chakwal, Punjab
7.	ChkBn249	Chakwal, Punjab
8.	LhrBn255	Lahore, Punjab
9.	LhrBn258	Lahore, Punjab
10.	LayBn220	Layyah, Punjab
11.	LayBn222	Layyah, Punjab
12.	JamBn225	Jampur, Punjab
13.	DerBn215	D. G. Khan, Punjab

TABLE 3.12: List of *B. napus* genotypes used for SSRs analysis (n=31)

Sr. No.	Genotypes	Source
14.	DerBn217	D. G. Khan, Punjab
15.	JamBn227	Jampur, Punjab
16.	SadBn230	Sadiqabad, Punjab
17.	SadBn231	Sadiqabad, Punjab
18.	SheBn253	Sheikhupura, Punjab
19.	NorBn233	Narowal, Punjab
20.	NorBn235	Narowal, Punjab
21.	NorBn237	Narowal, Punjab
22.	MuzBn207	Muzaffargarh, Punjab
23.	MuzBn210	Muzaffargarh, Punjab
24.	FaiBn201	Faisalabad, Punjab
25.	FaiBn203	Faisalabad, Punjab
26.	FaiBn204	Faisalabad, Punjab
27.	SheBn251	Sheikhupura, Punjab
28.	MulBn243	Multan, Punjab
29.	OkaBn261	Okara, Punjab
30.	MuzBn211	Muzaffargarh, Punjab
31.	A. canola	PGRI, Islamabad

TABLE 3.13: SSRs marker for diversity evaluation of *B. napus* genotypes

Sr No.	Primer	Forward primer (bp)	Reverse primer (bp)
1.	Na10-D03	ATGATTTGCCT TGAAATGCC	GATGAAACAAT AACCTGAGACA CAC
2.	Na10-G08	TTTCTTTTAACC TGATGTTTTGG	TCACTGTGTT TACTTGCGCC

TABLE 3.13: SSRs marker for diversity evaluation of *B. napus* genotypes

Sr No.	**Primer**	**Forward primer (bp)**	**Reverse primer (bp)**
3.	Na12-H02	CTATGGTTCATCTTTCGCCG	GCTGCACATCCATCTCTCG
4.	O110-B01	CCTCTTCAGTCGAGGTCTGG	AATTTGGAAACAGAGTCGCC
5.	O110-F12	TCCATGTTTCATGTTGGAGG	CTCTCCGGCTTCACTTTCC
6.	PBCESSRJU10	GCGGCGTAGGTACTGGAG	AGCCATCGAGCCATTCAG
7.	PBCESSRJU15	GGATCTCATGTTCACTGCTG	TGATTACATACCAAATATGAG
8.	PBCESSRNA3	ATCCCTTCTCACAGGTTTACT	GTCAAGTTTCTCTCCACACC
9.	PBCESSRNA8	ACTGAGAGCAACAACAACAAC	GTAGAGACGGAACCCTGA
10.	PBCESSRNA18	TTAAAATGAAACCCACCCGA	TGTTGGGCAACATCCATTTA

Table 3.14: Microsatellite PCR analysis (reaction mix)

Components	**Stock concetra -tion**	**Final concetra -tion**	**Vol/ Rxn**	**Samples**	**Total Vol.**
ddH_2O	-	-	10.7µl	x31	331.7µl
PCR Buffer plus 2mM MgCl2	10x	1 x	2.0µl	x31	62.0µl
dNTP Mixture	100 mM Each	2mM	2µl	x31	62µl
Forward Primer	20 pmoles/µl (20µM)	0.8 µM	0.8µl	x31	24.8µl
Reverse Primer	20 pmoles/µl (20µM)	0.8 µM	0.8µl	x31	24.8µl
Taq DNA Polymerase	5 Units/µl	1 unit/r x n	0.2µl	x31	6.2µl
Template DNA	20-50 ng/µl	20-50 ng/r x n	1.0µl	-	-
Total Volume	-	-	20.0µl	-	-

Table 3.15: PCR thermal cycler profile

Profile	**Temperature °C**	**Time**	**No. of Cycles**
Initial stand seperation	95	5 minutes	1
Denaturation	95	30 seconds	-
Annealing	55-60	30 seconds	35
Initial Extension	72	2 minutes	-
Final Extension	72	7 minutes	1

Chapter 4

Results and Discussions

4.1 Total Seed Storage Protein-based Variation in *B. napus* Genotypes through SDS-PAGE

Biochemical characterization has been used extensively by researchers for genomic improvements of *Brassica* species. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis is a reliable, efficient and cost-effective method for this purpose and has replaced many conventional assessment methods. Genetic diversity among species and phylogenetic relationships have been reported by using this technique but still, there is a limited knowledge known about *Brassica* variants of Punjab, Pakistan. The reason behind using SDS-PAGE system is because of its nature of separating protein subunits according to their molecular weight. A molecular marker comprising of weight of known proteins is used in this technique that determines the molecular weight of unknown proteins.

4.1.1 Genetic Diversity Anticipated through Total Seed Storage Protein-based Bands Analysis

In this experiment, thirty-one genotypes of *B. napus* were used to estimate the genetic diversity through SDS-PAGE. Maximum level of heterogeneity was observed

for tested genotypes. Almost all the genotypes showed polymorphism.

Protein band profile is shown in Figures 4.1 to 4.3. The genotypes used illustrated the bands of size ranged from 10 kDa to 180 kDa. A total of fourteen bands were recorded and about 92. 85% were polymorphic.

On the basis of their molecular weight the polypeptide bands were divided into four regions A, B, C and D as shown in Figures 4.1 to 4.3. Region 'A' contained high molecular weight proteins ranged from ∼130 to ∼180 kDa. Both polymorphic and monomorphic bands were present in this region. Similarly, region 'B' included proteins that were ranged from ∼100 to ∼130 kDa. Region 'C' contained medium size proteins which were ranged from ∼40 to ∼70 kDa. Lastly, region 'D' was the smaller polypeptides containing region with molecular weights of proteins ranged from ∼10 to ∼20 kDa Figures 4.1 to 4.3.

4.1.2 Similarity Co-efficient of *Brassica napus* Genotypes:

By following the standard protocols of Nei and Li [106], genetic diversity was estimated in thirty-one genotypes of *Brassica napus* through SDS-PAGE technique. All the genotypes tested were measured for similarity coefficient that was ranged from 26% to 95.24% as shown in Table 4.1.2. Highest similarity 95.24% was noted between OkaBn261 and SheBn253 accessions that indicated a substantial correlation among these genotypes. A significant level of similarity 94.74% was also noted between accessions OkaBn261 and FaiBn204, 91.67% between ChaBn245 and MuzBn207, SheBn251 and MuzBn207, 90.91% between ChaBn245 and MuzBn210, SheBn251 and ChaBn245, and 90% similarity between FaiBn204 and FaiBn201, NorBn235 and FaiBn201, ChaBn245 and DerBn217, ChaBn247 and MuzBn210, SheBn251 and SheBn250 and SheBn251 and DerBn217 was recorded among these tested genotypes.

However, low level of similarity 33.33% was also observed between accessions MulBn241 and JamBn225, 36.36% between MulBn241 and JamBn227, 37.50% between MulBn241 and MuzBn210, 40% between MulBn240 and DerBn217, MulBn

241 and SadBn231, MulBn243 and MulBn240, 42.86% between MulBn240 and LayBn 220, 46.15% between JamBn227 and JamBn225 and 47.06% between NorBn 233 and FaiBn204, ultimately, a very unique type of genotype MulBn241 was observed for its distinct behavior as it showed low level of similarity with almost all the genotypes. The least similarity coefficient values of 26% were between accessions SheBn250 and JamBn227. The results justified the variation in protein-based analysis via varying genotypes area to area.

4.1.3 Cluster Analysis

In the present study, Unweighted Pair Group Method with Arithmetic Averages was used to study the phylogenetic relationship among thirty-one *Brassica napus* genotypes. All the 31 genotypes were divided into seven clusters. All the Cluster I-VII contained 5, 10, 8, 1, 1, 4 and 2 genotypes, respectively. Cluster I contained a total of 5 genotypes, i.e., FaiBn201, NorBn235, SadBn230, SadBn231 and OkaBn260 Figure 4.4 and Table 4.1. The largest genotypes comprising group was Cluster II which included 10 genotypes that were FaiBn204, SheBn253, OkaBn261, MuzBn207, ChaBn245, SheBn251, DerBn217, A. Canola, MulBn243 and SheBn250. Cluster III contained 8 genotypes. Genotypes in Cluster III were FaiBn203, LayBn220, LayBn222, MuzBn210, ChaBn247, DerBn215, LhrBn255 and LhrBn258. Both the accessions from Lahore and Layyah lied in same group indicating that these two are in close relationship with each other. Cluster IV and V included only 1 diverse genotype each i.e., JamBn225 and ChaBn249, respectively, that indicated no similarity at all with any of the genotypes tested. Cluster VI contained low number of genotypes as compared to the first three clusters i.e., only four genotypes that were MuzBn211, NorBn233, JamBn227 and NorBn237.

Lastly, Cluster VII was also the minimum number containing group as it had only 2 genotypes that were also found unique and distinct from all other genotypes. Cluster VII genotypes were MulBn240 and MulBn241. All the groups were polymorphic. Genotypes MulBn240, MulBn241, JamBn225 and ChaBn249 of Cluster IV, V and VII indicated some exceptional kind of behavior therefore,

further analysis of all the genotypes through 2D and 3D system was observed for precise detection of diverse genotypes. Genotypes having similarity occupied the same groups. It indicated high level of seed protein-based diversity among *Brassica napus* genotypes.

4.1.4 PCoA Analysis

PCoA is a multivariate technique for identifying individuals based on genetic distance. To assess the genetic diversity of the 31 different canola genotypes more clearly, we used the DICE similarity coefficient matrix to distribute all genotypes into 2D and 3D scatterplots. PCoA-based 2D and 3D investigations show clear differences in each dimension. 2D and 3D studies can distinguish unique genotypes in vast populations. In this research, 2D plot categorized the 31 tested genotypes into four major groups. Diverse genotypes were observed in all groups, but some unique genotypes were seen on the peripheries that were SheBn250, FaiBn201, JamBn227, MulBn241, MulBn240 and NorBn237. These genotypes were highly diverged from the rest of genotypes Figure 4.5. Three-dimensional analysis further assessed genotypes from any other directions. The 3D analysis also indicated unique genotypes that were MulBn241 and MulBn240 Figure 4.6. Vector analysis is also shown in Figure 4.6.

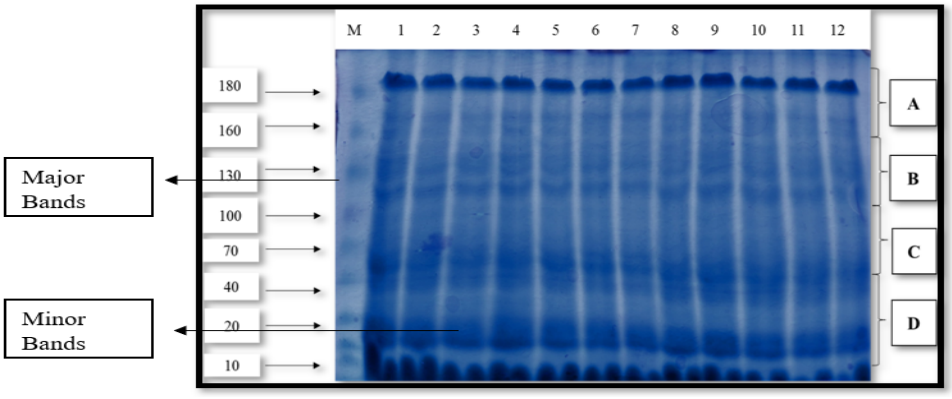

FIGURE 4.1: Electrophoretic banding pattern of *B. napus* genotypes generated through SDS-PAGE of total seed storage proteins 1-12. 'M' represents the molecular size marker, while numbers 1-12 indicate accessions, Bn201, Bn203, Bn204, Bn207, Bn210, Bn211, Bn215, Bn217, Bn220, Bn222, Bn225 and Bn227, respectively.

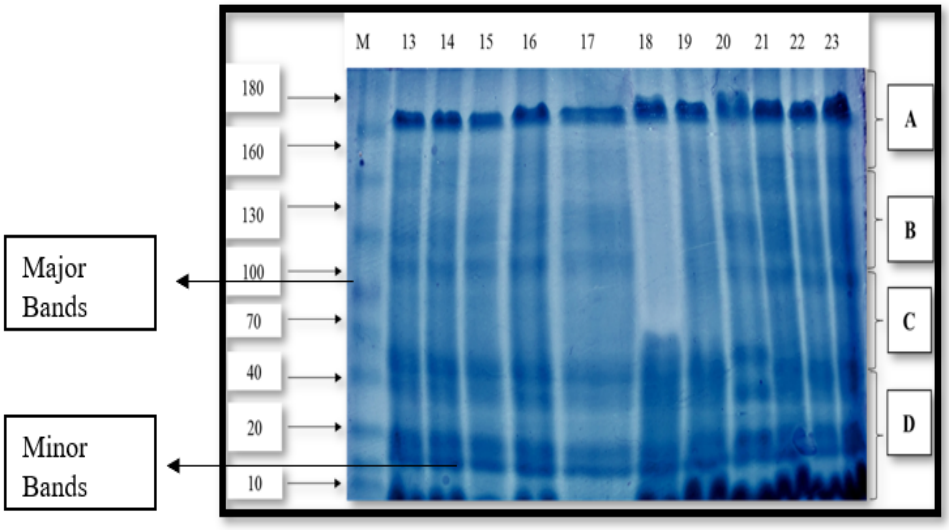

FIGURE 4.2: Electrophoretic banding pattern of *B. napus* genotypes generated through SDS-PAGE of total seed storage proteins 13-23.

The above figure 4.2 'M' represents the molecular size marker, while numbers 13-23 indicate accessions, Bn230, Bn231, Bn233, Bn235, Bn237, Bn240, Bn241, Bn243, Bn245, Bn247 and Bn249, respectively.

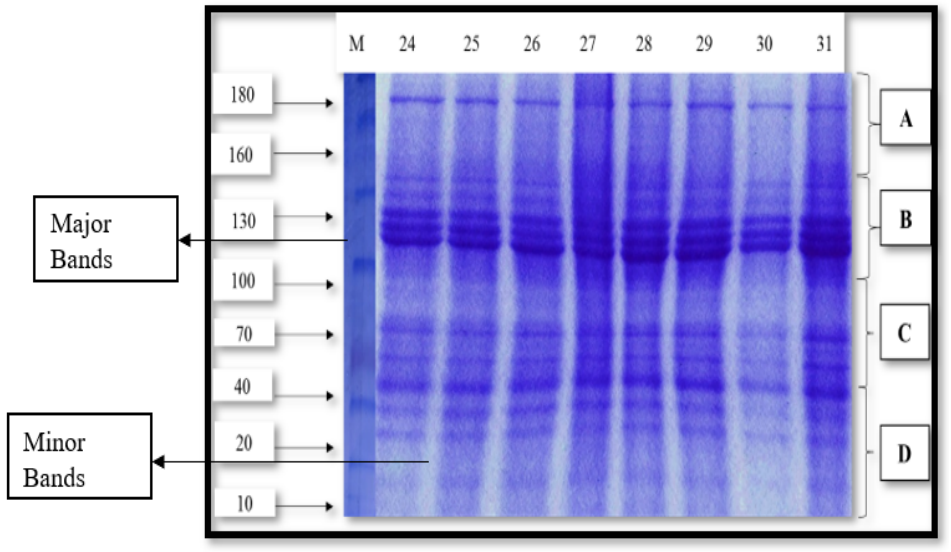

FIGURE 4.3: Electrophoretic banding pattern of *B. napus* genotypes generated through SDS-PAGE of total seed storage proteins 24-31.

The above figure 4.3 'M' represents the molecular size marker, while numbers 24-31 indicate accessions, Bn250, Bn251, Bn253, Bn255, Bn258, Bn260, Bn261 and A. Canola, respectively.

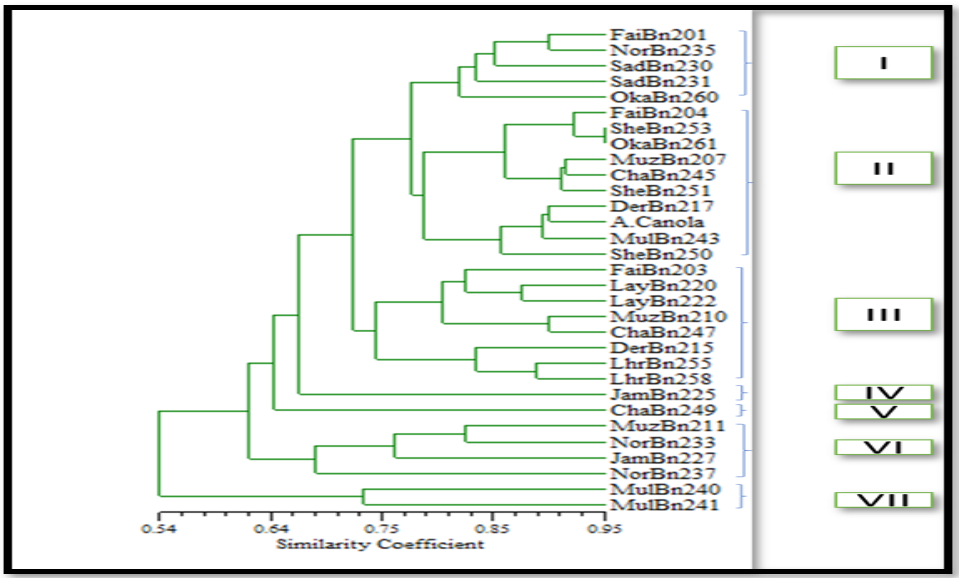

FIGURE 4.4: SDS-PAGE based diversity in 31 genotypes of *B. napus* using cluster analysis.

TABLE 4.1: Clusters of 31 *B. napus* genotypes based on SDS-PAGE method

Cluster	**No. of genotypes**	**Genotypes**
I.	5	FaiBn201, NorBn235, SadBn230, SadBn231 OkaBn260.
II.	10	FaiBn204, SheBn253, OkaBn261, MuzBn207, ChaBn245, SheBn251, DerBn217, A. Canola, MulBn243 SheBn250 MulBn243

TABLE 4.1: Clusters of 31 *B. napus* genotypes based on SDS-PAGE method

Cluster	No. of genotypes	Genotypes
III.	8	FaiBn203, LayBn220, LayBn222, MuzBn210, ChaBn247, DerBn215, LhrBn255 LhrBn258.
IV.	1	JamBn225.
V.	1	ChaBn249.
VI.	4	MuzBn211, NorBn233, JamBn227, NorBn237.
VII.	2	MulBn240 MulBn241.

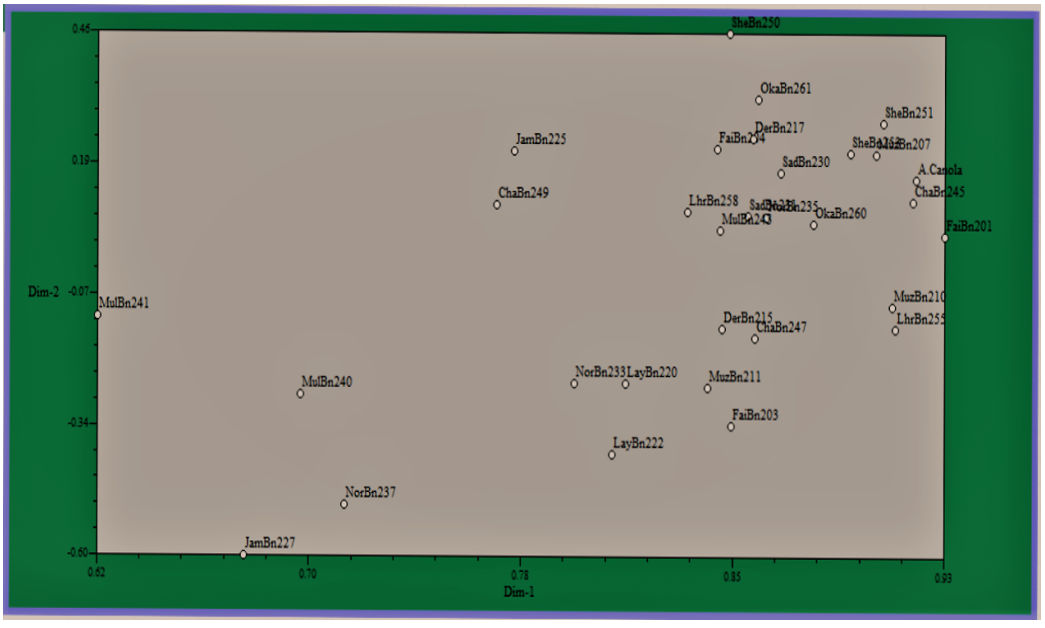

FIGURE 4.5: 2D analysis of 31 genotypes of *B. napus* based on SDS-PAGE.

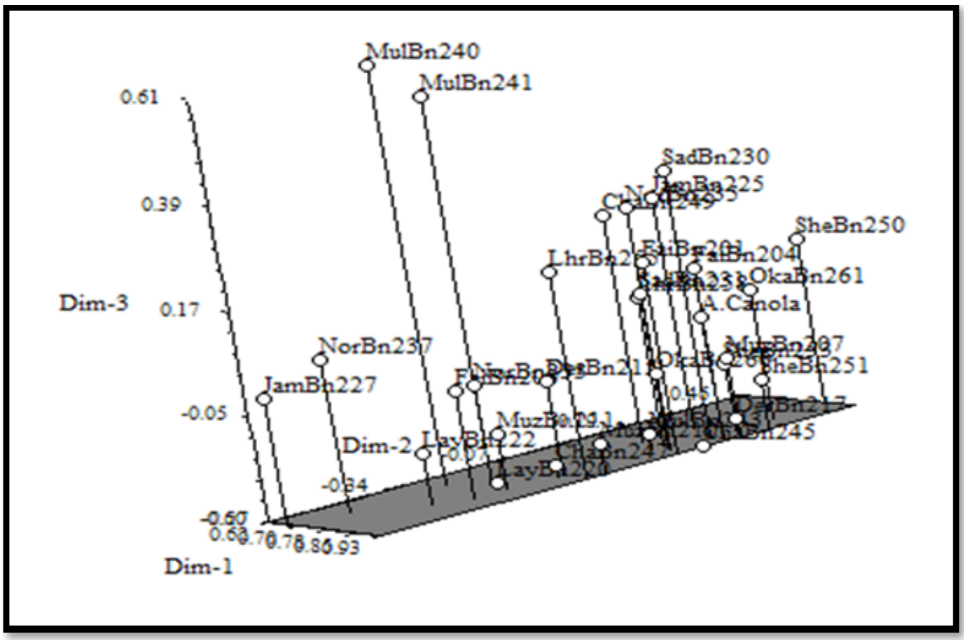

FIGURE 4.6: 3D representation of 31 genotypes of *B. napus* based on SDS-PAGE.

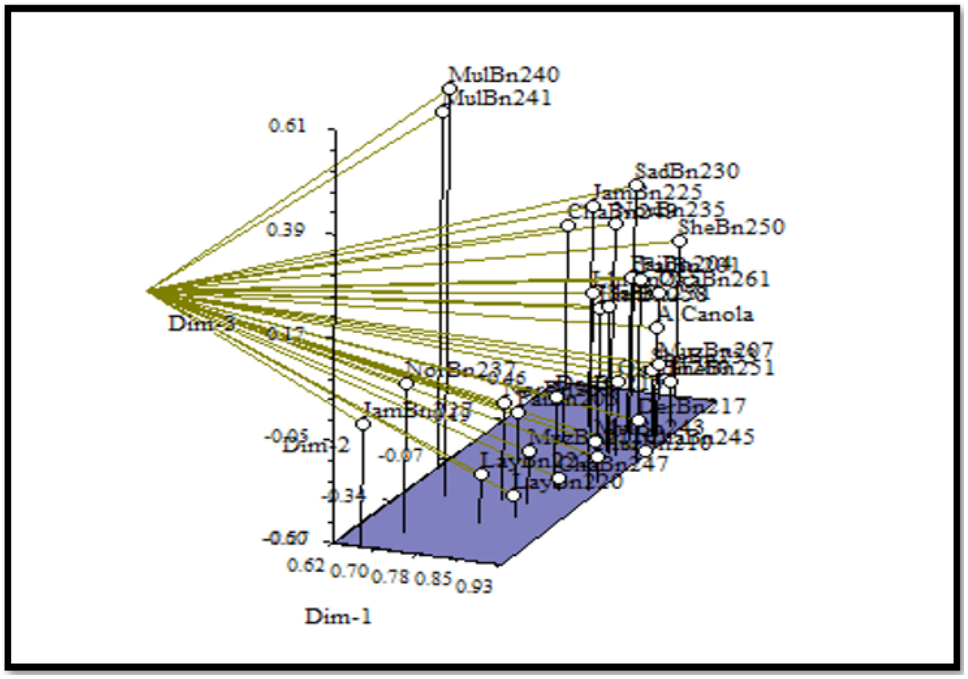

FIGURE 4.7: 2D and 3D vector analysis of 31 genotypes of *Brassica napus*.

TABLE 4.2: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SDS-PAGE analysis.

Acc	**FaiBn 201**	**FaiBn 203**	**FaiBn 204**	**MuzBn 207**	**MuzBn 210**	**MuzBn 211**	**DerBn 215**	**DerBn 217**
FaiBn201	1.00							
FaiBn203	0.80	1.00						
FaiBn204	0.90	0.67	1.00					
MuzBn207	0.83	0.73	0.82	1.00				
MuzBn210	0.82	0.80	0.70	0.83	1.00			
MuzBn211	0.80	0.78	0.67	0.73	0.80	1.00		
DerBn215	0.80	0.78	0.67	0.73	0.70	0.78	1.00	
DerBn217	0.70	0.67	0.67	0.82	0.80	0.67	0.67	1.00
LayBn220	0.74	0.82	0.71	0.67	0.74	0.71	0.82	0.71
LayBn222	0.74	0.82	0.71	0.67	0.84	0.71	0.71	0.59
JamBn225	0.78	0.50	0.75	0.70	0.67	0.63	0.63	0.63
JamBn227	0.59	0.80	0.40	0.53	0.71	0.80	0.67	0.40
SadBn230	0.86	0.74	0.74	0.78	0.76	0.63	0.63	0.74
SadBn231	0.86	0.63	0.74	0.78	0.86	0.74	0.74	0.63
NorBn233	0.63	0.71	0.47	0.67	0.74	0.82	0.71	0.71
NorBn235	0.90	0.67	0.78	0.73	0.70	0.78	0.78	0.67
NorBn237	0.59	0.67	0.53	0.53	0.71	0.67	0.53	0.53

TABLE 4.2: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SDS-PAGE analysis.

Acc	**FaiBn 201**	**FaiBn 203**	**FaiBn 204**	**MuzBn 207**	**MuzBn 210**	**MuzBn 211**	**DerBn 215**	**DerBn 217**
MulBn240	0.71	0.67	0.53	0.53	0.59	0.53	0.53	0.40
MulBn241	0.63	0.57	0.57	0.44	0.38	0.43	0.57	0.43
MulBn243	0.70	0.67	0.56	0.73	0.80	0.78	0.78	0.89
ChaBn245	0.82	0.80	0.80	0.92	0.91	0.80	0.70	0.90
ChaBn247	0.80	0.78	0.67	0.73	0.90	0.78	0.78	0.78
ChaBn249	0.63	0.59	0.59	0.76	0.74	0.59	0.47	0.71
SheBn250	0.80	0.56	0.78	0.82	0.70	0.56	0.67	0.89
SheBn251	0.82	0.70	0.80	0.92	0.82	0.70	0.80	0.90
SheBn253	0.82	0.70	0.90	0.92	0.82	0.70	0.70	0.80
LhrBn255	0.86	0.84	0.74	0.78	0.76	0.74	0.84	0.74
LhrBn258	0.74	0.71	0.71	0.76	0.63	0.59	0.82	0.82
OkaBn260	0.87	0.76	0.76	0.88	0.87	0.76	0.67	0.76
OkaBn261	0.86	0.63	0.95	0.87	0.76	0.63	0.63	0.74
A. Canola	0.82	0.70	0.70	0.83	0.82	0.70	0.80	0.90

TABLE 4.3: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SDS-PAGE analysis.

Acc	**LayBn220**	**LayBn222**	**JamBn225**	**JamBn227**	**SadBn230**	**SadBn 231**	**NorBn 233**	**NorBn 235**
LayBn 220	1.00							
LayBn 222	0.88	1.00						
JamBn 225	0.40	0.53	1.00					
JamBn 227	0.57	0.71	0.46	1.00				
SadBn 230	0.56	0.56	0.71	0.50	1.00			
SadBn 231	0.56	0.67	0.82	0.63	0.80	1.00		
NorBn 233	0.63	0.63	0.53	0.71	0.67	0.67	1.00	
NorBn 235	0.59	0.59	0.88	0.53	0.84	0.84	0.71	1.00

TABLE 4.3: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SDS-PAGE analysis.

Acc	**LayBn220**	**LayBn222**	**JamBn225**	**JamBn227**	**SadBn230**	**SadBn 231**	**NorBn 233**	**NorBn 235**
NorBn 237	0.71	0.86	0.46	0.67	0.50	0.50	0.71	0.53
MulBn 240	0.43	0.57	0.62	0.67	0.75	0.63	0.57	0.67
MulBn 241	0.62	0.46	0.33	0.36	0.67	0.40	0.46	0.57
MulBn 243	0.71	0.59	0.63	0.53	0.74	0.74	0.82	0.78
ChaBn 245	0.74	0.74	0.67	0.59	0.76	0.76	0.74	0.70
ChaBn 247	0.82	0.82	0.63	0.67	0.63	0.74	0.59	0.67
ChaBn 249	0.38	0.50	0.67	0.57	0.78	0.67	0.63	0.59
SheBn 250	0.59	0.47	0.75	0.27	0.84	0.74	0.59	0.78

Table 4.3: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SDS-PAGE analysis.

Acc	**LayBn220**	**LayBn222**	**JamBn225**	**JamBn227**	**SadBn230**	**SadBn 231**	**NorBn 233**	**NorBn 235**
SheBn 251	0.74	0.63	0.67	0.47	0.76	0.76	0.63	0.70
SheBn 253	0.74	0.74	0.67	0.47	0.76	0.76	0.63	0.70
LhrBn 255	0.78	0.78	0.71	0.63	0.80	0.70	0.78	0.84
LhrBn 258	0.75	0.63	0.67	0.43	0.67	0.56	0.63	0.71
OkaBn 260	0.70	0.70	0.63	0.56	0.82	0.82	0.70	0.76
OkaBn 261	0.67	0.67	0.71	0.38	0.80	0.80	0.56	0.74
A. Canola	0.74	0.63	0.67	0.47	0.86	0.76	0.74	0.80

TABLE 4.4: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SDS-PAGE analysis.

Acc	**NorBn237**	**MulBn240**	**MulBn241**	**MulBn243**	**ChaBn245**	**ChaBn247**	**ChaBn249**	**SheBn250**
NorBn237	1.00							
MulBn240	0.67	1.00						
MulBn241	0.55	0.73	1.00					
MulBn243	0.53	0.40	0.43	1.00				
ChaBn245	0.59	0.47	0.38	0.80	1.00			
ChaBn247	0.67	0.53	0.43	0.78	0.80	1.00		
ChaBn249	0.57	0.71	0.46	0.59	0.74	0.59	1.00	
SheBn250	0.40	0.53	0.57	0.78	0.80	0.67	0.71	1.00
SheBn251	0.47	0.47	0.50	0.80	0.91	0.80	0.74	0.90
SheBn253	0.59	0.47	0.50	0.70	0.91	0.70	0.74	0.80
LhrBn255	0.75	0.75	0.67	0.74	0.76	0.74	0.67	0.74
LhrBn258	0.57	0.57	0.62	0.71	0.74	0.71	0.63	0.82
OkaBn260	0.56	0.56	0.47	0.76	0.87	0.76	0.60	0.76
OkaBn261	0.50	0.50	0.53	0.63	0.86	0.63	0.67	0.84
A. Canola	0.59	0.59	0.63	0.90	0.82	0.80	0.74	0.90

TABLE 4.5: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SDS-PAGE analysis.

Acc	**SheBn 251**	**SheBn 253**	**LhrBn 255**	**LhrBn 258**	**OkaBn 260**	**OkaBn 261**	**A.Canola**
SheBn 251	1						
SheBn 253	0.91	1					
LhrBn 255	0.76	0.76	1				
LhrBn 258	0.84	0.74	0.89	1			
OkaBn 260	0.78	0.78	0.73	0.6	1		
OkaBn 261	0.86	0.95	0.7	0.67	0.82	1	
A. Canola	0.91	0.82	0.86	0.84	0.78	0.76	100

4.2 Microsatellite Based Inter-specific Variability Among *Brassica napus* Genotypes

A total of thirty-one (31) diverse *B. napus* genotypes were selected for SSRs based molecular analysis. Relative to 31 genotypes tested ten (10) microsatellite markers were used, obtained from previous literature. All the markers indicated maximum polymorphic banding pattern.

4.2.1 Inter-specific Variation Among *B. napus* Genotypes

For analyzing genomic variability in 31 genotypes of *B. napus* 10 SSRs markers were used. For this purpose, a molecular marker of known size was used to compare

the sizes of fragments obtained using these ten SSRs markers. The amplified fragments were noted down and a total of 12 alleles were obtained. The variability in number of alleles per primer was from 1-3. Almost all the primers showed polymorphic banding patterns Figures 4.9 - 4.12. Most of these primers amplified fragments ranged from 100-480 bp. Overall, 9 primers amplified 1 allele in all the tested *B. napus* genotypes except for one primer (PBCESSRNA3) that amplified 3 alleles Table 4.7.

4.2.2 Genomic Similarity and Phylogenetic Analysis

The genomic similarity ranged 0 to 100% was calculated among 31 genotypes of *B. napus*. The similarity value of 0% was recorded between genotypes of ChaBn249 and FaiBn201, SheBn250 and MuzBn211, SheBn253 and LayBn222, LhrBn255 and LayBn222, MulBn240 and FaiBn201, OkaBn260 and SadBn230, OkaBn261 and MuzBn207, A. Canola and LayBn222 etc. Among all these genotypes OkaBn260 showed maximum level of variation with ten genotypes. Minimum value of genetic similarity 0.00% was followed by 16.06% among OkaBn260 and LhrBn258 followed by 18.18% among genotypes OkaBn260 and JamBn227. Similarity of 18.18% was followed by 20% between genotypes SadBn230 and FaiBn201, SadBn231 and FaiBn201, NorBn237 and DerBn215, ChaBn247 and FaiBn201, ChaBn249 and LhrBn258, SheBn253 and NorBn235, MulBn240 and LayBn220, OkaBn260 and FaiBn203, A. canola and LayBn220 etc. This was followed by 93.33% between genotypes MulBn241 and LayBn220 and MulBn243 and JamBn227. The maximum similarity value of 100% was between genotypes DerBn217 and DerBn215. Results justified high level of genetic diversity among all the genotypes even though belongs to same origin Table 4.8.

All the 31 genotypes were categorized into five (5) main clusters based on UPGMA similarity analysis. The Clusters I to V had 6, 6, 9, 5 and 5 genotypes, respectively. The cluster I consisted of 6 genotypes i.e., FaiBn201, MuzBn210, MuzBn211, DerBn215, DerBn217 and LayBn222. Cluster II was also comprised of 6 genotypes namely FaiBn203, FaiBn204, MuzBn207, LayBn220, MulBn241 and LhrBn258.

However, cluster III was the highest genotypes containing group with 9 genotypes that were SadBn230, ChaBn245, JamBn227, MulBn243, ChaBn247, JamBn225, NorBn237, LhrBn255 and SheBn250. The genotypes of group III were found diverse. Cluster IV included 5 genotypes of NorBn233, SadBn231, NorBn235, SheBn251 and A. canola (Check variety).

Moreover, cluster V contained 5 genotypes also, but all the genotypes of group V were highly diverse and showed maximum level of variance than others. The genotypes belonging to cluster V were OkaBn261, SheBn253, ChaBn249, OkaBn260 and MulBn240. The results validated the high level of similarity among all the tested genotypes however, high degree of genetic variation among all the genotypes was also observed. These results could be used for future genomic variability study of *B. napus* Figure 4.8. and Table 4.6.

4.2.3 PCoA Analysis

To analyze the genetic diversity more clearly the modern techniques of 2D and 3D analysis are being used to investigate the most diverse genotypes. The method as a whole is known as Principal Coordinate Analysis (PCoA). It is a statistical tool that converts data on distance bases between items into a map-based visualization. It helps in better understanding of items which are closely related to each other and allows to identify groups or clusters. Although Clustering is somehow similar to PCoA, but clustering is based on similarity indices while PCoA works on the principle of both *dis* (similarity). However, PCoA tries to generate a 2D and tri-dimensional map while clustering merely groups data points. Also, PCoA focuses on distance and extract the dimensions that account for maximum distance.

In our findings of PCoA analysis, the 2D plot categorized the 31 genotypes into six major groups. Diverse genotypes were recorded in all of the groups. However, on the outskirts of 2D plot some unique genotypes were also observed like LayBn222, MuzBn211, LhrBn258, OkaBn260 and LhrBn255. These genotypes showed divergence from the rest (Figure 4.13). The genotypes were further visualized from

other directions through 3D analysis. It also indicated some unique genotypes such as MulBn241, LayBn220 and SheBn250 (Figure 4.14).

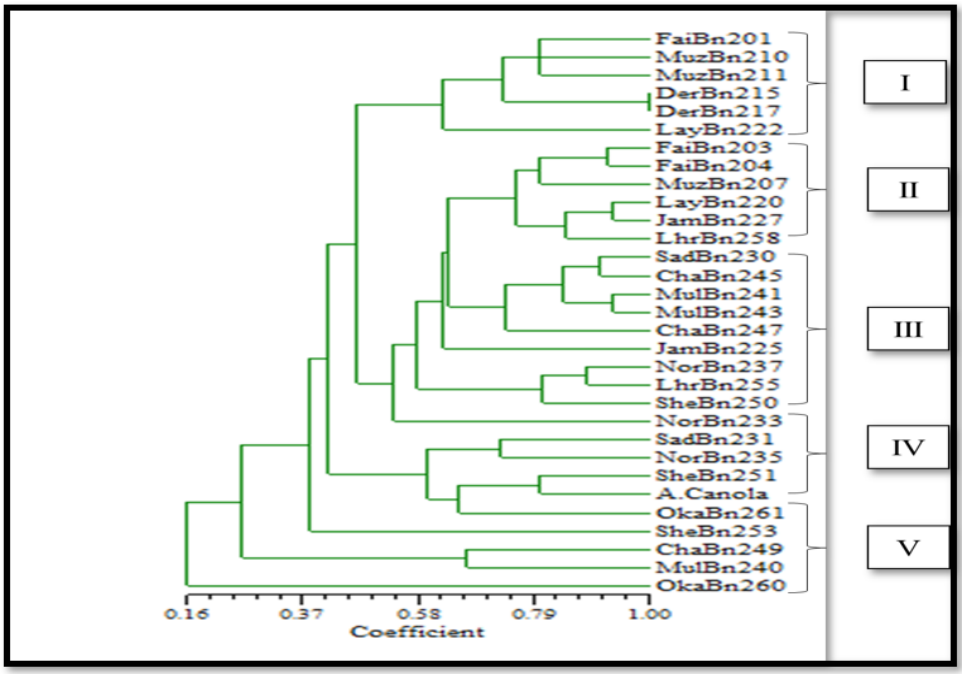

FIGURE 4.8: SSRs marker-based variability among 31 genotypes of *B. napus* using cluster analysis.

TABLE 4.6: Grouping of 31 genotypes of *Brassica napus* through cluster analysis based on SSRs marker.

Cluster	No. of genotypes	Genotypes	Origin
I	6	FaiBn201, MuzBn210, MuzBn211, DerBn215, DerBn217, LayBn222	Faisalabad, Muzaffargarh, Dera Ghazi Khan and, Layyah, Pakistan
II	6	FaiBn203, FaiBn204, MuzBn207, LayBn220, JamBn227, LhrBn258	Faisalabad, Muzaffargarh, Layyah, Jampur Lahore, Pakistan

TABLE 4.6: Grouping of 31 genotypes of *Brassica napus* through cluster analysis based on SSRs marker.

Cluster	**No. of genotypes**	**Genotypes**	**Origin**
III	9	SadBn230, ChaBn245, MulBn241, MulBn243, ChaBn247, JamBn225, NorBn237, LhrBn255, SheBn250	Sadiqabad, Chakwal, Multan, Jampur, Narowal, Lahore and, Sheikhupura, Pakistan
IV	5	NorBn233, SadBn231, NorBn235, SheBn251, A. canola	Narowal, Sadiqabad, Sheikhupura, Pakistan
V	5	OkaBn261, SheBn253, ChaBn249, MulBn240, OkaBn260	Okara, Sheikhupura, Chakwal and, Multan, Pakistan

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

Primers	**Total amplified alleles**	**Polymorphic alleles**	**% Poly morphism**	**Size range (bp)**	**Melting temperature (TM)°C**
Na10-D03	1	1	100	190-200	59

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

Primers	**Total amplified alleles**	**Polymorphic alleles**	**% Poly morphism**	**Size range (bp)**	**Melting temperature (TM)°C**
Na10-G08	1	1	100	280	57
Na12-H02	1	1	100	200	55
O110-B01	1	1	100	200	60
O110-F12	1	1	100	300-400	53.4
PBCES-SRJU10	1	1	100	110-120	54
PBCES-SRJU15	1	1	100	220-250	54
PBCES-SRNA3	3	3	100	100-120, 290, 480	55
PBCES-SRNA8	1	1	100	290	55
PBCES-SRNA18	1	1	100	100-110	56.0

The below figure 4.9 SSRs marker banding patterns of 23 genotypes of *B. napus* by SSRs primer Na10-D03, 1-23. (M = 100 bp molecular marker, 1=Bn201, 2=Bn203,

3=Bn204, 4=Bn207, 5=Bn210, 6=Bn211, 7=Bn215, 8=Bn217, 9=Bn220, 10=Bn 222, 11=Bn225, 12=Bn227, 13=Bn230, 14=Bn231, 15=Bn233, 16=Bn235, 17=Bn 237, 18=Bn240, 19=Bn241, 20=Bn243, 21=Bn245, 22=Bn247 and 23=Bn249).

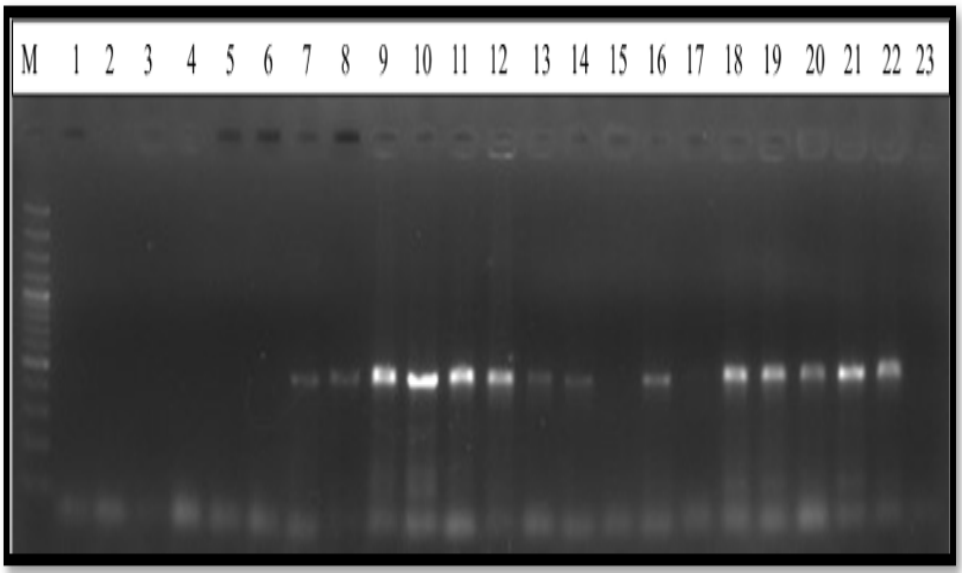

FIGURE 4.9: SSRs marker banding patterns of 23 genotypes of *B. napus*

The below figure 4.10 SSRs banding patterns of 8 genotypes of *B. napus* by SSRs primer Na10-D03, 24-31. (M = 100 bp molecular marker, 24= Bn250, 25=Bn251, 26=Bn253, 27=Bn255, 28=Bn258, 29=Bn260, 30Bn261 and 31=A. Canola).

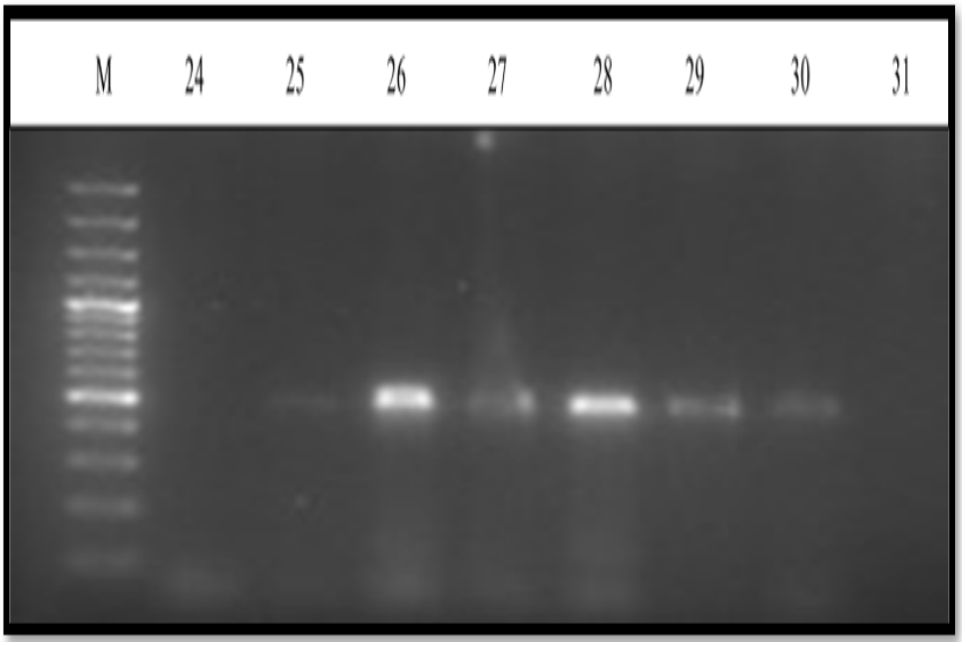

FIGURE 4.10: SSRs marker banding patterns of 8 genotypes of *B. napus*

The below figure 4.11 SSRs banding patterns of 23 genotypes of *B. napus* by SSRs primer Na12-H02, 1-23. (M = 100 bp molecular marker, 1=Bn201, 2=Bn203, 3=Bn204, 4=Bn207, 5=Bn210, 6=Bn211, 7=Bn215, 8=Bn217, 9=Bn220, 10=Bn 222, 11=Bn225, 12=Bn227, 13=Bn230, 14=Bn231, 15=Bn233, 16=Bn235, 17=Bn 237, 18=Bn240, 19=Bn241, 20=Bn243, 21=Bn245, 22=Bn247 and 23=Bn249).

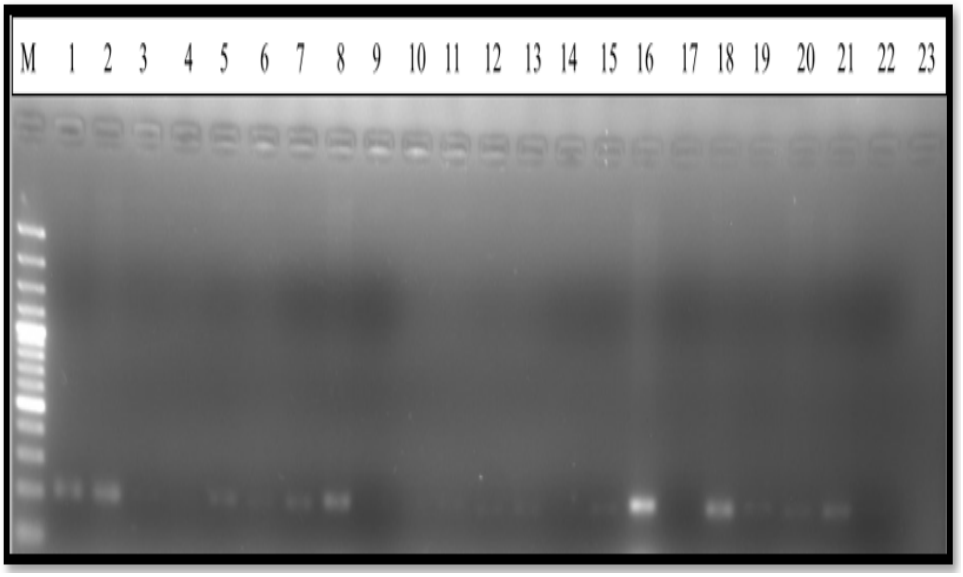

FIGURE 4.11: SSRs marker banding patterns of 23 genotypes of *B. napus*

The below figure 4.12 SSRs banding patterns of 8 genotypes of *B. napus* by SSRs primer Na12-H02, 24-31. (M = 100 bp molecular marker, 24= Bn250, 25=Bn251, 26=Bn253, 27=Bn255, 28=Bn258, 29=Bn260, 30Bn261 and 31=A. Canola).

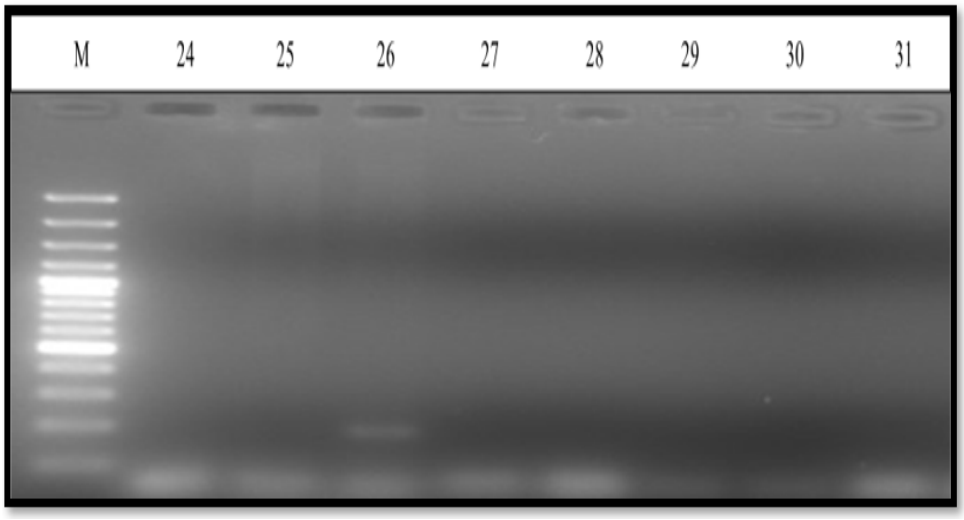

FIGURE 4.12: SSRs marker banding patterns of 8 genotypes of *B. napus*

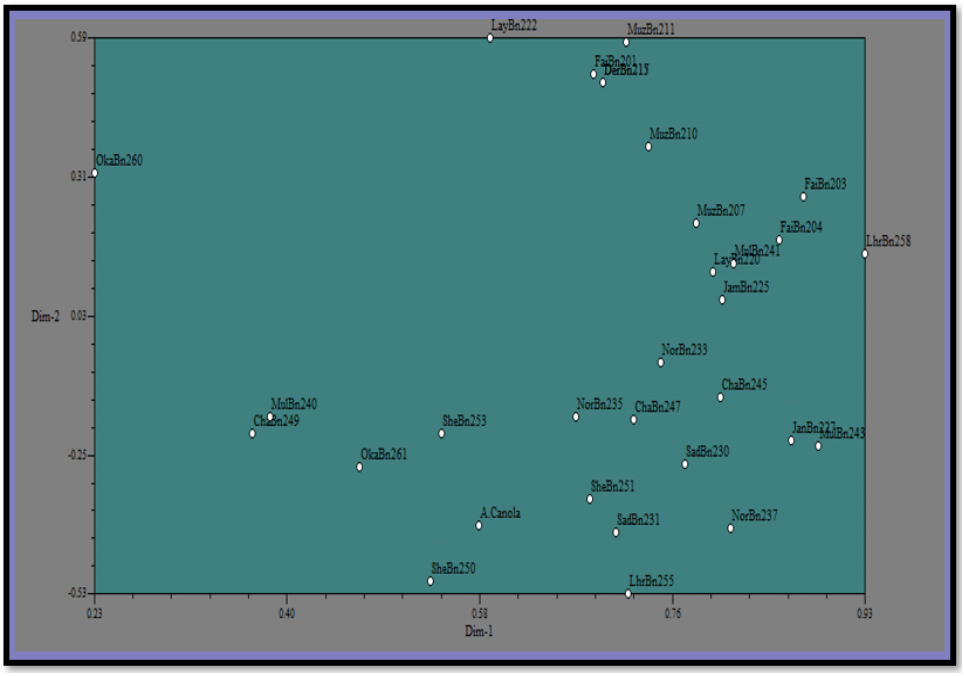

FIGURE 4.13: Two-dimensional (2D) diversity analysis of 31 genotypes of *B. napus* using SSRs primer.

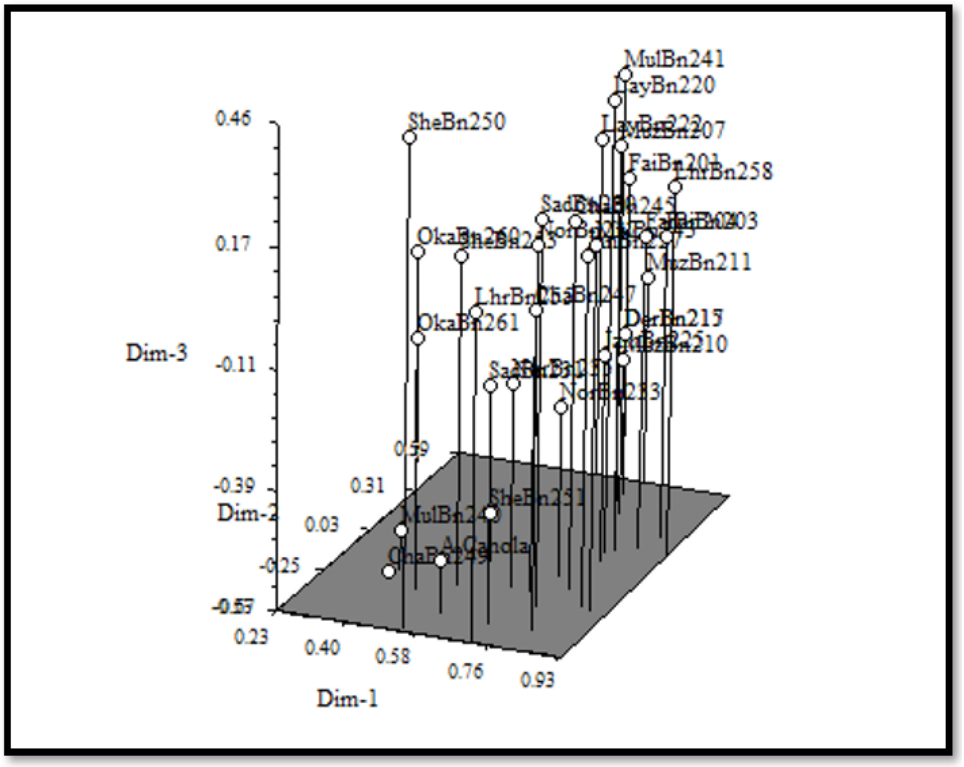

FIGURE 4.14: Two-dimensional (2D) diversity analysis of 31 genotypes of *B. napus* using SSRs primer.

TABLE 4.8: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SSRs.

Acc	**FaiBn201**	**FaiBn203**	**FaiBn204**	**MuzBn207**	**MuzBn210**	**MuzBn211**	**DerBn215**	**DerBn217**
FaiBn201	1							
FaiBn203	0.83	1						
FaiBn204	0.73	0.92	1					
MuzBn207	0.55	0.77	0.83	1				
MuzBn210	0.8	0.83	0.73	0.55	1			
MuzBn211	0.8	0.83	0.73	0.73	0.8	1		
DerBn215	0.6	0.67	0.55	0.55	0.8	0.8	1	
DerBn217	0.6	0.67	0.55	0.55	0.8	0.8	1	1
LayBn220	0.62	0.67	0.71	0.71	0.46	0.46	0.46	0.46
LayBn222	0.67	0.55	0.6	0.6	0.44	0.67	0.67	0.67
JamBn225	0.55	0.62	0.67	0.5	0.55	0.55	0.55	0.55
MulBn241	0.67	0.71	0.77	0.77	0.5	0.5	0.5	0.5
SadBn230	0.2	0.5	0.55	0.73	0.2	0.4	0.4	0.4
SadBn231	0.2	0.5	0.55	0.36	0.4	0.2	0.4	0.4
NorBn233	0.67	0.73	0.6	0.4	0.67	0.67	0.44	0.44
NorBn235	0.36	0.46	0.5	0.33	0.36	0.36	0.36	0.36
NorBn237	0.4	0.67	0.73	0.73	0.4	0.4	0.2	0.2

TABLE 4.8: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SSRs.

Acc	**FaiBn201**	**FaiBn203**	**FaiBn204**	**MuzBn207**	**MuzBn210**	**MuzBn211**	**DerBn215**	**DerBn217**
LhrBn258	0.71	0.88	0.8	0.8	0.71	0.71	0.71	0.71
JanBn227	0.46	0.67	0.57	0.57	0.46	0.46	0.46	0.46
MulBn243	0.5	0.71	0.62	0.62	0.5	0.5	0.5	0.5
ChaBn245	0.36	0.62	0.5	0.67	0.36	0.55	0.55	0.55
ChaBn247	0.2	0.5	0.55	0.55	0.2	0.4	0.4	0.4
ChaBn249	0	0.25	0.29	0.29	0.33	0.33	0.33	0.33
SheBn250	0.25	0.4	0.44	0.44	0.25	0	0	0
SheBn251	0.25	0.4	0.44	0.22	0.5	0.25	0.5	0.5
SheBn253	0.44	0.55	0.4	0.2	0.44	0.22	0.22	0.22
LhrBn255	0.22	0.55	0.6	0.6	0.44	0.22	0.22	0.22
MulBn240	0	0.22	0.25	0.25	0.29	0.29	0.29	0.29
OkaBn260	0.25	0.2	0.22	0.22	0.25	0.25	0.25	0.25
OkaBn261	0.29	0.22	0.25	0	0.29	0	0.29	0.29
A. Canola	0.29	0.44	0.5	0.25	0.57	0.29	0.29	0.29

TABLE 4.9: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SSRs.

Acc	**LayBn220**	**LayBn222**	**JamBn225**	**MulBn241**	**SadBn230**	**SadBn231**	**NorBn233**	**NorBn235**
LayBn220	1							
LayBn222	0.67	1						
JamBn225	0.71	0.6	1					
MulBn241	0.93	0.73	0.62	1				
SadBn230	0.62	0.44	0.55	0.67	1			
SadBn231	0.46	0.22	0.55	0.5	0.6	1		
NorBn233	0.33	0.25	0.6	0.36	0.44	0.44	1	
NorBn235	0.43	0.4	0.67	0.46	0.55	0.73	0.6	1
NorBn237	0.62	0.22	0.55	0.67	0.8	0.6	0.67	0.55
LhrBn258	0.82	0.62	0.67	0.88	0.71	0.57	0.62	0.53
JanBn227	0.75	0.33	0.71	0.67	0.77	0.62	0.67	0.57
MulBn243	0.67	0.36	0.62	0.71	0.83	0.67	0.73	0.62
ChaBn245	0.57	0.4	0.5	0.62	0.91	0.55	0.6	0.5
ChaBn247	0.62	0.44	0.73	0.5	0.8	0.6	0.44	0.55
ChaBn249	0	0	0.29	0	0.33	0.33	0.4	0.29

TABLE 4.9: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SSRs.

Acc	**LayBn220**	**LayBn222**	**JamBn225**	**MulBn241**	**SadBn230**	**SadBn231**	**NorBn233**	**NorBn235**
SheBn250	0.55	0	0.22	0.6	0.5	0.5	0.29	0.22
SheBn251	0.36	0.29	0.67	0.4	0.5	0.75	0.57	0.67
SheBn253	0.5	0	0.4	0.36	0.22	0.44	0.5	0.2
LhrBn255	0.5	0	0.4	0.55	0.67	0.67	0.5	0.4
MulBn240	0.2	0	0.5	0	0.29	0.29	0.33	0.25
OkaBn260	0.36	0.29	0.44	0.2	0	0	0	0.22
OkaBn261	0.4	0.33	0.5	0.44	0.29	0.57	0.33	0.5
A. Canola	0.2	0	0.5	0.22	0.29	0.57	0.67	0.5

TABLE 4.10: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SSRs.

Acc	**NorBn237**	**LhrBn258**	**JanBn227**	**MulBn243**	**ChaBn245**	**ChaBn247**	**ChaBn249**	**SheBn250**
NorBn237	1							
LhrBn258	0.71	1						
JanBn227	0.77	0.82	1					
MulBn243	0.83	0.88	0.93	1				
ChaBn245	0.73	0.8	0.86	0.92	1			
ChaBn247	0.6	0.57	0.77	0.67	0.73	1		
ChaBn249	0.33	0.2	0.22	0.25	0.29	0.33	1	
SheBn250	0.75	0.5	0.55	0.6	0.44	0.25	0	1
SheBn251	0.5	0.5	0.55	0.6	0.44	0.5	0.5	0.33
SheBn253	0.44	0.46	0.67	0.55	0.4	0.44	0	0.57
LhrBn255	0.89	0.62	0.67	0.73	0.6	0.44	0.4	0.86
MulBn240	0.29	0.18	0.4	0.22	0.25	0.57	0.67	0
OkaBn260	0	0.17	0.18	0	0	0.25	0	0
OkaBn261	0.29	0.36	0.4	0.44	0.25	0.29	0	0.4
A. Canola	0.57	0.36	0.4	0.44	0.25	0.29	0.67	0.4

TABLE 4.11: Dice coefficient of similarity among 31 *B. napus* genotypes on the basis of SSRs

Acc	**She Bn251**	**She Bn253**	**Lhr Bn255**	**Mul Bn240**	**Oka Bn260**	**Oka Bn261**	**A. Canola**
She Bn251	1						
She Bn253	0.29	1					
Lhr Bn255	0.57	0.5	1				
Mul Bn240	0.4	0.33	0.33	1			
Oka Bn260	0	0.29	0	0.4	1		
Oka Bn261	0.8	0.33	0.33	0	0	1	
A. Canola	0.8	0.33	0.67	0.5	0	0.5	100

4.3 Discussion

Brassica species are famous for their high oil content around the globe but still there is limited data available about the genetic diversity of these species. Exploration of genomic variation of a germplasm is vital for the conservation of genetic diversity [110]. For this purpose, proteins have been used as markers to assess the genomic variability. To analyze the genetic diversity among different plant species and sub-species total seed storage protein method appeared to be efficient and very useful. To study genetic diversity in plants many biochemical methods were being used but among all SDS-PAGE technique is one of the cost-effective, simple and efficient method [75] [111]. SDS-PAGE method not only elucidates

the diverse profiles of large number of plants but also characterize the taxonomic differences among plants [112].

Seed storage proteins are the source of identification and characterization of genetic diversity among different plant species [113]. The proteins found in seeds are considered more stable toward ecological influences in comparison to the proteins belonging to other body parts of plants [114]. The taxonomic information of plants is derived from seed storage proteins as it contains both salt soluble and insoluble proteins [115]. Moreover, SDS-PAGE technique used on the cotyledon proteins help in observing usable protein bands polymorphism and to cluster the genotypes on their specific banding patterns that could help in selection of parents for crosses for improvement of plant productivity [116]. As much diversity appear in plants it would be more favorable to survive by the harsh conditions of environment as it adopts the environment [117]. There occurs a broad range of polymorphism within *Brassica* species that compels the world to use modern methods to investigate it [118]. Evaluation of genetic diversity through protein marker have been discovered as a worthy tool in classification of many crop species [119].

In this study, 31 genotypes of *Brassica napus* were used to determine protein-based diversity among them. The standard protocol for quick and efficient SDS-PAGE was established for important oil seed *Brassica napus* species according to Jan *et al.* [105]. A low to moderate and high level of genetic variability was observed within genotypes. SDS-PAGE method resulted in 14 protein bands for 31 *B. napus* genotypes tested. Our results highly deviated from the findings of Akbar *et al.* [73], who found 20 polypeptide protein bands while analyzing 105 accessions of *Sesamum Indicum*. Among all the 20 bands only 14 (70%) were polymorphic with bands ranged 13.5-100 kDa that is far beyond than our observations. Turi *et al.* [85] also recorded 28 protein bands when they were investigating genetic diversity in 234 accessions of *Brassica*. A total of 60% polymorphism was recorded out of 28 bands rest were monomorphic. Zada *et al.* [120] observed 31 polypeptide subunits while estimating diversity in *B. carinata* via SDS-PAGE. Out of 31 bands only 14 were found polymorphic and the coefficient of similarity was ranged 0.50-1.00. Khan *et al.* [87] evaluated *Brassica napus* through SDS-PAGE and obtained

17 protein bands among which 10 were polymorphic and 7 were monomorphic that is also in no line with our results. However, Shinwari *et al.* [121] found 17 polypeptide bands while assessing *Eruca sativa* genotypes, using biochemical markers and found 16 polymorphic and one monomorphic protein band. The results of Shinwari *et al.* [121] were in strong agreement with our calculations but they estimated a 100% similarity within tested genotypes of *Eruca sativa*. Moreover, our results indicated close relatedness with the observations of Jan *et al.* [122] who obtained a total of 15 protein bands, whilst analyzing *Brassica rapa* genotypes. They recorded 14 polymorphic bands i.e., 93% that is equal to our analyzed values. Choudhary *et al.* [51] determined genetic diversity in 7 *Brassica napus* species and obtained 10 protein bands through SDS-PAGE technique. Their data indicated moderate level of genetic diversity among tested genotypes as they found only 5 polymorphic polypeptide fragments. Yousaf *et al.* [80] and Ibrahim *et al.* [77] estimated genomic variation in *Brassica compesrties* and *Brassica juncea* and found a total of 16 and 12 bands, that showed 75% and 58% polymorphism, respectively. There was a close agreement with previous investigation of Jan *et al.* [122] and Shinwari *et al.* [121] as we obtained 14 protein bands among which 13 are polymorphic i.e., our results could contribute to future breeding or research programs to help in conservation of genetic diversity of locally collected germplasm from Punjab region of Pakistan and for further evaluation of *Brassica napus* genotypes.

During the present study, *Brassica napus* genotypes were found to have 26-95.4% of genetic similarity. The recent data fully supported by findings of Jan *et al.* [123] who estimated maximum similarity of 96% among accessions Br-607/Br-560 and Br-589/Br-607 while electrophoretically analyzing the *Brassica rapa* sub-species. Our results also indicated closeness to the findings of Turi *et al.* [85] who estimated the genetic similarity of 99% among 234 accessions of *Brassica* species. Zada *et al.* [120] analyzed the maximum similarity ranged 54-83% when they were evaluating the *Brassica carinata* germplasms via SDS-PAGE. Similarly, the calculations of Shinwari *et al.* [121], Kakaei *et al.* [86] Jan *et al.* [78] and Ibrahim *et al.* [77] were somehow nearer to our observations. They all recorded

maximum genetic similarity of 100% while elucidating major plant species via protein markers. However, the genetic variation value of 26% was supported by the results of kakaei *et al.* [86] who estimated the genetic dissimilarity of 22% while investigating *Brassica napus* through SDS-PAGE method. Results of Jan *et al.* [78] and Sharma *et al.* [116] were also near to our least similarity value i.e., 20% and 16%, respectively. Overall, our results justify that SDS-PAGE is a reliable tool for estimation of genetic diversity among diverse genotypes but still there is a dire need of modern analytical techniques for better understanding of Diversity among different crop species.

The Cluster analysis of 31 diverse genotypes of *B. napus* divided all the tested genotypes into seven major groups via UPGMA clustering analysis. Our findings were in accordance with the results of Jan *et al.* [78] who characterized 24 GUAR (*Cyamopsis tetragonoloba*) via biochemical markers and clustered 24 genotypes into seven clusters that were highly diverse. The findings of Sharma *et al.* [116] were also in close agreement with our observations who clustered all the tested genotypes of Cowpea into seven clusters via UPGMA analysis while analyzing genetic diversity in Vigna unguiculata accessions using SDS-PAGE method. However, the observations of Khan *et al.* [87] and Yousaf *et al.* [80] were deviated from our findings. They clustered tested genotypes of *Brassica napus* and *Brassica carinata* into 4 clusters each using UPGMA clustering method, respectively. Similarly, the results of Jan *et al.* [122] also deviated from our calculations who categorized 65 accessions of *B. rapa* into 4 clusters while characterizing different ecotypes of *B. rapa* via SDS-PAGE method.

Although SDS-PAGE is a very reliable and certain method to investigate genetic differences but still alone it does not exhibit high level of intra-specific diversity that demands the further analysis of seed proteins using modern day methods. For this purpose, 2D and 3D protein analysis are recommended and used by many to investigate the diversity in crop species [111]. Khan *et al.* [114] analyzed more diverse genotypes using 2D and 3D protein analysis as compared to SDS-PAGE method while working on *Brassica napus* genotypes. The SDS-PAGE technique is not feasible because it cannot differentiate among closely related cultivars but

characterize those as a same banding pattern proteins falls in single common gene-pool [124] . Detailed agronomic and biochemical analysis of genotypes of similar banding pattern should be observed for better understanding and management of Gene Bank [125]. However, SDS-PAGE is still a very fruitful technique to identify diversity among different landraces of plants [126]. The PCoA analysis allowed to see a vivid depiction of all genotypes from various angles. But the 3D analysis was more comprehensive than the 2D representation. In the current findings, both applications resulted in specific, unique and diverse genotypes. Our findings were consistent to findings of Gupta *et al.* [127], who discovered seed protein diversity in 45 Chickpea genotypes using PCoA. Based on 3D analysis, their work revealed that FLIP-90-160 had a distinct relationship. Through Principle Coordinate Analysis, Mottaghi *et al.* [128] discovered maximum and clear protein-based divergence among Iranian Achillea species. They claim that the first three PCoA groups account for more than 83 percent of the variations.

However, change in number of polypeptide bands might be by value of differential extraction or disparity in solubility of protein or inadequacy of separation of varied sorts of proteins having identical migration rates as accessions of same species are governed through quantitative gene system.

Among vast flora of plant species, molecular variation plays an important role in characterization and identification of polymorphic variants. To analyze genomic variation many molecular techniques are being used for instance use of Random Amplified Polymorphic DNA, Amplified Fragment Length Polymorphism, Single Nucleotide Polymorphism etc. But among all the methods Simple Sequence Repeats (SSRs) are known worthy because of their quick and efficient genomic diversity analysis of many important crop species [129], [130]. The highly polymorphic nature and abundancy of SSRs markers throughout the plant genome gives it distinction over other DNA markers [131]. According to Ali *et al.* [104] SSRs can easily transfer among populations, easily automatized, differentiate closely related genotypes in no time and are require only low quantity of DNA. In the present study, 10 SSRs primers were used in analysis of 31 diverse genotypes of *B. napus* collected from various region of Punjab, Pakistan. High level of Polymorphism was

observed for all the tested genotypes. All the Primers used were highly polymorphic no monomorphic alleles were recorded. A total of 12 alleles were recorded for 10 diverse SSRs primers. The polymorphic alleles recorded per locus were ranged from 1-3 and 100% polymorphism was estimated for all the markers used. All the markers amplified one allele except for one marker (PBCESSRNA3) that amplified 3 alleles. Our results were in line with the previous findings of Verma *et al.* [132] who studied *Brassica juncea* using SSRs markers for investigation of genetic diversity in Indian mustard. They used a total of 11 SSRs markers for 43 germplasms of *B. juncea* among which five were cultivated varieties. 100% polymorphism was recorded for 11 SSRs primers used with 65 alleles in total. Yao *et al.* [133] also analyzed the *Brassica juncea* genotypes of China through 69 diverse SSRs markers. A total of 34 genotypes of *B. juncea* were studied for molecular analysis by 69 SSRs primers, which amplified 452 alleles i.e., 3-12 alleles per primer. They recorded 92.7% polymorphism. The results of Ghosh *et al.* [134] were highly diverged from our findings that recorded 50% polymorphism while elucidating 47 genotypes of *Brassica juncea* using 41 microsatellite markers. They found a total of 19 polymorphic alleles. Similar results were obtained by Sharma *et al.* [135] that investigated 59 accessions of leafy mustard *B. juncea var. rugosa* through 155 SSRs markers and recorded 482 alleles among which only 122 were polymorphic. Baghel *et al.* [136] also analyzed *Brassica juncea* (Indian mustard) via 20 diverse simple sequence repeats. Among 20 SSRs markers used only highly diverse 8 were picked to estimate the genetic diversity in 48 genotypes of *B. juncea*. They recorded 50% polymorphism that was much deviated from our findings.

Additionally, their data reveals that only one marker among 8 used was monomorphic and all the amplified fragments were ranged from 100-2000bp. Shyam *et al.* [137] observed 48 genotypes of *Brassica* species for low and high erucic acid content aided by SSRs markers. A total of 50 primer pairs were used that generated 109 alleles i.e., 4.47 alleles per primer and recorded 77% of polymorphism. Anyhow, their results and our findings justify that Simple Sequence Repeats are proved to be more reliable, quick, efficient and easily usable as compared to other markers. Li *et al.* [67] investigated *Brassica napus* through 16 AFLP and 22 SSRs

markers and recorded a total of 73% polymorphism. SSRs markers generated 134 fragments of which 54 were polymorphic and were ranged from 100-1200bp. Moreover, the primer Na12-A02 was the only polymorphic primer that recorded maximum polymorphic bands while FITO-063 was minimum polymorphic band generating primer. In the present study, similarity coefficient values revealed low to high level of genomic variation among *B. napus* genotypes. The similarity values ranged 0-100% for 31 tested *B. napus* genotypes. In fact, genotypes from same areas of origin also showed maximum heterozygosity with one another. However, some genotypes indicated huge similarity with each other. Our results showed deviation from findings of Ofori *et al.* [95] who recorded 83% of genetic similarity among *Brassica rapa* genotypes and 17% intra-specific variability. Similarly, results of Li *et al.* [67] are far from our similarity vales that estimated 69% of similarity among 25 hybrids of *B. napus* via AFLP and SSRs markers. Vinu *et al.* [138] were also short from our findings. They estimated 83.5% genetic similarity among 44 tested genotypes of *Brassica juncea* using SSRs markers. Moreover, the results of Abbasov *et al.* [99] were somehow near to our results. They used 11 SSRs primers for 139 genotypes of Diploid wheat (*Triticum* spp.). The maximum similarity value of 84% was observed between accessions of *T. boeoticum* and *T. monococcum*. However, the resultant data of Ali *et al.* [104] was in full agreement with our results. They estimated 100% similarity among 96 accessions of *Brassica rapa* collected from various regions of Khyber Pakhtunkhwa, Pakistan analyzed for diversity via 26 SSRs primers. Our results justify that genotype sharing same origin could be similar or varied. Cluster analysis categorized the 31 genotypes of *B. napus* into five groups on the basis of UPGMA method. Each group indicated distinct genotypes. Almost all of these groups had polymorphic genotypes. Yu *et al.* [139] investigated *Brassica rapa* subspecies and clustered the tested genotypes in five groups.

They identified five distinct groups for the important *B. rapa* subspecies known as Pak-choi. Using the UPGMA method, all 80 genotypes were classified into five major groups. Cluster I contained three types of Chinese cabbage, Cluster II contained smooth oval leafy types, Cluster III contained typical Chinese rape,

Cluster IV contained compact plant types, and Cluster V contained Wutacai subspecies. Bird *et al.* [140] analyzed five sub-populations (European turnip, Asian turnip, brown/yellow sarson, Chinese cabbage and bok choy, choy sum, and tatsoi). The phylogenic study using Single Nucleotide Polymorphism revealed a high level of variation among these morphotypes. Our results highly corelate with their findings. Also, the calculations of Vinu *et al.* [138], Hasan *et al.* [141] , Baghel *et al.* [136] and Zhu *et al.* [142] were close to our data. They all investigated *Brassica* species and sub-species and observed 4 clusters through UPGMA method while analyzing genetic variation aided by SSRs markers. Nevertheless, the observations of Abbasov *et al.* [99], Soengas *et al.* [143] and, Yao *et al.* [133] was far from our calculations. They estimated 10, 7 and 7 clusters using UPGMA analysis, respectively. In this study, all the groups indicated similarity within the genotypes of other areas of Punjab that showed they may have a similar evolutionary history. However, the genotypes of group V were highly diverse.

The Principal Coordinate Analysis was used in our study to investigate the diversity among 31 *B. napus* genotypes more clearly. PCoA analysis used for estimation of patterns of genetic variation within and among different germplasm types reveals a distinct relationship between different *Brassica* species [144]. In the present research, maximum level of genetic diversity was observed with the application of modern-day 2D and 3D techniques. Our work was confirmed by the results of Meeghakumbura *et al.* [145] that used PCoA method with GenA1Ex based on Nei's genetic distances to analyze the genetic relationship among *Tea* groups and found out 79.05% variation. Their PCoA results were also in line with STRUCTURE analysis results. Findings of Guzman *et al.* [146] also justifies our observations. They employed PCoA in software GenA1Ex to represent the *Capsicum* genotypes in multi-dimensional metric space investigating genetic diversity based on similarity in banding profiles.

PCoA revealed that 53.56% variation in 2D and a diverse genotype in 3D analysis i.e., *Capsicum baccatum* that show no evident close relation to other genotypes based on taxonomic species. Wang *et al.* [147] performed PCoA to estimate genetic relationship among 3 sections of *Carya* genus and *Rhysocaryan* of Juglans.

They found an overall variation of 44.1% among *Apo-carya, Carya* and *Sino-carya* of genus *Carya* and found close association among sections *Rhysocaryan* of Juglans, and 43.1% variation among Apo-carya and Sino-carya. Singh *et al.* [148] studied 88 genotypes of *B. juncea* via using 59 SSRs markers. They reported maximum level of genetic variation and few novels highly diverse genotypes using 2D and 3D methods. They suggested the use of modern day PCoA analysis could enhance the investigations based on diversity that would be a fruitful event for the rapidly evolving world to enter the new phase of development, capturing surplus food requirement of whole globe.

Chapter 5

Conclusions and Recommendations

From different areas of Punjab, Pakistan the diverse genotypes of *Brassica napus* were collected and characterized for seed storage protein profiling. Genotypes showed high to moderate level of diversity. The genotypes OkaBn261 and SheBn253 indicated maximum level of resemblance while genotypes SheBn250 and JamBn227 were highly diverged from others. The polymorphic data was further visualized via PCoA that generated 2D and 3D plots of genotypes. Through modern 2D and 3D techniques some unique genotypes were recorded. The seed protein-based diversity is attributed to geographical origin as well as unique morphological nature of genotypes. Our results established that biochemical diversity of *B. napus* genotypes could be utilized for exploration of genetic diversity. However, It is recommended that advanced 2D Gel-electrophoresis should be used to investigate diversity in *Brassica napus* germplasms. The SSRs analysis estimated some unique alleles in diverse *B. napus* genotypes. All the markers showed polymorphism and the genotypes ChaBn249, SheBn250, SheBn253, LhrBn255, MulBn240, OkaBn260 and OkaBn261 showed high level of variation with others. However, genotypes DerBn215 and DerBn217 showed maximum similarity with each other portraying that these might have same evolutionary history. These findings were further confirmed through PCoA analysis and the genotypes MulBn241, LayBn220

and SheBn250 showed maximum dissimilarity with all other genotypes.It is recommended that more modern methods like Genome Wide Association Study (GWAS) must be used along with SSRs to investigate these novel and unique genotypes of LayBn222, LayBn220, MuzBn211, LhrBn 258, OkaBn260, MulBn241,SheBn 250 and LhrBn255 obtained via PCoA analysis.

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