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Genetic Divergence among Local and Exotic *Brassica juncea* Germplasm as Revealed by Using Simple Sequence Repeats (SSRs)

by

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MY WORK IS DEDICATED TO THE ONLY GOD ALLAH ALMIGHTY, TO THE LAST HOLY PROPHET HAZRAT MUHAMMAD S.A.W.W. (PBUH) AND TO MY FAMILY

CERTIFICATE OF APPROVAL

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(Muhammad Mudassar Malik)

Abstract

Brassica juncea associate with the mustard family (Brassicaceae or Cruciferae), is known as a number of popular names, including Indian mustard and oriental mustard. Simple obovate-shaped leaves are present, as are bisexual flowers with four free sepals, two longer and two shorter stamens, and four sepal-free raceme inflorescences. *Brassica juncea* is a prominent tropical and subtropical oilseed crop in Asia. In the lack of sources for resistance, the extensive production of genetically similar types is known to attract fungal diseases that severely reduce yields. The traditional methods of illness management are frequently pricy, ineffective, and worse for the environment. Locating and using resistance sources in *Brassica* and other non-hosts helps maintain oil crop yields. For the purpose of analyzing genetic variation, a very effective SSR procedure was developed. The genetic diversity among the 23 *B. juncea* genotypes was rated as low, moderate, or high. Thirteen bands at most were detected, and every one of the (100%) was polymorphic. Twenty-three distinct *B. juncea* genotypes were analyzed for chromosomal variation using ten simple sequence repeat (SSR) markers in a molecular study. Primers designed specifically for *B. juncea* crop amplified fragments with sizes between 100 and 400 bps. There was complete polymorphism across all genotypes, as measured by ten separate microsatellites. Seven of the primers (70% of the total) detected a single allele, whereas the other two (20%) (Ni2-F02 and Ra2-A11) amplified two alleles each, and a single primer (Na10-D09) created three. Ten different primers were able to pick up 14 highly polymorphic alleles. All of 23 genotypes were grouped three distinct clusters using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Values for genetic similarity across different *B. juncea* genotypes ranged from 0.33 to 0.95 (33% to 95%). There were 6, 11, 5 and 1 different genotypes spread over clusters I through IV. Among groups I and III, only the Bj-1210 and K. Raya genotypes were found to be different from all others. Additional 2D and 3D representations of the genotypes were created. Genotypes Bj-1112, Bj-1130, Bj-1143, and Bj-1153 were identified by 2D analysis, whereas genotypes Bj-1137, Bj-1154, Bj-1199, and Bj-1194 were identified through 3D analysis. The findings presented here will serve as a foundational analysis for

further work on the characterization of *B. juncea* genotypes. These studies may be used as a starting point for further investigation and assessment.

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Abbreviations

AFLP:	Amplified fragment length polymorphism
***B. juncea*:**	*Brassica juncea*
***B. napus*:**	*Brassica napus*
***B. nigra*:**	*Brassica nigra*
***B. rapa*:**	*Brassica rapa*
EST:	Expressed Sequence Tags
FAO:	Food and Agricultural Organization
GSS:	Genomic Sequences
GWAS:	Genome Wide Association Study
InDels:	Insertions and deletions
ISSRs:	Inter-Simple Sequence Repeats
NARC:	National Agricultural Research Center
NGS:	Next Generation Sequencing
PCR:	Polymerase Chain Reaction
PCoA:	Principal coordinate analysis
QTLs:	Quantitative trait loci
RFLP:	Restriction Fragment Length Polymorphism
SCAR :	Special Sequence Characterized Amplified Region
SDS-PAGE:	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
SRAP:	Sequence-related amplified polymorphism
SSRs:	Simple Sequence Repeats
UN:	United Nation
UPGMA:	Unweighted Pair Group Method with Arithmetic Averages
WTO:	World Trade Organization

2D: Two Dimensional

3D: Three Dimensional

Chapter 1

Introduction

1.1 Background

The genetic variety of plant genotypes is critical for the invention of improved agricultural varieties to increase production and other appealing qualities, and the application of these valuable properties has greatly boosted the number and quality of crop plant backup tools in recent years. Many plant and animal breeders have sought genetic variety to attain desired yields and quality by carrying genes for desired characteristics and avoiding others from the early days of agriculture (about 10,000 years ago) [1].

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

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

1.2 *Brassicaceae*

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

1.3 *Brassica*

Generally, six unique species of *Brassica* are cultivated. Out of these six, three are diploid, *B. nigra* (2n = 16, genome BB), *B. oleracea* (2n = 18, genome CC), and *B. rapa* (2n = 20, AA) though the remaining three amphidiploid derivatives *Brassica carinata* (2n = 34, BBCC), *Brassica juncea* (2n = 36, AABB), and *Brassica napus* (2n = 38, AACC) [4]. These six *Brassica* crop species have a genomic relationship (called the triangle of U) nuclear DNA content, genome-specific markers used, sequence analysis, and pairing of chromosomes are shown in the figure (1.1).

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

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

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

Among amphidiploid species are *Brassica juncea, Brassica carinata,* and *Brassica napus*. The five most significant rapeseed and mustard species grown in Pakistan are *Eruca sativa, Brassica napus, Brassica carinata, Brassica campestris,* and *Brassica juncea*. Between these *B. juncea L.* is a very important species because its seed contains 40 to 45% oil content, 3.5% fats, and 0.35% phosphorous. *B. juncea* traits have a very limited genetic diversity but *B. rapa* and *B. oleracea* have incredible differences within their types and origin [5].

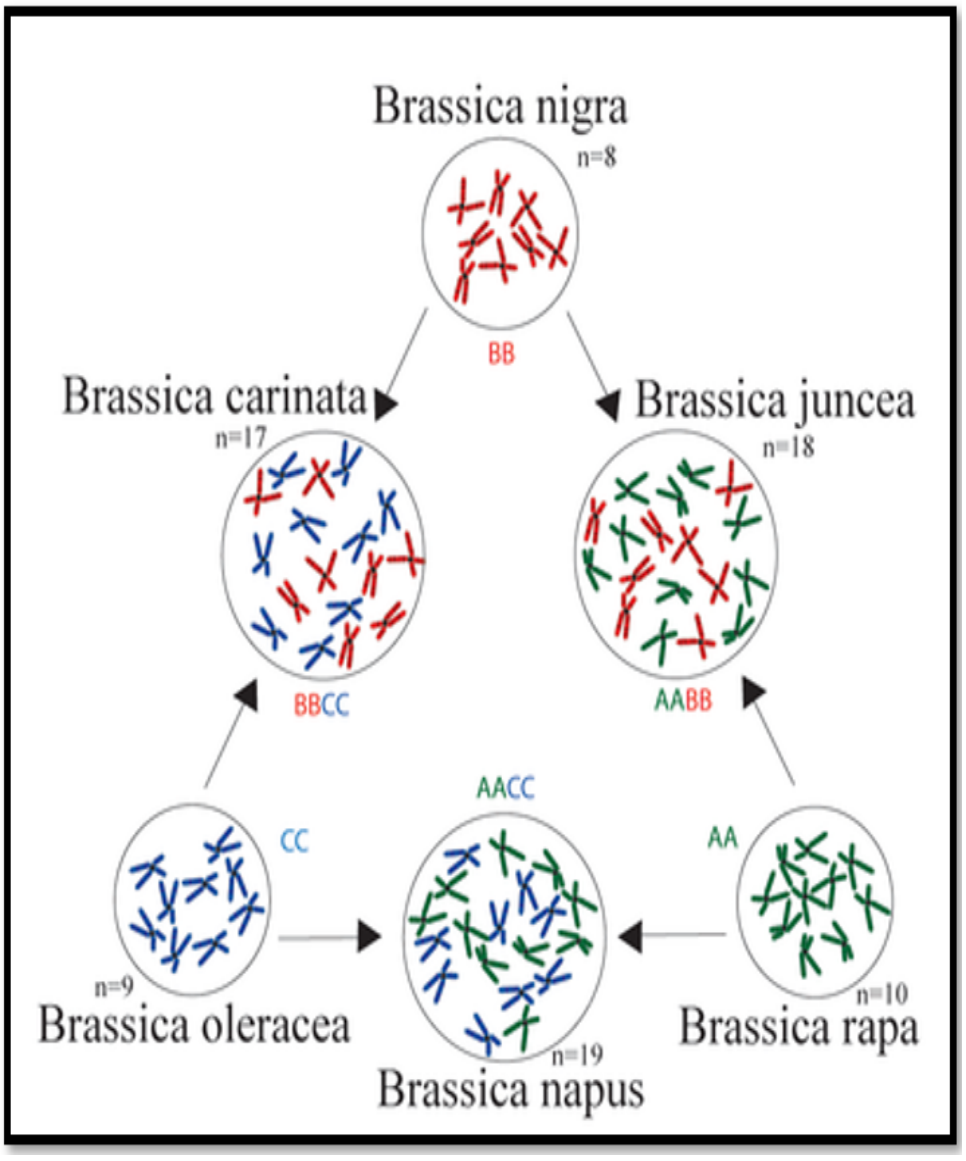

FIGURE 1.1: This diagram shows the genomic relationship between six *Brassica* crop species (called the triangle of U).

1.4 Background of *Brassica juncea*

Brassica juncea belong from the mustard family (Brassicaceae or Cruciferae), is known as a number of popular names, including Chinese mustard, brown mustard, and oriental mustard. Simple obovate-shaped leaves are present, as are bisexual flowers with four free sepals, two longer and two shorter stamens, and four sepal-free raceme inflorescences. *Brassica juncea* is a prominent tropical and subtropical oilseed crop in Asia. In the lack of sources for resistance, the extensive production of genetically similar types is known to attract fungal diseases that severely reduce yields. The traditional methods of illness management are frequently pricy, ineffective, and worse for the environment.

Locating and using resistance sources in *Brassica* and other non-hosts helps maintain oil crop yields. Chinese hybrids of *Brassica nigra* and other *Brassica* species include brown mustard seeds, while "oriental mustard" contains seeds that are yellow. This annual herb is an amphidiploid because it is a naturally hybrid of black mustard (*Brassica nigra L. Koch*) and turnip mustard (*Brassica rapa L.*), with both parents' whole genomes preserved. This species has a large number of variants and variants, including varietas *rugosa, gracilis,* and *juncea.* Although it is common in Europe and Africa according to a number of scholars, the primary genetic hubs for *Brassica juncea* are Eastern India, the Caucasus, and China [1].

Mustard is a low-cost, nutrient-rich food that also includes a significant amount of dietary fiber, beta-carotene, ascorbic acid, chlorophylls, minerals, and organic solvents. It also contains bioactive substances like glucosinolates and their degradation products, as well as polyphenols (flavonoids and anthocyanins). The distinct sharp, spicy, and pungent flavor of mustard makes it a popular spice. Additionally, mustard is widely used in pharmacy, and in folk medicine, the leaves can be utilized as a stimulant, diuretic, and expectorant. Particularly, research on fermented mustard has revealed a number of health advantages and illness preventive actions [2] and [3]. Despite the fact that numerous evaluations on the phytochemistry and biological activity of green vegetables have been produced, the mustard has not been thoroughly examined. Biological activities and phytochemicals of mustard,

as well as its plant properties, species, origin, distribution, and ingestion techniques, were examined in this study [4]. Approximately more than 3500 species and 336 genera make up the Brassicaceae family, which exhibits incredible diversity and is used as a supply of nutrition, oil, vegetables and mustard sauces. Their were different type of seed colors and type that were shown below in Figure 1.2

FIGURE 1.2: This diagram shows different type of *Brassica juncea*

Brassica juncea (L.) Czern & Coss is a naturally amphidiploid (AABB, 2n = 36) of *Brassica rapa* (AA, 2n = 20) and *Brassica nigra* (BB, 2n = 16) used for edible oil. During the 2018–2019 growing season, the estimated worldwide oil seed rape area, output, and yield were more than 35 Mha and 1980 kg/ha, accordingly. India

supplied 19.8 and 9.8%, respectively, of the world's acreage and production (USDA, 2020). This species is thought to have first appeared between 0.039 and 0.055 million years ago [5]. Despite chromosomal triplications and other reorganizations, juncea species have developed. The B linkage map *JUNEC* demonstrated that the genomes of both parents were preserved and unaltered since recombination.

The genomic assemblies of B lend support to this evolution scenario. *Rapa , B. Ngarra, B. juncea napus,* as well as B's pan-genomes. napus. the B genomes A and *B. juncea* contains the most varied genomes since it belongs to the two separate *Brassicaceae* lineages, Nigra and Rapa/Oleracea [6]. All cultivated types of B. are genetically identical. Juncea renders it susceptible to pathogen assaults. *Alternaria brassicae (Berk.) Sacc.; Alternaria juncea (A. A. Wiltshire; Brassicicola (Schwein.).* White rust, Clubroot, Powdery mildew, Blackleg, and Downy mildew are other plant diseases. Due to the existing cultivating varieties' lack of horizontal and vertical resistant strains, these diseases seriously harm the industrial supply of mustard.

The use of chemical fungicides to control disease is neither environmentally friendly nor cost-effective. Furthermore, very little effectiveness in illness control has been shown with cultural modifications. The most consistent and reliable method of illness prevention is the host's genetic immunity. Luckily, the Brassicaceae family contains the resistance gene(s) and quantitative trait loci (QTLs) for the majority of the above diseases [7].

Plant breeder's major goal is to explore the genetic variability of distinct genotypes of vital crops and to identify the promising genotypes for efficient yield. The information about genetic resources and genetic diversity of crops could help in the improvement of crops quality, production and in breeding techniques. The diversity evaluation and investigation of *Brassica* species would be a reason of identification of novel genes that are viable and resistant to abiotic stress [8].

The genetic diversity estimation of plant germplasm plays a crucial role in the improvement of crop varieties for better manufacturing and other desirable traits such as disease protection, insect and pest resistance, early shedding, heat and

cold tolerant. In the past, the harvesting, cultivation and yield of crop plants have increased and improved significantly because of using many morphological, biochemical and molecular methods.

Botanical researchers have been studying genomic diversity for ten thousand years ago, resulted in the birth of hybridization, in order to increase the yield and quality by exploring the genes of interest [9]. Hence it is understood that for the successful crop improvement genetic diversity analysis is crucial. Genomic studies helped in the security of food chain by determining the genes that were proven to be a cure for many agricultural problems. The genetic diversity analysis also provided us with advanced and improve breeding techniques that are a reason for modern known efficient genotypes [10]. Exploring a country's hidden and disregarded indigenous materials can help it thrive in socio-economic and scientific crisis. For future morpho-biochemical and molecular evaluations geographic information of selected germplasm is also critical.

According to the WTO, every country's customizing policy should be to get the most out of the global market by expanding their existing genetic resources. To measure the genetic variability in plant species, morpho-physiological, molecular, and biochemical markers are being used. In many crop species, now a day, plant breeders are utilizing molecular markers to study reproduction, speciation, and population spatial-temporal dynamics [11]. The *Brassicaceae* family is famous for economic significances among all plant families. *Brassica* vegetables are a dietary staple food in every part of the world with exception of tropical regions. *Brassica* crops like cauliflower, broccoli, and cabbage had a farm gate value of between 30 and 31 billion, cultivated on less than 40 million hectares according to 2013 report. So the origin and distribution of *Brassica* important.

1.5 Origin and Distribution of *Brassica*

The most significant genus of the *Brassicaceae* family is *Brassica*, which has approximately 3,500 species and 350 genera and is one of the top 10 geographically

and economically successful crops. However, the origin of *Brassica* is supposed to have originated in several locations throughout the globe, contemporary scientists believe that the wild varieties of this species are most likely to have originated in Europe. On the African continent, brassica is currently growing in Ethiopia, Tanzania, Kenya, Zimbabwe, and Mali. Among the Asian nations that cultivate it are Kazakhstan and some others. It is frequently cultivated in southern Australia's medium and high rainfall zones. *Brassica* is cultivated throughout the United States, including in Idaho, Minnesota, North Dakota, Montana, Oklahoma, Argentina, and Central America [12] .

1.6 Biochemical Based Diversity

In order to choose elite genotypes, it is crucial to evaluate the genetic diversity among various *Brassica* species. Different morpho-biochemical and physiological approaches are frequently employed to detect novel genotypes in plant species/-subspecies. Using biochemical techniques would allow for the greatest amount of polymorphism possible because they are not impacted by numerous environmental influences. It is widely used to examine the evolutionary connections between numerous important plant species or subspecies [13].

Modern techniques to quantify genetic diversity include the use of genetic indicators like total seed proteins, isotypes, and other biochemical markers. For *Brassica* species, genetic analysis is especially crucial since biochemically analyzed species differences may be crucial for identifying novel genotypes quickly and effectively. SDS-PAGE is an efficient, quick, and easy tool for examining genetic variation among organisms. The SDS-PAGE technique offers details on polypeptide profiles, which are thought to be a source of genetic diversity [14]. Their capacity to access genomic variation is tested by using random and EST-SSR markers to study genetic diversity. The markers qualifications will be obvious to you.

Crops can be genetically improved more quickly when there is a large genetic diversity and knowledge of the genetic resources that are available. To increase

oil production and health benefits, brassica germplasm may be investigated. The gathering of genetic resources and the evaluation of their genetic diversity should be planned as part of varietal development. Molecular markers aid in the investigation of cultivar differences and plant genetics. At any stage of plant development, DNA-based diversity analysis techniques can select traits like pest and disease resistance and produce highly reproducible results.

The only way to confirm the presence of numerous advantageous genes in a single variety and to reliably track advantageous traits throughout varietal selection is through DNA-based diversity analysis, cutting-edge genetic diversity analysis. The SDS-PAGE method has been used by researchers to analyses protein-based diversity among different *Brassica* species. For instance, the approach was employed to look at the protein levels of the *Eruca sativa* . The technique used to accomplish high resolution analytical separation of protein mixtures is called SDS-PAGE.

In order to examine genetic diversity, SDS-PAGE analysis of the important *Brassica* genus is useful. It is used to identify several kinds of protein components. The amount of oil consumed per person has increased due to population growth and rising levels of life. Genetic modifications must raise *B. juncea* production in order to meet demand for oil. The degree to which a species may be used for breeding and the variety of environments in which it can flourish are strongly correlated with the amount of genetic diversity that species contains. A set of parents and their offspring may be genetically distant from one another depending on two factors: the quantity of genes each parent contains and how those genes interact in a particular environment. Crop plant improvement can be impacted by genetic divergence and relatedness among breeding materials. If breeders and geneticists are able to predict which mattings will result in healthy, viable offspring and increase their genetic pool, they may benefit from a clearer grasp of *B. juncea's* genetic variety. The serious protein-based categorization of *Brassica* species is mandatory to monitor varied genotypes.

The SDS-PAGE process confers resourceful and rapid protein profiling of unusual crop varieties and it is harmless from every ecological cause. However, using

SDS PAGE alone for protein characterization of diverse *Brassica* varieties is not enough [15].

1.7 Molecular Markers Based Diversity

The history of *B. Juncea* is still not fully understood by previous studies. It may have originated from Europe, according to phylogenetic analyses employing RFLP markers, even though the most of its wild genotypes are located in Asia, from southern China to Japan. Therefore, East Asia did not experience turnip rape. Based on SSRs marker analysis three separate *B. rapa* groups were found in South Asia, Northern Europe, and Southern-Eastern Europe while the other two subgroups were only present in South Asia. Proper genomic research of this crop is necessary as *B. rapa* is crucial source of oil and food [16] .

Numerous studies of the genetic diversity of *B. juncea* based on molecular markers have shown that these species are frequently found in Asian and European habitats, with oil seed turnip rape typically occurring in Europe. Similar to this, a variety of this crop's vegetables were incorporated in the Asian variations, including turnips, bokchoy, tatsoi, etc. Understanding the genetic diversity across several *B. juncea* genotypes is vital for future genetic improvement [17]. The molecular markers can be used to compare species genetic differences. Also, those markers could be applied to detect chromosomal mutation that is caused by duplication, deletion, inversion, and insertion.
Molecular markers have little effect on the phenotypes as these are only present in close vicinity to the genes in the genome. The molecular markers have a lot of advantages over standard phenotypic markers as that are permanent and detectable in all tissues at stage of growth [18].

Additionally, a wide range of molecular methods have been created to assess genotypes in order to study genomic-based diversity. SSRs are among these markers because they are scattered throughout the genome, which is important for determining genotype divergence. Different markers have been examined for *B. juncea*,

however the SSRs marker has emerged as superior to others because of its durability, high polymorphism detection, and co-dominance production. SSRs are subsequently used as markers for more *Brassica* species, including safflower, Indian mustard, and others. Simple sequence repeats (SSRs), also known as microsatellites, are DNA-based markers found in eukaryotic organism's genomes that have 1-6 base pair tandem repeats in them. SSRs length variation is caused by high mutation rates ranging from 2-10 to 6-10 bp [19].

SSRs are codominant markers that are easy to use, predictable, and inexpensive. In closely related organisms, genomic variability is found using SSR-based analysis. SSR markers are now commonly utilized to characterize, use, and conserve crop varieties as well as to study wild populations. Recent years have seen the development and widespread use of molecular marker techniques for the study of genetic relationships among plants, phylogenetic relationship reconstruction, including identification of species, and gene mapping [20] .

Plant geneticists employ a variety of approaches to investigate genetic variation and relationships among plant assemblage members. Crop germplasm collection has engaged agricultural researchers and numerical taxonomists to categorize species and examine genetic relationships between and within species [21].

1.8 Research Gap

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

There is currently no detailed study available to describe *B. juncea* in this case of genotypes from the Province of Punjab, the objective of this study is to evaluate the genetic diversity and seed protein diversity of *B. juncea*. Comprehensive study is needed to correlated local Pakistan *B. juncea* genotypes with exotic genotype.

1.9 Scope

B.juncea is one of the imported oil seed crop of our country. *Brassica* breeding programmes can make use of potential genotypes that have been identified through the selection of elite genotypes applying molecular and biochemical methods. By doing this, it will be possible to increase domestic manufacturing of edible oil and lessen the need to import it.

1.10 Aim and Objectives

Aim

- To characterize distinct local and exotic *B. juncea L.* genotypes using efficient, highly reproducible and co-dominant SSRs markers.

Objectives

- Molecular markers-based characterization of diverse local and exotic *Brassica juncea L.* germplasm using Simple Sequence Repeats (SSRs)
- To identify promising *B. juncea L.* genotypes.

Chapter 2

Review of Literature

2.1 Biochemical Based Variability among Various Species

Brassica is the highly diverse genus of the plants belong to family Brassicaceae. These species are source of forage, edible oil, vegetables and ornamental that has been a cause of accelerating economies of many developing countries. The *Brassicas* are rich in oil content, essential metabolites and even its oil extracted press cakes are nutrients supply for animals. The specie contains a variety of members that are utilized as vegetables for human diets, such as Chinese cabbage, Pak-choi etc [22]. Its seeds contain 40% oil that is used as a human food i.e., a cooking medium, also in making pickles and many more businesses. Lightning lamps, oil-cakes, leather softening, and domestic animal feed are all made with the oils, and 1 Introduction 2 the seeds are used as medication for digestive disorders, swelling, cardiovascular diseases as an anticancer and so forth [23]. Rapeseed oil is widely used as edible oil and in breeding programmes because of its low glucosinolate concentration. The name erucic acid was coined in 1974 by Canadian oil, which is employed in human nutrition [24]. Among the crop families, the Brassicaceae family is crucial for producing edible oil of high quality and high yield. Brassica crops such as canola, *Brassica oleracea*, and mustard are cultivated

in Pakistan because of their high oil quality (44 to 46%). Its meal is made up of 38-40% protein and contains the essential amino acids for instance cystine, lysine, and methionine. After palm and soybean, *Brassica* oil seeds are the third most significant source of edible oil in the world, contributing almost 15% of edible vegetable oil (FAO, 2010). In Pakistan, all Brassica species are grown, but *B.napus, B.rapa, B. juncea* and *B.compesrtieset* are particularly more popular because of their high oil and protein content [25]. The most prominent member of Brassicaceae family is Brassica, which is among one of the top 10 economically and strategically important crops with over 3,500 species and 350 genera [26]. Many parts of the world are thought to be the center of origin of Brassica but according to modern scientist's community Europe is most likely to be the origin of wild types of this specie. Brassica is currently growing in Ethiopia, Tanzania, Kenya, Zimbabwe, and Mali on the African continent [27].

The crop *Brassica juncea* has been planted all over the world, but some of it has escaped and is now found as a noxious weed in uncultivated areas like wastelands and along the side of roads [28]. After fields have been harvested, any leftover seeds may sprout and threaten succeeding plantings. Australia, Japan, the Americas, and other Pacific Islands all consider it an invasive weed, but in Canada it is not a major problem. Since *B. juncea* extract hinders sunflower growth and some *Brassica* species use allelochemicals to inhibit other species, this plant may limit local biodiversity [29]. This species can be found all across the world, with the exception of the Arctic and Antarctic, where average annual temperatures fall below 6 degrees Celsius [30].

China, India, Myanmar, Iran, and the Near East have some of the most diverse populations. Its historic distribution covered Eastern Europe to China, where parent species overlap. Eurasia has long grown it. Bangladesh, Central Africa, China, India, Japan, Nepal, and Pakistan are major producers of this species. *Brassica juncea* is an amphidiploid (2n=36) hybrid. Linkage mapping revealed *B. juncea* genome is substantially unchanged after hybridization and includes both progenitor genomes [31]. This species is mostly self-fertilized [32], cross-pollinate 20-30% Cross-pollination occurs when racemes touch. At least 30 insect pollinators

from 10 families and four orders, including the cabbage white butterfly, visited brown mustard blooms in India [33]. Three bee species make up 88% of pollinators in Indonesian farmlands, together with Hymenoptera, Lepidoptera, Diptera, and Coleoptera species [34] .

Brassica juncea can be found growing in all of the world's life zones, ranging from Boreal Wet to Tropical Thorn to Tropical Wet Forest. It is said to be able to withstand annual precipitation ranging from more than 400 and less than 4300 millimeters, annual temperature ranging between 5 and 26 degrees Celsius, and pH ranging from 4.3 to 8.3. When the seed pods of the *Brassica juncea* plant dry out and shatter, the seeds inside are liberated. It demonstrates a marginally higher shattering resilience in comparison to other closely related species of Brassica, which may slow down the rate at which it disperses. There is a high volume of seed production, and these seeds, being relatively little, are easily carried by the air and the water [35].

Mathematical models of naturalized *B. juncea* populations in New Zealand showed that seed storage facilities and transit routes accounted for a large proportion of presence or absence variance. This points to the importance of human-mediated dispersal in the spread of wild populations [36] Hybrid species. *B. juncea's* top leaves clasp, unlike *B. napus* and *B. rapa.* *Brassica juncea* seeds are tiny and reddish-brown to orange. *B. juncea* has spherical seeds, while *B. nigra* has oval ones. *B. juncea* has thin, distinct lines that identify flat-bottomed interspaces. *B. juncea* seeds have obvious stipples, however *B. niger* seeds don't [37]. *Brassica juncea (L.)* Czern. is in the Cruciferae (Brassicaceae) or mustard family [38]. The blooms of the crucifer got their name because their four petals form a cross when viewed from above. The leaves of *B. juncea* are a light green in color, and they are hairy only on the youngest leaves. The leaf blades of *B. juncea* also end far up on the petiole. As they reach maturity, *B. juncea* plants can reach a height of two meters. The upper leaves are narrow and whole, while the lower ones are strongly lobed. Similar to its close relatives *B. napus* and *B. rapa, B. juncea* can be identified by the lack of a clasping top leaf rosette [39]. The inflorescence is shaped like an extended raceme, and its delicate, pale yellow flowers bloom in

ascending order from the raceme's base. The length of the pods, excluding the beak, ranges from 2.5 centimeters to 5 centimeters. The length of the beak is roughly 1 cm. The seeds are typically spherical and a range of colors, including yellow and brown. *Brassica juncea* has four subspecies with varied morphology, quality, and uses [40].

- Integrifolia, a plant whose leaves are consumed as a vegetable in Asia.
- Juncea, which is grown mostly for the seed it produces but can also be used as feed.
- Napiformis, a vegetable that is used for its roots and tubers.
- Taisai, In China, stalks, and leaves are all considered to be vegetables.

Oilseed *B. juncea* is a North American spice crop and an Asian cooking oil. Manitoba, Saskatchewan, and Alberta are Canada's key production areas [41] . Since World War II, when European supplies were hampered, Western Canada has become a significant mustard seed producer. The spice industry values the seed's appearance; thus, it's grown in warmer, drier areas where green seed is less likely. *B. juncea* is more drought-resistant than *B. napus* and *B. rapa*. This species' shape is preserved when swathed or sliced straight and connected, unlike *B. napus* [42]. Recent breeding efforts have resulted in cultivars with reduced glucosinolate and erucic acid content, making them suitable for use as a source of canola-quality cooking oil. Selective breeding has improved *Brassica juncea's* agronomic value, disease resistance, and quality. Yield, lodging, maturity, herbicide, drought, and shattering resistance are agronomic characteristics [43]. Blackleg, white rot, Alternaria blackspot, and Fusarium wilt resistance are bred. Whether canola or mustard cultivars are developed will determine quality improvements. Canola needs high oil, low glucosinolate, high protein, and a canola-quality fatty acid profile. Mustards should be low-oil, high-glucosinolate, and moderate-erucic acid [44]. *B. juncea* canola and mustard share agronomic methods. Both are grown in Canada's southern plains for their heat and drought endurance [41]. Mustard and canola are planted with cereal and pulses. Summer fallowing is a popular practice in

the southern grassland to store moisture and reduce weeds. Mustard and canola cultivated on weed-free fields. Wild mustard and cow cockle are hard to remove from harvested crops, degrading them. Mustard and canola diseases don't harm cereal crops, hence rotating both is useful for disease control [45]. Growing canola or mustard every three to four years helps reduce disease and weed concerns.

B. juncea grows annually. Self-pollination normally fertilizes ovules, however 20-30% outcrossing has been documented [46]. Pollen is heavy and sticky, therefore it's not moved far by wind. Flowering racemes cross-pollinate by touching. When resistant *B. juncea* was surrounded by non-resistant plants, outcrossing reached 35m. Due to *B. juncea's* similar floral structure, pollen volume, and outcrossing rate to *B. napus*, occasional outcrossing beyond 35m is likely [47], Outcrossing depends on recipient plant fertility, habitat, and pollinators. Outcrossing diminishes with field distance, but can occur at 200 m. *Beckie et al.* found 1.4% outcrossing at field boundaries and 0.04% at 400 m. A year revealed 800 m of gene flow. B. c. crops are field-grown. juncea seed may germinate before or after the next crop. The *B. Volunteer juncea* may weed. *Saskatchewan Brassica napus* losses average 3000 seeds m-2. *B. juncea* pod shatter resistance may reduce losses, although they're still large. *Bibbey* (1948) compared weed dormancy to cultivated species, including *B. juncea* [48]. He noticed that cultivated species germinated easily, even when buried, but weedy plants remained dormant. He attributed the discrepancy to weedy species' sensitivity to oxygen and CO2 concentrations. Since 1896, *Brassica juncea* has been described as an escape. *B. juncea* grows in prairies and parklands, although it's not a problem weed. Canada's Weed Seeds Order (2005) doesn't list it. Indian mustard ranked 131st out of 148 weeds in prairie weed surveys since 2000. Wild mustard ranked 24th and canola/rapeseed 14th. Given its lengthy history of cultivation in western Canada, *B. juncea* may be less prone to volunteer than *B. napus* and *B. rapa* [49]. Differences in cultivated acreage and, more recently, herbicide resistance in B. napus explain the different weed rankings across farmed species. *B. juncea's* shatter tolerance, small seeds, and thin seed coat in yellow-seeded cultivars may lessen its weediness compared to *B. napus* [43]. Rapeseed-mustard ranks third after soybean and palm.

Rapeseed-mustard adds 28.6% to India's oilseed production. After peanuts, it's India's second-most-important edible oilseed, accounting for 27.8% of the market. Rapeseed-mustard makes up 3% of India's total planted area. Rajasthan, UP, Haryana, MP, and Gujarat grow mustard. Karnataka, Tamil Nadu, and Andhra Pradesh grow it. Rain or irrigation can be used. Lotni and toria are brown sarsons. West Bengal, Assam, Bihar, and Orissa cultivate yellow sarson. Punjab, Haryana, UP, HP, and MP. Rajasthan, Haryana, and UP grow *Eruca sativa*. *Brassica napus L.* ssp. DC. Gobhi sarson grows in Haryana, Punjab, and Himachal Pradesh. It's greasy and effective. Karan rai is pest- and disease-free. From 1985–1986 to 1996–1997, production and productivity increased from 2.68 MT to 6.96 MT. India's 5.53 Mha of rapeseed-mustard yields 6.41 MT, or 1157 kg/ha [50].

TABLE 2.1: Featured rapeseed-mustard (Cruciferous) crop species [50].

Botanical name	**Common name**	**Maturity of days**	**Yield potential kg/ha**	**Oil%**
Brassica juncea	Indian mustard	105-160	1500-3000	38-42
Brassica nigra	Black mustard	70-90	1000-1200	40-41
Eruca sativa	Taramira	140-150	700-1400	34-38
Brassica napus	Gobhi sarson	145-180	1300-2700	37-45
Brassica rapa var. toria	Toria	70-100	600-1800	36-44

Mustard grows in temperate areas. In tropical and subtropical regions, it's a cold-weather crop. Indian mustard tolerates 500-4200 mm of annual precipitation, 6 to 27°C, and pH 4.3-8.3 [51]. Rapeseed-mustard assimilates carbon via C3. It photosynthesizes efficiently at 15–20°C. At this temperature, CO2 exchange peaks

and falls. Rai is a rainfed crop that tolerates acidic soil (pH 5.5 to 6.8) and drought. Mustard requires sandy loam. Rapeseed-mustard grows in rainfed systems with limited water (240–400 mm). 20% of these crops use rainwater. Review of rapeseed-mustard agronomic advances in India. rapeseed-mustard crops include brown sarson, raya, and toria. Rajasthan, UP, Haryana, MP, and Gujarat grow mustard. Karnataka, Tamil Nadu, and Andhra Pradesh grow it. Irrigated or rainfed crops are possible [52]. It responds better to fertilizers in irrigated conditions. Haryana, Punjab, and Himachal Pradesh grow Gobhi sarson. Slow-growing until late February, it's photo- and thermosensitive. It's oily and productive, rapeseed-mustard crop has eight crops; the main features have been shown in Table 2.1. Additionally, mustard oil is useful for therapeutic purposes. The leftover parts of the seed are used as animal feed and in the production of fertilizer. Even on soils that are polluted with heavy metals, the Indian mustard plant (*Brassica juncea*) is able to grow quickly and produce a large amount of biomass. Therefore, it is possible that this plant could be a viable choice for the Phyto filtration and/or Phyto stabilization of waste waters that are contaminated with heavy metals [53].

2.2 Molecular Markers Based Literatures

Simple-sequence repeats (SSRs), often called microsatellites, are small tandem repeating motifs [54]. SSR markers trump co-dominance Polymorphic, widespread, and easy to count. SSR markers are used to study genetic diversity, paternity, species identification, and genetic mapping [55]. Chrysanthemum nankingense has SSR markers. SSR markers have also been utilized to characterize Chinese ornamental chrysanthemum varieties [56].

Current genetic knowledge regarding Chinese traditional medicinal chrysanthemum variants is insufficient, hindering genetic conservation and enhancement of these endangered, commercially vital Chinese medicinal herbs. SSR markers have genome-wide coverage, good repeatability, co-dominant inheritance, high polymorphism, transferability between species, and low knowledge and instrumentation requirements. Small labs can employ SSR markers for low-cost plant genotyping [57].

SSR markers are used for fingerprinting, genetic diversity, population structure analysis, association mapping, and linkage maps. This technology advances genetics and plant breeding [58]. Many crops and plant diseases are developing SSR markers.

Making SSR markers was expensive and time-consuming. Many crops now have genome assemblies, and decreasing sequencing costs have made SSR markers timely and cost-effective [59]. With advances in sequencing technology, several commercially significant crops are re-sequenced. Reference genome assemblies and resequencing reads can detect and analyze many genome-wide SSR markers. LobSTR [60], RepeatSeq [61], STRViper [62], and HipSTR [63], use the reference assembly and whole-genome sequence data from several samples/individuals to find and genotype SSR alleles. Molecular markers accelerate plant breeding by analyze genetic diversity. ISSR, RAPD, RFLP, AFLP, SSR, and SRAP are frequent molecular markers [64]. Simple repeat sequences (SSRs) are a desirable candidate for genetic research since they are numerous, multi-allelic, highly polymorphic, and codominant [65]. RNA-sequencing is a viable alternative to a sequenced genome or model organism. Species-specific repeats (SSRs) from one species can reveal genetic variation in related species and genera. Genetic mapping, connection research, cultivar identification, and genetic diversity studies use SSR .SSR markers have been produced to study Zea mays [66], Citrullus lanatus [67], Triticum aestivum, and Cerasus species [68].

Molecular markers help identify individuals, define phylogenetic relationships, manage genetic material, and aid breeding. They use genome-based DNA polymorphisms [69]. Microsatellite markers, the grape plant's most common molecular marker system, use polymorphism based on simple sequence repetitions (SSR). Transferable, co-dominant SSR markers are important for identifying grapevine varieties and analyzing Vitis phylogeny. Most existing SSR markers fail to identify clones from a distinct single individual. Genomic techniques have also studied somatic changes causing clone diversity in grapevine varietals; 15 distinct Chardonnay clones have been identified by 1620 SNPs and InDels that can be used for clone-specific genotyping [70]. Plant genomes have many mobile components that

evolve quickly. They contribute to molecular polymorphism and are used in molecular marker systems. They exploit transposable elements' activity or structural variations. Sequence-specific amplification polymorphisms (SSAP) markers reveal the pattern of element insertion in the genus Vitis [71]. Retrotransposon polymorphism fingerprinting (RUP) shows a distinct pattern in all 94 Vitaceae accessions and is conserved between clones [72]. Using IRAP and REMAP, the white table grape cultivar Italia has been bred into many colored cultivars (Rubi, Benitaka, Brasil, and Black Star). Selective breeding and gene preservation use molecular markers in plant breeding [8].

Genetic mapping and map-based gene cloning require knowledge of molecular markers for breeding or genomic research [73]. SNPs, SSRs, and InDels are easy-to-use DNA markers. Microsatellites are tandem repeating motifs of 1–6 nucleotides [74]. Random distribution, high polymorphism, high clarity, cheap operational cost, reproducibility, hypervariability, ease of multiplexing, automation, and use with low-quality DNA make SSR markers practical. High-throughput sequencing has found unusual SNP, SSR, and InDel markers [75]. RNA-seq is next-generation sequencing of plant cDNA. RNA-seq and transcriptomics can gather molecular marker sequences. Protein-coding genes and transcribed areas cause RNA-seq read variations [76].

Transcriptome-based markers help understand the link between genes, phenotype, and function. Due to their great domain-to-domain conservation, closely related species can easily interchange them [77]. In addition to this, transcriptomic SSRs may provide vital information regarding the evolution of plant species as well as the conservation of their genetic variety [74].

Microsatellites tend to coexist with other satellites. For research of relationships, populations, and other topics, they are used as atomic markers in STR analysis [78]. Besides fingerprinting, marker-assisted determination, and the study of gene duplication/erasure, they can be used for a wide variety of other research purposes. To that end, OMICS Group International has become one of the most prominent Open Access Publishers, putting out 700+ peer-reviewed journals with

the help of 50,000+ editorial board members as editorial team. In addition to supporting 1000+ scientific organizations globally, OMICS Group organizes 3000+ international scientific conferences and events annually all over the world. Numerous gene investigations rely on SSR markers [79]. The use of genetic markers has greatly benefited plant breeding [80]. It is the gene or DNA sequence that controls a certain trait that serves as the genetic marker, and its placement on a chromosome is well-documented [81]. When searching for a specific gene, genetic markers can be used as signs or flags because of their close association with it. Classical markers and DNA/molecular markers are the two main types of genetic markers. DNA markers include RFLP, AFLP, and morphological, cytological, and biochemical markers [82].

Microsatellites are in DNA, chloroplasts, and mitochondria. Both coding and EST genes carry SSRs (ESTs). Small-Sample-Rate Fewer repetitive loci are seen in polymorphisms [83]. Variation in microsatellite repeats generates high polymorphism, which can be detected using PCR (PCR). DNA errors, recombination, retrotransposons, and mismatches cause SSRs. A, T, AT, GA, AGG, and AAAC are SSR motifs. Primers are developed from conserved sequences flanking SSRs. These primers are developed by sequencing a portion of a genetic library. See Kalia et al. for more on SSRs [84]. After creating an SSR library, certain microsatellites are isolated. After locating primer-designable sites, polymerase chain reaction is performed. Polymorphism research requires both banding and PCR analysis. SSR markers are the gold standard in plant mapping due to their co-dominance, repeatability, and genome abundance [85]. Furthermore, SSR markers can be applied effectively in a variety of different types of experiments [84]. SSRs are made up of repeating units that range in length from one to six base pairs [86]. Ancestry, genetic variation, molecular evolution, systematic taxonomy, linkage and comparative mapping, and functional diversity studies use SSR markers. SSR molecular markers are used in medicinal plant research. *Kherwar et al* [87]examined 24 SSRs in a total of 36 guava (*Psidium guajava L.*) varieties, in addition to analyzing wild species of the plant. Using RAPD and AFLP markers, investigations into the genetic diversity of guava, as well as genetic characterization of genotypes, cultivar

identification, and linkage mapping, were also carried out [88]. Technologies from the next generation of sequencing, or NGS, have also been used in the search for novel SSR markers. The Illumina Miseq NGS (next-generation sequencing) platform was used for the development of SSR markers for cumin (*Cuminum cyminum L.*) [89]. Identification of SSRs across the genome was also performed on the tea plant (Camellia sinensis) [90]. SSRs have been used in the investigation of not only terrestrial plant species but also aquatic plant varieties [91]. SSRs can be obtained through genomic sequencing or expressed sequence tag libraries (ESTs). Most genomic SSR markers come from intergenic DNA sequences whose genomic locations are unknown, making these approaches difficult and time-consuming. Transcriptome analysis and high-throughput DNA sequencing have helped find genic SSRs quickly and efficiently. This led to the discovery and research of SSR molecular markers in medicinal plants [92]. To date, however, only a limited number of SSR markers specific to Euphorbia have been reported. SSR loci in this plant have not been defined, despite the fact that studies have been done to characterize the transcriptome of Euphorbia fischeriana Steud [93].

Molecular markers are commonly employed to analyze genetic variation, including cultivar taxonomy, sex identification, and wood population genetic structure [94]. AFLP, RAPD, ISSSR, and SRAP are employed to determine sea buckthorn genetic diversity. Microsatellite markers are tandemly repeated 1-to-6-bp DNA segments. These mutations are codominant, randomly distributed across the genome, polymorphic, repeatable, and technically simple. Depending on how their detection sequences are structured, SSRs might be genomic or genic. Expressed genes contain SSRs from transcriptome or EST sequences. QTL analysis identifies phenotypic trait markers.

Transcriptome databases are important for SSR finding since high-throughput sequencing [95]. RNA-Seq SSRs are synthesized to analyze population genetics and species-level genetic connections. RNA-Seq SSRs are gene-based SSRs placed in the genome's coding region, giving them an advantage over genomic SSRs. Stable and transferable across taxonomies. RNA-Seq SSRs are widely used in plants due to their benefits. In sea buckthorn, nine genomic SSRs and 22 genic SSRs

have been produced [96]. No work employs RNA-Seq SSR markers to analyze sea buckthorn germplasm genetic links. Microsatellites are efficient molecular markers. Co-dominant genes have numerous alleles and are randomly dispersed across the genome. SSR in plants has been used for investigating genetic variety, developing genetic maps, improving crops with molecular markers, and identifying genotypes. SSRs are highly informative, codominant, multi-allele genetic markers that are repeatable and transferable among related species. SSRs are useful for studying genetic distance, gene flow and crossing over rates, and evolution, especially inferring infraspecific genetic linkages [97]. This applies especially to wild-animal investigations. SSRs are employed in cultivated plants to: I create linkage maps, map QTL loci, estimate kinship, use marker-assisted selection, and define cultivar DNA fingerprints. I make link maps. mapping quantitative trait loci (QTL). SSRs link phenotypic and genotypic variance. This is true for plant species where full-sib families are used to produce linkage maps and integrated genetic, physical, and sequence-based maps.

2.3 Genetic Diversity

Brassica sometimes called rapeseed-mustard, ranks third after soybeans and palm. China, India, Canada, Japan, and Germany cultivate rapeseed-mustard. After soybeans, they're India's second-most-important oilseed crop. India grows 20.23 percent of world rapeseed-mustard and produces 11.7% [98]. *Juncea, napus, rapa,* and B. India's 6.39 million hectares of carinata land produce 7.41 million tones [99]. It's more drought-tolerant and shatter-resistant than *B. napus* and *B. rapa*, therefore it's great for semi-arid locations. Population growth and improved living standards have raised per capita oil consumption. To meet oil needs, genetic interventions must improve *B. juncea* output. The quantity of genetic diversity that a species possesses is directly proportional to the extent to which it can be used for breeding and the variety of conditions in which it can thrive. The number of genes that each parent possesses and how those genes interact in a specific environment are two factors that might contribute to the genetic distance between

a set of parents and their offspring. Genetic divergence and relatedness among breeding materials can influence crop plant improvement. Breeders and geneticists may profit from a fuller understanding of *B. juncea's* genetic diversity if they can better forecast which mattings will produce healthy, viable offspring and expand their genetic pool [100]. Morphological, biochemical, and molecular techniques can identify genetic diversity. Many researchers have assessed *B. juncea's* genetic diversity using phenotypes. Isozyme loci have been used as genetic markers in *B. juncea* research. Environmental variables and plant development affect these metrics. *B. juncea's* genetic diversity is assessed using multiple marker systems. Molecular markers have been employed to study *Brassica's* diversity, genomic structure, and cultivar differences. SSRs are polymerase chain reaction markers that differentiate crops (PCR). Anyone can used this sign's trail map [101].

Although molecular markers are superior to other types of markers for genetic study in plants in terms of their ability to distinguish between closely related species and cultivars, many breeding groups still place more emphasis on physical features [102]. Their work was to use quantitative trait data and microsatellite markers to have a better idea of how closely related 44 *B. juncea* genotypes from different regions are to one another. It would be fascinating to compare the two methods' ability to identify *B. juncea* genotypes. In addition, genetic distances will aid in the identification of genetically varied genotypes, which can be put to use in the generation of useful selectable variation. To be acceptable for crop improvement breeding, a species must have a particular degree of genetic diversity. More importantly, thorough information must be obtained regarding the genetic divergence and relatedness of different breeding materials. Molecular markers are currently being used in an effective manner for the purpose of decoding the genetic variety that exists among populations [103]. Simple Sequence Repeat (SSR) markers are the most popular type of molecular marker due to their reproducibility, co-dominance, abundance, genome-wide distribution, straightforward scoring, and multi-allelic variation. SSR markers stand apart due to their distinctive features. Markers based on simple sequence repeats have been used for the purpose of conducting genetic diversity research on a variety of crops, including Indian

bread wheat [104]. In order to analyze the genetic variety in Indian mustard genotypes for tolerance against Alternaria blight, the present experiment was carried out using SSR markers. This allowed for the evaluation of genetic diversity. The locating of such genetically diverse or comparable genotypes will be of assistance in the process of introgression Alternaria blight resistance into other high-yielding cultivars.

Wild *B. rapa* and *B. nigra* likely bred several times in different locations across the Middle East and nearby regions, giving rise to *B. juncea*. It was also taken into account by Prakash and Hinata [105] that genuine wild forms of *B. juncea* exist in Asia Minor and that multiple distinct polyploidization events happened during the previous few thousand years. The results of taxonomic analyses, artificial synthesis experiments, molecular analyses, and chromosome mapping all point to its originating as an allotetraploid [106]. Molecular markers analyze oilseed B's genetic diversity. *Asian juncea*. 12th B. Using RAPD tests, we discovered less genetic variation in juncea than in 11 foreign accessions. *Khan et al.* [107] used RAPD markers to classify Indian mustard into two distinct groupings. Indian and Chinese lines clustered together according to AFLP markers, while lines from Australia, Canada, Eastern Europe, and Russia clustered together in a separate group [108].

Using RAPD analysis, scientists in southern and western China found a rich biodiversity of *B. juncea*. B-73. agroecological modifications influenced juncea landraces in southwestern China. 101 accessions from western China were investigated for molecular genetic diversity, and the results showed it was tied to local geological and ecological settings [109]. B winter genetic diversity. B's spring kinds and genetic diversity were lower than juncea's. Shaanxi and Xinjiang provinces had more juncea than Tibet. *Wu et al.* [110] evaluated oilseed B using SRAP markers. Molecular markers identify economically relevant genes and QTLs (QTLs). Pre-selection with molecular markers can lower a population's size and identify attractive genotypes in the early seedling stage [111]. SSR molecular markers are a powerful tool for characterizing germplasm collections. Co-dominant genes have many alleles, genome-wide distribution, high frequency, repeatability, and

high-throughput automated allele identification and quantification [112]. Due to a paucity of molecular markers, *B. juncea's* genetic diversity is poorly understood, which hinders crop improvement. The rapid development of sequencing data allows the discovery of genes and markers for molecular breeding and germplasm augmentation. In the case of *Brassica rapa*, SSR marker libraries have been generated, and these libraries have been used in the production of a genomic map for the species [113].

SSR markers derived from *B. rapa* are anticipated to be beneficial for detecting polymorphism in *B. juncea* as well because to the close evolutionary link between the two species. However, only a small number of SSRs derived from *B. juncea* have been identified up until this point, and a larger number of markers are necessary for comprehensive germplasm characterization and crop improvement. Because of this, the current study was carried out in order to determine the causes of disease resistance in order to generate disease-resistant cultivars that are of significant agronomic relevance.

DNA-based genetic markers are frequently used for quick cultivar identification, fingerprinting, and diversification studies due to advances in molecular biology and genomics. Greater genome coverage, more common, easy to reproduce, co-dominant inheritance pattern, and more polymorphisms [114], Plant breeders and biotechnologists use SSRs to measure genetic diversity and classify types. SSR markers are the most promising technology for generating a database of plant DNA fingerprints [115], doing genetic diversity research [116], using markers to aid in breeding [117], and determining whether crop types are pure. Since all Indian mustard types come from a small genetic pool, they all have a similar lack of polymorphism that makes it difficult to advance the crop through selective breeding. Large-scale polymorphism SSR markers equally placed across Indian mustard's linkage groups should improve molecular characterization. This would help identify mustard cultivars, protect plant breeders' rights, and investigate genetic variation. We also created molecular tags and DNA fingerprints to examine Indian mustard communities [118]. The cost of developing SSR markers for a crop limits their application in genetic investigations. Microsatellite development

involves creating a genomic library of DNA inserts, hybridizing using tandemly repeated oligonucleotides, and sequencing candidate clones. This method is expensive, time-consuming, and difficult. SSR transfer between closely related species or taxa solves this difficulty [119]. Many closely related species have substantially preserved genetic material and organization, and their SSR loci have sequence homology [84]. Because flanking sequences are preserved, SSR markers can detect microsatellite loci in related species.

This method of detecting and developing SSRs can be used in crops without sequence information. Several studies have revealed that specific sequence repeat (SSR) markers can be transferred within or between genera [120]. An amphidiploid, *Brassica juncea* (AABB) combines the genomes of two different species, in this case, B. rapa (AA) and B. nigra (BB) [121]. Numerous SSR markers have been created, with several demonstrating use within and between different *Brassica* species. *Brassica rapa* (AA). Transferable genomic SSRs will allow DNA fingerprinting, varietal identification markers, and other genomic studies on Indian mustard.

Molecular markers are currently being used in an effective manner for the purpose of interpreting the genetic variety that exists among populations. Simple Sequence Repeat (SSR) markers are the most extensively used type of molecular marker due to their repeatability, co-dominance, abundance, genome-wide distribution, simple score ability, and multi-allelic variation. SSR molecular markers are the most popular [103]. SSR have been used for the purpose of conducting genetic diversity studies on a variety of crops, including Indian bread wheat [122], rice [123], and maize. Both *B. rapa* and *B. napus* have had a tremendous amount of work put into developing and characterizing a large number of SSR markers. On the other hand, there are very few SSR markers detected for *B. juncea* [124]. *B. juncea's* variation and genetic structure have been studied using molecular markers. AFLP was used to investigate 16 *B. juncea* samples for genetic differences [125]. SRAP was used to compare *B. juncea* with *B. juncea var.* gracilis genetically [110]. Simple sequence repeat (SSR) was used to determine *B. juncea's* origin and distribution in India and China [109] and along the Yangtze River [126]. ISSR is used to determine

genetic diversity in wild *B. juncea* populations [127]. They skipped Tibetan and Xinjiang in favor of central and eastern China. Knowing it spread from the Middle East to West China isn't enough.

Molecular and phenotypic markers are used to identify plants. Phenotypic markers are based on the variety's fruit, leaves, and spines. When employed on closely related crops, these markers can be unreliable and imprecise. This is related to regional climate circumstances and natural fluctuation throughout plant growth [128]. Because molecular markers provide precise estimates of genetic line distance, DNA variation assessment has seen much innovation. Markers have different advantages, limitations, and traits that make them better for some jobs than others.

Codominant and dominant markers generate information. Using codominant markers, a diploid organism can identify heterozygosity from homozygosity. These markers distinguish two alleles in a diploid organism. Dominant markers are only examined based on allelic frequency or binary data. This means dominant markers are evaluated depending on a DNA fragment's length [129]. SSR markers based on genes have also been developed for diversity assessments and used in several research on plant genetic variety and organization. Ease of PCR amplification, co-dominance, and great allelic diversity have contributed to their appeal. SSR markers are the most effective, but their usage is limited since it depends on existing databases (EMBL-EBI, GenBank) or the identification and building of new SSR marker libraries.

These molecular indicators lack genotype x environment (G x E) interaction and seasonal constraint. The use of such markers helps assess a population's genetic diversity and characterize individuals and breeding lines, both of which are vital for making informed judgments on parental genotypes in breeding plans. Research shows that commercially grown peanuts have minimal genetic diversity. SSR markers have more DNA polymorphism in grown peanuts than RFLPs or RAPDs, are easier to amplify, and cost less [129].

Peanut's limited genetic variability and two genomes have slowed the identification and characterization of molecular markers compared to other commercially

important crops like soybean, maize, and rice. Cultivated peanuts are a hybrid of two separate species, thus they have two genomes. Molecular markers are used in phylogenetics, evolution, ecology, population genetics, and the study of complex plant and animal genomic features. Rapid advances in biological and molecular science technologies have made these applications possible [130]. Using DNA-based markers can help in plant genetic engineering [131]. Using different markers, each in its own way, can help determine a population's DNA diversity. These variants include amplified and restriction fragment length polymorphisms, SNPs, SSRs, and random amplified polymorphic DNA [132]. Plant breeders use molecular markers to choose plants with the desired traits. DNA markers offer benefits in plant molecular breeding due to their application, quantity, independence from time and stage, and lack of phenotypic bias [133]. Most PCR-based markers are microsatellites and SSRs. These co-dominant, locus-specific, hypervariable, multi-allelic, and durable markers have been successfully used across a wide range of plant species. SSR markers' extensive distribution across the genome makes them potential for high-quality genotyping and gene mapping [134].

This study focused on *Brassica* genetic diversity and genome polymorphism using PCR-based SSR markers (also known as PIC). Two of the three diploid species bred, forming amphidiploids. Molecular markers have considerably sped up global *Brassica* improvement. A expanding pool of sequencing data reveals the genetic origins of critical traits for molecular breeding and germplasm enhancement [135]. All three *Brassica* species (*B. napus, B. nigra,* and *B. rapa*) have distinct SSRs. Unfortunately, B. To characterize germplasm and improve crops, more juncea markers are needed. In India and China, *B. juncea's* genetic diversity was studied using molecular markers.

RAPD assays indicated that 12 Indian *B. juncea* accessions have lower genetic variability than 11 alien accessions [107]. *Khan et al.* (2008) classified Indian mustard using RAPD markers. AFLP markers showed that Indian and Chinese lines clustered together, while Australia, Canada, Eastern Europe, and Russia lines clustered together. RAPD study identified a lot of *B. juncea* in southern and western China. Southwest China's 73 *B. juncea* landraces were categorized

by agroecological adaptations. AFLP, SRAP, and SSR markers were used to link geological and biological conditions at collecting sites to 101 western China accessions' molecular genetic diversity. *B. juncea's* winter kinds are more diverse than its spring types, and its winter types in Shaanxi and Xinjiang are more diverse than those in *Tibet* [109]. *Wu et al.* [110] used SRAP markers to classify 67 Chinese oilseed *B. juncea* accessions, 10 international oilseed *B. juncea* accessions, and 18 non-oilseed *B. juncea* accessions based on their growth behaviour (spring or winter type).

Morphology, biochemistry, and molecular biology can determine genetic variety. Past study has evaluated *B. juncea's* genetic diversity using phenotypic characteristics. Numerous genetic studies have employed isozyme loci as a marker, including one on *B. juncea*. Climate and plant stage affect these features. Numerous marker systems have been used to explore *B. juncea's* genetic diversity [136]. Despite the fact that molecular markers are better to other markers for genetic studies in plants in terms of efficiency, precision, and dependability, many breeding groups still priorities morphological traits over molecular markers. This study was done to offer a reasonable estimate of 44 B's genetic diversity and to investigate the connection between juncea genotypes from different regions using quantitative trait data and microsatellite markers. These two ways of distinguishing B genotypes could be compared. juncea.

Genetic distances can be used to create selected variation by locating genotypes with genetic variety. DNA-based molecular markers have become prominent due to the rapid development of molecular biology techniques and genomic resources, allowing for the rapid identification of cultivars, fingerprinting of plants, and analysis of diversity. Because of their richness, repetition, simplicity, co-dominant inheritance, higher polymorphism rate, and larger genome coverage, simple sequence repeats (SSRs) have exceeded previous approaches for measuring genetic diversity and characterizing plant types. SSRs are polymorphism compared to other DNA-based markers. SSR markers are used to establish a plant DNA-fingerprint database, analyze genetic variation, aid in breeding, and identify varietal purity. SSR markers can sort agricultural homonyms, synonyms, and duplicates [137].

Scientists have studied Indian mustard's diverse mustard genotypes before. Most of these studies use tiny samples and uninformative RAPD or SSR markers. Some contend that randomly chosen markers overlook the functional diversity of the genome's coding regions. Plant breeding relies on detecting heterotic locus variation. Using random and EST-SSR markers to examine genetic diversity tests their ability to access genomic variation. You'll know the markers are qualified. Genetically upgrading crops is faster when there is a lot of genetic variety and information about available genetic resources. *Brassica* germplasm could be explored to boost oil production and health benefits. Varietal development should arrange the collecting of genetic resources and the assessment of their genetic variety. Molecular markers help researchers investigate plant genetics and differentiate cultivars.

DNA-based diversity analysis methods can select traits like pest and disease resistance and produce highly reproducible results at any stage of plant development. DNA-based diversity analysis is the sole way to establish the presence of many advantageous genes in a single variety, allowing for reliable tracking of beneficial traits throughout varietal selection. Genetic diversity analysis is cutting-edge [138]. Searching for putative plant miRNAs has evolved over time. Standard approaches include cloning size-fractionated RNAs, Sanger sequencing, and experimental validation. Due to the largely conserved nature and similar secondary structure of most miRNAs, new homologs can be easily predicted in species of interest. Following a conceptual procedure, we searched for probable miRNA homologs and their target genes in *B. juncea* using publicaly available transcriptome data, ESTs, and genomic sequences (GSS). To determine the biological role of projected miRNAs, we classified and enriched miRNA-targeted genes. We also analyzed predicted miRNAs, miR-markers (SSRs/SNPs), and their target genes for marker-assisted breeding of *B. juncea*.

Current agricultural methods and technologies have reduced crop genetic diversity, which has raised crop sensitivity to biotic and abiotic problems. This increases the likelihood of crop failure in these conditions. Climate change has made the situation harder to control. *Brassica* has 100 wild and weedy cousins. These

relatives provide agronomic and economic traits. To use genetic resources, one needs understand how they are organized and the genetic variety available. In this regard, molecular markers have contributed to breakthroughs in crop plant genetic analyses. Plant breeders often use SSR markers for molecular-based breeding. Gene-based SSR markers are excellent for this application since transcribed SSRs can become functional in genic regions [139]. In a previous work, we tested 161 genomic-SSR markers from *Brassica napus, Brassica nigra, Brassica rapa,* and *Brassica oleracea* in 11 other *Brassica* and allied taxa. Only 70 (43.5%) of 161 genomic-SSR markers were transferrable to at least one of 11 species. Since genic-SSR markers are created from the transcribed region of the genome, they are transferable between species and genera. DNA markers including microsatellites, SSRs, and ISSRs can estimate genetic diversity. They can overcome environmental impacts and work independently of tissue effects, making them more precise than other markers in characterizing genotypes and quantifying genetic connections. These studies don't focus on how to apply these new technologies to find exceptional varieties. Particularly difficult is confounding one of these elite genotypes with a randomly selected genotype from a broader pool and minimizing analysis costs (i.e., the number of amplifications, and consequently the number of primers).

Chapter 3

Materials and Methods

3.1 Research Methodology Flow Chart

To find out the promising genotype and of *Brassica juncea* following steps involved.

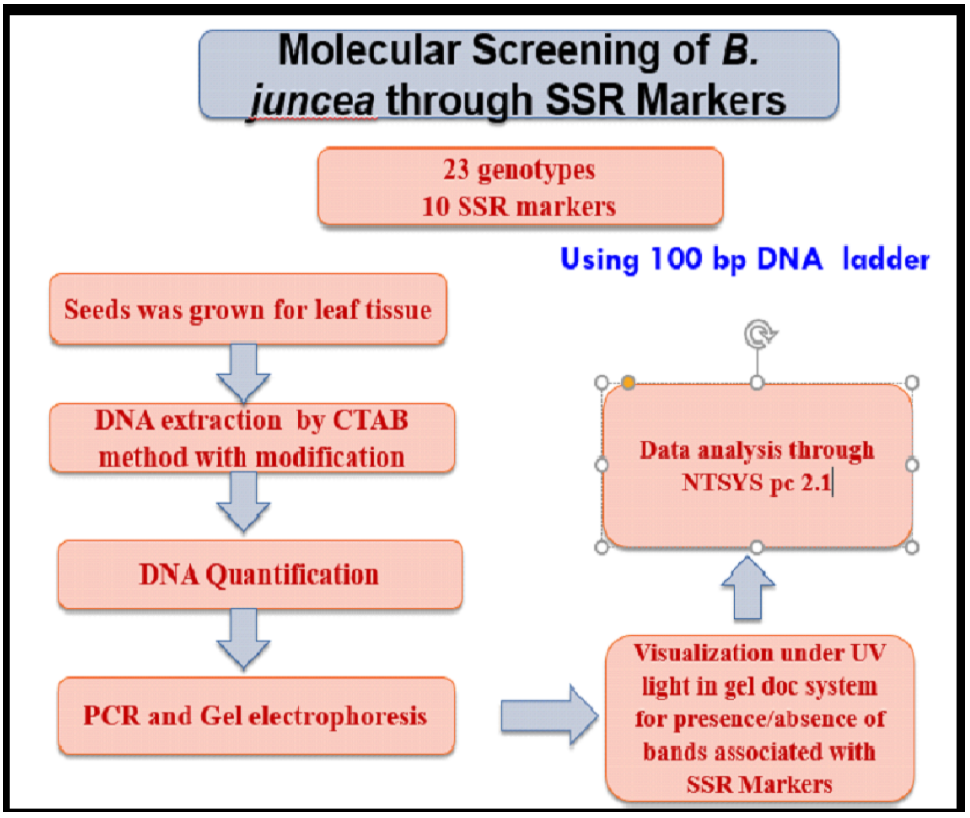

FIGURE 3.1: Overview of research methodology.

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

3.2.1 Experimental Material

Total 23 *B. juncea* genotypes local and exotic were collected of our country and Gene bank, NARC, Islamabad. Ten crop-specific SSR markers were used to analyze the genetic diversity of these genotypes. Tables 3.1 and 3.2 include *B. juncea* genotypes and markers used on samples.

TABLE 3.1: List of *B. juncea* genotypes used for SSR analysis (n=23).

Sr. No.	Acc. No.	Source
1.	*Bj1107*	Punjab, Pakistan
2.	*Bj1112*	Punjab, Pakistan
3.	*Bj1120*	Punjab, Pakistan
4.	*Bj1123*	Punjab, Pakistan
5.	*Bj1130*	Punjab, Pakistan
6.	*Bj1137*	Punjab, Pakistan
7.	*Bj1140*	Punjab, Pakistan
8.	*Bj1143*	Punjab, Pakistan
9.	*Bj1151*	KPK, Pakistan
10.	*Bj1153*	KPK, Pakistan
11.	*Bj1159*	KPK, Pakistan
12.	*Bj1163*	KPK, Pakistan
13.	*Bj1171*	KPK, Pakistan
14.	*Bj1173*	Sindh, Pakistan
15.	*Bj1182*	Sindh, Pakistan
16.	*Bj1187*	Sindh, Pakistan
17.	*Bj1190*	Germany
18.	*Bj1194*	Germany
19.	*Bj1199*	Germany

TABLE 3.1: List of *B. juncea* genotypes used for SSR analysis (n=23).

Sr. No.	**Acc. No.**	**Source**
20.	*Bj1201*	Netherlands
21.	*Bj1210*	Netherlands
22.	*Bj1216*	Netherlands
23.	*Khanpur Raya*	NARC, Islamabad, Pakistan

TABLE 3.2: SSRs primers for diversity evaluation of *B. juncea* germplasm.

Primer	**Forward Primer(bp)**	**Reverse Primer(bp)**
Na10-F06	CTCTTCG GTTCGAT CCTCG	TTTTTAA CAGGAA CGGTGGC
Ni3-G05	AGGAAGC ATTTGCGC TAGTC	TCTACAA CCACAAC GTCCAAG
PBCES SRJU2	TTCACAT CTTCTTC ATCTTCC	TTGCTAT TCGTTCT CAGTCTC
PBCES SRJU3	CCTCTTT TAATTCA AACAAG AAATCA	TTCGGAC AATGGCA GTGATA
PBCES SRJU6	TCTCTCA CCTGCCT TGTCT	ACTCCTC GGTAATG CCTC
PBCES SRJU12	AAGCTCA GATCGTT TGCG	AGATGAA TGTGAAA TAGGGGT
PBCES SRNA8	ACTGAGA GCAACAACAACAAC	GTAGAGA CGGAACCCTGA

TABLE 3.2: SSRs primers for diversity evaluation of *B. juncea* germplasm.

Primer	**Forward Primer(bp)**	**Reverse Primer(bp)**
Ra2-D04	TGGATTC	CAAACCA
	TCTTTACACACGCC	AAATGTGTGAAGCC
Ra2-E03	AGGTAGG	CCAAAAC
	CCCATCTCTCTCC	TTGCTCAAAACCC
Ra2-F11	TGAAACT	CTTCACC
	AGGGTTTCCAGCC	ATGGTTTTGTCCC

3.2.2 Sample Preparation

Each pot, was planted with 4-5 fresh seeds of the respective genotype. These pots were kept at glass house for 2-3 weeks, they were given many doses of water throughout that time. After a period of between two and three weeks following the germination, samples of the young leaves were collected. In preparation for potential usage in the future, the leaf samples were frozen at -4 °C.

3.2.3 DNA Extraction

All the stock solutions were prepared prior to DNA extraction (Table 3.3 and 3.4) and the DNA was extracted using CTAB method [140]. The DNA was extracted as follows:

TABLE 3.3: 10X TBE preparation.

Chemicals with Measurement
Tris = 188g
Boric Acid = 55g
EDTA= 7.45g
Agarose= 10g
Distilled water = Total volume of 1000 ml

Stored at room temperature (25°C)

Table 3.4: 2X C-TAB (100ml)

Chemical with Measurement
Tris= 10ml
EDTA= 0.4ml
NaCl= 28 ml
C-TAB (conc.)= 2g
PVP= 1g
ddH_2O= 58ml
Total volume of 100ml Stored in a refrigerator

- Three to four leaves from each genotype were mashed in a mortar and pestle with a 700 µL CTAB solution containing 30 µL of mercaptoethanol (1 ml CTAB solutions). Crushed samples were placed in a 1.5 ml Eppendorf tube.
- For 40 minutes at 65°C, all samples were rotated four times every five minutes in the water bath.
- The water bath rotated samples four times every five minutes for 40 minutes at 65°C.
- Samples were centrifuged at 13000 rpm for 10 mins at 4°C.
- The supernatant was transferred to 600 µL tubes for each sample and discarded.
- Then 350 µL of ice-chilled isopropanol was applied 2-3 times and frozen at 40°C for at least 30 mins.
- Centrifuged again for 10 minutes at 4°C at 13000 rpm.
- From each tube the small white pellets were collected.
- After carefully discarding the supernatant, each tube received 200 µL of 70% ethanol.

- Again, centrifugation takes 8 minutes at 13000rpm at room temperature.
- After carefully discarding the supernatant, the tubes were left open at room temperature on sterilized filter paper.
- The pellet was dried on filter paper for an hour until the ethanol smell disappeared.
- Then in every single tube 100 µL of fresh TE buffer was added and vortexed.
- Incubated each tube for 35 minutes at 40°C in a water bath with 1 µL of RNase A (10 mg/ml).
- Then at -20°C in freezer all the extracted DNA samples were stored.
- A Nano Drop ND-1000 Spectrophotometer at 260 and 280 nm assessed DNA sample quality and purity.
- All DNA samples were diluted to 20-50ng/µL for PCR accuracy.

3.2.4 Specification of the Primers

Ten simple sequence repeats (SSRs) markers were employed to examine genetic diversity among *B. juncea* strains. Primers designed specifically for *Brassica* resulted in both monomorphic and polymorphic bands, which can be found in the literature.

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

Based on primer data, the annealing temperature was tweaked to optimize PCR conditions. In table 3.5 lists PCR parameters and the 20 ul reaction volume. The primer's size determined the reaction's agarose quantity.The PCR profile parameter is given in table 3.6 PCR products separated clearly in a 2-3% agarose gel.

A 1xTBE buffer (Tris-Borate = 10mM and EDTA = 1 mM) and 5l ethidium bromide were used to make the high-resolution agarose gel. Cycles 3.14 and 3.15, PCR reagents/mix.

TABLE 3.5: Microsatellite PCR analysis (reaction mix)

Components	**Stock concetra -tion**	**Final concetra -tion**	**Vol/ Rxn**	**Samples**	**Total Vol.**
ddH_2O PCR Buffer	-	-	10.7µl	23	321µl
Minus $MgCl_2$	10x	1 x	2.0µl	23	46µl
dNTP Mixture	100 mM Each	2mM	2µl	23	46µl
Forward Primer	20 pmoles/µl (20µM)	0.8 µM	0.8µl	23	24µl
Reverse Primer	20 pmoles/µl (20µM)	0.8 µM	0.8µl	23	24µl
Taq DNA Polymerase	5 Units/µl	1 unit/r x n	0.2µl	23	4.6µl
Template DNA Polymerase	20-50 ng/µl	20-50 ng/r x n	1.0µl	-	-
Total Volume	-	-	20.0µl	-	-

TABLE 3.6: PCR thermal cycler profile

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

3.2.6 Electrophoresis of Amplified Products

Following the completion of the PCR process, 4 ul of a 6x loading dye was added to each of the PCR tubes. Each well received the 7 µl PCR sample that was placed into it. Using a DNA ladder with both 50 and 100 base pairs, the sizes of SSR markers were double confirmed. The PCR products' optimum sizes were measured and documented using the UVI Gel Doc Documentation System after the gels were viewed there.

3.2.7 Allele Scoring and Data Analysis

Each sample and primer had a single, two, or multiple banding pattern. Bands were 1 or 0. MS-Excel held all data. Only clear DNA bands were analyzed. Total allele presence, polymorphic allele count, and optimal annealing temperature were recorded for each primer. The dice algorithm calculated genetic similarity coefficients by comparing *B. juncea* genotypes with or without alleles [141]. A UPGMA-based phylogenetic tree 2D and 3D analysis was conducted by using Dice similarity coefficients value using NTSYS version 2.1 software [142].

Chapter 4

Results and Discussions

4.1 Microsatellite (SSR) Based Inter-specific Variability Among *B. juncea* Genotypes

Twenty-three unique *B. juncea* genotypes were selected as a foundation for SSR-based molecular analysis. 10 different SSR specific *B. juncea* markers were used to characterize the 23 tested genotypes.

4.1.1 Inter-specific Variations Among *B. juncea* Genotypes

Ten SSR markers examined the genetic diversity of twenty-three *B. juncea* genotypes. The desired amplified fragments were found by comparing these marker pieces to their known sizes. A locus had one to two alleles. Each primer showed polymorphic banding Figure 4.1 to 4.6 and Table 4.1.

TABLE 4.1: Grouping of 23 genotypes of *B. juncea* through cluster analysis.

Cluster	**No. of genotypes**	**Genotypes**	**Origin**
I	6	*Bj1107*	Punjab, Pakistan
		Bj1159	KPK, Pakistan

TABLE 4.1: Grouping of 23 genotypes of *B. juncea* through cluster analysis.

Cluster	**No. of genotypes**	**Genotypes**	**Origin**
		Bj1123	Punjab, Pakistan
		Bj1163	KPK, Pakistan
		Bj1199	Germany
		Bj1210	Netherlands
II	11	*Bj1151*	KPK, Pakistan
		Bj1153	KPK, Pakistan
		Bj1182	Sindh, Pakistan
		Bj1201	Netherlands
		K. Raya	NARC, Islamabad, Pakistan
		Bj1194	Germany
		BJ1171	KPK, Pakistan
		Bj1173	Sindh,Pakistan
		Bj1187	Sindh, Pakistan
		Bj1190	Germany
		Bj1216	Netherlands
		Bj1112	Punjab,
		Bj1130	Punjab,
III	5	*Bj1137*	Punjab,
		Bj1143	Punjab,
		Bj1140	Punjab,
IV	1	*Bj1120*	Punjab, Pakistan

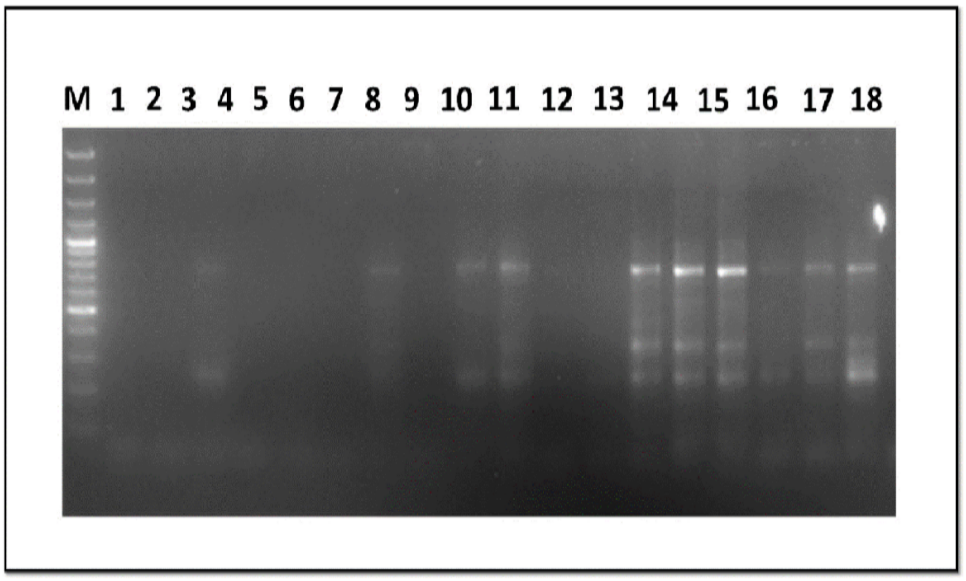

FIGURE 4.1: SSRs marker banding patterns of *B. juncea* that were explained.

In figure 4.1 SSRs marker banding patterns of 18 genotypes of *B. juncea* by SSRs primer Na10-C06, 1-23. M = 100 bp molecular marker, Bj-1107, Bj-1159, Bj-1123, Bj-1163, Bj-1199, Bj-1210, Bj-1151, Bj-1153, Bj-1182, Bj-1201, K. Raya, Bj-1194, BJ-1171, Bj-1173, Bj-1187, Bj-1190, Bj-1216 and Bj-1112 respectively.

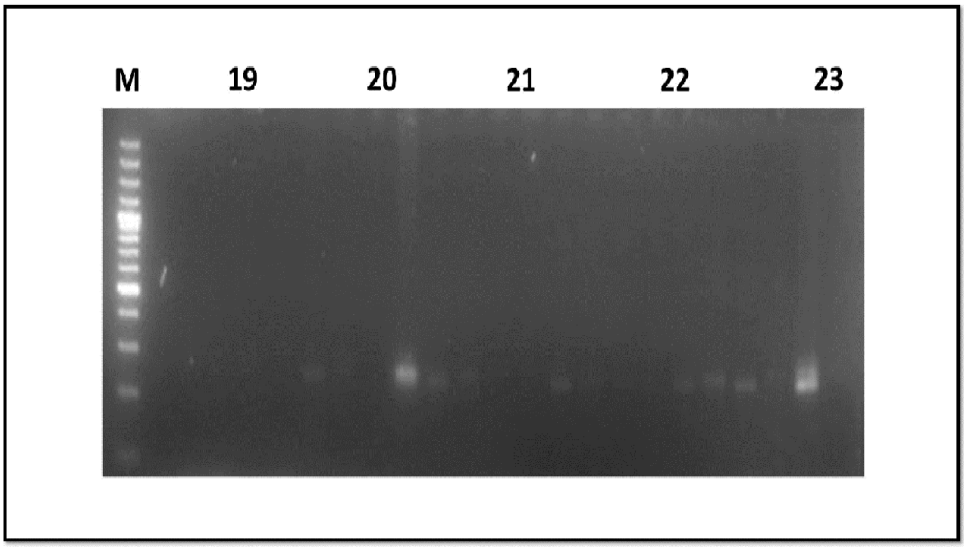

FIGURE 4.2: SSRs marker banding patterns of *B. juncea* that were explained.

In figure 4.2 SSR banding patterns of 5 genotypes of *B. juncea* by SSR primer Na10-C06, 24-30. M = 100 bp molecular marker, Bj-1130, Bj-1137, Bj-1143, Bj-1140, Bj-1120 and respectively.

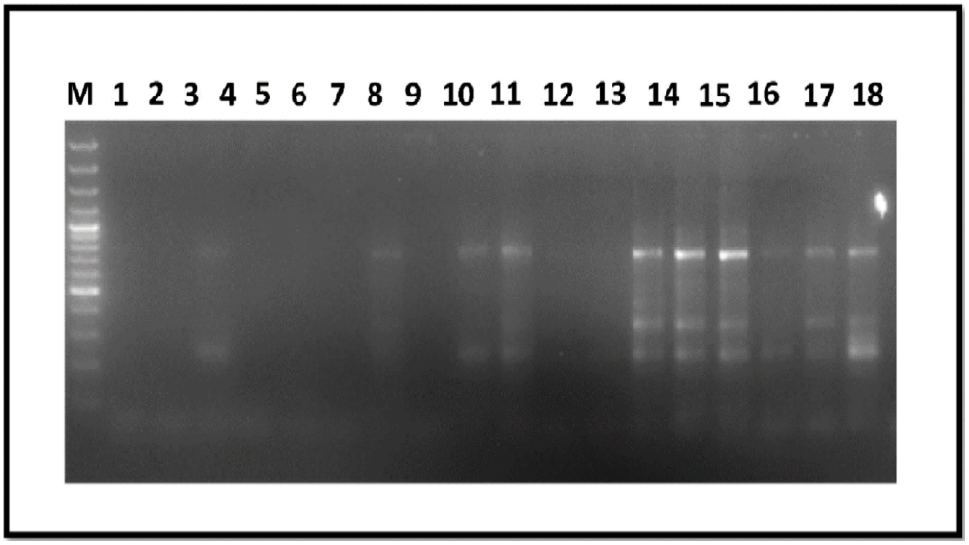

FIGURE 4.3: SSRs marker banding patterns of *B. juncea* that were explained.

In figure 4.3 SSRs marker banding patterns of 18 genotypes of *B. juncea* by SSRs primer Na10-C06, 1-23. M = 100 bp molecular marker, Bj-1107, Bj-1159, Bj-1123, Bj-1163, Bj-1199, Bj-1210, Bj-1151, Bj-1153, Bj-1182, Bj-1201, K. Raya, Bj-1194, BJ-1171, Bj-1173, Bj-1187, Bj-1190, Bj-1216 and Bj-1112 respectively.

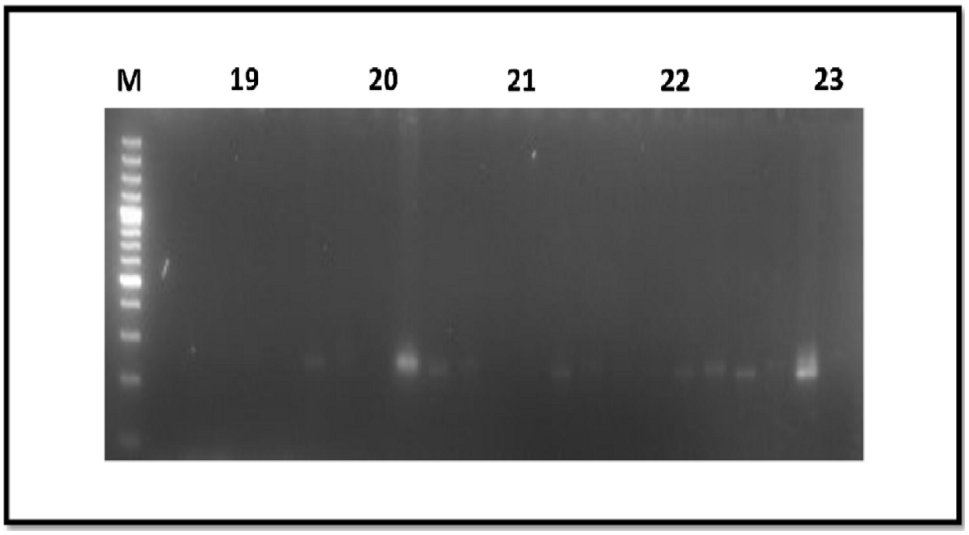

FIGURE 4.4: SSRs marker banding patterns of *B. juncea* that were explained.

In figure 4.4 SSR banding patterns of 5 genotypes of *B. juncea* by SSR primer Na10-C06, 24- 30. M = 100 bp molecular marker, Bj-1130, Bj-1137, Bj-1143, Bj-1140, Bj-1120 and respectively.

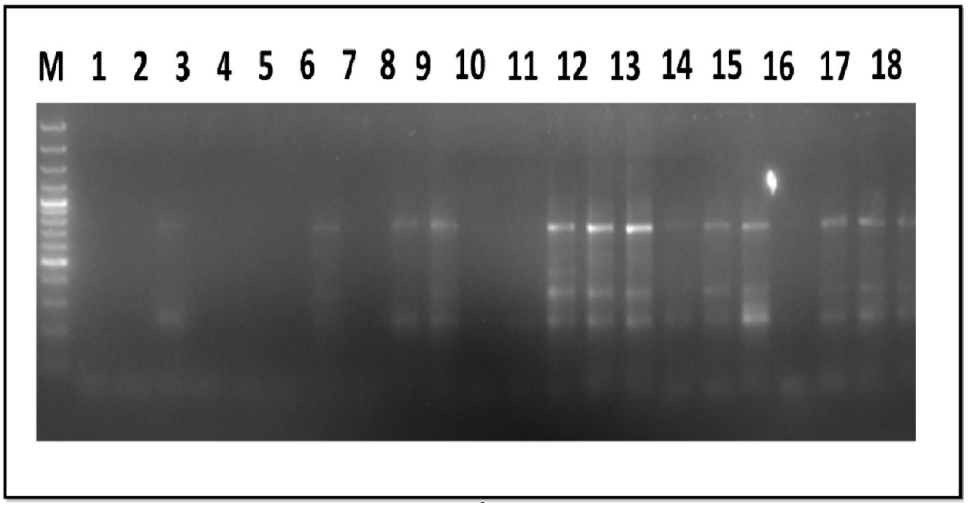

FIGURE 4.5: SSRs marker banding patterns of *B. juncea* that were explained.

In figure 4.5 SSRs marker banding patterns of 18 genotypes of *B. juncea* by SSRs primer Na10-C06, 1-23. M = 100 bp molecular marker, Bj-1107, Bj-1159, Bj-1123, Bj-1163, Bj-1199, Bj-1210, Bj-1151, Bj-1153, Bj-1182, Bj-1201, K. Raya, Bj-1194, BJ-1171, Bj-1173, Bj-1187, Bj-1190, Bj-1216 and Bj-1112 respectively.

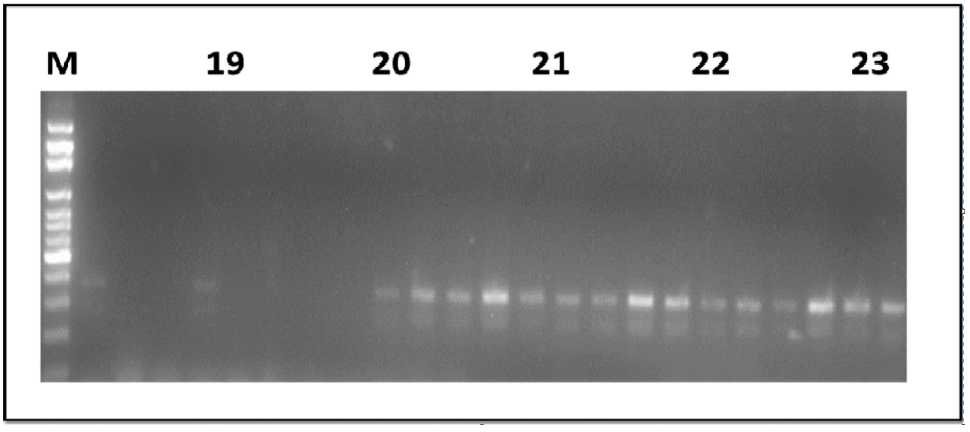

FIGURE 4.6: SSRs marker banding patterns of *B. juncea* that were explained.

In figure 4.6 SSR banding patterns of 5 genotypes of B. juncea by SSR primer Na10-C06, 24-30. M = 100 bp molecular marker, Bj-1130, Bj-1137, Bj-1143, Bj-1140, Bj-1120 and respectively. Most primers amplified 100-400 base pair fragments (bp). Seven out of ten primers (70%) revealed a single allele among the *B. juncea* genotypes tested.

4.1.2 SSRs Marker Banding Patterns of *B. juncea*

The overview of SSRs marker banding patterns of *B. juncea* were explained below:-

In figure 4.1 SSRs marker banding patterns of 18 genotypes of *B. juncea* by SSRs primer Na10-C06, 1-23. M = 100 bp molecular marker, Bj-1107, Bj-1159, Bj-1123, Bj-1163, Bj-1199, Bj-1210, Bj-1151, Bj-1153, Bj-1182, Bj-1201, K. Raya, Bj-1194, BJ-1171, Bj-1173, Bj-1187, Bj-1190, Bj-1216 and Bj-1112 respectively.
In figure 4.2 SSR banding patterns of 5 genotypes of *B. juncea* by SSR primer Na10-C06, 24-30. M = 100 bp molecular marker, Bj-1130, Bj-1137, Bj-1143, Bj-1140, Bj-1120 and respectively.
In figure 4.3 SSRs marker banding patterns of 18 genotypes of *B. juncea* by SSRs primer Na10-C06, 1-23. M = 100 bp molecular marker, Bj-1107, Bj-1159, Bj-1123, Bj-1163, Bj-1199, Bj-1210, Bj-1151, Bj-1153, Bj-1182, Bj-1201, K. Raya, Bj-1194, BJ-1171, Bj-1173, Bj-1187, Bj-1190, Bj-1216 and Bj-1112 respectively.
In figure 4.4 SSR banding patterns of 5 genotypes of *B. juncea* by SSR primer Na10-C06, 24- 30. M = 100 bp molecular marker, Bj-1130, Bj-1137, Bj-1143, Bj-1140, Bj-1120 and respectively.
In figure 4.5 SSRs marker banding patterns of 18 genotypes of *B. juncea* by SSRs primer Na10-C06, 1-23. M = 100 bp molecular marker, Bj-1107, Bj-1159, Bj-1123, Bj-1163, Bj-1199, Bj-1210, Bj-1151, Bj-1153, Bj-1182, Bj-1201, K. Raya, Bj-1194, BJ-1171, Bj-1173, Bj-1187, Bj-1190, Bj-1216 and Bj-1112 respectively.
In figure 4.6 SSR banding patterns of 5 genotypes of B. juncea by SSR primer Na10-C06, 24-30. M = 100 bp molecular marker, Bj-1130, Bj-1137, Bj-1143, Bj-1140, Bj-1120 and respectively. Most primers amplified 100-400 base pair fragments (bp). Seven out of ten primers (70%) revealed a single allele among the *B. juncea* genotypes tested. So these binding patterns of *B. juncea* where find with SSRs.

4.1.3 Genetic Similarity and Cluster Analysis

Different genotypes of *B. juncea* are related to one another at a rate of 0.33 to 0.95 (33% to 95%). The minimum similarity 0.33(33%) were note between Bj- 1137,

Bj -1120 and Bj- 1130 (Table 4.5). The genotypes are highly diverged from each other. The maximum similarities coefficient values 0.95(95%) were note between Bj- 1153 and Bj- 1151. These findings showed that these two genotypes are highly similar with each other as compared to other genotypes (Table 4.3).

UPGMA similarity was used to categorize the various genotypes into four distinct groups. The number of genotypes ranged from 6 in Cluster I, 11 in Cluster II, 5 in Cluster III and 1 in Cluster IV respectively. Bj-1160, Bj-1159, Bj-1123, Bj-1163, Bj-1199 and Bj-1210 are all examples of Group I genotypes. Bj-1151, Bj-1153, Bj-1182, Bj-1201, K. Raya, Bj-1194, Bj-1171, Bj-1173, Bj-1187, Bj-1190 and Bj-1216 are all members of Group II which is the largest group. Bj-1112, Bj-1130, Bj-1137, Bj-1143, Bj-1140 are the members of group III and Bj-1120 is only member of group IV (Table 4.2). Groups are usually very diverse (Fig 4.7; Table 4.4) Our study found great genetic variance and significant inter-specific similarity among genotypes. These findings may support genetic variability studies of important *Brassica* subspecies, particularly *B. juncea*.

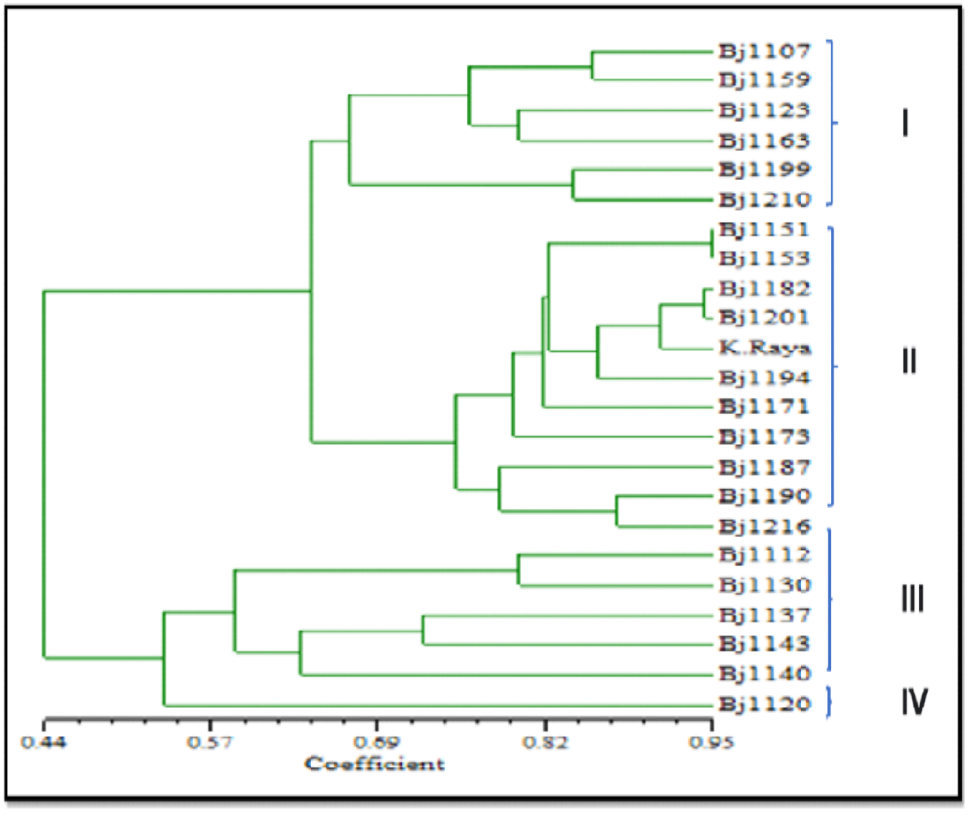

FIGURE 4.7: SSRs marker-based variability among 23 genotypes of *B. juncea* using cluster analysis.

TABLE 4.2: Details of SSRs markers used including total generated alleles, polymorphic alleles, % polymorphism and size of amplified alleles.

Primer Name	**Total amplified alleles (a)**	**Polymorphic allele (b)**	**%Percentage polymorphism (b x 100/a)**	**Size range (bp)**	**Melting temperature (Tm -C)**
Ni3-G05	3	3	100	∼200, ∼300, ∼800	55
PBCE-SSRJU2	1	1	100	∼110-∼120	55
PBCE-SSRJU3	1	1	100	∼290	54
PBCE-SSRJU6	2	2	100	∼310, ∼400	55
PBCE-SSRJU12	1	1	100	∼120	55
PBCE-SSRNA8	1	1	100	∼290-∼310	56
Ra2-D04	2	2	100	∼150, ∼200	59
Ra2-E03	1	1	100	∼200	59
Ra2-F11	1	1	100	∼200	58.5
Total= 14					
14					
100%					

TABLE 4.3: Dice similarity coefficient among 23 *B. juncea* genotypes on the basis of SRRs.

Acc	**Bj 1107**	**Bj 1112**	**Bj 1120**	**Bj 1123**	**Bj 1130**	**Bj 1137**	**Bj 1140**	**Bj 1143**	**Bj 1151**	**Bj 1153**
Bj 1107	1									
Bj 1112	0.67	1								
Bj 1120	0.5	0.67	1							
Bj 1123	0.83	0.67	0.5	1						
Bj 1130	0.5	0.8	0.5	0.5	1					
Bj 1137	0.5	0.44	0.33	0.5	0.5	1				
Bj 1140	0.4	0.57	0.6	0.6	0.67	0.6	1			
Bj 1143	0.73	0.75	0.55	0.3	0.57	0.73	0.67	1		
Bj 1151	0.67	0.67	0.67	0.8	0.36	0.53	0.62	0.57	1	
Bj 1153	0.75	0.75	0.63	0.75	0.33	0.63	0.57	0.67	0.95	1

The minimum similarity coefficient value 0.3 (30%) was recorded between Bj1143 and Bj1123 which the maximum similarity coefficient 0.95 (95%) was recorded between Bj1153 and Bj1151 that was shown above in the table 4.3.

TABLE 4.4: Dice similarity coefficient among 23 *B. juncea* genotypes on the basis of SRRs.

Acc	**Bj 1159**	**Bj 1163**	**Bj 1171**	**Bj 1173**	**Bj 1182**	**Bj 1187**	**Bj 1190**	**Bj 1194**	**Bj 1199**	**Bj 1201**
Bj 1159	1									
Bj 1163	0.71	1								
Bj 1171	0.56	0.74	1							
Bj 1173	0.63	0.59	0.78	1						
Bj 1182	0.59	0.67	0.84	0.82	1					
Bj 1187	0.43	0.67	0.75	0.57	0.8	1				
Bj 1190	0.5	0.71	0.78	0.63	0.82	0.86	1			
Bj 1194	0.74	0.7	0.76	0.74	0.9	0.71	0.84	1		
Bj 1199	0.71	0.78	0.63	0.47	0.67	0.53	0.59	0.7	1	
Bj1201	0.5	0.59	0.78	0.88	0.94	0.71	0.75	0.84	0.59	1

TABLE 4.5: Dice similarity coefficient among 23 *B. juncea* genotypes on the basis of SRRs.

Acc	**Bj1210**	**Bj1216**	**K. Raya**
Bj1210	1		
Bj1216	0.78	1	
K. Raya	0.78	0.75	1

4.1.4 PCoA Analysis

Genetic distances are used as a factor in a multivariate technique called principal coordinate analysis (PCoA) to classify people into distinct groups. All of the *B. juncea* genotypes were then visualized on 2D and 3D scatter plots using a DICE similarity coefficient matrix for an intuitive understanding of the species' genetic diversity. The 2D and 3D PCoA analysis both offer clear differences along any dimension. Genotypes in a vast population can be isolated thanks to 2D and 3D research (Fig 4.8 & 4.9).

Using a 2D dendrogram, we clustered all of the genotypes in our analysis into 3 distinct groups. The genetic make-up of each subset differs. However, other unusual, diverse genotypes were also recorded; they include the Bj-1112, Bj-1130 Bj-1143 and Bj-1153 K. Raya, etc. (Fig 4.8 & 4.9).

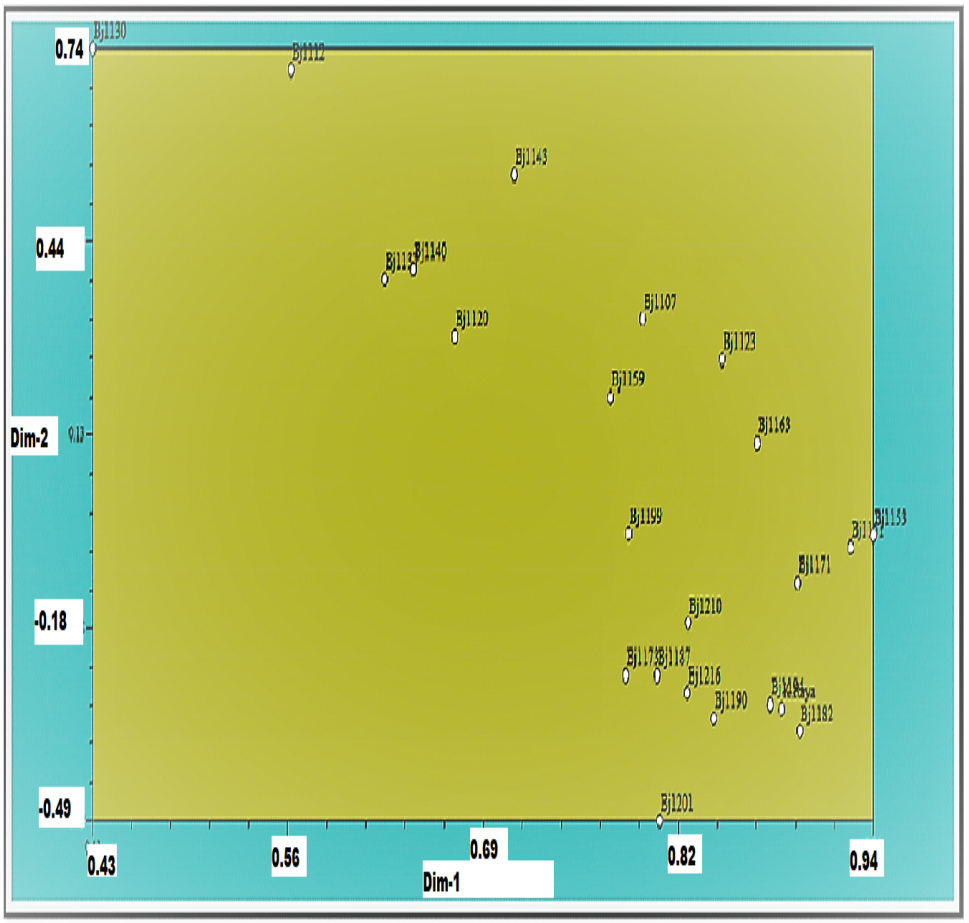

FIGURE 4.8: Two-dimensional (2D) diversity analysis of 23 genotypes of *B. juncea* using SSRs primer.

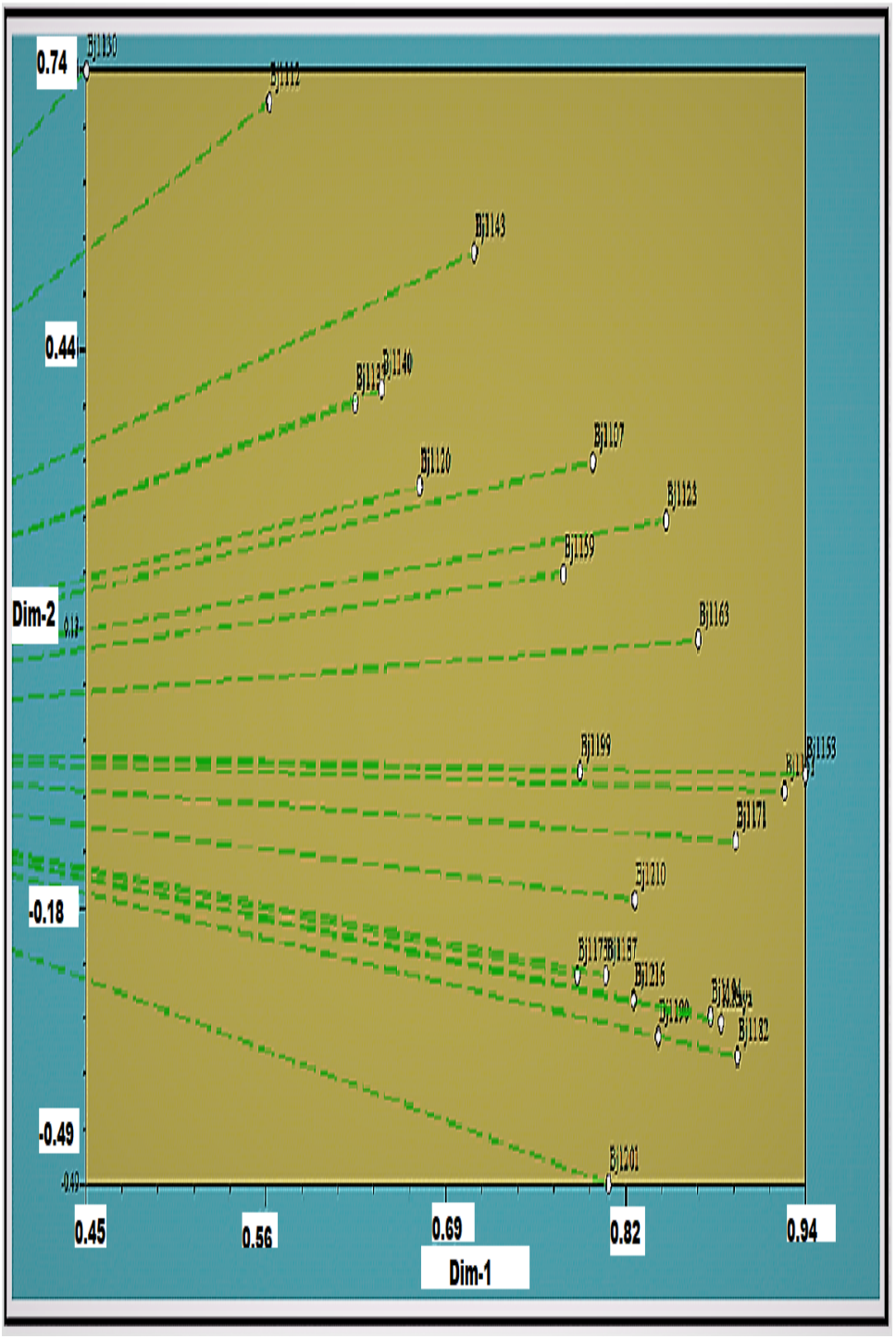

FIGURE 4.9: Two-dimensional (2D) diversity analysis of 23 genotypes of *B. juncea* using SSRs primer represented with dotted line.

The 3D Analysis were used for further confirmation results from different and closely angles. The 3D findings showed that genotypes Bj- 1199, Bj- 1159 and Bj- 1137 are highly diversel premises then rest ones as shown in (Fig 4.10, 4.11 & 4.12) respectively.

The below figure 4.10 explained the three-dimensional (3D) analysis of 23 *B. juncea genotypes* evaluated via SSRs with different angles. The 2D and 3D PCoA analysis both offer clear differences along any dimension. Genotypes in a vast population difference can be isolated.

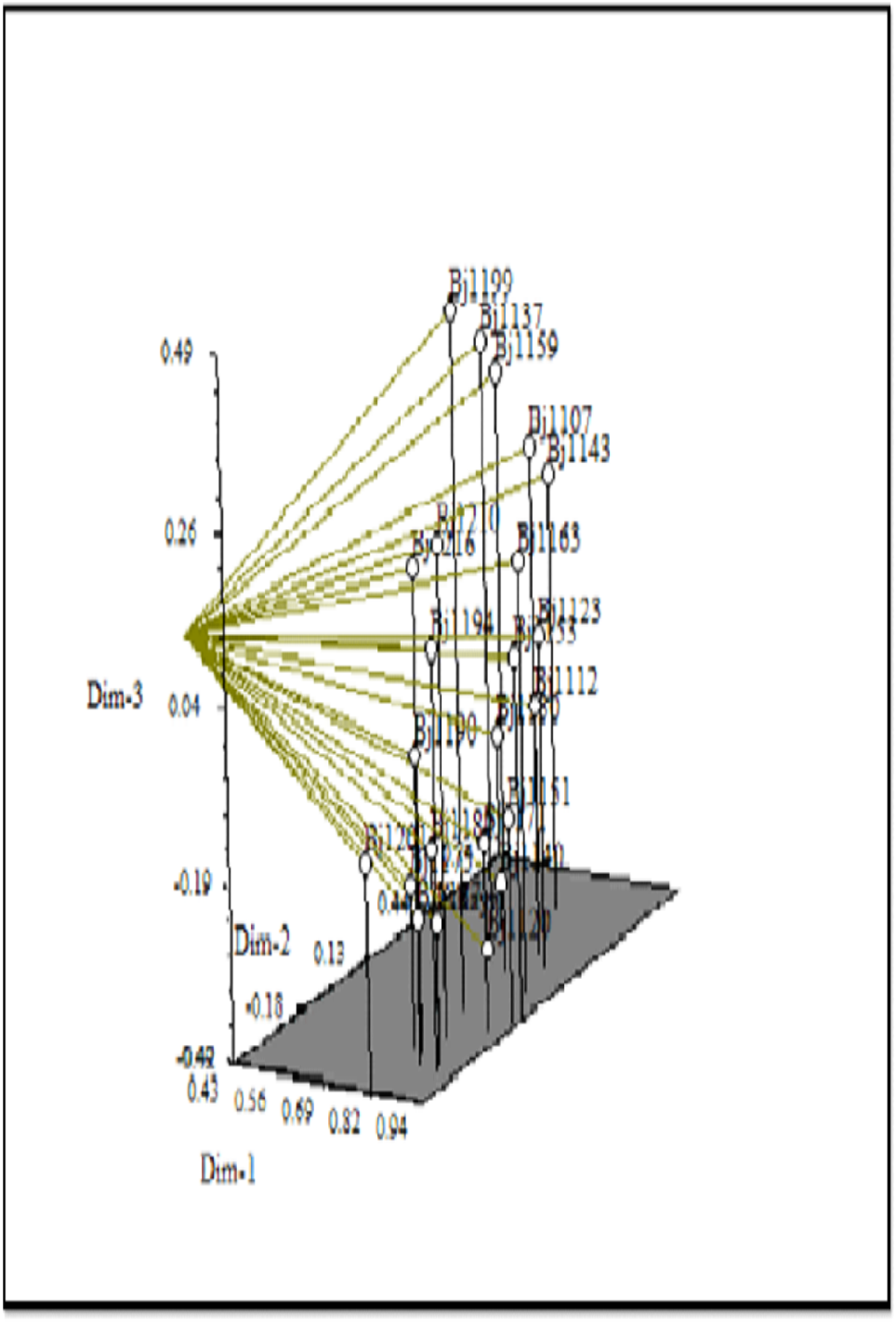

FIGURE 4.10: Three-dimensional (3D) analysis of 23 *B. juncea genotypes* evaluated via SSRs

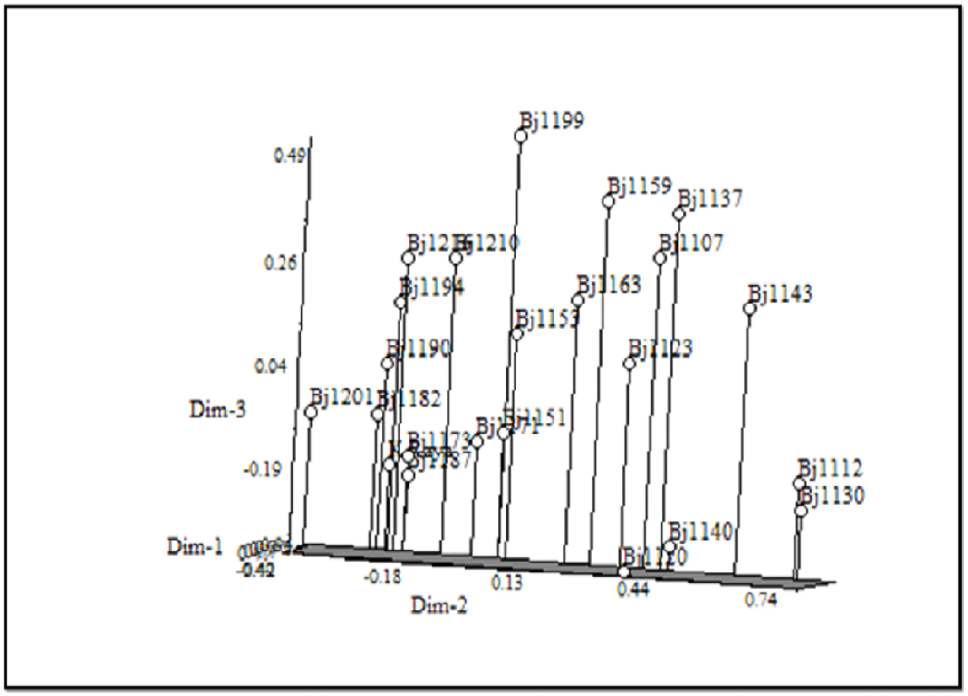

FIGURE 4.11: Three-dimensional (3D) analysis of 23 *B. juncea* genotypes evaluated via SSRs

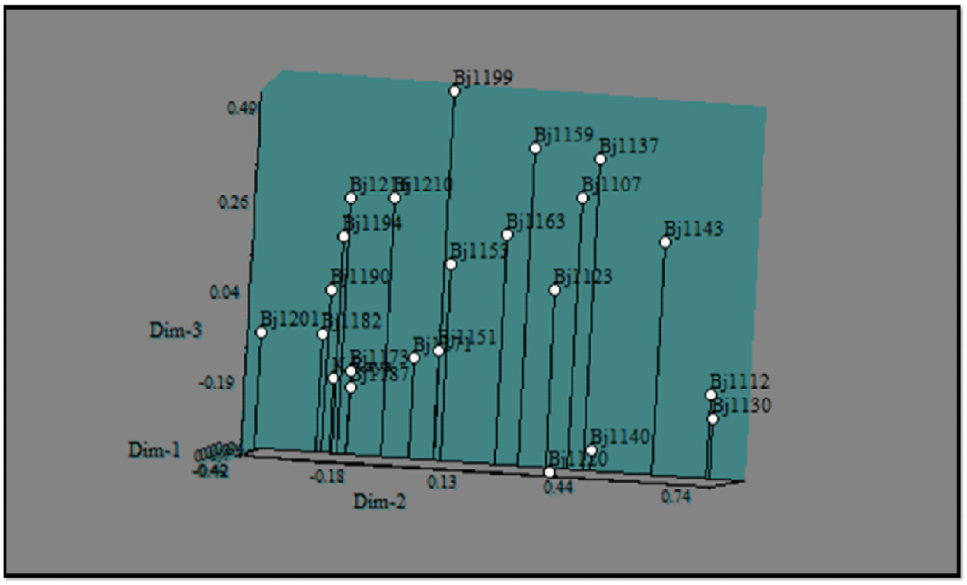

FIGURE 4.12: Three-dimensional (3D) analysis of 23 *B. juncea* genotypes evaluated via SSRs

The 3D findings showed that genotypes Bj- 1199, Bj- 1159 and Bj- 1137 are highly diversel premises then rest ones as shown in the above Figures 4.10, 4.11 & 4.12 respectively which represent the three-dimensional (3D) analysis of 23 *B. juncea* genotypes evaluated via SSRs. With the help of 2D dendrogram, we already clustered all of the genotypes in our analysis into 3 distinct groups.

4.2 Discussion

Protein storage classifies plants and assesses agricultural genetics. Total seed storage diversity helps distinguish plant species and subspecies. Its research enhances protein diversity in several plant populations [[140] to [143]]. Genetic variation research uses many biochemical approaches. SDS-PAGE is one of the most important protein size-based methodologies for differentiating *Brassica* species and subspecies. In this investigation, *B. juncea* genotypes showed the most variance. SDS-PAGE, the fastest and cheapest molecular method, maximizes protein-based genotype diversity. Biochemical and molecular methods study the genetic diversity of several crop species and subspecies [[144] to [145]]. SDS-PAGE measured *B. juncea* strain genetic variation. 13 bands showed polymorphism. Band diameters across genotypes show that seed total storage protein differs by plant subspecies. *Zada et al.*report [146] 31 polypeptide subunits in *B. carinata* strains [146]. *Shinwari et al.* [147] identified maximum polymorphism and little monomorphic proteins in *Eruca sativa* genotypes. *Turi et al.* discovered four proteins across many *Brassica* species using the same method. SDS-PAGE and RAPD soybean genotypes have the largest polymorphism [148]. *Ahmad et al.* investigated polypeptide-based polymorphism in Pakistani cultivar Hyppophaerhamnoides L. ssp Turkestanica [149]. Genotype determines protein bands [150]. Variations in genotypes, gel quantity, or data scoring protein subunits may explain protein subunit count differences. All 23 *B. juncea* genotypes were analyzed for genetic similarities. Genotypes have 33%–95% similarity. The phylogenetic tree classified the 23 genotypes into 4 groups. *Shinwari et al.* [147] found 60% to 100% commonality between Eruca sativa species, whereas our data are significantly less. Plant species cause this variation. *Mukhlesur et al.* [151] clustered *Brassica* cultivars using a similar method.

2D and 3D visualizations reveal genotype clustering. The 3D structure shows more information and depth than a 2D representation. Data from both categories can provide a wide range of genotypes in this study. Gupta et al. [152] used PCoA analysis to identify the 45 chickpea genotypes with the highest polypeptide-based

diversity. Our results concur. Their 3D research linked FLIP-90-160. Mottaghi et al. [152] found significant protein-based divergence among Iranian Achillea species using PCoA. The first three PCoA groupings explain about 83% of variance, they say. Twenty-three genotypes from different regions were analyzed using 10 SSR primers. These genotypes were distinct across marker sets. All genotypes showed polymorphic banding patterns. Our results match Chen et al. [147] and Agrama and Tuinstra [131]. They found the most *Brassica* SSR diversity. *Brassicaceae* microsatellite markers show chromosomal diversity. Ma et al. [154] examined genetic divergence in 20 Chinese and Japanese non-heading cabbages. The amplified allele had maximum polymorphism. Havlickova et al. [155] identified maximum genomic diversity in 94 Czech winter rapeseed genotypes using SSR and AFLP markers. Genotypes were 53%–100% same. Novel and different elite genotypes were also found. Gupta et al. discovered high RAPD and SSR-based genomic diversity in Indian mustard (*B. juncea*) and other *Brassica* species. Abbas et al. [156] catalogued 458 and 258 alleles using 10 RAPD and SSR markers. 250–2000 bp highly polymorphic bands were found. SSR fragments were smaller than RAPD markers.

The similarity coefficient values for all genotypes in this experiment ranged from very low (0% similarity) to very high (100% similarity). Ofori et al. [157] found 83% genetic variation across *B. rapa* winter genotypes. We discovered significantly less. Only 17% genetic variation was detected between species. They found the most polymorphism with 15 SSR markers, except for three genotypes. Find the highest polymorphism possible. Shen et al. [158] employed microsatellite markers to examine eleven non-heading Chinese cabbage genotypes, but we found similarity coefficients of 54–89%. The researchers compared cabbages.

Cluster analysis grouped genotypes into four groupings. The first batch had six genotypes from Punjab, KPK, Germany, and Netherlands. Group 2 had 11 genotypes, mostly from KPK, Sindh, and Germany. The third group had 5 genotypes from Punjab, Pakistan and the fourth group had only 1 genotype from Punjab, Pakistan. Das et al. [159] found similar genetic differences between B. campestris and cabbage genotypes. Framarzpour et al. examined 25 *B. napus* greenhouse

cultivars using 12 SRAP primer pairs. 96 polymorphic bands shared 33% of their genetics. PCoA clarifies B. napus genotype differences. The 2D and 3D analyses estimated that 83.66 percent of B. napus genome variation was due to poor SRAP marker distribution. Genotypes varied greatly in their principal component analysis. Singh et al. [160] identified 50% genetic similarity between 114 alleles (2.238 per primer) from 48 SSR markers. 2D and 3D analysis determined the best of 16 *B. Juncea* genotypes. They confirmed structural analysis genotype clustering with PCoA. They identified resistant and susceptible genotypes. Takahashi et al. analyzed 24 cultivars of *Brassica rapa, juncea, napus,* and *oleracea* using individual and bulked RAPD and ISSR markers [161]. Two genotypes of each *Brassica* cultivar showed full polymorphism across all techniques (305 bands and 422 bands, respectively). PCoA found more within-species variance than between. PCoA results matched UPGMA results.

Chapter 5

Conclusions and Recommendations

An investigation of the varying genotypes of *B. juncea* from different parts of Pakistan, Germany, Netherlands was carried out by SSR analysis. The genotypes showed similarity coefficients ranging from 33% to 95%. Principal Coordinate Analysis was utilized to produce 2D and 3D displays of the genotypes, which disclosed more detailed information about the polymorphism. The use of cutting-edge 2D and 3D technologies allowed for the identification of uncommon genotypes.

Our findings indicate that the variation in *B. juncea* genotypes could be used to analyze genetic diversity. However, we suggest using both 2-D gel electrophoresis and genetic markers to better understand the evolutionary roots of *B. juncea* germplasm differences. Further study is recommended for the unique genotypes Bj-1115, Bj-1120, K. Raya, Bj-1130, Bj-1137 and Bj-1216.

Our study uncovered some unusual alleles of *B. juncea* using SSR markers. The genetic similarity comparisons ranged from 33% to 95%. All the genotypes were polymorphic, with 7 out of 10 primers detecting one allele, 2 primers amplifying two alleles, and one primer amplifying all three. The study discovered new genotypes such as Bj-1163, Bj-1201, Bj-1151, Bj-1159, and Bj-1153. Further research

on *B. juncea* genotyping and genetic diversity should make use of both GWAS stands for genome and wide association study that help researches to identify the genetic variations and risk associated with the disease and specific traits as well as SSR markers that will help scientists and researchers in the future as they work to solve various problems related to plants such as *B. juncea*.

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