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Review

Molecular Markers Used to Reveal Genetic Diversity and Phylogenetic Relationships in Crop Plants

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Abstract

Genetic diversity allows plants to adapt to changing environmental conditions to survive and increases their ability to respond to yield, production, pests and diseases. The application of molecular markers developed due to developments in biochemistry, molecular biology, and plant technology has shed light on plant genetics and breeding studies and produced an enormous amount of knowledge. The theoretical knowledge will guide in determining the scope, amount, and distribution of different aspects of genetic diversity harbored in plants and how it is structured, determining what, where, and how to protect and management of the studies in practice. In plants, molecular markers have been used in the assessment of genetic diversity and population genetics, characterization of germplasm, investigation of phylogenetic relationships, identification of species, hybrids and varieties, ecology, evolutionary biology, taxonomy, selection and breeding studies based on molecular markers in the construction of gene maps and QTL maps in the last four decades. Each of the known molecular markers or their derivatives has different methodologies, advantages, or disadvantages. Comparative studies of different molecular markers performed in different plant species along with their wild and primitive relatives offer researchers the opportunity to determine and apply the most appropriate methodologies for future detailed studies. The

sustainability of life on earth depends first on the genetic diversity in individuals, second on the species diversity in the ecosystems, and finally on the ecosystem diversity. Deterioration or loss in any of these will disrupt the balance between living things.

Keywords
Crop plants; genetic diversity; molecular markers; hybridization-based markers; PCR-based markers; transposon markers

1. Introduction

After our hunter-gatherer ancestors discovered agriculture around 10,000 years ago, they settled down and began cultivating domesticated crop plant species in the Fertile Crescent in the Middle East. Traditional farmers have built genetic diversity into the genetic structure of landraces through selection over many generations, evolving as a defense against problems caused by genetic vulnerability [1]. However, in the 19th century, formal plant breeding studies of varieties with narrower and more homogeneous genome ranges [2] began to be carried out intensively to increase yield, quality, and resistance to biotic and abiotic stress factors. For example, wheat landrace germplasm has lost 75% of its genetic diversity [3], and the living conditions of the varieties produced due to breeding efforts becoming less suitable for existence and reproduction. Thus, it might be crucial to investigate the genetic diversity in cultivated crop species along with their wild and primitive relatives by reliable tools for the management of conservation and breeding studies.

1.1 Genetic Diversity

All the characteristics of living organisms are controlled by DNA molecules, which is the hereditary material. Differences in DNA sequences are defined as genetic diversity. Genetic diversity can be examined among individuals in a population, between different populations of a species, or between species [4, 5]. Genetic diversity is expressed as the frequency of different alleles resulting from mutation, genetic drift, or recombination within or between populations of a species. While the number of alleles shared by populations and their distribution might indicate gene flow and similarity, different allele numbers indicate genetic divergence and genetic differentiation between populations. The distribution of alleles also shows the distribution and adaptation abilities of populations in different eco-geographical conditions. Genetic diversity in the genomes of plants is shaped by natural selection and other factors in the natural environment or by artificial selection along with other factors affecting evolution if it is produced by farmers in the field [2, 6]. Genetic variability is the variation in alleles of genes or variation in DNA/RNA sequences in the gene pool of a species or population.

1.1.1 Why Is Genetic Diversity Important?

Genetic diversity is the presence of different allelic variants of genes or variations in DNA/RNA sequences in a population or species gene pool. If the genetic diversity is high, it may indicate a potentially high number of allelic variants harbored in the gene pool. We can see the expression of

different alleles in the phenotypes of living things, such as color, shape, and structural features [7]. Genetic diversity is important for defining the genetic structure of natural plants or modern cultivars of crop plant populations and determining how to arrange the genetic resources for future conservation and crop breeding studies. Evolution proceeds mainly based on genetic diversity in plant populations and other organisms. Additionally, mutation, genetic drift, migration into or out of a population, and natural selection are the driving forces of evolution and genetic diversity in a population [8].

Mutations occur due to changes in nucleotides in the DNA sequence by deletions, insertions, or rearrangements in the genome or chromosome numbers of individuals. These changes can occur spontaneously or as a result of an induced mutation. Mutations can be harmful or (rarely) beneficial to an organism or its descendants [9]. Mutations either have such a negligible effect on the phenotype that they are effectively neutral in selection, or they are so strongly harmful that they are rapidly eliminated from the population [10]. Mutations and recombination resulting from crossing over during meiosis, which provides one of the advantages of sexual reproduction, are sources of genetic diversity [11]. Mutations produce new alleles that are subject to the effects of the selection process, and the new alleles that survive and are well-adapted contribute to increased genetic diversity and adaptiveness in a population or populations of a species. Genetic bottleneck affects dramatically small-sized populations. Even the rare, unique alleles disappear in the gene pool of populations, decreasing adaptiveness and resulting in the extinction of populations.

Five characteristics influence the level and distribution of genetic diversity in plants: i) breeding system, ii) seed dispersal mechanisms, iii) life form, iv) geographic range, and v) taxonomic status [12]. In plants, two types of breeding systems evolved: selfing and outcrossing breeding systems. In a selfing system, a single parent produces offspring, and genetic diversity tends to decrease compared to outcrossing, in which the gametes of different parents unite to produce offspring. Plants have several types of seed dispersal mechanisms, such as wind (anemochory), water (hydrochory), animals (endozoochory), explosive release (ballochory), and gravity (barochory) [13]. Seed dispersal mechanisms affect the level of genetic diversity. If the seeds are dispersed around the mother plant, they share more or less similar gene combinations with the mother plant, and the plants in the same region even outcross; this will probably lead to inbreeding depression. Additionally, if the number of plants in the same area increases, demands for light, water, and soil increase and cause competition. If the seeds are transferred to long distances, they may be safeguarded and genetic diversity and adaptiveness are supposed to be increased. Life forms are annual, biennial and perennial observed in plants. Perennial forms accumulate many mutations because of long living compared to other forms. Many cereal crop plants, wheat, barley, bean, maize, etc., are consumed as food sources annually. The geographic range is any basic unit of biogeography. The structure and dynamics of geographic ranges, including sizes, shapes, boundaries, overlaps, and locations, are the scope of most biogeographic research. In analyses of genetic diversity, it is necessary to take into account the spatial patterns of the distribution of ranges, the temporal patterns of change in ranges, the relationships between ranges and phylogenies, and the processes that produce these patterns [14]. When explaining genetic diversity, species belonging to monocots or dicots in taxonomic categories are taken into account according to their breeding systems, seed dispersal patterns, life forms, and geographical ranges, and important knowledge is revealed [15]. In genetic diversity studies, comparative analyses based on the combinations of these five categories will shed light on how they affect genetic diversity and phylogenies in plant species.

1.1.2 Phylogenetic Relationships in Crop Plants

A population is a community of individuals of the same species in a particular geographical region and in a specific time, which can produce fertile offspring when they mate. Populations are the basic functional units studied in evolutionary biology, and the level of genetic diversity within and between populations is one of the evaluation parameters. Genetic changes should be measured at the population level, not individually, and transferable to the next generations to be considered evolutionary. While populations of different species live together in an ecosystem, there are also populations of the same species that have adapted to different eco-geographic conditions. Populations of each species interact with both the population dynamics within themselves and the physical factors of the species and the environment where they live together. Therefore, many factors affect the survival of a population. In plants, domesticated crop plants and commercial new varieties developed by breeding studies are consumed as food by humans, while their wild relatives live in the natural environment. However, some wild plant species, such as capers, cranberries, and blackberries, are also consumed by humans. Wild plant species are very important as genetic resources for crop improvement programs [16]. Because wild forms have adapted to the changes in the environment and managed to survive by struggling against all biological and physical stress factors applied by the environment, without being dependent on humans under natural conditions, for this reason, wild species have accumulated an enormous amount of rich genetic diversity in their gene pools for thousands of years [3]. Gene pools of wild ancestors are also used when developing new commercial varieties. Since homogenous monocultures produced through breeding studies have been developed to be compatible with certain environments and stress factors, they cannot compete at the same level as their wild relatives in harsh environmental conditions. For this reason, it is necessary to regularly monitor the status of wild and primitive landrace relatives of the cultivated plant species, especially as genetic resources, and to develop strategies to conserve those that are at extinction risk. It is necessary to determine the amount of genetic diversity, population genetic structures, genetic differentiation between populations, and levels of gene flow for the management of genetic resources. Various types of markers are used to determine these parameters. This review is focused on the genetic diversity and phylogenetic relations revealed by using different types of molecular markers in crop plants as significant food sources for humans.

2. Molecular Markers

Since the beginning of molecular markers, many genomic markers with different methodologies have been developed and used to study genetics in plants, pests, bacteria, fungi, animals, and humans. Molecular markers are actually miraculous tools that make visible what is invisible in a genome. Today, they are used in the diagnosis of genetic diseases in humans, forensic and criminal cases, determining parentage, and identification of corpses resulting from natural disasters such as earthquakes and fires. However, while molecular markers are most commonly used in genetic diversity, population genetics, and phylogenetic studies, with the development of new generation sequencing techniques, the locations of genes are determined, genomic libraries (cDNA, EST, STS, etc.) are developed, markers associated with phenotypes are developed, genome-wide association with traits studies, construction of physical, gene and QTL maps.

2.1 Types of Markers

2.1.1 Morphological Markers

These are the studies conducted according to the visually accessible traits such as flower color, plant height, seed color and shape, which are often susceptible to phenotypic plasticity. These markers still have the advantage of being used in species identification in phylogenetic and systematic studies [17]. Each plant species has its own unique morphological characteristics, such as seed shape, leaf shape, fruit shape, pollen shape, and placentation type in flower structure. However, in some cases, if species are morphologically very similar to each other and homoplasy is expected due to natural selection as seen in sibling species, morphological markers are not sufficient to differentiate the species. In this case, morphological data should be complemented by protein and DNA markers. Although there is no need for high and expensive technologies for morphological markers, situations such as the size of the populations to be examined, the sampling strategies, and the conduct of the studies in the field increase the cost of these studies and are also very time-consuming. If genetic diversity and phylogenetic relationships were investigated using only morphological markers, the results would be far from accurate classification.

2.1.2 Biochemical Markers

These proteins are found in the structure of organisms or play a role in metabolic functions. Gluten proteins found as seed storage proteins and isoenzymes in cereals are the most commonly used biochemical markers. Isoenzymes, also called allozymes, are isomorphs of an enzyme that have the same functions but different properties in terms of morphology or electrical charge (Figure 1). Depending on the different developmental stages of organisms, the types and forms of enzymes might be differentially expressed. The limitation in isoenzyme types causes the level of genetic diversity to be displayed lower than it currently is in genetic diversity studies. It does not allow accurate phylogenetic distinction between species that are very close to each other. Despite all these shortcomings, isoenzymes have been used extensively in population genetics and phylogenetic studies [18, 19].

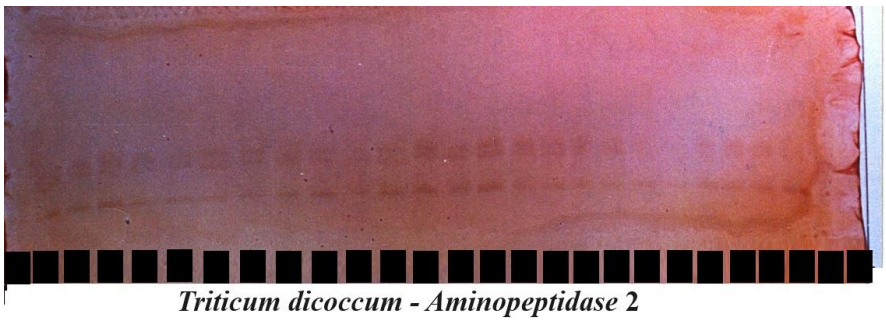

Figure 1 Isoelectrofocussing (IEF) gel image of *Aminopeptidase* 2 isoenzymes in a *Triticum dicoccum* population developed by substrate solution staining [20].

Gluten proteins, known as seed storage proteins in cereals, have also been used extensively in exploring the genetic diversity and phylogenetic relationships in cereal crops. In addition, some gluten proteins related to good quality bread making or pasta have been used as markers in wheat

breeding studies. Glutens are further divided into two classes of proteins: gliadins and glutenins. Gluten proteins are also analyzed by vertical electrophoresis in polyacrylamide gels. Gliadins are resolved by aluminum lactic acid polyacrylamide gel electrophoresis (A-PAGE). Glutenins are complex molecules of high molecular weight (HMW)- and low molecular weight (LMW)- glutenins, resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE), in which SDS is used as a denaturing agent to separate them into monomers (Figure 2 and Figure 3). Although the polymorphism rate is high compared to isoenzymes, some proteins are transferred in blocks, especially in gliadin proteins. This causes a limitation in diversity due to the linkage between the genes that control them and the low levels of recombination. Gliadin and glutenin proteins are used in the assessment of genetic diversity and phylogenetic relationships [21-26] in association with good bread-making and pasta quality studies [27, 28].

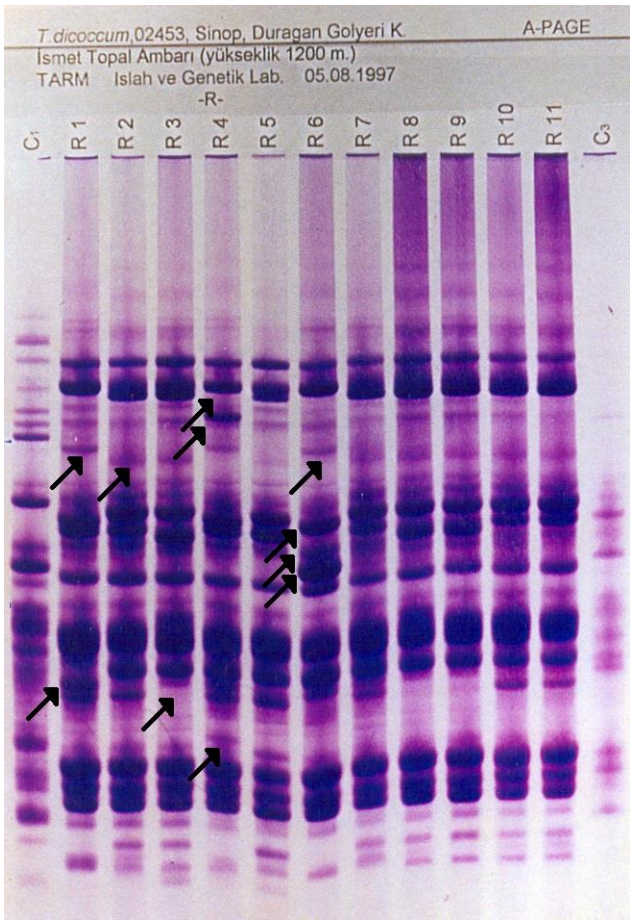

Figure 2 Gliadin band patterns developed by Coomassie brilliant blue 50 staining in a *Triticum dicoccum* population [20].

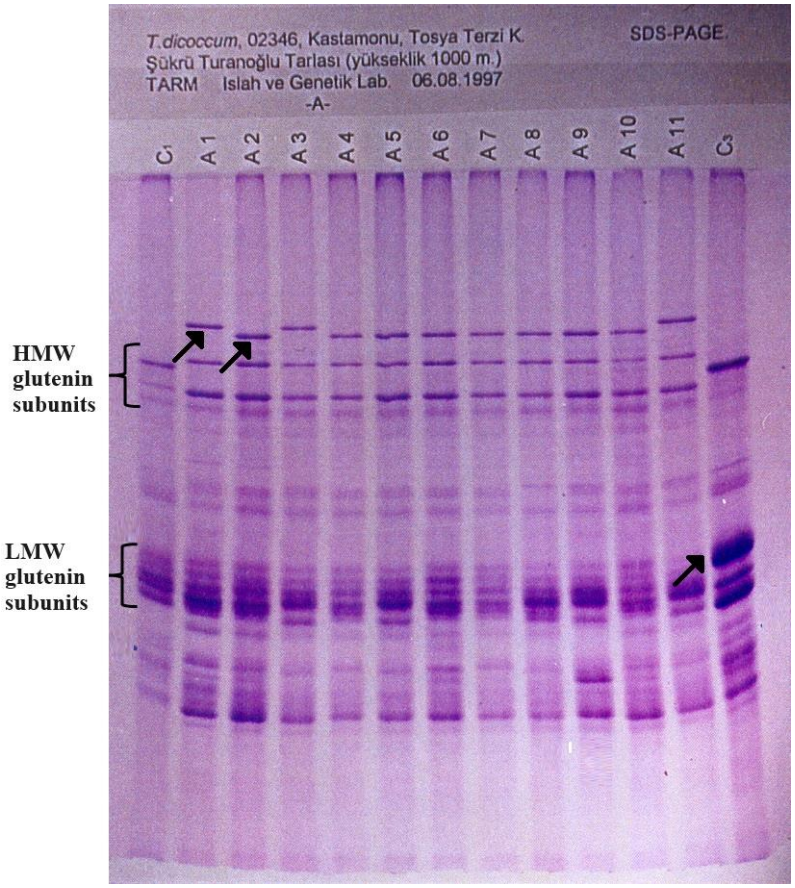

Figure 3 Glutenin band patterns developed by Coomassie brilliant blue 50 staining in a *Triticum dicoccum* population [20].

2.1.3 DNA (Molecular) Markers

A DNA (molecular) marker is a locus that detects differences between individuals and changes in nucleic acid sequences such as nucleotide insertions, deletions, replication errors, translocations, inversions, duplications, point mutations, chromosomal breaks or loss resulting from mutations. It can be located in coding DNA but usually in noncoding DNA regions [29, 30]. Genome structural features and the study's purpose are considered when developing DNA markers. Molecular markers are abundant in the genome, and in some methods, high polymorphism is detected, reproducibility levels are high, and the technology used, and cost effect may vary depending on each other. Molecular markers are used to determine polymorphism, that is, the differences in nucleic acid sequences between the genomes of individuals within a population or populations of a species, or even between species. Molecular markers show Mendelian (dominant/recessive) or codominant inheritance, as seen in the traits encoded by genes. They are not affected by environmental factors; therefore, they are considered neutral and do not have pleiotropic or epistatic effects. Codominant markers distinguish heterozygotes from homozygotes and are more informative than dominant markers [17].

Specific properties that ideal DNA markers are expected to have;

- High level of polymorphism,
- Distributed evenly throughout the genome,
- Codominant inheritance,

- High frequency in the genome,
- No need for prior knowledge of an organism's genome,
- Being linked to a specific phenotype,
- A small amount of tissue and DNA samples are sufficient,
- Selectively neutral behavior,
- Easy access (usability),
- Simple, easy, and fast analysis,
- High reproducibility,
- Easy data exchange between laboratories [31-33].

None of the molecular markers can have all the features that an ideal marker is expected to have. Therefore, depending on the content of the study of interest, markers that produce appropriate data should be used to investigate genetic diversity and population genetic structure, determine the gene's location in the genome, and investigate the relationships between organisms or genes in molecular phylogeny. Molecular markers are divided mainly into two groups according to the basis of the method: (i) Hybridization-based and (ii) PCR-based. Sequence-based and functional-based markers use the derived sequences from molecular markers such as EST, STS, SNP, and SSRs.

Hybridization Based Markers

(i) Restriction fragment length polymorphism (RFLP). The restriction fragment length polymorphism method was developed by Botstein et al. [34]. Since RFLP markers show codominant inheritance, they are very useful and reliable in distinguishing different genotypes, such as homozygous and heterozygous [35]. DNA probes used in hybridization must be prepared for each species from the DNA sequences in its own genome. Since sequence information is used when developing probes, they also provide information based on which chromosome or arm of the chromosomes they are located on. Until PCR-based DNA markers were created, they were used extensively in population genetics [36] and genetic diversity studies, in the investigation of phylogenetic relationships [37], and in the preparation of breeding and genome maps [38]. The stages of the method are briefly given below.

Restriction enzyme reaction: A high concentration (10-15 µg) of quality DNA isolated from plant samples is digested with one of the type II restriction enzymes appropriate to the study's content (1-6 hours at 37°C). Restriction fragments are run in agarose gel overnight to resolve them (Figure 4a).

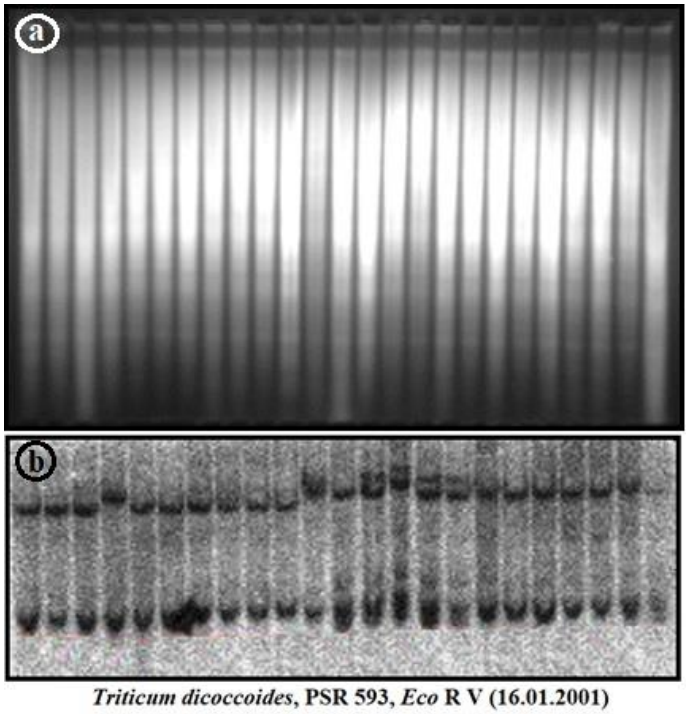

Figure 4 (a) Image of the genomic DNA of a group of *T. dicoccoides* L. samples under UV light after digesting with *Hind* III and electrophoresis in 0.8% agarose gel for 18 hours. (b) Band patterns of RFLP fragments of *Triticum dicoccoides* genomic DNA digested with *Eco* R V restriction enzyme and hybridized with PSR 593 probe [39].

Southern blot: The restriction fragments separated in an agarose gel are transferred from the gel to the nylon (positively charged) or nitrocellulose membrane overnight according to the blotting procedure (Figure 5).

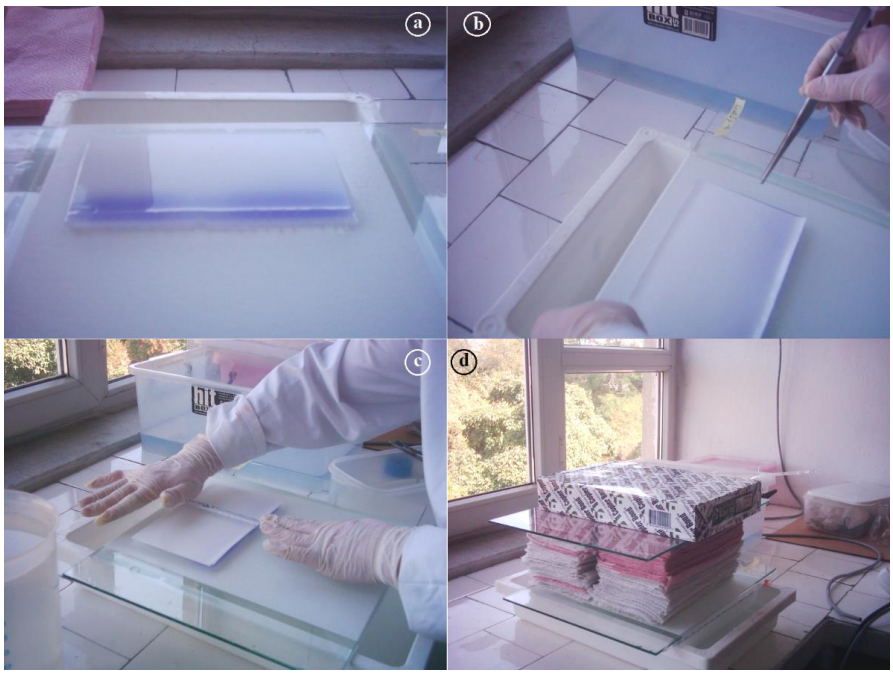

Figure 5 Southern blot transfer steps (a) Placing 3 MM chromatography paper on the glass plate, placing its edges into the solution and placing the gel, (b-c) Placing the

membrane on the gel and removing air bubbles, (d) Placing paper towel blocks and weight on the gel [39].

Hybridization and imaging: DNA or cDNA probes developed from the sequences on the organism's genome analyzed and labeled with radioactive ^{32}P are added to the hybridization solution and the hybridization process takes place with the fragments on the membrane (overnight at 65°C). After hybridization, probe DNAs that do not bind to the membrane may create a false signal are washed away. If there is an autoradiography or scanning device for imaging, a phosphor imaging analysis system is used. Phosphor imaging plates are put on the membrane, placed in a cassette, and left at room temperature overnight. The next day, the phosphor imaging plates are scanned in the scanning device to obtain and evaluate the image (Figure 5b).

The RFLP method, including Southern blot and hybridization stages, is quite complex, time-consuming, and labor-intensive and requires the use of radioactive labeling, expert personnel, and technical equipment. In addition, a large amount of clean and high-quality DNA is needed. For this reason, it is a method that is not preferred in routine studies but is still preferred in specific studies such as formal plant breeding programs. Nowadays, single nucleotide polymorphisms can also be detected by microarray and DArT, which include hybridization and restriction steps [40].

PCR-Based Molecular Markers

(i) Randomly amplified polymorphic DNA (RAPD). Randomly amplified polymorphic DNA is the first molecular marker method developed by Williams et al. [41]. RAPD markers show dominant inheritance, and polymorphism is based on scoring the presence or absence of bands. Since they do not require prior sequence information, they perform random amplification by scanning the entire genome and can be used in all organisms. Primers are very short oligonucleotides of 10-mer; therefore, their annealing temperatures are low, and only one primer is used in a PCR reaction. RAPD markers have high polymorphism rates, but reproducibility rates are lower than other molecular markers. Dominant inheritance of RAPDs cannot identify homozygous or heterozygous genotypes [42]. Despite the disadvantages, they have been used extensively in population genetics and genetic diversity studies, probably because they were the first simple, easy, and fast molecular markers.

(ii) Amplified fragment length polymorphism (AFLP). AFLP, a highly efficient genomic finger-printing method, was introduced by Vos et al. [43]. The technique is presented as a new technique that combines the high polymorphism and speed of the RAPD with the reliability of the RFLP. In general, AFLP markers show dominant inheritance, high level of polymorphism, and reproducibility since they screen the entire genome. The basis of the AFLP method begins with the digestion of genomic DNA into small fragments using two restriction enzymes (*Eco* R I and *Mse* I), and millions of restriction fragments are produced. Adapter sequences of known sequence are added to the ends of the restriction fragments, whose one end is cleaved by *Mse* I and the other by *Eco* R I and ligation using the DNA ligase enzyme. Adapter sequences and sequences recognized by restriction enzymes are designed as primers. AFLP primers have three parts: a core sequence consisting of adapter sequences, a restriction enzyme-specific sequence (RE), and a selective extension (SN) and *Eco* R I- and *Mse* I- primers with three selective nucleotides (SN, selective nucleotides denoted as NNN) are given in Figure 6 [44].

	CORE	RE	SN
Eco R I 5'-	GACTGCGTACC	AATTC	NNN-3
Mse I 5'-	GATGAGTCCTGAG	TAA	NNN-3

Figure 6 The parts of AFLP primers' sequences.

Pre-selective PCR is performed with or without adding +1 SN to the 3' end of the primer. Pre-selective PCR clones are diluted and used as template DNA in selective PCR. For selective PCR, while +3 more SN are added to the primer sequence designed in pre-selective PCR, preferably *Eco* R I, selective primer sequences are marked with different fluorescent dyes and selective PCR is performed. Alternatively, radioactive labeling can also be used instead of fluorescent labeling. DNA fragments amplified in selective PCR are denatured, and a number of fragments ranging from 40 to 200 bp are resolved in denatured polyacrylamide gel electrophoresis [45]. After electrophoresis, if fluorescent labeling is used, the images obtained by scanning with a scanner equipped with an analysis system that detects fluorescent signals can be evaluated through photographs (Figure 7). Alternatively, raw data can be obtained by reading the peaks formed according to fragment sizes in samples carried out with capillary electrophoresis. If radioactive labeling is used, it can be evaluated on x-ray films developed by autoradiography or images obtained using phosphor imaging analysis systems.

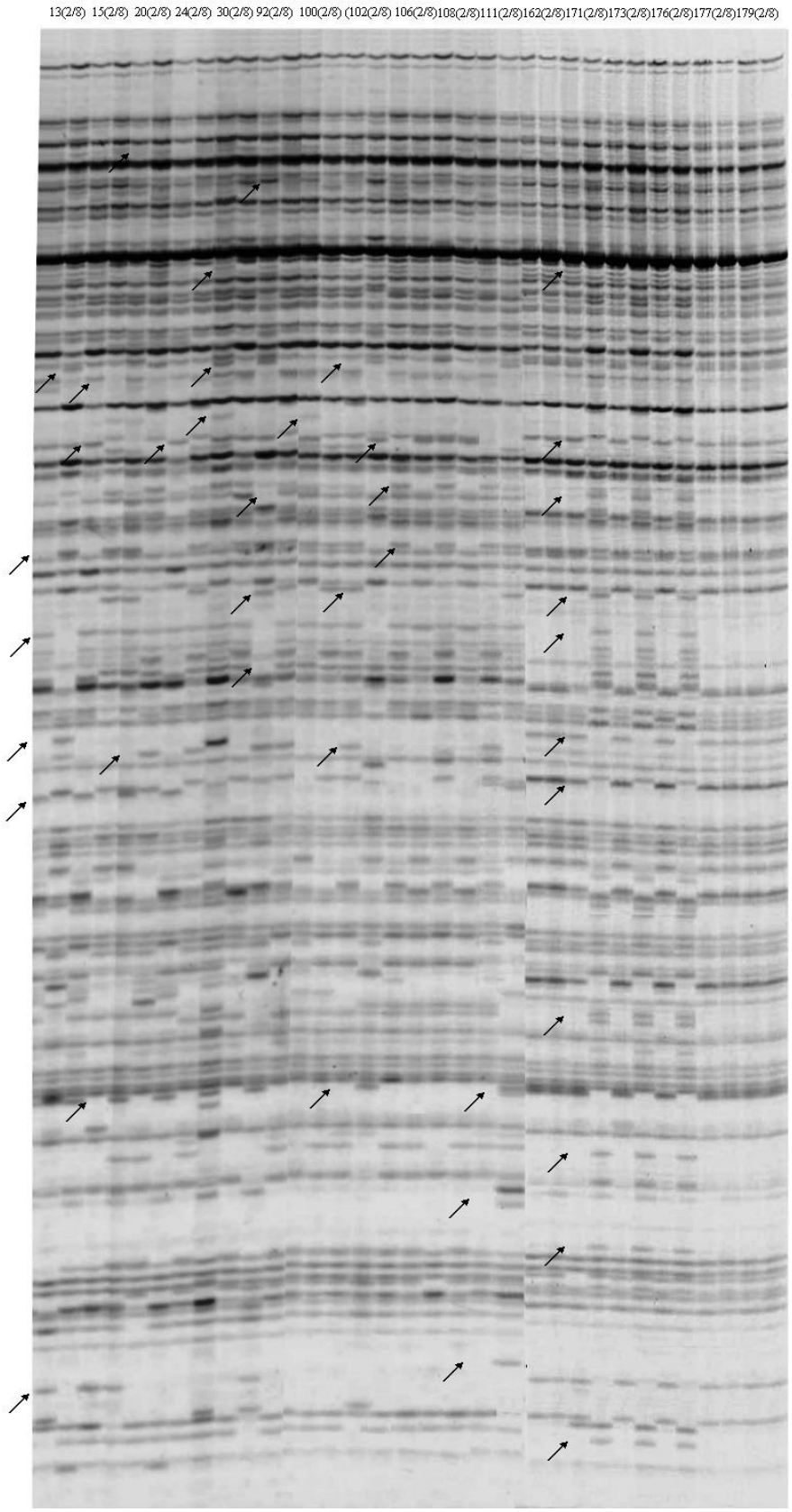

Figure 7 AFLP band patterns produced by ES2XMS1 primer combination of wild emmer wheat populations collected from six habitats in the Ammiad region of Israel between 1988 and 2002 [39]. *Arrows indicate present or absent bands in the band patterns of each individual.

The source of variation in AFLP molecular markers is the mutation in the sequences recognized by restriction enzymes or the formation of a new restriction site in a region, new nucleotide insertion, deletion, or duplications in the sequences of duplicated fragments, causing changes in the sequences of the fragments, increasing or decreasing their size [32]. AFLPs require the use of expensive methods such as silver staining, fluorescence or radioactivity in detection methods, large sequencing gels or automatic DNA sequencers in obtaining data, and expert personnel.

Although next-generation sequencing (NGS) has now become the predominant state-of-the-art technique for genotyping populations, AFLP DNA fingerprinting, the first method used to detect sequence polymorphism, remains a viable method due to its versatility, cost-effectiveness, independence from prior sequence information and broad applicability [46]. AFLPs are used to conduct studies on ecology, evolution, taxonomy, genetic mapping, population genetics, and phylogenetic relationships [32].

(iii) Simple sequence repeats (SSR). Simple sequence repeats are non-coding heterochromatic DNA sequences within the genome. They can usually be found scattered around the centromere and telomere regions and chromosome arms. They are short tandem repeat (STR) sequences, generally containing 1-6 bases, and were previously called simple sequences. The term "microsatellites" was used for the first time by Litt and Luty [47]. Several repeat sample units used for fingerprint and transcriptome analysis include the sequences (GATA/GACA), CA, (AT)n, (GAA)n, (TCC)n, (GGAT)n, (GGCA)n, and $(TAG)_n$ [45]. Microsatellites (STR) or simple sequence repeats (SSR) are abundantly distributed in the non-coding regions of eukaryotic and prokaryotic genomes. All SSRs occupy 3% of the human genome. They are widely distributed throughout the genome and are linked to many genes. It is believed that some microsatellites adjacent to the coding sequence regions play an important role in regulating gene expression by the formation of various secondary DNA structures and providing a DNA unwinding mechanism. The variation or polymorphism observed in SSRs is a result of polymerase slippage or unequal fragment exchange during DNA replication [48]. SSRs are not only very common but also highly variable in the number of repetitive DNA motifs in the genomes of eukaryotes [49].

SSR is a method based on PCR amplification, which is done by designing the flanking sequences of SSRs as primers. One of the advantages of SSRs is that they show codominant inheritance. Homozygous and heterozygous genotypes can be distinguished in phenotype since they are generally located in non-coding heterochromatic regions, where mutations are common and polymorphism rates are very high. SSRs provide important data due to their association with a trait of interest in marker-assisted selection (MAS) studies [50-53]. The disadvantage of SSR, the requirement of genome sequence information is time-consuming and very expensive, and genome-specific SSRs must be developed for each species.

In SSR-PCR, two primers are used, forward and reverse, and the annealing temperatures of the primers are quite high. Forward primers can be used in PCR by labeling them with radioactive or fluorescent dyes. When radioactive labeling is used, polyacrylamide sequencing gels resolve PCR clones by electrophoresis. Imaging can be evaluated on x-ray films developed using autoradiography or on the images obtained using phosphor imaging analysis systems. If fluorescent labeling is used, SSR fragments are resolved by capillary electrophoresis. In QTL mapping, SSRs are probably one of the most widely used molecular markers despite the limitation of genome sequence information. Codominant inheritance, high polymorphism, and reproducibility are attractive features of SSRs that

can be used extensively in the detection of genetic diversity, population genetics, and phylogenetic relationships, mostly in crop breeding studies.

(iv) Inter simple sequence repeats (ISSR). Inter simple sequence repeats (ISSR) are semi-random markers amplified by PCR of the regions between simple sequence repeats located in close locations [54-56]. Each amplified ISSR band corresponds to a delimited sequence between two simple sequences. ISSR-PCR combines simple sequence repeats and non-repetitive flanking sequences, ensuring that amplification is initiated at the same nucleotide position in each cycle [54]. ISSR primers are 15-35-mer long and have a high annealing temperature, and a single primer is used in a PCR reaction. It can be applied in all species as it does not require prior sequence information about the genome of interest; however, since the primer sequences are complements of some of the simple sequence repeats, they amplify known regions in the genome. ISSR markers show dominant inheritance, are multi-locus, and show high levels of polymorphism. Compared to RAPD, the reproducibility and polymorphism level are higher [44]. ISSR PCR clones can be resolved on regular agarose (Figure 8) or polyacrylamide gels.

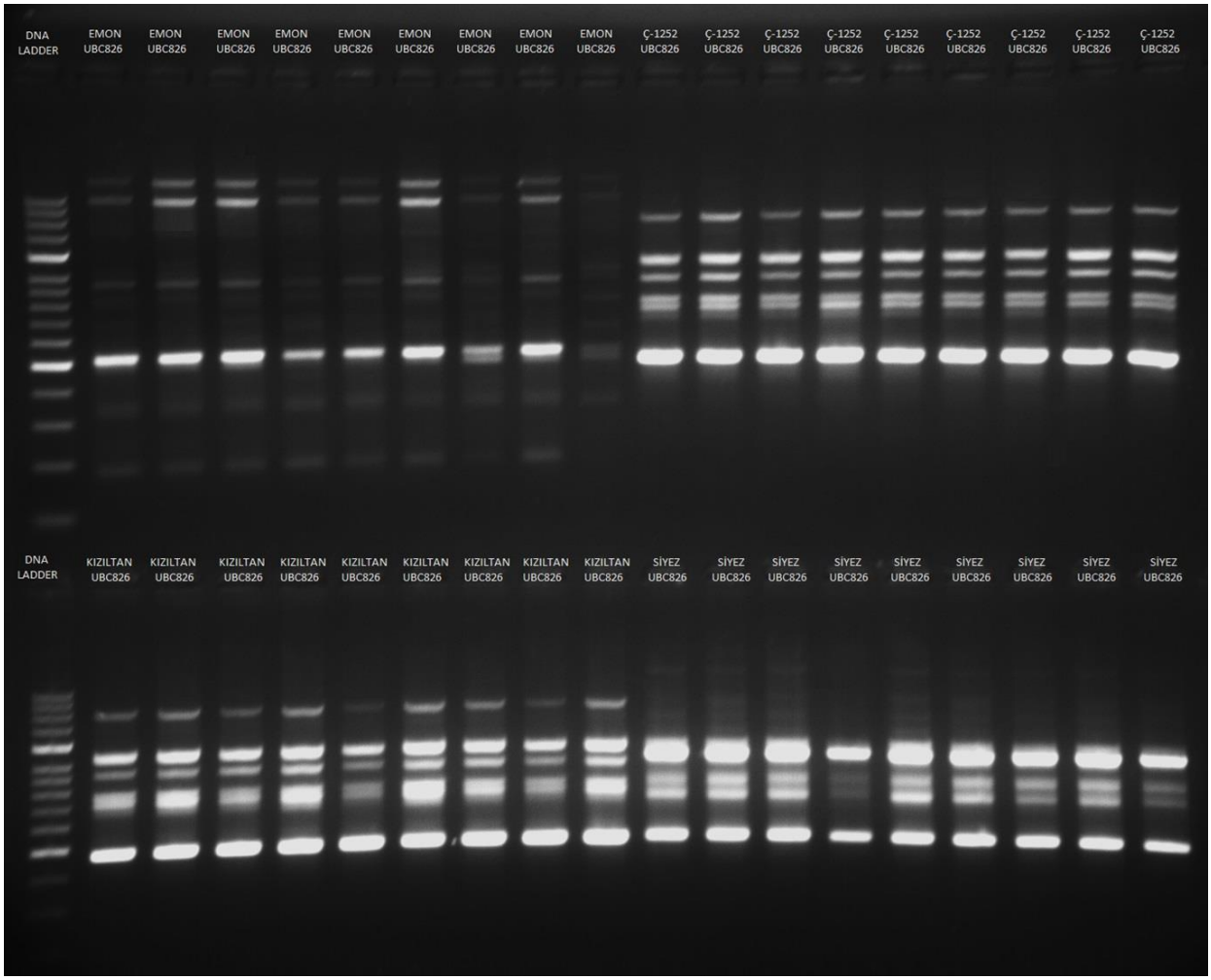

Figure 8 ISSR band patterns of barley (Emon), durum wheat varieties (Çeşit 1252 and Kızıltan 91) and einkorn wheat samples by primer UBC-826 [57].

(v) Sequence tagged site (STS). Sequence-tagged site (STS) markers, which are powerful molecular tools for locating genes in genome mapping, were used by Olsen et al. [58] for the human genome. However, today, it is also used in analyzing plant genomes. STSs are short sequences, 200-500 bp long, found as single copies in the genome and predicted to be strongly conserved in the gene groups within the same family. Since their locations in the genome are known, they can be used to construct genome mapping. Sequences of a few base pairs flanking the STS region can be designed as primers to amplify the STS region. STSs are derived from sequences known and expressed as an EST. For EST, cDNA is synthesized from purified mRNA using reverse transcriptase enzyme. A map showing the order of STS regions and the spaces between them on sequences of DNA fragments is called an STS map. STS and EST fragments can be from a single chromosome or the entire genome. In the STS approach, maps of wheat chromosomes were constructed by using RFLP clones in the design of STS primers, and some primers can be used to explain polymorphism in hexaploid wheat genotypes [59]. STSs can be used to transform a genetic map into a physical map and to identify a specific gene. Standard STS markers were developed in the rice genome with STS primers derived from RFLP clones [60].

(vi) Expressed sequence tag (EST). Adams et al. [61] used the term "Expressed Sequence Tag, EST" for the first time and started systematic sequencing of cDNA molecules obtained from brain cells as a project. ETSs are 200-800 bp long sequences and contain the identical sequences as the partial sequences of cDNA obtained by reverse transcription from mRNA. They are obtained from randomly selected cDNAs in the cDNA library. These are markers used in discovering new genes in the genome, determining the location of a gene in the genome, and SNP analysis. ETSs can be used effectively to identify genes expressed in cells in a specific tissue of an organism over a particular period of time. The ETS method consists of several stages. Constructing a genome map and locating a gene in an organism whose entire genome has not been sequenced is very complex, like looking for a needle in a haystack. ETS markers, powerful molecular tools developed for this purpose, have a high throughput in determining the location of genes and constructing genome maps.

The method uses mature mRNAs isolated from tissues or cells of interest to access the gene sequence. However, mRNA molecules are not helpful because they are unstable and degrade rapidly. For this purpose, the isolated mRNAs are synthesized into cDNA by reverse transcription using the reverse transcriptase enzyme under *in vitro* conditions. The second strand of cDNA can be synthesized using oligo (dT) primers and T7 DNA polymerase enzyme [62]. In the second step, the sequence of the cDNA is determined, and forward and reverse primer sequences are designed from the 5' and 3' ends. STR provides essential data in determining genes that are expressed differentially in plants' different growth and development stages. A total of 37 genes were reported to be identified as significantly differentially expressed between vegetative and reproductive stages of SAM in the shoot apical meristem of *T. monococcum* by STS markers. Investigation of differentially expressed genes revealed the importance of genes involved in energy metabolism, ubiquitin/26 S proteasome system, polyamine biosynthesis, and SAM differentiation in reactive oxygen species signaling towards the floral transition in *T. monococcum* [63]. Using EST-SSR markers derived from EST libraries and normal genomic SSR markers revealed significant genetic diversity in populations and varieties of Ethiopian finger millet landraces [64].

(vii) Internal transcribed spacer (ITS). In eukaryotes, genes encoding ribosomal RNAs are located in the "nucleolar organizer regions" (NOR) at the end of chromosomes [65]. When mitosis is completed in the cell cycle, and the chromosomes begin to unwind, the DNAs in the NOR region form the nucleolus. rDNAs are transcribed in the nucleolus by RNA Pol I into pre-rRNA, which encodes three rRNAs (35 S in plants and 45 S in yeast or mammals). 5 S rRNA is transcribed in the nucleoplasm by RNA Pol III and transferred to the nucleolus [66]. Internal transcribed spacer regions are sequences located within the gene that codes for ribosomal RNAs. ITS1 is located between 18 S and 5.8 S, while ITS2 is located between 5.8 S and 25 S sequences. ITS1, 5.8S, and ITS2 sequences are collectively called internal transcribed spacer [67]. ITS sequences are cleaved during pre-rRNA processing, producing mature 18 S, 5.8 S, and 25 S/28 S rRNAs. 18S rRNAs associate with ribosomal proteins (RPSs) of the small 40 S ribosomal subunit, while 5.8 S, 25 S/28 S and 5 S rRNA associate with ribosomal proteins (RPLs) to form the large 60S ribosomal subunit [66].

ITSs evolve rapidly and contain high levels of variation. They are used as molecular markers, especially in molecular systematics, in the investigation of phylogenetic relationships between related species and/or populations within a species [68]. ITS regions have become an essential nuclear locus for molecular systematic studies of Angiosperms at intergenic and interspecific levels. Universal PCR primers are positioned on conserved rRNA genes (18 S, 5.8 S, and 26 S) to amplify the entire ITS spacer regions [69]. The popularity of the ITS region can be attributed to the relatively high rate of nucleotide insertion in transcribed spacers, allowing systematic comparison of relatively recently diverged taxa [70].

ITS markers are frequently and reliably used to investigate genetic diversity and phylogenetic relationships. The genotype of Iranian wheat varieties (wild, native, and breed) was investigated using ITS markers, and it was observed that there were considerable nucleotide changes at the same position between diploid and hexaploid species. ITS markers can be used as the most appropriate evaluation tool to analyze inter- and intraspecific relationships in distinguishing different genotypes, as nucleotide changes decrease as evolution progresses so that only a few changes in nucleotides occur [71].

(viii) Sequence-related amplified polymorphism (SRAP). Li and Quiros [72] developed a simple marker method called sequence-related amplified polymorphism (SRAP). The method targeted the design of markers used in the amplification of open reading frames. In the method, two primers, forward and reverse, are used in a PCR reaction. The sequence number of SRAP primers is similar to AFLP primers, but a single PCR is performed [73]. The forward primer consists of 17-mer, while the reverse primer consists of 18-mer. The underlined sequences at 5’ end in both primers (F and R) are the core sequences, followed by the CCGG and AATT sequences (in red color) in F and R primers, respectively.

Forward: 5'- TGA GTC CAA A**CC GG**A TA-3',

Reverse: 3'- CAG **TTA A**GC ATG CGT CAG-5'

The PCR thermal program sets the primer annealing temperature to 35°C for the first five cycles to ensure that SRAP primers anneal to multiple loci on the target DNA. In contrast, in the following 30 cycles, the temperature is set to 50°C, increasing the reproducibility of SRAP. Thus, it is possible to achieve amplification similar to the band profile in AFLP without performing restriction digestion and pre-selective PCR. Amplified PCR clones are resolved by denaturing acrylamide gels and detected by autoradiography. Li et al. [74] employed the SRAP primers in a series of recombinant

inbred and double haploid lines of *Brassica oleracea* L., and they found that approximately 45% of the bands they isolated from the gel matched known genes in the Genbank database. Twenty percent of the SRAP markers were co-dominant. SRAP primers can be fluorescently labeled and combined with unlabeled SRAP primers so that SRAP PCR products can be resolved in capillary devices, which are the factors that increase the cost of the method.

(ix) Single nucleotide polymorphism (SNP). Single nucleotide polymorphism (SNP), a new molecular marker technology, was first proposed in the human genome by Lander [75]. When the complete base sequence of a DNA fragment is compared among individuals of a species, the variation that exists as a result of mutations at a position and the presence of different nucleotides is called single nucleotide polymorphism (SNP) and is pronounced as "Snips" [76]. SNPs arise as a result of transition, transversion, insertion, and deletion mutations occurring at a position on a DNA sequence [75], and for a variation to be considered as an SNP, it must be present in at least 1% or more of the population [77]. Among all types of SNP mutations, transitions are the most common (approximately 2/3) [78]. Four alleles represented by A, T, G, and C can be identified at each SNP locus in a DNA segment [76] but are usually biallelic. Due to their properties, SNPs are extremely useful in multiple analyses, as they can evaluate many loci and effectively distinguish between homozygous and heterozygous alleles. While some authors accept that SNPs occur only by transition or transversion mechanisms [79], others also consider single base indels (insertions or deletions) as SNPs [77, 80, 81]. New SNPs arise continuously in every cell of an organism, but most are eliminated by the action of the enzymatic process known as the mismatch repair (MMR) mechanism and therefore SNPs that become fixed in a germline and a population are mutations that escape the repair process [82]. In addition to considering individual SNPs, it is also necessary to consider SNPs in the context of the term ‘haplotype’. Any combination of closely linked SNPs inherited as a unit due to the lack of recombination between individual SNPs located in a given region is defined as a haplotype and represents a small fraction of the full genotype. Certain combinations of SNPs within a haplotype can be considered ‘associated’ with each other and, therefore, can be accepted as a single unit for genotyping purposes [83, 84]. SNPs can occur in both coding and non-coding regions of the genome. Gilchrist et al. [85] found SNP frequencies in poplar ranging from one SNP per 64 bp for non-coding regions to one SNP per 229 bp in coding regions. Therefore, targeting non-coding or intron-containing regions may be a more logical and profitable way of SNP discovery than scanning coding regions [82]. The characteristics and effects of SNPs are considered according to their location in the genome, whether in the coding or non-coding region. If a base change occurs within the exons in a coding region and does not cause an amino acid change, it is considered synonymous. If it causes a change, it is a missense, which can change the structure and function of the protein. It can also have negative consequences if it causes the formation of a new start or stop codon within the exon region. If a change occurs at the splice points in the structure of the gene, it can change the splice points in the gene's transcript, which can cause incorrect protein synthesis or failure to synthesize. SNPs occurring in transcription regulatory regions can change the regulation of gene expression. SNPs in protein-coding regions can affect post-translational modifications [86]. Some methods used to identify SNPs are non-sequencing (restriction-based methods are RFLP, CAPS and dCAPS; SSCP, DGG and TGGE based on DNA conformation; chip-based TILLING), sequencing (locus-specific PCR, whole genome shotgun, alignment of available genomic sequencing), re-sequencing (pyrosequencing) and bioinformatics

tools (dbSNP, POLYMORPH, ESTreedb, etc.) [87]. Since SNPs are abundant in the genome and their importance in genetic analyses has become more evident in recent years, they have begun to be used as an effective genetic marker in almost all studied species, both animal [88] and plant [89] in Garrido-Cardenas et al. [81]. Numerous studies have shown that the frequency of SNPs in plants varies from one SNP per 21 bp in potato to one SNP per 7000 bp in tomato [82]. Vegetative propagation and self-pollination in plants help plants protect themselves, but they also cause a decrease in genetic diversity. Although SNPs have a very high level of polymorphism since the methods used in SNP discovery are expensive, careful evaluation of plant materials and preliminary analysis of genetic diversity with more economical molecular markers will be beneficial in terms of time and economy [90]. Variations detected in the coding gene regions with SNP markers can be associated with determining different alleles and resistance to diseases and abiotic or biotic stress factors depending on the gene's function [91]. SNPs can be used in breeding programs to discover yield and quality genes, associate them with phenotype, and develop new varieties with high agronomic properties. The emergence of different alleles due to SNP variations that change gene expression may cause the production or non-production of products with different functions. Variations in some genes or genomic regions can be used effectively in studying phylogenetic relationships and in the evolutionary analysis of species [92]. Since SNPs can determine homozygous and heterozygous individuals, they will also allow the correct parental lines to be crossed in breeding studies [93].

(x) Diversity array technology (DArT). Diversity array technology (DArT) methodology was first used by Jaccoud et al. [40] and the details of the protocol were explained by applying it to the rice plant. Two types of fragments are identified as genomic representatives for DArT. (1) Constant fragment: Any representative fragment prepared from the DNA of an individual of a species. (2) Variable/Polymorphic fragment: Molecular markers present in some of the representatives but not all (source). For DArT analysis, first, genomic DNA is isolated from young plant seedlings, and then bulk DNA is prepared from the DNA of all individuals. Bulk DNA is digested with 1-3 restriction enzymes (Ex: *Pst* I, *Mse* I and *Eco* R I). Adapter sequences of restriction enzymes are added to the ends of the restriction fragments during the ligation reaction. Fragments with adapter sequences added in the PCR1 reaction are amplified using primers that match the adapter sequences and carry 1-3 selective bases. This process is done to reduce genomic complexity. PCR1 amplicons are cloned by ligation into a vector and then transferred into a recipient host, such as *E. coli*. In real-time PCR2, the target region is amplified using plasmid vectors carrying the insert and m13 primers, and PCR2 amplicons are purified and transferred to a 384-well plate. Using a microarrayer 6 copies per fragment are arrayed on a microscope slide. The probes used in hybridization are marked with fluorescent dyes, but no purification process is performed for the probes. Herring sperm DNA is thawed in the hybridization solution by keeping it at 96°C. Denatured probe DNAs are also added and mixed into the hybridization solution. Then, the solution taken from the hybridization solution with a pipette is applied directly to the slides prepared in the form of microarrays, and a microscope coverslip is placed on them. The slide prepared in this way is left for hybridization overnight at 65°C. After the hybridization process, the slide carrying the hybrids is washed at room temperature and dried by centrifugation (e.g. 1000 rpm, 1 min). The spots on the slide are screened and evaluated according to their signal intensity, and the data is analyzed [40]. Due to their advantageous features, such as not requiring sequence information, being economical, and being able to detect thousands

of SNPs in one study, DArT markers are used in many areas, such as genetic diversity, population genetic structure, phylogenetic relationships, and research on genes related to disease resistance. Mahboubi et al. [94] determined the genotypes by performing genetic diversity, population structure and linkage disequilibrium analyses with DArTseq (SilicoDArT and SNP) markers of 129 wheat genotypes with different origins worldwide. Although there was no relationship between the wheat grouping of the markers and the origins, they reported that the wheat genotypes examined showed a high level of diversity, with polymorphism information content (PIC) values ranging from 0.1 to 0.5. Such studies can be useful resources in breeding programs to improve grain yield and quality by discovering unique genes in the examined plant genotypes. DArT-based SNP markers were used to investigate upland rice germplasm's genetic structure and diversity. It was determined that gene flow was high between populations and genetic diversity was high within the population, and it was concluded that the genetic diversity of rice in upland areas could be beneficial for improving rice yield [95]. Knowledge of germplasm collections' genetic structure and diversity is crucial for sustainable genetic improvement through hybridization programs and rapid adaptation to changing breeding goals. A study applying SNP and DArT markers investigated the genetic diversity and population structure of soybean accessions. Molecular variance analyses revealed that both markers determine high variation levels and DArT markers' applicability in genetic diversity studies [96].

(xi) Cleaved amplified polymorphic sequence (CAPS). Cleaved amplified polymorphic sequence (CAPS) is the method used to detect SNPs that occur in sequences recognized by restriction endonucleases [97]. CAPS is a PCR-based method that uses DNA sequences of mapped RFLP markers and has been termed PCR-RFLP [98]. RFLP is used with the Southern blot method, but CAPS simplifies the method by eliminating the DNA blotting process [99]. The CAPS method consists of several stages. (1) There must be a restriction site in the target gene sequences, and a single fragment must be produced when PCR amplifies. Primer sequences for PCR are determined based on the sequence information available in the database of genomic or cDNA sequences or cloned RAPD bands. (2) Following the PCR process, locus-specific PCR amplicons are digested with one or more restriction enzymes. Digested fragments are resolved in agarose or polyacrylamide gels. (3) Band patterns of CAPSs are evaluated by comparing the size and number of bands of the wild type and mutant type. However, CAPS analysis is versatile. Its uses have been expanded by combining it with other methods, such as single-strand conformational polymorphism (SSCP), SCAR, AFLP, or RAPD analysis, to increase the probability of finding DNA polymorphisms. CAPS markers are locus-specific and show codominant inheritance [98, 100]. The disadvantage of the technique is that it is limited to the recognition site of the restriction enzyme.

(xii) Derived cleaved amplified polymorphic sequence (dCAPS). To overcome the limitation of the recognition site of the restriction enzyme, Michaels and Amasino [101] proposed a variant of the CAPS called dCAPS (derived cleaved amplified polymorphic sequence). In dCAPS analysis, a restriction enzyme recognition site containing the SNP is introduced into the PCR product by a primer containing one or more mismatches with the template DNA [102]. In the first reaction for dCAPS analysis, the target region sequence is amplified by PCR with specific primers specific to the genomic DNA template. When PCR products are resolved by electrophoresis, if there is a length polymorphism between the products, it is determined according to the DNA fragment length

polymorphism (DFLP) method. Otherwise, to look for sequence polymorphisms specifically for SNPs, PCR products are either directly sequenced or cloned into a suitable plasmid and then sequenced [103]. For dCAPS analysis, the primers are designed specifically to contain one or two mismatched nucleotides compared to CAPS primers [102]. In the second PCR reaction, amplification is performed by using the dCAPS primer together with one of the specific primers and a small amount of PCR product from the previous reaction instead of the genomic DNA template. During amplification in the second PCR reaction, the dCAPS primer introduces mutations into the target sequences and, together with the detected SNP, results in the formation of a unique restriction site in only one of the alleles examined in genetic analyses [101]. In the restriction endonuclease digestion reaction, PCR products amplified with the dCAPS primer are digested with a restriction enzyme and resolved by gel electrophoresis. Thus, wild type and mutant can be distinguished from each other.

(xiv) Sequence characterized amplified region (SCAR). A sequence-characterized amplified region (SCAR) marker is obtained by cloning and sequencing the two ends of randomly amplifying DNA markers (RAPD, AFLP, etc.) that have been shown to be diagnostic for certain purposes using a pair of specific oligonucleotide primers [103, 104]. The SCAR method consists of six stages. (1) PCR amplicons amplified by one of the DNA markers (RAPD, AFLP, SCoT, etc.) that randomly amplify a region known to be linked to the target gene or a locus relevant to determining the difference between varieties. The amplified PCR amplicons are resolved in agarose gel. (2) The excise of the band refers to the gene of interest, ligation of a plasmid, and transfer to a compatible *E. coli* bacterium and multiplication. (3) Selection of recombinant bacteria based on blue-white colonies containing x-gal in the medium. (4) propagation of single white colonies carrying the insert in a liquid medium. Then, pDNA was isolated from the bacteria and quantified. (5) Sequencing the insert amplified using primers specific to the plasmid. (6) After the target locus sequence, the SCAR primers are designed according to the target sequence. Polymorphic loci amplified by SCAR primers are called SCAR markers, which have advantages over RAPD markers because they detect only a single locus. Their amplification is less sensitive to reaction conditions and can potentially be converted into codominant markers [104]. Nunziata et al. [105] analyzed genetic diversity in wild potato species using AFLP and SCAR markers. They developed a new marker set that detected 8 new paralogous for the *Gro*1 locus with the SCAR markers and detected a wide variation with AFLP. The results indicate that SCAR markers can shed light on genetic diversity and phylogenetic studies in plants. Kamaluddin et al. [106] conducted a study to develop SCAR markers associated with stripe rust resistance and to determine genetic diversity in spring wheat (*Triticum aestivum* L. em Thell). The researchers revealed that the DNA fragments amplified by SCAR markers co-segregated with resistant wheat genotypes and could be used to distinguish susceptible and resistant genotypes in marker-assisted selection technology.

(xv) Start codon targeted (SCoT). Start codon targeted (SCoT) markers are new-generation molecular markers developed by Collard and Mackill [107], who designed 36 single SCoT primers from the region of plant genes containing short, conserved sequences surrounding the ATG start codon and introduced the method. SCoT primers are long, containing 18 mer, and have a high primer annealing temperature (50°C). In this method, fragments are amplified using a single primer and show dominant inheritance as in the RAPD and ISSR PCR but have a higher reproducibility rate. Resolving the amplified PCR fragments on a standard agarose gel simplifies the method and makes

it usable in many laboratories due to its low-cost effect. In addition, since primer sequences enable the amplification of the sequences of the gene that controls a trait, investigating the variations detected in the coding sequence regions and determining their effects on the adaptation of plants to environmental conditions will provide important data in the preparation of QTL maps, population genetics, genetic diversity and phylogenetic studies.

(xvi) Target region amplification polymorphism (TRAP). The target region amplification polymorphism (TRAP) method was developed by Hu and Vick [108]. The principle of the method is based on using bioinformatics tools and EST database information to create polymorphic markers around targeted candidate gene sequences. The two primers set used in the TRAP marker method are designed as a fixed/forward primer consisting of approximately 18 nucleotides from a target EST or gene sequence information in the database and random/reverse primer sequence containing AT- or GC-rich motif sequences of roughly the same length that hybridizes to intron or exon sequences [72, 108]. Random primers should be designed considering three principles: the addition of 3-4 selective nucleotides to the 3' end, the 4-6 nt AT or GC-rich core region, and the presence of filler sequences (around 11) forming the 5' end [72]. Additionally, basic rules of primer design, such as self-complementation and maintenance of 40% to 60% GC content, should also be considered when designing primers. TRAP PCR products are resolved in a 6.5% polyacrylamide sequencing gel [108]. TRAP markers were applied to infer genetic diversity in various plant species, chickpea (*Cicer arietinum* L.) [109].

(xvii) Transposon markers. Transposons are the mobile elements found in the genomes of organisms that can move from one location to another. Transposons were first discovered in maize by Barbara Mc Clintoc [110] and brought her the Nobel Prize in Physiology or Medicine in 1983. Then, transposons have been detected in all living organisms, from bacteria to plants, with a few exceptions [111]. When transposons are displaced, they can cause changes within the genome, and as a result, permanent mutations may occur [112, 113]. Among the genome changes due to the transposition of transposons, *Alu* or *L*1 insertions are both caused by hemophilia A in humans [114], glycogen storage disease, and duplication of the beta-globin gene [115], and it is associated with 15 diseases. They are divided into two groups, RNA transposons or retrotransposons (Class 1) and DNA transposons (Class II), according to their migration mechanisms within the genome [116]. After active retrotransposons are placed in the genome, they reproduce by the copy-paste mechanism. First, the mRNA molecule is synthesized by transcription, and the reverse transcriptase enzyme synthesizes cDNA from mRNA by reverse transcription. The DNA encoding the retrotransposon, which becomes double-stranded by DNA polymerase, is integrated at a different location in the genome [117]. DNA transposons move within the genome according to the cut-paste mechanism. A gene sequence located within a transposon encodes for an enzyme called transposase, which recognizes and cleaves the transposon according to the inverted repeat sequences at its ends, then moves the transposon to a different location in the genome, cleaves the DNA, and pastes the transposon into the cleaved site [118]. Transposons cause an increase in the size of the genome [110] or mutations [113]. In many species, retrotransposons occupy a large percentage of the genome: 45% in humans and rice [32], 75% in maize [119], and 80% in wheat and barley [119]. Methods in which transposons are used as markers can be divided into three major groups (i) inter

retrotransposon amplified polymorphism (IRAP) [120], (ii) retrotransposon microsatellite amplified polymorphism (REMAP) and (iii) retrotransposon based insertional polymorphism (RBIP) [121, 122].

i. Inter retrotransposon amplified polymorphism (IRAP)

Kalendar et al. [122] developed IRAP (Inter-Retrotransposon Amplified Polymorphism) markers, in which primers produced from the flanking of two neighboring LTRs bind to target sequences in the LTRs and amplify the genomic DNA sequences between them. Although multiple reliable and reproducible bands can be produced by IRAP and REMAP techniques, characterization of LTR sequences is necessary before marker development. Therefore, the availability of these methods depends on whether suitable LTR sequences are characterized [118]. After the primers are developed, the IRAP method is a simple PCR and the PCR clones are resolved by agarose gel electrophoresis. If the orientations of two retrotransposons are close to each other (head-to-head, tail-to-tail, or head-to-tail), then IRAP PCR can be performed with a single or two primer combinations [123].

ii. Retrotransposon microsatellite amplified polymorphism (REMAP)

Kalendar et al. [122] introduced retrotransposon-microsatellite amplified polymorphism (REMAP) markers, in which a regular IRAP primer and a primer matching microsatellite sequences were used to amplify the genomic region between LTRs and microsatellites adjacent to them.

iii. Retrotransposon-based insertional polymorphism (RBIP)

In the RBIP method, there can be two alleles. The first allele is the LTR retrotransposon that has been inserted in a region of the genome, and the second allele is without insertion. According to the method, the region between the retrotransposon-specific primer and one of the primers in the flanking regions is amplified with a simple PCR. However, no PCR product is synthesized between the primers developed from the areas on either side of the retrotransposon because the entire LTR retrotransposon is usually several thousand bases long. In the allele, in which the LTR retrotransposon is not added, it is amplified between primers developed from the flanking regions. The size of the PCR fragment produced from the allele containing the retrotransposon is larger than the other allele. A polymorphism is detected in a genome scanned with RBIP markers showing codominant inheritance. However, this method requires the LTR sequences and the sequences of the 5' and 3' flanking regions for marker development [118].

(xviii) Sequence-specific amplified polymorphism (S-SAP) markers. Sequence-specific amplified polymorphism (S-SAP) markers, developed by Stepanova et al. [124] based on *Cassandra* retrotransposon LTR sequences, to examine the genetic diversity and phylogeny of 65 representatives of seven species of the *Prunus* L. genus. The basis of the S-SAP method involves the amplification of the AFLP technique [43] using primers originating from the highly conserved terminus of the LTR. First, the genomic DNA of the organisms to be analyzed is digested with *Pst* I and *Mse* I restriction enzymes and the ends are cleaved with *Mse* I (5'-GACGATGAGTCCTGAG-3', 5'-TACTCAGGACTCAT-3') and *Pst* I (5'-CTCGTAGACTGCGTACA TGCA-3', 5'- TGTACGCAGTCTAC -3') adapters are added by ligation reaction. Pre-amplification PCR is performed using primer sequences homologous to adapter sequences. Using pre-amplification PCR products as templates, selective PCR is performed using [^{32}P] labeled LTR retrotransposon sequence-specific primers and P or M adapter-homologous primers carrying 0, 1, 2 or 3 selective nucleotides at their 3' ends. After selective PCR, the clones are denatured and resolved in polyacrylamide gel [125]. S-SAP markers developed from *Ty*1-*copia* and *Ty*3-*gypsy* group LTR retrotransposons were used to determine

genetic diversity in *Vicia* species. Three of the developed S-SAP markers, PDR1, Tps19 and Tvf4, were identified as useful markers in *V. faba* and *V. narbonensis*, while Tvf1 was determined to be productive only in *V. narbonensis*. Phylogenetic trees constructed according to the results obtained with S-SAP markers also tended to show long branch lengths with very little fine structure. It has been observed that clustering according to geographical origin is limited and there is no correlation between morphology-based taxonomic grouping and diversity for species [126]. S-SAP markers developed based on *Cassandra* retrotransposon LTR sequences were used to examine genetic diversity and phylogeny in seven species of the genus *Prunus* L. Between 29 and 185 polymorphic fragments, and 92.75% polymorphisms were identified per S-SAP markers developed. S-SAP markers have been reported to show high efficiency in assessing intra- and interspecific genetic diversity in members of the genus *Prunus* [124].

3. Assessment of Genetic Diversity

Wild and primitive relatives of plants are essential genetic resources due to the high level of genetic diversity they have accumulated in their gene pools for thousands of years and are used in breeding studies and in the development of modern new cultivars whose gene pools have narrowed. Researchers or breeders working on plant genomes primarily investigate the genetic diversity in the plant's gene pool of interest, agronomic traits related to quality, yield, and genes related to resistance to biotic and abiotic stress factors. Therefore, the conservation of genetic diversity in plant genetic resources is necessary because it allows plant breeders to select superior genotypes that they can use in studies such as the development of climate-resilient varieties and genetic stocks for hybridization programs or the release of a new crop variety [3]. Since the beginning of agriculture, traditional farmers have shaped the population structures of the plants they use by artificially selecting genotypes with characteristics such as high yield potential and large seeds using natural diversity in plants and natural selection. However, over time, the size of the population has increased as people have settled down and started to produce their own food through agriculture. While the increasing population has emerged with food shortage, different problems such as changing climatic conditions, extreme heat or cold, tolerance to various air and soil pollutants, and the emergence of new forms as a result of the evolutionary processes of insects, molds, bacteria and other pests harmful to plants have directed breeders towards pest and disease resistance and light sensitivity, etc. in plant breeding studies [7, 127]. Therefore, accurate evaluation, management and conservation of genetic diversity in germplasms of genetic resources is one of the essential issues for the future perspectives.

RFLP has long been used as the first molecular marker in the analysis of nuclear DNA. RFLPs can detect relatively large numbers of loci distributed throughout the genome and are not affected by environmental conditions. While RFLP markers are widely used in the study of genetic variation [128-135], and phylogenetic relationships among populations, species, and varieties [37], their popularity gradually decreased after the discovery of PCR-based molecular markers and due to reasons such as the time-consuming nature of the method, the use of radioactive labeling, relatively low polymorphism and high cost. Since short random primers that can amplify DNA fragments are used in RAPD analyses, it is necessary to prevent contamination of DNA samples. RAPD markers have been widely used in the analysis of genetic diversity [136-138] and phylogenetic relationships [139] because they do not require genome sequence information and are the first PCR-based

molecular markers. In RAPD analyses, the major drawbacks are across in transferring and comparing data obtained between laboratories, low reproducibility, not being locus-specific, band profiles not being interpretable in terms of loci and alleles (dominance of markers), and even fragments of similar size may not be homologous [140]. To estimate the spatiotemporal genetic diversity in populations of wild emmer wheat [*Triticum turgidum* ssp. *dicoccoides*], the ancestor of domesticated wheat, and AFLPs were used to analyze the contribution of spatial and temporal factors in maintaining genetic diversity in a population. Genetic diversity within populations revealed by AFLPs was much larger than between populations, and temporal genetic diversity was significantly smaller than spatial [141]. A comparison of genetic diversity in wild emmer wheat populations from Israel and Türkiye showed that AFLPs effectively differentiate the populations in both countries [142]. Moroccan sorghum landraces species (*Sorghum bicolor* L. Moench) and five sorghum races from the world collection (bicolor, durra, caudatum, guinea and kafir) were analyzed for genetic diversity and genetic structure using ISSR and AFLP markers. As a result, the data obtained using ISSR and AFLP markers together show that bicolor, durra and caudatum races are genetically closer to Moroccan sorghum landraces species than kafir and guinea races and the success of molecular markers used in binary combinations in revealing phylogenetic relationships is seen [143]. It has been described that in some crops, AFLPs are not completely randomly distributed in the genome and may be clustered in certain genomic regions such as centromeres [144]. SSRs have advantages as well as disadvantages that should be taken into account. Since sequence information is required for designing SSR primers, they cannot be applied to all plant species, which makes their application difficult. In plant species where sequence information is unavailable, sequence information from previously studied closely related species can be used, but developing species-specific primers is very time-consuming and expensive. Another problem is that mutations that may occur in the regions where the primers bind may result in null alleles, i.e., failure to amplify the targeted bands with PCR because the primer cannot bind, and may cause incorrect evaluation of the data. Band artifacts formed by DNA slippage during PCR amplification may confuse distinguishing heterozygotes and homozygotes [140]. Two alternative mutation models outline how new microsatellite alleles evolve. The infinite alleles model (IAM) assumes that all possible alleles have an equal probability of arising from a mutation. In contrast, the stepwise mutation model (SMM) describes microsatellite evolution as the stepwise addition or removal of single repeat units. The inferences about population genetic structure are sensitive to the assumed mutation model [145]. In addition, while the debate on mutation models continues, it is obvious that more studies need to be done on this subject. SSRs employed to investigate genetic diversity and population structure in Turkish emmer wheat [*Triticum turgidum* L. ssp. *dicoccon* (Schrank) Thell.] landraces [49], linkage analyses and genetic diversity studies in wheat genotypes [146], among 40 elite barley varieties [147], genetic diversity and phylogenetic relationships among chickpea core collection accessions for Western Himalayas [148], local maize genotypes collected from the Black Sea Region of Türkiye [149]. ISSR markers are still in demand by many researchers because they do not require sequence information and are designed using microsatellite sequences as primers, they are simple, economical, have relatively good reproducibility, and are practical. Plants where ISSR markers applied to investigate the genetic diversity are einkorn wheat [*Triticum monococcum* L. ssp. *monococcum*] landraces [150] and wheat cultivars [151]. STS markers were used alone or in combination with other markers (e.g. SCAR) to determine variation in genotypes sensitive or resistant to a specific trait such as leaf rust resistance [152]. In plants, the SRAP method is applied

to reveal the genetic diversity in barley [153], wheat [154], and *Avena macrostachya* Bal. ex Cross. et Durieu [155], bread wheat (*Triticum aestivum* L.) [156] and durum wheat landrace (*Triticum durum* desf.) [157]. SNP markers have been applied in plants due to their high level of polymorphism and abundance in the genome, including the worldwide durum wheat germplasm collection [158], allohexaploid and allotetraploid wheat populations [159], collard landraces [160], faba bean [93], rice germplasm [161], wheat [162], advanced breeding lines of bread wheat (*Triticum aestivum* L.) [163], and in Ethiopian barley (*Hordeum vulgare* L.) germplasm [164]. SilicoDArT markers generated by DArTseq were used to evaluate genetic diversity, population structure, and linkage disequilibrium in chickpea (*Cicer arietinum* L.) breeding lines. SilicoDArT markers can be used for future genomic studies such as large-scale diversity analysis in chickpeas, association studies with traits such as seed yield, resistance to biotic and abiotic stress factors, and genomic selection [165]. Ali et al. [166] investigated the genetic diversity for seed color polymorphism in a Pakistani barley core collection of 100 breeding lines and three approved barley varieties (*Hordeum vulgare* L.) based on 32 CAPS markers. CAPS markers shed light on genetic diversity in 100 barley breeding lines, finding high genetic diversity. SCoT markers revealed genetic diversity and genetic relationships in wheat [167, 168], *Aegilops triuncialis* [169], *Aegilops tauschii* Coss. (Poaceae) [170], rice [171], common bean [172], and barley [173]. TRAP markers were applied to infer genetic diversity in various plant species, chickpea (*Cicer arietinum* L.) [109]. Inter-priming binding sites (iPBS) -retrotransposon markers were used to determine the genetic diversity and population structure in Turkish wheat germplasm [174] and in common bean (*Phaseolus vulgaris* L.) [175]. Retrotransposon marker methods IRAP and REMAP were used to investigate genetic diversity in wild emmer wheat populations obtained from Türkiye and Israel, and the consistency of the results with other molecular markers used in previous studies also shows the suitability of retrotransposon markers in population genetic analyses [176]. IRAP markers were also employed to examine the genetic diversity of some Egyptian barley varieties and were found to produce 63% polymorphic bands. IRAP markers displayed a significant difference at the molecular level between barley varieties studied in Egypt [177]. Stress factors such as radioactivity can cause activation of mobile elements (TE) in the genome [178], and there is a hypothesis that they have a potential effect on genome remodeling [179]. It supports investigating genetic diversity resulting from genetic changes caused by radioactivity with retrotransposon markers. Conclusively, IRAP markers are a more suitable method for identifying LTR-retrotransposon polymorphism [180]. LTR retrotransposons, most of the maize genome, are used as high-throughput genetic markers. The genetic diversity of maize germplasm was examined using IRAPs. While 58% polymorphism was detected with IRAP markers, it was also determined that they produced high-quality and reproducible DNA fingerprints [181]. REMAP markers have been used in genetic diversity studies in maize [182]. All the studies published previously indicated that different varieties of molecular markers are successful in determining genetic diversity, population genetic structure, phylogenetic relationships among the taxa, plant breeding studies with MAS, discovering new genes, and QTL mapping in different plant species.

4. Phylogeny and Evolution

Phylogeny is the evolutionary history of a species or group in terms of its origin and relatedness to other species or groups [183]. It helps to organize biological diversity and to understand the evolution of traits. Phylogenies are important for answering various biological questions based on

DNA, RNA, or protein sequences derived from phylogenetics, such as relationships among species or genes, the origin and spread of viral infections, demographic changes in species, and migration patterns [184]. The questions that phylogeneticists seek to answer are: what is the evolutionary history of the relationships among species, individuals, and genes? How have their sequences evolved, and how can the processes of sequence evolution be revealed and described? To investigate the answers to these questions, examining the phylogenetic relationships among species, individuals, and genes is necessary. Morphological, cytological, and biochemical markers have investigated phylogenetic relationships among crop plant species [185]. However, since these features are affected by environmental factors, molecular markers have been published in previous studies to provide more reliable results. Investigating phylogenetic relationships covers studies to determine species' degree of kinship and origin. It allows species to be placed in the right place in the taxonomy and to benefit from them most efficiently. Determining the genetic diversity, genetic differentiation or similarity, and genetic distances of plant species according to the data obtained by using any marker method or a combination of several methods sheds light on the explanation of phylogenetic relationships between modern cultivated plants and their wild or primitive relatives. Phylogenetic relationships between species or the investigation of parameters such as genetic diversity, genetic differentiation and genetic distance using data obtained by molecular methods from different populations of a species adapted to different ecological conditions and geographical regions, and the creation of phylogenetic trees in which the evolutionary relationship is depicted in the form of a tree diagram according to cluster analyses, make it possible to explain phylogenetic relationships. When investigating the gene or gene groups responsible for the expression of a trait in plants, knowing the genetic relationships between closely related species can also be informative in determining whether the gene or gene groups of interest are orthologous [186] or emerged after the speciation event and determining their evolutionary processes. With the development of high throughput, economical, and automation-compatible methods in DNA sequence analysis, such as next-generation sequencing, the entire genome sequences of many plant species, especially most plants consumed by humans as a food source, have been sequenced. However, there are also those whose wild relatives or different species have not yet had their genome sequences sequenced. In the investigation of genes related to yield, quality, or tolerance in species whose genome sequences have not been sequenced among closely related species, it is possible to determine them by modeling with molecular methods and using bioinformatics tools from species whose complete genome sequences have been sequenced, based on phylogenetic relationships. In this way, when the relevant genes are identified, superior genotypes found in closely related species can be determined and used in plant breeding studies to develop varieties with desirable characteristics [187]. In economically important plant species, to use a species, its phylogeny must be known precisely and its genetic characterization must be completed. Considering the species in terms of their phylogenetic status and eliminating uncertainties may provide a new perspective for breeding studies to develop new varieties with desired characteristics in the future [188]. Molecular markers have revolutionized the timescale over which closely related species have diverged and the types of genetic variation associated with the formation of new species [189]. An essential first step in determining whether any germplasm is part of the primary, secondary, or tertiary gene pool of the system in question is taxonomic classification [190].

5. Conclusion

Analysis of genetic diversity is the first stage of studies on taxonomy, phylogenetics, breeding and development of new varieties with desired characteristics, such as yield, quality and resistance. Therefore, genetic diversity studies are a process that should be evaluated carefully. Each of the molecular markers has its own characteristic features. No type of marker covers the entire genome, and each of them scans only certain regions. Even if the genetic diversity detected between populations of a species or within a population using any molecular marker is detected as high when evaluated alone, this will only express the variation in the regions scanned by the marker of interest. In order to assess genetic diversity accurately and reliably, the combined use of more than one molecular marker and genomic data generated through high-throughput sequencing technologies will allow for a more comprehensive detection of diversity at the genome-wide level. With the development of bioinformatics tools and cost-effective technologies such as genome sequencing, a new generation of molecular markers with high throughput, automation, and more advanced gene targeting is expected to be developed. The information that will be produced by different omics techniques and computational tools to be developed in the future will provide a more accurate characterization of more diverse plant germplasms (wild, landraces, cultivated forms, etc.) in a short time with limited time and resources, and will provide significant contributions to the understanding of plant genetics and genomics. Using these data, the complexities related to the evolutionary history and phylogenetic relationships of plant species will be resolved and will also lead to new questions to be investigated. Interpreting the data obtained with morphological markers by combining them with the data obtained with molecular markers will increase the efficiency of breeding studies and strengthen the effectiveness of the markers. According to the data obtained with molecular markers, it will be possible to determine how and when a species or closely related species diverged evolutionarily and according to which regions of the genome they differed, and that different gene pools have different genetic bases, with parameters such as genetic diversity, genetic differentiation and gene flow. Genetic diversity and phylogenetic studies will provide functional and usable information for the identification, conservation, and management of modern cultivars of cereal plants, which are the primary sources of calories in human nutrition, and for the development of broad-based and climate-resilient cultivars or hybrids that will meet the breeding challenges of the near future. Genetic diversity is not only a biological concept. Still, it has evolved into a parameter that is considered in addressing global concerns such as climate change and population growth, which pose challenges to food production and sustainability for humans. Therefore, researchers and decision-makers working on food security can use the information provided by the markers to strengthen food security and increase agricultural yields. Although many molecular markers have been developed based on different methods, the high cost of devices and chemicals used for molecular markers based on PCR and DNA sequence analysis still remains an obstacle to small research groups accessing these technologies, particularly in developing countries. The development of new high throughput marker methods would be great if they require less labor but reveal more polymorphisms, ensure that the results obtained are comparable to other methods, and are affordable for every researcher, which will enable researchers to conduct similar studies in different laboratories and compare their results.

Author Contributions

The author did all the research work, writing, reviewing and editing of this study.

Competing Interests

The author has declared that no competing interests exist.

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