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# Molecular Marker Techniques and Their Novel Applications in Crop Improvement: a Review Article

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**Abstract:** Recently, considerable emphasis has been placed on the development of molecular marker technology to be used for a variety of applications including in both basic plant research and plant breeding programs. Basically, there are three different types of genetic markers viz., morphological, biochemical and molecular markers. Morphological markers have certain constraints i.e. narrow diversity, influenced by environment, problem with epistasis, pleiotropy, incomplete penetrance and variable expressivity. Biochemical marker analysis is based on the separation of proteins into specific banding patterns however, only a limited number of enzymes loci are available in the genome and thus, the resolution of diversity is limited. However, the use of molecular markers has been playing a vital role in improvement of crops and their genetic studies. Thus, molecular markers have the advantage of improving the effectiveness of conventional breeding through the selection of desirable characteristics based on the presence of molecular markers, which are linked to the particular traits of interest since these markers are not influenced by the environment and can be scored at all stages of plant growth. Therefore, this review attempts to give an account of the application of most of the available molecular markers that can be routinely employed in various aspects of plant genome analysis such as genetic diversity analysis, DNA fingerprinting, gene tagging, QTL analysis and marker assisted selection.

**Key words:** Molecular Markers · Novel Applications · Crop Improvement

## INTRODUCTION

Current plant breeding practices have been very successful in producing a continuous range of improved crop varieties. Thus, the most important way of crop improvement through conventional approaches is primarily depends on (i) discovery and generation of new genetic variability in agronomic traits (e.g., genes for biotic and abiotic stress resistance etc.) and (ii) precise selection of desired genotypes with favorable characteristics, as a product of a recombination among superior alleles at different loci [1]. Additionally, in all crop improvement programs, the breeders are interested to maximize the efficiency of the selection that permits differentiation of the best genotypes [2]. It has also been reported that where the selection is based only upon the phenotypic values, the breeders commonly confront the problem of genotype-environment interaction, which may mask favorable genotypes making selection more difficult [3]. Thus, the breeders tried to minimize the genotype-environment interaction by stratifying the selection plot [4] or using the correlation among agronomic characters for making an indirect yield selection [5] as well as to increase the efficiency of selection through progeny test [6]. Overall, these conventional techniques have been contributed to reach more accuracy and precise during selection; however due to the increased use of resources or the time spent in screening on the one hand and new exigencies and challenges for increasing the yield of crops on the other have led to the development of new molecular tools that have enhanced the effectiveness of selection [2]. Recently, the use of several molecular marker techniques, which permits establishment of linkage maps in crop plants [7, 8] and these linkage maps allow breeders to conduct applied research so as to identify and characterize genetic variability in economically important plant species [9, 10].

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Thus, the main reasons supporting the utilization of molecular marker techniques in national crop improvement programs are; the heritability nature of the markers and their cost, which is potentially lower than the conventional selection methods [11] as well as the proposed uses of molecular markers into crop improvement are their potential applications onto selection for traits with low heritability, identification of complex inheritance like resistance or tolerance for biotic or abiotic stresses and the gene introgression coming from native or exotic germplasm [12]. In addition to this, other potential uses of the markers such as estimation of genetic relatedness among population and breeding materials [13], identification and fingerprinting of genotypes [14] and the detection of quantitative trait loci (QTL) linked to molecular markers, which control important agronomic traits [15], marker-assisted selection (MAS) and identification of sequences of useful candidate genes for future research in plant breeding programs [14].

The aim of this review is therefore to present clear examples of the role of molecular markers in crop improvement and establishes potential uses for future research in plant breeding programs.

**Genetic Markers: Concepts and Types:** Genetic marker is a gene or DNA sequence with a known location on a chromosome that can detect variation in either a protein or DNA sequence. Any difference, whether phenotypic or genotypic, may act as a genetic marker if it identifies characteristics of an individual's genotype and/or phenotype and if its inheritance can be followed through different generations [16]. The analysis of genetic diversity within and among populations routinely involves the use of different genetic markers. Nowadays, genetic markers are applicable in both basic plant research and plant breeding programs to characterize plant germplasm, for gene isolation, marker assisted introgression of favorable alleles, production of improved varieties [17] and to obtain information about the genetic variation of populations. Generally, there are two classes of traits to measure genetic variations. These are (i) traits controlled by many gene loci (polygenic traits/minor genes) and (ii) traits controlled at the single gene loci (monogenic traits/major genes). Therefore, to fully characterize the level and pattern of genetic diversity in a species and its evolutionary causes, studies involving both classes of traits are necessary. Thus, basically, there are three types of genetic markers. These are morphological, biochemical and molecular markers [16, 18, 19].

**Morphological Markers:** The use of morphological markers as an important tool to select the plants with desired traits had started in breeding long time ago. During the evolution of plant breeding, the markers used mainly included visible traits, such as leaf shape, flower color, pubescence color, pod color, seed color, seed shape, awn type and length, fruit shape, flesh color, stem length, etc.). As reported by Jiang [20] these markers generally represent genetic polymorphisms which are easily identified and manipulated. Some of these markers are linked with other agronomic traits and thus can be used as indirect selection criteria in practical breeding. In the green revolution, selection of semi-dwarfism in rice and wheat was one of the critical factors that contributed to the success of high-yielding cultivars [20]. This could be considered as an example for successful use of morphological markers to modern crop breeding. This is ascertained by Liu [21] in wheat breeding, the dwarfism governed by gene Rht10 was introgressed into Taigu nuclear male-sterile wheat by backcrossing and a tight linkage was generated between Rht10 and the male-sterility gene Ta1. Then, the dwarfism was used as the marker for identification and selection of the male-sterile plants in breeding populations.

Characterization and evaluation of genetic diversity in crop species is based on variation in quantitative and qualitative characters [22, 23], this might be due to the morpho-agronomic traits does not need any advanced equipment or complex experiments. They are simple, rapid and inexpensive to score and measure. Phenotypic estimates are used to present the degree of genetic relationship and difference between lines; it is presumed that similarity in phenotype characteristics reflects genetic similarity of genotypes [24]. However, morphological determinations need to be taken by an expert in the species, they are subject to changes due to environmental factors and may vary at different developmental stages and their number is limited [16, 19].

In general, morphological markers cannot be reliable measures of genetic differences because of the influence of the environment on gene expression; therefore methods to assess and detect genetic diversity have been extended to include biochemical and molecular analysis [25].

**Biochemical Markers:** To address the limitations of morphological markers, other markers have been developed for example the protein markers [16]. Protein markers are usually named 'biochemical markers' but, more than often, they are mistakenly considered as a common class under the so-called 'DNA based markers'

[16]. Biochemical analysis is based on the separation of proteins into specific banding patterns. It is a fast method which requires only small amounts of biological material. However, only a limited number of enzymes are available and thus, the resolution of diversity is limited [19]. Allozymes, being allelic variants of enzymes, provide an estimate of gene and genotypic frequencies within and between populations [19]. This information can be used to measure genetic diversity, gene flow, genetic structure of species, population structure and population divergence [26].

Major advantages of these types of markers include: codominant inheritance, ease of use, low costs of the assay and general applicability, while limitations include: (i) there are only few isozyme systems per species (no more than 30) with correspondingly few markers, (ii) the number of polymorphic enzymatic systems available is limited and the enzymatic loci represent only a small and not random part of the genome (the expressed part) therefore, the observed variability may not be representative of the entire genome, (iii) although these markers allow large numbers of samples to be analyzed; comparisons of samples from different species, loci and laboratories are problematic, since they are affected by extraction methodology, plant tissue and developmental stage of the plant [19].

**Molecular Markers:** Molecular markers are defined as a fragment of DNA revealing mutations/variations, which can be used to detect polymorphism between different genotypes or alleles of a gene for a particular sequence of DNA in a population or gene pool. Such fragments are associated with a certain location within the genome and may be detected by means of certain molecular technology [20]. These markers are based on naturally occurring polymorphisms in DNA sequences due to base pair deletions, insertions and substitutions [27]. Molecular markers are superior to both morphological and biochemical markers because they are highly polymorphic and heritable (their expression is not affected by environmental variability), abundant throughout the genome even in a highly inbred cultivars, completely independent of environmental conditions and can be detected at virtually any stage of plant development [27].

The rapid development of molecular marker techniques, over the last few decades, now offers a palette of technical approaches for plant genotyping or genome analysis. Which technique is most appropriate depends upon (i) the extent of genetic polymorphism required; (ii) the analytical or statistical approaches available for the technique's application and (iii) the pragmatics of time and costs of materials [28]. The discovery of the polymerase chain reaction (PCR) was a landmark in molecular marker evolution and has proved to be a unique process for the development and utilization of a battery of new very sensitive and quick approaches, such as amplified fragment length polymorphism (AFLP) or microsatellites [29].

An ideal molecular marker technique must have some desirable properties. Below are the ideal properties of molecular markers as described by De Vicente and Fulton [16] and Xu [30] (i) high level of genetic polymorphism: that is, it is variable among individuals and the degree of polymorphism detected depends on the technology used to measure it, (ii) codominance: depending on the type of application, the selected technology must be able to detect the marker's different forms, distinguishing between homozygotes and heterozygotes (codominant inheritance) so that heterozygotes can be distinguished from homozygotes, (iii) discriminating: that is, able to detect differences between closely related individuals, (iv) evenly distribution on the entire genome: the more distributed and dense genome coverage is, the better the assessment of polymorphism, (v) neutral selection: the allele present at the marker locus is independent of and has no pleiotropic effect on, the selection pressure exerted on the individual, (vi) easy detection: so that the whole process can be automated, (vii) low cost of marker development and genotyping, (viii) high duplicability/reproducibility: so that the data can be accumulated and shared between laboratories and (viiii) not subject to environmental influences: the inference of a marker's genotype should be independent of the environment in which the individual lives or its developmental stage. In general, it is extremely difficult to find a molecular marker which would meet all the above criteria. However, depending on the type of study to be undertaken a marker system can be identified that would fulfill at least a few of the above characteristics [30].

**Types of Molecular Marker Techniques:** A wide range of molecular marker techniques is available that detect polymorphism at the DNA level. They have been grouped into the following categories based on the method of their detection. There are mainly two types of molecular markers i.e. (i) Non PCR-based marker for example, Restriction Fragment Length Polymorphism (RFLP) [31] and (ii) PCR-based marker systems including, Random Amplified Polymorphic DNA (RAPD) [32], Amplified Fragment Length Polymorphism (AFLP) [33], Inter Simple Sequence Repeats (ISSR) [34] and Simple Sequence Repeats (SSR) [35].

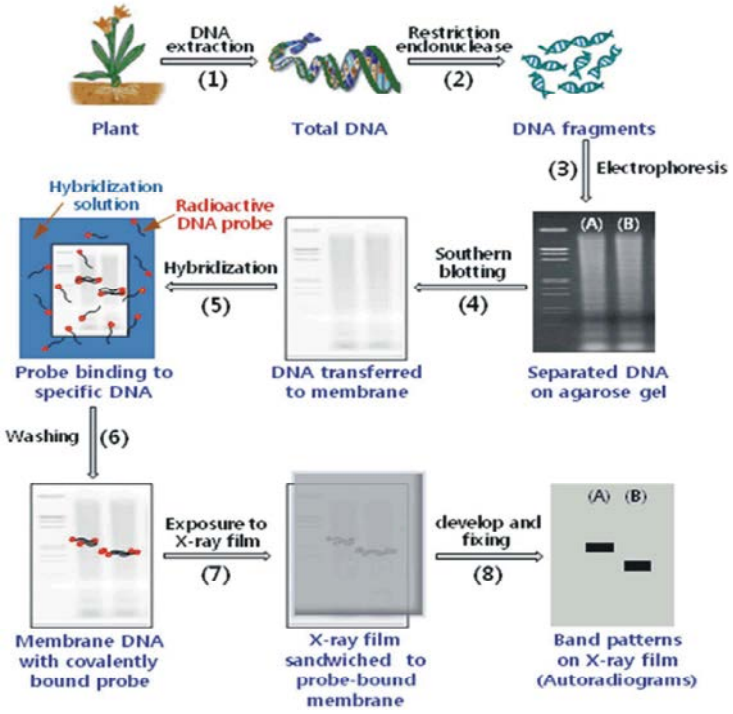


Fig. 1: Restriction Fragment Length Polymorphism (RFLP) procedure. (i) Extract DNA from individuals A and B. (ii) Use restriction enzymes to cut DNA. (iii) Electrophoreses DNA fragments on agarose gel to separate them by size. (iv) Transfer the DNA in the gel to a nylon membrane by Southern blot. (v) Use radioactively labeled DNA fragments as probes to hybridize to the DNA. (vi) Remove non-specifically bound or unbound probes by washing the nylon membrane. (vii) Expose the washed membrane to X-ray film. (viii) Develop the X-ray film to observe DNA polymorphisms. Source: [38]

**Non PCR-Based Molecular Marker Technique**

**Restriction Fragment Length Polymorphism (RFLP):** Restriction Fragment Length Polymorphism (RFLP) was developed first and was initially used for human genome mapping [31]. Later, RFLP markers are one of the most important tools for plant genome mapping [20] and they are classified as hybridization-based markers. RFLP involves the extraction of genomic DNA followed by its digestion with specific restriction enzymes, which cut the DNA into fragments. RFLP results when there is variation in restriction enzyme cleavage sites, arising due to base substitutions, insertions, deletions or translocations in the genomic DNA [36].

The major strength of RFLP markers are their high reproducibility, codominant inheritance and good transferability between laboratories which provides locus-specific markers that allow synteny (conserved order of genes between related organisms) studies [37, 20]. For this, no sequence information is required and they are relatively easy to score due to large size differences between fragments. Still, there are several limitations for RFLP analysis: it requires the presence of high quantity and quality of template DNA. The requirement of radioactive isotope makes the analysis relatively expensive as well as the assay is time consuming and labor intensive [37]. RFLPs can be applied in diversity and phylogenetic studies ranging from individuals within populations or species, to closely related species. It is widely used in gene mapping studies because of their high genomic abundance due to the ample availability of different restriction enzymes and random distribution throughout the genome [37].

**PCR-Based Molecular Marker Techniques:** Some of the advantages of PCR-based marker systems are: (i) PCR requires only small amounts of template DNA and often crude miniprep procedures yield DNA of sufficient quantity and quality, (ii) PCR is relatively quick to perform and technically straightforward, once PCR conditions have been established and (iii) the range of primer sequences possible gives PCR based techniques great diagnostic power [39]. Thus, various PCR-based molecular marker techniques are as follows;

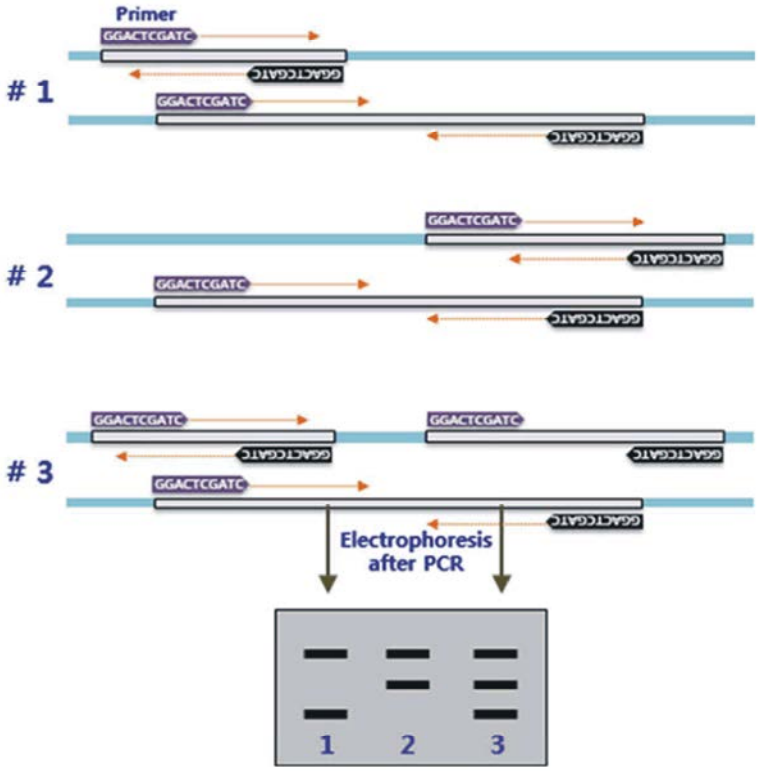


Fig. 2: Principle of Random Amplified Polymorphic DNA (RAPD) markers. A single random primer binds to complementary sites in the genome. Difference RAPD profiles on an agarose gel reveals polymorphism among individuals. Source: [38]

**Random Amplified Polymorphic DNA (RAPD):** Random Amplified Polymorphic DNA (RAPD) is based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequence, usually 10 bp long [32]. These primers detect polymorphisms in the absence of specific nucleotide sequence information and the polymorphisms function as genetic markers and can be used to construct genetic maps. RAPD polymorphisms arise when genomic regions vary for the presence or absence of complementary primer annealing sites due to insertion or deletion between two priming sites, which results in different lengths of the amplification products [40]. Since most of the RAPD markers are dominant, it is not possible to distinguish whether the amplified DNA fragment is heterozygous (two different copies) or homozygous (two identical copies) at a particular locus [41].

RAPD analysis has many potential applications and may be used to assess kinship relationships or genetic diversity [42], construct genetic maps [40]. The main advantages of the RAPD technology include: (i) suitability for work on anonymous genomes, (ii) applicability to problems where only limited quantities of DNA are available and (iii) low expense [42]. However, there are various limitations and considerations in RAPD analysis, which include specificity of the marker in genome scanning [42], reproducibility of amplification products [32] and unclear and non-reproducible fragments [43]. Consequently, the analytical power of RAPD markers is not competitive with analysis using sequence information or single locus probe fingerprinting technologies and as such is not suitable for applications such as extensive fingerprinting analysis [44] or definitive parentage determination.

**Amplified Fragment Length Polymorphism (AFLP):** Amplified Fragment Length Polymorphism (AFLP) method was originally developed as a universal DNA fingerprinting analysis [33] and is robust and relatively insensitive to PCR reaction conditions. AFLP is a restriction digestion and PCR-based marker technique for the rapid screening of genetic diversity and intraspecific variation. It is a potent fingerprinting technique for genomic DNAs of any origin or complexity and rapidly generates a number of highly replicable markers that allow high resolution genotyping.

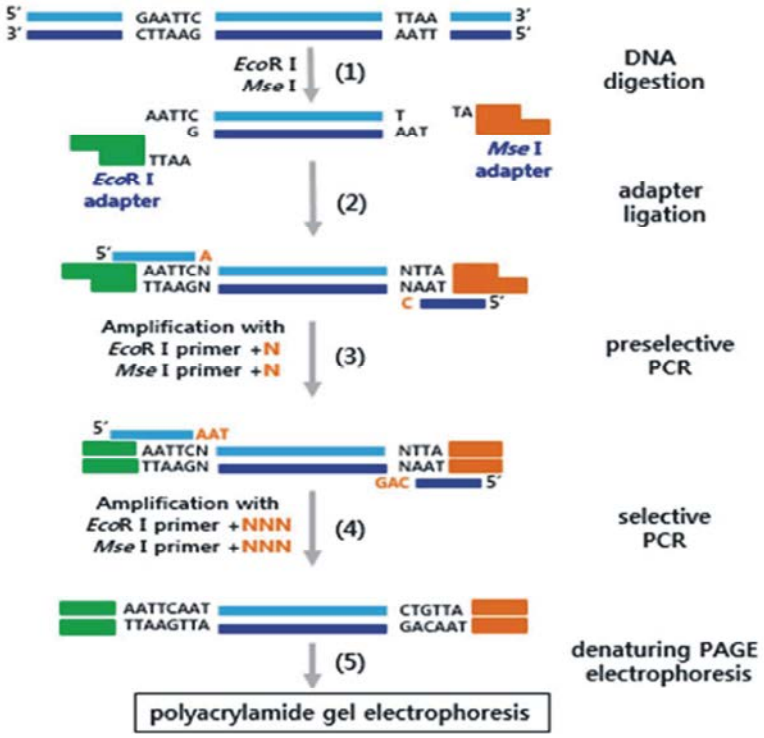


Fig. 3: Schematic diagram showing the Amplified Fragment Length Polymorphism (AFLP) technique. (1) The DNA sample is digested with two types of restriction enzymes. (2) Adaptors are attached to each side of DNA fragment. (3) Pre-amplification by PCR using primers that have the adaptor sequence and one random additional base (+1 selective nucleotide). (4) Selective amplification with primers that have the adaptor sequence and +2–4 selective nucleotides. (5) Electrophoresis of AFLP fragments on an acrylamide gel. Different individuals have different restriction recognition sites and selective nucleotides, giving different AFLP fragments. Source: [38]

The strength of AFLPs includes its high genomic abundance, high reproducibility, highly polymorphic, generation of many informative bands per reaction; small amount of template DNA is needed and the fact that no sequence information for primer construction is required [45]. Possible reasons for AFLP polymorphisms are: (i) sequence variations in a restriction site, (ii) insertions or deletions within an amplified fragment and (iii) differences in the nucleotide sequence immediately adjoining the restriction site. AFLPs have been used for the analysis of genetic diversity [46], DNA fingerprinting [47], the construction of linkage maps [48] and to locate traits of interest [49].

According to Semagn *et al*. [18], in contrast to RAPD, the limitations of AFLP include: a) It requires more number of steps to produce the result. b) It requires template DNA free of inhibitor compounds that interferes with the restriction enzyme. c) The technique requires the use of polyacrylamide gel in combination with AgNO3 staining, radioactivity, or fluorescent methods of detection, which will be more expensive and laborious than agarose gels. d) It involves additional cost to purchase both restriction and ligation enzymes as well as adapters. e) Like RAPD, most AFLP loci are dominant, which does not differentiate dominant homozygotes from heterozygotes. This reduces the accuracy of AFLP markers in population genetic analysis, genetic mapping and marker assisted selection.

**Inter Simple Sequence Repeat (ISSR):** Inter Simple Sequence Repeat (ISSR) is a PCR based technique, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction[50, 51]. ISSR markers were introduced in 1994 [34] for assessing genetic variation below the species level, mainly in studying population structure and differentiation of cultivated plants. The technique uses microsatellites, usually 16-25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetra-nucleotide, or penta-nucleotide [51 -53].

ISSR markers are inherited in a dominant or codominant Mendelian fashion [54]. However, they are interpreted as dominant markers and scored di-allelically with presence (1) or absence (0) of bands similar to RAPD markers [53]. The absence of a band is interpreted as sequence divergence at the site of primer binding. This occurs by loss of a locus through mutation i.e. the deletion of the site or chromosomal rearrangement which could prevent amplification of a fragment and thus give a presence or absence polymorphism or the amplified region would result in the absence of a product [55].

ISSRs have high reproducibility possibly due to the use of longer primers (16-25 bp) as compared to RAPD primers (10 bp) which permits the subsequent use of high annealing temperature (45-60°C) leading to higher stringency. Various studies on reproducibility of ISSR markers were carried out. Among these, Fang and Roose [52] reported a reproducibility level of more than 99 % after performing repeatability tests for ISSR markers by using DNA samples of the same cultivar grown in different locations, DNA extracted from different aged leaves of the same individual and by performing separate PCR runs. In other cases, the reproducibility of ISSRs amplification products ranged from 86 to 94 % [56].

ISSRs exhibit the specificity of microsatellite markers, but do not need sequence information for primer designing enjoying the advantage of random markers [50]. Furthermore, the potential advantages of this technique are: (i) simple and fast, (ii) low quantity of template DNA required, (iii) randomly distributed throughout the genome, (iv) small reaction volumes and amounts of enzyme are needed for PCR, (v) the hypervariability of banding patterns, (vi) large quantities of plant material for DNA extraction are not required, (vii) no specialized apparatus needed (e.g. automated sequencer, autorad development, etc.), (viii) banding patterns are easily scorable and (ix) the higher annealing temperatures used for ISSR reactions may reduce the amount of template-primer mismatch artifacts than may be encountered with RAPD markers, which generally rely on lower annealing temperatures. However, limitations of the technique are: (i) pure template DNA and similar concentrations among samples are required for standardization of reactions, (ii) optimization of initial reactions is needed, (iii) bands are scored as dominant markers and genetic diversity estimates are based on diallelic characters [53].

ISSR markers have been used for different applications such as genetic diversity analysis, fingerprinting and genome mapping, parentage analysis, phylogenetic studies, gene tagging, clone and strain identification and taxonomic studies of closely related species [34]. ISSRs are widely used in genetic diversity analyses because of no genome sequence information is required for primer designing and the primers are not proprietary and can be synthesized by anyone, their development costs are low, highly discriminative, reproducible, informative, reliable and the laboratory procedures can easily be applied to any plant species [34].

**Simple Sequence Repeat (SSR):** Simple Sequence Repeats (SSR) are one of the most powerful molecular marker classes and they are very short motifs (about 1-6 bp) usually characterized by a high degree of repetition and occur at many thousand loci in the nuclear genome [25]. They are ubiquitous and highly polymorphic, owing to the mutation affecting the number of repeat units. The hypervariability of SSRs among related organisms makes them an informative and excellent choice of markers for a wide range of applications in population and evolutionary biology [57], which include estimate genetic diversity [58], identify and test the paternity of cultivars [59], study population structure and gene flow [60] and develop gene mapping [61].

With the advent of polymerase chain reaction (PCR) technology, this property was converted into a highly versatile molecular marker [62] and became the basis for SSR-based DNA fingerprinting. Products of different length can be amplified with primers flanking the variable microsatellite region and single locus are typically amplified, resulting in one or two bands, depending on the homozygous or heterozygous state in diploid organisms. Therefore, SSRs are considered locus-specific and codominant markers. The popularity of microsatellites stems from a unique combination of several important advantages: the relatively abundance with uniform genome coverage, the enormous extent of allelic diversity, the hypervariability, the codominant inheritance, the ease of detection by PCR using pair of flanking primers and requirement for only a small amount of template DNA [57, 25].

Numerous molecular marker strategies have been developed, but the most common employs sequence information of repeat-flanking regions to design locus-specific PCR primer pairs. The necessity of sequence information for primer design is the more serious obstacle of this technique, in addition to the possible presence of undetected null alleles, which can interfere with the interpretation of molecular data. Null alleles can be due to mutations in one or both primer binding sites [62] and these mutations can prevent PCR amplification. Homozygous individuals for a null allele do

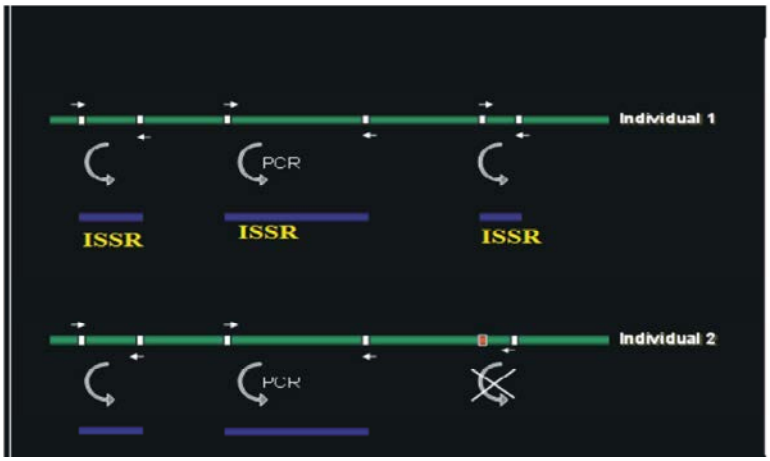


Fig. 4: Principle of Inter Simple Sequence Repeats (ISSR) markers. ISSRs are DNA fragments located between adjacent oppositely oriented microsatellites and based on PCR amplification of inter-SSR sequences to target multiple loci in the genome.

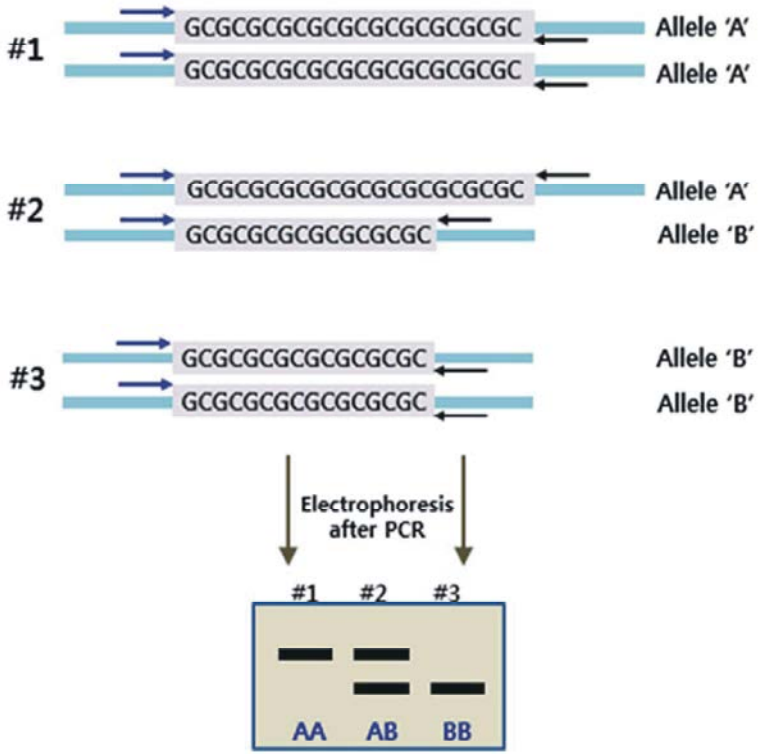


Fig. 5: Principle of Simple Sequence Repeats (SSR) markers. Amplifying the SSR sequence by PCR using primers just outside the sequence reveals polymorphisms in amplicon size. Source: [38]

not show any band at all, whereas heterozygotes have only one band and therefore mimic a homozygote on the electrophoresis gel. Erroneous interpretations due to null alleles may be solved by redesigning primer pairs for the locus, avoiding the mutated primer binding site and by examining multiple microsatellite loci, reducing the influence of null alleles.

**Cleaved Amplified Polymorphic Sequences (CAPS):** Cleaved amplified polymorphic sequences (CAPS) are a class of molecular markers with the combination of the PCR and RFLP [63]. The technique involves amplification of a target DNA through PCR using specific 20-25 bp primers, followed by digestion of the PCR products with a restriction enzyme. Subsequently, length

Table 1: Comparison of the most commonly used molecular marker systems for their features in crop plants [66, 18, 37]

| Feature | RFLP | RAPD | AFLP | SSR | ISSR | SNP |
|---|---|---|---|---|---|---|
| Amount of DNA required | high | low | medium | low | low | low |
| DNA quality | high | high | moderate | moderate | Medium-high | high |
| PCR based | no | yes | yes | yes | yes | yes |
| No. of polymorphic loci analyzed | 1.0-3.0 | 1.5-50 | 20-100 | 1.0-3.0 | 1.0-20 | 1.0 |
| Ease of use | not easy | easy | easy | easy | easy | easy |
| Genomic abundance | high | Very high | Very high | medium | medium | medium |
| Amenable to automation | low | moderate | moderate | high | moderate | high |
| Reproducibility | high | unreliable | high | high | Medium-high | high |
| Utility for genetic mapping | species specific | across-species | across-species | species specific | across-species | speciesspecific |
| Development cost | low | low | moderate | high | low | high |
| Cost per analysis | high | low | moderate | low | Low-medium | low |
| Cloning and/or sequencing | yes | no | no | yes | no | yes |
| Inheritance | Codominant | dominant | dominant | Codominant | dominant | Codominant |

polymorphisms resulting from variation in the occurrence of restriction sites are identified by gel electrophoresis of the digested products. Advantages of CAPS include the involvement of PCR requiring only low quantities of template DNA (50-100 ng), the co-dominance of alleles and the high reproducibility. Compared to RFLPs, CAPS analysis does not include the laborious and technically demanding steps of southern blot hybridization and radioactive detection procedures. However, CAPS show only low levels of polymorphism that is more difficult to find because of the limited size of the amplified fragments (300-1800 bp), only assesses variation at one locus and needs sequence information for designing of the primers.

**Single Nucleotide Polymorphisms (SNP):** This approach can be considered as one of newest and highly automated genotyping techniques that can detect changes in single nucleotide sequences [64]. Single nucleotide polymorphisms (SNPs) and insertions and deletions (indels) are highly abundant and distributed throughout the genome in various species including plants. The abundance of these polymorphisms in plant genomes makes the SNP marker system an attractive tool for gene mapping, marker-assisted breeding and map-based cloning. Hence, in contrast to all previous techniques, allele discrimination cannot be based on size differences on a gel [65].

**Novel Applications of Molecular Marker Techniques in Crop Improvement:** Recently, novel molecular tools have therefore started to demonstrate their usefulness into practical plant breeding for facilitating the identification, characterization and manipulation of the genetic variation on economically important agronomic traits. Therefore, the novel applications of molecular markers in crop improvement programs are listed below:

- Genetic diversity analysis
- Identification and fingerprinting of genotypes
- Gene tagging/mapping
- Quantitative trait loci (QTL) analysis
- Marker assisted selection (MAS)

**Genetic Diversity Analysis:** Analyses of the extent and distribution of genetic variation in a crop species are very essential in understanding the evolutionary relationships between accessions and to sample genetic resources in a more systematic fashion for breeding and conservation purposes. Thus, molecular markers have proved to be excellent tools for analysis of genetic diversity in a wide range of plant species. The information is often of direct utility to plant breeders since it is indicative of the performance, adaptation or other agronomic qualities of the germplasm [14]. Since molecular markers have provided very useful information about the overall genetic range of crop germplasm. For breeders, this molecular information is very important to take decisions regarding the utility of germplasm particularly in search for rare and unique genes. A crop species of narrow genetic base is obviously unlikely to harbour novel genes e.g. those conferring resistance to biotic and abiotic stresses.

Different types of molecular marker techniques have been used for genetic studies in crop plants including RFLPs, RAPDs, AFLPs and SSRs, among different classes of molecular markers, SSR markers are useful in variety of applications like plant genetics and breeding because of their reproducibility, multiallelic nature, codominant inheritance, relative abundance and good genome coverage. SSR markers have been useful for integrating the genetic, physical and sequence-based physical maps in plant species and simultaneously have provided breeders and geneticists with an efficient tool to link phenotypic and genotypic variation.

Table 2: Comparison of molecular markers based on their advantages and disadvantages [18, 37, 41]

| Type of molecular markers | Advantages | Disadvantages |
|---|---|---|
| Inter Simple Sequence Repeats (ISSR) | ▪ Quick, simple and cheap<br>▪ Multiple loci from a single primer<br>▪ Small amount of DNA required<br>▪ No sequence information | ▪ Dominant marker<br>▪ Non-homology of similar sized fragments appeared |
| Restriction Fragment Length Polymorphism (RFLP) | ▪ High genomic abundance<br>▪ Co-dominant markers<br>▪ Highly reproducible<br>▪ Good genome coverage<br>▪ Can be used across species<br>▪ No sequence information<br>▪ Can be used in plants reliably<br>▪ Needed for map based cloning | ▪ Need large amount of good quality DNA<br>▪ Laborious (compared to RAPD)<br>▪ Difficult to automate<br>▪ Need radioactive labeling<br>▪ Cloning and characterization of probe are required |
| Randomly Amplified Polymorphic DNA (RAPD) | ▪ High genomic abundance<br>▪ Good genome coverage<br>▪ No sequence information<br>▪ Less amount of DNA (poor DNA acceptable)<br>▪ No radioactive labeling<br>▪ Relatively faster<br>▪ Can be used across species | ▪ Use random primer-non-informative<br>▪ Dominant markers<br>▪ Not reproducible |
| Amplified Fragment Length Polymorphism (AFLP) | ▪ High genomic abundance<br>▪ High polymorphism<br>▪ No need for sequence information<br>▪ Can be used across species<br>▪ Work with smaller RFLP fragments | ▪ Very tricky due to changes in patterns with respect to materials used<br>▪ Cannot get consistent map (not reproducible)<br>▪ Need to have very good primers |
| Cleaved Amplified Polymorphic Sequences (CAPS) | ▪ Involvement of PCR requiring only low quantities of template DNA (50-100 ng)<br>▪ The co-dominance of alleles<br>▪ The high reproducibility<br>▪ Compared to RFLPs, CAPS analysis does not include the laborious and technically demanding steps of southern blot hybridization and radioactive detection procedures<br>▪ Only assesses variation at one locus and needs sequence information for designing of the primers. | ▪ Show only low levels of polymorphism that is more difficult to find because of the limited size of the amplified fragments (300-1800 bp) |
| Simple Sequence Repeats (SSR) | ▪ High genomic abundance<br>▪ Highly reproducible<br>▪ Fairly good genome coverage<br>▪ High polymorphism<br>▪ No radioactive labeling<br>▪ Easy to automate<br>▪ Multiple alleles | ▪ Cannot be used across species<br>▪ Need sequence information |
| Single Nucleotide Polymorphism (SNP) | ▪ Most abundant polymorphism in any organism<br>▪ Adaptable to automation<br>▪ Reveal hidden polymorphism not detected by other markers and methods<br>▪ Inherited as co-dominant markers. | ▪ Need specialized equipment<br>▪ Analytical procedures require sequence information for the design of allele-specific PCR primers. |

Elias *et al.* [67] was assessed the genetic diversity of 31 varieties of cassava using AFLP markers. Sreekumar *et al.* [68] have used five selective AFLP primer combinations on 60 samples of bread fruit and reported a total of 414 bands in which 85% were polymorphic. The values of genetic distance varied from 0.0044 to 0.3376. Analysis of molecular variance (AMOVA) revealed most of the variation within populations (57.45%) than (42.55%) among populations. RAPD markers also proved to be very useful in the genetic diversity study, resulting in

important implications for cassava germplasm collections and genetic breeding [69]. The 53 wheat cultivars have been genotyped using 24 SSR markers in order to evaluate genetic similarities among Polish Wheat's. One SSR marker allowed identifying DNA polymorphisms, giving in total 166 alleles, from 3 to 13 alleles per marker with mean of 7.22. The study revealed spring cultivars were less diverse than winter cultivars [70]. Teklu *et al*. [71] have studied genetic diversity in 73 accessions of Emmer Wheat from 11 geographical regions using a set of 29 SSR markers. The SSR primers amplified a total of 357 different alleles with an average of 12.31 alleles per locus. The coefficient of gene differentiation showed that the genetic variation within and among the 11 geographical regions was 73 % and 27 %, respectively. During the same period, in a separate study from North Oman, the genetic diversity of hexaploid wheat landraces in relation to their geographic origin was demonstrated through SSR based diversity analysis [72]. Pinto *et al.* [73] have conducted analysis of genetic diversity of maize populations using 30 microsatellite markers. The population did not differ significantly regarding the amount of genetic diversity as a consequence of selection genetic variability losses in terms of mean number of alleles per locus. The genetic distances confirm the favorable effects of one cycle of recurrent selection, as the synthetic become more isolated in comparison to the original population [73]. Adebabay *et al.* [74] has also been genotyped 82 introduced genotypes of sugarcane from 7 geographical regions using a set of 12 ISSR markers so as to evaluate the extent of molecular genetic diversity. The ISSR primers amplified a total of 149 different alleles with an average of 12.41 alleles per locus. Thus, the ISSR fingerprint in this study identified genetically unique genotypes that are potentially important source of diverse sugarcane germplasm [74].

**Identification and Fingerprinting of Genotypes:** DNA fingerprinting profile is very important for varietal identification as well as for ascertaining variability in the germplasm. Thus, the fingerprinting information is useful for quantification of genetic diversity, characterization of accessions in plant germplasm collections and for protection of property of germplasm [14]. Molecular marker has been used widely for DNA fingerprinting of cultivars and breeding lines in a number of crops species. Molecular marker technology has great potential for enhancing purity assessment in crop hybrids. For instance, genetic purity of three F1 chilli hybrid varieties was determined using two molecular techniques RAPD and ISSR by Mongkolporn *et al.* [75]. They found that RAPD analysis successfully detected all three F1 hybridity, while ISSR detected only two. This was due to the RAPD marker system producing a greater number of markers/alleles than the ISSR system. In general, all the available molecular tools from the simplest and readily useable RAPDs, ISSRs, DNA amplification fingerprinting (DAF) to the more precise and robust AFLPs, microsatellites/SSRs and RFLP analyses have been utilized for discrimination of closely related lines as well as high yielding varieties. Thus, various molecular marker techniques are ideal for distinguishing closely related genotypes that differ in few morphological traits.

**Gene Tagging/Mapping:** The most interesting application of molecular markers at present time, as a tool for locating genes governing agronomically important characters via linkage to mapped DNA sequences. Gene tagging refer to mapping of genes of economic importance close to known markers [14]. Phenotypic evaluation at the whole plant level or at the cellular level provides information, which can be used to determine the chromosomal location of the genes that confer the phenotype of interest. Thus, a molecular marker very closely linked to gene act as a tag that can be used for indirect selection of gene in breeding programs with the construction of molecular map, several genes of economic importance such as disease resistance, stress tolerance, insect resistance, fertility restoration genes, yield attributing traits have been tagged. Huang *et al.* [76] tagged powdery mildew resistance gene ol-1 on chromosome 6 of tomato using RAPD and SCAR markers. Gene tagging is a pre-requisite step for marker assisted selection and map based gene cloning [14].

**QTL Analysis:** Genetic factors that are responsible for a part of the observed phenotypic variation for a quantitative trait can be called quantitative trait loci (QTL). Although similar to a gene, a QTL merely indicates a region on the genome and could be comprised of one or more functional genes. QTL-mapping association between observed trait values and presence/absence of alleles of markers that have been mapped onto a linkage map is analyzed. When it is significantly clear that the correlation that is observed did not result from some random process, it is proclaimed that a QTL is detected. Also the size of the allelic effect of the detected QTL can be estimated. A breeder can analyze QTL occurrences and use this knowledge to his advantage, for instance by using indirect selection. When selection is based on genetic information retrieved through the application of molecular markers this is called marker-assisted selection.

Thus, according to Datta *et al.* [77], QTL analysis requires the followings;

- Make cross and generate mapping population
- Identify markers those are polymorphic between the parents
- Generate marker data
- Generate linkage maps of molecular markers
- Collect phenotypic measurements of QTL trait
- Map QTLs (Association of QTL with marker)

**Marker Assisted Selection (MAS):** With the advent of marker-assisted selection (MAS), a new breeding tool is available to make more accurate and useful selections in breeding populations. MAS allow heritable traits to be linked to the DNA segments that are responsible for controlling that trait. These segments of DNA or QTLs can be detected through specific laboratory techniques. Instead of selecting for a trait, the breeder can select for a marker that can be detected very easily in the selection scheme.

According to Datta *et al*. [77], the essential requirements for marker assisted selection (MAS) in plant breeding program are as follows;

- Search of molecular markers that are linked to the trait of interest
- Validate the available markers in parents and breeding population
- If markers are not available, it has to be designed and validated before use (if mapping populations are not available in hand it may take 2-4 years to generate and validate markers)
- Design a selection scheme and breeding strategy
- Fix the minimum population to be assayed to capture all beneficial alleles
- Meticulous record keeping
- Progeny testing for fixation of traits

**Steps Involved in MAS**

- Validation of molecular markers. Extract the DNA from test individuals and find out whether there is one to one relationship with marker and the trait.
- Extract the DNA of breeding population at the seedling stage and apply MAS. Select the individuals on the basis of presence of desired molecular markers for the concerned trait. For other traits, selection is based on classical breeding methods. Minimum individuals to be assayed should be as per the defined strategy and statistical considerations [77].

**Limitations of MAS**

- Cost factor
- Requirement of technical skill
- Automated techniques for maximum benefit
- DNA markers are not affected by environment but traits may be affected by the environment and show genotype-environment interactions. Therefore, while developing markers, phenotyping should be carried out in multiple environments and implications of G x E should be understood and markers should be used judiciously.
- DNA marker has to be validated for each of the breeding population.

Regarding with the application of MAS, several activities have been conducted for example in soybean; soybean cyst nematode (SCN) (*Heterodera glycines* Inchinoe) may be taken as a good example of MAS for major genes. This pathogen is the most economically significant soybean pest. The principal strategy to reduce or eliminate damage from this pest is the use of resistant cultivars [78]. However, identifying resistant segregants in breeding populations is a difficult and expensive process. A widely used phenotypic assay takes five weeks, requires a large greenhouse space and about 5 to 10 h of labor for every 100 plant samples processed [79]. Fortunately, the SSR marker Satt309 has been identified to be located only 1-2 cM away from the resistance gene rhg1 [78], which forms the basis of many public and commercial breeding efforts. In a direct comparison, genotypic selection with Satt309 was 99% accurate in predicting lines that were susceptible in subsequent greenhouse assays for two test populations and 80% accurate in a third population, each with a different source of SCN resistance [79]. In soybean, Shi et al. [80] also reported that using molecular markers in a cross J05xV94-5152, they developed five F4:5 lines that were homozygous for all eight marker alleles linked to the genes/loci of resistance to soybean mosaic virus (SMV). These lines exhibited resistance to SMV strains G1 and G7 and presumably carried all three resistance genes (Rsv1, Rsv3 and Rsv4) that would potentially provide broad and durable resistance to soybean mosaic virus (SMV).

Therefore, the integration of MAS into conventional plant breeding programs will be an optimistic strategy for crop improvement in the future. It can be expected that the drawbacks of MAS will be gradually overcome, as its theory, technology and application are further developed and improved. This should lead to a wide adoption and use of MAS in practical plant breeding programs for more crop species and for the improvement of important agronomic traits.

## CONCLUSION

The new challenges of plant breeding urge to integrate the latest innovations in molecular biology and genetics to enhance crop improvement. Recent advances in molecular biology and biotechnology indicates that the development and application of DNA based markers using high throughput marker discovery and genotyping assay is still a relatively young field and more exciting advances are expected in the future. One of the great promises of DNA markers, using high throughput approaches, is that the ability to carry out comprehensive genomic analyses easily, inexpensively, accurately and rapidly with high sensitivity should create a new generation of routine genomic tools to assist the crop breeding. Molecular markers are important genetic tools for plant breeders to detect the genetic variation available in the germplasm collection. During the last two decades, varieties of molecular markers and in large numbers have been developed for almost all major crop species. Genetic variation detected by molecular markers has been useful for understanding the genome dynamics as well improving the breeding efficiency. For instance, these markers have been utilized extensively for the preparation of saturated molecular maps (genetic and physical) and their association with genes/QTLs controlling the traits of economic importance has been utilized in several cases for marker assisted selection (MAS). As a result of extensive efforts undertaken at international level to identify molecular markers tightly linked with a large number of agronomic traits as well as tolerance/resistance to abiotic and biotic stresses in major crop species, it has been possible to realize the potential of molecular markers to track loci and genome regions in several crop breeding programs.

Other important uses of molecular markers include germplasm characterization, genetic diagnostics, genome organization studies and phylogenetic analysis. The major application of molecular markers lies in the strategic research for rapid understanding of basic genetic mechanisms and genome organization at molecular level. The success of molecular marker technology for bringing genetic improvement in crops would depend on close interaction between plant breeders and biotechnologists, availability of skilled man power and substantial financial investment on research.

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