General Impact Factor (GIF): 0.875
Scientific Journal Impact Factor: 1.205

e- ISSN: 2394-5532
p- ISSN: 2394-823X

International Journal of Applied And Pure Science and Agriculture
www.ijapsa.com

MOLECULAR MARKERS: APPLICATION IN PLANT IMPROVEMENT PROGRAMMES

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Abstract

The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics. The presence of various types of molecular markers, and differences in their principles, methodologies, and applications require careful consideration in choosing one or more of such methods. The idea of using genetic markers appeared very early in literature but the development of electrophoretic assays of isozymes and molecular markers have greatly improved our understanding in biological sciences. Molecular detection of plant pathogens and characterization of genetic variability by using different DNA markers have offered additional tools in the hands of plant pathologists and plant breeders. This article provides detail review for different molecular marker methods: restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSRs), sequence characterized regions (SCARs), sequence tag sites (STSs), cleaved amplified polymorphic sequences (CAPS), microsatellites or simple sequence repeats (SSRs), expressed sequence tags (ESTs) and single nucleotide polymorphisms (SNPs)

Key words:AFLP,DNA markers, hybridization, ISSR, polymerase chain reaction, RAPD, RFLP, SNP

Introduction

The differences that distinguish one plant from another are encoded in the plant's genetic material, the deoxyribonucleic acid (DNA). DNA is packaged in chromosome pairs (strands of genetic material; Figure 1), one coming from each parent. The genes, which control a plant's characteristics, are located on specific segments of each chromosome. All of the genes carried by a single gamete (i.e., by a single representative of each of all chromosome pairs) is known as genome (King and Stansfield, 1997). Although the whole genome sequence is now available for a few plant species such as *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000) and rice (The Rice Genome Mapping Project, 2005), to help identify specific genes located on a particular chromosome, most scientists use an indirect method called genetic markers. A genetic marker can be defined in one of the following ways: (a) a chromosomal landmark or allele that allows for the tracing of a specific region of DNA; (b) a specific piece of DNA with a known position on the genome; or (c) a gene whose phenotypic expression is usually easily discerned, used to identify an individual or a cell that carries it, or as a probe to mark a nucleus, chromosomes, or locus (King and Stansfield, 1990). Since the markers and the genes they mark are close together on the same chromosome, they tend to stay together as each generation of plants is produced. As scientists learn where markers occur on a chromosome, and how close they are to specific genes, they can create a genetic linkage map. Such genetic maps serve several purposes, including detailed analysis of associations between economically important traits and genes or quantitative trait

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loci (QTLs) and facilitate the introgression of desirable genes or QTLs through marker-assisted selection. Genetic markers fall into one of the three broad classes: those based on visually assessable traits (morphological and agronomic traits), those based on gene product (biochemical markers), and those relying on a DNA assay (molecular markers). The idea of using genetic markers appeared very early in literatures (Sax, 1932; Wexelsen, 1933) but the development of electrophoretic assays of isozymes (Markert and Moller, 1959) and molecular markers (Botstein et al., 1980; Nakamura et al., 1987; Welsh and McClelland, 1990; Williams et al., 1990; Adams et al., 1991; Caetano-Anolles et al., 1991; Akkaya et al., 1992; Akopyanz et al., 1992; Jordan and Humphries, 1994; Zietkiewicz et al., 1994; Vos et al., 1995; Jaccoud et al., 2001) have greatly improved our understanding in biological sciences. Molecular markers should not be considered as normal genes, as they usually do not have any biological effect, and instead can be thought of as constant landmarks in the genome. They are identifiable DNA sequences, found at specific locations of the genome, and transmitted by the standard laws of inheritance from one generation to the next. The existence of various molecular techniques and differences in their principles and methodologies require careful consideration in choosing one or more of such marker types. This review article deals on the basic principles, requirements, and advantages and disadvantages of the most widely used molecular markers for genetic diversity studies, genetic mapping, marker-trait association studies, and marker assisted selection programs.

Markers play an essential role today in the study of variability and diversity, in the construction of linkage maps, and in the diagnosis of individuals or lines carrying certain linked genes. Within this context, the limitations of morphological markers became quickly apparent. They tend to be restricted to relatively few traits, display a low degree of polymorphism, are often environmentally variable in their manifestation, and can depend on the expression of several unlinked genes. Furthermore, some may affect plant viability or seed set, distorting gene frequencies in the progeny. The emergence of marker systems has, for the last 30 years, closely tracked developments in biochemistry and molecular biology. Morphological markers were largely supplanted by biochemical markers, particularly isoenzymes that could be easily scored by electrophoresis (Ganapathy et al., 1973). The limitations of isoenzmyes as markers, in particular both the limited number of polymorphic enzymes that can be conveniently stained and the environmental effects on expression pattern, were apparent already twenty years ago (Tanksley, 1983). The shortcomings drove the development of markers based on DNA polymorphisms. These marker types generate ‘‘fingerprints,’’ distinctive patterns of DNA fragments resolved by electrophoresis and detected by staining or labeling. A molecular marker is in essence a nucleotide sequence corresponding to a particular physical location in the genome. Its sequence needs to be polymorphic enough between plant accessions to allow its pattern of inheritance to be easily followed.

Genetic marker

A genetic marker is a measurable character that can detect variation in either a protein or DNA sequence. A 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. A genetic trait may not have necessarily observable consequences on an individual’s performance. Sometimes, however, this trait may be linked to, or correlated with, other traits that are more difficult to measure and do affect the individual’s performance. In such cases, these unobservable genetic traits may be used as genetic markers for the linked traits because they indirectly indicate the presence of the characteristics of interest. The two measures can be correlated, using an analysis of inheritance and studying the characteristics in both parents and offspring. Molecular markers specially RAPD are being applied to a greater extent to study genetic diversity and contributes about

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25% of the total molecular markers (Fig. 1) used in biotech activities (FAO, 2004). Molecular markers assess variations in the nucleotide sequence of DNA of different individuals. Molecular markers are numerous and therefore a large genome can be easily assayed for existence of any variation, such genetic markers are easy to score. Use of molecular markers therefore provides an objective assessment of genetic diversity in a plant species and enables unequivocal identification of elite genotypes.

Types of Genetic markers:

1. **Morphological markers**
2. **Protein (biochemical) markers**
3. **DNA (molecular) markers**

1. Morphological markers

Traditionally, diversity within and between populations was determined by assessing differences in morphology. These measures have the advantage of being readily available, do not require sophisticated equipment and are the most direct measure of phenotype, thus they are available for immediate use, an important attribute. 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.

2. Protein (biochemical) markers

To overcome the limitations of morphological traits, other markers have been developed at both the protein level (phenotype) and the DNA level (genotype). Protein markers are usually named biochemical markers but, more and more; they are mistakenly considered as a common class under the so-called 'molecular markers'.

Protein markers (seed storage proteins and isozymes) are generated through electrophoresis, taking advantage of the migrational properties of proteins and enzymes, and revealed by histochemical stains specific to the enzymes being assayed. Detecting polymorphisms i.e. detectable differences at a given marker occurring among individuals in protein markers is a technique that shares some of the advantages of using morphological ones. However, protein markers are also limited by being influenced by the environment and changes in different developmental stages. Even so, isozymes are a robust complement to the simple morphometric analysis of variation.

3. DNA (molecular) markers

DNA polymorphisms can be detected in nuclear and organelle DNA, which is found in mitochondria and chloroplasts. Molecular markers concern the DNA molecule itself and, as such, are considered to be objective measures of variation. They are not subject to environmental influences; tests can be carried out at any time during plant development; and, best of all, they have the potential of existing in unlimited numbers, covering the entire genome.

Characteristics of good marker:

1. **Polymorphic,** that is, it is variable among individuals. The degree of polymorphism detected depends on the technology used to measure it.
2. **Reproducible** in any laboratory experiment, whether within experimental events in the same laboratory or between different laboratories performing identical experiments.
3. **Codominant.** 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

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inheritance). A heterozygous individual shows simultaneously the combined genotype of the two homozygous parents.

4. **Evenly distributed throughout the genome**. The more distributed and dense genome coverage is, the better the assessment of polymorphism.
5. **Discriminating,** that is, able to detect differences between closely related individuals.
6. **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.
7. **Neutral.** The allele present at the marker locus is independent of, and has no effect on, the selection pressure exerted on the individual. This is usually an assumption, because no data are usually available to confirm or deny this property.
8. **Inexpensive, Easy,** fast and cheap in detecting across numerous individuals. If possible, the equipment should be of multipurpose use in the experiment.

Molecular markers:

(Bousquet *et al.,* 1992) distinguished between two classes of molecular marker viz. molecular genetic markers (those derived from direct analysis of polymorphism in DNA sequences), and biochemical markers (those derived from study of the chemical products of gene expression).

i. Molecular markers consist of specific molecules, which show easily detectable differences among different strains of a species or among different species.
ii. Molecular markers analyze genes directly.
iii. Molecular markers reveal neutral sites of variation at the DNA sequence level. These variations do not necessarily show themselves in the phenotypes.
iv. Use of linked molecular markers would allow indirect selection for desirable traits in early segregating generations at the seedling stage and independent of environmental influences.
v. This, in turns will save time and other resources that are needed.
vi. They have the big advantage that they are much more numerous than morphological markers.

Classification of molecular markers based on the basic strategy

1. **Non PCR based approaches**
   a. RFLP (Restriction fragment length polymorphism)
2. **PCR-based approaches**
   a. RAPD (Random Amplified Polymorphic DNA)
   b. SSR (Simple Sequence Repeat)
   c. SCAR (Sequence characterized amplified regions)
   d. CAPS (Cleaved amplified polymorphic sequence)
   e. ISSR (Inter-simple sequence repeats)
   f. AFLP (Amplified fragment length polymorphism)

RFLP (Restriction fragment length polymorphism)

Restriction fragment length polymorphism (RFLP) analysis was one of the first techniques to be widely used for detecting variation at the DNA sequence level. The principle behind the technology rests on the possibility of comparing band profiles generated after restriction enzyme digestion in DNA molecules of different individuals. Diverse mutations that might have occurred affect DNA molecules in different ways, producing fragments of variable lengths. These differences in fragment lengths can be seen after gel electrophoresis, hybridization (Southern, 1975) and visualization.

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Advantages of RFLPs

i. Highly robust methodology with good transferability between laboratories.
ii. Codominantly inherited and, as such, can estimate heterozygosity.
iii. No sequence information required.
iv. Because based on sequence homology, highly recommended for phylogenetic analysis between related species.
v. Well suited for constructing genetic linkage maps.
vi. Locus-specific markers, which allow synteny studies.
vii. Discriminatory power can be at the species and/or population levels (single-locus probes), or individual level (multi-locus probes).
viii. Simplicity given the availability of suitable probes, the technique can readily be applied to any plant.

Disadvantages of RFLPs

i. Large amounts of DNA required.
ii. Automation not possible.
iii. Low levels of polymorphism in some species.
iv. Few loci detected per assay.
v. Need a suitable probe library.
vi. Time consuming, especially with single-copy probes.
vii. Costly.
viii. Distribution of probes to collaborating laboratories required.
ix. Moderately demanding technically.
x. Different probe/enzyme combinations may be needed.

Random Amplified Polymorphic DNA (RAPD) technique

First developed independently by (Welsh and McClelland 1990) and (Williams *et al.,* 1990) and considered the most widely used molecular marker type in molecular studies (White *et al*., 2007). It is a first PCR-based marker. The random amplified polymorphic DNA (RAPD) technique is a PCR-based method that uses a short primer (usually 10 bases) to amplify anonymous stretches of DNA. With this technique, there is no specific target DNA, so each particular primer will adhere to the template DNA randomly. As a result, the nature of the obtained products will be unknown. The DNA fragments generated are then separated and detected by gel electrophoresis. RAPDs can be detected by running PCR products through electrophoresis on an agarose or acrylamide gel.

In both cases, the gel is stained with ethidium bromide. The difference obtained by running RAPD products in acrylamide versus agarose lies only in the degree of resolution of bands. In most cases, agarose gel electrophoresis gives sufficient resolution.

Main features:

Specific regions of the DNA molecule are amplified for analysis of variation by the PCR, using primers (usually 10 bases),

i. Random stretches of the genome will be sampled and amplified and used as the basis for variation analysis
ii. The no. of amplified fragments generated by PCR will depend on the length and sequence of the primer and the genome size

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iii. In most plant s a 10 nucleotide primer will generate on an average 2-10 amplification products (Waugh and Powell, 1992).

Many different fragments (corresponding to multiple loci dispersed throughout the genome) are normally amplified, using each single primer. The technique is therefore rapid in detecting polymorphisms. Although most commercially produced primers result in several fragments, some primers may fail to give amplification fragments from some material. The technique is simple. RAPD analysis does not require expertise to handle hybridization of DNA or other highly technical activities.

Advantages of RAPDs

i. High number of fragments are generated.
ii. Simple.
iii. Arbitrary primers are easily purchased, with no need for initial genetic or genomic information.
iv. Only tiny quantities of target DNA are required.
v. Unit costs per assay are low.

Disadvantages of RAPDs

i. RAPD markers are dominant. Amplification either occurs at a locus or it does not, leading to scores based on band presence or absence. This means that homozygotes and heterozygotes cannot be distinguished. In addition, the absence of a band through lack of a target sequence cannot be distinguished from that occurring through the lack of amplification for other reasons (e.g. poor quality DNA), contributing to ambiguity in the interpretation of results.
ii. Nothing is known about the identity of the amplification products unless the studies are supported by pedigree analysis.
iii. Problems with reproducibility result as RAPD suffers from sensitivity to changes in the quality of DNA, PCR components and PCR conditions, resulting in changes of the amplified fragments. Reproducible results may be obtained if care is taken to standardize the conditions used.
iv. Problems of co-migration raise questions like 'Do equal-sized bands correspond to the same DNA fragment?'
v. The presence of a band of identical molecular weight in different individuals is not evidence per se that the individuals share the same (homologous) DNA fragment.
vi. A band detected on a gel as being single can comprise different amplification products. This is because the type of gel electrophoresis used, while able to separate DNA quantitatively (i.e. according to size), cannot separate equal-sized fragments qualitatively (i.e. according to base sequence).

Sequence characterized amplified regions (SCARs)

i. SCARs take advantage of a band generated through a RAPD experiment.
ii. They use 16-24 bp primers designed from the ends of cloned RAPD markers.
iii. This technique converts a band prone to difficulties in interpretation and/or reproducibility into being a very reliable marker (Paran and Michelmore 1993)

Advantages:

i. Simpler patterns than RAPDs
ii. Robust assay due to the design of specific long primers
iii. Mendelian inheritance.

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iv. Sometimes convertible to codominant markers

Disadvantages:

i. Require at least a small degree of sequence knowledge
ii. Require effort and expense in designing specific primers for each locus

Because the primers used are longer than is usual for RAPDs, SCARs are typically more reproducible than the RAPDs from which they were derived. SCARs are usually codominant, although not if one or both primers overlap the site of sequence variation.

Microsatellites (SSRs, STMS or SSRPs)

Microsatellites are also called simple sequence repeats (SSRs) and, occasionally, sequence-tagged microsatellite sites (STMS) or simple sequence repeat polymorphisms (SSRPs). The term "Microsatellites" was coined by Litt and Lutty (1989). SSRs are short tandem repeats, their length being 1 to 10 bp, most typically, 2-3 bp. SSRs are highly variable and evenly distributed throughout the genome. This type of repeated DNA is common in eukaryotes, their number of repeated units varying widely among organisms to as high as 50 copies of the repeated unit. These polymorphisms are identified by constructing PCR primers for the DNA flanking the microsatellite region. The flanking regions tend to be conserved within the species, although sometimes they may also be conserved in higher taxonomic levels.

PCR product size variation is caused by differences in the number of microsatellite repeat units. SSR polymorphisms can be visualised by agarose or polyacrylamide gel electrophoresis. Microsatellite alleles can be detected, using various methods: ethidium bromide, silver staining, radioisotopes or fluorescence.

If fluorescence-labelled primers are used, and the products are different enough in size and not overlapping, then multiplexing that is, loading more than one sample per lane of reaction products can greatly increase the already high efficiency of these markers.

Advantages:

i. Require very little and not necessarily high quality DNA.
ii. Highly polymorphic.
iii. The loci identified are usually multi-allelic and codominant.
iv. Bands can be scored either in a codominant manner, or as present or absent.
v. Evenly distributed throughout the genome.
vi. Interpretation of result is simple.
vii. Easily automated, allowing multiplexing.
viii. Good analytical resolution and high reproducibility.

Disadvantages:

i. Complex discovery procedure.
ii. Costly.

Cleaved amplified polymorphic sequence (CAPS)

Cleaved amplified polymorphic sequences (CAPS) are like SCARs, but with an additional step of a restriction digest to help identify polymorphisms that may not be identifiable from whole PCR products. Both SCARs and CAPS are based on the presence of nucleotide changes or insertions and/or deletions causing differences between the test sequences. One drawback of both is that they detect

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polymorphism only over a small range of the genome, the area between the primers being typically less than 5 kb.

i. This method is based on the design of specific primers, amplification of DNA fragments, and generation of smaller, possibly variable, fragments by means of a restriction enzyme.
ii. CAPS are based on the presence of nucleotide changes or insertions and/or deletions causing differences between the test sequences.
iii. This technique aims to convert an amplified band that does not show variation into a polymorphic one.

Advantages:

i. Robust assay because specific long primers are designed.
ii. Codominant markers.
iii. Benefit from markers that may have already been mapped.
iv. Identify polymorphisms in markers that were previously not informative.

Disadvantages:

i. Require at least a small amount of sequence knowledge.
ii. Effort and expense required to design specific primers for each locus.

Inter-simple sequence repeats (ISSRs)

ISSRs are reported by Zietkiewicz *et al* .,1994. They are regions found between microsatellite repeats. The technique is based on PCR amplification of inter-microsatellite sequences. Because of the known abundance of repeat sequences spread all over the genome, it targets multiple loci.

Advantages:

i. Do not require prior sequence information.
ii. Variation within unique regions of the genome may be found at several loci simultaneously.
iii. Tend to identify significant levels of variation.
iv. Microsatellite sequence-specific.
v. Very useful for DNA profiling, especially for closely related species.

Disadvantages:

i. Dominant markers.
ii. Polyacrylamide gel electrophoresis and detection with silver staining or radioisotopes may be needed.

Expressed sequence tag markers (EST):

This term was introduced by Adams *et al.,* 1991. Such markers are obtained by partial sequencing of random cDNA clones. Once generated, they are useful in cloning specific genes of interest and synteny mapping of functional genes in various related organisms. ESTs are popularly used in full genome sequencing and mapping programmes. An EST that appears to be unique helps to isolate new genes. EST markers are identified to a large extent for rice, Arabidopsis, etc. wherein thousands of functional cDNA clones are being converted in to EST markers.

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Amplified fragment length polymorphism (AFLP)

This is a highly sensitive method for detecting polymorphism throughout the genome and it is becoming increasingly popular. It is essentially a combination of RFLP and RAPD methods, and it is applicable universally and is highly reproducible. It is based on PCR amplification of restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of few nucleotide bases (Vos et al., 1995) It is a novel DNA fingerprinting technique. DNA fingerprinting involves the display of a set of DNA sample. Fingerprints are produced without prior sequence knowledge, using a limited set of genetic primers. AFLP technique uses stringent reaction conditions for primer annealing and combines the reliability of RFLP technique with the power of PCR technique (Fig:2).

Interpreting AFLP bands:

i. The AFLP technique detects polymorphisms arising from changes (presence or size) in the restriction sites or adjacent to those.
ii. Different restriction enzymes can be used, and different combinations of pre- and selective nucleotides will increase the probability of finding useful polymorphisms.
iii. The more selective bases, the less polymorphism will be detected.
iv. Bands are usually scored as either present or absent.
v. Heterozygous versus homozygous bands may be detected, based on the thickness of the signal, although this can be tricky.

Advantages:

i. AFLPs allow a quick scan of the whole genome for polymorphisms.
ii. Because of the large number of bands generated, each marker gives a highly informative fingerprint.
iii. They are also highly reproducible.
iv. No prior sequence information or probe generation is needed.
v. Extremely useful in creating quick genetic maps.
vi. Transcript profiling.

Disadvantages:

i. AFLPs generate huge quantities of information, which may need automated analysis and therefore computer technology.
ii. AFLP markers display dominance.
iii. In genetic mapping, AFLPs often cluster at the centromeres and telomeres.
iv. They are technically demanding in the laboratory and, especially, in data analysis.

Advantages of Molecular Markers:

i. Increases the Effectiveness of selection as it avoids the confusing effects of the environment.
ii. Permit pyramiding of disease resistant genes, which is difficult through conventional breeding.
iii. They permit screening of the traits linked to them even during seedling stage.
iv. Indirect selection for QTL's requires much less time.
v. Used for negative selection, i.e., for elimination of undesirable traits. They allow mapping of QTL's.

Disadvantages of Molecular markers:

i. High cost is a serious limitation.
ii. Require some to considerable sophistication and expertise.
iii. Marker like SSR's requires considerable and concerted effort and investment of resources.

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COMPARISON OF COMMONLY USED GENETIC MARKERS

Characteristic	Isozymes	RFLP	SSR	RAPD	AFLP
Number of loci	30-50	Unlimited	10000	Unlimited	Unlimited
Degree of polymorphism	Low- moderate	Moderate- high	Very high	Moderate- high	Moderate –high
Dominance	Co-dominant	co-dominant	Co-dominant	Dominant	Dominant
Null allele	Rare	Extremely rare	Occasionally	Presence or absence	Presence or absence
Transferability of loci	Across family	Among related genera	Within sub-genera	Within sps	Within sps
Reliability	Very high	Very high	High	Low-medium	Medium – high
Amount sample required	Mg of tissue	2-10 mg of DNA/ lane	25-50 ng DNA	5-10 ng of DNA	25 ng DNA
Ease of assay	Easy	Difficult	Easy to moderately difficult	Easy	Moderately difficult
Can be automated	Difficult	Difficult	Yes	Yes	Yes
Multiplexing	Gel slices	1-3	1-9	5-20	20-100
Equipment	cheap	Expensive	Expensive to very expensive	Moderate	Expensive

(Glubitz *et al.* 2000)

BEST DNA MARKERS FOR VARIOUS APPLICATIONS:
Applications that require large number of loci:
I. Measuring genetic diversity and differentiation.
II. Estimating rates of gene flow or migration (between population).
III. Genetic linkage mapping or quantitative trait loci localization.
Markers of Choice- AFLP's OR RFLP's
Applications that require high discrimination power:
I. Characterizing mating systems.
II. Analyzing paternity or parentage.
III. Characterizing patterns of gene flow or migrating within populations.
IV. Assessing seed orchard efficiency.
V. Quality control in breeding, DNA fingerprinting or cross verification.
Markers of Choice- Microsatellites
Applications that require DNA sequence information:
I. Phylogeny and taxonomy
Markers of Choice- PCR and sequencing

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Marker assisted selection (MAS):

It is the selection of trees with desirable traits based on their molecular genotype. It can be used alone or in combination with the classical methods of selection.

Benefits:

i. Early selection that can potentially decrease the breeding cycle time.
ii. Decrease cost by reducing expensive progeny test establishment, maintenance and measurement.
iii. Increasing selection intensity because more individuals can possibly be evaluated.
iv. Increasing the relative efficiency of selection on low heritability traits (Lande and Thompson, 1990)

MOLECULAR MARKERS FOR TESTING GENETIC FIDELITY:

More recently, molecular markers have also been used for testing the genetic fidelity during micropropagation/*ex situ* conservation on the one hand, and for characterization of plant genetic resources on the other. This aspect of the use of molecular markers has received attention in recent years due to the significance that is being attached to micropropagation of elite genotypes and to the *in situ* and *ex situ* conservation of plant genetic resources (PGRs). Molecular markers have particularly been suggested to be useful for confirmation of genetic fidelity in micropropagated tree species, where life span is quite long and performance of micropropagated plants could only be ascertained after their long juvenile stage in field conditions. a study on *Picea* the genetic integrity during somatic embryogenesis has been studied using RAPDs. In India also, an extensive study on genetic fidelity and molecular diagnostics in micropropagation systems was carried out where several molecular markers including RFLPs (using rDNA probes and mtDNA probes), RAPDs, MP-PCR and oligonucleotide in-gel hybridization were used in micropropagated clones of 4 tree species namely *Populus deltoides, Eucalyptus tereticornis, E. camaldulensis* and *Coffea Arabica*(Rani *et al* 1998).
RFLPs (using nDNA and cpDNA probes) and RAPDs were also used for characterization and identification of genetic resources of perennial crops like *Musa* and to solve problems related to plant genetic diversity conservation (Bhat 1997).

MOLECULAR MARKERS FOR ESTABLISHING DISTINCTNESS IN VEGETATIVELY PROPAGATED CROPS

Distinctness, uniformity and stability (DUS) testing of varieties is usually required to apply for Plant Breeders' Rights. This exam is currently carried out using morphological traits, where the establishment of distinctness through a minimum distance is the key issue. The possibility of using microsatellite markers for establishing the minimum distance in a vegetatively propagated crop (grapevine) has been evaluated. A collection of 991 accessions have been studied with nine microsatellite markers and pair-wise compared, and the highest intra-variety distance and the lowest inter-variety distance determined. The collection included 489 di Verent genotypes, and synonyms and sports. Average values for number of alleles per locus (19), Polymorphic Information Content (0.764) and heterozygosities observed (0.773) and expected (0.785) indicated the high level of polymorphism existing in grapevine. The maximum intra-variety variability found was one allele between two accessions of the same variety, of a total of 3,171 pair-wise comparisons. The minimum inter-variety variability found was two alleles between two pairs of varieties, of a total of 119,316 pair-wise comparisons. In base to these results, the minimum distance required to set distinctness in grapevine with the nine microsatellite markers used could be established in two alleles (Ibanez *et al*., 2009).

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APPLICATION OF DNA FINGERPRINTING IN IPR PROTECTION:

Since the last nearly two decades, DNA fingerprinting techniques have found wide pseudocereals (*Echinochloa* spp., *Hordeum* spp., *Oryza* spp., *Secale cereale, Triticum* spp., *Zea mays*), oilseeds (*Arachis* spp., *Brassica* spp., *Glycine* spp.), pulses (*Cicer* spp., *Lens culinaris, Pisum sativum*, *Phaseolus* spp., *Vigna* spp.), sugar yielding plants (*Beta* spp., *Saccharum* spp.), vegetables (*Capsicum* spp., *Cucumis sativus., Lycopersicon esculentum.*, *Solanum* spp., *Raphanus sativus*) and fruits and nuts (*Anacardium occidentale, Citrus* spp., *Mangifera indica, Malus* spp., *Musa* spp., *Prunus* spp., *Pyrus* spp., *Rubus* spp. *Vitis* spp.). The National Research Centre on DNA Fingerprinting has been entrusted with the responsibility of fingerprinting released varieties and important germplasm in crops of national importance.

List of the crops fingerprinted at the NRC using different molecular techniques.

Species	Varieties	Technique used
Mango	30	ISSR, RAPD, AFLP
Citrus	34	ISSR, AFLP
Cashew	140	ISSR, RAPD, AFLP
Neem	69	ISSR, RAPD, AFLP
Vetiver	24	RAPD, AFLP
Saffron	13	AFLP
Pleurotus	8	RAPD

Within the context of IPR protection, DNA fingerprinting can have application in patenting of genes/gene fragments, aiding variety registration and in detecting infringement of breeder's rights and biopiracy. The advantages of fingerprinting based on molecular markers over morphological character is as include; i) high degree of non-tissue specific polymorphism, (ii) minimal influence of environment and, (iii) simple inheritance pattern. UPOV has constituted a Working Group on Biochemical and Molecular Techniques and DNA Profiling in Particular (BMT) to study the utility of molecular markers in the variety registration system. The BMT in its Seventh Session (2001, BMT/7/2) recommended that the current greatest need for development of molecular techniques is in pre-screening in the process of examining distinctness, rather than the final decision of distinctness. Pre-screening is a part of the process of examining distinctness, (i.e. establishing distinctness between a candidate variety and others prior to a growing trial).

Misidentification of clones in seed orchard establishment or crossing programmes is probably very common. Incorrectly identified ramets in seed orchards have been estimated to comprise 2–13% in Douglas fir, 7–10% in Scots pine, and 10% in loblolly pine (Wheeler and Jech 1992), no doubt resulting in some loss of genetic gain. The economic implications are likely to be even greater if these errors occur in clonal forestry programmes. Molecular markers will therefore have an important role in quality control in advanced breeding programmes. Although isozymes will be satisfactory for some verification problems, DNA markers are much more powerful for genotype identification, and will be necessary where identification is to be more or less exclusive. Such identification would be required for Plant Variety Rights (PVR) registration. It seems unlikely though that PVR would be of widespread importance in forestry - registration of all of the clones of interest to a program would be expensive, and Genotype × Environment interaction (GXE) is likely to limit the extent to which clones could confidently be transferred from one program to another without prior testing.

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MOLECULAR MARKERS FOR DETECTION OF PLANT PATHOGENS

Management of plant diseases has been a high priority area of research worldwide. In India, during the post Green Revolution era, productivity of high yielding cultivars of various crops has been seriously affected by pest and diseases, which can only be sustained by the use of better plant protection strategies Molecular detection and identification of pathogens using nucleic acids based methods have been in use for the past few years. These methods overcome various problems associated with microscopical and immunological detection of plant pathogens. Most importantly, DNA based methods can be used at any developmental stage of the pathogen, since every pathogen propagule contains the entire set of nucleic acids of the organism. Precise detection and identification of plant pathogens can be performed by the use of specific DNA probes in infected tissues and identification at genus/species or even at race level. There are various methods used for making DNA probes (Sharma *et al*.,2002 a). These probes have been used by various workers for pathogen detection(Sharma *et al*.,1999). Various non-radioactive DNA probes used in the detection of plant pathogens by dot blot hybridization can also be developed by PCR(Sharma *et al*.,2002a). Such probes have already been developed and used for the detection of many pathogens including *Pythium ultimum* (Levesque *et al*., 1994) and *Alternaria brassicae* (Sharma and Tewari.,1996a,1998).

All the disease management strategies based on host resistance require the knowledge of variability in pathogens. Traditional markers used to study the variability in pathogens are based on the use of differential hosts, culture characteristics, morphological markers and biochemical tests. These markers distinguish pathogens on the basis of their physiological characters i.e., pathogenecity and growth behaviors and can group them according to their similarity for these particular characters. However, these markers are highly influenced by the host age, inoculums quality and environmental conditions. The techniques are time consuming and laborious. Moreover, differential hosts are not available in most of the host- pathogen systems, thus variability cannot be assessed. In such cases, molecular markers are used for the characterization of genetic variability in plant pathogens (Sharma *et al*., 1999).

In India, twenty isolates of *A. brassicae* collected from geographically distinct regions of the world and different host species with RAPD markers have also been analyzed (Sharma and Tewari, 1995, 1998). Out of the five primers tested, primers OPA 07 and OPA 09 could not distinguish variation among these isolates. However, three primers e.g. OPA 03, OPA 04 and OPA 18 were efficient in the detection of inter and intra regional variation among the isolates of *A. brassicae*.

In *Magnaporthe grisea,* 14 MGR-DNA fingerprint lineages have been reported from 55 isolates from South India (Sivaraj *et al*., 1994). Seventy six isolates characterized into 7 lineages (Sridhar *et al*., 1996). These studies could not establish definite relationship between clonal lineages and pathotype diversity in *M. grisea.* PCR based DNA fingerprinting using RAPD markers has revealed high genetic variability among 250 isolates of *M.grisea* populations of Himachal Pradesh (Sharma *et al*.,2002). The groups reported in this study were not region specific probably because *M.grisea* isolates were collected from different locations of the geographically distinct regions, where wide genetic diversity exists among the rice cultivars grown in those areas.

MOLECULAR MARKERS IN MUSHROOM RESEARCH

The application of molecular biology techniques to the genetic improvement programmes has introduced innovative DNA based markers and provided much- needed fillip to the on-going developments in genetics and breeding of cultivated mushrooms. These DNA based markers have been used in the following areas concerned with molecular breeding of mushrooms:

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1. Identification of homokaryons and confirmation of hybridizations
2. Assessment of genetic diversity and selection of parents
3. Genomic inter- relationship
4. Construction of linkage map
5. Breeding behavior and sexuality

USE OF ISSSR MARKERS FOR STRAIN IDENTIFICATION IN THE BUTTON MUSHROOM, *Agaricus bisporus*

The white button mushroom *Agaricus bisporus* is the most widely cultivated species of edible mushrooms all over the world. Originating from a limited heritage line, commercial strains of *A.bisporus* are supposed to be genetically very similar. Many highly polymorphic molecular markers have been exploiting for strain identification in wild and commercial strains of this mushroom. ISSR marker with the whole- genome coverage, accuracy and reproducibility as well as robustness has proved to be a promising marker for genetic diversity analysis of many crops. The objective of this work was to evaluate the potential of ISSR markers for genotype identification in common button mushroom, *A. bisporus*. For this purpose, 18 *A. bisporus* genotypes, including four cultivars, 13 hybrid strains and their single spore progenies along with an indigenous wild strain, were assessed for their similarity using 20 ISSR primers. Out of 20 primers, 10 proved to be discriminative in *A. bisporus,* producing 110 scorable and 76 polymorphic bands. ISSR primers successfully identified every single individual; however high similarity was detected among genotypes. The wild genotype Dezful exhibited little relatedness with other genotypes and placed in a separate individual group. Genotypes IM0037 and Dezful with similarity coefficient of 0.937, were accordingly the least and the most similarity genotypes. The result demonstated that ISSR markers are powerful enough for detection of polymorphism among closely related genotypes of *A. bisporus* (Malekhzadeh et al., 2011).

OYSTER MUSHROOMS SPECIES DIFFERENTIATION THROUGH MOLECULAR MARKERS RAPD

A RAPD methodology for differentiation and characterization of the fungal edible species *Pleurotus ostreatus* and *Pleurotus sajor- caju* was developed. Two of the most usual methods of DNA extraction were studied. The first was based in the simultaneous precipitation of proteins and polysaccharides by presence of the SDS (sodium dodecyl sulphate) and sodium acetate and the second by use of cetyltrimethylammonium bromide (CTAB). Both methods presented satisfactory results and the best extraction products were obtained by the one that used SDS and sodium acetate. The study with RAPDs produced consistent DNA fragments, reproducible in different gels, serving as good markers for characterization and genetic differentiation of the studied species of mushrooms. The PCR conditions for obtaining RAPD markers were optimized with good interpretation (Fonseca et al., 2008).

In this study, utilizing the RAPD methodology, 19 different primers were tested for the two species of mushroom in study, being obtained a large number of DNA fragments (bands) consistent and reproduced in several gels. Among the tested primers, 6(AC-04, AD-08, AD-09, B-14, D-20,G-02) produced amplifications for both species allowing the differentiation of same ones, 5(S-03, D-06, G-07, G-08, B-15) produced amplifications only for *Pleurotus osreatus*, the primer S-17 produced amplifications only for Pleurotus sajor-caju and 7 primers (AC-09, B-02, B-05, B-07, D-05, G-06, G-09) did not amplify any fragment.

The number of amplification products for each primer varied from 1 to 12, totaling 87, being that 77 were polymorphic and 10 were monomorphic (present in both species)

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MOLECULAR MARKERS IN VARIETAL PROTECTION

Basmati is nearly twice as expensive as normal rice in the European market. Adulteration of basmati with large grain rice has frequently been reported. Until recently, determining a rice variety was limited to skilled visual inspection, combined with fragrance assessment, or sample grain measurements. DNA technology has now been applied to detect the presence of non-basmati grains in samples of Basmati rice and quantify non-Basmati grains in a rice sample. A recent study by the British Food standards Agency (FSA) using DNA technique revealed that of 363 samples collected from a range of outlets in UK, 17% contained over 20% conventional rice, and of these, 9% contained more that 60% non-Basmati. European Union has recently imposed strict controls on Basmati quality including use of DNA-analysis. Five rice samples labeled as Basmati of US origin were examined using SSR markers. The DNA fingerprinting data were compared with a database of alleles derived from a collection of USA and India/Pakistani rice varieties. The genetic markers of four of the five samples were related to other US rice varieties, but the fifth sample was more closely related to the Indian Pusa Basmati variety. The study highlighted the potential of DNA technology in establishing the origin of a variety.

The case relates to the unauthorized commercial sale of seeds of three spurious chilly varieties marketed under the brand name of an elite variety. Fingerprinting results proved that the four chilly samples were different from each other although they were being marketed under the name of one elite variety (Agro BioForum 2005).

Indian scenario for development of molecular markers in crop improvement programmes

i. Agriculture is one of the most important occupations in India with almost 70% of the population being dependent on it. A noteworthy research in conventional breeding for several years has made this country self-sufficient in many respects. However, the ever-increasing population has alarmed food security in India and attempts have been initiated to integrate modern biotechnology tools in conventional breeding to improve the most important crops such as rice, wheat and legumes.
ii. Extensive research using DNA markers is in progress in many institutions all over India. Markers tagged and mapped with specific genes have been identified and some such examples are:-
    resistance genes for blast - rice
    leaf rust resistance gene - wheat
    pre-harvest sprout tolerance gene - wheat
    Heterosis- rice
iii. Germplasm analysis to study genetic diversity is another important area in which a lot of efforts have been put in. Fingerprinting of crops like rice, wheat, chickpea, pearl millet etc. is being carried out extensively. This information has potential in strategic planning of future breeding towards crop sustainability in India.

FUTURE PERSPECTIVES:

i. DNA fingerprinting, through its high precision in identifying plant genotypes, holds considerable promise as a reliable tool of intellectual property protection of crop varieties and germplasm. In order to fully harness the potential of this technology, it is necessary to establish a network of DNA fingerprinting laboratories using uniform set of standardised
ii. Emphasis should be on the use of microsatellite markers for fingerprinting and their development in crops for which these are not available.

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iii. There is also a need for creating scientific and legal awareness regarding the authenticity and reliability of DNA fingerprinting in plants.
iv. UPOV is testing the scope and application of these markers for rapid identification and protection of crop varieties or for verifying varietal identity. It has included electrophoresis of seed proteins in barley and wheat and of isozymes in maize, soybean and sunflower as additional characters for establishing distinctness of varieties.

Protocols for each crop.

v. In India also similar sort of coordinated efforts are required to test the suitability and application of these marker techniques for molecular profiling of varieties and parental lines of hybrids.
vi. Molecular marker technologies can be used to attack trade secrets by rapid identification of female parent inbred line contaminants in bags of hybrid seed. These inbred lines might then be used directly as parents of hybrids or as parents for further breeding.
vii. Molecular marker technology can be used to identify segregating molecular characteristics in an otherwise uniform variety and thus to select a distinct "new" variety from the segregating source without any breeding effort being expended.
viii. Plant breeders may seek to strengthen their claim for protection of new varieties by including molecular profiles as supplementary information to establish the distinctness of their varieties.
ix. Microsatellite markers have been used successfully to determine the degree of relatedness among individuals or groups of accessions, to clarify the genetic structure, or partitioning of variation among individuals, accessions, population and species of rice and similar trends can be applied in the field of forestry for the registration of hydrids.

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