IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 9, Issue 2 (Mar. – Apr. 2023), PP 58-70
www.iosrjournals.org

A Review On Genetic Markers In Plant Identification

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Abstract
Genetic marker is useful in identification of various genetic variations. The development of DNA- based genetic markers had a revolutionary impact on genetic study. The development of molecular genetics has put the preparations for genomics. It has introduced new generationsof molecular markers for use in the genetic improvement of plants. These markers provide more accurate genetic information and better understanding of plants. The review represent the different types of molecular marker by introducing a brief summary on the development of genetic markers including both the classical genetic markers and more advanced DNA- based molecular markers.

Date of Submission: 16-04-2023 Date of Acceptance: 29-04-2023

I. Introduction-

The demand for herbal medicines is increasing day-to-day because their wide biological activity, higher safety margin than synthetic drugs, hence it is important to characterize the medicinal herbs.medicinal plants have been usedto maintain health and to treat diseases.

molecular markers can be act as characterization, genetic marker have acted as very useful tools in various fields like taxonomy, physiology, embryology, plant breeding, ecology, genetic engineering etc. detail about the genetic difference present insideand joining various plant populations and their structure level can play a important role in the efficient utilization of plants. Genetic markers are important developments in the field of plant breeding.development and utilization of molecular markers for the identification of plant genetic diversity.

A genetic marker is "a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or a character". It can be described as a variation, which may arise due to mutation or alteration of nucleotide in genomic loci that can be observed.

Genetic markers are broadly grouped into two categories.Classical markers and DNA/molecular markers.

Morphological, Cytological, Biochemical markers are classical markers. DNA/molecular markers are Random amplification of polymorphic DNA(RAPD), Restriction fragment length polymorphism(RFLP), Amplified fragment length polymorphism(AFLP), Simple sequence repeats(SSRs), Single-nucleotide polymorphism(SNP), Sequence characterization of amplified region (SCAR), Inter- simple sequence repeats(ISSR), Loop mediated isothermal amplification(LAMP), Next generation sequencing(NGS), Variable number tandem repeats(VNTR), Inter-Retrotransposon amplified polymorphism (IRAP), Retrotransposon microsatellite amplification polymorphisms (REMAP), Cleaved amplified polymorphic sequences (CAPS), Expressed Sequence Tags (EST).

II. Classical markers -

Morphological marker-

Morphological markers are easy to use with no requirement for specific instruments, they do not require any specialized biochemical and molecular technique. breeders have used this typeof markers successfully in the breeding programmes forvarious crops. main disadvantages of morphological markers are: they are limited in number, influenced by the plant growth stages and various environmental factors.

Cytological markers-

These are related with difference present in the numbers, banding patterns, size, shape, order and position of chromosomes are known as cytological markers. These difference disclosed differences in the distributions of euchromatin and heterochromatin.

Biochemical markers-

Biochemical markers, or isoenzymes, are multi-molecular forms of enzymes which are coded by various genes, but have the same functions. Biochemical markers have been strongly applied in the detection of genetic diversity, population structure, gene flow and they are co-dominant, easy to use and costly.

Molecular markers-

Molecular markers are nucleotide sequences and its investigated through the polymorphism present between the nucleotide sequences of different individuals. molecular markers used in many different areas such as genetic mapping, paternal tests, detect mutant genes which are connected to hereditary diseases, cultivars identification, marker assisted breeding of crops, population history, epidemiology and food safety, population studies.

III. Restriction Fragment Length Polymorphism (RFLP)–

Restriction Fragment LengthPolymorphism was the first molecular marker technique in this system based on hybridization. Isolation of pure DNA is the first step in the RFLP method. This DNA is mixed with restriction enzymes this are isolated from bacteria and these enzymes are used to cut DNA at particular site. This results in vast number of fragments with different length. Gel electrophoresis and southern blotting is applied for the separation of these fragments by producing a series of bands.Specificpatterns of banding are then visualized by hybridization with labeled probe. Each band represents a fragment having different lengths. Base-pair deletions, mutations, inversions, translocations and transpositions are the main causes for the variation resulting in the RFLP pattern. These variations lead to the gain or loss of recognition sites, resulting in fragments of various lengthand polymorphism. The restriction enzymes will not cut the fragment if a single base-pair variation occurs in the recognition site. Denaturation, annealing and extension are the most important steps involved in PCR reactions. The detection of variation in length of segments of DNA by use of restriction enzymes has to identification of new class of genetic markers RFLPs.

Steps of RFLP

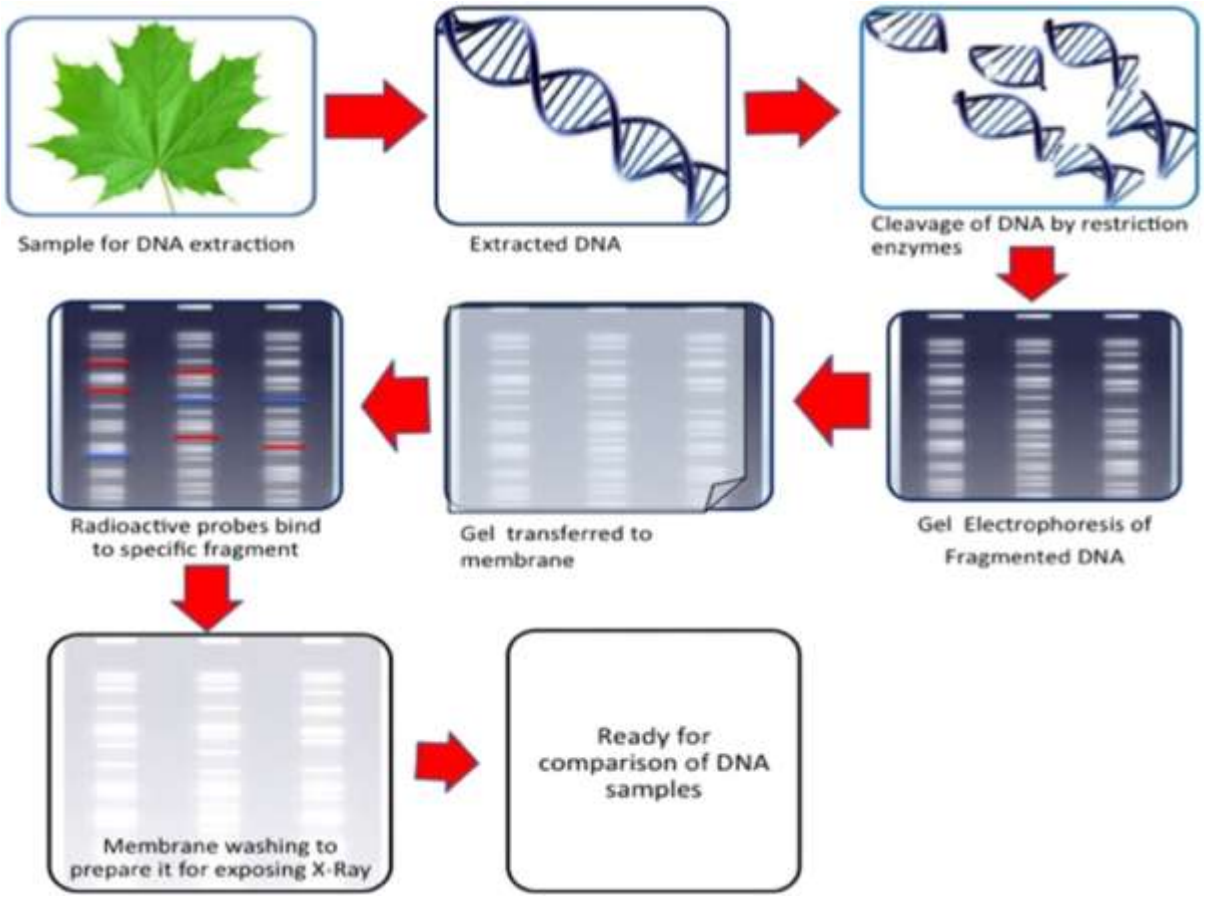


Fig. 01

The primer efficiency depends on the following main factors:
(1) Primer–template duplex association and dissociation during the annealing step and the extension temperature;
(2) Stability of the duplex to mismatched nucleotides;
(3) Efficiency of polymerase in identification and extension of mismatched duplex. Primer length, GC%, melting and annealing temperature, 3' end specificity and 5' end stability are important features playing an important role in the efficiency of a primer.

Primer length is also critical for a good PCR and normally primers of 18–30 nucleotides in length are considered the best primers. Melting temperatures (Tm) in the range of 52–58 ^{0} C gives good results. The GC content is the most important factor affecting the efficiency of a primer; 45%–60% is optimum GC% for a good primer.

Advantages of RFLP markers-

I. RFLP markers used for constructing genetic maps.
II. RFLPs are co dominant and reliable markers in linkage analysis, breeding.
III. Can be easily determined in homozygous or heterozygous state of an individual.

Disadvantages of RFLP markers-

I. The large amount of DNA required for restriction digestion and southern blotting.
II. Expensive, time-consuming and hazardous.

Limitation of RFLPs-

- The limited sensitivity of detection associated with RFLPs is the serious problem; because it is very difficult to get valid profiles from trace biological evidence or from samples that are too aged or have significantly compromised environmental insult.
- RFLPs involves radioactivity, and is laborious and difficult to automate. However, this can be overcome by PCR-based DNA typing systems.

IV. Random amplification of polymorphic DNA(RAPD)

A single and short oligonucleotide primer is binds to many different location, it is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size ofboth the primer and the target genome. The assumption is made that a given DNA sequence(complementary to that of the primer)will occur in the genome, on opposite DNA strands, in opposite orientation within a distance

that is readily amplifiable by PCR. these amplified products (of up to 3.0 kb) are usually separated on agarose gels (1.5-2.0%) and visualized by ethidium bromide staining. The use of a single decamer oligonucleotide forward the generation of several distinct DNA products and these are considered to originate from different genetic loci. Polymorphisms from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band.this means that RAPDs are dominant markers and, therefore, cannot be used to identify heterozygote's.DNA products if these priming sites are within an amplifiable distance of each other (Fig. 1). The profile of amplified

DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs gives result in the presence or absence of bands because of changes in the priming sites.

Limitations of RAPDs

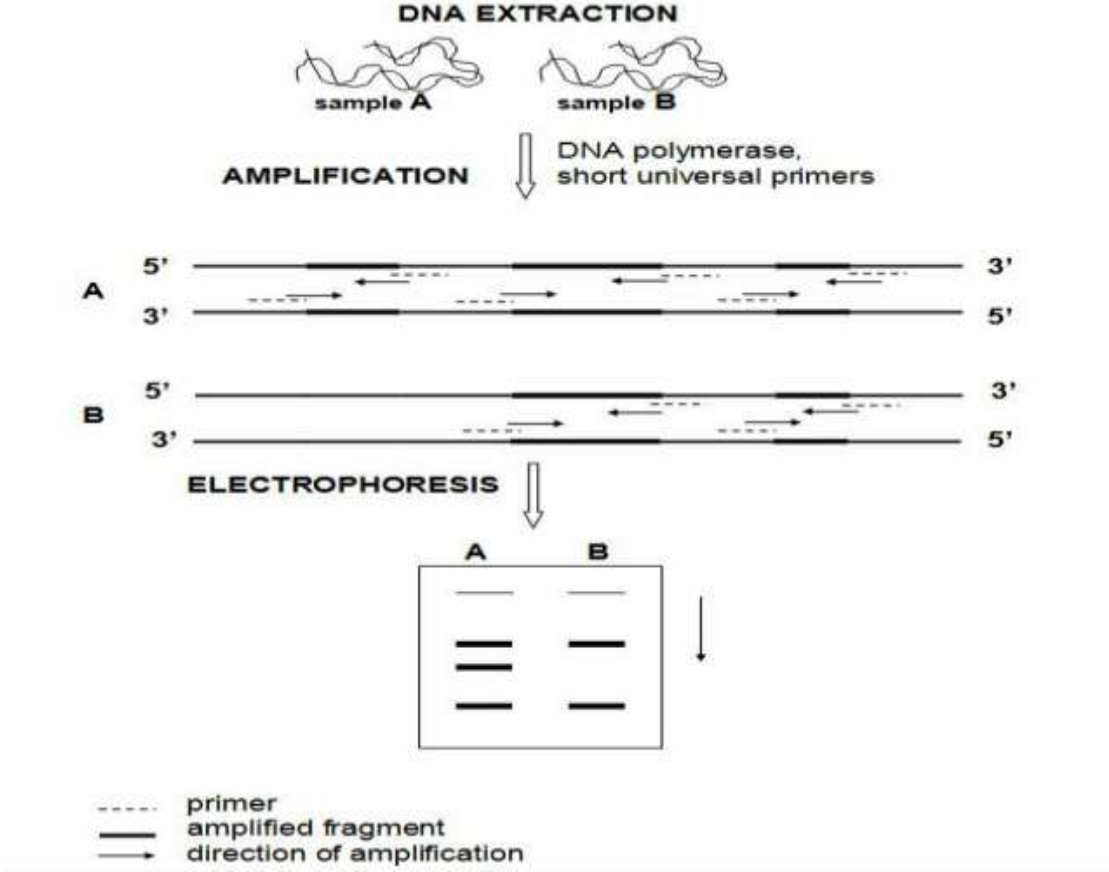
- RAPD is a less reproducible marker. Because the annealing temperature
- for such marker is low (28–38°C) therefore, there are chancesof wrong annealing.
- It is a dominant marker.

Advantages of RAPD

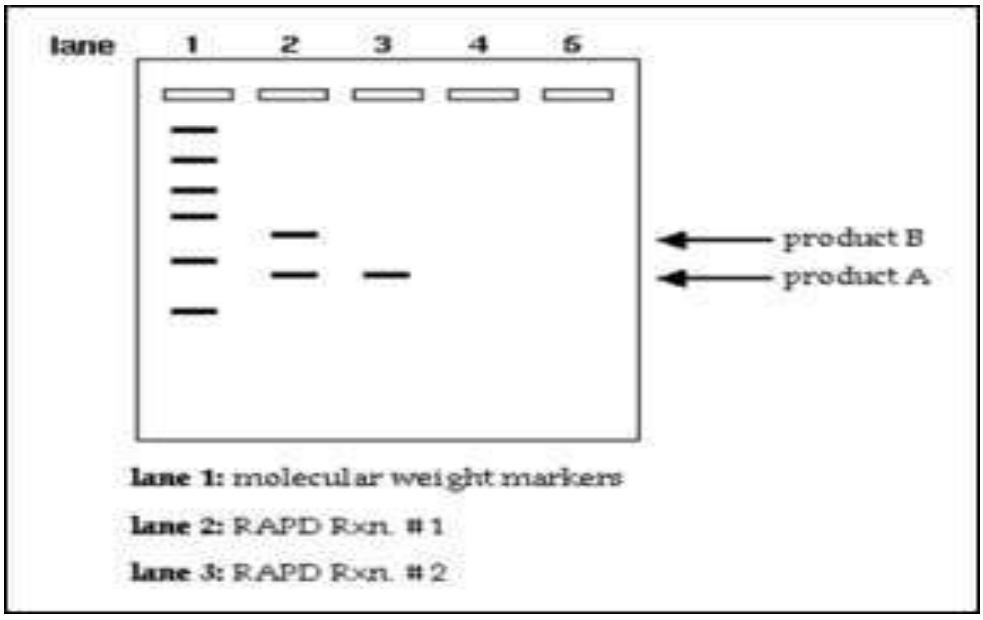
RAPD has been used widely because of the following advantages:

- It requires no DNA probes and sequence information for the design of specific primers.
- It involves no blotting or hybridization steps, hence, it is quick, simple and efficient.
- It requires only small amounts of DNA (about 10 ng per reaction) and the procedure can be automated.
- High number of fragments.
- Arbitrary primers are easily purchased.

- Unit costs per assay are low compared to other marker technologies.



Steps of RAPD



RAPD reactions on an agarose gel

Disadvantages of RAPD

- Nearly all RAPD markers are dominant,
- Lack of a prior knowledge on the identity of the amplification products.
- Problems with reproducibility (sensitive to changes in the quality of DNA, PCR components and PCR conditions).

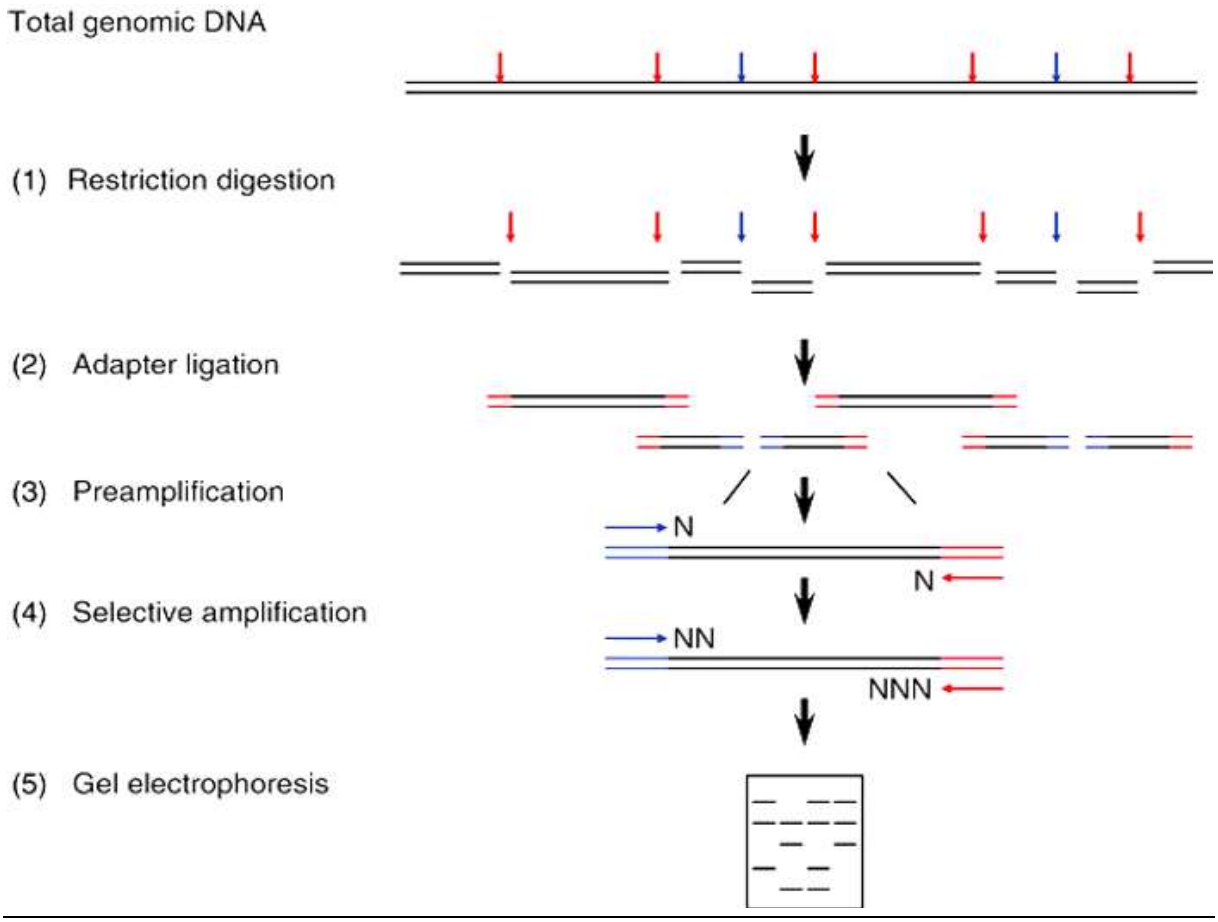
Application of RAPD

It has become widely used in the study of

- genetic diversity/polymorphism,
- germplasm characterization,
- genetic structure of populations, domestication,
- detection of somaclonal variation,
- cultivar identification,
- hybrid purity,
- genome mapping,
- developing genetic markers linked to a trait in question,
- population and evolutionary genetics,
- plant and animal breeding,
- animal-plant-microbe interactions,
- pesticide/herbicide resistance,
- animal behavior study, and forensic use.

V. Amplified fragment length polymorphism(AFLP)

AFLP analysis involves restriction digestion of genomic DNA with a combination of rare cutter (EcoRI or PstI) and frequent cutter (MseI or TaqI) restriction enzymes. Double-stranded oligonucleotide adaptors are then designed in such a way that the initial restriction site is not restored after ligation. Such adaptors are ligated to both ends of the fragments to provide known sequences for PCR amplification. PCR amplification will only occur where the primers are able to anneal to fragments which have the adaptor sequence plus the complementary base pairs to the additional nucleotides called selective nucleotides. The first PCR (preamplification) is performed with primer combinations containing a single bp extension, while final (selective) amplification is performed using primer pairs with up to 3-bp extension. Because of the high selectivity, primers differing by only a single base in the AFLP extension amplify a different subset of fragments. AFLP fragments are visualized or on denaturing polyacrylamide gels with autoradiography, or on agarose gel, or automatic DNA sequences.



Steps of AFLP

Advantages of AFLP

(i) It is highly reproducible and reliable.

(ii) It does not require any DNA sequence information from the organism under study.

(iii) It is information - due to its ability to analyze a large number of polymorphic location simultaneously with a single primer combination on a single gel as compared to RFLPs and microsatellites.

Limitations of AFLPs

- Like RAPD markers AFLPs are also dominant.
- The method needs purified and highmolecular weight DNA. Also, the procedure involves harmful radioactive materials; however, this can be overcome by using fluorescent tags.

VI. Sequence characterization of amplified region (SCAR)

Michelmore (1991) and Martin (1991) was the first to introduce this technique, in which RAPD marker termini are sequenced and longer primers of 22–24 nucleotide bases long are designed for specific amplification of a particular locus. It shows similar with STS markers in construction and application. The presence or absence of the band represent variation in sequence.

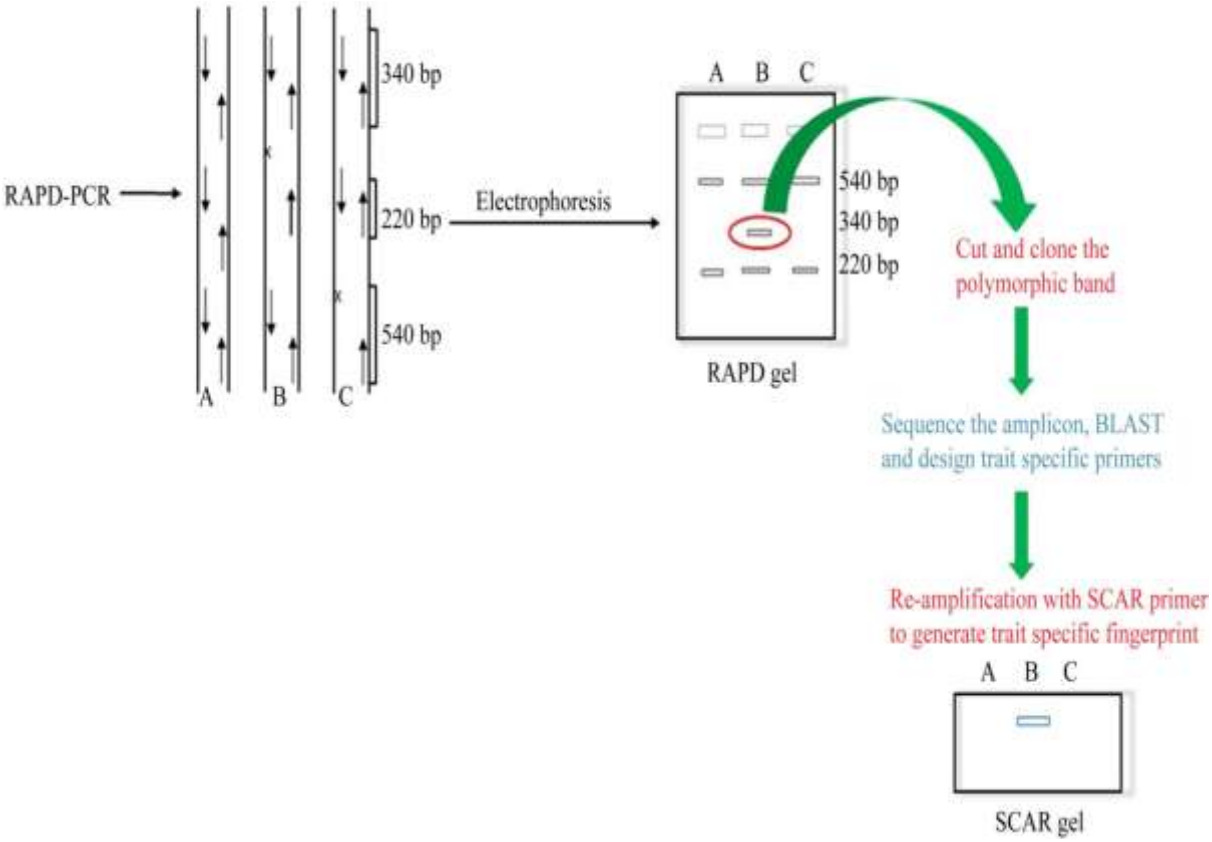
Advantages of SCARs markers

a) SCARs are advantageous over RAPD markers as

- They detect only a single locus.
- Their amplification is less sensitive to reaction conditions.
- They can potentially be converted into codominant markers (Paran andMichel more 1993).

b) SCARs compared to arbitrary primers

- SCARs exhibit several advantages in mapping studies (codominant SCARs are informative for genetic mapping than dominant RAPDs)
- map based cloning as they can be used to screen pooled genomic libraries by PCR.
- locus specificity and physical mapping.



Steps of SCAR

VII. Single-nucleotide polymorphism (SNP) -

Single nucleotide polymorphism is also called as minisatalite. Is a DNA sequence variation occurring when a single nucleotide (A, T, G or C) differs among members of a species. SNP is the most abundant marker system both in animal and plant genomes and has recently emerged as the new generation molecular markers for various applications. A single-nucleotide base is the smallest unit of inheritance and SNP can provide the simplest and maximum number of markers. SNPs are present in abundance in plants and animals and the SNP frequency in plants ranges between 1 SNP in every 100–300 bp. SNPs are widely distributed within the genome and can be found in coding or non-coding regions of genes or between two genes (intergenic region) with different frequencies. SNP marker have becomeextreamely popular in plant molecular genetics due to their genome – wide abundance and amenability for high – to ultra- high – throughput detection platforms.

VIII. Simple sequence repeats (SSR) –

Simple sequence repeats(SSR) are also known as microsatellite. Simple Sequence Repeats are comprised of 2–5 bp DNA monomeric units that are repeated multiple times at a specific locus. Such markers are completely co-dominant; used for fingerprinting, marker assisted selection, breeding behavior such as selfing and out crossing, establish population structure etc. Microsatellites are locally tandemly duplicated, and are also found dispersed throughout the genome. Thus it becomes important to amplify a specific microsatellite in a locus specific manner using locus specific primers. The primer designing involves identifying microsatellites, either is manual examination in case the sequences are small or of limited length, or using automated tools for locating microsatellite such as microsatellite finders. However, depending upon the research objectives, either unique flanking regions can be used for primer designing where the products are co-dominant, or microsatellites can themselves be used as primers where the products generated act as dominant markers.

Plant breeders have become increasingly interested in marker – assisted selection for efficient and precise transfer of genes conditioning important agronomic traits. Because SSR markers are codominant, more polymorphic and stable,and much easier to assay compared with RFLP or AFLP markers, they should be very useful for genomic mapping and gene tagging.

Advantages of SSRs markers

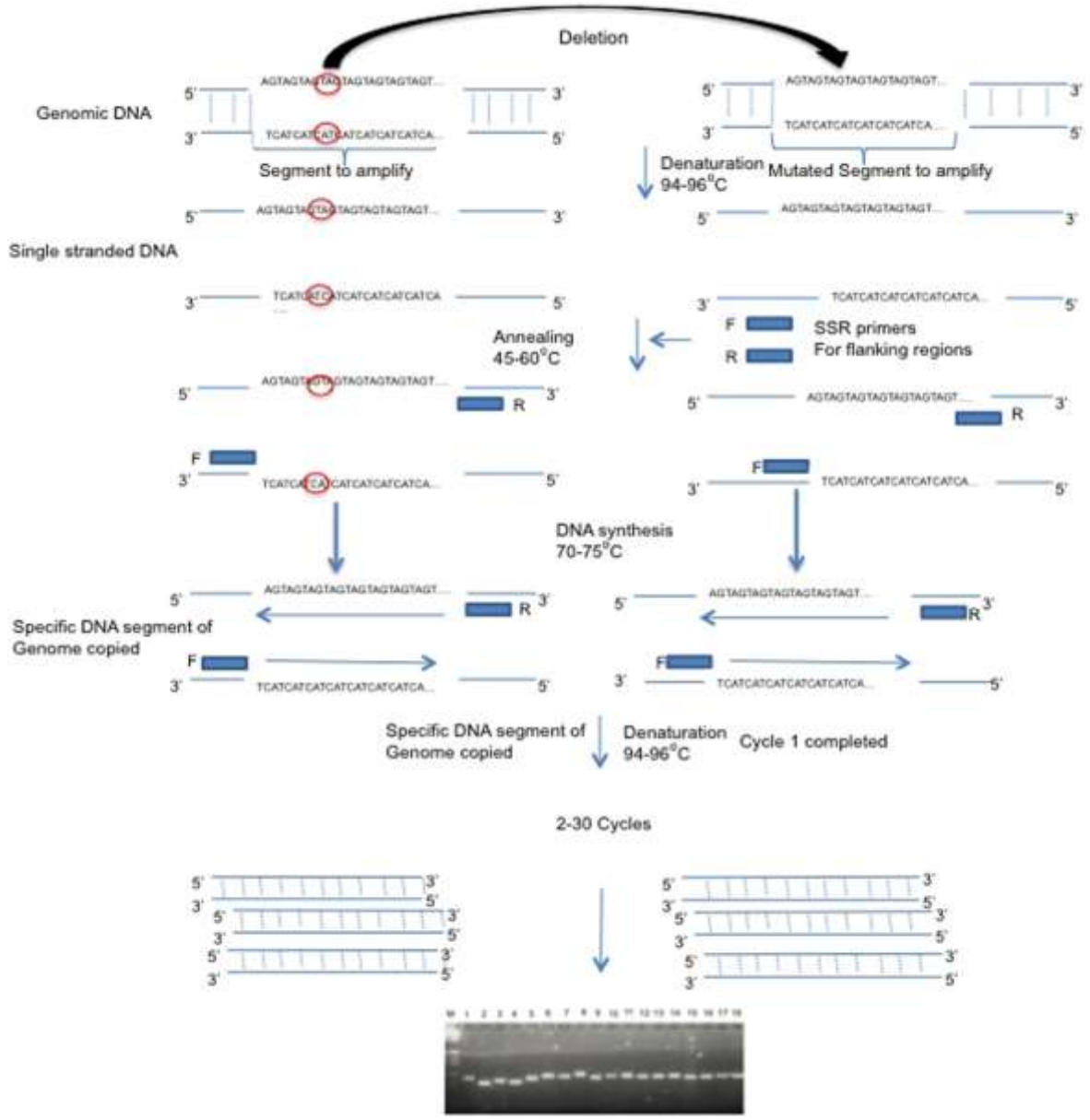
- SSR are used for plant breeding, conservation biology and population genetics as forensics, paternity analysis and gene mapping (Coates and Byrne 2005).
- Require little amount of DNA, which does not have to be of high quality.
- The simple interpretation of results (de Vicente and Fulton 2003).

Disadvantage of SSRs markers

- The requirement of a known sequence to be amplified (Weising et al. 2005).
- Developing new microsatellites are expensive and time consuming (Coates and Byrne 2005).

Limitation of SSR

- It requires much time and cost to isolate and characterize each SSR
- locus when the DNA sequence of a plant species is not available.
- Another drawback is the occurrence of null alleles. This may be due to
- the poor primer annealing because of nucleotide sequence divergence, inconsistent DNA quality or low DNA quantity or it might be due to mutations in the primer binding site.
- This can cause difficulty in the determination of allelic and genotypic.



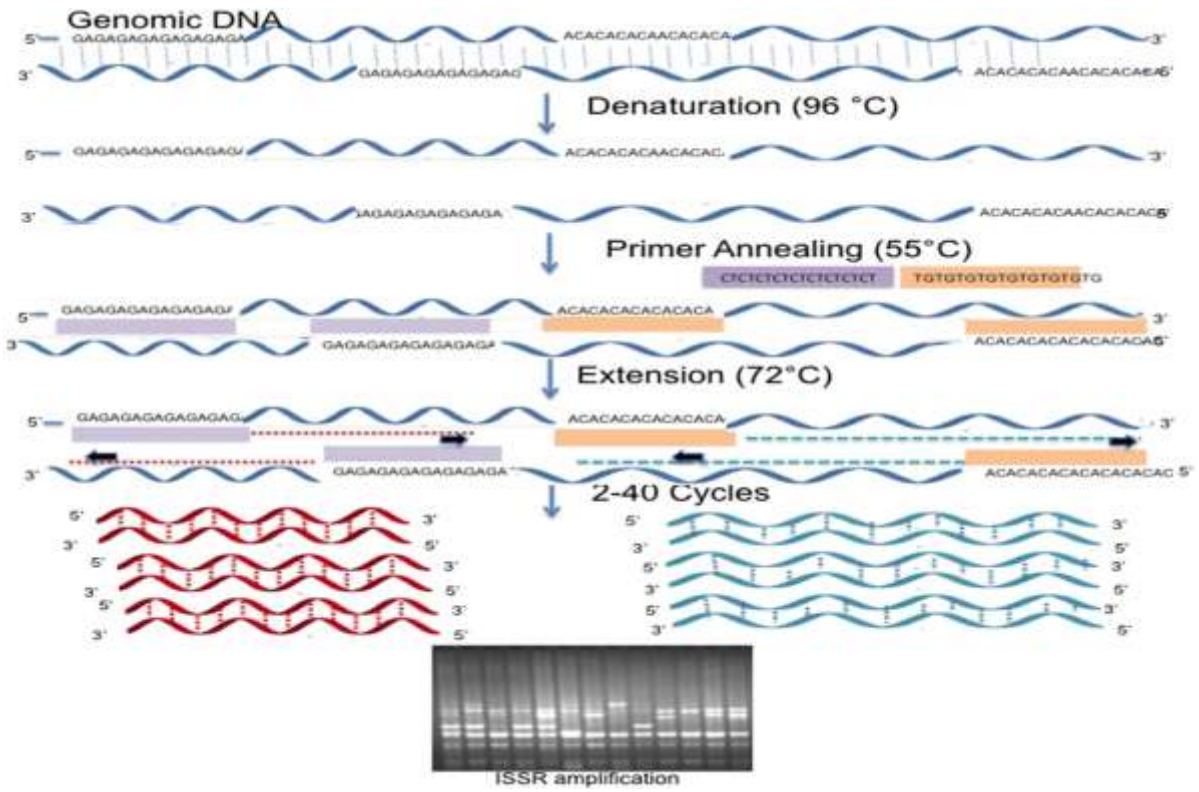
Steps of SSR

IX. Inter- simple sequence repeats(ISSR)

Inter-Simple Sequence Repeat **(**ISSR) is one of dominant markers involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. Inter-Simple Sequence Repeat usually 16-25 bp long as primers in a single primer PCR reaction targeting multiple genomic loci to amplify different sizes of inter-SSR sequences. The microsatellite repeats used as primer can be either di-nucleotides or tri-nucleotides. ISSR markers are highly polymorphic and are used on genetic diversity, gene tagging, phylogeny, evolutionary biology and genome mapping studies. ISSR PCR is a technique, which overcomes the problems like high cost of AFLP, low reproducibility of RAPD, and the flanking sequences to develop species specific primers for SSR polymorphism.

Advantages of ISSR

- ISSR is quick, simple, highly reproducible and the use of radioactivity is not essential.
- ISSR markers usually show high polymorphism, and with the most important advantage that no prior information about genomic sequence is required.



Steps of ISSR

Limitations of ISSRs

- ISSR markers do have more value compared to RAPDs; however, the marker has the reproducibility issues.
- This marker is also dominant.

X. Cleaved amplified polymorphic sequences (CAPS) –

CAPS are a merge of the RFLP and PCR and it was originally named PCRRFLP (Maeda et al., 1990). The technique involves amplification of a target DNA through PCR, followed by digesting with restriction enzymes (Michaels and Amasino 1998). Hence, CAPS markers rely on differences in restriction enzyme digestion patterns of PCR fragments caused by nucleotide polymorphism between samples. Critical steps in the CAPS marker application involve DNA extraction, and the number or distribution of polymorphic sites, and PCR conditions.

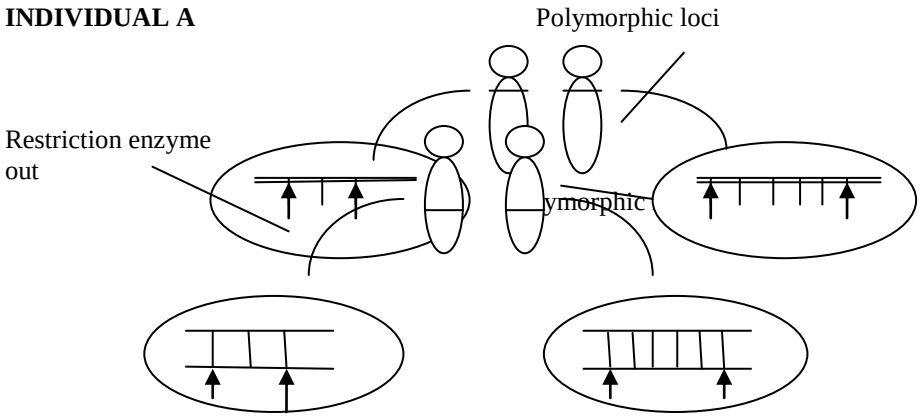
Advantage of CAPS -

(i) Since analysis of restriction fragment length polymorphisms is based on PCR amplification, it is much easier and less time-consuming than analyzing alternative types of markers that require southern hybridizations.
(ii) CAPS primers developed from ESTs are more useful as genetic markers for comparative mapping study than those markers derived from non-functional sequences such as genomic microsatellite markers.
(iii) CAPS markers are co-dominant markers.

XI. Variable number tandem repeats (VNTR) –

A marker system based on restriction fragment length polymorphism (RFLP) is often used. When DNA is digested with a restriction enzyme that cuts at sites flanking the VNTR locus (but not within the repeats), the length of the DNA fragments produced will vary with individuals depending on the number of repeats in the locus. The unique length patterns of the restriction fragments provide a DNA fingerprint of an individual.

The DNA fragments obtained by restriction digestion are separated into bands according to their sizes by gel electrophoresis. The resolved bands are transferred by southern blot to a nitrocellulose membrane, which is subject to DNA hybridization using a radiolabeled probe having sequence complementary to the repeats of a VNTR locus.



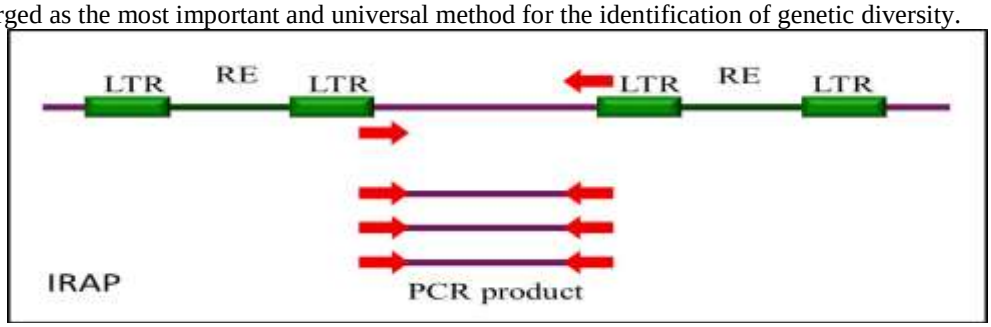
The use of VNTR in generating fingerprints.

Application-

- VNTR application in paratuberculosis.
- VNTR application in Haemophilus influenza type B.
- Multiplelocus VNTR analysis (MLVA) application in salmonella enteric subsp. Enteric.

XII. Inter-Retrotransposon amplified polymorphism (IRAP) –

Inter-retrotransposon amplified polymorphism (IRAP) is a Retrotransposon-type marker developed by Kalendar et al. Sequences present between two adjacent LTR Retrotransposon are amplified by the IRAP system through the application of primers which are complementary to the LTR sequence 3' end. The orientation of these LTR sequences can be (1) tail–tail, (2) head–head and (3) head–tail. Identical sequences are present in different strands that are separated by small inter-genic distances in headto- head arrangement and are transcribed away from each other. The arrangement is opposite to the head-to-head one and they are transcribed towards each other. Both 5' and 3' primers are used for head-to-tail LTRs, while a single present with the other retrotransposons and result in close occurrence of PBS sequences with each other. PBS is a universal method, as they occur in all LTR-based Retrotransposon sequences. Recently, inter-primer binding site (iPBS) markers have emerged as the most important and universal method for the identification of genetic diversity.



Product from IRAP amplification.

XIII. Retrotransposon microsatellite amplification polymorphisms (REMAP) –

The presence or absence of Retrotransposon sequences is investigated, which can be used as molecular marker in this technique, DNA amplification is achieved through a primer having 3' and 5' end regions flanking the Retrotransposon insertion site. Detection of the presence of insertion is achieved through the development of primer from LTR. Sequence information about the regions flanking the Retrotransposon insertion sites is needed in this technique and it results in the typing of a single locus as compared to other Retrotransposon-based markers Agarose gel electrophoresis is used for the detection of polymorphism. Tagged microarray marker, which is based upon fluorescent microarray marker scoring, is used for high-throughput Retrotransposon-based insertion polymorphism (RBIP) analysis.

XIV. Loop mediated isothermal amplification (LAMP)–

Loop-mediated isothermal amplification (LAMP) is amolecular biology technique (Notomiet al.2000) thatdepend upon auto-cycling strand displacement DNA synthesis with Best DNA polymerase. DNA synthesis occurs within 1 hr. at a single temperature which is in range of 60–66°C.

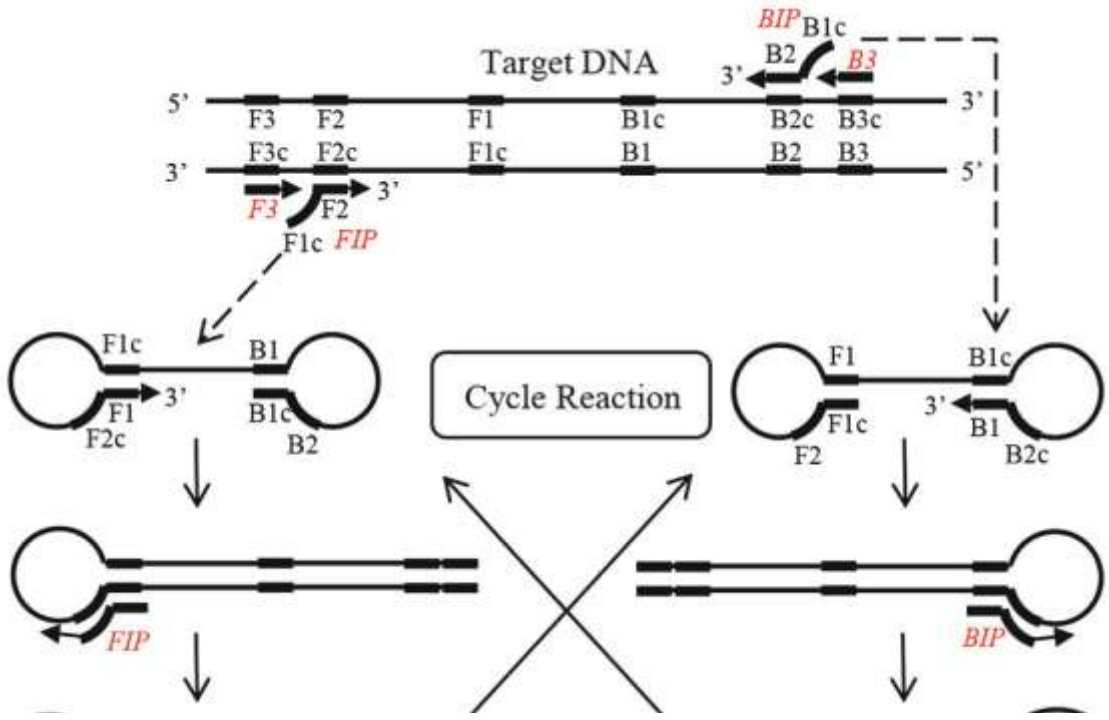
In the LAMP reaction, gene amplification proceeds through repet of two types of elongation reactions that occur through the loop regions (i.e., template self-elongation from the stem loop structure formed at the 3'-terminal and subsequent binding and elongation of new primers to the loop region). In this reaction, pairs of inner and outer primers are used. Each of the inner primers possesses a sequence complementary to one chain of the amplification region at the 3'-terminal and identical to the inner region of the same chain at the 5'-terminal.The elongation reactions are sequentially repeated by DNA polymerase-mediated strand-displacement synthesis using the aforementioned stem loop regions as a stage. This method operates on the fundamental principle of the production of a large quantity of DNA amplification products with a mutually complementary sequence and an alternating, repeated structure. Gel electrophoresis is also not mandatory since LAMP products can be detected by the turbidity that come to light due to alarge amount of by-product, pyrophosphate ion, being produced, yielding an insoluble white precipitate of magnesium pyrophosphate in thereaction mixture.

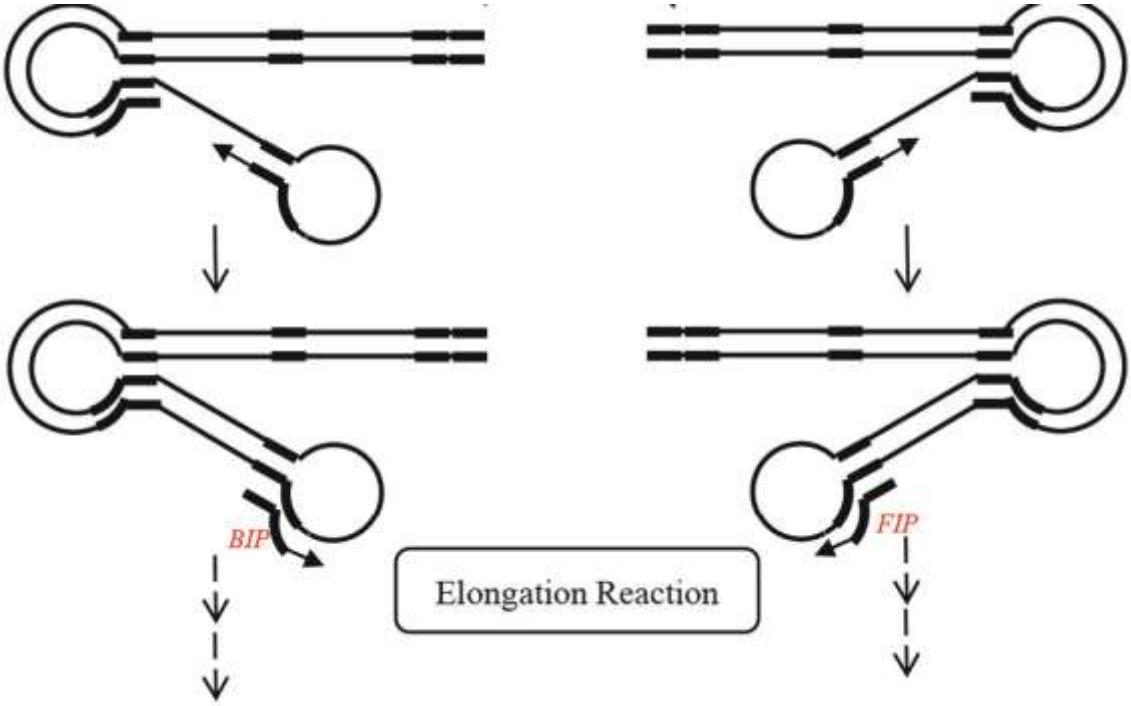
Limitations of LAMP markers

• Primer designing in LAMP technology is complex; a minimum of twoprimer pairs is required to identify six different regions of target gene/DNA sequence.

Application of LAMP-

- Development of detection reagents for influenza virus the LAMP reaction is relatively resistant to the effects of inhibitors produced by specimens.
- Detection of tuberculosis.





Schematic representation of the LAMPmechanism.

XV. Next generation sequencing (NGS) –

The development in this techniques increased the demand for extensive throughput sequencing at a low cost. This demand led to the development of NGS and currently this technique produces millions of sequences. The technique is based on the principle that DNA templates are first fragmented and thereafter immobilized on a solid support. These fragments are to be amplified and sequenced.This technique has the ability to produce several hundreds of millions to several hundreds of billions of DNA bases per run. Many organizations have developed this technique successfully and they provide their services commercially, such as illumine MiSeq and HiSeq 2500, Roche 454 FLX Titanium and Ion Torrent PGM. These NGSs resulted in low prices with covering whole genome more precisely, similar methodology is used in all NGS techniques for the preparation of template DNA, where fragments of DNA are randomly sheared andligated at both ends with universal adapters. This sequencing is performed in constant channel and one or more nucleotides are incorporated, resulting in the release of a signal that is detected by a sequencer. The technology uses pyrosequencing in which pyrophosphate ions released are detected during the addition of nucleotides by DNA polymerase.

Application-

- Personal genomics, project human diversity in 1000 genomes.
- RNA sequencing, analysis of gene expression.
- Chromatin immune precipitation, ChiP-seq technique.

Advantages of NGS–

- NGS is more accurate to older sequencing methods and
- low in cost with high throughput.
- Recently, this technique is being used in whole genome sequencing in order to investigate the maximum numbers of SNPs and for consideration of diversity present within the species, construction of linkage/ halophyte maps and in genome-wide association studies (GWAS).
- Sequencing of older DNA samples is also performed by NGS and this technique has strengthened the field of metagenomics.

XVI. Expressed Sequence Tags (EST) –

Expressed sequence tags are obtained by single – pass sequencing of cDNA clones, usually randomly selected from a cDNA library, which represents a tissue of interest. Withcurrently available capillary - based sequencing techniques it results in a sequence of about 100-700 bp (depending on cDNA quality and sequencing reaction preparation). EST is a partial sequence representative of a corresponding cDNA clone and can be used for its characterizing with various bioinformatics tools. Standard analyses focus on EST identification by homology searches in existing protein and DNA sequence database. Each gene must be converted or transcribed into messenger RNA (mRNA) that serves as a template for protein synthesis. The resulting mRNA then guides the synthesis of a protein through a process called translation. The problem is that mRNA is very unstable outside of a cell; therefore, scientists use an enzyme called reverse transcriptase to convert mRNA to complementary DNA (cDNA). cDNA production is the reverse of the usual process of transcription in cells because the procedure uses mRNA as a template rather than DNA. cDNA is a much more stable compound and it represents only expressed DNA sequence because it is generated from mRNA that represents exons by excising (splicing) introns. Once cDNA representing an expressed gene has been isolated, scientists can then sequence a few hundred nucleotides from either the 5' or 3' end to create 5' expressed sequence tags (5' ESTs) and 3' ESTs, respectively. A 5' EST is obtained from the portion of a transcript (exons) that usually codes for a protein. These regions tend to be conserved across species and do not change much within a gene family. The 3' ESTs are likely to fall within non-coding (introns) or untranslated regions (UTRs), and therefore tend to exhibit less cross-species conservation than do coding sequences. The challenge associated with identifying genes from genomic sequences varies among organisms and is dependent upon genome size as well as the presence or absence of introns, which are the intervening DNA sequences interrupting the protein coding sequence of a gene. The production of ESTs starts with the construction of cDNA libraries. The identification of ESTs has proceeded rapidly, with over 6 million ESTs now available in computerized databases.

XVII. CONCLUSION-

The study of genetic marker their primary target is conservation utilization of plant. Different methods have been developed and tested at DNA sequence level. These method provide large number of markers and opening up new opportunities for evaluating diversity in plant genetic resources. the present study suggested that mtDNA and RAPD are valuable molecular tools for species characterization. DNA markers are useful in many aspects of studying genetic polymorphism in plants.

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