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An Overview of Molecular Markers for Probing Genetic Diversity

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ABSTRACT

Molecular markers are indispensable tools for identifying a particular sequence of DNA in a pool of unknown DNA. Use of Molecular markers are generally based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for various purposes such as species identification, genetic diagnostics, characterization of transformants, study of genome organization, germplasm characterization and phylogenic analysis. There are various types of markers viz. morphological, biochemical and DNA based molecular markers. The molecular markers are selected for their appropriate purpose on the basis of high level of accuracy and reproducibility. The main properties which helps in choosing the most reliable marker are: Genetic abundance, level of polymorphism, locus-specificity, co-dominancy, technical demands, operational costs and many more. The DNA based molecular markers are again divided in to two i.e. hybridization based and PCR based molecular markers. RFLP belongs to the first non PCR based techniques and PCR based technique includes RAPD and AFLP. In this review, we discuss about the molecular markers with their merits and demerits.

Keywords: Molecular markers, Polymorphism, RAPD, RFLP, AFLP, Hybridization

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INTRODUCTION

Genetic diversity is the genetic characteristic that varies between individuals and populations when compared with other individuals. This difference is due to the genetic material, Deoxyribonucleic acid (DNA) and it is packaged in chromosomes pairs, inherited from each parent. The genes that are responsible for the phenotypic characteristics are located on specific locus. It is well accepted that decline in genetic variation results in the reduction of capacity of population to adopt to environmental stress and as a result its long term survival also get decreased, which ultimately results in lower individual fitness and poor adaptability. [1, 2, 3, 4]

Molecular markers are DNA sequences that can be readily used to trace the desired genes and whose inheritance can be easily monitored. Its use depends on naturally occurring polymorphism between DNA sequences and thus can be applied for different purposes such as to study species diversity, ecological diversity, morphological diversity, etc. A molecular marker should to be polymorphic i.e. it must exist in different forms so that chromosome carrying the mutant genes can be distinguished from the chromosome carrying the normal gene. Genetic polymorphism is defined as the occurrence of two or more clearly different phenotypes existing in the same population of two discontinuous variants or genotypes. DNA markers seem to be the best candidates for efficient evaluation of genetic polymorphism.[5, 6, 7]There are different types of markers such as morphological, biochemical and DNA based markers. DNA based markers can be categorized in to 2 types: First is Hybridization based markers such as RFLP and second is PCR based RAPD and AFLP. The majority of these molecular markers has been developed either from genomic DNA libraries (e.g. RFLP Markers) or from random PCR amplification of genomic DNA (e.g. RAPDs) or both (e.g. AFLP). These molecular markers can be used extensively for molecular marker assisted selection, gene isolation, genetic evolutionary studies, diagnostics, germplasm characterization and varietal identification etc. Thus, in this review we discuss different types of molecular markers and future potential of these markers.[5, 8]

HYBRIDIZATION BASED MOLECULAR MARKERS

RFLP (Restriction Fragment Length Polymorphism):

RFLP was the first to develop among the various molecular markers and was first used in 1975 to identify DNA sequence polymorphisms for genetic mapping of a temperature-sensitive mutation of adeno-virus serotypes.[9] It was then adopted for human genome mapping[10] and later used for plant genomes.[11, 12]

Variations found in the characteristic pattern of a RFLP digest can be caused by base pair deletions, mutations, inversions, translocations and transpositions which results in the loss or gain of recognition site for restriction enzymes, resulting in a fragment of different length and polymorphism. Only a single base pair difference in the recognition site will cause the restriction enzyme not to cut at the specified site. If the base pair mutation is present in one chromosome but not the other, both fragment bands will be present on the gel, and the sample is said to heterozygous for the marker. Only co-dominant markers exhibit this behavior which is highly desirable, whereas dominant markers exhibit a present/absent behavior which can limit data available for analysis. RFLP has some limitations since it is time consuming.[13]

RFLP denotes that restriction enzymes produces fragments of different lengths from the same stretch of DNA from different related species or different strains of a species. The procedure for RFLP is summarized as follows:

DNA isolation:

Significant amount of genomic DNA must be isolated from the sample and purified to a fairly stringent degree so that contaminants would not interfere with the restriction enzyme.

Restriction Digestion:

Restriction enzyme is added to purified genomic DNA under buffered conditions and it cuts at recognition sites throughout the genome and leaves behind hundreds of thousands of fragments.

Gel electrophoresis:

Fragments obtained through restriction digestion are separated through gel electrophoresis.

Southern Blotting:

Separated fragments obtained from agarose gel electrophoresis are transferred to nitrocellulose or nylon membrane.

Probe Visualization:

For Detection of specific fragments from large number of fragments, probes are constructed to visualize specific bands. Probes consist of radio labeled oligonucleotide sequences which will anneal to the fragment sequences for which it is complementary so that that they may be visualized on photographic paper using a technique called autoradiography. The free probes (not involved in hybridization) are washed out.

Analysis:

Number of RFLP loci can be analyzed after autoradiography [1, 5, 6]

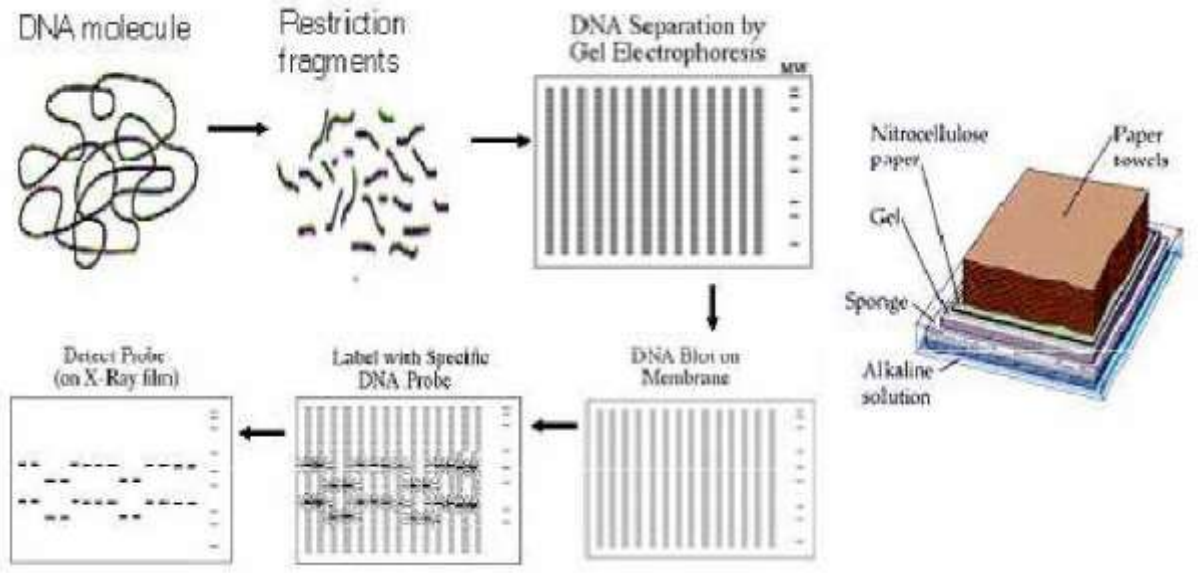

Figure 1. Outline diagram of different steps of Restriction Fragment Length Polymorphism (RFLP) (Source: Semagn *et al*, 2006)[1]

Advantages:

- RFLPs are codominant markers and are highly reproducible.
- RFLPs, being codominant markers, can detect coupling phase of DNA molecules, as DNA fragments from all homologous chromosomes are detected.
- RFLP are reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in individual, information highly desirable for recessive traits.
- Even Quantitative trait loci can be mapped, which is virtually impossible through conventional linkage mapping [6, 13]

Disadvantages:

- It requires large quantities of purified and high molecular weight DNA
- Use of Radioactive isotopes makes the analysis relatively expensive and hazardous.
- It is time consuming, expensive and labor-intensive
- Level of polymorphism is low and few loci are detected per assay, which is highly inconvenient especially for crosses between closely related species [1, 6]

PCR- BASED MOLECULAR MARKERS

Polymerase Chain Reaction:

The development of PCR is a milestone in terms of scientific development that actually deserves time worm superlatives such as 'Revolutionary' and 'breakthrough' in the analysis of genome.[14]

PCR is a technique in which small quantities of DNA is enzymatically amplified without using a living organism. Polymerase Chain Reaction was developed in 1984 by Kary Mullis and received the Nobel Prize and the Japan Prize for the development of PCR in 1993. However the basic principle of replicating a piece of DNA had already been described using two primers by Gobind Khorana and his group in 1971, but the concept was limited by primer synthesis and polymerase purification issues.[15] The basic concept was started for the first time with *Klenow polymerase* but the real breakthrough came when *Taq polymerase* was isolated and purified, a thermostable DNA polymerase.[16] The basic procedure for PCR is as follows:

Denaturation:

Double-stranded DNA is denatured at high temperature (92-95^{o}C) for 2 min to form single stranded DNA strands (templates).

Annealing:

Short single stranded oliginucleotides (known as primers) bind at a lower annealing temperature (generally 40^{o}-60^{o}C for 1 min) to the single stranded complementary templates at ends flanking the target sequences, these sequences are located at the 3' ends of the two ends of the desired segments.

Primer Extension:

The temperature is adjusted so that the DNA polymerase catalyze the template-directed syntheses of new double-stranded DNA molecules that are identical in sequence to the starting material. The duration of primer extension is usually 2 min for 72^{0}C (sometimes 68^{0}C).

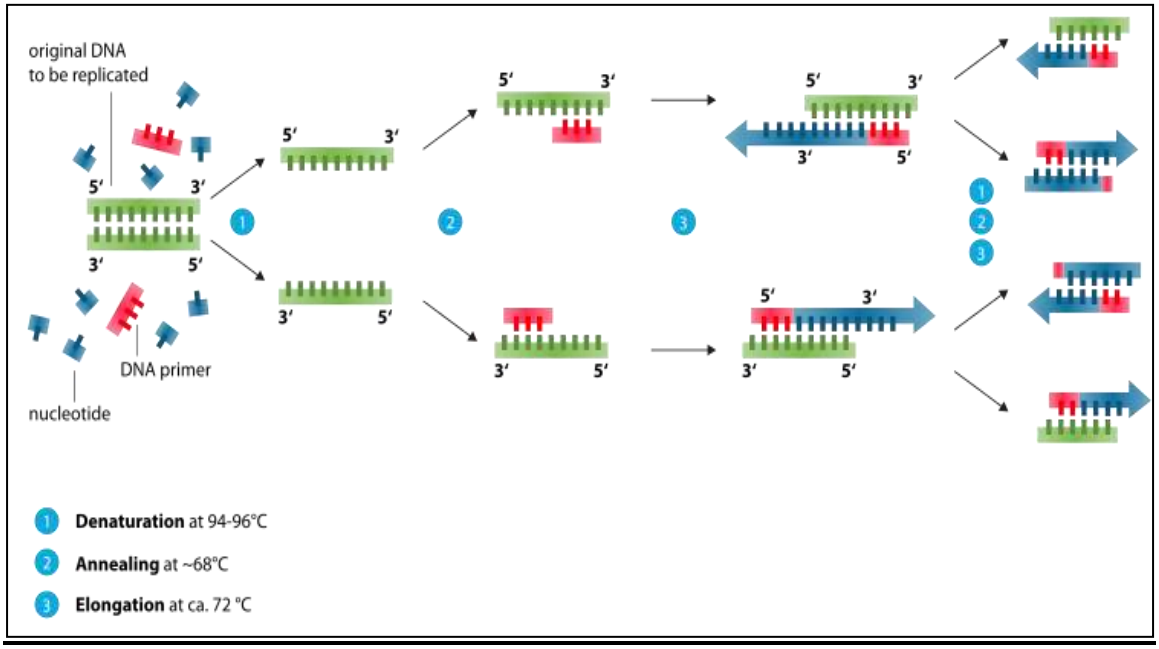

Figure 2: Diagram showing the basic protocol of Polymerase Chain Reaction (PCR)

The main advantages of PCR based molecular techniques as compared to Hybridization based techniques are as follows:

1) Small amount of DNA is required
2) Eliminates the use of radioisotopes
3) DNA sequences can also be amplified from preserved tissues.
4) Can be accessed by small labs in terms of expertise, equipment, facilities, and cost.
5) No prior sequence knowledge is required
6) High polymorphism rate that enables to generate many genetic markers within a short period of time[1, 17]

PCR based molecular markers are of two types depending on the type of primer used for amplification:

1. PCR techniques that emerged without any prior sequence information : Arbitrary or semi-arbitrary primer are used (RAPD, AFLP)
2. PCR techniques that emerged from known DNA sequences : Site Targeted primers are used

RAPD (RANDOM AMPLIFIED POLYMORPHIC DNA):

RAPD utilizes arbitrary (random) primers to amplify DNA sequences without any prior knowledge of DNA sequences and is mainly executed by the interaction between primer, template annealing sites and enzymes. RAPD primers are usually 10 nucleotides long, binds to site on opposite strands of the genomic DNA that are within an amplifiable distance of generally less than 3,000 bp (base pairs).[1]

It is easy to perform, cheap and fast technique as compared to RFLP and it can be performed by anyone without expertise. It is applicable to many different applications because universal set of primers is used and it includes DNA polymorphism study different groups of organism, mapping, variety identification and analysis of parentage [1, 7]

RAPD protocol usually involves following steps:

1. Isolation of genomic DNA
2. Annealing was performed at constant low temperature (34 -37^{o}C) and arbitrarily designed 10 nucleotides long primers are used. Although the primers are randomly chosen but 2 basic criteria must be considered- Generally the percentage of GC content should be 50-80% and palindromic sequence must be absent (base sequence that exactly reads the same from right to left as from left to right). Because three hydrogen bonds are present in G-C bonding and only 2 are there in A-T, so to withstand 72^{o}C temperature at

which DNA elongation takes place by DNA polymerase, primer-DNA hybrid must have 50% GC content.

3. After several cycles of amplification, the PCR products are subjected to Gel electrophoresis at 1.5 to 2.0% of Agarose and stained with Ethidium Bromide or Polyacrylamide gels in combination with either $AgNO_3$ staining

RAPD bands are formed due to amplification of segments and two types of polymorphism occur- the band may be present or absent. A distinct band is observed in those samples in which both the ends of DNA sequences are homologous to the primers used, whereas band will be absent from those in which one or both the ends of the DNA sequence is either deleted or modified.[1,6,18]

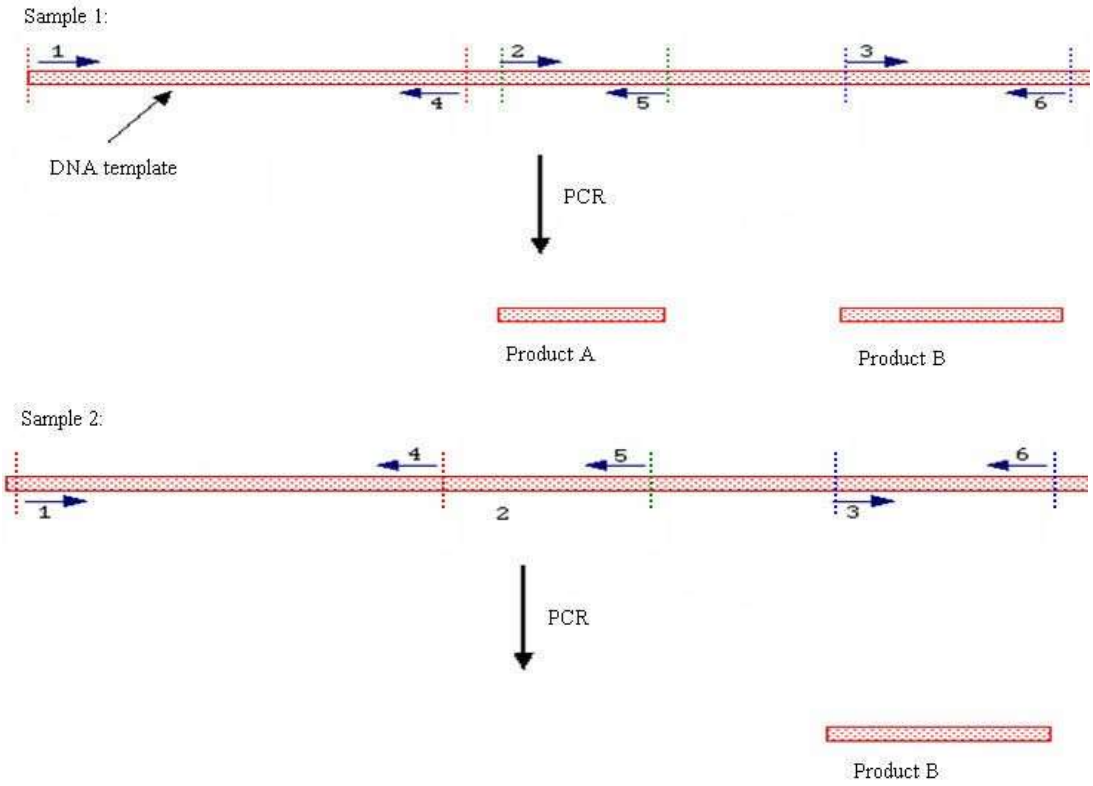

Figure 3: Schematic representation of RAPD analysis (In Sample 1, two PCR products are formed due to amplification with a set of primers for position 2-5 and 3-6 primers whereas in Sample 2, the DNA sequences is either modified or deleted for the position 2-5, so this region resulted in no PCR product and only Product B is formed) [Source: Semagn *et al*, 2006][1]

RAPD has three limitations:

Reproducibility:

Reproducibility of RAPD outcome is greatly influenced by quality and quantity of template DNA, PCR buffer, concentration of magnesium chloride, primer to template ratio, annealing temperature, DNA polymerase enzyme and PCR cycling conditions.

Dominant Marker:

RAPD markers are dominant i.e it is not possible to distinguish dominant homozygotes from heterozygotes because it produce fragments from homozygous dominant or heterozygous alleles, but no fragment is produced from homozygous recessive alleles because amplification is disrupted in both alleles.

Homology:

Assumption that co-migrating bands (bands that migrate equal distance) represent homologous DNA fragment during pair wise comparison of RAPD fragments because equal length equals homology may not be true, especially in case of polyploidy and it has been reported that more accurate resolution of fragment size can be obtained by using polyacrylamide gels and $AgNO_3$ staining) to reduce errors of holmology. [19,20,21,22]

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

AFLP is based on combined strengths of both RFLP and RAPD. It is highly sensitive method applicable for fingerprinting genomic DNA within any organism, giving reproducible results. AFLP is based on selectively amplifying PCR products, generated by restriction digestion of genomic DNA with restriction endonucleases. Detection of Polymorphism are done on the basis of differences in the length of the amplified fragments by polyacrylamide gel electrophoresis (PAGE) or by capillary electrophoresis[23,24,5,6,7]. The specific feature of AFLP is its capacity for "genome representation", the simultaneous screening of representative DNA sequences distributed randomly throughout the genome. AFLP markers can be generated for DNA of any organism without prior information of sequences and initial investment in primer development[6].

The technique involves 4 steps and is depicted in Figure 4:

Step 1: Preparing the AFLP Template

Genomic DNA was isolated and it is then digested with a pair of restriction enzymes, one frequent cutter (4 bp recognition site) and one rare cutter (6 bp recognition site), generally *Mse*I and *Eco*RI. *Mse*I recognizes 5′-TTAA-3′ and cleaves after the first 5′-T, whereas *Eco*RI recognizes 5′-GAATTC-3′ and cleaves after the 5′-G. *Mse*I and *Eco*RI generate DNA fragments with 5′ overhangs (5′-TA-3′ and 5′-AATT-3′, respectively) that are distinct from each other and are non complementary.

Step 2: Ligation Reaction with Restriction Fragments and Adaptors

Oligonucleotide "adaptors" are ligated to the ends of each fragment. One end with a complimentary sequence for the frequent cutter and the other with the complimentary sequence for

the rare cutter. This way only fragments which have been cut by the frequent cutter and rare cutter will be amplified.

Step 3: Selective PCR Amplification

Primers are designed from the known sequence of the adaptor and 1-3 selective nucleotides are added at the end of each primer which permits the amplification of a subset of genomic DNA fragments. Sequences not matching these selective nucleotides in the primer will not be amplified during PCR.

Step 4: Visualization of Amplified DNA Fragments

These fragments are viewed on denaturing polyacrylamide gels either through autoradiography or fluorescent methodologies and are generally scored as dominant markers. [25,5,6,7]

Advantages:

- It is highly reliable, sensitive and reproducible technique
- It does not require any prior DNA sequence information.
- It is information-rich due to its ability to analyze a large number of polymorphic markers or loci by single reaction
- Co-migrating (bands that migrate equal distance) AFLP products are mostly homologous and locus specific with exceptions in polyploidy species[5,6]

Disadvantages:

- It requires purified and high molecular weight DNA template free of inhibitor compounds that interferes with the restriction enzyme
- It requires number of steps to produce the results
- The technique requires the use of polyacrylamide gel in combination with $AgNO_3$ staining or fluorescent methods of detection, which will be more expensive and laborious than agarose gels.
- It also involves additional cost to purchase restriction endonucleases and ligation enzymes as well as adaptors.
- Most AFLP loci are dominant, which does not differentiate dominant homozygotes from heterozygotes. [5,6]

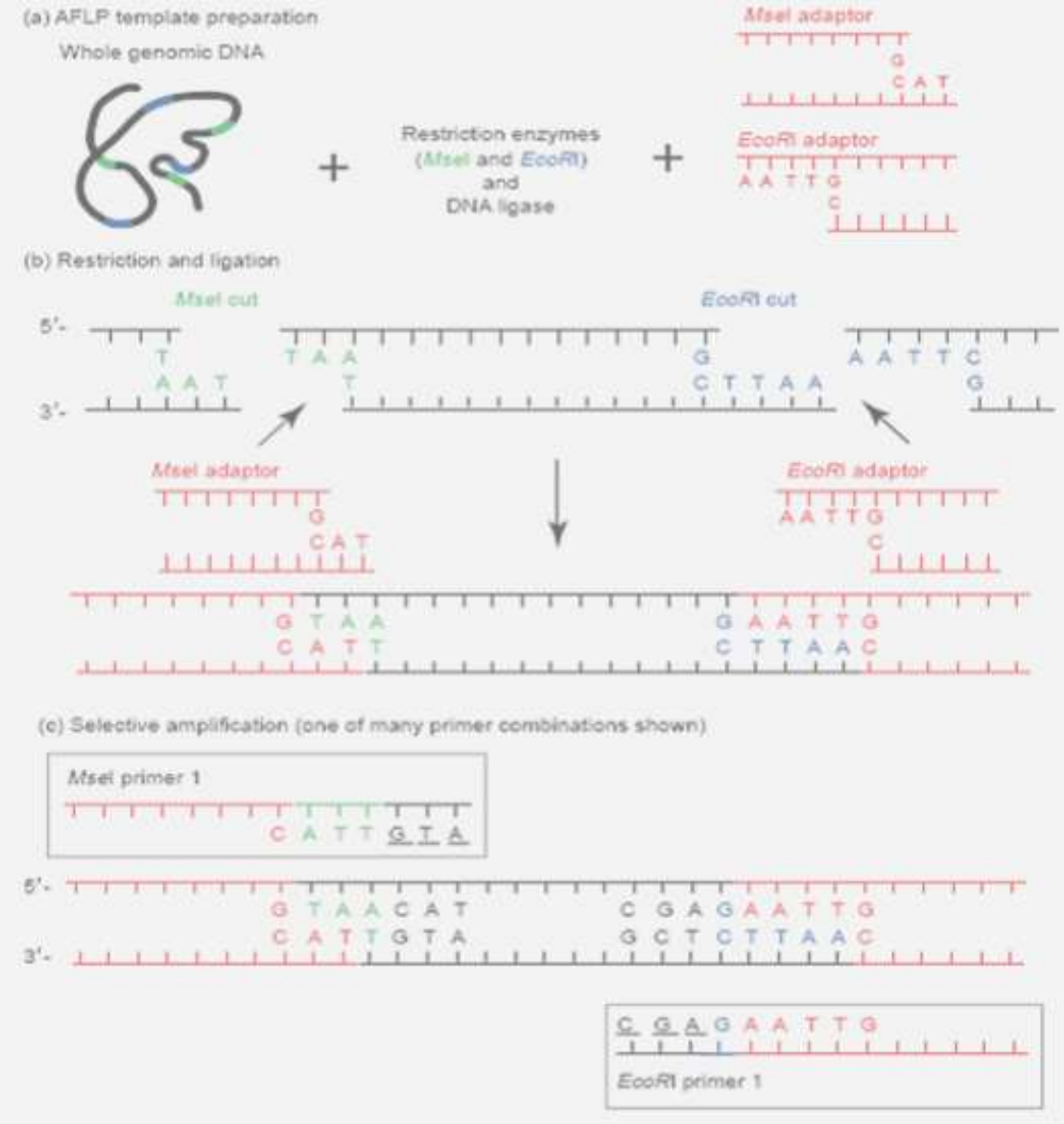

Figure 4: Protocol for Amplified fragment length polymorphism (AFLP) [Source Mueller & Wolfenbarger, 1999] [25]

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