Review article

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Potential of Molecular Markers in Plant Biotechnology

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Abstract

During the last few decades, the use of molecular markers, revealing polymorphism at the DNA level, has been playing an increasing part in plant biotechnology and their genetics studies. There are different types of markers viz. morphological, biochemical and DNA based molecular markers. These DNA based markers are differentiates in two types first non PCR based (RFLP) and second is PCR based markers (RAPD, AFLP, SSR, SNP etc.), amongst others, the microsatellite DNA marker has been the most widely used, due to its easy use by simple PCR, followed by a denaturing gel electrophoresis for allele size determination, and to the high degree of information provided by its large number of alleles per locus. Despite this, a new marker type, named SNP, for Single Nucleotide Polymorphism, is now on the scene and has gained high popularity, even though it is only a bi-allelic type of marker. Day by day development of such new and specific types of markers makes their importance in understanding the genomic variability and the diversity between the same as well as different species of the plants. In this review, we will discuss about the biochemical and molecular markers their Advantages, disadvantages and the applications of the marker in comparison with other markers types.

Keywords: Molecular markers; plant biotechnology; genetic diversity; polymorphism; isozymes; polymerase chain reactions (PCR).

Introduction

In current scenario, the DNA markers become the marker of choice for the study of crop genetic diversity has become routine, to revolutionized the plant biotechnology. Increasingly, techniques are being developed to more precisely, quickly and cheaply assess genetic variation. In this reviews basic qualities of molecular markers, their characteristics, the advantages and disadvantages of their applications, and analytical techniques, and provides some examples of their use. There is no single molecular approach for many of the problems facing gene bank managers, and many techniques complement each other. However, some techniques are clearly more appropriate than others for some specific applications like wise crop diversity and taxonomy studies. Our goal is to update DNA marker based techniques from this review, to conclude DNA markers and their application and provide base platform information to the researchers working in the area to be more efficiently expertise. Due to the rapid developments in the field of molecular genetics, varieties of different techniques have emerged to

analyze genetic variation during the last few decayed. These genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate genetic marker has depend on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and know-how, time constraints and financial limitations. The classification main marker technologies that have been widely applied during the last decades are summarized in Table-1.

A. Biochemical Marker - Allozymes (Isozyme)

Introduction: Isozymes analysis has been used for over 60 years for various research purposes in biology, viz. to delineate phylogenetic relationships, to estimate genetic variability and taxonomy, to study population genetics and developmental biology, to characterization in plant genetic resources management and plant breeding (Bretting & Widrlechner 1995, Staub & Serquen 1996). Isozymes were defined as structurally different molecular forms of an enzyme with, qualitatively, the same catalytic function. Isozymes originate through amino acid alterations, which cause changes in net charge, or the spatial structure (conformation) of the enzyme molecules and also, therefore, their electrophoretic mobility. After specific staining the isozyme profile of individual samples can be observed (Hadačová & Ondřej 1972, Vallejos 1983, Soltis & Soltis 1989).

Allozymes are allelic variants of enzymes encoded by structural genes. Enzymes are proteins consisting of amino acids, some of which are electrically charged. As a result, enzymes have a net electric charge, depending on the stretch of amino acids comprising the protein. When a mutation in the DNA results in an amino acid being replaced, the net electric charge of the protein may be modified, and the overall shape (conformation) of the molecule can change. Because of changes in electric charge and conformation can affect the migration rate of proteins in an electric field, allelic variation can be detected by gel electrophoresis and subsequent enzyme-specific stains that contain substrate for the enzyme, cofactors and an oxidized salt (e.g. nitro-blue tetrazolium). Usually two, or sometimes even more loci can be distinguished for an enzyme and these are termed isoloci. Therefore, allozyme variation is often also referred to as isozyme variation (Kephart 1990, May 1992) isozymes have been proven to be reliable genetic markers in breeding and genetic studies of plant species (Heinz, 1987), due to their consistency in their expression, irrespective of environmental factors.

Advantages: The strength of allozymes is simplicity. Because allozyme analysis does not require DNA extraction or the availability of sequence information, primers or probes, they are quick and easy to use. Some species, however, can require considerable optimization of techniques for certain enzymes. Simple analytical procedures, allow some allozymes to be applied at relatively low costs, depending on the enzyme staining reagents used. Isoenzyme markers are the oldest among the molecular markers. Isozymes markers have been successfully used in several crop improvement programmes (Vallejos 1983, Glaszmann et al. 1989, Baes & Custsem 1993). Allozymes are codominant markers that have high reproducibility. Zymograms (the banding pattern of isozymes) can be readily interpreted in terms of loci and alleles, or they may require segregation analysis of progeny of known parental crosses for interpretation. Sometimes, however, zymograms present complex banding profiles arising from polyploidy or duplicated genes and the formation of intergenic heterodimers, which may complicate interpretation.

Disadvantages: The main weakness of allozymes is their relatively low abundance and low level of polymorphism. Moreover, proteins with identical electrophoretic mobility (co-migration) may not be homologous for distantly related germplasm. In addition, their selective neutrality may be in question (Berry & Kreitman 1993, Hudson et al. 1994, Krieger & Ross 2002). Lastly, often allozymes are considered molecular markers since they represent enzyme variants, and enzymes are molecules. However, allozymes are in fact phenotypic markers, and as such they may be affected by environmental conditions. For example, the banding profile obtained for a particular allozyme marker may change depending on the type of tissue used for the analysis (e.g. root vs. leaf). This is because a gene that is being expressed in one tissue might not be expressed in other tissues. On the contrary, molecular markers, because they are based on differences in the DNA sequence, are not

environmentally influenced, which means that the same banding profiles can be expected at all times for the same genotype.

Applications: Allozymes have been applied in many population genetics studies, including measurements of out crossing rates (Erskine & Muehlenbauer 1991), (sub) population structure and population divergence (Freville et al. 2001). Allozymes are particularly useful at the level of conspecific populations and closely related species, and are therefore useful to study diversity in crops and their relatives (Hamrick & Godt 1997). They have been used, often in concert with other markers, for fingerprinting purposes (Tao & Sugiura 1987, Maass & Ocampo 1995), and diversity studies (Lamboy et al. 1994, Ronning & Schnell 1994, Manjunatha et al. 2003), to study interspecific relationships (Garvin & Weeden 1994), the mode of genetic inheritance (Warnke et al. 1998), and allelic frequencies in germplasm collections over serial increase cycles in germplasm banks (Reedy et al. 1995), and to identify parents in hybrids (Parani et al. 1997).

B. Molecular Markers: A molecular markers a DNA sequence that is readily detected and whose inheritance can be easily be monitored. The uses of molecular markers are based on the naturally occurring DNA polymorphism, which forms basis for designing strategies to exploit for applied purposes. A marker must to be polymorphic i.e. it must exit in different forms so that chromosome carrying the mutant genes can be distinguished from the chromosomes with the normal gene by a marker it also carries. Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two discontinuous variants or genotypes. DNA markers seem to be the best candidates for efficient evaluation and selection of plant material. Unlike protein markers, DNA markers segregate as single genes and they are not affected by the environment. DNA is easily extracted from plant materials and its analysis can be cost and labour effective. The first such DNA markers to be utilized were fragments produced by restriction digestion –the restriction fragment length polymorphism (RFLP) based genes marker. Consequently, several markers system has been developed.

What is an ideal DNA marker?

An ideal molecular marker must have some desirable properties.
1) Highly polymorphic nature: It must be polymorphic as it is polymorphism that is measured for genetic diversity studies.
2) Codominant inheritance: determination of homozygous and heterozygous states of diploid organisms.
3) Frequent occurrence in genome: A marker should be evenly and frequently distributed throughout the genome.
4) Selective neutral behaviours: The DNA sequences of any organism are neutral to environmental conditions or management practices.
5) Easy access (availability): It should be easy, fast and cheap to detect.
6) Easy and fast assay
7) High reproducibility
8) Easy exchange of data between laboratories.

It is extremely difficult to find a molecular marker, which would meet all the above criteria. A wide range of molecular techniques is available that detects polymorphism at the DNA level. Depending on the type of study to be undertaken, a marker system can be identified that would fulfill at least a few of the above characteristics (Weising et al. 1995). Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labeled probe, which is a DNA fragment of known origin or sequence. PCR-based markers involve *in vitro* amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermos table DNA polymerase enzyme. The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining and autoradiography. PCR is a versatile technique invented during the mid-1980s (Saiki et al. 1985). Ever since thermos table DNA polymerase was introduced in 1988 (Saiki et al. 1985), the use of PCR in research and clinical laboratories has increased tremendously. The primer sequences are chosen to allow base-specific binding to the template in reverse

orientation. PCR is extremely sensitive and operates at a very high speed. Its application for diverse purposes has opened up a multitude of new possibilities in the field of molecular biology.

Restriction Fragment Length Polymorphism (RFLP)

Introduction: Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of particular *Restriction Endonucleases*, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. This technique is mainly based on the special class of enzyme i.e. Restriction Endonucleases.

They have their origin in the DNA rearrangements that occur due to evolutionary processes, point mutations within the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and unequal crossing over (Schlotterer & Tautz, 1992). Size fractionation is achieved by gel electrophoresis and, after transfer to a membrane by Southern blotting; fragments of interest are identified by hybridization with radioactive labeled probe. Different sizes or lengths of restriction fragments are typically produced when different individuals are tested. Such a polymorphism can by used to distinguish plant species, genotypes and, in some cases, individual plants (Karp et al. 1998). In RFLP analysis, restriction enzyme-digested genomic DNA is resolved by gel electrophoresis and then blotted (Southern 1975) on to a nitrocellulose membrane. Specific banding patterns are then visualized by hybridization with labeled probe. Labeling of the probe may be performed with a radioactive isotope or with alternative non-radioactive stains, such as digoxigenin or fluorescein. These probes are mostly species-specific single locus probes of about 0.5–3.0 kb in size, obtained from a cDNA library or a genomic library. Though genomic library probes may exhibit greater variability than gene probes from cDNA libraries, a few studies reveal the converse (Miller & Tanksley 1990, Landry & Michelmore 1987).

Advantages: RFLPs are generally found to be moderately polymorphic. In addition to their high genomic abundance and their random distribution, RFLPs have the advantages of showing codominant alleles and having high reproducibility. RFLP markers were used for the first time in the construction of genetic maps by Botstein et al. (1980). RFLPs, being codominant markers, can detect coupling phase of DNA molecules, as DNA fragments from all homologous chromosomes are detected. They are very 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 (Winter & Kahl, 1995).

Disadvantages: The of utility RFLPs has been hampered due to the large quantities (1–10 μg) of purified, high molecular weight DNA are required for each DNA digestion and Southern blotting. Larger quantities are needed for species with larger genomes, and for the greater number of times needed to probe each blot. The requirement of radioactive isotope makes the analysis relatively expensive and hazardous. The assay is time-consuming and labour-intensive and only one out of several markers may be polymorphic, which is highly inconvenient especially for crosses between closely related species. Their inability to detect single base changes restricts their use in detecting point mutations occurring within the regions at which they are detecting polymorphism.

Applications: RFLPs can be applied in diversity and phylogenetic studies ranging from individuals within populations or species, to closely related species. RFLPs have been widely used in gene mapping studies because of their high genomic abundance due to the ample availability of different restriction enzymes and random distribution throughout the genome (Neale & Williams 1991). They also have been used to investigate relationships of closely related taxa (Miller & Tanksley 1990; Lanner et al. 1997), as fingerprinting tools (Fang et al. 1997), for diversity studies (Debreuil et al. 1996), and for studies of hybridization and introgression, including studies of gene flow between crops and weeds (Brubaker & Wendel 1994, Clausen & Spooner 1998, Desplanque et al. 1999). RFLP markers were used for the first time in the construction of genetic maps by Botstein et al.1980. A set of RFLP genetic markers

provided the opportunity to develop a detailed genetic map of lettuce (Landry et al. 1987).

Random Amplified Polymorphic DNA (RAPD)

Introduction: RAPD is a PCR-based technology. The method is based on enzymatic amplification of target or random DNA segments with arbitrary primers. In 1991 Welsh and McClelland developed a new PCR-based genetic assay namely randomly amplified polymorphic DNA (RAPD). This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermo cyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals (William et al.1993). However, due to the stoichastic nature of DNA amplification with random sequence primers, it is important to optimize and maintain consistent reaction conditions for reproducible DNA amplification. RAPDs are DNA fragments amplified by the PCR using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1–10 genomic sites simultaneously. Amplified products (usually within the 0.5–5 kb size range) are separated on agarose gels in the presence of ethidium bromide and view under ultraviolet light (Jones et al. 1997) and presence and absence of band will be observed. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites. Each product is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands that are complementary to the primer. Kesseli et al. (1994) compared the levels of polymorphism of two types of molecular markers, RFLP and RAPDs, as detected between two cultivars of lettuce in the construction of a genetic linkage map. RFLP and RAPD markers showed similar distributions throughout the genome, both identified similar levels of polymorphism. RAPD loci, however, were identified more rapidly.

Advantages: The main advantage of RAPDs is that they are quick and easy to assay. Because PCR is involved, only low quantities of template DNA are required, usually 5–50 ng per reaction. Since random primers are commercially available, no sequence data for primer construction are needed. Moreover, RAPDs have a very high genomic abundance and are randomly distributed throughout the genome. They are dominant markers and hence have limitations in their use as markers for mapping, which can be overcome to some extent by selecting those markers that are linked in coupling (Williams et al. 1993). RAPD assay has been used by several groups as efficient tools for identification of markers linked to agronomically important traits, which are introgressed during the development of near isogenic lines.

Disadvantages: The main drawback of RAPDs is their low reproducibility (Schierwater & Ender 1993), and hence highly standardized experimental procedures are needed because of their sensitivity to the reaction conditions. RAPD analyses generally require purified, high molecular weight DNA, and precautions are needed to avoid contamination of DNA samples because short random primers are used that are able to amplify DNA fragments in a variety of organisms. Altogether, the inherent problems of reproducibility make RAPDs unsuitable markers for transference or comparison of results among research teams working in a similar species and subject. As for most other multilocus techniques, RAPD markers are not locus-specific, band profiles cannot be interpreted in terms of loci and alleles (dominance of markers), and similar sized fragments may not be homologous. RAPD markers were found to be easy to perform by different laboratories, but reproducibility was not achieved to a satisfactory level (Jones et al. 1997) and, therefore, the method was utilized less for routine identifications. RAPD marker diversity was used also applied for diversity studies within and among some other Asteraceae species (Esselman et al. 2000).

Applications: The application of RAPDs and their related modified markers in variability analysis and individual-specific genotyping has largely been carried out, but is less popular due to problems such

as poor reproducibility faint or fuzzy products, and difficulty in scoring bands, which lead to inappropriate inferences. RAPDs have been used for many purposes, ranging from studies at the individual level (e.g. genetic identity) to studies involving closely related species. RAPDs have also been applied in gene mapping studies to fill gaps not covered by other markers (Williams et al. 1990, Hadrys et al. 1992). Monteleone et al. (2006) used this technique for the distinguish *mugo* and *uncinata* their subspecies. Variants of the RAPD technique include Arbitrarily Primed Polymerase Chain Reaction (AP-PCR), which uses longer arbitrary primers than RAPDs, and DNA Amplification Fingerprinting (DAF) that uses shorter, 5–8 bp primers to generate a larger number of fragments. Multiple Arbitrary Amplicon Profiling (MAAP) is the collective term for techniques using single arbitrary primers.

AFLP (Amplified Fragment Length Polymorphism)

Introduction: Amplified fragment length polymerphism (AFLP), which is essentially intermediate between RFLPs and PCR. AFLP is based on a selectively amplifying a subset of restriction fragments from a complex mixture of DNA fragments obtained after digestion of genomic DNA with restriction endonucleases. Polymorphisms are detected from differences in the length of the amplified fragments by polyacrylamide gel electrophoresis (PAGE) (Matthes *et al.* 1998) or by capillary electrophoresis. The technique involves four steps: (1) restriction of DNA and ligation of oligonucletide adapters (2) preselective amplification (3) selective amplification (4) gel analysis of amplified fragments. AFLP is a DNA fingerprinting technique, which detects DNA restriction fragments by means of PCR amplification. AFLP involves the restriction of genomic DNA, followed by ligation of adaptors complementary to the restriction sites and selective PCR amplification of a subset of the adapted restriction fragments. These fragments are viewed on denaturing polyacrylamide gels either through autoradiographic or fluorescence methodologies (Vos et al. 1995, Jones et al. 1997). AFLPs are DNA fragments (80–500 bp) obtained from digestion with restriction enzymes, followed by ligation of oligonucleotide adapters to the digestion products and selective amplification by the PCR. AFLPs therefore involve both RFLP and PCR. The PCR primers consist of a core sequence (part of the adapter), and a restriction enzyme specific sequence and 1–5 selective nucleotides (the higher the number of selective nucleotides, the lower the number of bands obtained per profile). The AFLP banding profiles are the result of variations in the restriction sites or in the intervening region. The AFLP technique simultaneously generates fragments from many genomic sites (usually 50–100 fragments per reaction) that are separated by polyacrylamide gel electrophoresis and that are generally scored as dominant markers.

Selective Fragment Length Amplification (SFLA) and Selective Restriction Fragment Amplification (SRFA) are synonyms sometimes used to refer to AFLPs. A variation of the AFLP technique is known as Selectively Amplified Microsatellite Polymorphic Locus (SAMPL). Witsenboer et al. (1997) studied the potential of SAMPL (Selectively Amplified Microsatellite Polymorphic Locus) analysis in lettuce to detect PCR-based codominant microsatellite markers. SAMPL is a method of amplifying microsatellite loci using general PCR primers. SAMPL analysis uses one AFLP primer in combination with a primer complementary to microsatellite sequences (Witsenboer et al. 1997). This technology amplifies microsatellite loci by using a single AFLP primer in combination with a primer complementary to compound microsatellite sequences, which do not require prior cloning and characterization.

Advantages: The strengths of AFLPs lie in their high genomic abundance, considerable reproducibility, the generation of many informative bands per reaction, their wide range of applications, and the fact that no sequence data for primer construction are required. AFLPs may not be totally randomly distributed around the genome as clustering in certain genomic regions, such as centromers, has been reported for some crops (Alonso-Blanco et al. 1998, Young et al. 1999, Saal & Wricke 2002). AFLPs can be analyzed on automatic sequencers, but software problems concerning the scoring of AFLPs are encountered on some systems. The use of AFLP in genetic marker technologies has become the main tool due to its capability to disclose a high number of polymorphic markers by single reaction (Vos et al. 1995).

Disadvantages: Disadvantages include the need for purified, high molecular weight DNA, the dominance of alleles, and the possible non-homology of comigrating fragments belonging to different loci. In addition, due to the high number and different intensity of bands per primer combination, there is the need to adopt certain strict but subjectively determined criteria for acceptance of bands in the analysis. Special attention should be paid to the fact that AFLP bands are not always independent. For example, in case of an insertion between two restriction sites the amplified DNA fragment results in increased band size. This will be interpreted as the loss of a small band and at the same time as the gain of a larger band. This is important for the analysis of genetic relatedness, because it would enhance the weight of non-independent bands compared to the other bands. However, the major disadvantage of AFLP markers is that these are dominant markers.

Applications: AFLPs can be applied in studies involving genetic identity, parentage and identification of clones and cultivars, and phylogenetic studies of closely related species because of the highly informative fingerprinting profiles generally obtained. Their high genomic abundance and generally random distribution throughout the genome make AFLPs a widely valued technology for gene mapping studies (Vos et al. 1995). AFLP markers have successfully been used for analyzing genetic diversity in some other plant species such as peanut (Herselman, 2003), soybean (Ude et al. 2003), and maize (Lübberstedt et al. 2000). This technique is useful for breeders to accelerate plant improvement for a variety of criteria, by using molecular genetics maps to undertake marker-assisted selection and positional cloning for special characters. Molecular markers are more reliable for genetic studies than morphological characteristics because the environment does not affect them. SAMPL is considered more applicable to intraspecific than to interspecific studies due to frequent null alleles. AFLP markers are useful in genetic studies, such as biodiversity evaluation, analysis of germplasm collections, genotyping of individuals and genetic distance analyses. The availability of many different restriction enzymes and corresponding primer combinations provides a great deal of flexibility, enabling the direct manipulation of AFLP fragment generation for defined applications (e.g. polymorphism screening, QTL analysis, genetic mapping).

Minisatellites, Variable Number of Tandem Repeats (VNTR)

Introduction: The term minisatellites was introduced by Jeffrey et al. (1985). These loci contain tandem repeats that vary in the number of repeat units between genotypes and are referred to as variable number of tandem repeats (VNTRs) (i.e. a single locus that contains variable number of tandem repeats between individuals) or hypervariable regions (HVRs) (i.e. numerous loci containing tandem repeats within a genome generating high levels of polymorphism between individuals). Minisatellites are a conceptually very different class of marker. They consist of chromosomal regions containing tandem repeat units of a 10–50 base motif, flanked by conserved DNA restriction sites. A minisatellite profile consisting of many bands, usually within a 4–20 kb size range, is generated by using common multilocus probes that are able to hybridize to minisatellite sequences in different species. Locus specific probes can be developed by molecular cloning of DNA restriction fragments, subsequent screening with a multilocus minisatellite probe and isolation of specific fragments. Variation in the number of repeat units, due to unequal crossing over or gene conversion, is considered to be the main cause of length polymorphisms. Due to the high mutation rate of minisatellites, the level of polymorphism is substantial, generally resulting in unique multilocus profiles for different individuals within a population.

Advantages: The main advantages of minisatellites are their high level of polymorphism and high reproducibility.

Disadvantages: Disadvantages of minisatellites are similar to RFLPs due to the high similarity in methodological procedures. If multilocus probes are used, highly informative profiles are generally observed due to the generation of many informative bands per reaction. In that case, band profiles can not be interpreted in terms of loci and alleles and similar sized fragments may be non-homologous. In addition, the random distribution of minisatellites across the genome has been questioned (Schlötterer 2004).

Applications: The term DNA fingerprinting was introduced for minisatellites, though DNA fingerprinting is now used in a more general way to refer to a DNA-based assay to uniquely identify individuals. Minisatellites are particularly useful in studies involving genetic identity, parentage, clonal growth and structure, and identification of varieties and cultivars (Jeffreys et al. 1985a&b, Zhou et al. 1997), and for population-level studies (Wolff et al.1994). Minisatellites are of reduced value for taxonomic studies because of hypervariability.

Polymerase Chain Reaction (PCR)-sequencing

Introduction: The process of determining the order of the nucleotide bases along a DNA strand is called Sequencing. DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of all i.e. the exact order of the bases A, T, C and G in a segment of DNA.

In 1974, an American team and an English team independently developed two methods. The Americans, team was lead by Maxam and Gilbert, who used "chemical cleavage protocol", while the English, team was lead by Sanger, designed a procedure similar to the natural process of DNA replication. These methods are known as and the chemical degradation the chain termination method and were equally popular to begin with and even both teams shared the 1980 Nobel Prize, but Sanger's method became the standard because of its practicality.

PCR was a major breakthrough for molecular markers in that for the first time, any genomic region could be amplified and analyzed in many individuals without the requirement for cloning and isolating large amounts of ultra-pure genomic DNA (Schlötterer 2004). PCR sequencing involves determination of the nucleotide sequence within a DNA fragment amplified by the PCR, using primers specific for a particular genomic site. The method that has been most commonly used to determine nucleotide sequences is based on the termination of in vitro DNA replication.

Sanger's chain termination method

This method is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis.

The key to the method is the use of modified bases called Dideoxy nucleotide, due to which this method is also known as **"Sanger's Dideoxy sequencing method"**. The dideoxy method gets its name from the critical role played by these synthetic nucleotides that lack the -OH at the 3′ carbon atom of De-oxy ribose sugar. A dideoxynucleotide-for ex-dideoxythymidine triphosphate or ddTTP can be added to the growing DNA strand but when, chain elongation stops as there is no 3′ -OH for the next nucleotide to be attached. Hence, the dideoxy method is also called the chain termination method.

The procedure is initiated by annealing a primer to the amplified DNA fragment, followed by dividing the mixture into four subsamples. Subsequently, DNA is replicated in vitro by adding the four deoxynucleotides (adenine, cytocine, guanine, thymidine; dA, dC, dG and dT), a single dideoxynucleotide (ddA, ddC, ddG or ddT) and the enzyme DNA polymerase to each reaction. Sequence extension occurs as long as deoxynucleotides are incorporated in the newly synthesized DNA strand. However, when a dideoxynucleotide is incorporated, DNA replication is terminated. Because each reaction contains many DNA molecules and incorporation of dideoxynucleotides occurs at random, each of the four subsamples contains fragments of varying length terminated at any occurrence of the particular dideoxy base used in the subsample. Finally, the fragments in each of the four subsamples are separated by gel electrophoresis.

Advantages: Because all possible sequence differences within the amplified fragment can be resolved between individuals, PCR sequencing provides the ultimate measurement of genetic variation. Universal primer pairs to target specific sequences in a wide range of species are available for the chloroplast, mitochondria and ribosomal genomes. Advantages of PCR sequencing include its high reproducibility and the fact that sequences of known identity are studied, increasing the chance of detecting truly homologous differences. Due to the amplification of fragments by PCR only low quantities of template DNA (the "target"º DNA used for the initial reaction) are required, e.g. 10–100 ng per reaction. Moreover, most of the technical procedures are amenable to automation.

Disadvantages: Disadvantages include low genome coverage and low levels of variation below the species level. In the event that primers for a genomic region of interest are unavailable, high development costs are involved. If sequences are visualized by polyacrylamide gel electrophoresis and autoradiography, analytical procedures are laborious and technically demanding. Fluorescent detection systems and reliable analytical software to score base pairs using automated sequencers are now widely applied. This requires considerable investments for equipment or substantial costs in the case of outsourcing. Because sequencing is costly and time-consuming, most studies have focused on only one or a few loci. This restricts genome coverage and together with the fact that different genes may evolve at different rates, the extent to which the estimated gene diversity reflects overall genetic diversity is yet to be determined.

Applications*:* In general, insufficient nucleotide variation is detected below the species level, and PCR sequencing is most useful to address questions of interspecific and intergeneric relationships (Sanger et al. 1977, Clegg 1993a). Until recently, chloroplast DNA and nuclear ribosomal DNA have provided the major datasets for phylogenetic inference because of the ease of obtaining data due to high copy number. Recently, single- to low-copy nuclear DNA markers have been developed as powerful new tools for phylogenetic analyses (Mort & Crawford 2004, Small et al. 2004). Low-copy nuclear markers generally circumvent problems of uniparental inheritance frequently found in plastid markers (Corriveau & Coleman1988) and concerted evolution found in nuclear ribosomal DNA (Arnheim1983) that limits their utility and reliability in phylogenetic studies (Bailey et al. 2003). In addition to biparental inheritance, low-copy nuclear markers exhibit higher rates of evolution (particularly in intron regions) than cpDNA and nrDNA markers (Wolfe et al. 1987, Small et al. 2004) making them useful for closely related species. Yet another advantage is that low-copy sequences generally evolve independently of paralogous sequences and tend to be stable in position and copy number.

Microsatellites or Simple sequence Repeat (SSR)

Introduction: The term microsatellites was coined by Litt & Lutty (1989)and it also known as Simple Sequence Repeats (SSRs), are sections of DNA, consisting of tandemly repeating mono-, di-, tri-, tetra- or penta-nucleotide units that are arranged throughout the genomes of most eukaryotic species (Powell et al. 1996). Microsatellite markers, developed from genomic libraries, can belong to either the transcribed region or the non transcribed region of the genome, and rarely is there information available regarding their functions. Microsatellite sequences are especially suited to distinguish closely related genotypes; because of their high degree of variability, they are, therefore, favoured in population studies (Smith & Devey 1994) and for the identification of closely related cultivars (Vosman et al. 1992). Microsatellite polymorphism can be detected by Southern hybridisation or PCR. Microsatellites, like minisatellites, represent tandem repeats, but their repeat motifs are shorter (1–6 base pairs). If nucleotide sequences in the flanking regions of the microsatellite are known, specific primers (generally 20–25 bp) can be designed to amplify the microsatellite by PCR. Microsatellites and their flanking sequences can be identified by constructing a small-insert genomic library, screening the library with a synthetically labelled oligonucleotide repeat and sequencing the positive clones. Alternatively, microsatellite may be identified by screening sequence databases for microsatellite sequence motifs from which adjacent primers may then be designed. In addition, primers may be used that have already been designed for closely related species. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation in the number of repeat units of a microsatellite, resulting in length polymorphisms that can be detected by gel electrophoresis. Other causes have also been reported (Matsuoka et al. 2002).

Advantages: The strengths of microsatellites include the codominance of alleles, their high genomic abundance in eukaryotes and their random distribution throughout the genome, with preferential association in low-copy regions (Morgante et al. 2002). Because the technique is PCR-based, only low quantities of template DNA (10–100 ng per reaction) are required. Due to the use of long PCR primers, the reproducibility of microsatellites is high and analyses do not require high quality DNA. Although microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the

size ranges of the alleles of different loci do not overlap (Ghislain et al. 2004). This decreases significantly the analytical costs. Furthermore, the screening of microsatellite variation can be automated, if the use of automatic sequencers is an option EST-SSR markers are one class of marker that can contribute to 'direct allele selection', if they are shown to be completely associated or even responsible for a targeted trait (Sorrells & Wilson 1997).Yu et al. (2004) identified two EST-SSR markers linked to the photoperiod response gene (ppd) in wheat. In recent years, the EST-SSR loci have been integrated, or genome-wide genetic maps have been prepared, in several plant (mainly cereal) species. A large number of genic SSRs have been placed on the genetic maps of wheat (Yu et al.2004, Nicot et al. 2004, Holton et al. 2002, Gao et al. 2004).

Microsatellites can also be implemented as monolocus, codominant markers by converting individual microsatellite loci into PCR-based markers by designing primers from unique sequences flanking the microsatellite. Microsatellite containing genomic fragment have to be cloned and sequenced in order to design primers for specific PCR amplification. This approach was called sequence-tagged microsatellite site (STMS) (Beckmann & Soller 1990).. In the longer term, development of allele-specific markers for the genes controlling agronomic traits will be important for advancing the science of plant breeding. In this context, genic microsatellites are but one class of marker that can be deployed, along with single nucleotide polymorphisms and other types of markers that target functional polymorphisms within genes. The choice of the most appropriate marker system needs to be decided upon on a case by case basis and will depend on many issues, including the availability of technology platforms, costs for marker development, species transferability, information content and ease of documentation.

Disadvantages: One of the main drawbacks of microsatellites is that high development costs are involved if adequate primer sequences for the species of interest are unavailable, making them difficult to apply to unstudied groups. Although microsatellites are in principle codominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring. The potential presence of null alleles increases with the use of microsatellite primers generated from germplasm unrelated to the species used to generate the microsatellite primers (poor "crossspecies amplification"). Null alleles may result in a biased estimate of the allelic and genotypic frequencies and an underestimation of heterozygosity. Furthermore, the underlying mutation model of microsatellites (infinite allele model or stepwise mutation model) is still under debate. Homoplasy may occur at microsatellite loci due to different forward and backward mutations, which may cause underestimation of genetic divergence. A very common observation in microsatellite analysis is the appearance of stutter bands that are artifacts in the technique that occur by DNA slippage during PCR amplification. These can complicate the interpretation of the band profiles because size determination of the fragments is more difficult and heterozygotes may be confused with homozygotes. However, the interpretation may be clarified by including appropriate reference genotypes of known band sizes in the experiment.

Applications: In general, microsatellites show a high level of polymorphism. As a consequence, they are very informative markers that can be used for many population genetics studies, ranging from the individual level (e.g. clone and strain identification) to that of closely related species. Conversely, their high mutation rate makes them unsuitable for studies involving higher taxonomic levels. Microsatellites are also considered ideal markers in gene mapping studies (Hearne et al. 1992, Morgante & Olivieri 1993, Jarne & Lagoda 1996). Molecular markers have proven useful for assessment of genetic variation in germplasm collections (Mohammadi & Prasanna 2003). Expansion and contraction of SSR repeats in genes of known function can be tested for association with phenotypic variation or, more desirably, biological function (Ayers et al.1997). Several studies have found that genic SSRs are useful for estimating genetic relationship and at the same time provide opportunities to examine functional diversity in relation to adaptive variation (Eujayl et al.2001, Russell et al. 2004).

Inter Simple Sequence Repeats (ISSR)

Introduction: ISSRs are DNA fragments of about 100–3000 bp located between adjacent, oppositely oriented microsatellite regions. This technique, reported by Zietkiewicz et al. (1994) primers based

Table 1. Classification of markers.

S.No.	Name of the Technique		Discoverer
A.	**Biochemical markers**	Allozymes	Tanksley and Orton 1983; Kephart 1990; May 1992
B.	**Molecular markers**		
	i) Non-PCR[2] based techniques	Restriction Fragment Length Polymorphisms (RFLP)	Botstein *et al.* 1980; Neale and Williams 1991
		Minisatellites or Variable Number of Tandem Repeats (VNTR)	Jeffreys *et al.*. 1985
	ii) PCR-based techniques		
	DNA sequencing	Multi-copy DNA, Internal Transcribed Spacer regions of nuclear ribosomal genes (ITS)	Takaiwa *et al.* 1985; Dillon *et al.* 2001
		Single-copy DNA, including both introns and exons	Sanger *et al.* 1977; Clegg 1993a
	Sequence-Tagged Sites (STS)	Microsatellites, Simple Sequence Repeat (SSR), Short Tandem Repeat (STR), Sequence Tagged Microsatellite (STMS) or Simple Sequence Length Polymorphism (SSLP)	Litt and Lutty (1989),Hearne *et al.* 1992; Morgante and Olivieri 1993; Jarne and Lagoda 1996
		Amplified Sequence Length Polymorphism (ASLP)	Maughan *et al.* 1995
		Sequence Characterized Amplified Region (SCAR)	Michelmore *et al.* (1991); Martin *et al.* (1991); Paran and Michelmore 1993
		Cleaved Amplified Polymorphic Sequence (CAPS)	Akopyanz *et al.* 1992; Konieczny and Ausubel 1993
		Single-Strand Conformation Polymorphism (SSCP)	Hayashi 1992
		Denaturing Gradient Gel Electrophoresis (DGGE)	Riedel *et al.* 1990
		Thermal Gradient Gel Electrophoresis (TGGE)	Riesner *et al.* 1989
		Heteroduplex Analysis (HDA)	Perez *et al.* 1999; Schneider *et al.* 1999
		Denaturing High Performance Liquid Chromatography (DHPLC)	Hauser *et al.* 1998; Steinmetz *et al.* 2000; Kota *et al.* 2001
	Multiple Arbitrary Amplicon Profiling (MAAP) Caetano-Anolles 1996; Caetano-Anolles *et al.* 1992		
		Random Amplified Polymorphic DNA (RAPD)	Williams *et al.* 1990; Hadrys *et al.* 1992
		DNA Amplification Fingerprinting (DAF)	Caetano-Anolles *et al.* 1991
		Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)	Welsh and McClelland 1990; Williams *et al.* 1990
		Inter-Simple Sequence Repeat (ISSR)	Zietkiewicz *et al.* 1994; Godwin *et al.* 1997
		Single Primer Amplification Reaction (SPAR)	Staub *et al.* 1996
		Directed Amplification of Minisatellites DNA (DAMD)	Heath *et al.* 1993; Somers and Demmon 2002
		Amplified Fragment Length Polymorphism (AFLP)	Vos *et al.* 1995
		Selectively Amplified Microsatellite Polymorphic Loci (SAMPL)	Witsenboer *et al.* 1997

on microsatellites are utilized to amplify inter-SSR DNA sequences. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16–18 bp). About 10–60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size. Techniques related to ISSR analysis are Single Primer Amplification Reaction (SPAR) that uses a single

primer containing only the core motif of a microsatellite, and Directed Amplification of Minisatellite region DNA (DAMD) that uses a single primer containing only the core motif of a minisatellite.

Advantages: The main advantage of ISSRs is that no sequence data for primer construction are needed. Because the analytical procedures include PCR, only low quantities of template DNA are required (5–50 ng per reaction). Furthermore, ISSRs are randomly distributed throughout the genome. This is mostly dominant marker, though occasionally its exhibits as codominance.

Disadvantages: Because ISSR is a multilocus technique; disadvantages include the possible non-homology of similar sized fragments. Moreover, ISSRs, like RAPDs, can have reproducibility problems.

Applications: Because of the multilocus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Godwin et al. 1997, Zietkiewicz et al. 1994, Gupta et al. 1994).

Single-Strand Conformation Polymorphism (SSCP)

Introduction: SSCPs are DNA fragments of about 200–800 bp amplified by PCR using specific primers of 20–25 bp. Gel electrophoresis of single-strand DNA is used to detect nucleotide sequence variation among the amplified fragments. The method is based on the fact that the electrophoretic mobility of single-strand DNA depends on the secondary structure (conformation) of the molecule, which is changed significantly with mutation. Thus, SSCP provides a method to detect nucleotide variation among DNA samples without having to perform sequence reactions. In SSCP the amplified DNA is first denatured, and then subject to non-denaturing gel electrophoresis. Related techniques to SSCP are Denaturing Gradient Gel Electrophoresis (DGGE) that uses double stranded DNA which is converted to single stranded DNA in an increasingly denaturing physical environment during gel electrophoresis, and Thermal Gradient Gel Electrophoresis (TGGE) which uses temperature gradients to denature double stranded DNA during electrophoresis.

Advantages: Advantages of SSCP are the codominance of alleles and the low quantities of template DNA required (10–100 ng per reaction) due to the fact that the technique is PCR-based.

Disadvantages: Drawbacks include the need for sequence data to design PCR primers and the necessity of highly standardized electrophoretic conditions in order to obtain reproducible results. Furthermore, some mutations may remain undetected, and hence absence of mutation cannot be proven.

Applications: SSCPs have been used to detect mutations in genes using gene sequence information for primer construction (Hayashi 1992).

Cleaved Amplified Polymorphic Sequence (CAPS)

Introduction: CAPS are DNA fragments amplified by PCR using specific 20–25 bp primers, followed by digestion of the PCR products with a restriction enzyme. Subsequently, length polymorphisms resulting from variation in the occurrence of restriction sites are identified by gel electrophoresis of the digested products. CAPS have also been referred to as PCR-Restriction Fragment Length Polymorphism (PCR-RFLP).

Advantages: Advantages of CAPS include the involvement of PCR requiring only low quantities of template DNA (50–100 ng per reaction), the codominance of alleles and the high reproducibility. Compared to RFLPs, CAPS analysis does not include the laborious and technically demanding steps of Southern blot hybridization and radioactive detection procedures. These markers are codominant in nature.

Disadvantages: In comparison with RFLP analysis, CAPS polymorphisms are more difficult to find because of the limited size of the amplified fragments (300–1800 bp). Sequence data needed for synthesis of the primers.

Applications: CAPS markers have been applied predominantly in gene mapping studies (Akopyanz et al. 1992, Konieczny & Ausubel 1993).

Sequence Characterized Amplified Region (SCAR)

Introduction: Michelmore et al. and Martin et al. (1991) introduced this technique wherein the RAPD marker termini are sequenced and longer primers are designed (22–24 nucleotide bases long) for specific amplification of a particular locus. SCARs are DNA fragments amplified by the PCR using specific 15–30 bp primers, designed from nucleotide sequences established from cloned RAPD fragments linked to a trait of interest. By using longer PCR primers, SCARs do not face the problem of low reproducibility generally encountered with RAPDs. Obtaining a codominant marker may be an additional advantage of converting RAPDs into SCARs, although SCARs may exhibit dominance when one or both primers partially overlap the site of sequence variation. Length polymorphisms are detected by gel electrophoresis.

Advantages: The main advantage of SCARs is that they are quick and easy to use. In addition, SCARs have a high reproducibility and are locus-specific. Due to the use of PCR, only low quantities of template DNA are required (10–100 ng per reaction).

Disadvantages: Disadvantages include the need for sequence data to design the PCR primers.

Applications: SCARs are locus specific and have been applied in gene mapping studies and marker assisted selection (Paran & Michelmore 1993).

Single Nucleotide Polymorphism (SNP)

Introduction: A noval class of DNA markers namely single nucleotide polymorphism in genome (SNPs) has recently become highly proffered in genomic studies. The fact that in many organisms most polymorphisms result from changes in a single nucleotide position (point mutations), has led to the development of techniques to study single nucleotide polymorphisms (SNPs). Analytical procedures require sequence information for the design of allele-specific PCR primers or oligonucleotide probes. SNPs and flanking sequences can be found by library construction and sequencing or through the screening of readily available sequence databases. Once the location of SNPs is identified and appropriate primers designed, one of the advantages they offer is the possibility of high throughput automation. To achieve high sample throughput, multiplex PCR and hybridization to oligonucleotide microarrays or analysis on automated sequencers are often used to interrogate the presence of SNPs. SNP analysis may be useful for cultivar discrimination in crops where it is difficult to find polymorphisms, such as in the cultivated tomato. SNPs may also be used to saturate linkage maps in order to locate relevant traits in the genome. For instance, in *Arabidopsis thaliana* a highdensity linkage map for easy to score DNA-markers was lacking until SNPs became available (Cho et al. 1999). To date, SNP markers are not yet routinely applied in genebanks, in particular because of the high costs involved. Retrotransposon-based markers Retrotransposons consist of long terminal repeats (LTR) with a highly conserved terminus, which is exploited for primer design in the development of retrotransposon-based markers. Retrotransposons have been found to comprise the most common class of transposable elements in eukaryotes, and to occur in high copy number in plant genomes. Several of these elements have been sequenced and were found to display a high degree of heterogeneity and insertional polymorphism, both within and between species. Because retrotransposon insertions are irreversible (Minghetti & Dugaiczyk 1993, Shimamura et al. 1997), they are considered particularly useful in phylogenetic studies. In addition, their widespread occurrence throughout the genome can be exploited in gene mapping studies, and they are frequently observed in regions adjacent to known plant genes. Several variations of retrotransposon-based markers exist. Sequence-Specific Amplified Polymorphism (S-SAP) is a dominant, multiplex marker system for the detection of variation in DNA flanking the retrotransposon insertion site. Retrotransposon containing fragments are amplified by PCR, using one primer designed from the conserved terminus of the LTR and one based on the presence of a nearby restriction endonucleases site. Experimental procedures resemble those used for AFLP analysis and they are usually dominant markers. Compared to AFLP, S-SAP generally yields fewer fragments but higher levels of polymorphism (Waugh et al. 1997). Inter-retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon- Microsatellite Amplified Polymorphism (REMAP) are dominant, multiplex marker systems that examine variation in retrotransposon insertion sites. With IRAP, fragments between two retrotransposons are isolated by PCR, using outward-

facing primers annealing to LTR target sequences. In the case of REMAP, fragments between retrotransposons and microsatellites are amplified by PCR, using one primer based on a LTR target sequence and one based on a simple sequence repeat motif. IRAP as well as REMAP fragments can be separated by high-resolution agarose gel electrophoresis (Kalendar et al. 1999). Retrotrans-poson-Based Insertional Polymorphism (RBIP) is a codominant marker system that uses PCR primers designed from the retrotransposon and its flanking DNA to examine insertional polymorphisms for individual retrotransposons. Presence or absence of insertion is investigated by two PCRs, the first using one primer from the retrotransposon and one from the flanking DNA, the second using primers designed from both flanking regions. Polymorphisms are detected by simple agarose gel electrophoresis or by dot hybridization assays. A drawback of the method is that sequence data of the flanking regions is required for primer design.

Comparative qualities of marker techniques: DNA provides many advantages that make it especially attractive in studies of diversity and relationships. These advantages have included: (1) Freedom from environmental and pleiotropic effects. Molecular markers do not exhibit phenotypic plasticity, while morphological and biochemical markers can vary in different environments. DNA characters have a much better chance of providing homologous traits. Most morphological or biochemical markers, in contrast, are under polygenic control, and subject to epistatic control and environmental modification (plasticity); (2) A potentially unlimited number of independent markers are available, unlike morphological or biochemical data; (3) DNA characters can be more easily scored as discrete states of alleles or DNA base pairs, while some morphological, biochemical and field evaluation data must be scored as continuously variable characters that are less amenable to robust analytical methods; (4) Many molecular markers are selectively neutral. These advantages do not imply that other more traditional data used to characterize biodiversity are not valuable. On the contrary, morphological, ecological and other "traditional" data will continue to provide practical and often critical information needed to characterize genetic resources. Molecular markers differ in many qualities and must therefore be carefully chosen and analyzed differently with their differences in mind. To assist in choosing the appropriate marker technique, an overview of the main properties of the marker technologies described in **Table 2.**

Genomic abundance: The number of markers that can be generated is determined mainly by the frequency at which the sites of interest occur within the genome. RFLPs and AFLPs generate abundant markers due to the large number of restriction enzymes available and the frequent occurrence of their recognition sites within genomes. Within eukaryotic genomes, microsatellites have also been found to occur frequently. RAPD markers are even more abundant because numerous random sequences can be used for primer construction.
In contrast, the number of allozyme markers is restricted due to the limited number (about 30) of enzyme detection systems available for analysis. To investigate specific genomic regions by PCR sequencing, SSCP, CAPS or SCAR, sequence data of the sites of interest (structural genes mainly) are required for primer construction. Although, in principle, many sites of interest may occur within genomes, the proportion of the genome covered by PCR sequencing, SSCP, CAPS and SCAR in studies reported to date is limited. However, this is expected to change due to the wealth of sequence information that is becoming increasingly available for different crops. Genomic abundance is essential to studies where a large fraction of the genome needs to be covered, e.g. for the development of high-density linkage maps in gene mapping studies.

If, in addition to genomic abundance, genome coverage is also sought, caution should be taken in marker selection. While some markers are known to be scattered quite evenly across the genomes, others, such as some AFLP markers, sometimes cluster in certain genomic regions. For example, clustering of AFLP markers has been reported in centromeric regions of *Arabidopsis thaliana* (Alonso-Blanco et al. 1998), soybean (Young et al. 1999) and rye (Saal & Wricke 2002).

Level of polymorphism: The resolving power of genetic markers is determined by the level of polymorphism detected, which is determined by the mutation rate at the genomic sites involved. Variation at allozyme loci is caused by point mutations, which occur at low frequency (<10–6 per meiosis). Moreover, only mutations modifying the net electric

Table 2. *Summary Advantage and disadvantage of Some commonly used markers.*

Type of markers	Advantages	Disadvantages
Restriction Fragment Length Polymorphism (RFLP)	-High genomic abundance -Co-dominant markers -Highly reproducible -Can use filters many times -Good genome coverage -Can be used across species -No sequence information -Can be used in plants reliably (well-tested) -Needed for map based cloning	-Need large amount of good quality DNA -Laborious (compared to RAPD) -Difficult to automate -Need radioactive labeling -Cloning and characterization of probe are required
Randomly Amplified Polymorphic DNA (RAPD)	-High genomic abundance -Good genome coverage -No sequence information -Ideal for automation -Less amount of DNA (poor DNA acceptable) -No radioactive labeling -Relatively faster	-No probe or primer information -Dominant markers -Not reproducible -Can not be used across species -Not very well-tested
Simple Sequence Repeat (SSR)	-High genomic abundance -Highly reproducible -Fairly good genome coverage -High polymorphism -No radioactive labeling -Easy to automate -Multiple alleles	-Can not be used across species -Need sequence information -Not well-tested
Amplified Fragment Length Polymorphism (AFLP)	-High genomic abundance -High polymorphism -No need for sequence information -Can be used across species -Work with smaller RFLP fragments -Useful in preparing contig maps	-Very tricky due to changes in patterns with respect to materials used -Cannot get consistent map (not reproducible) -Need to have very good primers
Sequence-Tagged Site (STS)	-Useful in preparing contig maps -No radioactive labeling -Fairly good genome coverage -Highly reproducible -Can use filters many times	-Laborious -Cannot detect mutations out of the target sites -Need sequence information -Cloning and characterization of probe are required
ISOZYMES	-Useful for evolutionary studies -Isolation lot easier than that of DNA -Can be used across species -No radioactive labeling -No need for sequence information	-Laborious -Limited in polymorphism -Expensive (each system is unique) -Have to know the location of the tissue -Not easily automated

charge and conformation of proteins can be detected, reducing the resolving power of allozymes.

The other markers generally show intermediate levels of polymorphism, resulting from base substitutions, insertions or deletions which may alter primer annealing sites and recognition sites of restriction enzymes, or change the size of restriction fragments and amplified products. In choosing the appropriate technique, the level of polymorphism detected by the marker needs to be considered in relation to the presumed degree of genetic relatedness within the material to be studied. Higher resolving power is required when samples are more closely related. For example, analyses within species or among closely related species may call for fast evolving markers such as microsatellites. However if the objective is to study genetic relatedness at higher taxonomic levels (such as congeneric species), AFLPs or RFLPs may be a better choice because co-migrating fast-evolving markers will have less chance of being homologous. A primary guiding principle in marker selection is that more conservative markers (those having slower evolutionary rates) are needed with increasing evolutionary distance and vice-versa.

Table 3. *Comparison of the most common Used Markers*

S.N.	Feature	RFLP	RAPD	AFLP	SSRs	SNPs
1	DNA Require (µg)	10	.02	.5-1.0	.05	.05
2	DNA quality	High	High	Moderate	Moderate	High
3	PCR based	No	Yes	YES	YES	YES
4	No. of Polymorph loci analyzed	1-3	1.5-50	20-100	1-3	1
5	Ease of use	Not Easy	Easy	Easy	Easy	Easy
6	Amenable to automation	Low	Moderate	Moderate	High	High
7	Reproducibility	High	Unreliable	High	High	High
8	Development Cost	Low	Low	Moderate	High	High
9	Cost per analysis	High	Low	Moderate	Low	Low

Locus-specificity: Genetic markers using multi locus probes or primers benefit from the fact that multiple polymorphisms, representing various genomic regions, are generated simultaneously. However, a major drawback is that in general the band profiles cannot be interpreted in terms of loci and alleles, but are scored as the presence or absence of bands of a particular size. As a consequence, similar sized fragments may represent alleles from different loci and not be homologous. Therefore, locus-specific markers should be considered for questions of phylogeny or genetic relatedness. Alternatively, markers for fingerprinting studies rely on differences only, and homology is not a concern. In general, locus-specific markers generate polymorphisms of known identity, however in most cases sequencing data are needed for their development.

Codominance of alleles: Codominant markers are markers for which both alleles are expressed when co-occurring in an individual. Therefore, with codominant markers, heterozygotes can be distinguished from homozygotes, allowing the determination of genotypes and allele frequencies at loci. In contrast, band profiles of dominant markers are scored as the presence or absence of fragments of a particular size, and heterozygosity cannot be determined directly.

As a consequence, only an approximation of allele frequency can be obtained by assuming Hardy-Weinberg equilibrium in a population and estimating allele frequency from the proportion of individuals with the absent phenotype (homozygous recessive). For predominantly self-fertilizing species, heterozygosity could be disregarded and allele frequencies be considered equal to observed band frequencies. Codominant markers are preferred for most applications. The majority of codominant markers are single locus markers and hence the degree of information per assay is usually lower compared to the multilocus techniques.

Reproducibility: Reproducibility is always an important property of markers, but even more important with collaborative projects, involving the generation of data by different labs whose results need to be assembled. To obtain reproducible results, the extraction of purified, high quality DNA is a prerequisite for the majority of the marker techniques. For example, degraded and/or unpurified DNA may affect the amplification or restriction of DNA, resulting in unspecific polymorphisms. Even when purified and high molecular weight DNA is used, RAPDs often fail to show reproducible results. This is because RAPD primers are very short (10 bp), which can result in alterations in their annealing behaviors to the template DNA and the resulting band profiles as a result of small deviations in experimental conditions. Therefore, highly standardized experimental procedures are required when RAPD markers are being used. This implies the need for including repeated samples and also the inclusion of reference genotypes that represent bands of known size. Problems with reproducibility in RAPD analysis could be overcome by focusing on mapped markers for which their inheritance has already been verified.

Labour-intensity: RFLPs and minisatellites are labour-intensive markers because their analysis includes the time-consuming steps of Southern blotting, labelling of probes and hybridization. Therefore, PCR based techniques are currently preferred, some of which can even be automated to decrease the labour-intensity. PCR sequencing may still be quite labour-intensive if performed by the old

time consuming method of performing four separate sequence reactions per sample. However, automated procedures have greatly reduced labour-intensity of PCR-sequencing. The labour-intensity of the other PCR-based techniques presented varies from low to medium, depending on the methodological procedures required in addition to PCR.

Technical demands: RFLPs, minisatellites and manual PCR sequencing require higher technical skills and facilities for analysis. RFLP and minisatellite analyses require Southern blot hybridizations and may include radioactive labelling. This calls for expertise and exclusive facilities needed to comply with special legal and safety requirements. These technologies are therefore among the most technically demanding markers. Another type of technical demand arises from the use of polyacrylamide gels and automated equipment. Allozymes and PCR-based markers analyzed on agarose gels (e.g. RAPD, SCAR and microsatellites) are the least technically demanding.

Operational costs: Wages, laboratory facilities, technical equipment and consumables all contribute to the operational costs of the technologies. Relatively expensive consumables include Taq-polymerase needed for all PCR based marker types, restriction enzymes (for RFLPs, minisatellites and CAPS, and particularly the restriction enzyme MseI often used in AFLPs) and isotopes where polymorphisms are visualized by means of radioactive labelling. Polyacrylamide gels are more expensive to run than agarose gels and require visualization of polymorphisms by autoradiography or silver staining procedures, which are more costly compared to ethidium-bromide staining. Laborious and technically demanding markers, such as RFLPs, minisatellites, PCR sequencing, and those techniques being performed by automated equipment, are quite expensive. Costs of performing RAPD analyses are usually considered low. However, if measures to ensure reproducibility and low numbers of markers per primer are taken into account, costs may increase to the level of the more complex technologies. In general, operational costs of markers will vary depending on the methodology. Regarding automated procedures and technologies, while purchasing the equipment is usually very expensive and the technical expertise required is high, a significant increase in throughput may be obtained through multiplexing. An additional consideration is the emergence of cost effective "outsourcing" companies to generate marker-based and DNA sequencing data, as service laboratories keep up with efficient equipment developments. Outsourcing allows researchers to concentrate on defining questions, experimental design, data analysis and interpretation. The relative costs/benefits of outsourcing will vary in different labs according to local labour and supply costs, availability of equipment, the benefit of generating your own data for quality control or educational purposes, and the legal requirements to ship crop germplasm DNA out of a country.

Development costs: Marker development may be very time-consuming and costly when suitable probes or sequence data for primer construction are unavailable. Development of suitable probes for Southern blot hybridizations (e.g. for RFLP analysis) requires the construction of either genomic or cDNA libraries and the examination of various probe/restriction enzyme combinations for their ability to detect polymorphisms. The development of site-specific PCR primers (e.g. for microsatellite analysis) also requires the construction of libraries, which then need to be screened to identify the fragments of interest. Subsequently, the identified fragments need to be sequenced to verify their suitability and to design primers. Therefore, the investment required for marker development should be evaluated in relation to the intended range of application of the technique.
Alternatively, new genomic tools are allowing probes, primers and sequence data to be obtained from genome databases of other species, with the understanding, as in all DNA tools, that their usefulness may decrease with increasing evolutionary distance between the species.

Quantity of DNA required*:* Because only small quantities of template DNA (5–100 ng per reaction) are required, techniques, which are based on the PCR, are currently preferred. Although RFLPs and minisatellites require the largest amount of DNA (5–10 µg per reaction), Southern blot membranes may be probed several times. Intermediate quantities of DNA are needed for AFLP-analysis (0.3–1 µg per reaction) because restriction of the DNA precedes the PCR reaction. In general, consideration should be given to the use of PCR-based markers if only small amounts of DNA can be obtained **(Table-3).**

Amenability to automation: Currently, if adequate equipment and resources are available, techniques that can be automated are highly preferred because of the potential for high sample throughput. Although considerable financial investment is still required, automation may be cost effective when techniques are applied on a routine basis. As pointed out above, outsourcing of data generation may also be an alternative strategy. Nearly all techniques that are based on the PCR are amenable to a certain degree of automation.

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