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Molecular markers: A tool to identify hidden science with especial emphasis on agricultural crops

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Abstract

Spectacular development of molecular science in the field of genetics provide new era of genomics. Genetic improvement of the plant has been done through development of new generation of genetic markers. At the same time accomplishment of DNA sequence provide information which use in the identification of new species and unknown species. There are various molecular markers are used which have different principles, methodologies and application. So for choosing one of them they require careful consideration. The emergent pattern makes its unique feature for the analysis of individualistic and presently reekon to be the absolute tool for biological specification. Continuous development of novel molecular markers introduced the importance in the genomic variability and genetic diversity between the intra-specific and inter-specific species of the plant. In this chapter we discuss different types of molecular markers and their application in plants comparing other types of molecular markers.

Keywords: molecular markers, biotechnology, genetic diversity, polymorphism, PCR

Introduction

Molecular markers are genetic loci that can be simply traced and quantified in a population, correlated with a particular gene of interest [3]. In general markers identify a locus/location on map, which use a standard image displaying custom image which is referred as icon. In biological science marker is the gene or DNA sequence in which location of chromosome is known and can be used to identify the individual or the species known as genetic markers, which can be used for analysis of genetic variation (*i.e.* variation caused by mutation or alteration of gene in the genomics loci) which may observed. These genetic markers can also be used to describe the genetic diversity in organism and plant at the level of single genes. The development of the discipline of genetics would not have been possible without genetic markers such as the visible characters in peas and Drosophila, tree unfortunately does not have large number of visible Mendelian characters, this was the limitation of forest research. To overcome this limitation biochemical marker were developed for trees such as terpenes and allozymes. These biochemical markers were applied to an array of research problems. Biochemical markers have some limitation in which the major limitation is the use of small number of different markers. Different marker loci; therefore, genetic information obtained from such markers may not be very representative of genes throughout the genome. The application of plant biotechnology can be categorised into two class of one of them have same goal as convention breeding method *e.g.* improvement of crop plant for high yield, quality improvement, resistance to diseases and abiotic tolerance and other is completely novel application where plant use as bioreactor for the development of pharmaceuticals vaccine and biodegradable plastic.

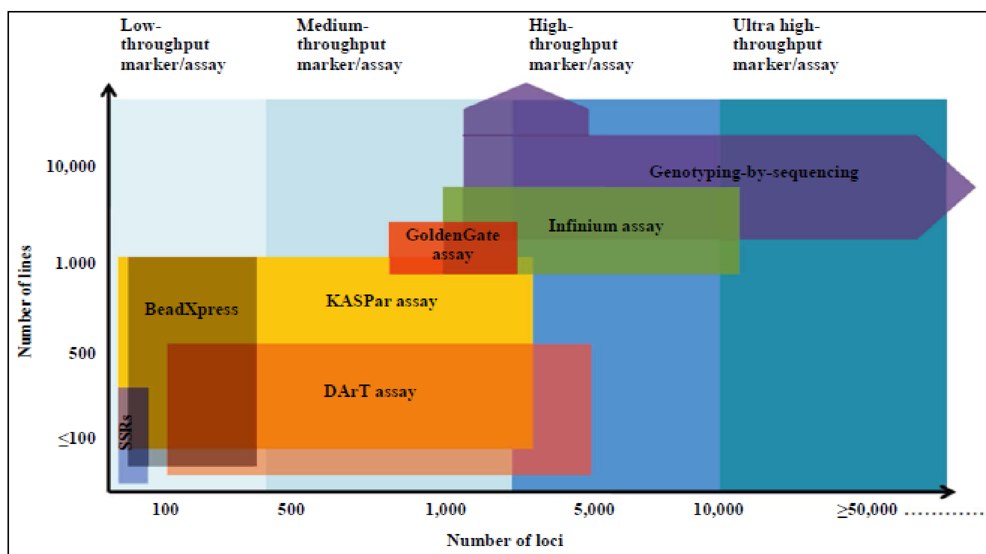
In this effort the discovery of PCR was landmark in 1990s. Now a days PCR become unique method which brought a new class of DNA profiling markers. This alleviate the development of gene tags, genetic maps, map based cloning of agronomically genetic diversity studies. Thus PCR give new opportunity to plant research and marker aided selection that reduces the time period taken in the development of novel varities. In comparison to traditional phenotypic markers, DNA markers have several advantages such as providing data which can be analysed. Present day different types of molecular markers, and have different principle and methodologies (Table 1).

Table 1: Classification of markers

Class	Marker system	Abbreviation	Remarks	References
Biochemical markers	Allozymes	Isozymes	allelic variants of enzymes encoded by structural genes	19,45
First Generation Molecular Markers	Restriction Fragment Length Polymorphism	RFLP	Based on restriction digestion and hybridization with probe	86
	Sequence Tagged Sites	STS	RFLP probes sequenced and converted in to PCR based STS markers	87
	Random Amplified Polymorphic DNA	RAPD	Random primers for PCR amplification	88
	Sequence Characterized Amplified Regions	SCAR	RAPD marker termini sequenced for designing longer primer	89
	Arbitrary Primed PCR	AP-PCR	RAPD primers of 10-15 bases in length for discrete amplification	88
	DNA Amplification Fingerprinting	DAF	Single random primer of 5 bases short length	90

Second Generation Molecular Markers	Simple Sequence Length Polymorphism	SSLP	Based on tandem repeat flanking sequence	91
	Variable Number of Tandem Repeats	VNTRs	Based on tandem repeat sequence hybridization by probe	92
	Random Amplified Micro satellite Polymorphism	RAMPO	Random primers used for amplification and then hybridized with micro satellite oligonucleotides probe	93
	Cleaved Amplified Polymorphic Products	CAPs	PCR amplified products digested by restriction enzymes	94
	Inter Simple Sequence Repeat	ISSR	Single primer based on SSR motif	95
	Amplified Fragment Length Polymorphism	AFLP	Detection of genomic restriction fragment by PCR amplification	96
	Allele Specific Associated Primers	ASAP	Specific allele sequenced and primers designed for amplification	97
Third Generation Molecular Markers	Expressed Sequence Tag markers	EST	Sequencing of random DNA clones	98
	Single Nucleotide Polymorphism	SNP	Non-gel based marker system and DNA sequence differs by single base	98
	Miniature Inverted Repeat Transposable Elements	MITE	Non autonomous transposable elements with strong target site preference	

More importantly, steady plant productivity to bestow required food for the increasing human population has become a core issue in this epoch of uncertain sudden global climatic changes, various stresses, and land confinement [68]. The emphasis of this chapter therefore reflects these changes and is focused more on the application of plant biotechnology rather than the detailed molecular biology which underlies those applications. Mostly molecular markers was developed during the last two decade of molecular biology research and have various applications mostly in crop improvement. Presently progressively developed marker systems shifted in different generation of molecular markers such as first, second, third and fourth generation. First generation and second generation include RFLP, RAPD, SSR and AFLP and third and fourth generation include SNP, DArT assay and GBS (Figure 1).



Source: http://dx.doi.org/10.1007/978-94-007-5687-8_11

Fig 1: Low to ultra-high throughout cost effective marker assay platforms for genotyping. Number of loci represent on Horizontal axis that express in a single experiment and number of lines per samples lies on vertical axis that could be genotyped in high throughout manner at low cost.

Ideal Properties

DNA markers have desirable properties such as highly polymorphic in nature, co-dominant inheritance, selective neutral behaviour, frequent occurrence in genome, easy and fast assay. Therefore, it is very difficult to find out molecular markers have all these criteria. However, depending upon the types of study to be carried out. Molecular markers when fulfilled at least few of all above can be used. Different types of molecular markers are used to analyse DNA polymorphism, generally classified as PCR-based markers and hybridization-based markers. Electrophoresis method are used to separate amplified fragments and banding pattern are detected by various method such as autoradiography and staining. Their application for variant purposes has developed multitude of new possibilities in the molecular biology fields.

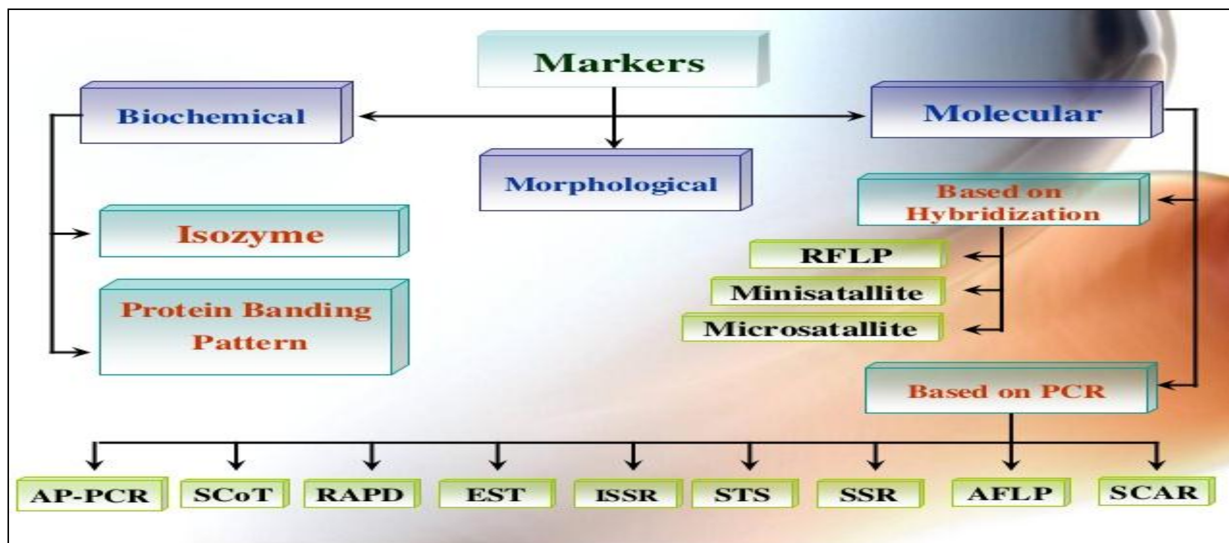
Molecular Markers

Generally, in nature a marker should be polymorphic which means that it will exist in different forms, so they can differentiate the chromosome which carry mutant gene or chromosome with normal gene in the form of marker it also carries. This polymorphism in the marker can be detected at three levels *i.e* phenotypic level (by Morphological markers), difference in proteins (by Biochemical markers) or difference in the nucleotide sequence of DNA (by Molecular markers) (Figure.2).

Morphological Markers

Morphological markers generally qualitative traits that can be observed visually. They have naturally occurring or resultant of mutagenesis experiments. These markers are highly influenced by environmental factors. The conclusions

reached through applying morphological markers are often not completely accurate when they used for the evaluation of farm plant genetics, because these markers based on subjective judgments and descriptions.



Source: http://www.slideshare.net/FAOoftheUN/molecular-markers-types-and-applications

Fig 2: Molecular markers systemic roadmap.

Biochemical Markers (*e.g.* Isozymes)

These markers are proteins based produced by gene expression. These proteins can be identified and isolated through the electrophoresis and staining technique. Isozyme the different molecular form of same enzyme that catalyses the same reaction, are proteins, that can be identified through the enzymatic associated coloured reaction. This process can be done on electropherogram and these are the product of various alleles of one or several genes. Isozyme (or isoenzyme) was the first biochemical markers extensively used to study of plant, animals and insects *etc.* [47]. Initially isozymes were first utilised for the taxonomy study of plant pathogenic fungi [41, 63]. During this method crude protein is extracted which further separated through electrophoresis on starch or non-denaturing Poly acrylamide gels (PAGE). Appropriate enzymes are added which is mandatory for the specific activity of each enzyme and for the visualization of isozymes zones. And resulting isozymes banding patterns (zymograms) are used to find out genetic relationships. By comparing 18 isozymes loci, three *Phytophthora* species (*P. cactorum, P. cambivora*, *P. cinnamomi*) could be distinguished and systematic of 12 papillate *Phytophthora* species were re-evaluated [47].

D.N.A Based Molecular Markers

Molecular markers are also known as DNA markers. The sequence of DNA was easily detected and whose inheritance can be monitored very easily. Molecular markers are used on the bases on naturally occurring DNA polymorphism, that's forms the basis for designing strategies to exploit applied purpose. A marker has some desirable features like

- It must be polymorphic
- Co-dominant inheritance
- It should be evenly and frequently distributed
- It should be reproducible, fast, cheap and easy to detect.

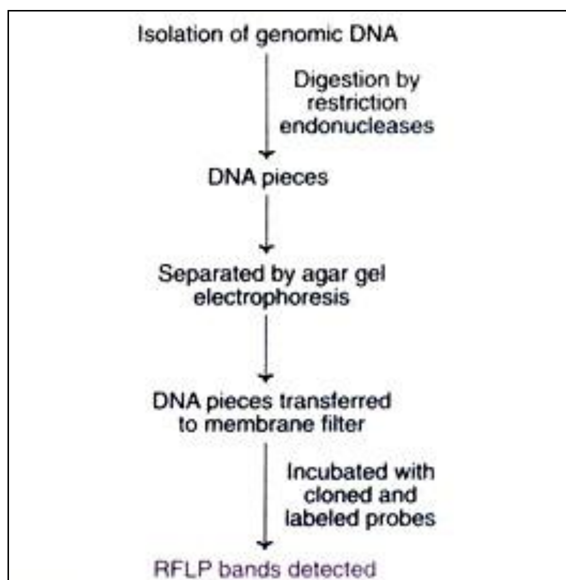
Now a days there are various protocols for extraction of DNA from different samples. In the phylogenetic analysis of fungal pathogens, DNA probes were used among the first molecular markers [9, 45]. DNA isolation is most important step in DNA based molecular markers study. Generally fungal biomass is collected through liquid culture and crushed with help of liquid nitrogen to isolate genomic DNA. But in some case like those organism which cannot be culture from these species the DNA is extracted directly from their spores collected from infected plant tissue.

DNA Fingerprinting

DNA fingerprinting is also known as a DNA profile. This technique is useful in many areas: paternity tests, criminal cases, evolution studies, evaluation of biodiversity, mapping of genes, and genetic tests. Additionally, there are several methods for accessing the DNA profile of an individual, depending on the type of test being done.

Restriction Fragment Length Polymorphism (RFLP)

Developed by Alec Jeffreys in England, in the beginning of the 1980s, this technique is based on the distance between restriction sites in the DNA. The RFLP technique uses special enzymes called *restriction enzymes* to cut DNA into different fragments. These restriction enzymes recognise a very specific short specific sequence of DNA and make cleavage in the DNA sequence at recognise site. After the DNA is treated with a restriction enzyme, it is cut into fragments of various sizes. The number and size of the fragments is unique to each individual. The restriction (cut) sites of a person, a corn variety, or a sheep are as unique as a fingerprint, allowing unequivocal identification of the individual. If the DNA of two individuals is cut with the same enzyme, *Eco*R V for example, two patterns of DNA fragments are produced, making it possible to distinguish them on the basis of the variation in the length of the fragments because each pattern of fragments is unique to each individual. The occurrence of many patterns of fragments with different lengths is called RFLP (Figure.3).



Source: http://www.biologydiscussion.com/plants/ molecular-marker-study-notes/10883

Fig 3: A small outline of restriction fragment length polymorphism (RFLP).

Randomly Amplified Polymorphic DNA (RAPD) Markers

These markers are generally based on random DNA segments, amplified by PCR, with single, which is a typically short primer of arbitrary nucleotide sequence [71]. Gel electrophoresis techniques are used to visualise DNA amplified product. Since, the primers are randomly selected, no prior knowledge of the DNA sequence is required. RAPDs show few limitation with data reproducibility but have major advantage such as the independence of any prior DNA sequence information and simplicity in technique [68, 11].

Arbitrarily Primed-PCR (AP-PCR)

Some variant of RAPD such as AP-PCR and DAF techniques are independently developed methodologies. In which [68] a single primer (about 10–15 nucleotides long) is used and involve amplification for initial two PCR cycles at low stringency. There after increasing the annealing temperature the remaining cycle was carried out at higher stringency. AP-PCR and DAF are simplified as fragments and can be fractioned by using agarose gel electrophoresis but it is not popular because it involved autoradiography while in DAF technique involved the usage of a single arbitrary primers shorter than ten nucleotides for PCR amplification [5] and the PCR amplicons are analysed using polyacrylamide gel thereafter with silver staining dye.

Start Codon Targeted (ScoT) Marker

In recent years, there are other new alternative and promising markers techniques which have emerged, now a days. SCoT polymorphisms PCR use a single primer (18mer) and more annealing temperature (Tm), 50°C [45]. Markers are observed by agarose gel electrophoresis and staining making this method applicable for many plant research labs with common equipments [45]. Low recombination and random markers such as SSRs and RAPDs making it possible to be used directly in marker-assisted breeding programs [9]. These markers have been easily used to identify cultivars, to know genetic diversity, for quantitative trait loci (QTL) mapping and DNA fingerprinting in different species, including peanut, garbanzo and rice etc. [45, 73].

Amplified Fragment Length Polymorphism (AFLP®) Markers

These marker is the combination of both RFLP and RAPD markers. During initiation, genomic DNA is directly restricted and at the both end of these restriction fragments oligonucleotide adapters are ligated. During second step now, these fragments are selectively PCR amplified. Lastly, the amplicons are separated on gel electrophoresis and visualized, resulting in a unique reproducible fingerprint for every individuals.

Specialized algorithms and software have been developed now a days, which are able to find such markers and scoring them co-dominantly [40]. In addition the literature wrapping AFLP marker development since 1995 [11, 47, 65, 68] a review by [38] also describe the new method for AFLP technique in the genomic era.

Simple Sequence Repeats (SSR)

SSR also known as microsatellite were developed during 1990s. These are the random tandem repeats of short nucleotides (2-6bp). These markers are mostly highly polymorphic normally found in animals and plant species [28]. SSR markers are taken from non-coding genomic regions, such as GSSs and BACs, that's why, development of microsatellite markers used to be expensive and highly laborious [42]. This method is inexpensive, easy and simple detection through gel electrophoresis for large number of samples. Polymorphism is depend on the differences in the number of repeats in different genotypes [13]. Longer penta repeats polymorphism are easier to differentiate in a method of detection as well as longer repeat be more steely [30].

Recently, for various plant species, SSR markers simply produced *in silico* by the easily availability of the large scale (expressed sequence tag) EST. Since EST sequencing projects have produced sequence data which is present online and can be screened for characterization of SSR [68]. Compared with RFPL and RAPD, the high degree of polymorphism of SSR markers their locus specific nature and co-dominant nature make them unique for different purposes as well as in practical plant breeding. Therefore due to the vast genome coverage and higher variable nature SSR or microsatellite markers become markers of choice for any array of application in plant (Figure.4).

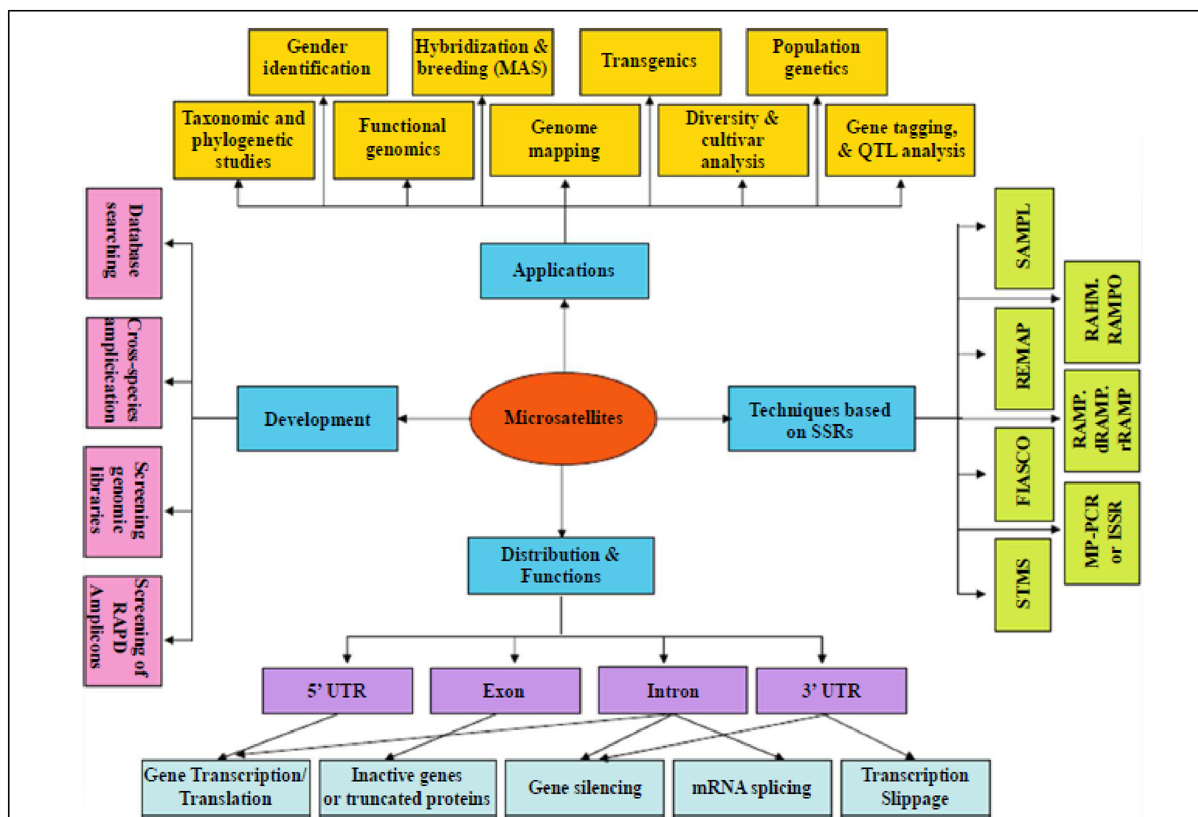


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Fig 4: Microsatellites-a summary of development, distribution, functions and applications.

Inter Simple Sequence Repeat (ISSR)

In eukaryotic genomes, microsatellites or simple sequence repeats (SSRs) are universal.

This marker include DNA segments amplification available in between two identical microsatellite repeat regions oriented in an opposite direction. This uses SSR as primers in a single primer PCR reaction targeting multiple genomic loci. For ISSRs, these microsatellite repeats used as primers. The used primers can be unanchored [19, 39, 72] or mostly anchored at 3` or 5` end [76]. As compared to RAPD primers, ISSRs use longer primers (15–30 mers), which allow the further use of high Tm resulting to higher stringency. Tm depends on the GC content of the used primer and that vary from 45 to 65˚C. These amplicons are 200–2000 bp long and can be detected by both polyacrylamide gel electrophoresis and agarose gel electrophoresis. ISSRs need no prior primer sequence information [27]. This technique is quick, simple, and radioactivity use is not mandatory. Usually, ISSR markers show high polymorphism [31]. ISSRs segregate mostly as dominant markers [19, 33, 62, 66], albeit co-dominant segregation also has been found in few cases [1, 58, 66, 72]. Like RAPD, there is also a possibility that fragments have the same mobility originate from regions which are non-homologous [57]. It was reported that the reproducibility level of this technique ranged 86% to 99% [43].

Expressed Sequence TAGS (EST)

During this marker each gene must be converted or transcribed into messenger RNA (mRNA) that serves as a template for new protein synthesis, resulting mRNA then guides the synthesis of a protein through a process called translation. The problem, however, is that mRNA is very unstable outside of a cell; to overcome this problem. Once the cDNA has been isolated, researchers can then sequence a few hundred nucleotides from either the 5' or 3' end to create 5' ESTs and 3' ESTs, respectively. This 5' EST is obtained from the exon (coding sequence) part of a transcript. On the otherhand 3' ESTs are obtained from Intron part (non-coding region) of the transcript. ESTs have been used is used. ESTs also allow the masterfull development of low-copy RFLP markers developed from ESTs (EST-RFLP) have been widely used for the fabric of high-density genetic linkage maps [23, 10] and physical maps [33]. Often EST-based RFLP markers allow comparative mapping across different species, because sequence conservation is high in the coding regions. Hence, marker development and map based cloning in one species will profit directly from data, which are available in any other species. ESTs also allow a computational approach to the development of SSR and SNP markers [8, 13], which are cheaper than the previous development strategies.

Single Nucleotide Polymorphism (SNP) Markers

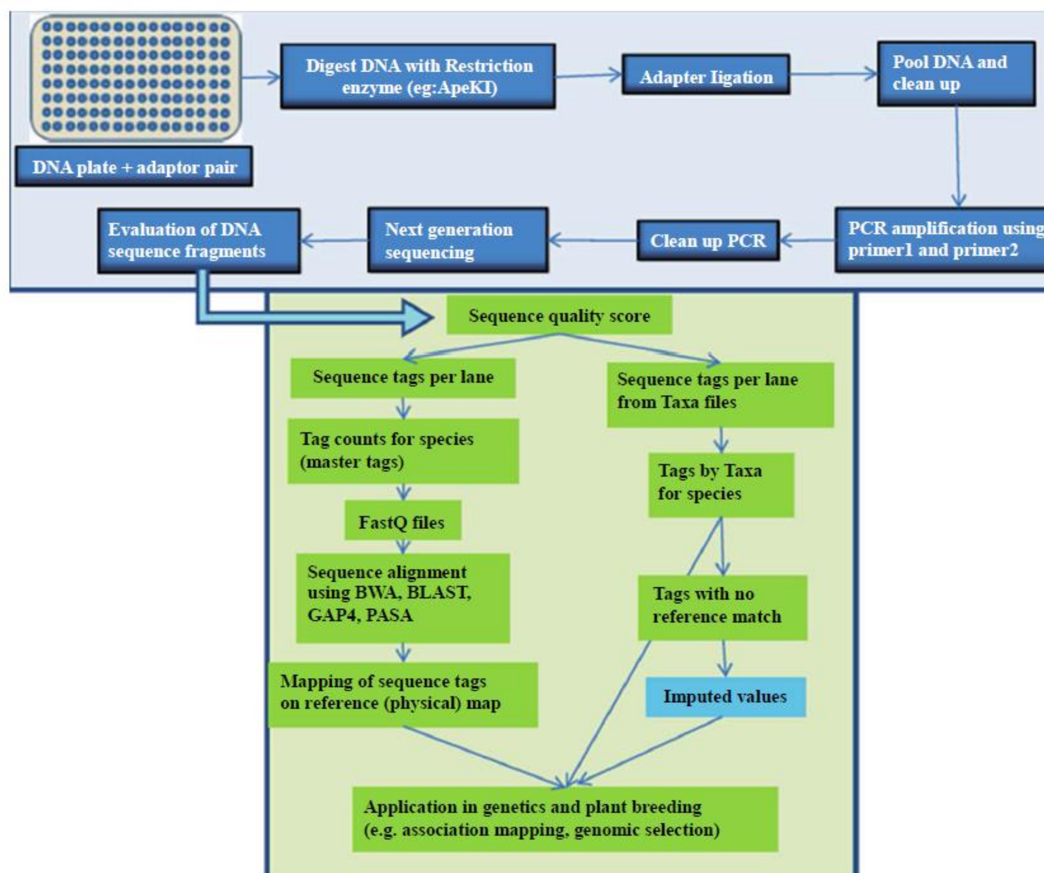
These markers are generally based on single-base pair position differences in genome sequence. Single nucleotide exchanges in genomes are in large numbers, hence these SNP markers produce a great marker density. SNP is not gel based technology that is the important advantage. In marker assisted breeding programs, large scale genotyping is required. The high density of SNP markers in a genome sequence enhance the number of chance to find polymorphisms in a target gene that are closely linked to the locus of interest and not within that provide a huge advantage over previous markers. In linkage case, it can simply happen that a linkage is lost when a marker is employed to other

populations with distinct recombination samples [74].

Genotyping by Sequencing (GBS)

With invention of new technologies day by day, rapid development and increased of NGS scafold, re-sequencing for genome broad study for genetic diversity became pertinent and affordable [70]. While in genetic diversity estimation across the genome in both coding and non-coding region this assay is bioinformatically challenging. Restriction enzymes are used in this assay for decreasing the subtlety of genomes ensured by targeted sequencing of very low proportions. A workflow of GBS has been represented in Figure. 5.

Mainly construction of GBS libraries was based on the minimisinf genome complexity with the use of restriction enzymes that allow to reach important region of genome that are unreachable to sequence capture approach [64]. One of the major drawback of this method is that it reduce the chance of data point missing and improved SNP [50].



Source: http://dx.doi.org/10.1371/journal.pone.0032253

Fig 5: A systematic representation for genotyping by sequence approach and various step involved in GBS approach has been shown (Adopted from Poland, Brown).

Cleaved Amplified Polymorphic Sequence (CAPS)

Konieczny and Ashubel [32] were the first persons who adapted the cleaved amplified polymorphic sequence (CAPS) concept for genetic mapping by developing a set of CAPS markers for use with *Arabidopsis.* These CAPS are PCR amplification of DNA fragments using specific 20-25bp primers followed by restriction of PCR amplicons using restriction endonucleases. One of the main advantages of CAPS is that PCR requires a very low concentration of template DNA (50–100 ng) and the high reproducibility. In comparison to RFLPs, it is easy and does not require technically demanding steps such as radioactive detection procedures and Southern blot hybridization. These genetic markers are co-dominant in nature. Analysis when compared with RFLP-CAPS, because of the limited size of the amplified fragments (300–1800 bp) polymorphisms are more difficult to find. Sequence data is required for primers synthesis. CAPS markers have been used dominantly in the studies of gene mapping [2, 32].

Sequence Tagged Site (STS)

In the physical mapping of the human genome and later adopted in the plants, STS was a landmark in science developed by Olsen [46]. STS markers are technical defiance in comparison to AFLP conversion. They are technically simple to use, co-dominant, suitable for high throughput, and automation.

Sequence Characterized Amplified Region (SCAR)

This technique was introduced by Michelmore [37] and Martin [40] in which the RAPD marker PCR amplified products are sequenced and longer primers are redesigned for specific amplification of a particular specific locus. These markers are DNA fragments PCR amplified by using specific 15–30 bp primers, designed from nucleotide sequences established from cloned RAPD fragments which are linked to a trait of

interest/choice. These SCARs markers have high reproducibility unlike RAPDs. Agarose Gel electrophoresis method used for identification of length polymorphism. SCARs are unique and easy to use which is the main advantage and additionally has high reducibility and locus specific. Due to the use of PCR, only low quantities (10–100 ng per reaction) of template DNA are required. It also has some limitations such as, to design the PCR primers there is a need for sequence data. Now a day's, in gene mapping studies and marker assisted selection, SCARs have been applied [49].

Diversity Arrays Technology (DArT)

DArT is the recent developed molecular markers approach/technique. With a great potential for genetic diversity and mapping studies in a number of 'orphan' crops in the Third World countries and especially used in Cassava, Wheat, Rice, Barley, Arabidopsis, Eucalyptus, and Pigeon-pea and it is non-exclusive method based on microarray hybridization, able to the typing of large number of polymorphic loci simultaneously spread all around the genome [24]. Restriction enzymes (PstI and TaqI) digestion are used for the preparation of genomic representation which is then ligated with restricted fragments to adapters. The complexity of genome is minimised by PCR using primers with complementary sequences to the adapter and selective over hangs. Labelled genomic representations included in the pool are hybridized to the discovery array [24]. For different individuals variable hybridization signal intensities are shown by polymorphic clone (DArT markers). DArT does not require prior sequence information for the species to be studies, so are appropriate to all species regardless DNA sequence information. It is cheaper and gives high reproducibility. However, it has some limitations such as it require ment of software such as, DArTdb and DArT soft, and skilled manpower for the genomic data analysis.

Table 2: Comparison of the five widely used DNA markers in plants [13, 47].

	RFLP	RAPD	AFLP	SSR	SNP
Genomic coverage	Low copy coding region	Whole genome	Whole genome	Whole genome	Whole genome
Amount of DNA required	10 µg–50	100 ng–1	100 ng–1	120 ng–50	≥ 50 ng
Quality of DNA required	High	Low	High	Medium high	High
Type of polymorphism	Single base changes, indels	Single base changes, indels	Single base changes, Indels	Changes in length of repeats	Single base changes, indels
Level of polymorphism	Medium	High	High	High	High
Effective multiplex ratio	Low	Medium	High	High	Medium to high
Inheritance	Co-dominant	Dominant	Dominant/ co-dominant	Co-dominant	Co-dominant
Type of probes/primers	Low copy DNA cDNA clone	Usually 10 bp random nucleotides	Specific Sequence	Specific sequence	Allele-specific PCR primers
Technically demanding	High	Low	Medium	Low	High
Radioactive detection	Usually yes	No	Usually yes	Usually no	No
Reproducibility	High	Low to medium	High	High	High
Time demanding	High	Low	Medium	Low	Low
Automation	Low	Medium	High	High	High
Development/start-up cost	High	Low	Medium	High	High
Proprietary rights Required	No	Yes and licensed	Yes and licensed	Yes and some licensed	Yes and some licensed
Suitable utility in diversity, genetics and breeding	Genetics	Diversity	Diversity and Genetics	All purposes	All purposes

Applications

Diagnostic in Molecular biology:

For the developments of molecular markers, same principles can be applied for the various molecular diagnostic purposes in plant including following points mentioned below.

i) Identification of plant pathogens (viruses, fungi, nematodes, bacteria, insects);
ii) Studying population structure/variations in pathogens;
iii) Identifying the presence and quantifying the presence of transgenes in transgenic foods;
iv) Following possible pollen transfer of transgenes.

All that is needed is to identify specific nucleic acid (RNA or DNA) sequences unique to the target organism and then to develop a reliable extraction/PCR analysis system such that a DNA fragments is only amplified if the target organism or target sequence is present in a sample. The methods and scale by which such analyses (and also marker-assisted selection) can be carried out are advancing rapidly.

Conclusion

New-new or advance technologies are continuously being developed to enhance by large-scale genotyping of genetic resources. New promising approach such as allele mining associated genetic and comparative genomics to obtain insight the organization and variation of genes that affect relevant phenotypic traits. Development of new technology and approach sucked up by the combining expertise of several disciplines, including statistics, molecular genetics, and bioinformatics *etc.* A variety of technique has been developed due to the intense developments in the field of molecular genetics for the analysis of genetic variation in germplasm and gene-bank arrangements especially during few decades. Molecular markers have some desirable properties such as they are highly polymorphic, co-dominant inheritance, exubrant occurrence, selectively neutral behaviour, open access, even distribution throughout the genome, easy, fast, and cheap with high reproducibility. To fulfil all these requirements no molecular markers are available yet, therefore it needs careful selection of molecular marker having atleast few of required properties.

Moreover, differential hosts are available only for a few host pathogen systems thus limiting the analysis of pathogen variability. In such cases use of molecular markers has been advocated for characterization for genetic variability in phyto

pathogenic microbes. In many ways plant pathologists are faced with more difficult diagnostic problems than are in their counter parts in human and veterinary medicine. Plant pathologists deal with many crop species and hundreds of pathogens ranging from viriods through parasitic plants and have access to fewer products to assist in diagnosis. Agriculture lacks the extensive and highly develop infrastructure as medical field for disease diagnosis which includes a wide array of practioners and supporting laboratories where sophisticated tests can be run properly. Only a handful of companies worldwide have developed products for plant disease diagnosis. However many new techniques have been bring forward during the last two decades and becoming available for application in practical disease diagnosis.

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