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Molecular Markers: New Prospects in Plant Genome Analysis

A. Sharma, Ajay G. Namdeo* and K.R.Mahadik

Department of Pharmacognosy, Poona College of Pharmacy,
Bharati Vidyapeeth University, Pune 411038 (Maharashtra) INDIA.
***Author for Correspondence** : ajay_namdeo@rediffmail.com ; ajaysharmapharma@rediffmail.com*

ABSTRACT

Accurate authentication is constantly necessary to prevent the adulteration of target plant with other plant species. For the standardization of botanical preparations chromatographic techniques (HPLC, TLC, HPTLC, UV spectroscopy, mass spectroscopy, gas chromatography, infrared and NMR spectroscopy) have limitations because the compositions and relative amount of chemicals in a species varies with growing conditions, harvesting periods, post-harvest processes and storage conditions. This can be misleading if the samples are deliberately adulterated with a marker compound. Also, it is difficult to distinguish closely related species due to similar chemical compounds. Ordinary chemical authentication was not reliable enough to produce easy-to-interpret results. Therefore, it is necessary to develop a more effective, accurate, reliable and sensitive technology for the authentication of herbs. In recent years DNA manipulation techniques have been adapted for the authentication of herbs which comprise of the molecular markers, sequencing of specific genes, and sophisticated hybridization setups such as DNA microarrays. DNA-based molecular markers have acted as resourceful tools in various fields like taxonomy, physiology, embryology, plant breeding, ecology, genetic engineering etc. The innovation of polymerase chain reaction was a milestone in the development of DNA markers. This facilitated the development of marker based gene tags, cloning of agronomically important genes, variability studies, phylogenetic analysis, selection of wanted genotypes, etc. Thus DNA markers offer several advantages over conventional phenotypic markers, as they provide data that can be analyzed objectively.

KEY WORDS

Molecular authentification, DNA markers, Polymerase chain reaction, RAPD.

ABBREVIATIONS

PCR- polymerase chain reaction; AFLP- amplified fragment length polymorphism; HPLC- high performance liquid chromatography; TLC- thin layer chromatography; HPTLC- high performance thin layer chromatography; UV- ultraviolet spectroscopy; NMR- nuclear magnetic resonance spectroscopy; DNA- deoxy ribonucleic acid; RFLP- restriction fragment length polymorphism; STS- sequence tagged sites; ASAP- allele specific associated primers; EST- expressed sequence tag markers; SSCP- single strand conformation polymorphism; RLGS- restriction landmark genomic scanning ; VNTRs- variable number of tandem repeats; HVRs- hypervariable regions; RAPD- randomly amplified polymorphic DNA; SCAR- sequence characterized amplified regions for amplification of specific band; RAMPO- randomly amplified microsatellite polymorphisms; CAPs- cleaved amplified polymorphic sequences.

INTRODUCTION

Molecular markers are biochemical constituents (e.g. secondary metabolites in plants) and macromolecules, viz. proteins and deoxyribonucleic acids (DNA) that plays a very important role in taxonomy, physiology, embryology, plant breeding, ecology, genetic engineering etc. Analysis of secondary metabolites is restricted to those plants that produce a suitable range of metabolites which can be easily analyzed and which can be distinguish between varieties. These metabolites which are being used as markers should be ideally neutral to environmental changes. Thus among all molecular markers DNA markers, are more suitable and ubiquitous to most of the living organisms.

DNA Based Molecular Markers

Genetic polymorphism is defined as the instantaneous episodes of a trait in the same population with two or more genotypes. DNA sequencing is expensive and painstaking technique but it is a clear-cut approach for identifying variations at a locus. Numerous techniques have been developed in the recent years for visualizing DNA sequence polymorphism.

The term DNA fingerprinting describes bar code like DNA fragment patterns generated by multilocus probes after electrophoretic separation of genomic DNA fragments (1). Recently, the term DNA fingerprinting/profiling is used to describe the combined use of several single locus detection systems and is used as resourceful tools for investigating diverse aspects of plant genomes. These include characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, plant breeding, and diagnostics. DNA-based markers have now become a popular means for the identification and authentication of plant genome.

Desirable Properties of Ideal DNA Markers

- Easily available
- Assay is easy and rapid
- Highly polymorphic and reproducible
- Codominant inheritance and recurrent occurrence in genome

- Selectively neutral to environmental conditions or management practices
- Data exchange between different laboratories should be easy.

It is really difficult to get molecular marker of above criteria. Depending on the type of study undertaken, a marker system can be recognized that would fulfill the above characteristics. Different types of molecular markers are utilized to evaluate DNA polymorphism and are classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In hybridization based markers DNA profiles are visualized by hybridizing the restriction endonuclease digested DNA fragment, to a labelled probe, which is a DNA fragment of known sequence while PCR based markers involve *in vitro* amplification of particular DNA sequences with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified DNA fragments are separated by electrophoresis and banding patterns are detected by different methods such as staining (using ethidium bromide dye) and autoradiography.

With the advent of thermostable DNA polymerase the use of PCR in research and clinical laboratories has increased tremendously. 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 (2, 3).

Types and Description of DNA Markers

1. Single or low copy probes

a) Restriction fragment length polymorphism (RFLP)

RFLPs are inherited naturally occurring Mendelian characters. They have their DNA rearrangements due to evolutionary processes, point mutations within the restriction enzyme recognition site, mutations within the fragments, and unequal crossing over (4).

In RFLP analysis, restriction enzyme digested genomic DNA is resolved by gel electrophoresis and western blotting (5). Specific banding patterns are then visualized by hybridization with labeled probe. These probes are mostly species-specific of about 0.5–3.0 kb in size, obtained from a cDNA library or a genomic library. The genomic libraries are easy to construct but a large number of scattered duplicates are found in inserts that makes complex patterns. This problem can be overcome by using methylation sensitive restriction enzyme *Pst*I which facilitates DNA sequences of small sizes, preferred in RFLP analysis (6-8). In contrast cDNA libraries are difficult to construct, however, they are more popular as actual genes are analyzed and they contain fewer repeat sequences (8, 9).

RFLP markers were used for constructing genetic maps. RFLPs are codominant and reliable markers in linkage analysis and breeding and can be easily determined in homozygous or heterozygous state of an individual. However, their utility has been restricted due to the large amount of DNA required for restriction digestion and Southern blotting, expensive and hazardous, time-consuming, and only one marker may be polymorphic, which is highly inconvenient especially for crosses between closely-related species and their inability to detect point mutations and polymorphism (10, 11).

b) RFLP markers converted in to PCR based-markers: Sequence-tagged sites (STS)

This approach is extremely useful for studying the relationship between various species. and are linked to some specific traits e.g. powdery mildew resistance gene or stem rust resistance gene in barley (12-14). RFLP probes specifically linked to a desired trait can be converted into PCR-based STS markers, based on nucleotide sequence of the probe giving polymorphic band pattern, to obtain specific amplicon. Tedious hybridization procedures involved in RFLP analysis can be overcome using this technique.

c) Allele-specific associated primers (ASAPs)

Allele-specific marker, (either in homozygous or heterozygous state) is sequenced and specific primers are designed for amplification of DNA template to generate a single fragment at inflexible annealing temperatures. These markers tag specific alleles in the genome and are more or less similar to SCARs (15).

d) Expressed sequence tag markers (EST)

This term was introduced by Adams *et al*. (16). These markers are obtained by partial sequencing of random cDNA clones and are useful in cloning specific genes of interest and synteny mapping of functional genes in various related organisms. ESTs are popularly used in full genome sequencing and mapping programmes for a number of organisms and for identifying active genes thus helpful in the identification of diagnostic markers. EST also helps to isolate new genes. EST markers are identified to a large extent for rice and *arabidopsis* etc (17, 18).

e) Single strand conformation polymorphism (SSCP)

This is a powerful technique for the detection of point mutations and typing of DNA polymorphism (19). SSCP (asymmetric-PCR) can identify heterozygosity of DNA fragments of the same molecular weight and detect changes in nucleotide bases due to its conformational change (20). This technique was developed to overcome the problems of reannealing and complex banding patterns wherein the denaturation step was eliminated and a large-sized sample could be loaded for gel electrophoresis, making it a potential tool for DNA polymorphism. It was found useful in the detection of heritable human diseases. It is not well developed in plants but once the suitable primers are designed for agronomically important traits selective progenies can be exploited (21).

f) Restriction landmark genomic scanning (RLGS)

This method is used for the analysis of genomic DNA of higher organisms. It uses direct labeling of genomic DNA at the restriction site and two-dimensional electrophoresis to determine restriction enzyme. This technique analyze DNA of closely-related species for obtaining polymorphic markers which can be cloned by spot target method. It has been used as a new fingerprinting technique for rice cultivars (22-24).

2. Multi locus probes

a) Repetitive DNA

About 30–90% of the genome of all the species is constituted by repetitive DNA, which is highly polymorphic in nature.

These DNA contain multi loci comprising several hundred alleles, different from each other with respect to length, sequence or both and they are interspersed in tandem arrays universally. The repetitive DNA regions play a very important role in absorbing mutations in the genome. Thus repetitive DNA and mutational forces functional in nature together form the basis of a number of marker systems that are useful for various applications in plant genome analysis. The markers belonging to this class are both hybridization-based and PCR-based.

b) Microsatellites and minisatellites

This term were coined by Lutty and Jeffrey respectively (1, 25). Both are multilocus probes creating complex banding patterns and are usually non-species specific occurring universally. They are repetitive DNA. Fingerprints generated by these probes are also known as oligonucleotide fingerprints.

Minisatellites are tandem repeats with a monomer repeat of length about 11–60 bp, while microsatellites or short tandem repeats/simple sequence repeats (STRs/SSRs) consist of 1 to 6 bp long monomer sequence that is repeated several times. 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) or hypervariable regions (HVRs) (26). Microsatellites and minisatellites thus form an ideal marker system creating complex banding patterns by simultaneously detecting multiple DNA loci. These markers are dominant fingerprinting markers and codominant STMS (sequence tagged microsatellites) markers.

c) Minisatellite and microsatellite sequences converted into PCR-based markers

Sequence-tagged microsatellite site markers (STMS)

This method includes DNA polymorphism using specific primers designed from the sequence data of a specific locus. Primers opposite to the contiguous regions of the simple sequence repeat loci yield highly polymorphic amplification products (27). Tri- and tetranucleotide microsatellites are more popular for STMS analysis because it gives clear banding pattern after PCR and gel electrophoresis (28). However, dinucleotides are generally abundant in genomes and have been used as markers (29).

Direct amplification of minisatellite DNA markers (DAMD-PCR)

This technique generates DNA probes used to detect polymorphism. DAMD-PCR clones can yield individual-specific DNA fingerprinting pattern and thus have the potential as markers for species differentiation and cultivar identification (30, 31).

d) Inter simple sequence repeat markers (ISSR)

In this technique primers based on microsatellites are utilized to amplify inter-SSR DNA sequences. These are mostly dominant markers occasionally few of them exhibit codominance (32). An unlimited number of primers can be synthesized for various combinations of di-, tri-, tetra- and pentanucleotides [(4)3 = 64, (4)4 = 256].

3) New repetitive DNA markers

a) Transposable elements -A large number of transposable repeat elements have been studied in plants, however, only a few have been used as molecular markers. Retrotransposon events promoting unequal crossing over and mediates fingerprinting has been shown to be an efficient method for detecting genetic differences between different species.

b) Alu-repeats

This approach was developed to fingerprint genotypes using semispecific primers, complementary to repetitive DNA elements called 'Alu-repeats', in human genome analysis. *Alu* repeats are a class of randomly repeated interspersed DNA that reveals extensive levels of polymorphism. These short and long interspersed nuclear elements are known as *SINES*. *Alu* elements are approximately 300 bp in size and have been suggested to be originated from special RNA species. These repeats have been studied largely in humans, while their function in plants remains largely unexplored (33).

c) Repeat complementary primers

These are the primers complementary to repetitive sequence elements used for the generation of polymorphisms, e.g. introns/exons splice junctions, tRNA genes, 5sRNA genes and Zn-finger protein genes. Primers complementary to specific exons, resulting in the amplification of the intervening introns. One of the strengths of these new strategies is that they are more amenable to automation than the conventional hybridization-based techniques (34-38).

d) Arbitrary sequence markers

Randomly amplified polymorphic DNA markers (RAPD)

RAPD markers can provide simple and reproducible fingerprint of germplasm by using single arbitrary chosen primers (39). These markers detect 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 thermocyclic amplification (40). 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 (41). RAPD assay has been used by several groups as efficient tools for identification of markers linked to agronomically important traits. These markers can detect genetic polymorphism and when linked to major genes it can be potentially used to identify morphological traits. It is also used in variability analysis and individual-specific genotyping but is less popular due to problems such as poor reproducibility, faint products, and difficulty in scoring bands, that leads to inappropriate inferences. RAPD and Eastern blotting analyses using ginsenoside Rb1 and Rgl monoclonal antibodies were employed to identify *Panax notoginseng*, *P. quinquefolius* and *P. japonicus*. RAPD was first used to differentiate the species of *Panax* spp. and thus the absence of ginsenoside Rc in the extract of *P. notoginseng* in the Eastern blot confirmed the identity of this species (42).

Arbitrary primed polymerase chain reaction (AP-PCR)

This is a special case of RAPD, wherein discrete amplification patterns are generated by employing single primers of 10–50 bases in length in PCR of genomic DNA. In the first two cycles, annealing is under non-stringent conditions. The final

products are structurally similar to RAPD products. Compared to DAF, this variant of RAPD is not very popular as it involves autoradiography. Recently, however, it has been simplified by separating the fragments on agarose gels and using ethidium bromide staining for visualization (43).

Sequence characterized amplified regions for amplification of specific band (SCAR)

This technique was introduced by Michelmore (1991) and Martin (1991) in which RAPD marker termini are sequenced and longer primers are designed (22–24 nucleotide bases long) for specific amplification of a particular locus. It shows similarity with STS markers in construction and application. The presence or absence of the band indicates variation in sequence. These are better reproducible than RAPDs and can be converted into codominant markers by digesting them with tetra cutting restriction enzymes and polymorphism can be deduced by either denaturing gel electrophoresis or SSCP. Compared to arbitrary primers, SCARs exhibit several advantages in mapping studies (codominant SCARs are informative for genetic mapping than dominant RAPDs), map-based cloning as they can be used to screen pooled genomic libraries by PCR, physical mapping, locus specificity, etc. SCARs also allow comparative mapping or homology studies among related species, thus making it an extremely adaptable concept in the near future (44-46).

Amplified fragment length polymorphism (AFLP)

This technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for DNA of any origin or complexity (47). The fingerprints are produced, without any prior knowledge of sequence, using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. This technique is reliable and is extremely useful in the detection of polymorphism between closely related genotypes (48). AFLPs are extremely useful as tools for DNA fingerprinting, cloning and mapping of variety-specific genomic DNA sequences (49-51). AFLP procedure consists of - Cutting of DNA using restriction enzyme, selective amplifications of sets of restriction fragments designed primers and gel analysis of the amplified fragments.

Randomly amplified microsatellite polymorphisms (RAMPO)

In this PCR-based strategy, genomic DNA is first amplified using arbitrary (RAPD) primers. The amplified products are then electrophoretically separated and the dried gel is hybridized with microsatellite oligonucleotide probes. Several advantages of oligonucleotide fingerprinting (52), RAPD (53) and microsatellite-primed PCR (54) are thus combined, these being the speed of the assay, the high sensitivity, the high level of variability detected and the non-requirement of prior DNA sequence information (55). This technique has been successfully employed in the genetic fingerprinting of tomato, kiwi fruit and closely-related genotypes of *D. bulbifera*.

Cleaved amplified polymorphic sequences (CAPs)

These polymorphic patterns are generated by restriction enzyme digestion of PCR products. Such digests are compared for their differential migration during electrophoresis (56, 57). PCR primer for this process can be synthesized based on the sequence information available in databank of genomic or cDNA sequences or cloned RAPD bands. These markers are codominant in nature.

Limitation of Molecular Markers in Quality Control

There are several limitations. Firstly, it is not easy to extract DNA from drug using general methods. Secondly, although the differentiation of the geographical origins by molecular markers, such as the chloroplast *mat*K gene sequence (58). ITS from nuclear rDNA (59), 18S rDNA gene (60) has been established from time to time so DNA markers may not correspond to the chemical profiles. Therefore, DNA markers together with the chemical fingerprint for quality control of drug have been investigated. For example, three species of rhizome *Curcuma* (Ezhu) including *C. wenyujin*, *C. phaeocaulis*, and *C. kwangsiensis* have been used as medicinal materials. Chemical components such as curdione, curcumol, and germacrone in the essential oil are considered as the active constituents in *R. Curcumae*. The amount of these chemicals varies among samples from different species or samples from the same species but from different regions of cultivation. Chemical fingerprints were generated from these species as the identification markers. At the same time, the 5S rDNA spacer domains of five *Curcuma* species, including adulterants were sequenced. The chemical fingerprint together with the sequence data could serve as the marker for quality control of *Curcuma species* (61). To identify the origin of *Panax notoginseng* and its seven adulterants, and to analyze *P. notoginseng* in different localities, the nuclear 18S rDNA and chloroplast genes were sequenced. HPLC fingerprinting was also used to correlate the chemical composition and geographical distribution. This study concluded that DNA markers can be applied to authenticate the easily-confused species and can help to trace their geographical origins (62). For the quality evaluation of *Pogostemon cablin* cultivated in Guangdong and Hainan, two sequences, 1.2 kb of plastid matK gene and 1.8 kb nuclear 18S rDNA gene, and two chemotypes (pogostone-type and Patchouliol-type in essential oil composition) were compared. The result showed that the sequence divergence in both matK and 18S rDNA genes among six samples of *P. cablin* were well correlated with their regions of cultivation and intraspecific chemotypes of essential oil compositions (63). Testing for unknown contaminants is extremely difficult. These limitations are expected to be eliminated by advancement of molecular technology in the future.

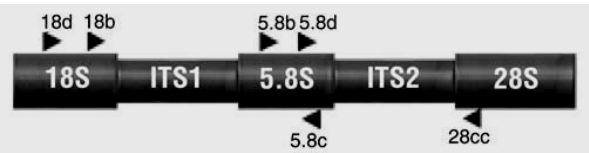

Fig. 1: ***Schematic diagram of the nuclear rDNA internal transcribed spacer region.***

18b: 5'-TAG AGG AGG GAG AAG TCG TA-3'; 18d: 5'-CAC ACC GCC CGT CGC TCC TAC CGA-3'; 28cc: 5'-ACT CGC CGT TAC TAG GGG AA-3'; 5.8b: 5'-TGA AGA ACG TAG CGA AAT GCG-3'; 5.8d: 5'-AAC CAT CGA GTC TTT GAA CGC A-3'.

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Table 1: List of plant species studied by different molecular technologies

Markers	Drugs	Comments	Refs.
RAPD	*Glycyrrhiza* species	Differentiation of four species	(64)
	Zaocys dhumnades	Identification of crude snake drugs	(65)
	Anoectochilus	Identification of two species	(66)
	Atractylodes plants	Revealed intraspecific variation	(67)
	Astragalus medicines	Differentiation of the two species	(68)
	Rabdosin serra plants	Authentication	(69)
	Amomun villosum species	Analysis of A. *villosum* and adulterants	(70)
	Scutellaria plants	Discrimination of the three species	(71)
	Panax notoginseng	Authentication of *P. notoginseng*	(72)
	Yu-ping-feng san	Identification of components	(73)
	Aconitum plants	Differentiation of *A. noveboracense* and *A. columbianum*	(74)
	Ginkgo biloba	Differentiation of the nine populations	(75)
RFLP	*Atractylodes lancea*	Revealed intraspecific variation	(76)
	Panax species	Differentiation of *P. ginseng* and *P. quinquefolius*	(77)
	Fritillaria pallidiflora	Identification	(78)
rbcL	*Belamcanda chinensis*	Analysis of *B. chinensis* and related plants	(79)
*trn*K	*Curcuma* drugs	Authentication	(80)
	Atractylodes drugs	Authentication of derived crude drugs	(81)
	Atractylodes plants	Phylogenetic analysis	(82)
*mat*K	*Panax vietnamensis*	Phylogenetic analysis	(83)
18S	*Panax notoginseng*	Analysis of homology	(84)
ITS	*Saussurea medusa*	Comparison on ITS sequences	(85)
	Herba Hedyotis Diffusae	Authentication	(86)
	Hypericum species	Genetic profiling	(87)
	Zanthexylum bungeanum Maxin	Authentication of population and adulterants	(88)
5S	*Fritillaria* species	Molecular diversity	(89)
	Radix Astragali	Species identification	(100)
	Ephedra plants	Phylogenetic analysis	(101)
12S	*Snake gallbladder*	Identification	(102)
Cyt b	*Oviductus ranae*	Authentication of original animals	(103)

Table 2: Comparsion between different molecular methods for plant genome analysis.

PARAMETERS	AFLP	RAPD	ISSR	MACROSCOPIC IDENIFICATION	MICROSCOPIC IDENIFICATION	CHEMICAL ANALYSIS
Cost	High	Low	Low	Low	High/low	Medium
Time	High	Low	Low	Low	low	Low
Reproducibility	High	Good	Good	Good	Medium	Good
Accuracy	High	Good	Good	Good	medium	Good
Starting material	Any part preferably young and fresh material	Any part preferably young and fresh material	Any part preferably young and fresh material	Whole root, leaves, fruit etc.	Whole, fragmented or powdered	Botanical extracts
Database	Electronic	Electronic	Electronic	Herbarium	Text	Text
Identification level	Intraspecific differences	Intraspecific differences	Intraspecific differences	Might not allow to the species level	Might not allow to the species level	Might not allow to the species level
Storage space	Low	Low	Low	Very high	Low	Low
Possible problems	DNAdamage, affected by secondary metabolite	DNAdamage, affected by secondary metabolite	DNAdamage, affected by secondary metabolite	Require plant in particular development stage	Lack of references for comparision	Seasional, environmental and individual variation affect chemical profile

The three rDNA subunits: 18S, 5.8S and 28S are separated by internal transcribed spacers (ITS1 and ITS2). Arrows indicate the annealing sites of the primers used for PCR amplification.

Applications of Molecular Markers in Plant Genome Analysis and Breeding

Molecular markers have large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They are extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. Genome analysis based on molecular markers becomes popularize.

Mapping and labeling of genes: Generating tools for marker-assisted selection in plant breeding

Plant improvement, either by natural selection or by plant breeding, has always depended on creating, evaluating and selecting the right combination of alleles. The exploitation of a large number of genes is required for the improvement of characteristics (90). Molecular markers trace valuable alleles in a segregating population and mapping them. After mapping these markers facilitate the analysis of complex traits into component genetic units more accurately, thus providing breeders to manage these complex units more efficiently in plant breeding programme (91).

Using RFLP markers first genome map was reported in maize, rice, *Arabidopsis*, potato, barley, banana and members of Brassicaceae (92-96). Once the outline of maps is generated, a large number of markers are used to saturate the maps. Microsatellite markers, especially STMS markers extremely useful in this regard. Owing to their quality of following clear Mendelian inheritance. About 30 microsatellites have already been assigned to five linkage groups in Arabidopsis, while their integration into the genetic linkage maps is still in progress in rice, soyabean, and maize.

Once mapped, these markers are efficiently employed in tagging several individual traits that are extremely important for a breeding programme like yield, disease resistance, stress tolerance, seed quality, etc. A large number of monogenic and polygenic loci for various traits have been identified in a number of plants, which are currently being exploited by breeders and molecular biologists to get marker-assisted selection come true. Tagging of useful genes like the ones responsible for conferring resistance to plant pathogen, synthesis of plant hormones, drought tolerance and a variety of other important developmental pathway genes, is a major target. Such tagged genes can also be used for detecting the presence of useful genes in the new genotypes generated in a hybrid programme or by other methods like transformation,etc. RFLP markers have proved their importance as markers for gene tagging and are very useful in locating and manipulating quantitative trait loci (QTL) in a number of crops (97-99). The very first reports on gene tagging were from tomato, availing the means for identification of markers linked to genes involved in several traits like water use efficiency, resistance to *Fusarium oxysporum* (the 12 gene), leaf rust resistance genes LR 9 and 24, and root knot nematodes (*Meliodogyne* sp.) (the mi gene) (100-106). Allele-specific associated primers have also exhibited their utility in genotyping of allelic variants of loci that result from both size differences and point mutations. Example leaf rust resistance wheat, resistance to the root cyst nematode *Globodera rostochiensis* in potato, and powdery mildew resistance in cereals. AFLP and SSR markers have been identified to be associated with quantitative resistance to *Globodera pallida* (stone) in tetraploid potato (107-113).

STMS markers have displayed a potential use as diagnostic markers for important traits in plant breeding programmes, e.g. (AT) 15 repeat has been located within a soybean heat shock protein gene conferring resistance to soybean mosaic virus. Several resistance genes including peanut mottle virus

(Rpv), phytopthera (Rps3) and Javanese rootknot nematode are clustered in this region of the soybean genome (114). Similar to specific markers like RFLPs, STMS and ASAPs, arbitrary markers like RAPDs have also played important role in saturation of the genetic linkage maps and gene tagging. RAPD markers used in the saturation of genetic maps (115). They have proven utility in construction of linkage maps among species where there has been inherent difficulty in producing F2 segregating populations and have large genome size, e.g. conifers. RAPD markers in near isogenic lines can be converted into SCARs and used as diagnostic markers (116, 117). SCAR/STS marker linked to the translocated segment of bread wheat carrying the *Lr28* gene (118). Newly, ISSRs(arbitrary marker) have been employed as a reliable tool for gene tagging. An ISSR marker (AG) 8YC linked with rice nuclear restorer gene, RF1 for fertility. RF1 is essential for hybrid rice production and this marker not only used for breeding but also for the purity management of hybrid rice seeds. Similarly ISSR marker $(AC)_8$ YT linked to the gene for resistance to fusarium wilt race in chickpea (119, 120). Another important use of RFLP markers has been observed in detecting gene introgression in a backcross breeding programme, and synteny mapping among closely related species. Similar utility of STMS markers has been observed for reliable preselection in a backcross breeding programme. Apart from specific markers, DAMD-based DNA fingerprinting in wheat has also been useful for monitoring backcross-mediated genome introgression in hexaploid wheat (121, 122).

Phylogeny and evolution

Earlier the theories of evolution were based on morphological and geographical variations between organisms. The techniques of molecular biology provide path-breaking information regarding time scale on which closely related species have diverged and type of genetic variations are associated with species formation (123). RFLPs have been used in evolutionary studies for assuming the relationship between the hexaploid genome of bread wheat and its ancestors (124). Similarly a number of transposon elements like *tos1-1, tos2-1* and *tos3-1* retrotransposons have been used to detect the genetic differences between different species of rice and distinguish the cultivars of Asian and African rice, *O. sativa* and *O. glaberrima*. Retroelement *Wis-2* has been found to detect genomic variation within individual plants of wheat variety and also within and between varieties of wheat. This element has also been found to occur in the genomes of other grasses like barley, rye, oats and *Aegilops* species, indicating common ancestral elements in grasses. Though RFLPs, microsatellites, minisatellites and transposons are useful for carrying out genetic variability analysis, the trend is now shifting towards the use of PCR-based markers. Specific markers are preferred over arbitrary primers. However, arbitrary primers are found to be the markers of choice in the analysis of complex genomes like wheat, where genetic variation is extremely difficult to dissect. RAPD and ISSR markers used in evolutionary studies of wheat and rice, respectively (125, 126). Specific markers like STMS (sequence-tagged microsatellite markers) ALPs (Amplicon length polymorphisms) or STS markers have proved to be extremely valuable in the analysis of gene pool variation of crops during the process of cultivar development, and classification of germplasm. These markers are extremely sensitive and can detect allelic variability during cultivar development. STS markers useful in providing seed and pollen specific markers which can be utilized for the detection of length and provide haplotype data and genotypically unique individual plants (127-131).

Diversity analysis of exotic germplasm

Following domestication, genetic variation in crop plants has continued to narrow due to continuous selection pressure for specific traits i.e. yield, thus rendering them more vulnerable to disease and insect epidemics and jeopardizing the potential for sustained genetic improvement over a long term (132, 133). Thus it is extremely important to study the genetic composition of the germplasm of existing modern-day cultivars in comparison with their ancestors and related species. This will not only provide information regarding phylogenetic relationship but also indicate a chance of finding new and useful genes. The exotic germplasm for breeding is selected on the basis of certain characteristic features such as (a) the exotic germplasm must possess a significant number of unique DNA polymorphisms (b) each exotic germplasm has to be genetically dissimilar. This is necessary to understand the genetic variations between the existing cultivars (134). STMS markers may provide useful criteria for elevating the genepool of crop plants and determine how efficient plant breeders have been in accessing preexisting forms of variation (135). AFLP has gained popularity as marker for the study of genetic polymorphism especially in species where polymorphism is extremely rare using other types of markers Example APLF studying variation in wild barley with reference to salt tolerance and associated ecogeography (136, 137). Similarly ISSR markers have been used for diversity analysis of pine, rice and also in wheat. These studies have helped in the classification of existing biodiversity among plants, which can be further useful in wild gene introgression programs (138-140).

Genotyping of cultivars

The repetitive and arbitrary DNA markers used in the genotyping of cultivars. Microsatellites like $(CT)_{10}$, $(GAA)_5$, $(AAGG)_4$, $(AAT)_6$ (ref. 123), $(GATA)_4$, $(CAC)_5$ and minisatellites used in DNA fingerprinting for the detection of genetic variation, cultivar identification and genotyping, thus useful for quantification of genetic diversity, characterization of accessions in plant germplasm collections and taxonomic studies (141-151). The first application of microsatellites in plants has been in cultivar identification, wherein microsatellites have been used to genotype unequivocally diverse materials like rice, wheat, grapevine (*Vitis vinifera*) and soybean etc. This is important especially for protection of proprietary germplasm. Similarly microsatellite markers have also been advantageous in pedigree analysis. The multi allelism of these markers facilitates comparative allelic variability detection reliably across a wide range of germplasm and allows individuals to be ubiquitously genotyped, so that gene flow and paternity can be

established. One of the most recent applications of these markers has been shown in sex identification of dioecious plants wherein microsatellite probe $(GATA)_4$ is found to reveal sex-specific differences in Southern analysis and can be used as a diagnostic marker in this system where male and female plants do not show any sex-specific morphological difference until flowering. Similarly RAPD DNA marker used for pseudo-autosomal plant sex chromosome in *Silene dioica* (L.) (152-154).

Medicinal plant breeding

ISSR-PCR has been found to be an efficient and reliable technique for the identification of zygotic plantlets in citrus interploid crosses (155). Molecular markers have been used as a tool to verify sexual and apomictic offspring of intraspecific crosses in Hypericum perforatum, a well-known antihelminthic and diuretic (156). An attempt has been made towards marker-assisted selection of fertile clones of garlic with the help of RAPD markers (157). RAPD markers have been successively used for selection of micropropogated plants of *Piper longum* for conservation (158).

Detection of adulteration/substitution

RAPD technique was adopted to identify eight types of dried Coptis rhizomes and one type of Picrorrhiza rhizome (159). P. ginseng is often substituted by *P. quinquefolius* (American ginseng). Sequence characterized amplified region (SCAR), AP-PCR, RAPD and RFLP have been successfully applied for differentiation of these plants and to detect substitution by other closely related species (160-162). Characterization of *Echinacea* species and detection of possible adulterations have been done using RAPD technique (163).

Applications in foods and nutraceuticals

DNA-based molecular markers have been used extensively for a wide range of applications in food crops and horticultural plants (164-166). These applications include study of genetic variation, cultivar identification, genotyping, cross-breeding studies, identification of disease-resistant genes, identification of quantitative-trait loci, diversity analysis of exotic germplasms, sex identification of dioecious plants, phylogenetic analysis, etc. Recently, the application of DNA-based molecular markers is being explored in the field of nutraceuticals. According to the new European Council legislation, the labelling of food or food ingredients produced from, or containing licensed genetically modified organisms must indicate the inclusion of these ingredients where they are present at or above a level of 1%. In compliance with the labelling regulation for GM foods, several countries in Europe such as Germany and Switzerland, have extensively developed PCR methods for both identification and quantification purposes. In response to reports of unlicensed GM ingredients in food in the international market, the Food Safety Authority of Ireland has completed a survey to determine the levels of GM maize ingredients in tortilla chips and taco shells on sale in Ireland, using the PCR technique. Where sufficient GM DNA was present in the sample, quantitative analysis was undertaken using real time PCR.

DNA markers as new pharmacognostic tool

Traditionally, pharmacognosy mainly addressed quality-related issues using routine botanical and organoleptic parameters of crude drugs. Pharmacognosy became more interdisciplinary because of subsequent advances in analytical chemistry. These developments added emphasis on chemoprofiling assisted characterization with chromatographic and spectroscopic techniques. The new pharmacognosy includes all aspects of drug development and discovery, where biotechnology-driven applications will play an important role.

Extensive research on DNA-based molecular markers is in progress in many research institutes all over the world. This technique remains important in plant genome research with its applications in pharmacognostic identification and analysis. Chinese researchers have applied DNA markers extensively for characterization of botanicals from the Chinese materia medica. These markers have shown remarkable utility in quality control of commercially important botanicals like *Ginseng, Echinacea, Atractylodes*. In India several agricultural universities and research institutes are actively involved in exploring DNA-based techniques in genotyping of medicinal plants. Although considerable progress has been made in DNA marker technology, applications of these techniques for characterizing semi-processed and processed botanical formulations to ensure the desirable quality remain under-utilized.

Although DNA analysis is currently considered to be cutting-edge technology, it has certain limitations due to which its use has been limited to academia. In order to establish a marker for identification of a particular species, DNA analysis of closely related species and/or varieties and common botanical contaminants and adulterants is necessary, which is a costly and time consuming process. Isolation of good-quality DNA suitable for analysis from semi-processed or processed botanicals is also a challenge. Another important issue is that DNA fingerprint will remain the same irrespective of the plant part used, while the phytochemical content will vary with the plant part used, physiology and environment. DNA fingerprinting ensures presence of the correct genotype but does not reveal the contents of the active principle or chemical constituents. Hence DNA analysis and pharmacognostic techniques for chemoprofiling such as TLC, HPTLC, etc. will have to be used hand in hand rather than in isolation. Identification of quantitative-trait loci that are closely linked to a biologically active phytochemical will prove to be useful. Several attempts have been made in recent years, to correlate DNA markers with qualitative and quantitative variations in phytochemical composition among closely related species (167-172). Proper integration of molecular techniques and analytical tools will lead to the development of a comprehensive system of botanical characterization that can be conveniently applied at the industry level for quality control of botanicals. Ayurvedic classification of medicinal plant is based on basic principles and therapeutic characters that may have a genetic basis. We have undertaken an exploratory study on the use of molecular markers for quick identification of botanical materials in crude, semi-processed and processed herbal formulations. Our strategy involves identification of species-specific marker after screening a number of species and/or varieties of the

medicinal plant using random oligonucleotide primers, followed by cloning and subsequently converting it to SCAR markers for better specificity and reproducibility. Also, application of RAPD markers has been explored for standardization of botanical formulations containing ayurvedic medicines like *Emblica officinalis* and *Tinospora cordifolia*.

Indian scenario for development of molecular markers in crop improvement programmes

Agriculture is one of the most important occupations in India with almost 70% of the population being dependent on it. A noteworthy research in conventional breeding for several years has made this country self-sufficient in many respects. However, the ever-increasing population has alarmed food security in India and attempts have been initiated to integrate modern biotechnology tools in conventional breeding to improve the most important crops such as rice, wheat and legumes. Extensive research using DNA markers is in progress in many institutions all over India. Markers tagged and mapped with specific genes have been identified and some such examples include resistance genes for blast and gall midge using RFLP- and PCR-based approaches in rice. Similarly, in wheat, leaf rust resistance gene *LR* 28 and pre-harvest sprout tolerance gene have been tagged. QTLs such as protein content in wheat and heterosis in rice have also been identified. While efforts for tagging genes providing resistance to BPH, WBPH, sheath rot and drought are going on, many attempts are also being made towards pyramiding different resistance genes for a specific disease or pest attack like blast, bacterial blight, gall midge, BPH, WBPH, etc. in rice in order to increase the field life of the crop.

Germplasm analysis to study genetic diversity is another important area in which a lot of efforts have been put in. Fingerprinting of crops like rice, wheat, chickpea, pigeonpea, pearl millet etc. is being carried out extensively. This information has potential in strategic planning of future breeding towards crop sustainability in India. Apart from use of molecular markers in crop plants, efforts are also underway in other horticultural plants. Early identification of sex in dioecious papaya using molecular marker is such example (173-181). Thus, tissue culture, plant breeding and other modern biotechnological advances have unrestrained scope in Indian agriculture.

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