

Review Article

Molecular markers in characterization of medicinal plants: An overview

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The demand for herbal medicines is increasing day-to-day because of their wide biological activity, higher safety margin than synthetic drugs. Hence, it is important to characterize the medicinal herbs. For the characterization of medicinal plants it is necessary to develop the sensitive and effective technology. Molecular markers can be employed for characterization, since the progress made in genetic markers has been exciting. DNA-based molecular markers have acted as very useful tools in various fields like taxonomy, physiology, embryology, plant breeding, ecology, genetic engineering etc. DNA based markers have their applications in fingerprinting genotypes, determining the seed purity and in phylogenetic analysis by which the conservation of the plant can be made easy. The innovation of Polymerase Chain Reaction (PCR) made the development of DNA based markers easier. Extensive research on molecular markers is in progress in many research institutes all over the world. DNA-based molecular markers have a great utility in the drug analysis and widely used for the characterization of medicinally important plant species. This review is an attempt to evaluate critically the role of different DNA based markers in the characterization of medicinal plants.

A *genetic marker* is "a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or a character". It can be described as a variation, which may arise due to mutation or alteration of nucleotide in the genomic loci that can be observed (Srivatsava *et al.*, 2009). DNA-based molecular marker helps in improvement of medicinal plant species. DNA markers are more reliable because the genetic information is unique for each species and is independent of age, physiological conditions and environmental factors (Kalpana *et al.*, 2004). The molecular marker technique efficiency is based on the amount of polymorphism it can detect in the given accessions (Leela *et al.*, 2009). Restriction

fragment length polymorphism (RFLPs) were one of the earliest molecular marker technique used for detecting variations in DNA sequence by restriction digestion of DNA with restriction enzymes. Later-on many methods were developed. A more recent way of detecting individual variations in DNA nucleotides directly is through Single Nucleotide Polymorphism (SNPs). All these methods have expanded the availability of molecular markers which facilitated the creation of genetic maps.

Description of Molecular Markers:

- RFLP (Restriction fragment length polymorphism)
- AFLP (Amplified fragment length polymorphism)

- RAPD (Random amplification of polymorphic DNA)
- VNTR (Variable number tandem repeat)
- SSRs (Simple sequence repeats)
- SNP (Single nucleotide polymorphism)
- STR (Short Tandem Repeats)

Various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphism. These are hybridization-based methods, polymerase chain reaction (PCR) based methods and sequencing-based methods.

Based on the development of these techniques in the last three decades, classified into three classes (Gupta *et al.*, 2001):

- a) The first generation molecular markers, including RFLPs, RAPDs and their modifications;
- b) The second generation molecular markers, including SSRs, AFLPs and their modified forms,
- c) The third generation molecular markers including ESTs and SNPs.

A **Restriction Fragment Length Polymorphism**, or RFLP, (commonly pronounced "rif lip"), is a technique that exploits variations in homologous DNA sequences. It refers to difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites (Gnavi *et al.*, 2010). Even though DNA characterization can be done very thoroughly by DNA sequencing techniques, RFLP analysis was developed first and was cheap enough to see wide application in localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting, and paternity testing (Srivatsava and Nidhi, 2009). Jana and Eva (1997) performed the RFLP analysis of the genomic DNA of *Hypericum perforatum* L. They stated that the occurrence of the identical RFLP patterns in some R₀

somaclones and seed-derived plants and their progenies were related to the apomictic mode of reproduction which is assumed to be prevalent in *H. perforatum*. The differences in RFLP patterns of progenies when compared with the maternal plants (1 out of 10 progenies of one control plant and 1 out of 8 progenies of one somaclone) indicated that some progenies have originated through sexual reproduction.

The phylogenetic relationship among the congeneric species (Anthemideae, Asteraceae) in Egypt has studied using RFLP markers along with RAPD and ISSR markers. In this case twenty six RFLP bands were detected after *EcoRI* and *BamHI* digestion. The average of similarity co-efficient was 0.0 to 0.5. The band products of restricted DNA of *Achillea fragrantissima*, *Artemisia arborescens*, *Artemisia Judaica*, *Matricaria aurea*, *Glebionis coronaria*, *Cotula barbata* and *Cotula cinerea* were 6, 4, 5, 4, 3, 2 and 2 bands respectively, while *Achillea santolina*, *Anacyclus monanthos* and *Matricaria recutita* showed no bands. Based on this study the species were classified as *Matricaria recutita* as out group; *Achillea fragrantissima* and *Artemisia arborescens* were closely related among the group of seven species with less genetic distance (Abd EI-Twab and Zahran, 2010). Using phenotypic evaluation and Southern blot experiments the genomic instability of tobacco plants derived from protoplast was studied. DNA polymorphism (RFLP) was displayed by three regenerates upon Southern blot hybridization and thus confirmed that in such plants alterations in the genome structure could be found and that genotypes of protoplast derived plants frequently differ from the parental genotype (Vyskot *et al.*, 1991)

The taxonomy and phylogeny of Indian *Citrus* is revisited using PCR-RFLP with the *trnD-trnT* and *rbcL-ORF* regions as well as sequence data analysis of the *trnL-trnF* intergenic spacer region of

cpDNA by Satya Narayan *et al* (2009). The study was based on 50 accessions of *Citrus* genotypes collected from wild, semi-wild and domesticated stocks. PCR-RFLP analysis resulted in a well resolved phylogenetic tree with branches supported by moderate to high bootstrap values, while the trnL-trnF sequence-based trees showed only moderate to low bootstrap support for the internal tree branches, indicating uncertain origin of some *Citrus* genotypes. However, PCR-RFLP and trnL-trnF data supported the recognition of *Citrus maxima*, *C. medica*, and *C. reticulata* as the basal species of edible citrus.

Amplified fragment length polymorphism (AFLP) is a technique based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Dorothea *et al*, 1996). The technique involves three steps:

1. Digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half-site specific adaptors to all restriction fragments.
2. Selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction site specific sequences.
3. Electrophoretic separation of amplicons on a gel matrix, followed by visualization of the band pattern.

PCR amplification of restriction fragments is achieved by using the adaptor and restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence. The method allows the specific co-amplification of high numbers of restriction

fragments. The number of fragments that can be analyzed simultaneously, however, is dependent on the resolution of the detection system. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels (Vos *et al.*, 1991)

AFLP represents dominant marker system among the different marker systems available at present and also provides multi-locus and genome wide marker profiles. These features make the AFLP technology more suitable for molecular characterization and DNA fingerprinting of any germplasm collection (Leela *et al.*, 2009). AFLP not only has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques, but it also has the capability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification. As a result, AFLP has become extremely beneficial in the study of taxa including bacteria, fungi, and plants, where much is still unknown about the genomic makeup of various organisms (Hao *et al.*, 2010). AFLPs can be used to measure recombination rates which can lead to a genetic map with the distance between AFLP loci measured in CentiMorgans (Terashima *et al.*, 2002).

AFLP markers have been successfully employed for the development of a high-density linkage map of ryegrass (*Lolium perenne* L.). The application of AFLP markers to genome mapping, in *Lolium* as a prelude to quantitative trait locus (QTL) identification for diverse agronomic traits in ryegrass and for marker-assisted plant breeding was carried out (Bert *et al.*, 1999). The usefulness of molecular markers of DNA polymorphism, based on AFLP analysis, to unravel the disputed attributions of *Ocimum* genus has been conducted by Labra *et al.* (2004). The *Ocimum* genus includes more than 150 species. They concluded that the combined analysis of morphological traits, volatile oil

composition and molecular markers represents the optimal approach to verify taxonomy and to correlate it with agronomic traits. In this case the genetic distance was computed by using the Nei and Li's index. This index is more appropriate for AFLP data than others, such as Jaccard's similarity index or the simple band matching distance indexes (Labra et al., 2004). Baydar et al (2004) investigated the genetic relationships among *Rosa damascena* plants grown in Turkey by using microsatellite and AFLP markers. Twenty three AFLP and nine microsatellite primer pairs were used for this experiment. No polymorphism was detected among the plants, as the marker patterns obtained from different plants are identical. The conclusion from these data is that all *R. damascena* plants studied are derived from the same original genotype by vegetative propagation.

Rapid Amplification of Polymorphic DNA (RAPD) is a type of PCR reaction, but the segments of DNA that are amplified are random (Srivatsava et al., 2009). RAPD markers are amplification products of anonymous DNA sequences as primers to amplify nanogram (ng) amounts of total genomic DNA under low annealing temperature. RAPD does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers sequence (Bardakci, 2001). It is used to analyze the genetic diversity of an individual by using random primers (Arif et al., 2010). Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely (Salim khan et al., 2010).

The RAPD (Random Amplified Polymorphic DNA) technique was employed for authentication of *Glycyrrhiza glabra* L. from its adulterant *Abrus precatorius* L. by Salim khan et al (2009). Fifty two decamer oligonucleotide primers were screened in the RAPD analysis for identification of genuine and adulterant samples. Out of fifty two primers, sixteen primers gave species specific reproducible unique amplicons. The obtained unique amplicons in PCR amplification clearly distinguished genuine as well as adulterant samples having similar morphology and thus, RAPD helping to serve as a complementary tool for quality control. (Salim khan et al., 2009).

In a case study by Mehrnia et al (2009) the fresh leaves of 20 individual plants belonging to Iranian *Astragalus microcephalus* were collected and analyzed for RAPD markers. A total of 218 bands from eight out of thirty decamer primers were selected. The results showed that RAPDs can be used to study the systematic relationship among closely related species and sub-specific taxa (Mehrnia et al., 2005). Similar type of work has been done on *Podophyllum hexandrum* collected from high altitude regions of North Western Himalayas. Random Amplified Polymorphic DNA (RAPD) analysis revealed a high degree of genetic diversity among the 12 collected accessions, attributed to their geographical and climatic conditions. The study demonstrated that RAPD markers are very useful tools to compare the genetic relationship and pattern of variation among such prioritized and endangered medicinal plants (Sultan et al., 2010).

Variable Number Tandem Repeats (VNTR) A family of genetic loci found in eukaryotes consisting of short (15-100 bp) sequences of DNA repeated in tandem arrays. The alleles for any particular locus all have the same sequence but differ as to how many times the sequence is repeated. VNTR loci contribute to

repetitive DNA and have proved valuable in DNA fingerprinting, especially in forensic science. VNTRs were an important source of RFLP markers used in linkage analysis (mapping) of genomes. Now that many genomes have been sequenced, VNTRs have become essential to forensic crime investigations, via DNA fingerprinting and the CODIS database. The VNTR sequences can be released intact from a DNA sample using restriction endonuclease enzymes and identified using gene probes with Southern blotting. Alternatively, the VNTR alleles can be amplified by the polymerase chain reaction, separated by gel electrophoresis, and the resultant patterns compared without the need for special gene probes. Since each VNTR locus typically has many different alleles, the likelihood of two individuals having identical sets of alleles for even a few such loci is very remote. Hence, a DNA sample can be ascribed to a particular person with a high degree of certainty. The number of repeats can be gauged by dividing the entire molecular weight of the repeated sequence.

VNTRs are similar to Short Tandem Repeats, (STRs) the difference being that in a VNTR, the repeated sequence is no longer about 10-100 base pairs long (Rogstad, 1993). Satsuki reported the VNTR loci found in the mitochondrial DNAs (mt DNAs) of sugar beet and its close relatives. This raises the possibility that the plant mitochondrial genomes may also contain minisatellite like VNTRs. They found four unrelated tandem repeat loci (TR1, TR2, TR3 and TR4) in the mitochondrial genomes of beets with TR1 locus embedded within a three membered family of recombining repeat sequences (the *rrn26*-repeat) (Satsuki *et al.*, 2000).

Microsatellites, or Simple Sequence Repeats (SSRs), are stretches of 1 to 6 nucleotide units repeated in tandem and randomly spread in

eukaryotic genomes. SSRs are very polymorphic due to the high mutation rate affecting the number of repeat units. It is believed that when DNA is being replicated, errors occur in the process and extra sets of these repeated sequences are added to the strand. Over time, these repeated sequences vary in length between one cultivar and another. Such length-polymorphisms can be easily detected on high resolution gels (for example, sequencing gels), by running PCR amplified fragments obtained using a unique pair of primers flanking the repeat. Using PCR to detect DNA polymorphisms offers improved sensitivity and speed compared with standard blotting and hybridization techniques (Weber and May, 1989).

They are used as molecular markers which have wide-ranging applications in the field of genetics, including kinship and population studies. SSRs have several advantages over other molecular markers. For example, (i) microsatellites allow the identification of many alleles at a single locus, (ii) they are evenly distributed all over the genome, (iii) they are co-dominant, (iv) little DNA is required and (v) the analysis can be semi-automated and performed without the need of radioactivity. With the above mentioned features microsatellites have become the genetic markers of choice in Mammalian and many plant systems (Weber and May, 1989).

The genetic characterization of *Rhodiola rosea* L. using gene specific SSR molecular markers was performed. The phylogenetic relationships of 30 *Rhodiola rosea* collected from three different valleys were analyzed using 10 PCR markers (5 gene specific SSRs and 5 CAPS). The gene specific SSR analysis yielded 12 polymorphic loci, with an average of 1.8 polymorphic bands per primer. The genetic differentiation observed was considerable low, indicating a high level of

gene flow, which had a strong influence on the genetic structure. The results indicate considerable gene flow among *R. rosea* population that might be a result of seed dispersal rather than cross-pollination (Soni et al., 2010).

Guilford et al., (1997) reported that screening of an apple genomic library with (GA) 15 and (GT) 15 probes demonstrated that these repeats are abundant, occurring about every 120 and 190 kb, respectively. As few as three microsatellite markers were sufficient to differentiate between all 21 cultivars.

Table 1: Different types of markers used in the study of medicinal plants

Plant	Marker type	No. of markers	Total no. of bands	Poly-morphic fragments	Poly-morphic percentage	Average bands/primer	Ref.
<i>Jatropha curcas</i>	AFLP	7 pairs	770	680	88	110	Leela et al. 2009
<i>Achillea</i> spp	AFLP	9 pairs	313	301	96.1	33.4	Rahim malek et al. 2009
<i>Incarvillea young husbandii</i>	AFLP	7 pairs	332	185	55.7	20	Zhu et al. 2009
<i>Rosa damascena</i>	AFLP	23 pairs	966	-	-	42	Baydar et al. 2004
<i>Huperzia serrata</i>	AFLP	7 pairs	615	532	86.5	38	Huang and He, 2010
<i>Gardenia jasminoides</i>	AFLP	11 pairs	244	165	67.6	15	Han et al., 2007
<i>Ocimum</i> spp.	AFLP	8 pairs	253	150	59.5	31	Moghaddam et al., 2011
<i>Calathea</i> Spp.	AFLP	6 pairs	733	497	67.8	41.1	Chao et al., 2005
<i>Oroxylum indicum</i>	RAPD	40	387	188	49.6	9.6	Jayaram & Prasad 2008
<i>Zeyheria montana</i>	RAPD	9	105	65	61.9	11.6	Bertoni et al. 2007
<i>Anisodus tanguticus</i>	RAPD	12	92	76	82.6	7.67	Zheng et al. 2008
<i>Swertia przewalskii</i>	RAPD	13	166	156	94	12	Zhang et al. 2007
<i>Astragalus</i> spp	RAPD	6	98	98	100	16.3	Adiguzel et al. 2006
<i>Podophyllum hexandrum</i>	RAPD	3	116	109	93.9	36.3	Sultan et al. 2010
<i>Astragalus microcephalus</i>	RAPD	8	218	218	100	27.25	Mehrnia et al. 2005
<i>Pongamia pinnata</i>	RAPD	18	210	22	10.4	1.2	Kesari & Rangan, 2011
<i>Jatropha curcas</i>	RAPD	23	90	53	58.8	3.9	Dhakshnamoorti et al., 2011
<i>Ocimum basilicum</i>	RFLP	9	163	83	50.9	9.22	Labra et al. 2004
<i>Rhodiola rosea</i>	SSR	5 pairs	12	12	100	1.8	Soni et al. 2010
<i>Lycium chinense</i>	SSR	18 pairs	108	91.4	84.7	6	Zhao et al., 2010
<i>Punica granatum</i>	SSR	12 pairs	34	12	35	3	Hasnaoui et al., 2012
<i>Liviope</i>	SSR	67	22	8	32.8	4	Li et al., 2011
<i>Androsace tapets</i>	ISSR	9	60	50	83	6.6	Geng et al. 2009
<i>Rhodiola chrysanthemifolia</i>	ISSR	13	116	104	89.7	8	Xia et al. 2009
<i>Rheum tanguticum</i>	ISSR	13	329	326	92.9	25	Hu et al., 2010
<i>Chrysanthemum</i> spp.	ISSR	22	182	148	81.8	6.7	Shao et al., 2010
<i>Magnolia officinalis</i>	ISSR	12	137	114	83.2	9	Yu et al., 2010
<i>Hemitelia littoralis</i>	ISSR	11	117	89	76	8	Jian et al., 2010

Single-nucleotide polymorphisms or SNPs (pronounced “snips”) are DNA sequence variations that occur when a single nucleotide (A, T, G and C) in the genome sequence is altered. SNPs constitute the most abundant of molecular markers. The high density and mutational stability of SNPs make them particularly useful DNA markers for population genetics and for mapping candidate genes for disease resistances and abiotic stress tolerance (Alves *et al.*, 2008).

For example a SNP might change the DNA sequence AAGGCTAA to ATGGCTAA. For a variation to be considered a SNP, it must occur in at least 1% of the population (Coles *et al.*, 2005). SNPs can occur in both coding and non-coding regions of the genome. SNPs within a coding sequence do not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. SNPs that are not in protein-coding regions may still affect gene splicing, transcription factor binding, or the sequence of non-coding RNA (Rafalski *et al.*, 2002). Several approaches can be used for discovery of new SNPs and about a dozen different methods are now available for SNP genotyping. Some of these methods are also suitable for automation and high throughput approaches. These methods, in principle, make a distinction between a perfect match and a mismatch (at the SNP site) between a probe of known sequence and the target DNA containing the SNP site. The target DNA in most of these methods is a PCR product, except in some cases like ‘invasive cleavage assay’, and ‘Reduced Representation Shotgun (RRS)’ devised and used recently (Gupta *et al.*, 2001).

Coles *et al.* (2005) used an expressed sequenced tag library in quinoa (*Chenopodium quinoa* Willd.) for the discovery of Single nucleotide polymorphism. The SNP markers identified in this work are of particular value in ongoing efforts to characterize gene

expression and regulation associated with seed development, while the SNPs identified have immediate application for genetic mapping experiments, germplasm development, and the investigation of evolutionary relationships within the genus *Chenopodium*.

Short Tandem Repeats (STRs) are DNA regions with short repeat units (usually 2-6 base pairs in length). The repeated DNA sequences of a genome come in various sizes and are classified according to the length of the core repeat units, the number of contiguous repeat units, and/or the overall length of the repeat region (Gilmore *et al.*, 2003). STRs are found surrounding the chromosomal centromere (the structural center of the chromosomes). STRs have proven to have several benefits that make them especially suitable for identification (Jin *et al.*, 2003). The smaller size of STR alleles makes STR markers better candidates for use in forensic applications, in which degraded DNA is common (Gilmore *et al.*, 2003). PCR amplification of degraded DNA samples can be better accomplished with smaller target product sizes. By examining enough STR loci and counting how many repeats of a specific STR sequence there are at a given locus, it is possible to create a unique genetic profile of an individual (Nevin *et al.*, 2005). Length variations in simple sequence tandem repeats are being given increased attention in plant genetics. Some short tandem repeats (STRs) from a few plant species, mainly those at the dinucleotide level, have been demonstrated to show polymorphisms and Mendelian inheritance (Wang *et al.*, 1994).

Authentication of medicinal plants:

DNA based techniques have been widely used for authentication of important medicinal plant species. Molecular biology offers an assortment of techniques that can be very useful for authentication of medicinal

plants. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable (Ngan et al., 1999). Some researchers verified the capacity of the AFLP approach to analyze genetic distances among *Ocimum basilicum* L. varieties and to verify the occurrence of any correlation among genetic distance, essential oil profile and morphological description (Labra et al., 2004). The RAPD technique has also been used for determining the components of three herbs (*Astragalus membranaceus* (Fisch.) Bge, *Ledebouriella seseloides* Wolff and *Atractylodes macrocephala* Koidz). The components in the herbal formulation have been detected using a single RAPD primer (Adiguzel et al., 2006).

Three RAPD primers have been identified that could successfully discriminate between three species of *Podophyllum*, from North Western Himalayas (Sultan et al., 2010). In another study, three random primers were used to reveal the genetic variability of *Astragalus* medicine materials sold in Taiwan market (Adiguzel et al., 2006). SSCP analysis was also conducted on PCR products from the ITS-1 region of rDNA in order to differentiate the two *Astragalus* species (Mehrnia et al., 2005). Primers have been designed for hybridization with the hyper variable ends of microsatellite loci that could reveal DNA-polymorphism among five *Eucalyptus* species (De Melis et al., 1999). RAPD analysis has been used for authentication of *Glycyrrhiza glabra* L. from its *Abrus precatorius* L. Fifty two decamer oligonucleotide primers were screened in the RAPD analysis for identification of genuine and adulterant samples. The obtained unique amplicons in PCR amplification clearly distinguished genuine as well as adulterant samples having similar morphology and thus, RAPD helping to serve as a

complementary tool for quality control (Salim Khan et al., 2009).

A DNA microarray for detecting processed medicinal *Dendrobium* species (*Herba dendrobii*) was constructed by incorporating the ITS1-5.8s- ITS2 sequences of *Dendrobium* species on a glass slide. The established microarray could detect the presence of *D. nobile* in a Chinese medicinal formulation containing nine herbal components (Hao et al., 2010). Molecular authentication of the medicinal plant *Angelica sinensis* was carried out based on internal transcribed spacer of nrDNA. The internal transcribed spacer (ITS) regions of *A. sinensis* and seven other *Angelica* species used as adulterants were sequenced. A pair of specific primers was designed from the polymorphic ITS regions to distinguish *A. sinensis* from the adulterants and regional substitutes. The result showed that there were four samples adulterating *A. sinensis* with regional substitutes. This indicated that *A. sinensis* could be accurately distinguished from the adulterants and regional substitutes. Therefore, the method of molecular authentication based on the ITS sequences may be contributed to raw material production and quality control of *A. sinensis* (Feng et al., 2010).

Conclusion:

It can be concluded that markers differ in their ability to differentiate among individuals, in the mechanism of detecting polymorphism, in genome coverage, and in the ease of application. Therefore, they could complement one another depending on technical availability. Molecular technology provides an independent approach for the characterization of medicinal plant materials. It will be more fruitful if a concentrated effort is made to integrate the existing molecular fingerprinting data and to co-ordinate the projects of molecular characterization of medicinal plants.

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