Genetic Markers - A Cutting-Edge Technology in Herbal Drug Research

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Abstract

There is a great demand for herbal medicines in the developed and developing countries because of their wide biological activity, higher safety margin than synthetic drugs as a result of this Herbal drugs have a great potential in the global market. Herbal drug technology is used for converting botanicals materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important .Extensive research on DNA-based molecular markers is in progress in many research institutes all over the world.DNA -based molecular have a great utility in the herbal drug analysis and widely used for the authentification of plant species of medicinal importance.

Key Words: DNA-based molecular markers, Genetic markers, Herbal drugs, Herbal medicine technology

Introduction

Natural products have been our single most successful source of medicines. Each plant is like factory capable of synthesizing unlimited number of highly complex and unusual chemical substances whose structures could otherwise escape the imagination forever [1]. There are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in the world, while several other drugs are simple synthetic modifications of the natural products [2]. Despite the long history of success in discovering drugs from natural sources, natural products have fallen out of favor in current high throughput screening. In 2002, spending on medicines exceeded $ 400 bn worldwide. Completion of human genome project and role of genomic and proteomics have revolutionized natural products based drug discovery. Over 50% of the best-selling pharmaceuticals in use today are derived from natural products[3]. In natural product drug discovery the conventional approach of extraction, isolation, separation, identification, characterization and test for the desired biological activity suffers from problems like lower yields, de-replication, difficult separation and inconsistent biological activity. However with the introduction of innovative technologies like high throughput screening (HTS) and recent advances in extraction, chromatography, electrophoresis and spectroscopy have revolutionized the entire scenario of pharmacognosy [4].

Chromatographic techniques like TLC, HPTLC and HPLC uses chemical markers which may not be therapeutically active and has its own limitation. Secondary metabolites that are used as marker may change due to environmental factors and hence correct identification of botanicals is difficult task. Marker compound must not be present widely (like primary metabolites) and should neutral to environmental or other effects[5].

Each herb contains large number of compounds, so it is not possible to analyze for presence or absence either quantitatively or qualitatively for all compounds. These serious difficulties in testing for active principles or chemical constituents are well known. Various national and international guidelines have suggested that test material should be free from pesticides, heavy metals and aflatoxins.Estimation of these contaminants requires advanced analytical techniques. Secondly countries with ethno botanical practices are engaged in developing monographs of their indigenous plants [4-5]. Monographs preparation involves chemical as well as biological markers. Marker development requires advanced analytical tools. Due to faster dereplication rates these techniques will able monograph preparation of even those botanicals whose chemical identification or characterization was not possible. These markers developed by these techniques will further be useful in stability testing and biological standardization. According to general guidelines for methodologies on research and evaluation of traditional medicines by WHO, first step in assuring quality, safety, and efficacy of traditional medicines is correct identification and this can be done very successfully with the application of molecular markers[6].

Pharmacognosy, since 1990 has become molecular science, as molecular markers prove important tool in revolution of biochemical constituents and macromolecules, viz. proteins and deoxyribonucleic acids (DNA). Molecular markers especially genetic marker identify plant at genomic level and establish new standards in standardization and quality control of botanicals. Hence, are more suitable and ubiquitous to most of plants. Molecular markers have highly polymorphic nature, show co dominant inheritance, occur frequently in genome, unbiased to environmental conditions or management practices and easily available, highly reproducible and allow easy exchange of data between laboratories [7].

Genetic Markers: An Introduction

For many years, gene mapping was limited in most organisms by traditional genetic markers which include genes that encode easily observable characteristics such as blood types or seed shapes. The insufficient amount of these types of characteristics in several organisms limited the mapping efforts that could be done.New molecular techniques in 1980's, made it possible to examine variations in DNA, providing unlimited number of genetic markers that can be used for creating genetic maps as well as for studying linkages between diseases and genetic inheritance.[7-8] Restriction fragment length polymorphism (RFLPs) were one of the earliest molecular marker methods used which detect variations in DNA sequencing by cutting the DNA with restriction enzymes. More methods were developed later that detected variable numbers of short DNA sequences repeated in tandem, called microsatellites. Single nucleotide polymorphisms (SNPs) were a more recent way of detecting individual variations in DNA nucleotides directly. All of these methods have expanded the availability of genetic markers hence facilitating the creation of genetic maps [8].
A *genetic marker* is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. It can be described as a variation, which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may

be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism SNP), or a long one, like minisatellites.
Some commonly used types of genetic markers are

- RFLP (or Restriction fragment length polymorphism)
- AFLP (or Amplified fragment length polymorphism)
- RAPD (or Random amplification of polymorphic DNA)
- VNTR (or Variable number tandem repeat)
- Micro satellite polymorphism
- SNP (or Single nucleotide polymorphism)
- STR (or Short tandem repeat)
- SFP (or Single feature polymorphism)
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They can be further categorized as dominant or co-dominant. Dominant markers allow for analyzing many loci at one time, e.g. RAPD. A primer amplifying a dominant marker could amplify at many loci in one sample of DNA with one PCR reaction. Co-dominant markers analyze one locus at a time. A primer amplifying a co-dominant marker would yield one targeted product [8].

A ***restriction fragment length polymorphism***, or RFLP, (commonly pronounced "rif lip"), is a variation in the DNA sequence of a genome that can be detected by breaking the DNA into pieces with restriction enzymes and analyzing the size of the resulting fragments by gel electrophoresis. It is the sequence that makes DNA from different sources different, and RFLP analysis is a technique that can identify some differences in sequence (when they occur in a restriction site). Though DNA sequencing techniques can characterize DNA very thoroughly, RFLP analysis was developed first and was cheap enough to see wide application [8-9]. Analysis of RFLP variation was an important tool in genome mapping, localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting, and paternity testing. The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis, and transferred to a membrane via the Southern blot procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe. A RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele, and can be used in genetic analysis [9].Analysis of RFLP variation in genomes was vital tool in genome mapping and genetic disease analysis. If researchers were trying to initially determine the chromosomal location of a particular disease gene, they would analyze the DNA of members of a family afflicted by the disease, and look for RFLP alleles that show a similar pattern of inheritance as that of the disease (see Genetic linkage). Once a disease gene was localized, RFLP analysis of other families could reveal who was at risk for the disease, or who was likely to be carriers of the mutant gene. RFLP analysis was also the basis for early methods of Genetic fingerprinting, useful in the identification of samples retrieved from crime scenes, in the determination of paternity, and in the characterization of genetic diversity or breeding patterns in animal populations[10-12]

Amplified fragment length polymorphism **PCR** (or **AFLP-PCR** or just **AFLP®**) is a PCR-based tool used in genetics research, DNA fingerprinting, and in the practice of genetic engineering. Developed in the early 1990's by Keygene, AFLP uses restriction enzymes to

cut genomic DNA, followed by legation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers complementary to the adaptor and part of the restriction site fragments (as described in detail below). The amplified fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies [12].

AFLP-PCR is a highly sensitive method for detecting polymorphisms in DNA.In detail, the procedure of this technique is divided into 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 visualisation of the band pattern.
4.

A variation on AFLP is cDNA-AFLP, which is used to quantify differences in gene expression levels. Another variation on AFLP is TE Display, used to detect transposable element mobility. The AFLP technology has the capability to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. As a result, AFLP has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, and bacteria. The AFLP technology has been used in criminal and paternity tests, in population genetics to determine slight differences within populations, and in linkage studies to generate maps for quantitative trait locus (QTL) analysis. There are many advantages to AFLP when compared to other marker technologies including randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and micro satellites. 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 [10-12]. As a result, AFLP has become extremely beneficial in the study of taxas including bacteria, fungi, and plants, where much is still unknown about the genomic makeup of various organisms.

RAPD (pronounced "rapid") stands for **R**andom **A**mplification of **P**olymorphic **D**NA. It is a type of PCR reaction, but the segments of DNA that are amplified are random RAPD markers are decamer (10 nucleotide length) DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way [11].

A ***Variable Number Tandem Repeats*** (or **VNTR**) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and often show variations in length between individuals. Each variant acts as an inherited allele, allowing them to be used for personal or parental identification. Their analysis is useful in genetics and biology research, forensics, and DNA fingerprinting.

Microsatellites*, or* ***Simple Sequence Repeats*** (SSRs), are polymorphic loci present in nuclear and organellar DNA that consist of repeating units of 1-6 base pairs in length. They are typically neutral, co-dominant and are used as molecular markers which have wide-ranging applications in the field of genetics, including kinship and population studies. Microsatellites

can also be used to study gene dosage (looking for duplications or deletions of a particular genetic region) [11-12]

A ***single-nucleotide polymorphism*** (**SNP**, pronounced *snip*) is a DNA sequence variation occurring when a single nucleotide — A, T, C or G— in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual). For example, two sequenced DNA fragments from different individuals, AAGC**C**TA to AAGC**T**TA, contain a difference in a single nucleotide. In this case we say that there are two *alleles* : C and T. Almost all common SNPs have only two alleles.Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, drugs, vaccines, and other agents. SNPs are also thought to be key enablers in realizing the concept of personalized medicine.However, their greatest importance in biomedical research is for comparing regions of the genome between cohorts (such as with matched cohorts with and without a disease).The study of single-nucleotide polymorphisms is also important in crop and livestock breeding programs (see genotyping). See SNP genotyping for details on the various methods used to identify SNPs. They are usually biallelic and thus easily assayed.

A ***Short Tandem Repeat*** (STR) in DNA is a class of polymorphisms that occurs when a pattern of two or more nucleotides are repeated and the repeated sequences are directly adjacent to each other. The pattern can range in length from 2 to 10 base pairs (bp) (for example (CATG)n in a genomic region) and is typically in the non-coding intron region, making it junk DNA. 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. There are currently over 10,000 published STR sequences in the human genome. STR analysis has become the prevalent analysis method for determining genetic profiles in forensic cases. **STR** analysis is a relatively new technology in the field of forensics, having come into popularity in the mid-to-late 1990s. It is used for the genetic fingerprinting of individuals. The STRs in use today for forensic analysis are all tetra- or penta-nucleotide repeats (4 or 5 repeat units), as these give a high degree of error-free data while being robust enough to survive degradation in non-ideal conditions. Shorter repeat sequences tend to suffer from artifacts such as PCR stutter and preferential amplification, as well as the fact that several genetic diseases are associated with tri-nucleotide repeats such as Huntington's disease. Longer repeat sequences will suffer more highly from environmental degradation and do not amplify by PCR as well as shorter sequences[12-13].

***Uses*:** Genetic markers can be used to study the relationship between an inherited disease and its genetic cause (for example, a particular mutation of a gene that results in a defective protein). It is known that pieces of DNA that lie near each other on a chromosome tend to be inherited together. This property enables the use of a marker, which can then be used to determine the precise inheritance pattern of the gene that has not yet been exactly localized.Genetic markers have to be easily identifiable, associated with a specific locus, and highly polymorphic, because homozygotes do not provide any information. Detection of the marker can be direct by RNA sequencing, or indirect using allozymes.Some of the methods used to study the genome or phylogenetics are RFLP, Amplified fragment length polymorphism (AFLP), RAPD, SSR. They can be used to create genetic maps of whatever organism is being studied. There was a debate over what the transmissible agent of (canine transmissible venereal tumor, CTVT) was. Genetic Markers have also been used to measure the genomic response to selection in livestock. Natural and artificial selection leads to a change in the genetic makeup of the cell. The presence of different alleles due to a distorted

segregation at the genetic markers is indicative of the difference between selected and non-selected livestock. [14]

Molecular Markers in Plant Genetic Research for increasing the productivity *[15]*

Molecular markers, sometimes called DNA markers, should be thought of as signs along the DNA trail that pinpoint the location of desirable genetic traits or indicate specific genetic differences. Just as smoke rising into the sky makes it easier to locate a forest fire, a gene of interest is easier to locate when a researcher starts with a nearby marker. "To successfully use a specific marker to follow a specific trait, the marker must be found close enough to the gene of interest that variations (alleles) of both the marker and the gene can be inherited together," This was said by Dr. Jim Register, who was research coordinator of analytical nucleic acid technologies at Pioneer. This ca be further explained and understand by experiment carried out by Dr. Jim Register at Pioneer shown in figure.

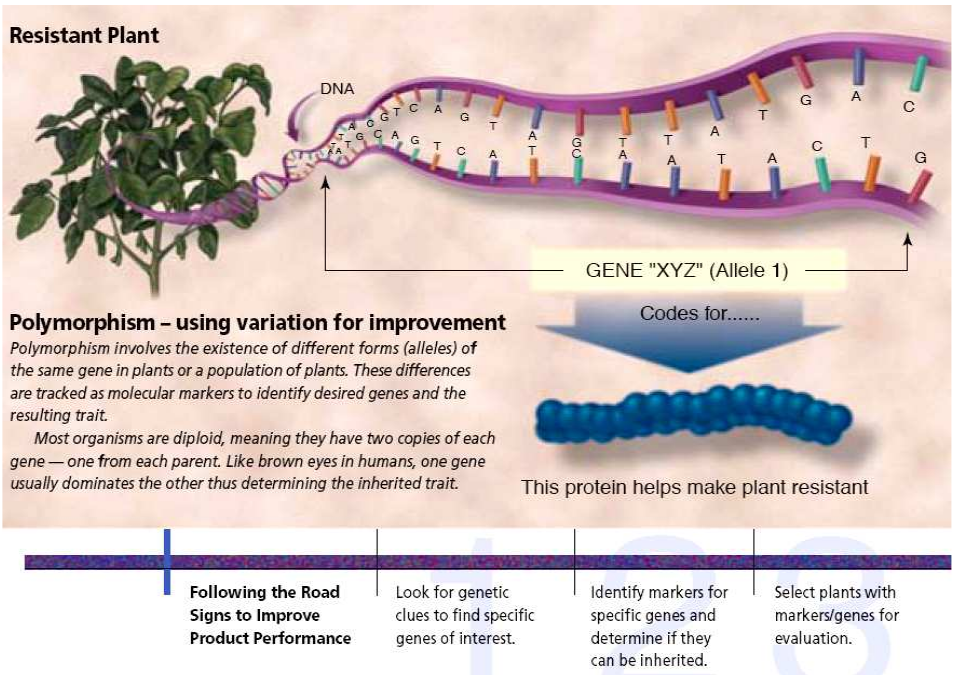


Figure 4: Experimentation shows the use of genetic marker to identify the genes for evaluation

Molecular markers are first identified as short fragments or "strings" of DNA located in a specific position on a chromosome. "We are able to use a particular fragment of DNA as a marker when we can detect differences in that fragment's DNA sequence between multiple plants or plant lines," Register says. According to Register, these variations in DNA sequence, called polymorphisms, can be associated or linked with different forms (alleles) of nearby genes involved with particular traits. The polymorphism, or difference, is the clue researchers need to find the gene of interest. For example, markers associated with genes involved in disease resistance have been identified in corn and soybeans. Differences between the DNA sequences of these genes can be responsible for making a plant sensitive or resistant to a particular disease. And differences in DNA sequences near the gene can be used as markers to locate the gene and track the desired results in breeding programs. Besides disease resistance research, this same technology can be used to localize genes and follow markers associated with other traits such as maturity, plant height, insect resistance, grain oil content, etc. Register said that Getting the desired results "Using the latest marker technologies, scientists are able to determine right in the lab which plants have economically

beneficial traits faster," faster," "This allows us to select plants based on the traits they possess even before going to field trials. The time saved allows us to move improved products to the market faster."

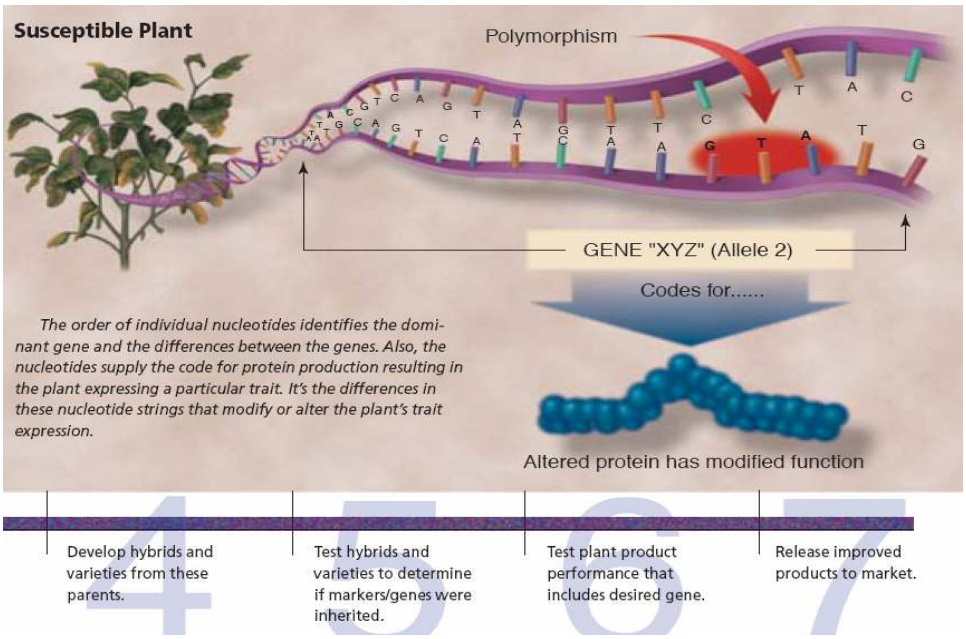


Figure 5: Experimentation shows the use of genetic marker to identify the genes for evaluation

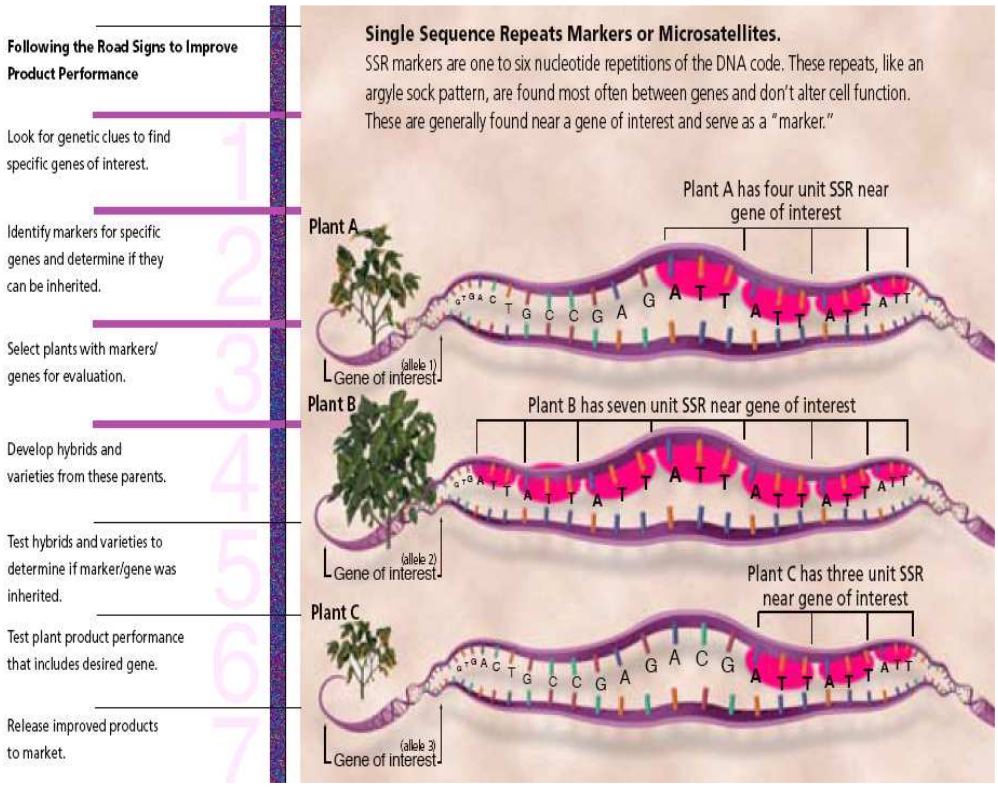
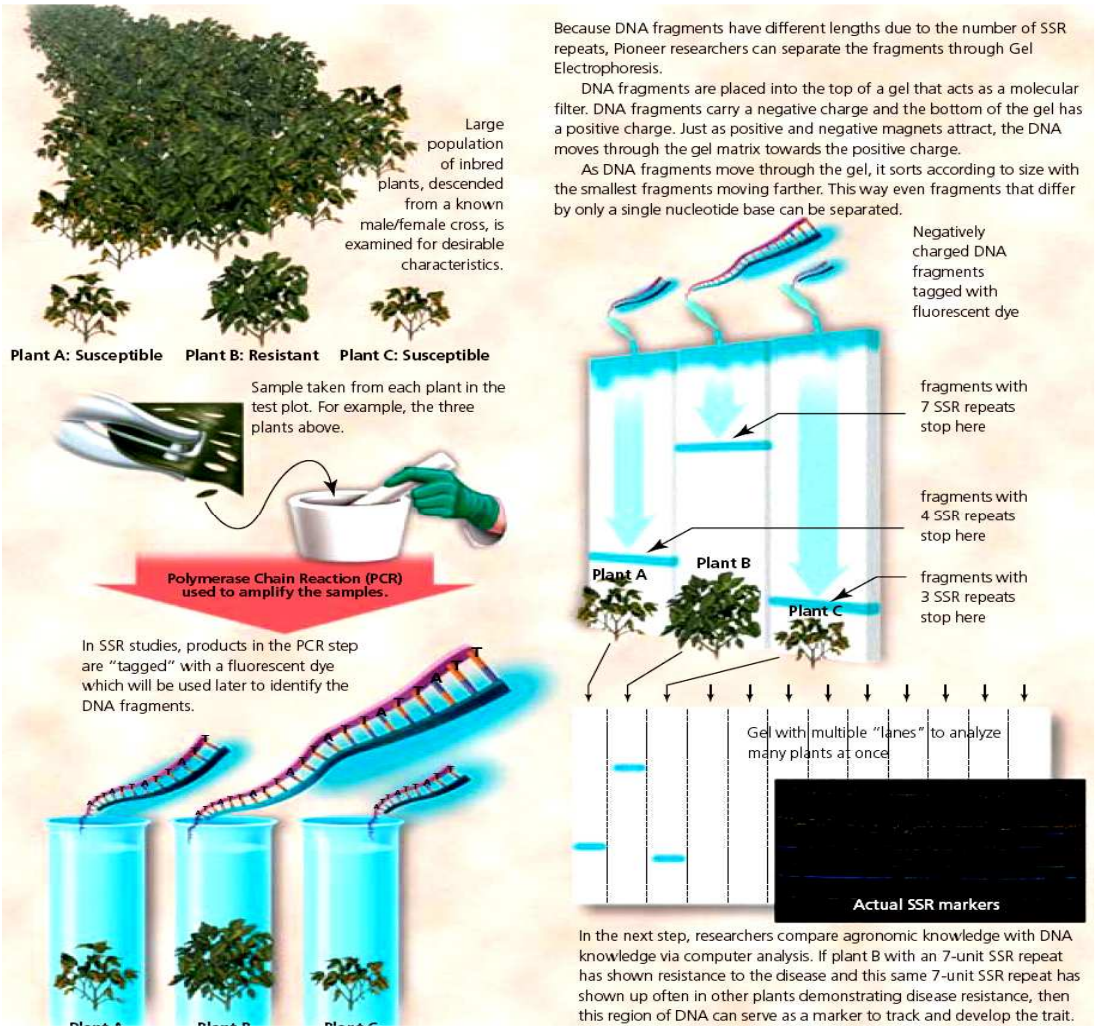


Figure 6: SSR Markers or Micro satellites to improve the product performance

Simple Sequence Repeats (SSR) markers, or microsatellite markers, are one of the most advanced marker technologies available in genetic research today and area key part of

research efforts underway at Pioneer. SSR markers are stretches of DNA in which the same short nucleotide sequence is repeated over and over. Polymorphism, or variation, among SSR markers is determined by the number of times the base sequence repeats (e.g. AGTTAGTT vs.AGTTAGTTAGTTAGTT). "This variation in DNA sequence can be used just like other types of DNA sequence variation to locate nearby genes and follow specific forms of those genes through product development," says Dr. Jim Register, research coordinator of analytical nucleic acid technologies at Pioneer. (Experiment conducted shown in figure 6 and 7 using SSR techniques to improve the product performance). "SSR markers are considered highly polymorphic as the number of repeats can vary greatly among plants. This allows us to detect many different alleles for that marker." These highly polymorphic SSR markers are also excellent tools for comparing our germplasm with other competitors'," he concluded this technology protects our intellectual property and the investment of the researchers puts into product development research for its customers." With SSR markers, researchers are routinely able to analyze the location of genes on chromosomes (positions of genes on chromosomes) hundreds of plants at one time.



Plant A **Plant B** **Plant C**

Figure 7: Use of SSR techniques through gel electrophoresis

Types of DNA markers used in plant genome analysis

Various types of DNA-based molecular techniques [7, 16] are utilized to evaluate DNA polymorphism. These are hybridization-based methods, polymerase chain reaction (PCR)-based methods and sequencing-based methods.

Hybridization-based methods

Hybridization-based methods include restriction fragment length polymorphism (RFLP) [16]. and variable number tandem repeats [17]. Labelled probes such as random genomic clones, cDNA clones, probes for micro satellite [18] and minisatellite [19] sequences are hybridized to filters containing DNA, which has been digested with restriction enzymes. Polymorphisms are detected by presence or absence of bands upon hybridization.

PCR-based methods

PCR-based markers involve *in vitro* amplification of particular DNA sequences or loci, with the help of specific or arbitrary oligonucleotide primers and the thermo stable
DNA polymerase enzyme. PCR-based techniques where random primers are used, include random amplified polymorphic DNA (RAPD) [20], arbitrarily primed PCR (AP–PCR)[21] and DNA amplification fingerprinting (DAF)[21-22]. Inter simple sequence repeats (ISSRs) [22]polymorphism is a specific primer-based polymorphism detection system, where a terminally anchored primer specific to a particular simple sequence repeat (SSR) is used to amplify the DNA between two opposed SSRs of the same type. Polymorphism occurs whenever one genome is missing in one of the SSRs or has a deletion or insertion that modifies the distance between the repeats. A recent approach known as amplified fragment length polymorphism (AFLP) [23] is a technique that is based on the detection of genomic restriction fragments by PCR amplification. Adaptors are ligated to the ends of restriction fragments followed by amplification with adaptor-homologous primers. AFLP has the capacity to detect thousands of independent loci and can be used for DNAs of any origin or complexity [24].

Sequencing-based markers

DNA sequencing can also be used as a definitive means for identifying species. Variations due to transversion, insertion or deletion can be assessed directly and information on a defined locus can be obtained. Genetic variation occurs extensively at the single nucleotide level. Direct sequencing can efficiently identify such single nucleotide polymorphisms that usually depend on how closely related are the organisms being compared. Other sequencing-based strategies include analysis of the variable internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA). The ITS region of 18s–26s rDNA has proved to be a useful sequence for phylogenetic studies in many angiosperm families. The level of ITS sequence variation suitable for phylogenetic analysis is found at various taxonomic levels within families, depending on the linkage.
A number of researchers have also sequenced other regions of DNA such as trnK of chloroplast and spacer region of 5s rDNA as diagnostic tools for authentication [21-24].

Genetic Markers in Herbal drug technology

Genetic markers have proved their utility in fields like taxonomy, physiology, embryology, genetics, etc. As the science of plant genetics progressed, researchers have tried to explore these molecular markers techniques for their applications in commercially important plants such as food crops, horticultural plants, etc. and recently in pharmacognostic characterization of herbal medicine.

Genetic variation/genotyping

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. If we look at the history of the development of these markers, it is evident that they have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders. Genome analysis based on molecular markers has generated a vast amount of information and a number of databases are being generated to preserve and popularize it.

It has been well documented that geographical conditions affect the active constituents of the medicinal plant and hence their activity profiles [25]. Many researchers have studied geographical variation at the genetic level. Estimates of genetic diversity are also important in designing crop improvement programmes for management of germ plasm and evolving conservation strategies. RAPD-based molecular markers have been found to be useful in differentiating different accessions of *Taxus wallichiana*[26], neem[27], *Juniperus communis* L.[26], *Codonopsis pilosula*[28], *Allium schoenoprasum* L.[29], *Andrographis paniculata*[29] collected from different geographical regions. Similarly, different accessions of *Cannabis sativa*[30] have been discriminated using ISSR markers and those of *Arabidopsis thaliana* L. Heynh[30] have been differentiated using cleaved amplified polymorphic sequence and ISSR markers.Inter- and intra-species variation has also been studied using DNA-based molecular markers. Interspecies variation has been studied using RFLP and RAPD in different genera such as *Glycerrhiza(34)*, Echinacea[31] and *Arabidopsis*[32]. RAPD and RFLP have also been applied for characterization of *Epimedium* [33] species at the genetic level. Members of three different species of *Scutellaria*[33], Chinese medicinal plants and three subspecies of *Melissa officinalis*[34] have been discriminated using RAPD. Varietals characterization of Kenaf has been done with the help of agronomical and RAPD data. Varietals identification and genetic purity test in pepper and *Capsicum annuum* were carried out using RAPD markers. RFLP technique was used for interspecific genetic variation within the genus *Capsicum* and also for DNA fingerprinting of pepper cultivars[35]. An attempt has been made to understand population structure of *Podophyllum peltatum* to establish commercial level propagation of useful secondary metabolites using molecular markers [36]. Also, high genetic diversity has been shown in *Podophyllum hexandrum* species from Himachal Pradesh, India [37]. Genetic variation and relationships among and within *Withania* species [38], and genetic relationships among papaya and its wild relatives have been revealed using AFLP markers. Genetic variation within *Brassica campestris* cultivars has been studied using AFLP and RAPD markers [39]. Phylogenetic relationship has been studied among citrus and its relatives using SSR markers [40]. RAPD has been used to construct genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* [41]. RAPD markers have been developed for genetic mapping of Pacific yew (*Taxus bravifolia* Nutt.)[42]. An attempt has been made to develop a physical AFLP map of the complex *Arabidopsis* genome by combining gel based AFLP analysis with *in silico* restriction fragment analysis using the published genome sequence[43].

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.

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, pigeon pea, pearlmillet etc[41-43] 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 one such example. One of the most recent applications of these markers has been shown in sex identification of dioeceous 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. Thus in the last few years there are many reports of amalgamation of classical breeding and modern biotechnological approaches which have unlimited scope in Indian agriculture. [43-48]

Authentication of medicinal plants

DNA-based techniques have been widely used for authentication of plant species of medicinal importance. 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.

Dried fruit samples of *Lycium barbarum* were differentiated from its related species using RAPD markers .The RAPD technique has also been used for determining the components of a Chinese herbal prescription, yu-pingfeng san. In this study the presence of three herbs (*Astragalus membanaceus* (Fisch.) Bge., *Ledebouriella seseloides* Wolff and *Atractylodes macrocephala* Koidz) in the formulation have been detected using a single RAPD primer[48-49]

Three RAPD primers have been identified that could successfully discriminate between three species of *Atractylodes*, from Chinese formulation purchased from local markets[49]. In another study, three random primers were used to reveal the genetic variability of *Astragalus* medicine materials sold in Taiwan market. SSCP analysis was also conducted on PCR products from the ITS-1 region of rDNA in order to differentiate the two *Astragalus* species[50]. Primers have been designed for hybridization with the hypervariable ends of microsatellite loci that could reveal DNA-polymorphism among five *Eucalyptus* species[49-50].DAF has been used to identify the Chinese traditional medicine, *Magnoliae officinalis*, its counterfeits and substitutes[49]. An RAPD primer that is selective for an elite strain Aizu K-111 of *Panax ginseng*, including its cultured tissues has been identified[51]. RAPD and PCR–RFLP analysis have been used for authentication of *P. ginseng* among ginseng populations[49-51]. Some researchers have used a new approach called Direct Amplification of Length Polymorphism (DALP) for authentication of *Panax ginseng* and *Panax quinquefolius.* Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of rDNA has been done successfully [50-52]. 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[53]. Molecular authentication of *Atractylodes*-derived crude drugs (Jutsu) was done with the help of PCR–RFLP and direct sequencing of chloroplast trnK. Two regions (Region1 and Region2) inside the chloroplast trnK were selected as molecular markers for identification and discrimination of *Atractylodes*

rhizome (Byaku-jutsu) and *Atractylodes* Lancea rhizome (So-jutsu). Based on polymorphism in the restiction site for *Hinf*1 in Region1 fragment (260 bp), it was possible to discriminate between the two species. By direct sequencing of Region 2(436 bp) and comparison of the nucleotide sequence datasets, we could not only discriminate Byaku-jutsu and Sojutsu, but also identify the original plant species of each crude drug specimen [53-54].

Detection of adulteration/substitution

RAPD technique was adopted to identify eight types of dried *Coptis* rhizomes and one type of *Picrorrhiza* rhizome, a substitute for the former in the Chinese herbal market[55]. *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[56]. Characterization of *Echinacea* species and detection of possible adulterations have been done using RAPD technique. DNA fingerprinting and polymorphism in the Chinese drug 'Ku-Di-Dan' (herba elephantopi) and its substitutes were studied using AP–PCR and RAPD. The results were used for authentication of 'Ku-Di-Dan' and its substitutes. DNA fingerprinting of *Taraxacum mongolicum* (herba taraxaci) and its adulterants of six species of Compositae was demonstrated using AP–PCR and RAPD. Bulb of *Fritillaria cirrhosa*, an official drug of *Chinese Pharmacopoeia* (1995), is commonly used as an antitussive and expectorant. It has often been adulterated with similar bulbs of other related species. Specific DNA-based primers have been designed for authentication of *F. cirrhosa* at the genomic level. A molecular marker that is specific to medicinal rhubarb- based on chloroplast trnL/trnF sequence which is absent in its adulterants has been identified. DNA sequence analysis of rDNA ITS and PCR–RFLP were explored for their application in differentiating four medicinal *Codonopsis* species from their related adulterants, *Campanumoea javania* and *Platycodon grandiflorus*. The technique allowed effective and reliable differentiation of *Codonopsis* from the adulterants [57].

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. 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.An attempt has been made towards marker-assisted selection of fertile clones of garlic with the help of RAPD markers. RAPD markers have been successively used for selection of micropropogated plants of *Piper longum* for conservation. [58-59]

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. 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 dioeceous plants, phylogenetic analysis, etc. Recently, the application of DNA-based molecular markers is being explored in the field of nutraceuticals [60-61]

Regulatory aspects of plant genetic markers

According to the new European Council legislation, the labeling 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 above 1%.

In compliance with the labeling 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.Primers specific for inserted genes in Roundup ReadyTM soybean have been found to be suitable for detection and discrimination of GM soybean from non-GM products. In another study, Roundup Ready soybeans, But maize and Cecropin D capsicum have been successfully discriminated from non-GM products using primers specific for inserted genes and crop endogenous genes [62].

Influence of Genetic Markers in Herbal Drug Research

Quality control and standardization of medicinal plant materials [63]
Correct identification and quality assurance of the starting material is an essential pre-requisite in herbal medicines to ensure reproducible quality of herbal medicine which contribute to its safety and efficacy.

Most of the regulatory guidelines and pharmacopeias suggest microscopic and macroscopic evaluation and chemical profiling of the botanical materials for quality control and standardization. Chemical profiling establishes a characteristic chemical pattern for a plant materials its fractions or extracts. Thin layer chromatography (TLC) and (HPTLC) are routinely used as valuable tools for qualitative determination of small amounts of impurities.

In order to ensure efficacy, selection of the correct chemo type of the plant is necessary even when there are many known chemotypes of a plant species, selection of the right chemo type to which clinical effects are attributed is difficult. Another difficulty encountered in the selection of the correct plant material is to establish the identity of certain species that may be known by different binomial botanical names in different regions. In view of these limitations there is need for anew approach that can complement or in certain situations serve as an alternative. Molecular markers generally refer to biochemical constituents including primary and secondary metabolites and other macromolecules such as nucleic acid. Secondary metabolites as markers have been extensively used in quality control and standardization of botanical drugs.DNA markers are reliable for informative polymorphism as the genetic composition is unique for each species and is not effected by age, physiological condition as well as environmental factors .DNA can be extracted from fresh or dried organic tissue of the botanical material; hence the physical form of the sample for assessment does not restrict detection. 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.

Hybridization-based methods include restriction fragment length polymorphism (RFLP) and variable number tandem repeats.Labelled probes such as random genomic clones,cDNA clones, probes for microsatellites and minisatellites sequences are hybridized to filters containing DNA,which has been digested with restriction enzymes.Polymorphism are detected by presence or absence of bands upon hybridization.

PCR-based markers involve in vitro amplification of particular DNA sequences or loci, with the help of specific or arbitary oligonucleotide primers and the thermostable DNA

polymerase4 enzyme.PCR-based techniques where random primers are used, include random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR) and DNA amplification fingerprinting (DAF). A recent approach known as amplified fragment length polymorphism (AFLP) is a technique that is based on the detection of genomic restriction fragments by PCR amplification. Adaptors are ligated to the ends of restriction fragments followed by amplification with adaptor-homologous primers. AFLP has the capacity to detect thousands of independent loci and can be used for DNAs of any origin or complexity [63].

Genomic profiling of medicinal plants by DNA Micro array Technique

DNA microarray was developed in response to the need for a high-throughput, efficient and comprehensive strategy that can simultaneously measure all the genes or a large defined subset, encoded by a genome [64-65] .Several methodologies including differential display PCR,northern blots, Quantitative PCR are used along side micro arrays as research tools. DNA micro arrays is an orderly arrangement of thousands of oligonucleotides or identified sequenced genes printed on an impermeable solid support, usually glass, silicon chips or nylon membrane. Novel technologies such as SAGE [66-67] and TIGR orthologous gene alignments (TOGA) [68-69] are used alongside microarray as a research tools. DNA microarray allows rapid and detailed analysis of thousands of transcripts, providing a revolutionary approach to the investigation of gene expression. There are three main application of DNA micro array:

a) In pharmacodynamics for the discovery of new diagnostic and prognostic indicators and biomarkers of therapeutic response; elucidation of molecular mechanism of action of an herb, its formulations or its phytochemical components and identification and validation of new molecular targets for herbal drug development.
b) In pharmacogenomics for prediction of potential side-effects of the herbal drug during preclinical activity and safety studies; identification of genes involved in conferring drug sensitivity or resistance and prediction of patients most likely to benefit from drug and use in general pharmacogenomics studies.
c) In pharmacognosy for correct botanical identification and authentification of crude plant materials as part of standardization and quality control.

The identification of genes modulated by compounds isolated from *Centella asiatica* with the help of gene microarray provides the basis the basis for a molecular understanding of *Centella's* bioactivity,opportunities for the quantitative correlation of this activity with clinical effectiveness at molecular level [70].Similarly,the antiproliferative activity of *Coptidis* rhizome a medicinal herb and its major component berberine was investigated in human pancreatic cancer cell lines and it was possible to identify common and distinct genes related to anti-proliferative activities[71]. High density oligonucleotide micro array have been used for pioneering studies on the multiple gene expression effects exhibited by *Ginkgo biloba* leaf extract EGb 761 and various studies on diets supplemented with *G.biloba* extract that have notable neuromodulatory effects in vivo and illustrates the utility of genome wide expression monitoring to investigate the biological actions of complex extracts [72].
cDNA microarray analyses have shown that exposure of human breast cancer cells to a Ginkgo extract altered the expression of genes that are involved in the regulation of cell proliferation, cell differentiation or apoptosis and that exposure of human bladder cancer cells to a Ginkgo extract produced an adaptive transcriptional response that augments antioxidant status and inhibits DNA damage [73]. Recent studies have highlighted the concurrent use of herbs which may mimic, magnify or oppose the effect of drugs [74-75].DNA microarray can

be used for studying herb-drug interactions, and the mechanisms underlying these interactions.

Marker assisted Breeding

Marker assisted breeding involves the use of DNA markers linked with DNA sequences of interest. Inheritance pattern of sequences/ traits can be confirmed even prior to expression using RFLP, RAPD, AFLP and minisatellites markers [76-77]. A high content artimisinin producing plant variety "CIM-Arogya" was developed at CIMAP, Lucknow through marker assisted breeding. Selection of genotype with increased biomass led to selection of higher artimisinin yielding variety [78]. An increase in artimisinin from 0.15% to1.16% on dry weight basis was recorded in this variety. Thus marker assisted breeding could also be used in developing high secondary metabolite yielding plants.

Thus DNA- based molecular markers have acted as versatile tools in plant genome analysis and are specifically important in differentiating different plant species and their varieties. Various techniques like RFLP, RAPD have been successfully applied for characterization of semi-processed and processed herbal drug materials. Being environmentally stable and specific, DNA markers could gain wide popularity in quality control and standardization of medicinal plant materials

Limitations of Genetic Markers in Herbal Drug Technology

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. 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 numberof 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*.[79]

Conclusion

Here we concluded that DNA markers, which may have several advantages over typical phenotype markers. DNA markers are reliable for informative polymorphisms as the genetic composition is unique for each species and is not affected by age, physiological conditions as well as environmental factors20. DNA can be extracted from fresh or dried organic tissue of the botanical material; hence the physical form of the sample for assessment does not restrict detection. Various DNA-based methods for species characterization and adulteration detection in medicinal plants; agricultural crops and genetically modified (GM) foods have been published. Molecular techniques can be used in pharmacognosy for cultivation of medicinal plants, identification, and detection of adulterants and to discourage its fraudulent commercialization in herbal formulations. Newer biotechnological tools like cloning, sequencing, gene expression, gene manipulation can be used to increase yield of secondary metabolite. With the evolution of these molecular approach role of pharmacognosy is likely to be more challenging in forthcoming years.

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