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Original Research Article

Random Amplified Polymorphic DNA (RAPD) Markers in Anticancer Drug Plants

M. Shivashankar*

Department of Life Science, Janan Bharathi, Bangalore University, 560056-Bangaluru, India
**Corresponding author*

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RAPD analysis, Turmeric, Anticancer Drug Plants

A B S T R A C T

RAPD analysis of Turmeric revealed 19 RAPD polymorphic and monomorphic bands was generated by the use of selected primers like OPF 01 – 11 and the same has been represented in the Fig .1and 2. Traditional methods using morphological traits are not very successful in establishing the diversity and relationship among different species level because environmental influence PCR based molecular marker method RAPD was employed to study the genetic diversity and inter relationships among 36 anticancer medicinal plants. The sizes of the amplified products ranged from 100-3000 base pairs. RAPD analysis identified species/accession specific amplification products, which will be useful in medicinal plant germplasm classification and introgression studies. These results suggest that RAPD based marker are useful for genetic characterization of medicinal species/accession.

Introduction

India a country of immense biotic wealth has more than 7000 species reportedly used for medicinal purposes (Groombridge, 1992) most of which are being exploited recklessly for the extraction of drugs. It will be prudent to study species of indigenous medicinal plants at genetic and molecular levels for efficient conservation and management of genetic diversity. Study of inter and intraspecific variation at the molecular level provides an efficient tool for taxonomic and evolutionary studies and for devising strategies to protect genetic diversity of species. Genetic variability also can be exploited to select useful genotypes that could be utilized as cultivars to avoid batch to batch variation in extraction of standard drugs. Recent global emphasize on exploitation of herbal resources and instances of patenting of developing country plants by developed countries emphasize the need to generate databases on indigenous medicinal plants which can be used for future reference. Himalyan May apple (Podophyllum hexandrum Royle), an endangered medicinal herb, grows wild in the interior Himalayan ranges of India. It is recognized for its anticancer properties.

The advent of polymerase chain reaction (PCR) has resulted in the development of a large number of molecular techniques which have already made rapid impact in population genetics, analysis of biodiversity, genetic mapping and studies of relationships among populations at different levels (Nagaraju and Singh,1997).Many PCR based molecular techniques like RAPD's, SSRs and AFLPs have been developed and utilized to characterize genetically the plant and animal populations (Panaud, *et al*., 1995,Plaschke *et al.,* 1995, Provan et al.1996, Akagi *et al.,*1997, Pejic *et al.,*1998; Butler *et al.,*1999).

These molecular markers are known to provide unambiguous estimates of genetic variability of populations since they are independent of confounding effects of environment. Many of these molecular techniques have recently been employed to characterize different silkworm populations. Nagaraja and Nagaraju (1995) used random amplified polymorphic DNA (RAPD) technique to study the DNA profile of thirteen silkworm genotypes. Nagaraju and Singh (1997) applied RAPD and microsatellite (SSR) techniques to study the genetic diversity in silkworm, *Bombyx mori* L. Reddy *et al.,* (1999a) used SSRs to study DNA polymorphism in silkworm and Tan et al. (2001) employed amplified fragment length polymorphism (AFLP) to construct a linkage map and for mapping genes underlying agronomically important traits in silkworm, *Bombyx mori* L. Keeping these objectives in view, a very recent and a reliable molecular technique viz., inter-simple sequence repeats (ISSRs) was employed to study the DNA polymorphism in fourteen selected genotypes of silkworm.

Randomly-amplified polymorphic DNA markers (RAPD). In 1991 Welsh and McClelland developed a new PCR-based genetic assay namely randomly amplified polymorphic DNA (RAPD).This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermocyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals. However, due to the stoichastic nature of DNA amplification with random sequence primers, it is important to optimize and maintain consistent reaction conditions for reproducible DNA amplification. 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 RAPD assay has been used by several groups as efficient tools for identification of markers linked to agronomically important traits, which are introgressed during the development of near isogenic lines. The application of RAPDs and their related modified markers in variability analysis and individual-specific genotyping has largely been carried out,but is less popular due to problems such as poor reproducibility faint or fuzzy products, and difficulty in scoring bands, which lead to inappropriate inferences.

DNA amplification fingerprinting (DAF).Caetano-Anolles et al employed single arbitrary primers as short as 5 bases to amplify DNA using polymerase chain reaction. In a spectrum of products obtained, simple patterns are useful as genetic markers for mapping while more

complex patterns are useful for DNA fingerprinting. Band patterns are reproducible and can be analysed using polyacrylamide gel electrophoresis and silver staining. DAF requires careful optimization of parameters; however, it is extremely amenable to automation and fluorescent tagging of primers for early and easy determination of amplified products.DAF profiles can be tailored by employing various modifications such as predigesting of template. This technique has been useful in genetic typing and mapping.

Today one fourth of all pharmaceuticals are of plant origin either used as a pure compound or chemically engineered to form appropriate derivatives. These include compounds such as morphine, codeine and several anticancer drugs. The plant *Catharanthus roseus* (Madagaskar Periwinkle) is the source of a compound that is used in the treatment of advanced breast cancer and leukaemia, for example.

Cancer is one of the commonest causes of death not only in our country rather throughout the world. Thus many scientists and clinicians are working in many countries for decades to find out its etiopathogenesis and management though the incidence of cancer is more in developed countries also do not lag behind. Rather incidence of some types of cancer are reported higher e.g. oral cancer uterine cancer, oesophageal cancer etc. in Karnataka (Bonoshree *et al.,* 1978), leukaemia in U.P. (Kushawaha,1978) and breast cancer in West Bengal(Pal *et al.,* 1980). It shows the importance of the problem in our country where the large population belongs to rural area with the minimum health consciousness and facilities particularly for this disease. Most of the patients in our country attend the hospital at a later stage, when little help could be provided to them. However, the present protocol of treatment like radiotherapy and multichemotherapy are, not available to many of them, because imported drugs have become costly. Moreover, one of the main disadvantage of these normal cells but also all other normal cells of the body. Many a time, the mortality becomes more due to these chemotherapeutic agents than to the disease itself. Thus there is a great need to find out a drug which is effective on cancer cells only without damaging the other normal cells of the body. Cancer, one of the most dreaded diseases, continues to spread with increasing incidents. Statisticians estimate that over 10 million new cases of cancer appeared worldwide, with over 6 million deaths in the year 2000.Since 1990 cancer incidence and mortality have increased 22% based on information until 2003;the four most frequently occurring cancer are lung, breast, colorectal and stomach and the four most deadly comprise lung, stomach, liver and colorectal cancers. Only cardiovascular disease surpasses cancer that is the second leading cause of death in the United States.

The word cancer is derived from the Greek word meaning “CRAB” which was used in medical sciences for along time as a technical term cancer is applied to eroding ulcers. This medical use of the term was inspired by the larger number of prominent veins surrounding a growth giving a picture like claws of crab. W.R. Belt suggested that the term cancer is used with such an obstinancy to the part of i.e. like a crab and they cannot be separated from each other. The identification and differentiation of the malignant disease have been enlightened much later than the description available in the ancient literature. The earliest and foremost record could be seen in Atharva Veda where the disease was named as ‘APACIT’ in later period Sushruta has elaborated it as multiple swellings at

different places in the body in the body. It does not mean that the ancient Indian clinicians were unaware about this disease; rather they depicted their views as a swelling superficially or in deeper structure or sometimes as chronic incurable disease. Such swellings or lumps have been categorised under the heading of Arbuda, Gulma, or asadhya vrana, Asadhya Galgand, Asadhya-Kamal etc. Depending entirely on its type stages and the varying clinical symptomatology.

The marker based on RAPD and protein have been used extensively to characterize the genetic resources (Sharma et al. 2004) and to study the phylogenetic relationship in Podophyllum anticancer medicinal plant varietals RAPD or protein variants require separation by electrophoresis and are visualized by enzyme activity assay for the relevant enzyme. They are generally relatively neutral well distributed over the genome and are co-dominant. The variation of these often do not have any noticeable effect on phenotype, thus making them suitable markers. However, number of genetic marker provided by the isozyme analysis is barely sufficient for many applications in anticancer medicinal plant crop improvement programme.

The advent of recombinant DNA technology heralded a completely approach to define potentially polymorphic DNA sequences. This new technology promises to revolutionize some areas of plant genetics and plant breeding. The markers based on DNA sequences have introduced a new dimension to the development of genetic maps and mapping of agronomically and physiologically important characters.

The major strength of DNA markers is that they have a potential to reveal almost unlimited number of polymorphisms covering the whole genome. The DNA markers have very unique features like ubiquitous nature, detection at any developmental stage and independent of environmental effects and management practices, and hence have direct applicability to breeding programmes.The most important of the DNA marker technology is the generation of saturated linkage maps which have been extremely useful for mapping and tagging of genes of agronomically important traits.

The development of the Polymerase Chain Reaction (PCR) for amplifying DNA led to a revolution in the applicability of molecular methods and a range of new technologies were developed which can overcome many of the technical limitations of RFLPs such as developing probes for RFLP analysis, Southern hybridization and use of radioactive isotopes, which render them unsuitable for large scale genomic studies. A subset of the latter involves the use of a single 'arbitrary' primer which results in amplification of several discrete DNA products. The method is referred to as Random Amplified Polymorphic DNA (RAPD) analysis.

Genetic analysis with RAPD markers is more rapid and simpler than RFLP analysis and requires small amounts of DNA. Moreover, it does not require cloning of DNA or Southern hybridization. However, unlike RFLPs, most of the RAPD markers are usually scored as dominant and hetrozygotes cannot be identified from homozygotes.

In India there are hundreds of collections of anticancer medicinal plants accessions whose genotypic status is not known. Besides an in-depth molecular marker genetic analysis of anticancer plants has not been carried out so far. By making use of the

molecular marker technology based on PCR approach, the diverse anticancer medicinal plants were analyzed to realize the following objectives. The collection of anticancer medicinal plants, PCR amplification of genomic DNA. To study dendrogram of the anticancer medicinal plants. To find out the genetic distance by Ward's method. Since the molecular marker is one of the recent methods, the literature pertaining to genetic diversity and relationships of anticancer drug plants are limited. However, efforts have been made here to review the literature pertaining to the molecular marker studies in anticancer drug plants and other crop plants under various environmental factors.

Kalpana et al. (2004) worked out Molecular marker in some of the herbal plants, that DNA based molecular marker have utility in the fields like taxonomy, physiology, embryology, genetics etc.DNA based techniques have been widely used for authentication of plant species of medicinal importance. This view provides a brief account of DNA based technologies that are useful in genotyping and quick identification of botanicals in to medicines.

Materials and Methods

The present investigation was carried out in the Department of Horticulture, Biotechnology Centre, DNA Fingerprinting Laboratory, Govt of Karnataka, Hulimavu, Banerghata Road, Bangalore, Karnataka, India, during the year January-2013 to December-2013. The materials used and methods followed in this study are presented here.

Presently anticancer drug plants described based on the morphological and phenotypic characteristic that are highly variable and environment dependent. These limitations were for the use of reliable DNA based technique for varietals characterization. The polymorphic DNA markers revealed by DNA marker assays could be used to construct high density mapping of anticancer drug plants genome and genetic fingerprinting of diverse varieties.

The present study selected leaf samples of the thirty six anticancer drug plants that were collected from the conservatory of Biotechnology Centre, Hulimavu, Department of Horticulture, Bangalore, Karnataka, India. Which may represent the wide variation prevalent in the genome? The recently matured leaves were collected and used for DNA extraction.

DNA Amplification

The basic protocol reported by Williams et al. (1990) for PCR was followed with slight modifications. A single decamer of arbitrary sequence was used in each PCR reaction. With the PCR reaction conditions optimized, informative and reproducible fingerprint profiles were carried out in 25 µl reaction mixture containing template DNA (25 ng), 10 pmol of primer (Operon technologies USA, Inc.), 2.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl and 0.1% Triton X-100. One unit of Taq DNA polymerase (Bangalore Genie) and 250 µM of each dNTPs (Bangalore Genie). Amplification was performed in a thermal cycler (Eppendorf, Master cycler) for 42 cycles after an initial denaturation at 94º C for 5 min. In each cycle, denaturation for 1 min at 94º C, annealing for 1 min at 33º C and extension for 2 min at 72º C was programmed with a final extension step at 72º C for 8 min after the 40 cycles.

DNA Electrophoresis

Amplified DNA fragments were separated out on 1.2% agarose gel stained with

ethidium bromide. Running buffer containing Tris-buffer, Acetic acid and EDTA (pH 8.0) was used for electrophoresis and for preparing gels. Wells were loaded with 25µl reaction volume and 5 µl of loading buffer (Sucrose, Bromophenol blue and Xylene cyanol) together. Electrophoresis was conducted at 45 volts for 3 hours and the gel photographed under UV light using gel dock system (Herolab).

Statistical Analysis

Binary coding was used to score gel and each band of primer was scored of 6 species and 12 primers with 100 to 1000 base pairs Marker level pair wise squared Euclidean species was calculated and utilizing these distances, species were clustered following Wards method. The segregation of species was also assessed through principle component analysis, the Statistica version 5.0 a computer application was used to generate dendrogram using squared Euclidean distance and Wards method.

Porbeski *et al*. (1997) described a relatively quick, inexpensive and consistent protocol for extraction of DNA from expanded leaf material containing large quantities of polyphenols, tannins and polysaccharides. Mature strawberry leaves, which contain high levels of the secondary components, were used as a study group. The method involved a modified CTAB extraction, employing high salt concentrations to remove polysaccharides, the use of polyvinyl pyrrolidone (PVP) to remove phenols, an extended RNAse treatment and phenol-chloroform extraction. Average yields ranged from 20-84µg/g, mature leaf tissue for both wild and cultivated octoploid and diploid *Fragaria* spp. Results from 60 plants wee examined and were consistently amplifiable in the RAPD reaction with as little as 0.5ng DNA per 25µ reaction. Presently this is the first procedure for the isolation of DNA from mature strawberry leaf tissue that produces consistent results for a variety of different species, both octoploid and diploid, and is both stable and PCR amplifiable before and after extended storage.

Polymorphisms deviating from genetic expectations were mainly observed in root and fresh leaf DNA, indicating that some RAPD markers may not present satisfactory leaves of reproducibility. Judicious and uniform selection of DNA purification method as well as tissue source for DNA extraction is, therefore, important considerations for reliable RAPD based DNA fingerprinting analysis in carrot. In addition, the studies allowed the identification of a better combination of procedures for use in routine manipulations of carrot DNA such as RFLP –RAPD based cultivar fingerprinting, molecular mapping, screening of transgenic plants, construction of genomic libraries, and gene cloning.

Quantification of DNA

DNA quantification can be done by flurometry, spectrometry and agarose gel electrophoresis with standard DNA concentrations (Boiteux *et al.,* 1999). The quality can be assessed by restriction digestion with restriction endonucleases (EcoRI, HindIII etc.,) electroporation and spectral properties. Quality is that to what extent the DNA is pure of secondary metabolites and other substances, which hinder further use of DNA in molecular techniques. A good DNA preparation generally exhibits the following spectral properties. It will have A_{230}, A_{230}/A_{260}, A_{280}/A_{260} or A_{260}/A_{280} rations of less than 0.10, less than 0.45, less than 1.65 or more than 1.80, respectively (Shantha *et al.,* 1998). If a DNA preparation exhibits

A_{260}/A_{280} more than 1.80, it shows the presence of RNA and if it is less than 1.65 or less indicates protein contamination (Sambrook *et al.,* 1989).

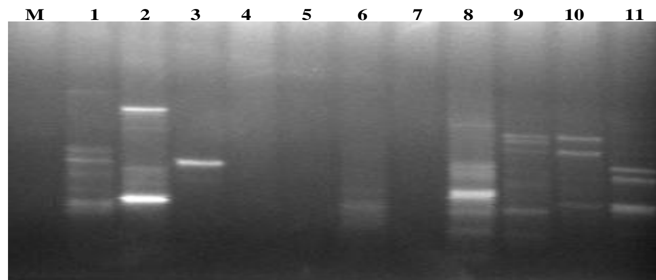
Results and Discussion

The data obtained in the present study regarding the RAPD molecular markers in anticancer drug plants are recorded. The genomic DNA was extracted as described in materials and methods from dry leaves of anticancer drug plants.The extraction method yield a very good high quality of DNA on test gel.It gave a good resolution.The quantity of DNA per gram of dry leaves tissue and relative ratio of DNA to proteins are given in Further PCR amplification of genomic DNA of anticancer plants with known universal primers was carried out. Than the amplified products was subjected to agarose gel electrophoretic isolation of random amplified polymorphic DNA.

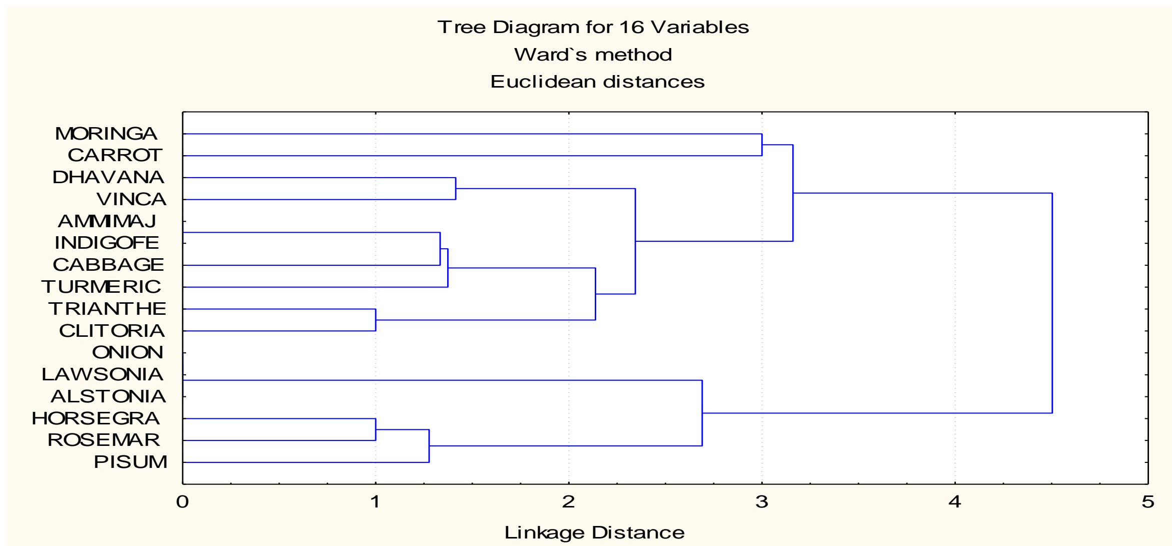
The following are the details of the individual plant parameters examined in this present work.

RAPD analysis of Turmeric (*Curcuma longa*).In this observation of the result 19 RAPD polymorphic and monomorphic bands was generated by the use of selected primers like OPF 01 – 11 and the same has been represented in the Fig .1and 2

Fig.1 and 2 Showed RAPD Bands and Dendrogram of Turmaric Amplified with Primers OPF-01 to OPF-11



Gel profile of Turmeric (*Curcuma longa*) amplified with selected primers like OPF-01 to OPF-11 series



RAPD amplification is performed in conditions resembling those of polymerase chain reaction using genomic DNA from the species of interest and a single short oligonucleotide primer (usually a 10-mer). Most genomes (from bacteria through humans) contain enough perfect or imperfect binding sites for a short primer orarbitrary sequence so that a subset of them will lie in inverted orientation to each other within a distance of up to a few thousand bp. As a result, a number of bands are amplified with each arbitrary sequence primer through the action of a DNA polymerase. A DNA sequence difference between individuals in a primer binding site result in the failure of the primer to bind, and hence in the absence of a particular band among the amplification products. The reaction products are conveniently analysed on agarose gels, and no radioactivity is needed. Each RAPD band results from the match of a primer to two sites in the genome (18-20 nt) while a RFLP band is produced by the action of a restriction endonuclease which recognises two hexanucleotide sites (12 nt). A RAPD reaction which produces 5 amplified bands may therefore screen for polymorphisms in 90-100 bp of or somatic embryos are very suitable. For a detailed discussions of the informativeness of different populations for mapping with RAPD.

The assessment of the anticancer medicinal plants germplasm diversity and the management of the genetic resources is pivotal for anticancer medicinal plant breeding in introgressing exotic genes and characterized into established cultivars Tanksley and Mc Cough 1997.Further more fingerprinting genotype offers an opportunity for the removal of any duplicaters that have been introduced through mislabeling or during multiplication in clonely propagated plants. When compared with other major crops of economic importance. The collection and conservation of anticancer medicinal plants has received worldwide attention. Morphological and physiological characteristics are not sufficient to differentiate some of the anticancer medicinal plants because the differences between them are often subtle. Assessment of germplasm resources is also made difficult because hybridization occurs commonly in nature and the relatedness of many genotype of anticancer plants is scanty (Sharma *et al.,* 2004).

RAPD Analysis of Turmeric (*Curcuma longa*).In this observation of the result 19 RAPD polymorphic and monomorphic bands was generated by the use of selected primers like OPF 01 – 11 and the same has been represented in the Fig .1and 2

These findings were similar to coffee plant reported by Sera *et al.,*2003 and Michalik *et al.,*2003 reported similar investigations in 26 carrot accessions as shown the fig 14.dia 14.table 14.However in the plant like.

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