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RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
AFLP	Amplified fragment length polymorphism
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
NMPB	National Medicinal Plants Board
TIFAC	Technology Information Forecasting and Assessment Council
SSR	Simple sequence repeat
UPGMA	Unweighted Pair Group Method with Arithmetic mean
NTSYS	Numerical Taxonomy and Multivariate Analysis System
CTAB	Cetyl trimethyl ammonium bromide
DAF	DNA Amplification Fingerprinting
VNTR	Variable Number Tandem Repeats
SNP	Single Nucleotide Polymorphism

ABSTRACT

Acknowledging the importance, increase in demand and exploitation of medicinal plants, a genuine endevour has been made in this report to examine genetic diversity among 15 accessions of economically important medicinal plant *Bacopa monnieri* using RAPD-PCR technique. Genomic DNA was isolated using the CTAB method with slight modifications. The amplification of isolated genomic DNA was accomplished by using 10 random oligonucleotide primers and an appropriate PCR program. Clear and unique bands were obtained implies that RAPD is an easy and reliable tool for plant genome analysis. Out of 10 RAPD primers, 8 primers proved to be reproducible. During the procedure of RAPD-PCR profiling, the amplification of genomic DNA of the 15 Brahmi (*B.monnieri*) samples of varied geographical origin was established by 8 primers generating 35 bands, of which 23 were polymorphic with 65.75% polymorphism. A dendogram was constructed and utilized using the UPGMA algorithm with the aid of NTSYS-pc software (SAHN module). Cluster analysis of RAPD data placed 15 Brahmi accessions into two main groups. The analysis grouped all the accessions having different geographical origins into different clusters. The results indicated that RAPD marker system can be efficiently utilized in determination of genetic variability among Brahmi accessions having different geographical origins. It can be concluded that this study prove to be useful for plant breeders in selecting wild type parents of *B.monnieri* in breeding programmes in the future, in designing sampling strategies for in situ and ex situ conservation of this commercially significant medicinal plant and also in authentication of this economically important medicinal plant for quality assurance of herbal material which is an essential prerequisite to ensure safety of herbal medicines obtained from Brahmi for human consumption. To our best of knowledge only few reports till date are assessible on the utility of RAPD markers for *B.monnieri* (Darokar *et al*., 2001; Tripathi *et al*., 2012; Karthikeyan *et al*., 2011).

1. INTRODUCTION

1.1 *Bacopa monnieri* - A medicinal plant

Bacopa monnieri is a small prostrate herb of family Scrophulariaceae and is extensively widely spread in the warmer regions of Asia, America and Australia. In India, it is commonly known as 'Brahmi' or the 'thinking person's herb'. *B.monnieri* is a perennial, creeping herb with fleshy leaves and light purple flowers that grows significantly in marshy, swampy, wet and damp fields throughout India (Chopra, HS. 1958 and Bone, K.1996). *Bacopa* is easily cultivated if adequate water is available and the whole plant is medicinally useful. In a report by Thejavathi *et al*.,1997 and Shrivastava *et al*., 1999, *B.monnieri* was second in the priority list of the most valuable Indian medicinal plants assessed on the basis of their medicinal value, commercial importance and potential for further research and development.

1.2 Economical Importance

B.monnieri (Brahmi) is one of the reputed drugs of Ayurveda and it has been extensively used since ancient times as a traditional orthodox medicine for enhancing memory and as anti inflammatory, antipyretic, analgesic sedative, anti epileptic agent. It also possess anticancerous properties (Ganjewala *et al.*, 1966). Brahmi has been used for centuries to prepare ayurvedic formulations like 'Brahmighritam' and 'Brahmirasayanam' (Tripathi *et al*., 2012). Pharmacologically, Brahmi has constituents that are helpful in curing mental illnesses and beneficial in the treatment of convulsive disorders like epilepsy. The plant has also been used in India from centuries as a cardiac aid, digestive tonic, and to improve respirative function in cases of broncho-constriction (Ganjewala *et al*., 1966). It is used in all sorts of skin problems – eczema, psoriasis, ulceration and it is said to induce the growth of skin, hair and nails. The extract of Brahmi has been found to be very profuse in the treatment of anxiety, neurosis and mental fatigue (Satyavati *et al*., 1976). It is widely used by students as it improves mental clarity, confidence and memory recall. It has been found to be effective against neurosis, Alzheimer's disease, diabetes, cancer etc. Brahmi is effective in treatment of Alzheimer's since it is used for cognitive impairment (Verma *et al*., 2013). *B.monnieri* contains different types of saponins such

as bacoside A, B, C and D of which two saponins, bacoside A and B are mostly influential active compounds of Brahmi. They are commonly called as 'memory chemicals' that are responsible for cognitive effect of this herb. In addition to the bacosides, *Bacopa* contains a wide variety of medically active substances which include sapogenins, stigmasterol, and flavonoids etc. Some other compounds include triterpenoid saponins. *Bacopa* also contains beta-sitosterol, D mannitol, octacosane, betulic acid, nicotine, and amino acids such as aspartic acid, alpha-alanine, glutamic acid, and serine (Singh *et al*., 1997). Alkaloids like Brahmines, Herpestine and an association of three alkaloids were reported from the leaves of this plant.

1.3 Commerical Value

According to NMPB and TIFAC, the yearly market demand of *Bacopa* during the year 2004-2005 was 6621.8 tons with an annual growth rate of 7% annually. This is rising rapidly in view of the popularity of the *Bacopa* based drugs due to its significant usage in ayurvedic medicine system to treat different ailments. The increase in demand of *B.monnieri* for its use in commercial formulations is because of high memory enhancing properties of bacosides. Due to this rising demand, NMPB and TIFAC has identified Brahmi among seven most important medicinal plants recommended for contiguous attention and have been included in the IUCN red list of endangered medicinal plant of India. In view of the wider market demand, there is straightaway necessity to conserve the wild stocks of *B. monnieri*. There is over exploitation of the natural populations of *B.monneri* because of commercial increase in the sale of herbal drugs like Memory Plus in the market (Ahmad *et al*.,1993). There is thus an immediate need for assessing the natural populations, developing protocols for agronomical practices at lower cost (Mathur *et al*., 1998). In recent years it has become difficult to maintain ample supply of medicinal plants due to ruthless exploitation, lack of conservation of the environment, increasing labor costs, economic and technical problems associated with the cultivation of medicinal plants.

1.4 Genetic diversity

Genetic diversity is significantly important for long duration survival and proliferation of species and it is a critical characteristic in conservation. For proper conservation and management, the gene pool of the species in different geographic factors needs to be assessed and analyzed. Morphological traits are affected by environmental factors. Therefore, precise identification based on morphological characteristics only becomes unmanageable and emphasize on the importance of molecular markers for accurate identification. Recently, RAPD based fingerprinting analysis have been increasingly utilized for analyzing genetic polymorphism in different plant genera. Due to technically simple and rapid nature, RAPD methodology has been extensively used in diversity analysis in many medicinal plant species (Padamlatha *et al*., 2007). PCR based RAPD marker was widely used in assessing genetic variation with in a species by measuring genetic diversity in many species, including medicinal plants. The most common uses of the technique for scientific and commercial purposes include species characterization and adulteration detection in medicinal plants and determination of genetic variation in wild and cultivated populations (Kumar *et al*. 2011).

1.5 DNA markers used in plant genome analysis

DNA markers have many advantages over phenotypic markers and they provide reliable information on DNA polymorphisms as the genetic pool is unique for each individual species and is not influenced by age, environmental factors as well as physiological conditions. There are different types of DNA based molecular markers which are used to assess DNA polymorphism in order to analyze genetic variability. They are hybridization based methods, PCR based techniques and methods based on sequencing (Joshi *et al*., 2004).

Hybridization based methods include RFLP, VNTRs and microarray or chip based. Polymorphism is detected by the presence or absence of bands upon binding.

PCR based methods require in vitro amplification of DNA sequences with the help of arbitrary or specific oligonucleotide primers and thermostable DNA polymerase enzyme. PCR based methods where random primers are used include RAPD, AP-

PCR and DAF. On the other hand, PCR based methods where specific primers are used include ISSR. AFLP is a technique which is based on the identification of genomic restriction fragments amplified by PCR. DNA sequencing is also used as effective means for genetic variability analysis. Genetic variations occur at the single nucleotide level. Direct sequencing can effectively detect such single nucleotide polymorphisms (Srivastava *et al*, 2009).

The genetic diversity analysis of Indian herb *B.monnieri* accessions using molecular markers is precisely less. Only few reports on the use of RAPD marker for medicinal herb *B.monnieri* are available (Tripathi *et al*. 2012).

In this report with PCR-based DNA (RAPD) markers, we will exclusively detail the feasibility for the identification of phylogenetic relationship among collected *B. monnieri* accessions. The objective of the present study is to assess genetic diversity of different accessions of *B.monnieri* which is economically an important medicinal plant of Central India and to provide genetic information and a theoretical baseline for protection of the species.

2. REVIEW OF LITERATURE

2.1 Herbal Medicines

According to the WHO, "Herbal Medicines include herbs, herbal preparations and herbal finished products". Herbal formulations and preparations may also include powdered herbal materials or extracts and fatty oils of herbal materials and form basis for finished herbal products (Srivastava *et al*., 2009). Herbal drugs are integrated part of traditional medicine system and modern medicine system especially in developing countries. Herbal drugs include crude plant parts, extracts, oils and mixtures of extracts. Use of drugs from plant origin forms a major part of complementary and alternative medicine (CAM). Herbal drug technology comprises all the steps that are required in converting botanical material into herbal medicine, where quality assurance with appropriate integration of modern techniques and traditional knowledge remain significant (Anonymous, 2002). Herbal drugs may vary in properties and compositions, unlike conventional pharmaceutical drugs, which are prepared from chemically pure materials by manufacturing techniques. Correct identification and quality control of herbal material is important to check quality of herbal medicine, which contributes to its safety and the root cause lies mainly with the starting material where the adulteration takes place (Joshi *et al*., 2004).

2.2 Current trends in herbal medicine

Medicinal plants play a key role in world health. In spite of recent advancement in modern medicine system, herbal plants still make a significant contribution to worldwide healthcare needs. There are minimum 120 discrete chemical substances obtained from plants considered as important drugs currently used worldwide. Demand for herbal medicines in the developed and developing countries is increasing due of their wide biological activity, higher safety standards than usual synthetic drugs. Consequently, Herbal drugs have a great potential in the global market. The herbal medicine world market including raw material and herbal products has been estimated to have a yearly growth rate of 5-15%. Total global herbal drug market is

expected to rise to US $5 trillion by 2050 (Joshi *et al*., 2004). According to the WHO, 75-80% total world population is dependent on herbal drugs mainly because of their high compatibility with human body, ease of availability and lesser side effects (Nikam *et al*., 2012). India has vast wealth of traditional knowledge and the Indian herbal medicinal plant industry is expanding at the rate of approximately 15% yearly. The medicinal plant related trade value in India is estimated at Rs. 5000 crores per year (Biswas *et al.,* 2014). India has recognized over 3000 plants for their medicinal importance. Traditionally, over 6000 plants are in use for preparing herbal formulations. Globally increasing demands of medicinal plants for pharmaceuticals, nutraceuticals, cosmetics and other products has provided an opportunity for Indian trade (Joshi *et al.,* 2004). About 90% of different medicinal plant species currently used are collected from wild and about only 10% of medicinal plants used commercially are cultivated. Recently, herbal drugs are prepared mostly by using eco-friendly methods from plants as starting material and can be defined as preparations containing bioactive constituents of medicinal importance. Herbal medicines are also often called as phytomedicines (Banerjee *et al*., 2012).

2.3 Molecular markers in medicinal plants

Currently, research on DNA based molecular markers is in expanding in various research institutes worldwide. DNA markers have a high utility in the herbal medicine analysis and are widely used for the authentication of plant varieties of medicinal importance (Biswas *et al*., 2014). Molecular markers specifically genetic marker detect plant at genomic level and establish new standards in quality assurance of botanicals. Hence, molecular markers are more reliable and suitable for herbal drug analysis. In general, molecular markers are highly polymorphic, occur frequently in genome and remain unaffected by environmental conditions or management practices and are easily available and highly reproducible (Kumar *et al*., 2009). In 1980's, new molecular techniques have made it possible to identify variations in DNA, leading to the use of unlimited number of genetic markers which can be used for creating genetic maps as well as for studying phlyogenetic linkages and genetic inheritance (Shaqha *et al*., 2014).

A molecular marker is a genetic or a DNA sequence with a known location on a chromosome. A molecular marker may be a short DNA sequence like SNP or a long sequence like that of minisatellites (Srivastava *et al*., 2009). In other words, a molecular marker can be defined as a DNA sequence which is readily detected and whose inheritance can be easily be monitored. A molecular marker should be polymorphic in characteristics i.e. it must have its existence in different forms so that chromosome carrying the mutant genes can be distinguished from the chromosomes with the normal gene by a marker. Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two discontinuous variants or genotypes. DNA markers perhaps seem to be one of the best candidates for efficient and effective evaluation and selection of plant material as they are not affected by the environment (Gantait *et al*., 2014). The extraction of DNA from plant materials is quite easy and its analysis can be cost and labor effective.

There are some desirable properties which an ideal molecular marker must have. It should be highly polymorphic in nature. The basis of measuring genetic variability is polymorphism. A marker should occur frequently throughout the genome and should be evenly distributed. It should be fast and easy to detect. The marker assay should be cheap and easy. It must have high reproducibility. (Kumar *et al*., 2012). However, it is not easy to find a molecular marker having all the desirable properties. Depending upon the aim of the study, a marker system can be selected that will fulfill some of the above mentioned characteristics.

There are various different varities of markers viz. biochemical, morphological and DNA based molecular markers. These DNA based markers are differentiated into two kinds one as non PCR based (RFLP) and other as PCR based markers (RAPD, AFLP, SSR, SNP etc.). They can be further classified as dominant or co-dominant. Dominant markers analyze many loci simultaneously at one time, e.g. RAPD. Co-dominant markers allow analysis of one locus at one given time (Gantait *et al*., 2014)

2.4 Randomly Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is a type of PCR based molecular marker technique. These random oligonucleotide primers detect polymorphism

without having any prior information about the nucleotide sequence. It is unlikely to differentiate whether amplified DNA sequence is homozygous or heterozygous since RAPD primers are mostly dominant. A basic RAPD – PCR technique involves five steps (i) isolation of pure DNA (ii) polymerase chain reaction using RAPD primers (iii) separation of amplified fragments using gel electrophoresis (iv) visualisation of amplified RAPD- PCR fragments under UV light after ethidium bromide staining (v) determination of fragmented band size by comparing with known molecular marker using suitable gel analysis software. This technique does not involve any hybridization or blotting step. The technique only requires a thermocycler, electrophoresis unit and relevant chemicals which are easily available as commercial kits (Ibrahim *et al*., 2010). The technique is simple, efficient, quick, cost friendly and does not require genomic information. Another advantage of RAPD technique is that only small amount of template DNA samples is required (10-50 ng per reaction) because PCR is involved. RAPD markers have limited usage in genome mapping since it is a dominant marker (Kumar *et al*., 2009).

Random amplified polymorphic DNA (RAPD) proficiency has been widely utilized in many plant species for analysis of varieties, studies on populations and phylogenetic linkage mapping. Optimization of the RAPD protocol depends on selection of primers. Although, the RAPD method uses arbitrary sequences of primer, all but many of these primers must be put to screening in order to pick primers that provide varied useful amplification products. RAPD is a non-specific marker and hence, it is easier to put it to use for minor and under-exploited species (Zothansangi *et al*., 2011)
An attempt was made to investigate true variation among the collected sampled different species of *Asparagus* by using RAPD markers. RAPD markers are usually based on the amplification of characteristically unknown DNA sequences using single, short, random oligonucleotide primers. Henceforth, RAPD polymorphism is the mirror reflection of variation of the whole genomic DNA and would be a more efficient parameter to measure the genetic diversity pattern of the rare and endangered plants. (Sairkar *et al*., 2013)

2.5 APPLICATIONS OF MOLECULAR MARKERS IN PLANT GENOME ANALYSIS

2.5.1 GENETIC VARIATIONS

Due to over exploitation of medicinal and ornamental plants for commercial preparations the medicinal plants are under great threat of getting extinct. There is an immediate need of novel methods for conservation of medicinal plants. Aromatic and medicinal plant utilizations have attracted worldwide concern due to their over exploitation. (Tripathi *et al*., 2012) One of the widely accepted methods is the in vitro propagation technique. However, in vitro plants are usually vulnerable to genetic changes because of environmental conditions or culture stress. Several techniques have been employed to check such genetic variations and to authenticate genetic stability of micro propagated plants. One such novel method is the use of molecular markers techniques.

In a study, Random Amplified Polymorphic DNA (RAPD) markers were used to analyze genetic fidelity of the in vitro propagated plants to confirm the genetic homogeneity of micro propagated plantlets. (Raji *et al*., 2014) It is necessary to examine the amount of variation prevailing within and between populations since genetic variability is a requirement for strain selection programmes. (Govarthanan *et al*., 2011) The geographical conditions influence the bioactive components of the medicinal plants and therefore their activities. Genetic variations at the genomic level have been studied by many researchers. Different accessions of *Taxus wallichiana, neem* and *Allium schoenoprasum* obtained from different geographical regions have been differentiated using RAPD markers. Estimation of genetic variations is necessary in designing crop improvement programmes for germplasm management and is also helpful in conservation strategies outline.

In a report by Joshi *et al*, 2004, RAPD and RFLP has been used to study interspecies variation in different genera such as *Arabidopsis*, *Curcuma* and *Echinacea* and have also been utilized for characterization of *Epimedium* species at genomic level. In a report RAPD markers have been utilized to test genetic purity in *Capsicum annuum*

and pepper. RAPD-PCR technique was used as an effective tool for genetic variability detection in Jojoba and tea. Molecular markers have been used to study population structure of *Podophyllum Peltatum* to analyze propagation of important secondary metabolites for commercial preparations. Genetic variation and relationship has been studied among *Brassica campestris* using RAPD and AFLP. An attempt has been made to construct linkage maps of *Eucalyptus urophylla* and *Eucalyptus grandis*. Genetic map of Pacific yew (*Taxus Bravifolia Nutt*.) has been developed using RAPD molecular markers (Joshi *et al*., 2004).

In a report by Tripathi *et al,* 2012 genetic variations of 15 accessions of *B.monnieri* were assessed using 22 RAPD and 25 ISSR markers. RAPD and ISSR can be efficiently utilized in detecting genetic relationship among different Brahmi accessions obtained from different areas of central India. The data of genetic diversity and likeliness among *Bacopa* accessions is essential for breeding and conservation programs. RAPD markers have been used in the identification of genotypes (Tripathi *et al*., 2012).

In a study by Govarthanan *et al,* 2011 RAPD banding pattern analysis was used to examine genetic diversity within *Coleus sp*. and to determine the best suitable quality for commercial purpose and human consumption. In a study carried out by Shaw *et al* 2008 PCR based DNA markers RAPD and ISSR were used to examine genetic variability among different cultivars of *Catharanthus roseus*. In this investigation, 11 RAPD and 4 ISSR markers were used to evaluate genetic diversity between 17 cultivars of *Catharanthus roseus*. This report proved to be helpful for designing breeding programmes of improved quality.(Shaw *et al*., 2009). RAPD analysis has been used to achieve high density genetic maps of medicinal plants like *Withania somnifera* and *Chlorophytum borivilianum*. Ramesh *et al.,* 2011 used RAPD fingerprinting technique to analyze genetic stability of 19 different in vitro propagated clones with wild type mother plant (Gantait *et al*., 2014).

In a report by Pathak *et al.,* 2013 somaclonal variants between in vitro and in vivo plantlets of *Tylophora indica* and *Bacopa monnieri* were detected using RAPD markers. Plant tissue culture techniques usually give rise to somaclonal variations which makes it a necessity to test the homogeneity of plants. Variants can be

distinguished at the morphological and molecular levels. However morphological variations can only be detected when the plant matures and they occur at very low frequency. On the other hand molecular markers are highly effective and preferred as novel tools for analyzing genetic stability as environmental factors have no influence. RAPD markers are widely used to detect somaclonal variants (Pathak *et al*., 2013).

RAPD has served as a tool to determine genetic relationship and molecular characterization within 9 varieties of *Hibiscus* species (Prasad, MP. 2014). RAPD analysis in association with morphological traits can be utilized in the identification and determination of the genetic variability among the different varieties of *Hibiscus*. Thus, RAPD markers are capable of identifying and analyzing genetic variability within the varieties of a species. RAPD marker based molecular characterization is useful in determining the existence of gene flow and allele movement between and within populations. This investigation is helpful in studying phylogenetic relationships.

Random Amplified Polymorphic DNA (RAPD) has been most effectively used to elucidate molecular genetic fingerprints of 18 medicinal plant species *Hemigraphis colorata, Marjorana hortensis, Artemisia vulgaris, Artemisia pallens, Ocimum sanctum, Ocimum basilicum, Ocimum gratissimum, Mentha piparita, Mentha citrata, Mentha spicata, Acorus calamus, Centella asiatica, Bacopa monnieri, Piper longum, Piper nigrum, Clitoriaternatea, Aloe Vera, Stevia rebaudiana* collected from different areas of Karnataka. 20 RAPD primers were used. This method of assessment proved to be useful in giving broadness to the germplasm of medicinal plants and in selecting diverse parents in plant breeding programs in future (Radhika *et al*., 2012).

In a study, evaluation of genetic diversity in a population of 40 *Jatropha curcas* L. genotypes collected from different geographical areas of India were carried out using 44 RAPD marker. Breeders can easily identify diverse parent genotypes from genetic relationships and cluster analysis and can effectively utilize in future breeding programmes (Ikbal *et al*., 2010). In a report carried out by Raji S *et al* 2014, 10 RAPD primers were used to analyze genetic fidelity of in vitro propagated plants and callus of *Justicia tranquebariensis* L.f. which is a well established medicinal plant. The results showed high frequency shoot multiplication and high molecular stability which

ensures suitability of the developed protocol for production and conservation of this economically important herb. Genetic variability in two important medicinal plants collected from seven different geographical regions of Madhya Pradesh, India of economic value *Withania somnifera* and *Rauwolfia serentina* was analyzed using RAPD markers. *R.serpentina* revealed high genetic diversity whereas *W. somnifera* revealed low genetic diversity. High diversity implies good sign of existence of species whereas low diversity shows immediate need for appropriate conservation pragrammes to be implemented (Sairkar *et al*., 2013).

An attempt has been made to analyze variations between five different varieties of *Asparagus* of Asparagaceae obtained from Gujarat, India using 20 RAPD markers. Because of its high therapeutic and nutraceutical properties, *Asparagus* is an economically important plant. In this evaluation, low level of similarity and high level of diversity was observed. This data proved to be helpful in inter breeding programmes and for authentication of taxa which is important for consumers and breeders to ensure protection of IPR (Lal *et al*., 2011). Hence, RAPD markers are intensely used in determination of genotyping and identification of genetic varieties of *Asparagus spp*.

Differences between genotypes are reflected as a result of differences in banding patterns. In a study, RAPD technology was utilized to examine stages of variations between rice (*Oryza sativa*) germplasm collection (Virk *et al.*, 1995). In another study carried out by Domyati *et al* 2011 molecular markers such as ISSR, RAPD and AFLP were used to fingerprint selected germplasm of seven medicinal plants *Artemisia judaica* L., *Arnebia hispidissima* (Lehm.) DC., *Aerva javanica* (Burm. f.) Juss, *Balanites aegyptiaca* (L.) Del, *Cleome droserifolia* (Forssk.) Delile, *Zygophyllum simplex* L., *Zilla spinosa* (L.) Prantl along the Western Red Sea coast of Sinai.

RAPD profiling has been effectively utilized to examine molecular characterization of cultivated Pawpaw (*Asimina triloba*). 34 varieties of cultivated pawpaw were investigated using 71 RAPD primers to establish genetic relatedness which was further examined using UPGMA cluster analysis. The data obtained from RAPD profiling provides useful information in sampling strategy decision making and

management of germplasm. It also helps breeders to decide which parent to select for breeding in future (Huang *et al*., 2003)

2.5.2 AUTHENTICATION OF MEDICINAL PLANTS

The authentication of herbal material on the DNA level is based on accurate identification of species, which is a key to ensure efficacy, safety, therapeutic potency, minimizing trading fraud, herbal drug quality and to increase consumer confidence in herbal medicines (Joshi *et al*., 2004). Herbal material usually contains dried and processed material which makes their identification difficult as useful characters are lost in the drying process (Biswas *et al*., 2014). Morphological characters and phytochemical characters form the basis of identification of medicinal plants. Molecular marker serves as a reliable tool for authentication of medicinal plants at DNA level owing to the advancement in plant genetics and molecular biology in the past decades. Authentication at DNA level is essential for quality assurance and for reliable, successful clinical application of medicinal plants. Unlike chemical fingerprinting techniques, DNA is not affected by external factors such as age, environmental conditions, area, drying conditions, storage and harvesting period since DNA is highly stable molecule (Matsubara *et al*., 2012)

In a report by Matsubara K *et al* 2012 an efficient method was developed to authenticate *Angelica acutiloba* var. *acutiloba* and *A. acutiloba* var. *sugiyamae* from one another and among other *Angelica* varieties using RAPD analysis. It was used to determine whether the Japanese *Angelica* root material used is a pure variety or not.

The randomly amplified polymorphic DNA (RAPD) – PCR technique was effectively employed in authentication of *Cuscuta reflexa* and its adulterant *Cuscata chinensis*. The results obtained thus showed that RAPD can serve as complementary tool for quality assurance. The authentication of medicinal plants is highly crucial issue to provide protection to the consumers. The consumption of wrong herb may harm the health of patients which can sometimes prove to be fatal. The consumption of substituted and adulterated herbal drug may be extremely dangerous. For example, traditional Chinese medicine (TCM) was found to be contaminated with *Aristolochia* that caused epidemic of subacute intestinal nephropathy. This made the patients

undergo kidney transplantation in Belgium. Many pharmaceutical industries obtain herbal plant materials from traders who have collected them from unskilled collectors from forest areas or rural places. This gives rise to increase in adulteration of herbal materials which ultimately leads to bad quality of herbal preparations. Wrong identification of herbs can be intentional or non intentional. Example of intentional misidentification is when profit driven traders sometimes substitute expensive herbs with less expensive similar varieties. For example, *Swertia chirata* is often adulterated by easily available, morphologically similar and inexpensive *Andrographis paniculata* (Joshi *et al* 2004). Non intentional identification can happen when it is extremely difficult to differentiate processed plant parts. Thus, to maintain the safety and efficacy of herbal drug formulations, it is extremely essential to rely on DNA based molecular markers (Khan *et al*., 2009).

The morphological traits are not reliable for authentication of medicinal plants whether in dried state or fresh state as they vary with environmental conditions and surroundings. In a report by Joshi *et al* 2004, dried samples of fruit *Lycium barbarum* were distinguished using RAPD markers from its related species. The RAPD –PCR technique has been effectively used to determine components of yu-ping-feng san which is a Chinese herbal formulation. In this study a single RAPD primer was used to identify the presence of 3 herbs (*Astragalus membanaceus, Ledebouriella seseloides Wolff* and *Atractylodes macrocephala Koidz*) in the formulation yu-ping-feng san.

Increase in worldwide popularity of herbal drugs has lead to increase in their adulteration at commercial level leading to poor quality of herbal medicines. Adulteration can also occur due to ignorance. Genetic analysis helps in useful identification of genotypes which improve efficacy of standard drug formulations. Hence, DNA based marker analysis have gained popularity in quality assurance to authenticate crude materials. In a study 32 RAPD markers were used to identify *Senna angustifolia, Senna acutifolia, Senna tora* and *Senna sophera* (Khan *et al*., 2011).

In a study RAPD-PCR technique was employed to determine the presence of different herbal ingredients in an ayurvedic formulation, *Rasayana churna* using 120 decamer

oligonucleotide primers. Three ayurvedic components (dried stem of *Tinospora cordifolia*, dried fruit of *Tribulus terestris* and dried fruit of *Emblica officinalis*) were identified in the RAPD analysis. (Shinde *et al* 2007). This assay proved to be helpful in ensuring quality control standards of herbal medicine at commercial level.

In another report, RAPD markers were successfully employed to authenticate an important ayurvedic drug Shankhpushpi. Because of high price and high demand, shankhpushpi is often adulterated by trader with cheaper related species. The aim of this study was to identify *Convolvulus pluricaulis, Evolvulus alsinoides* and *Clitoria ternatea* which are the common sources of shankhpushpi, and to analyze the market samples of Shankhpushpi to ensure their safety (Ganie *et al.*, 2011).

2.5.3 MARKER ASSISTED SELECTION OF DESIRABLE CHEMOTYPE

Most of the pharmaceutical companies rely on microscopic and macroscopic estimation and chemical profiling of herbal materials for standardization and quality assurance in their regulatory guidelines. Microscopic evaluation involves inspection of crude as well as powdered herbal materials based on parameters like color, shape, size, texture, odor, taste etc that are compared to a standard reference material. Many analytical techniques including thin layer chromatography (TLC), gas chromatography, high performance liquid chromatography (HPLC), spectrophotometry are used for standardization and quality control of the herbal material to ensure its safety and efficacy for human consumption. However, because of chemical complexity, chromatographic technique has limitation in standardization of botanical preparations. Many intrinsic and extrinsic factors affect the chemical profile of medicinal herbs. Intrinsic factors include genetics and extrinsic factors include harvesting, drying, cultivation and storage conditions. Use of specific DNA based markers remains a preferred option for selection of desirable chemotypes. The markers used are ideally neutral to environmental conditions, surroundings and management practices. Selection of desirable and correct chemotype of herbal plant is essential to ensure efficacy. Identification of right type of chemotype of herbal plant is difficult. For example, in a study, there are three chemotypes of *Withania somnifera* on the basis of presence of similar steroidal lactones like withaferin A, withanolides etc. The content of biologically active compounds differ depending upon the genotype

of plant, environment, time at which plant material was collected etc. As a result it is important to select right chemotype to ensure safety and therapeutic efficacy. (Joshi *et al*., 2004)

For effective quality control parameters to use herbal material for commercial purposes, prediction of accurate concentration of bioactive chemicals is required. DNA marker based fingerprinting techniques have been extensively utilized in selection of correct chemotype of herbal plant. In a study, AFLP markers have been used to predict phytochemical markers in varieties of *Echinacea purpurea* (Joshi *et al.,* 2004).

In another study, RAPD markers were used to characterize chemotypes of *Artemisia annua* variants. *A.annua* is a source of antimalarial compound artemisinin. The content of this bioactive compound varies in different varieties of *A. annua* all over India. A study has been carried out to analyze variations in essential oil constituents using RAPD marker technique. 12 basil (*Ocimum gratissimum*) accessions were analyzed to study differences in morphological, genetic and chemical properties to determine whether volatile oils and flavonoids can be used to analyze relation between RAPDs and these chemical markers.

2.6.4 OTHER APPLICATIONS

Today molecular markers are used as effective tools in medicinal plant breeding programmes. In a study ISSR-PCR technique has been successfully employed to identify zygotic plantlets in citrus interploid crosses. Molecular markers have proved to be a reliable tool to verify sexual offsprings of intraspecific crosses in a very well known diuretic, *Hypericum perforatum*. RAPD markers have been efficiently employed towards marker assisted identification of fertile clones of garlic. Micropropogated plants of *Piper longum* have been effectively selected for conservation using RAPD- PCR technique. (Joshi *et al* 2004)

Recently, molecular markers are being explored in the field of food industries and nutraceuticals. There are wide range of applications of molecular markers in the food crops and horticultural plants. These include genetic variability studies, diversity

analysis of exotic germplasms, phylogenetic analysis, cultivar identification, genotyping, identification of disease resistant genes etc. In a survey, PCR technique has been successfully utilized by Food Safety Authority of Ireland to evaluate the levels of GM maize ingredients in taco shells and tortilla chips in Ireland. In another study, primers specific for inserted genes have been found to be suitable for successful discrimination of Bt 176 maize, *Cecropin capsicum* and Roundup Ready soybeans from non GM crops.

Recently, DNA markers have found to play an important role in the field of pharmacognosy. This field deals with all aspects of drug discovery and drug development. Molecular markers play important role in plant genome analysis with its use in pharmacognostic identification and research. Several agricultural and research institutes in India are actively utilizing DNA marker based techniques in genotyping of medicinal plants. Proper utilization of molecular markers and analytical techniques will lead to development of a reliable system of standardization which can be conveniently utilized for quality control of herbal preparations at industrial level.

3. METHODOLOGY

The materials required and the methodology of the present work was carried out at Plant Tissue Culture laboratory, Department of Biotechnology, Delhi Technological University, Delhi, India. The materials required and the methods involved are presented here.

3.1 Plant materials

A total of 15 Brahmi *(B.monnieri*) accessions having different geographical origins were collected from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow and maintained at Plant Tissue culture lab, Department of Biotechnology, Delhi Technological University, Delhi. Fresh, young, disease free leaves were collected for genetic variability assessment using RAPD.

3.2 DNA extraction

1. Fresh, young leaves were collected from each plant of every accession and immediately kept on ice to reduce the nuclease activity.
2. The DNA was extracted using the CTAB method of Doyle and Doyle (1990) with certain modifications.
3. 1 g of leaf sample along with 30mg polyvinylpyrrolidone (PVP) was grinded in liquid nitrogen to fine powder.
4. The powdered plant material was placed in centrifuge tubes and 1 ml of CTAB extraction buffer (prewarmed at 65°C) and 10µl of 0.2% mercaptoethanol was added.
5. This mixture was incubated at 65°C for about 45min-1hr in water bath with intermittent shaking after every 10 min.
6. The suspension was then cooled to room temperature and equal volume of chloroform and isoamyl alcohol (24:1) was added to supernatant.
7. The mixture was then subjected to centrifugation at 10,000 rpm for 15 min.
8. The aqueous phase was taken, and 2 volume of cold isopropanol was added to it and incubated at –20°C for 1 h or at 4°C for overnight.

9. The sample was centrifuged at 10,000rpm for 10 min to obtain DNA pellet.
10. Discard the supernatant and the pellet was washed with ice cold 70% ethanol and centrifuge at 10,000 rpm for 10 min.
11. Dry the pellet at 37°C for 30-45min and dissolve in 100µl TE buffer and keep at 4°C for overnight.
12. The isolated DNA was treated with RNase A (1mg/ml) at 37°C for 1hr.
13. Equal volume of chloroform and isoamyl alcohol was mixed and was further subjected to centrifugation at 13,000rpm for 10 min.
14. $1/10^{th}$ volume of 3M sodium acetate along with 3 volume of ethanol was poured to supernatant.
15. Keep at 4°C for overnight.
16. At 13,000rpm it was centrifuged for 10 min.
17. Supernatant was discarded and 200µl 70% ethanol was added and was further subjected to centrifugation at 13,000rpm for 5min.
18. The pellet was dried at 37°C for 30-45min till ethanol evaporated and was dissolved in TE buffer.

3.3 Estimation of DNA on quantitative and qualitative basis

1. Quantity of DNA was assessed using Nanodrop Spectrophotometer at the ratio of absorbance at 260 nm and 280 nm such that pure DNA preparation is determined at a value of 1.8. A value less than 1.8 signifies presence of either proteins or phenol as impurities. Quantity of DNA samples was calculated using the following formula:

   Concentration of DNA= A260 X 50µg X dilution factor

   Purity of DNA = A260: A280 ratio = A260/ A280
2. The DNA quality is also determined using agarose gel electro ethidium bromide staining.

RAPD primers	Sequence(5'- 3')	mer	nmol	µl for 100µM
OPA-11	CAATCGCCGT	10	55.9	559
OPB-08	GTCCACACGG	10	42.2	422
OPA-10	CTGCTGGGAC	10	41.3	413
OPC-05	GATGACCGCC	10	41.7	417
OPC-09	CTCACCGTCC	10	83.3	833
OPAC-12	GGCGAGTGTG	10	42.3	423
OPAB-07	GTAAAGCGCC	10	35.5	355
OPAA-04	AGGACTGCTC	10	39.3	393
OPAD-06	AAGTGCACGG	10	32.2	322
OPAH-05	TTGCAGGCAG	10	31.1	311

Table.1 Sequence information of RAPD primers used for amplification in present study.

3.4 PCR Amplification

1. The PCR amplification was performed according to the method developed by McClelland et al. (1995).
2. Aliquot stock solution of RAPD primers is prepared by adding 10 times Mili Q water of the nanogram concentration of RAPD primers to the primer stock to make stock solution volume of 100 pmol. Working solution of primers is made by adding 10ml of primer stock solution and 90 ml of distilled water. Final working stock solution volume is 10 pmol.
3. PCR reaction was carried out in 25 µl reaction tubes, using 10 random primers.
4. PCR reaction mixture contains 10X Taq buffer, 25mM $MgCl_2$, 10X dNTPs, Taq polymerase, miliQ and primers.
5. Amplification was performed in a thermo cycler.
6. A standard PCR cycle was used: an initial denaturation step at 94°C for 5 min, followed by 45 cycles of 94°C for 1 min, 37°C for 1 min, and 72°C for 2 min; the final extension was held at 72°C for 7 min.

Components	Volume for 1 reaction set up (µl)	Volume used for 16 reactions set up (µl)
Taq buffer (10X)	2.5	40
$MgCl_2$ (25mM)	1.5	24
dNTPs (10X)	0.5	8
Taq polymerase (10X)	0.1	1.6
RAPD marker	5.0	80
Mili Q H_20	13.4	214.4

Table.2 PCR reaction set-up

Temperature	Duration	Step	Number of cycles
94°C	5 min	Initial denaturation	1
94°C	1 min	Denaturation	
37°C	1 min	Annealing	42
72°C	2 min	Extension	
72°C	7 min	Final extension	1
4 °C	Overnight	Hold	overnight

Table.3 Temperature – time profile used for RAPD

3.5 Agarose gel electrophoresis

1. Agarose gels were prepared using an agarose concentration appropriate to the size of DNA fragments to be separated. For genomic DNA 0.8% concentration of agarose gel was used whereas for PCR product 1% agarose gel was used.
2. PCR products were resolved on 1% agarose gel in 1X TAE buffer along with standard 1kb ladder.
3. The gel was run for 45 min- 1 hour at 80V.
4. The agarose gel containing 0.5 μgml^{-1} ethidium bromide is visualized in GEL-DOC and photograph is taken.

3.6 Data analysis

1. Evaluation of fragment patterns is carried out by similarity index.
2. DNA banding patterns generated by RAPD are scored from the photograph as '1' for the presence of band and '0' for its absence. Only clear bands were considered. The bands are considered polymorphic when they are absent in some samples. On the other hand, bands are considered monomorphic when they are present in all samples. Change in band intensity is not considered as polymorphism.
3. A final RAPD data is generated in form of MS Excel sheet which is used to calculate pairwise similarity co-efficients using the SIMQUAL format of NTSYS-pc software package.
4. Cluster analysis was performed and the resulting similarity co-efficients were used to construct dendogram using the SAHN module of computer package program Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc).

4. RESULTS

4.1 Extraction of genomic DNA

15 accessions of *B.monnieri* having different geographical origins were collected from CIMAP, Lucknow, India and maintained at plant tissue culture laboratory of Department of Biotechnology, Delhi Technological University, Delhi. Genomic DNA of 15 samples was successfully extracted using a modified protocol of CTAB method of Doyle and Doyle. DNA extraction is the first step for all types of molecular marker analysis. DNA was extracted using fresh green leaves of *B.monnieri*. However, DNA can also be extracted from dried, preserved or lyophilized samples but it is recommended to use fresh material to have good quality DNA. The yield and quantity of isolated DNA from different accessions of *B.monnieri* was evaluated using Nanodrop Spectrophotometer. The yield obtained by Nanodrop Spectrophotometer was approximately in the range 150-260 ng/µl. An absorbance (A_{260}/A_{280}) ratio of 1.6-2.0 indicated insignificant levels of contaminating proteins and polysaccharides in DNA. Therefore, isolated DNA was treated with RNAase A in order to obtain DNA free from contaminating polysaccharides and proteins. The pure DNA samples thus obtained were then separated on 0.8% agarose gel for the quality of DNA. Clear and visible bands were obtained after gel electrophoresis of plant genomic DNA.

4.2 Screening of RAPD primers

Choosing suitable primers is extremely important step of RAPD- PCR technique to get viable, clear and good quality bands. In this study, the 10 selected primers were OPA-11, OPB-08, OPA-10, OPC-05, OPC-09, OPAC-12, OPAB-07, OPAA-04, OPAD-06 and OPAH-05. The primers were selected to generate RAPD pattern for all accessions of *B.monnieri* samples. The number of fragments generated depends on the primer sequence rather than the nucleotide length. In the present study, PCR amplification of 15 DNA samples was performed with 10 RAPD primers. Amplification reaction was performed with a reaction volume of 25 µl containing following components: 2.5 µl of Taq buffer (10X), 1.5 µl of $MgCl_2$ (25mM), 0.5 µl of dNTPs (10X), 0.1 µl of Taq polymerase (5U/µl), 5 µl of RAPD primer and 2 µl of DNA template. Final volume was made upto 25 µl using Mili Q water. The PCR

amplification was achieved in a ThermoCycler using following conditions: Initial denaturation was done at 94°C for 5 min, 42 cycles of denaturation were futher done at 94°C for 1 min, annealing at 37°C for 1 min, extension step at 72°C for 2 min. The samples were made to subject to a final extension step at 72°C for 7 min. The products of PCR were then made to resolve on 1% agarose gel, visualized and photographed under GEL-DOC spectrophotometer. Out of 10 random oligonucleotide primers, the amplification of only 8 RAPD primers (OPA-11, OPB-08, OPA-10, OPC-05, OPAB-07, OPAA-04, OPAD-06, OPAH-05) were satisfactory. The most valid reason for the non amplification of the 2 primers (OPAC-12, OPC-09) could be that the amplified DNA had no binding site for the primers.

4.3 RAPD analysis

The amplification patterns of 8 RAPD primers have been depicted in Fig.1 to Fig.8 and the details of RAPD analysis in Table no. 4. Different primers produced fragments of different patterns. Out of 10 primers, 8 primers displayed amplification of 35 bands out of which 23 bands (65.75%) were polymorphic while 12 bands (34.28%) were monomorphic, indicating the presence of a relatively high degree of genetic variability in studied accessions of *B.monnieri*. All 8 RAPD primers were found to be polymorphic, out of which 1 primer (OPB-08) was found to have 80% polymorphism, 4 primers (OPC-05, OPAB-07, OPAA-04 and OPAD-06) were found to have 75% polymorphism. Primer OPA-11 and primer OPA-10 were found to have polymorphism of 50% and 66.6% respectively. In a report by Darokar *et al* 2001 48% polymorphism was reported among distinct accessions of *B.monnieri* using RAPD markers. It was concluded that the vegetative propagation of *B.monnieri* species could have contributed to the narrow genomic base in population of *B.monnieri*. The lack of RAPD variations can be due to heterozygous plants. In another study by Tripathi N *et al* 2012 genetic diversity in *B.monnieri* was established using RAPD and ISSR markers. High level of polymorphism was found which made RAPD markers as an effective tool for genetic diversity analysis.

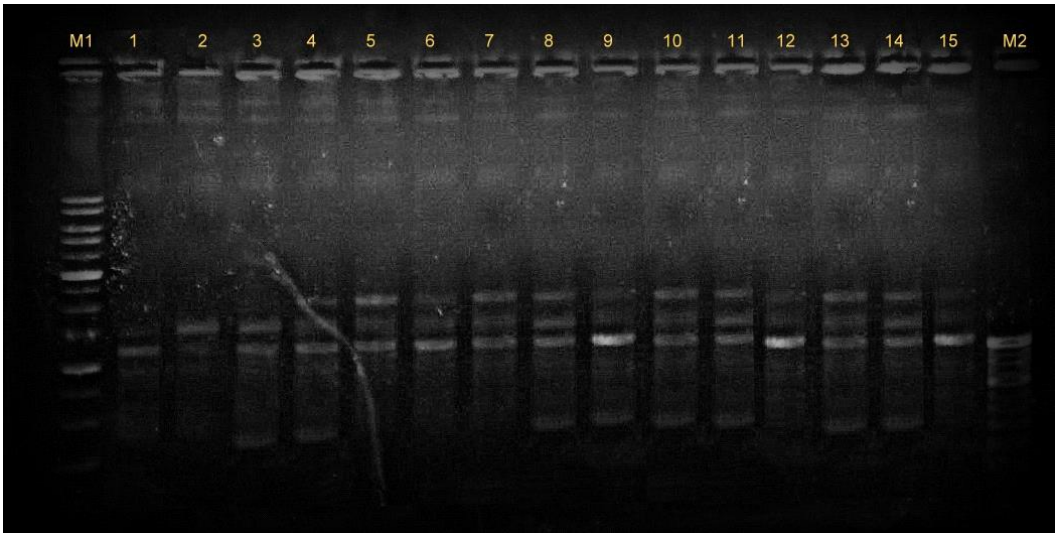

Fig1. RAPD-PCR product amplified by OPA-11 primer. M1= 1kb ladder, M2= 100bp ladder, lanes 1-15 represent accessions of *B.monnieri*

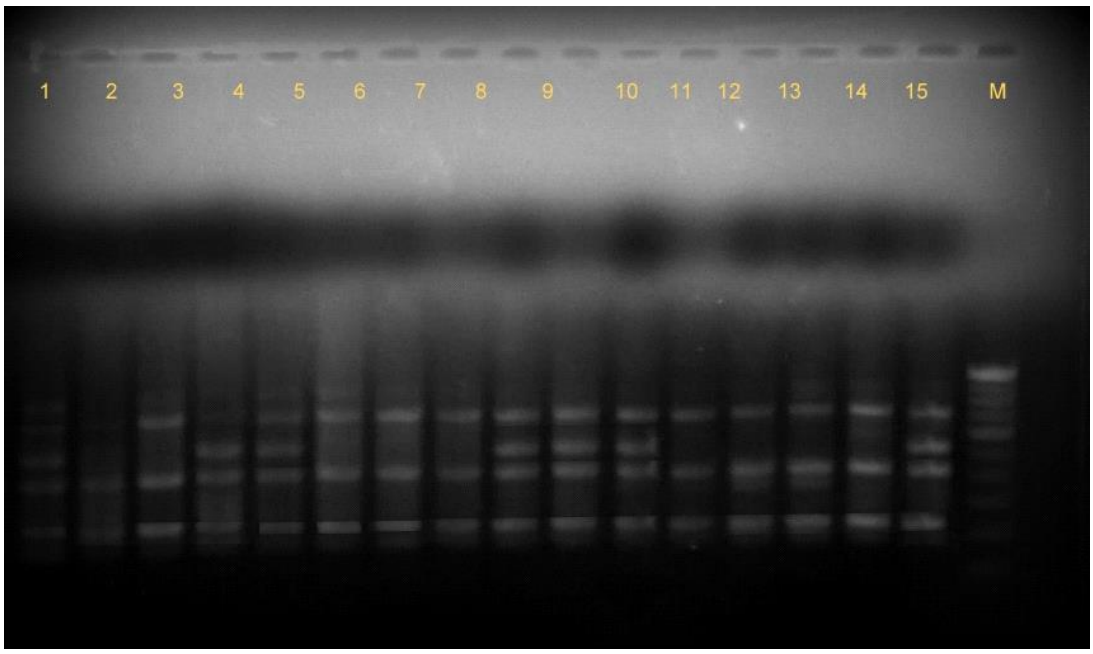

Fig2. RAPD-PCR product amplified by OPB-08 primer. M= 1kb ladder, lanes 1-15 represent accessions of *B.monnieri*

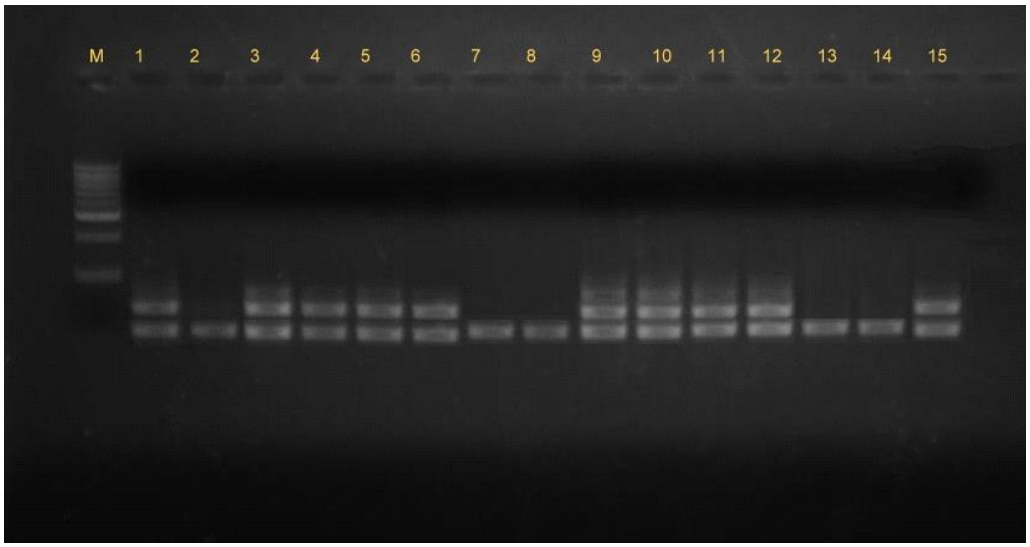

Fig3. RAPD-PCR product amplified by OPA-10 primer. M= 1kb ladder, lanes 1-15 represent accessions of *B.monnieri*

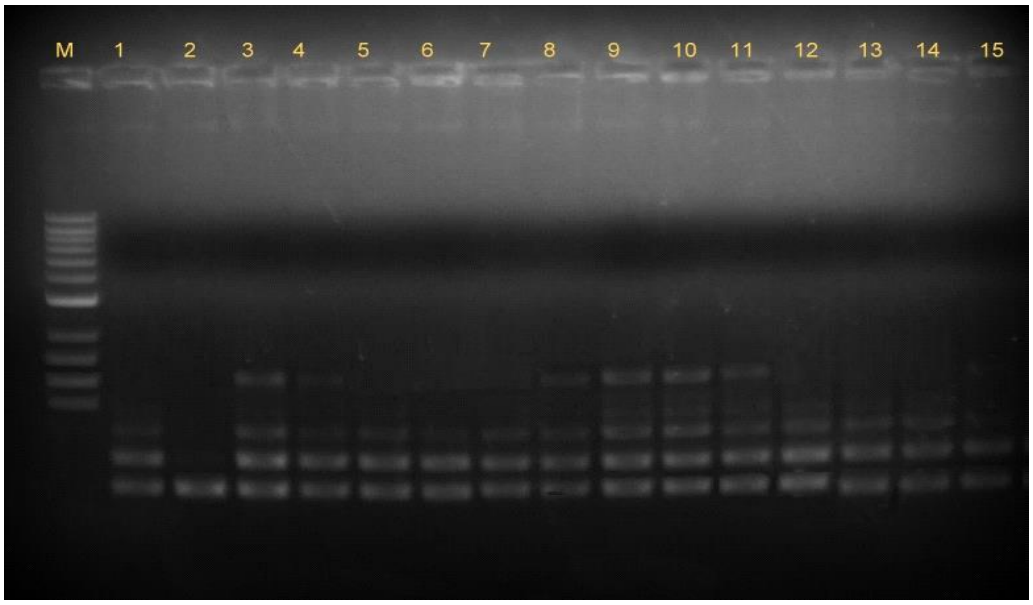

Fig.4 RAPD-PCR product amplified by OPC-05 primer. M= 1kb ladder, lanes 1-15 represent accessions of *B.monnieri*

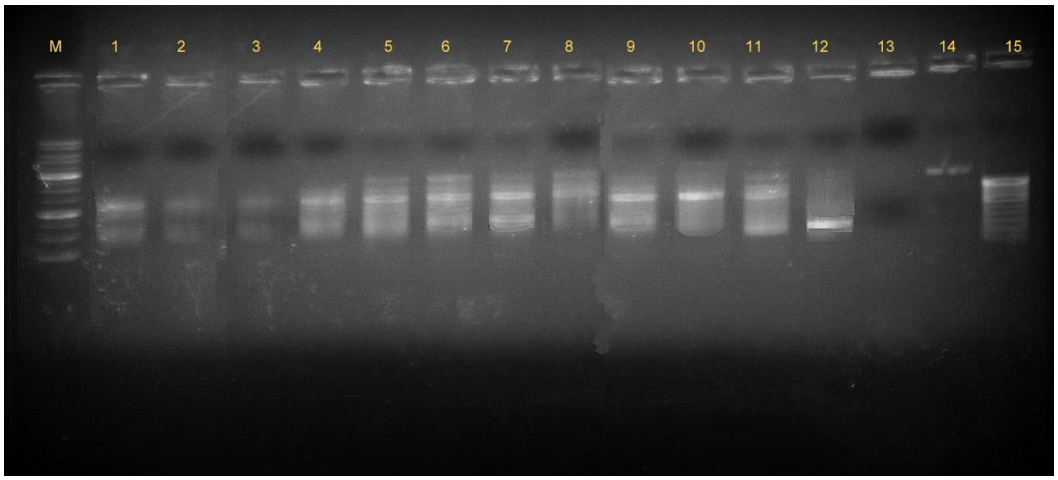

Fig5. RAPD-PCR product amplified by OPAB-07 primer. M= 1kb ladder, lanes 1-15 represent accessions of *B.monnieri*

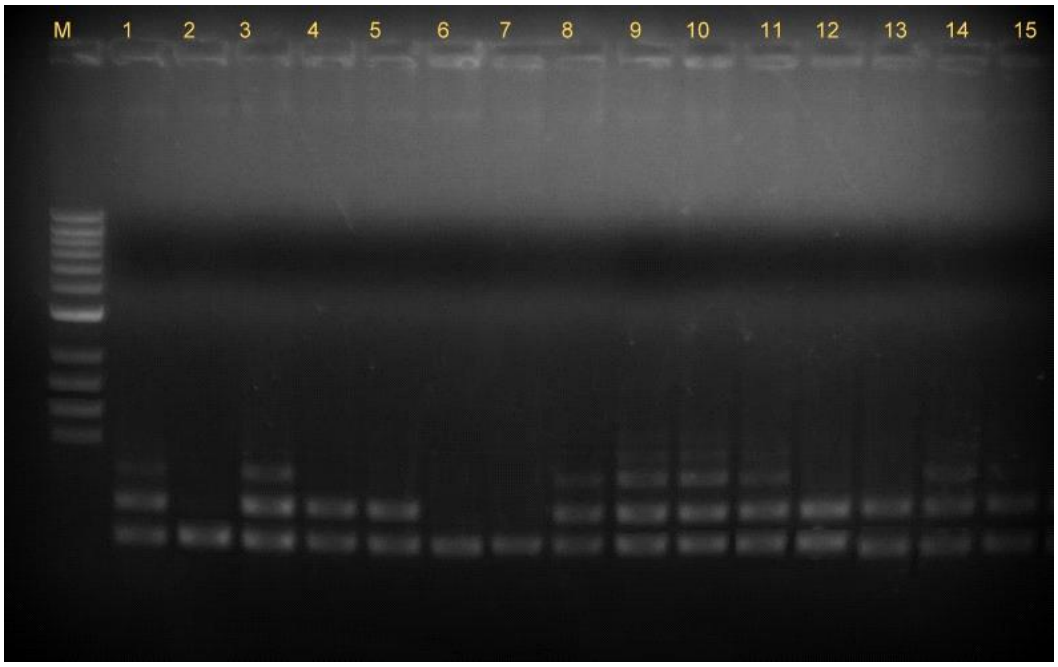

Fig6. RAPD-PCR product amplified by OPAA-04 primer. M= 1kb ladder, lanes 1-15 represent accessions of *B.monnieri*

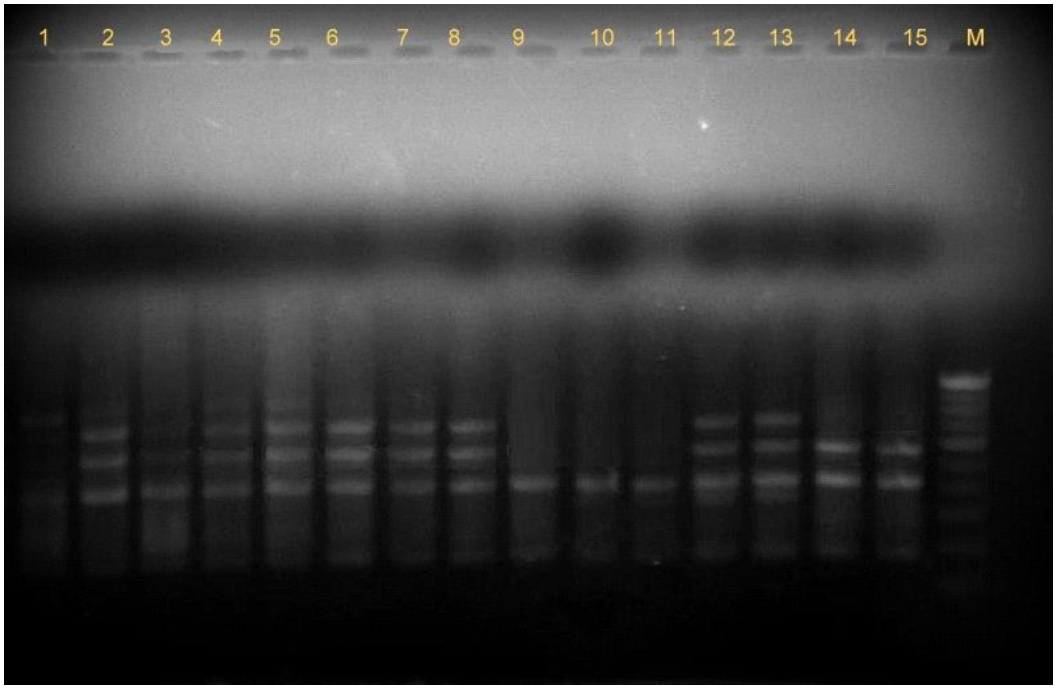

Fig7. RAPD-PCR product amplified by OPAD-06 primer. M= 1kb ladder, lanes 1-15 represent accessions of *B.monnieri*

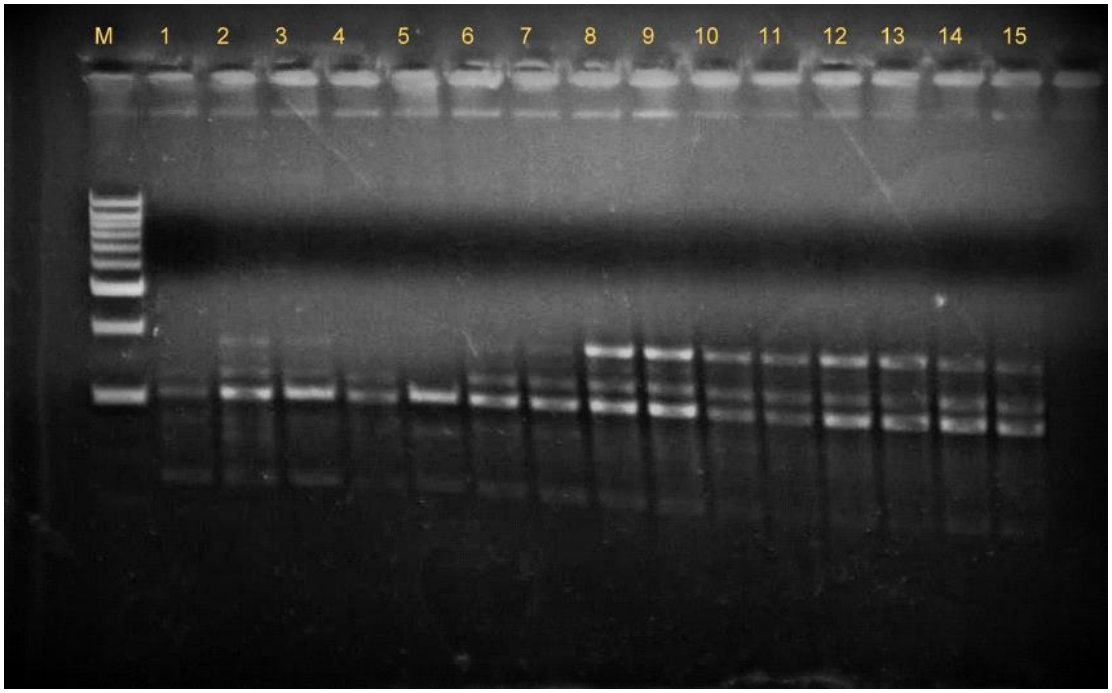

Fig.8 RAPD-PCR product amplified by OPAH-05 primer. M= 1kb ladder, lanes 1-15 represent accessions of *B.monnieri*

Primer	Sequence(5'-3')	Total bands	No. of polymorphic bands	No. of monomorphic bands	% polymorphism
OPA-11	CAATCGCCGT	4	2	2	50%
OPB-08	GTCCACACGG	7	4	3	80%
OPA-10	CTGCTGGGAC	3	2	1	66.67%
OPC-05	GATGACCGCC	4	3	1	75%
OPAB-07	GTAAAGCGCC	4	3	1	75%
OPAA-04	AGGACTGCTC	4	3	1	75%
OPAD-06	AAGTGCACGG	4	3	1	75%
OPAH-05	TTGCAGGCAG	5	3	2	60%
Total		**35**	**23**	**12**	**65.75%**

Table.4 Details of banding pattern of RAPD analysis in 15 accessions of B.monnieri

4.4 Dendogram cluster analysis

Genetic variability was determined by converting RAPD data into a dendogram by using NTSYS-pc software. The dendogram prepared from cluster analysis using NTSYS-pc software divided all the 15 accessions of Brahmi genotypes taken in the present study into 2 major groups A and B. Major group A was further subdivided into 2 sub groups. First subgroup comprises of 3 accessions C1, C2 and C4, all of them geographically belonging to Madhya Pradesh. The second subgroup comprises of 8 accessions from different geographical regions of Central India such as C8 (Kerala), C9 (Uttar Pradesh), C10 (Delhi), C11 (Punjab), C12 (Madhya Pradesh), C13

(Jharkhand), C14 (Jharkhand) and C15 (Orissa). In this subgroup, C8 and C14 showed much divergence from other Brahmi accessions. Major group B was divided into 2 sub groups. First subgroup comprise of only 1 accession C3 (Jammu and Kashmir) and second sub group comprises of 3 accessions C5, C7 both from Karnataka and C6 (Kerala).

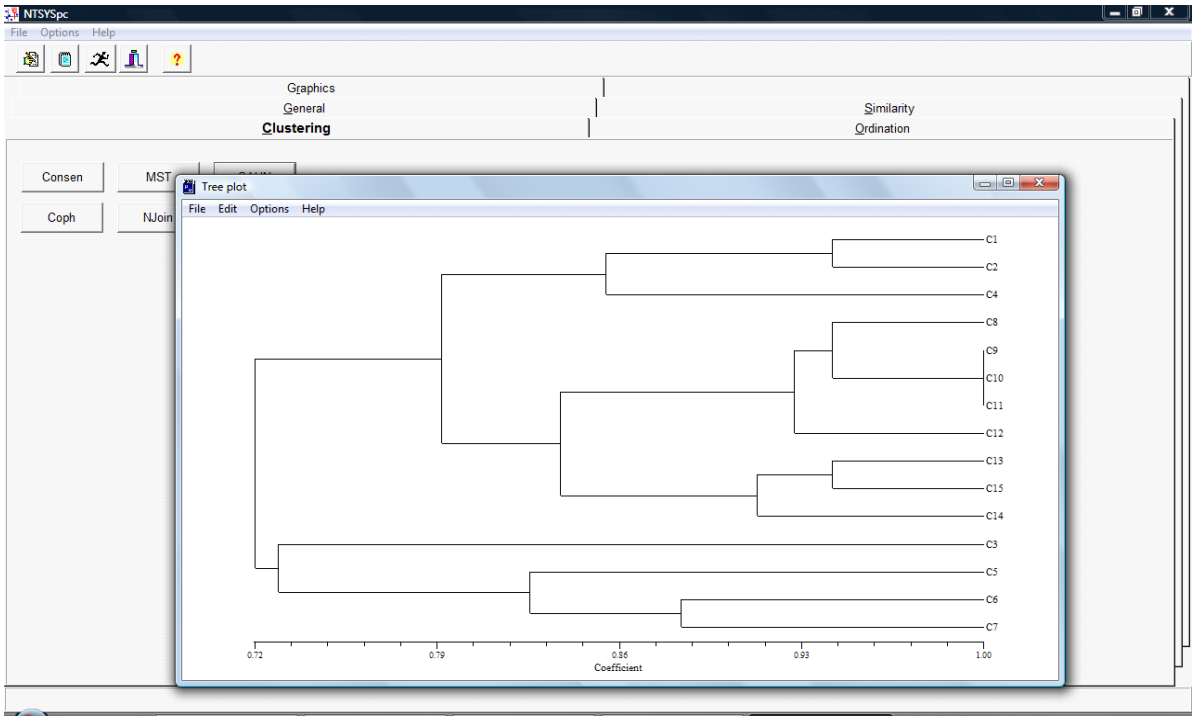

Fig.9 Dendogram of *B.monnieri* generated by primers OPA-11, OPB-08, OPA-10, OPC-05, OPAB-07, OPAA-04, OPAD-06 and OPAH-05

The phylogenetic cluster of *Bacopa* plant populations highlight the co relation of many different processes such as mutation, gene flow, mating system, selection, evolutionary background of the species for example habitat fragmentation and isolation of population (Slatkin 1987, Schaal *et al.,* 1998). Genetic diversity analysis is important for sustainability of population of medicinal plants (Wang *et al.,* 2007). Genetic variability patterns provide effective knowledge required for conservation of endangered plant species. Cluster analysis provides suitable information for sampling strategies for in situ as well as ex situ conservation (Torre *et al.,* 2008). Many reasons may give rise to polymorphism in RAPD analysis such as deletion, substitution of base within the primer binding site of the sequence. (William *et al.,* 1990). Higher the diversity higher is the adaptation to the environment which is important for

propagation and domestication of wild species and conservation of resources (Sarwat *et al* 2008). This study aims to establish genetic diversity background in an economically important medicinal plant *Bacopa monnieri* using RAPD marker analysis which provides detailed information to understand every aspect of genetic divergence. RAPD makers were found to be suitable for genetic variability studies. RAPD-PCR technique has been successfully used for identification of herbal medicinal constituents and identification of herbal medicinal plants. In a report, RAPD technique was successful employed for genotyping of ornamental plants (Shinde *et al* 2007).

5. DISCUSSION AND FUTURE PERSPECTIVE

Characterization has been based primarly upon morphological traits. Morphological traits such as leaf color (green, dark green and yellow-green), stem (thickness and color) and leaf size (large and small) were used to identify *B. monnieri* accessions. However, variability of morphological characteristics is often restricted, characters may not always be present at all stages of the development of plant and appearance may be affected and resulted by the environment. Nowadays, varied genetic markers have been suggested to assess genetic variability as a complementary strategy and blueprint to more traditional and orthodox approaches in genetic resources management. Molecular tools provide valuable data on diversity through their ability to detect variations at the DNA level. Genetic resources which are available for medicinal plant improvement are themselves abundant within the plant species. Even though a handful species of medicinal plants occur naturally in India, many cultivated medicinal plant species tend to find their origin within the country especially India. Barring few, almost all the cultivated and naturally occurring medicinal plants which are classified under different family and species, cross pollinate with each other and produce fertile offspring showing no signs at all of sexual incompatibility characteristic of medicinal plant species. This said fact suggest that an near genetic or non-genetic relationship exists among the medicinal plants.

In this present study, *Bacopa monnieri* which is an economically important medicinal plant was chosen to study genetic variability using RAPD-PCR technique. Cluster analysis of 15 *B.monnieri* accessions with RAPD-PCR technique revealed that the accessions belonging to different geographical regions fell in the same cluster and those from similar regions fell in different clusters. Occasionally, few accessions from similar geographical regions fell in same cluster. The RAPD analysis results inferred that individuals from more varied populations can be taken so that the diversity can be conserved for future (Govardhan *et al.,* 2011). The banding patterns and band distribution is important and distinct to understand the value of breeding patterns in medicinal plants. The minimum numbers of clusters indicate that gene flow is very low in medicinal plants (Banerjee *et al.,* 1999). The slight differences in the banding patterns among 15 accessions of *B.monnieri* with 8 RAPD primers reflect polymorphism that might have independently occurred while long term maintenance

of these medicinal accessions (Matsubara *et al*. 2012). In this study we found that quantity and purity of isolated DNA plays a crucial role in optimization of PCR parameters and so in the variability analysis (Qari, SH. 2010). The present study shows that when assay conditions are controlled carefully, RAPD-PCR technique can provide an effective, cheap and rapid mean to assess the genetic variability among different varieties of medicinal plants and help in designing sampling strategies for in situ as well as ex situ conservation programmes (Maltas *et al.,* 2015). From the dendogram clustering pattern obtained by RAPD analysis, plant breeders can easily identify the diverse varieties from different clusters and utilize them in effective breeding programmes in future for selection of wild type parents (Kawar *et al*., 2009). High diversity reflects the good sign for the existence of plant species whereas low diversity reflects the need for immediate steps to be taken for conservation of species. Poor breeding practices by plant breeders and inappropriate conservation of plants maybe responsible for low diversity (Sairkar *et al*., 2013). The results obtained in present study demonstrate the usage of RAPD markers to examine germplasm variation in *B.monnieri* varieties. The findings of present work can also be useful in identifying potential sources of germplasm material of wild type and also to identify the presence of adulterants or substitutes in herbal material samples. This report may prove to be helpful in devising molecular marker tools to identify different components present in herbal preparations.

In a similar study carried out by Tripathi *et al.*, 22 RAPD and 25 ISSR markers for identification of phylogenetic relationship among *B. monnieri* accessions. The RAPD analysis was carried out using decamer primers of different series of OPERON Technologies (OPA, OPAA, OPAB, OPAC, OPAD, OPAH, OPAI, OPB, OPBB and OPC) for DNA amplifications through PCR, out of which only 22 primers responded to all the accessions of *B.monnieri*. Per primer, average no. of bands was 8.95, while average number of polymorphic bands was 8.50. The PIC value ranged from 0.363 (OPAI-03) to 0.908 (OPA-11) with an average 0.644. On the other hand, Darokar *et al*. (2001) reported only 48 % polymorphism with RAPD markers among geographically distinct accessions of *B. monnieri* and they concluded that the narrow genetic base in *B. monnieri* population could be attributed to the vegetative propagation of the species.

In another study carried out by Pathak *et al* (2012) a total 10 arbitrary primers were screened to assess the genetic fidelity of the *in vitro* shoots, regenerated from the leaf explants of *B.monnieri*. Profiles of the amplified RAPD markers for the *B.monnieri* showed that wild plant and *in vitro* had different banding patterns of amplified DNA. In *B. monnieri*, 9 out of 10 primers gave reproducible results of total 58 amplification products in the size range of 150 to 1250 bp. Total 8 products were found to be polymorphic (13.19%) and 50 products were monomorphic (86.21%). The genetic variations which are known to occur in tissue culture plants can be either pre-existing or induced. Tissue culture induced variations include mode of regeneration, type of explant, type and concentration of the plant growth regulators as well as number and duration of subculture (Bairu *et al.*, 2011).

RAPD technique has been used frequently in herbal drug technology for detection of genetic variability and authentication of medicinal plants. Due to its simplicity, rapidity and ease RAPD has gained much importance. Also RAPD technique requires no prior information about the plant. Irrespective of plant age or source, RAPD patterns are consistent. These characteristics of RAPD are advantageous for selection of herbal medicines since less DNA is present in dried material (Shinde *et al*., 2007). Both coding and non coding regions of the plant genome are reflected by RAPD analysis. However, some of the limitations of RAPD include low reproducibility. In a finding, it is stated that if the overall temperature time profiles inside the PCR tubes are identical, reproducible RAPD fragments are likely to be produced (Kawar *et al*., 2009). It is an extremely important point in RAPD technique to take into consideration that the GC content of the primer should be 50% as the DNA primer hybrid will not withstand the polymerization if GC content is less than 50% (Ganie *et al*., 2012). Primers and chemicals required for PCR used in this study are readily available on the market. Our study showed that the PCR protocol used worked well for 8 primers out of 10 RAPD primers.

Molecular techniques have been effectively used for authentication of medicinal plants based on phylogenetic variations. DNA markers have proved to be useful tool for the detection of adulterants and to ensure quality control in medicinal plant genome analysis as well as in the manufacturing, clinical use, and forensic analysis of herbal medicines for their safest for human consumption. So, there is straightaway

need to develop the genomic tools so as to ensure safety of herbal drug formulations for their broad use commercially. The present findings can prove to be useful in finding adulterations/ substitutions of *Bacopa monnieri* to prevent forgery in the marketing of this valuable medicinal plant.

Adulteration is a major issue in the herbal drugs market and therefore authentication and standardization is prerequisite for them to minimize the unfair trade of all traditional herbal medicines. Much of this is carried out by chemical fingerprinting which is not always reliable due to variation in the metabolite content. DNA markers are more reliable and can authenticate plants up to the varietal level. However some of these techniques like microarray and sequencing are expensive but other DNA marker based methods such a RAPD can be used for reliable authentication. It would be appropriate to choose and start with more feasible techniques like RAPD and then move on to the more complex techniques as these techniques can reveal the identity even at the species level. Another aspect has to be taken care that the active metabolite may not be present in desirable quantity in the authenticated plants. This can be therefore combined with the chemical analysis and also the authenticated plants can be cultivated in the geographical regions where it is known to produce high amount of the required active metabolite (Biswas *et al*., 2014).

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