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LIST OF ABBREVIATIONS

CIMAP – Central institute for medicinal and aromatic plants

CTAB- cetyl trimethyammonium bromide

DNA - deoxyribonucleic acid

dNTP- deoxynucleotide triphosphate

^{o}C - degree Celcius

EtBr- ethidium bromide

ISSR - Inter Simple Sequence Repeat

µg - microgram

µL- microlitre

mM- millimolar

ng- nanogram

ml- milli litre

NMPB - National Medicinal Plants Board

NTSYSpc- **N**umerical **T**axonomy **SYS**tem for **p**ersonal **c**omputer

PCR - Polymerase Chain Reaction

PVP- polyvinyl pyrrolidone

RAPD- Random Amplified Polymorphic DNA

RNA- deoxyribonucleic acid

SSR- Simple Sequence Repeat

TAE- Tris acetate EDTA

TE- Tris EDTA

UPGMA- unweighted pair group method with the arithmetic averaging algorithm

UV- ultraviolet

WHO- world health organization

Assessment of genetic variation using ISSR markers in accessions of *Bacopa monnieri*

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ABSTRACT

Bacopa monnieri is an important medicinal plant with high demand in pharmaceutical industries due to its numerous therapeutic properties. It is known by the name *Brahmi* and has been used as brain tonic in the Indian ayurvedic medicinal system to enhance memory development and concentration. A large number of *Bacopa monnieri* based commercial ayurvedic products are available in the market due to its therapeutic values and the raw material is taken mostly from the available natural wild resources. The quality of raw material of medicinal plants collected from wild populations is likely to be affected by genotype. Molecular markers are a powerful tool to verify the genetic consistency and to test whether in vitro regenerated plants are true-to-type. In the present study fifteen accessions of *Bacopa monnieri* belonging to different regions of India were analysed using ISSR molecular markers in order to derive the genetic relationships among them. With the 5 ISSR primers analysed, 25 distinct bands, lying in the size range of 200–2000 bp were observed of which 14 (56%) were polymorphic while rest 11 (44%) were monomorphic. Cluster analysis was performed on the basis of similarity co-efficient computed from ISSR marker data to create a dendrogram by using UPGMA method of the NTSYS-pc software.

1. INTRODUCTION

1.1 Medicinal Plants

There has been a great increase in use of herbal products in recent times, around the world (Gohil and Patel, 2010). Numerous drugs and their precursors that are currently being used originate from plant sources. About 28% of all new chemical compounds that are launched into the market in the last 20 years are accounted for by the natural products or natural product-derived drugs (Verma *et al*., 2012). The world health organization (WHO) also stated that more than 80% of the world's population relies on traditional medicines for their essential health care needs (Singh, 2012).

The medicinal value of plants is due to the presence of specific chemical substances that produce a definite physiological effect on the human body. These chemical constituents can either be therapeutically active or inactive and are known as active constituents and inert chemical constituents respectively. Some of these bioactive constituents of plants include alkaloids, phenols, flavonoids and tannins (Iyengar, 1995).
The information about the chemical constituents of plants can help in the discovery of various therapeutic agents and can also be useful because in providing information that may lead to revealing of new sources of economically important materials such as tannins, oils, gums, and precursors required for the synthesis of complex chemical substances (Singh, 2012).

Medicinal plant-based drugs have advantage that they are simple, effective and offer a broad spectrum of activity with well-documented therapeutic actions. Furthermore they have minimised adverse side effects as compared to various chemotherapeutic agents (Verma *et al*., 2012). It has also been found that available psychotherapeutics does not efficiently meet the therapeutic demand of vast majority of patients with mental health problems and the herbal remedies provide useful alternative in such cases around the world (Gohil *et al*., 2010).

India is rich in medicinal plant diversity and since the ancient times use of the drugs of herbal origin is prevalent in traditional systems of medicines such as *Ayurveda* and *Unani*. There are about 426 biomes comprising of different habitat diversities that give rise to one of the richest centres for plant genetic resources in the world (Verma *et al*., 2012). Out of 18,665 flowering plant species, the classic systems of medicines such as Ayurveda, Siddha, and Unani are known to make use of only about 3000 plants in various formulations (Schippmann *et al*., 2006).

1.2 *Bacopa monnieri*

Bacopa monnieri is one of the most important medicinal plants widely used for its therapeutic properties and is a well known nootropic (Gohil *et al*., 2010). *Bacopa monnieri* is a perennial, creeping herb that inhabits damp and marshy areas, known by the name *Brahmi,* and has been used in the Indian Ayurvedic medicinal system as nerve and brain tonic to enhance memory development, concentration and learning for treatment of patients with anxiety or epileptic

disorders (Asha *et al*., 2013). It also has anti-inflammatory, analgesic, anti-pyretic, anti-cancer and anti-oxidant properties.

Bacopa is cultivated as valuable drug resource whose price is determined on the basis of quality and active constituents. There has been a decline in natural population B. monnieri from the natural habitat due to the continuous exploitation which necessitates this important medicinal herb to be conserved (Tripathi, Niraj*, et al*. 2012). A large number of *Bacopa monnieri* based commercial ayurvedic products are available in the market due to its therapeutic values (Pravina *et al*. 2007) and the raw material is taken mostly from the available wild resources (Ramesh *et al*., 2011). It has a wide distribution pattern and the quality of raw material collected from wild populations is likely to be affected by genotype (Nadeem *et al*. 2002) which in turn is affected by environmental factors. Therefore, there is a need for identification of elite accessions (based on active principle content) and molecular characterization of wild population for diversity documentation as well as the optimization of in vitro propagation protocol (applicable for range of populations) for conservation (Bansal, *et al*.2014.

Molecular markers are a powerful tool establish genetic relationship and help determine similarities or variations among species based on their genetic makeup. They can also be used to verify the genetic consistency and to test whether in vitro regenerated plants are true-to-type (Gantait *et al*., 2014). The DNA marker systems have been found to be useful for authentication of plants with medicinal value as they enable to identify plant species that are either substituted or adulterated with other species or varieties that are morphologically or phytochemically difficult to distinguish.

Therefore in the present study fifteen accessions of *Bacopa monnieri* belonging to different regions of India were analysed using ISSR molecular markers in order to determine the genetic relationships among them.

REVIEW OF LITERATURE

2.1 Bacopa monnieri

Bacopa monnieri is a perennial, creeping herb that inhabits wetlands and muddy shores (Asha et al., 2013). It is known by the name *Brahmi* and *Nir-brahmi* in Sanskrit, *Brihmi-sak* and *Jalanimba* in Bengali, *Brahmi* in Hindi, *Nirbrahmi* in Malayalam, Marathi and Tamil, *Sambranichettu* in Telugu and *Nirubrahmi* in Kannada. It is an important medicinal plant widely used for its therapeutic properties and is a well known nootropic (Gohil *et al*., 2010). Brahmi has been traditionally used in India as a brain tonic and to prepare widely known ayurvedic preparations like ‘‘Brahmighritam’’, and ‘‘Brahmirasayanam’’ (Govindarajan *et al*., 2005; Prasad *et al.* 2008). It belongs to the family Scrophulariaceae and has been used in the Indian Ayurvedic medicinal system as nerve and brain tonic to enhance memory development, concentration and improve learning for the treatment of patients with anxiety or epileptic disorders. It has also been used as a cardiac tonic and in cases of broncho-constriction to improve respiratory function as well as a digestive aid (Elangovan *et al*., 1995). It also has anti-inflammatory, analgesic, anti-pyretic, anti-cancer and anti-oxidant properties (Rastogi *et al*.1994).
There has been a rapid expansion of the pharmaceutical industry along with popularization of brahmi based drugs like ‘Mentat’, ‘Memory Plus’ and ‘Memory Perfect’ in the Indian and global market. In addition, due to the recent report of anti-cancer activity of the herb extracts using Sarcosoma cell culture, the pharmaceutical requirement for Bacopa has further increased dramatically (Elangovan *et al.* 1995; Mathur and Kumar, 1998).

Based on its medicinal value due to its varied therapeutic properties and potential for future research and development, *Bacopa monnieri* has been placed second in a priority species list of the most important medicinal plants (Rajani 2008) and has also been listed among 32 medicinal plants identified for cultivation and conservation by the NMPB (National Medicinal Plants Board 2004), Government of India. *Bacopa monnieri* is also one of the 7 medicinal plants recommended for immediate attention and categorised as highly endangered medicinal plants in India by NMPB and Technology Information Forecasting and Assessment Council (TIFAC) (http://www.nmpb.nic.in/prioritisemedicinalplants.htm). During 2007 the annual market demand estimated for brahmi was found to be around 1,000 tonnes (National Medicinal Plants Board 2007) and is likely to increase in the approaching years due to its numerous therapeutic uses (Ramesh, Manikandan, et al. 2011).

Conventional propagation of *Bacopa monnieri* in nature via seed or vegetative propagation is slow and is hampered due to seedling death at 2-leaved stage and specific habitat requirements (Sharma *et al.,* 2010). Due to poor performance of propagules the vegetative propagation is also slow. Due to the increasing demand for herbal drugs in pharmaceutical markets, medicinal plants such as *Bacopa monnieri* are threatened with overexploitation (Mehta *et al*., 2012)**.** Thus for the rapid clonal propagation and conservation of valuable and threatened germplasm of medicinal importance, use of *in vitro* techniques has increased over the last two decades (Sharma *et al.,* 2010). Development of a fast clonal propagation method

can help reduce the pressure on existing natural plant populations and to meet requirement of constant supply of plant material for pharmaceutical industry (Tiwari and Singh, 2010).

There has been a decline in natural population *B. monnieri* from the natural habitat due to the continuous exploitation which necessitates this important medicinal herb to be conserved (Tripathi, Niraj, *et al*. 2012). A number of *Bacopa monnieri* based commercial ayurvedic products are available in the market due to its therapeutic values (Pravina *et al.* 2007) and the raw material for their production is taken mostly from the available wild resources (Ramesh *et al*., 2011). The quality of raw material of medicinal plants collected from wild populations is likely to be affected by genotype (Nadeem *et al.* 2002). In addition, morphological characteristics are found to be substantially affected by the environmental factors. The accurate identification of a specific plant variety or species based merely on morphological traits becomes difficult and affirms the importance of molecular markers for precise identification (Tripathi, Niraj, *et al*. 2012). Therefore, there is a need for identification of elite accessions (based on active principle content) and molecular characterization of wild population for diversity documentation as well as the optimization of in vitro propagation protocol (applicable for range of populations) for conservation (Bansal, *et al*.2014).

Classification of *Bacopa monnieri*:
Kingdom- Plantae

Division -Angiospermae

Class -Dicotyledonae

Order -Tubiflorae

Family- Scrophulariaceae

Genus- *Bacopa*

Species -*monnieri*

2.2 Chemical constituents:

Active constituents present in *Bacopa monniera* include many phytochemicals such as alkaloids, saponins, bacosides and nicotine (Patil et al., 2012). It contains saponins like bacosides A, B, C and, alkaloids like nicotine, brahmine and herpestine. Bacosides A and B are responsible for the potent nervine activity (Rastogi et al.1994; Tiwari et al. 2001). It also contains flavonoids (such as luteolin and apigenin), stigmasterol, beta-sitosterol, betulinic acid, and bacopasaponins (Ali et al., 1999; Chatterji et al., 1963, 1965). Other minor components that are found to be present include bacopasaponin F, bacopasaponin E, bacopaside N1, bacopaside III, bacopaside IV and bacopaside V (Karatas *et al*., 2013). Bacoside A is found to usually co-occur with bacoside B and known to differ only in the optical activity. Bacoside A is laevorotatory and bacoside B is dextrorotatory. Bacosides upon acid hydrolyis yields bacogenins A1, A2, A3 and A4 and two sapogenins jujubogenin and pseudojujubogenin (Rameshwari et al., 2013).

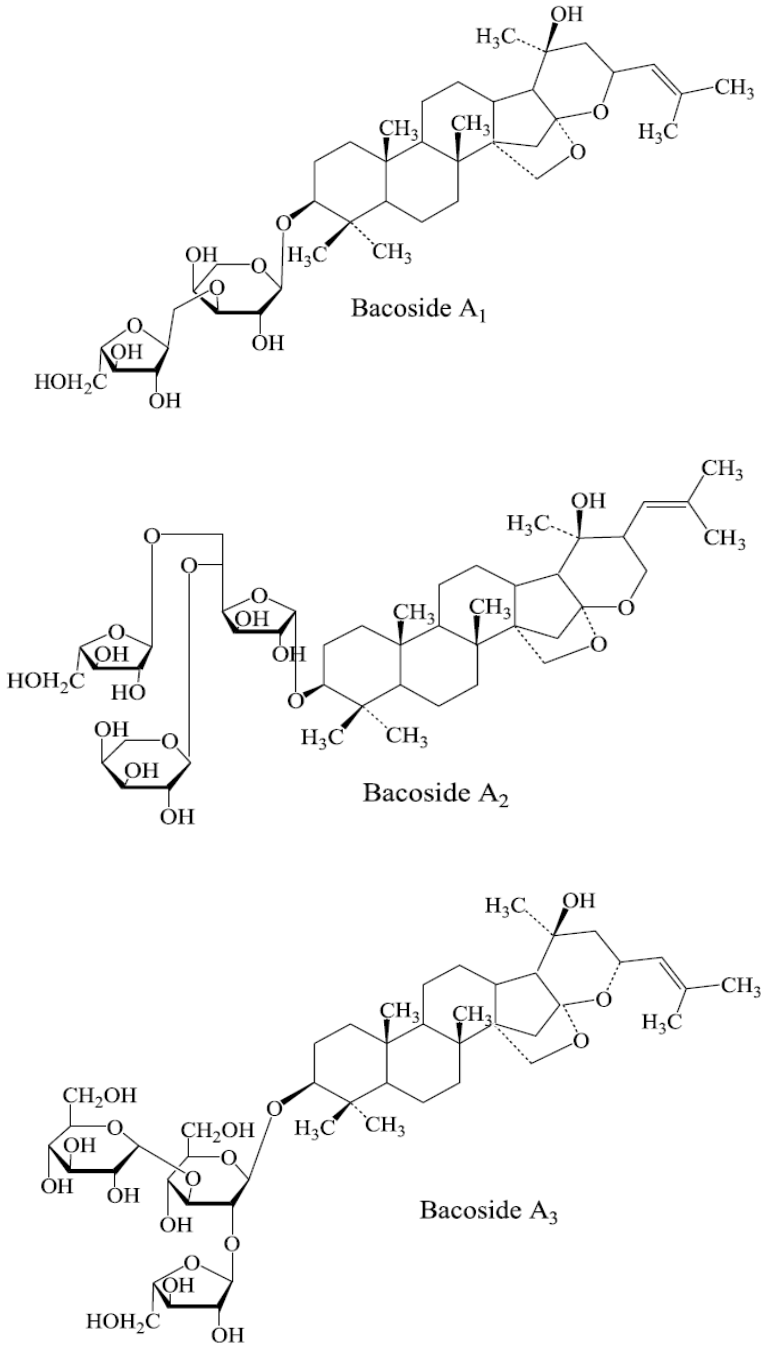

Fig.1 Some chemical constituents of *Bacopa monnieri* – saponins (Ramawat KG, & Mérillon JM, 2008)

2.3 Morphological characterization

Morphologically, *Bacopa monnieri* is known to be a small perrineal herb, creeper in nature with number of branches, small oblong leaves and bearing flowers either light purple or small and white, with 4-5petals. It is found to occur throughout the Indian subcontinent in wetlands, damp and marshy or sandy areas nearby streams or tropical regions (Russo and Borrelli, 2005).

The plant has a wide range of distribution pattern and has been now cultivated as valuable raw drug resource whose price is determined on the basis of quality, and most importantly on the basis of its active constituents. The various morphological characteristics (such as colour of stem, colour of flower, shape of leaves, leaf length and inter nodal length) and biochemical traits (such as total saponin and bacoside A) of *Bacopa monnieri* from different agro climatic regions can be used for the screening of superior accessions so as to make the systematic cultivation of *Bacopa monnieri* a profitable venture (Roshni, et al.2014).

Some of the morphological characteristics of *Bacopa monnieri* are as follows (API, 1989)*:*

(a) Root - Thin, wiry, small, branched creamish-yellow.

(b) Stem - Thin, green or purplish green, 1-2 mm thick, soft, nodes and internodes prominent, glabrous; taste, slightly bitter.

(c) Leaf - Simple, opposite, decussate, green, sessile, about 1-2 cm long, obovate-oblong; taste, slightly bitter.

(d) Flower - Small, axillary and solitary, pedicels 6-30 mm long, bracteoles shorter than pedicels.

(e) Fruit - Capsules upto 5 mm long, ovoid and glabrous.

2.4 Use of Molecular Markers in *Bacopa monnieri*

The use of molecular markers makes it feasible to derive inferences related to genetic divergence as well as inter-relationships amongst organisms at DNA level and offer the advantage that they are free from the misleading environmental influences or fallible pedigree accounts. The advancement in the range of molecular marker systems available has greatly assisted in evaluating genetic constitution of plant populations for taxonomic, evolutionary, and ecological research purposes. Each molecular marker system works based on distinctive principles and selection of an apt DNA marker depends on several factors. The selection of a molecular marker system is commonly based on simplicity and consistency of analysis, statistical rule and efficiency of detecting polymorphisms. Molecular marker systems have numerous applications such as characterization of genetic diversity amongst several plant varieties or species for the evaluation of genetic fidelity, identification of genes of commercial and agronomic interests, and enhancement using genetic transformation system (Gantait *et al*., 2014). Detection of genetic variation also finds important application in case of micropropagation and in vitro germplasm conservation to help eradicate undesirable somaclonal variations. Molecular markers are a powerful tool that can help in verification of genetic consistency and to test whether in vitro regenerated plants are true-to-type (Gantait *et al*., 2014).

RAPD markers have been utilised for studying the genetic variation in different accessions of *Bacopa monnieri* belonging to Southern Indian states, along with their in vitro micropropagated plant samples (Karthikeyan *et. al*, 2011). RAPD markers were used to evaluate the genetic integrity of micropropagated plants of *Bacopa monnieri* obtained by two-stage culture procedure (Ceasar et. al, 2010). RAPD based have also been used by Ramesh *et al* (2011) for the assessment of genetic stability among micropropagated, synthetic seed derived and hardened plants of *Bacopa monnieri*. Genetic diversity among fourteen wild

accessions *of Bacopa monnieri* collected from different parts of India has been reported using RAPD and ISSR markers by Bansal *e.t al* (2014). Smilarly a study of genetic variations in fifteen accessions of Brahmi from Central India using RAPD and ISSR markers has also been reported by Trpathi *et al (*2012). RAPD markers have been used for detecting somaclonal variants in *Bacopa monnieri* and *Tylophora indica by* Pathak *et. al* (2013).

RAPD-based SCAR marker has been developed to identify *Bacopa monnieri* from its adulterant candidates namely *Centella asiatica*, *Eclipta alba* and *Malva rotundifolia* (Yadav *et al*., 2012).

2.5 Inter Simple Sequence Repeat (ISSR) Marker

ISSR are dominant markers and that are used to detect polymorphisms in the microsatellite and regions between microsatellite loci without the requirement of any prior information of DNA sequences (Zietkiewicz et al. 1994). It is a PCR based technique in which amplification of DNA fragment lying between two identical SSR regions that are oriented in opposite direction. Microsatellites or simple sequence repeats (SSR) are short stretches of DNA (usually 10-20 bp) that show high variabilility and found to occur as different variants within the populations as well as across different species. They comprise of mono-, di- or tri-nucleotide repeats with 4-10 units arranged side by side. ISSR marker system makes use of 15-20 bp primers having nucleotide sequence complementary to microsatellite sequences found throughout eukaryotic genomes (Reddy *et al*., 2002). ISSR marker system thus helps to determine variation in the numerous SSR regions distributed across the genome (Semagn et al., 2006). ISSR-PCR makes use of a single primer comprising of a di-or trinucleotide simple sequence repeat and primers that have SSR motif attached at the 5' or 3' end by 2-4 arbitrary nucleotides (Zietkiewicz *et al*., 1994). There is no need for prior knowledge of genome sequence in case of ISSR markers as opposed to SSR markers that requires knowledge of sequence of the region flanking the SSR region. Several advantages associated with used of ISSR marker system include multiple polymorphic loci detection, low cost of operation and high throughput. This technique is rapid and can be used to differentiate between closely related individuals. (Bakkappa *et. al*, 2011) ISSR analysis is fast and amplifies and detects a greater number of bands per primer and thus ISSR markers reveal higher levels of polymorphism. (Godwin *et al*.1997). This technique is a powerful tool that can be used to determine intra-genomic and inter-genomic diversity as it can reveal variation within the genome at several loci simultaneously. Various properties of microsatellite such as high variability among taxa, high copy number and ubiquitous occurrence in eukaryotic genomes make ISSR markers extremely useful (Morgante *et al*., 1996).

ISSR marker system have several advantages over other molecular markers such as AFLP and SSR, which include: 1) does not require information of genome sequence, 2) simple and quick, 3) amenable to laboratory level, 4) high stability, 5) use of radioactivity is not required, and 6) ability to detect high level of polymorphism.

ISSR markers are universal in nature as the microsatellite repeats are found to occur in every eukaryotic genome thus are widely applicable. Due to the longer lengths of ISSR primers the use of higher annealing temperature is permitted, which in turn reduces non-specific binding

and results in higher stringency (Bornet and Branchard, 2004; Qian et al., 2001). The amplification products obtained by ISSR primers usually ranges from 100-2000bp long and can be detected by both agarose and polyacrylamide gel electrophoresis (Reddy *et al.,* 2002).

Various previous studies using ISSR markers have been successfully utilised for the assessment of genetic diversity among a number of medicinal plants such as *Tribulus terrestris* (Sarwat *et al.* 2008), *Papaver somniferum* L. (Acharya and Sharma 2009), *Anoectochilus formosanus* (Zhang et al.2010), and *Rheum officinale* (Wang 2011). ISSR markers have also been used to investigate the genetic variation in wild and cultivated varieties of Chinese medicinal plants such as Rhizoma corydalis by Qiu et al. (2009) and Coptis chinensis by Shi *et al*. (2008). Other medicinal plants where application of ISSR markers has been successful include Swertia chirayita (Joshi and Dhawan 2007), Tribulus terrestris (Sarwat *et al.* 2008), Pleurotus citrinopileatus (Zhang *et al.* 2012).

2.6 Applications of molecular markers

The molecular marker techniques have been found to have numerous applications in plants with commercial value such as food crops, horticultural plants, etc. Some of these are mentioned below:

Genetic variation/genotyping

Active constituents within the medicinal plants are known to be affected by geographical conditions which in turn affect their activity profiles (Oleszek et al., 2002). Therefore geographical variation at the genetic level has been widely studied. Studies of genetic diversity are useful in developing crop improvement programmes for the management of germplasm and formulating conservation strategies.

Detection of genetic variation also finds important application in case of micropropagation and in vitro germplasm conservation to help eradicate undesirable somaclonal variations. *In vitro* propagation techniques are known to generate somaclonal variations that are manifested in the form of chromosome reorganizations, DNA methylation and point mutations (Phillips *et al.,* 1994).

The RAPD markers have been widely used to evaluate DNA polymorphisms that arise due to rearrangements or deletions at or in between the primer binding sites across the genome using short random oligonucleotide primers (about 10 bp long) (Williams *et al.* 1991). The main limitation of this approach is that the marker profiles are affected by the reaction conditions that may fluctuate amidst laboratories and since multiple distinct loci in the genome are amplified by each RAPD primer, they are incapable of differentiating between heterozygous and homozygous members (Bardakci, 2001). However, since the RAPD analysis is swift and effective, it has been used for genetic mapping in a number of medicinal plant species such as *Pueraria montana* (Heider et al. 2007), *Atalantia species* (Ranade *et al.* 2009), *Chlorophytum borivilianum* (Samantaray and Maiti 2010), *Swertia chirata* (Balaraju *et al.* 2011), *Withania somnifera* (L.) *Dunal* (Rana *et al*. 2012). RAPD-based molecular markers have been successfully used for differentiating different accessions of many plants such as *Taxus wallichiana* (Shasany et. al,1999), neem (Farooqui et. al, 2002) , *Juniperus*

communis L.(Adams et. al, 2002), *Codonopsis pilosula* (Fu et.al ,1999) and *Andrographis paniculata* (Padmesh et. al, 1999) collected from different geographical regions.
Similarly ISSR markers have been used to differentiate various accessions of *Cannabis sativa* have been and cleaved amplified polymorphic sequence and ISSR markers in case of *Arabidopsis thaliana.* Inter-species as well as intra-species variation have also been studied using DNA-based molecular markers such as RFLP and RAPD. Phylogenetic relationship between various plant varieties have also been studied using molecular markers (Joshi *et a,l* 2004).

Authentication of medicinal plants

Molecular marker systems have also been found useful for authentication of plants with medicinal value. They enable to identify cases of those plant species that have either been substituted or adulterated with undesirable plant species or varieties that are morphologically or phytochemically difficult to distinguish. RAPD-based SCAR marker has been developed to identify *Bacopa monnieri* from its adulterant candidates namely *Centella asiatica*, *Eclipta alba* and *Malva rotundifolia* (Yadav et al, 2012). RAPD markers were used in a study of dried fruit samples of *Lycium barbarum* to differentiate it from other related species (Zhang *et al*, 2001). Three RAPD primers have also been used to successfully differentiate between three species of *Atractylodes*, from Chinese formulation purchased from local markets (Chen *et al.*, 2001). Sequence characterized amplified region (SCAR), RAPD and RFLP marker systems have been successfully applied for differentiating these plants and detecting substitution of *P. ginseng* by *P. quinquefolius* (American ginseng) or other closely related species(Joshi *et al* 2004).

Marker assisted selection of desirable chemotypes

Determination of plant varieties with greater production of active phytochemical compounds can be extremely useful for pharmaceutical purposes. In such cases, DNA markers that can help provide a means to correlate DNA fingerprinting data with quantity of desired phytochemical marker of the particular plant, would be immensely useful in pharmaceutical application for quality control of raw materials. AFLP analysis has been successfully used in *Echinacea purpurea* germplasm and some related species for determining phytochemical markers (Baum *et. al*, 2001). To differentiate between chemotypes varying in oil quality produce of three different genotypes of *Pelargonium graveolens* (Shasany et. al, 2002) and the flavonoid composition of *Aconitum* species (Fico et. al, 2003) RAPD fingerprint have been successfully developed.

Medicinal plant breeding

Medicinal plant breeding is another important application of molecular markers. ISSR–PCR technique has been found to be useful for identification of zygotic plantlets in citrus interploid crosses (Rongwen *et al* 1995). In intraspecific crosses of *Hypericum perforatum* molecular markers have been employed to verify sexual and apomictic offspring (Morjane *et al* 1994). In case of of *Piper longum,* RAPD markers systems have also been successively applied for selection of micropropogated plants for the purpose of conservation.

2.7 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a powerful scientific technique in molecular biology that is used to generate multiple of copies of a particular DNA sequence. It was developed by the American biochemist, Kary Mullis in 1984 (Bartlett and Stirling, 2003). It is quick, inexpensive and simple technique for amplifying specific DNA fragments (Erlich, 1989). The technique involves three major steps which are denaturation, annealing, and extension which are carried out in thermocycler. In the denaturation step, the DNA is denatured at high temperatures (about 90-97 °C). This disrupts the hydrogen bonding between complementary bases due to heat leaving single-stranded DNA. In the next step, primers recognize and anneal to complementary sequences on template DNA at about 50-60°C. In the last step, dNTPs or nucleotides are added by DNA polymerase causing extension to occur at the end of the annealed primers thus creating a complementary copy of template DNA strand. Towards the end of the process duplication of the original DNA occurs and each of the new molecules contain one old and one new strand of DNA. Each of these strands can further act as template and can be used to generate two new copies and so on (Ochman *et al*., 1988).

2.8 NTSYSpc, Version 2.2

NTSYSpc stands for "**N**umerical **T**axonomy **SYS**tem for **p**ersonal **c**omputer". It is a system of programs that can be used to determine pattern and structure in multivariate data. It can be used by an individual to discover whether a sample of data points may have come from two or more distinct populations or to estimate a phylogenetic tree using the neighbor-joining or UPGMA methods for constructing dendrograms. NTSYSpc is one of the most popular softwares being used in genetic diversity analysis.

The NTedit program which is included within the NTSYSpc software can be used to prepare data files of proper format. The NTS files of NTSYSpc are ordinary ASCII files (txt files). Furthermore, NTSYSpc can also read data matrices from Excel spreadsheets (*.XLS files) and trees from Nexus format files (Rohlf F. J., 1992).

3. METHODOLOGY

3.1 Plant Material:

Fifteen accessions of *Bacopa monnieri* belonging to different regions of India were obtained from CIMAP, Lucknow and were maintained at the plant tissue culture laboratory of Department of biotechnology, Delhi Technological University.

3.2 DNA Isolation:

Genomic DNA of Bacopa Monnieri was isolated using conventional CTAB method of Doyle and Doyle (1990) with minor modifications.

About 2gm of young or recently matured leaves were collected and washed with distilled water and were then blotted dry. It was then ground into a fine powder in mortar and pestle using liquid nitrogen and PVP. Due care was taken to prevent the thawing of the material. The powdered material was immediately transferred to an autoclaved eppendorf tube with 800µl of extraction buffer (2% CTAB) with 0.2% β-mercaptoethanol (i.e.20µl). These sample tubes were then incubated at 65˚C in water bath for one hour with gentle end to end inverting of each tube at an interval of 10 minutes.

The tubes were cooled to room temperature and equal volume (800µl) of Chloroform: Isoamyl Alcohol (CIA 24:1) was added and gently mixed by inverting for 10 minutes. These tubes were then centrifuged at 10,000 rpm for 15 mins at room temperature. The top aqueous layer was separated and transferred to a new set of centrifuge tubes using a cut tip. To the above solution 2 volume of chilled Isopropanol was added and kept at -20˚C for 2 hours (or at 4˚C overnight).

The tubes were then centrifuged at 10,000 rpm for 10 minutes at 4˚C. The supernatant carefully was discarded and the pellet was washed with cold 70% ethanol (500µl).This was followed by centrifugation at 10,000 rpm for 10 minutes at 4˚C. The pellet was then dried at 37˚C in an incubator for 20-45 minutes until alcohol was completely removed.

The pellet obtained was dissolved in 200µl-300µl of T.E. buffer and left overnight at 4˚C.

Purification of DNA isolated:

3-4µl of RNase stock 100mg/ml was added to the DNA sample and was incubated for one hour at 37˚C. Equal volume of Chloroform: Isoamyl alcohol was then added and centrifuged at 13,000 rpm for 10 minutes at 26˚C in refrigerated centrifuge. The upper aqueous layer was transferred to a new eppendorf tube and 0.1 volume of 3M Sodium Acetate and 3 volumes of chilled absolute ethanol was added and incubated at -20˚C for 1-2 hours. The tubes were centrifuged at 13,000 rpm for 5 minutes at 4˚C. The supernatant obtained was then discarded and washed by adding 100µl of 70% ethanol and centrifuging at 12,000 rpm for 12 minutes. (If the impurities remain, this step was repeated.) The supernatant was discarded carefully and pellet was retained. The pellet was allowed to dry at 37˚C for 20 minutes. Finally the pellet was dissolved in 100µl of T.E. buffer. The DNA stock samples were stored at 4˚C or at -20˚C for long duration.

3.3 DNA Quantification and Quality test using Agarose:

After isolation genomic DNA were estimated for both quantity and quality, quantity was estimated using spectrophotometer in which the UV absorbance at 230, 260 and 280 nm were recorded and compared to determine the concentration as well as the quality of DNA. In principle nucleic acid (both RNA and DNA) has the maximum absorbance at the wavelength 260 nm and 1 OD is equivalent to 50µg/ml DNA. The ratio of absorbance obtained at 260 and 280 nm wavelength is used to assess the purity of DNA and RNA. A ratio in between the range of 1.8-2.0 is generally accepted as "pure" for DNA. A ratio significantly lower than this range indicates the presence of any contaminants such as protein or phenols that absorb at or near 280 nm. The value of 2.0 and greater than 2.0 shows RNA contamination.

DNA quality test using Agarose Gel Electrophoresis:

Using agarose gel electrophoresis the DNA fragments can be separated, identified and purified. The DNA within the gel can be located either by staining with low concentration of fluorescent intercalating dyes, such as EtBr. This technique is simple to perform, rapid, and is capable of resolving fragments of DNA that cannot be separated by other procedures.

Agarose Gel Electrophoresis:

Agarose gel was prepared by adding agarose to a desired buffer and melting it until a clear, transparent solution was achieved. The melted solution was then poured into a mould and allowed to cool down and form solidified gel. After the solidification of gel the sample of DNA were loaded into the wells. When an electric field is applied across the gel, the negatively charged DNA fragments that are at neutral pH begin to migrate towards the anode.

Preparation of gel

Add 0.8gm of agarose in 100ml (1XTAE buffer)

↓

Boiled in microwave

↓

Allowed to cool

↓

Add5µl EtBr and mixed it properly

↓

Allow it to solidify in gel plate

3.4 Amplification of DNA by PCR:

Optimization of PCR conditions for ISSR analysis:

The PCR condition for ISSR amplification on *Bacopa monnieri* was done. The components of PCR were Taq DNA polymerase, PCR Buffer, dNTP, $MgCl_2$, primer and template DNA.

Screening of Primers:

Ten ISSR primers were screened against the *Bacopa monnieri* DNA samples for optimum amplification. The primers which produced amplification were selected for the further study.

S.no.	Marker	Sequence
1	ISSR 1	ATATATATATATATAT
2	ISSR 2	GAGAGAGAGAGAGAGAC
3	ISSR 3	CTCTCTCTCTCTCTCTG
4	ISSR 4	GCGCGCGCGCGCGCGCT
5	ISSR 5	GCGCGCGCGCGCGCGCA
6	ISSR 6	GAGAGAGAGAGAGAGACG
7	ISSR 7	AGAGAGAGAGAGAGAGA
8	ISSR 8	ACACACACACACACACGG
9	ISSR 9	AGAGAGAGAGAGAGAGT
10	ISSR 10	ACACACACACACACACCG

Table1: Nucleotide Sequences of ISSR markers

PCR Amplification:

The final PCR reactions are performed in 25µl mixture by adding 2µl from the dilution of genomic DNA, 0.5µl of (10X) dNTP Mix, 2.5µl of 10X Dream Taq assay buffer, 1.5µl of 25mM MgCl2, 3µl of ISSR primer, 0.1µl of Taq polymerase. ISSR primers were used to amplify the DNA using thermal cycler.

The standardized PCR reaction conditions were: initial denaturation step at 94°C for 4minutes followed by 41 cycles comprising of denaturation step at 94°C for 1 minute, a primer annealing step of 55°C for 45seconds and an extension step at 72°C for 90seconds terminated with a final extension at 72°C for 5minutes and final hold at 4°C for infinity.

S.no.	Reagents	Volume
1	(10X) Dream Taq assay buffer	2.5 µl
2	25mM MgCl2	1.5 µl
3	(10X) dNTP Mix	0.5 µl
4	ISSR primer	3 µl
5	Taq polymerase	0.1 µl
6	Template DNA	2 µl
7	Distilled water	15.4 µl
	Total	**25 µl**

Table2: PCR ingredients for 25 µl reaction

The PCR products (5-10µl) are then checked for PCR amplification by adding 2-3µl of 6X loading dye along with 6µl of 100bp and 1kb ladder on agarose gel (2%) containing ethidium bromide in a 1X TAE buffer. The PCR amplified products in gel were then visualized and photographed under UV light.

3.5 ISSR Analysis

All bands amplified by each ISSR markers were used to assign loci and scored for presence as “1” or absence as “0”. The information obtained in form of binary data from all ISSR markers were used to produce a combined score matrix using Microsoft Excel 2007 before transferring it to NTedit 1.07c program. The binary data produced was then used to calculate the genetic similarity matrix. The analysis was performed using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software version 2.02. Dendrograms showing the phylogenetic relationships between various accessions of *Bacopa monnieri* were constructed with unweighted pair group method (UPGMA) using the SAHN subroutine and Tree plot of NTSYS-pc.

RESULTS:

Genomic DNA isolation

15 accessions of *Bacopa monnieri* belonging to different regions of India, obtained from CIMAP, Lucknow and maintained at the plant tissue culture laboratory of Department of biotechnology, Delhi Technological University were used to perform genomic DNA isolation. Genomic DNA from fresh green leaves of 15 samples was successfully extracted using a modified protocol of CTAB method of Doyle and Doyle. The yield and quality of isolated DNA was evaluated using Spectrophotometer and the isolated DNA was then treated with RNAase A in order to obtain DNA free from contamination.

Screening of ISSR primers

A total of 10 ISSR primers were screened against the *Bacopa monnieri* DNA samples. Out of these, only 5 primers (ISSR2, ISSR6, ISSR8, ISSR9, ISSR10) were found to produce multiple bands successfully and were subsequently used for further analysis of the 15 accessions of *Bacopa monnieri*.

ISSR analysis

The results of the ISSR-PCR reactions performed for the fifteen *Bacopa monnieri* accessions were used to produce a score matrix where bands were marked as ‘‘1’’ for presence and ‘‘0’’ for absence for each primer. The number of monomorphic and polymorphic bands generated by each primer was estimated. The score matrix of binary data thus produced was used to determine levels of polymorphism by dividing the number of polymorphic bands by the total number of bands scored.

The amplification patterns obtained from 5 ISSR primers have been depicted in Fig.1 to Fig.6. These primers showed variation in the percentage of polymorphism as shown in the Table3 with maximum polymorphism observed in case of ISSR10 (71.4%) and least by ISSR9 (33.3%). The results from ISSR analysis of the 15 accessions of *Bacopa monnieri* in the presennt study clearly showed polymorphism and the amplified bands produced by the 5 ISSR primers were found to range in size from 200bp to 2000bp. Many reasons may be responsible for occurrence of polymorphism such as deletion, substitution of base within the primer binding site of the sequence (William *et al.,* 1990).

S.no.	Marker	Sequence	Total no. of loci	No. of Polymorphic loci	Percentage Polymorphism %
1	ISSR 2	GAGAGAGAGAGAGAGAC	5	2	40
2	ISSR 6	GAGAGAGAGAGAGAGACG	4	2	50
3	ISSR 8	ACACACACACACACACGG	7	5	71.4
4	ISSR 9	AGAGAGAGAGAGAGAGT	3	1	33.3
5	ISSR 10	ACACACACACACACACCG	6	4	66.6
		Total	**25**	**14**	**56%**

Table3: Percentage Polymorphism generated with ISSR primers.

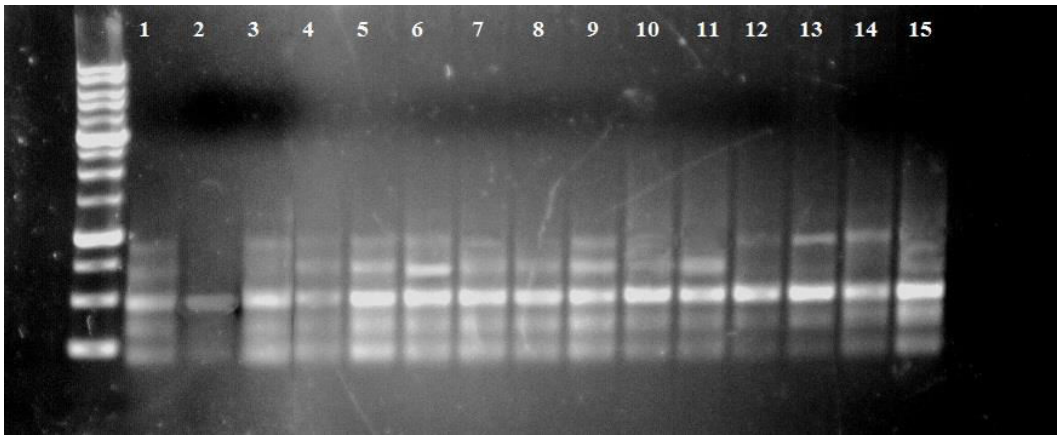

Figure2: ISSR-PCR product amplified by with primer ISSR2**,** lanes 1-15 represent accessions of *B.monnieri*

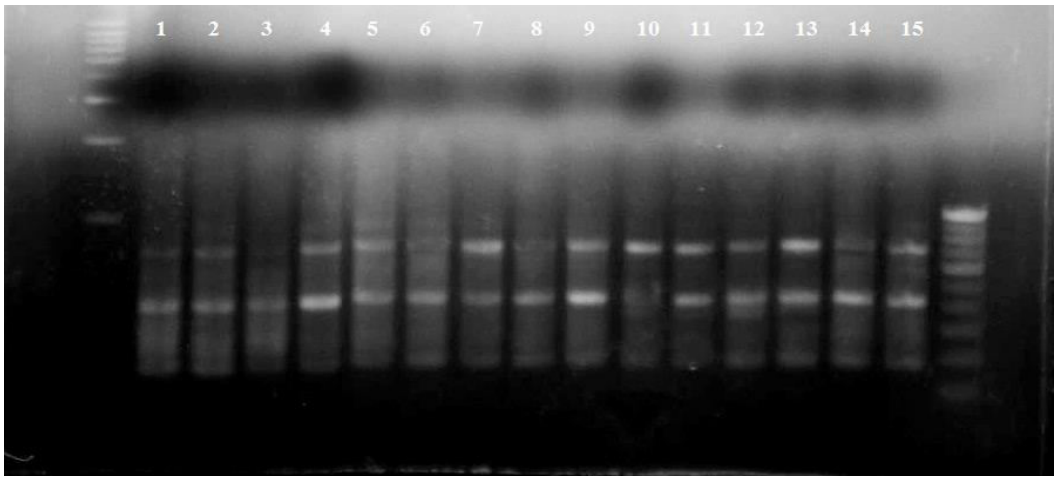

Figure3: ISSR-PCR product amplified with primer ISSR 6, lanes 1-15 represent accessions of *B.monnieri*

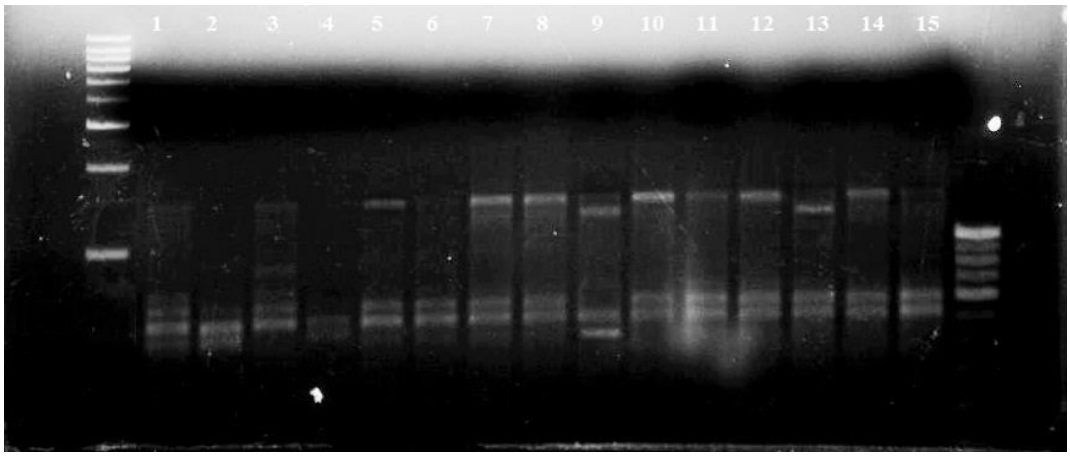

Figure4: ISSR-PCR product amplified with primer ISSR 8, lanes 1-15 represent accessions of *B.monnieri*

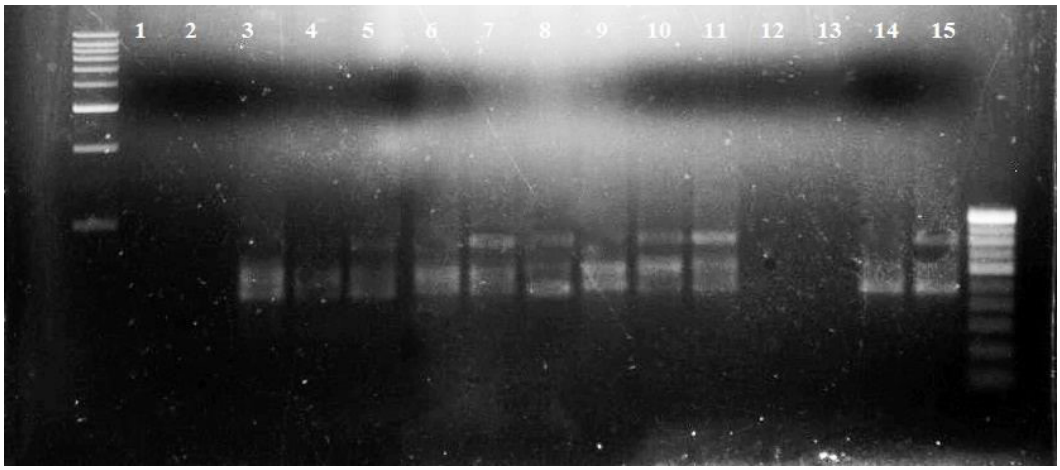

Figure5: ISSR-PCR product amplified with primer ISSR 9, lanes 1-15 represent accessions of *B.monnieri*

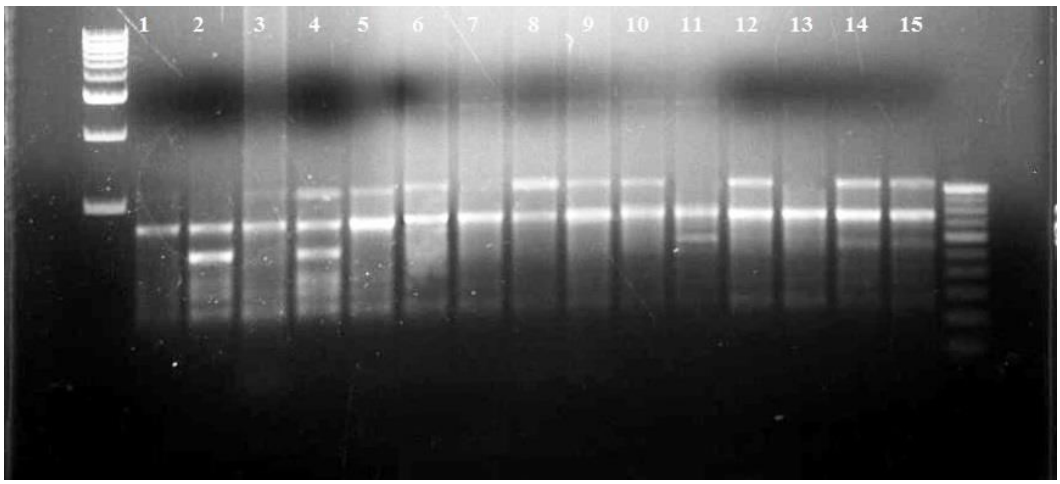

Figure6: ISSR-PCR product amplified with primer ISSR 10, lanes 1-15 represent accessions of *B.monnieri*

Clustering and UPGMA analysis

Genetic variability was determined by converting ISSR data into a dendogram by using NTSYS-pc software. The dendogram was prepared from cluster analysis using NTSYS-pc software ISSR profiles obtained were used to generate similarity co-efficient for cluster analysis. The similarity coefficients thus generated were used as input data to derive a dendrogram by UPGMA method of cluster analysis using NTSYS-pc program in order to determine the genetic relationships among the different accessions of *Bacopa monnieri*.

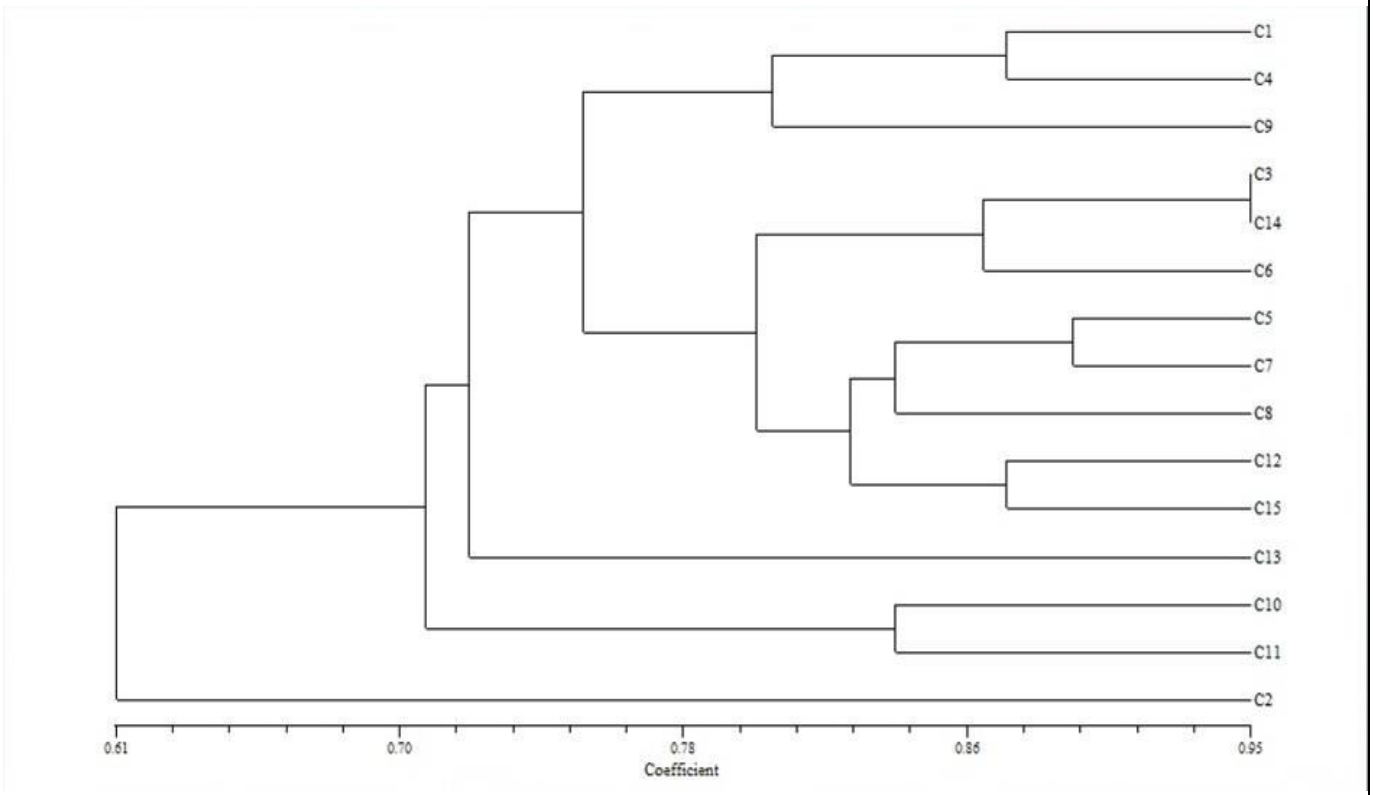

Figure 8: Dendrogram resulting from UPGMA analysis of fifteen accessions of *Bacopa monnieri* from different regions of India using the data generated from ISSR marker data

The dendogram showed that cluster analysis resulted in the accessions belonging to same geographical regions being grouped into same cluster. The cluster analysis grouped all the *Bacopa monnieri* accessions under study in three main groups (Fig.8). The first major group A was divided into two sub groups, first subgroup containing three accessions C1 (Madhya Pradesh), C4 (Madhya Pradesh), and C9 (Madhya Pradesh). Second subgroup contains eight accessions clustered as C3 (Madhya Pradesh), C14 (Jharkhand) and C6 (Jharkhand) together and C5 (Karnataka), C7 (Karnataka), C8 (Orissa), C12 (Kerala) and C15 (Kerala) in another cluster. Here C3 and C14 are shown to be closely related accessions with high similarity. The second major Group B was found to contain only two accessions, namely, C10 (Delhi) and C11 (Punjab) while group C shows C13 (Uttar Pradesh) separately. Lastly accession C2 (Jammu and Kashmere) has been shown separately indicating that it is genetically diverse from other *Bacopa monnieri* accessions and is hence placed at end of the cluster. All accessions were found to lie within the range 0.6 to 0.95 of similarity.

DISCUSSION AND FUTURE PERSPECTIVE:

In the present study fifteen accessions of *Bacopa monnieri* belonging to different regions of India were analysed using ISSR molecular markers in order to determine the genetic relationships among them. The cluster analysis showed that the accessions belonging to same geographical regions were grouped into same cluster. With the 5 ISSR primers analysed, 25 distinct bands, in the size range of 200–2000 bp were observed of which 14 (56%) were polymorphic while rest 11 (44%) were monomorphic. The cluster analysis grouped all the Brahmi genotypes under study in 3main groups (Fig.8). Major group A was divided into two sub groups, first subgroup containing only three accessions C1, C4 and C9 belonging to Madhya Pradessh. Second subgroup contains eight accessions clustered as C3 (Madhya Pradesh), C14 (Jharkhand) and C6 (Jharkhand) together and C5 (Karnataka), C7 (Karnataka), C8 (Orissa), C12 (Kerala) and C15 (Kerala) in another cluster. Here C3 and C14 are shown to be closely related accessions with high similarity. Group B contains only two accessions, namely, C10 (Delhi) and C11 (Punjab) while group C shows C13 (Uttar Pradesh) separately. Lastly C2 (Jammu and Kashmere) has been shown separately indicating that it is diverse from other accessions and hence being placed at end of the cluster. All accessions were found to lie within the range 0.6 to 0.95 of similarity. The phylogenetic cluster of *Bacopa* plant populations are helpful in highlighting the variation that might have resulted from many different processes such as mutation, gene flow, mating system, selection and evolutionary background of the species for example habitat fragmentation and isolation of population.

Bacopa monnieri is one of the most important ayurvedic medicinal plant of global interest due to its numerous medicinally valuable compounds. It is cultivated as valuable drug resource whose price is determined on the basis of quality and active constituents. It has a wide geographic distribution pattern and quality of raw material from wild populations is affected by genotype which in turn is affected by environmental factors. Thus there is a need for diversity documentation. Molecular markers are powerful tool to establish genetic relationship and help determine similarities or variations among species based on their genetic makeup. The identification of elite *Bacopa monnieri* accessions with high concentrations of valuable compounds is important in pharmaceutical industries as it greatly affects the quality pharma products. Molecular markers provide an important means by which these can be identified by screening the available germplasm for range of genetic diversity. Detection of genetic variation also finds important application in case of micropropagation and in vitro germplasm conservation to help eradicate undesirable somaclonal variations.

The use of molecular markers makes it feasible to derive inferences related to genetic divergence as well as inter-relationships amongst organisms at DNA level and offer the advantage that they are free from the misleading environmental influences or fallible pedigree accounts. The advancement in the range of molecular marker systems available has greatly assisted in evaluating genetic constitution of plant populations for taxonomic, evolutionary, and ecological research purposes. Molecular marker systems have numerous applications such as characterization of genetic diversity amongst several plant varieties or species for the evaluation of genetic fidelity, identification of genes of commercial and agronomic interests,

and enhancement using genetic transformation system. Molecular markers are a powerful tool that can help in verification of genetic consistency and to test whether in vitro regenerated plants are true-to-type.

ISSR marker system has several advantages over other molecular markers such as: no requirement for information of genome sequence, simplicity of operation, high stability, and ability to detect high level of polymorphism.
Therefore various previous studies using ISSR markers have been successfully utilised for the assessment of genetic diversity among a number of medicinal plants such as *Tribulus terrestris* (Sarwat *et al.* 2008), *Papaver somniferum* L. (Acharya and Sharma 2009), *Anoectochilus formosanus* (Zhang et al.2010), and *Rheum officinale* (Wang 2011). ISSR markers have also been used to investigate the genetic variation in wild and cultivated varieties of Chinese medicinal plants such as Rhizoma corydalis by Qiu et al. (2009) and Coptis chinensis by Shi *et al*. (2008). Other medicinal plants where application of ISSR markers has been successful include Swertia chirayita (Joshi and Dhawan 2007), Tribulus terrestris (Sarwat *et al.* 2008), Pleurotus citrinopileatus (Zhang *et al.* 2012).

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