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Research article

A STUDY ON ASSESSMENT OF GENETIC DIVERSITY AND RELATIONSHIPS OF MEDICINAL PLANTS USING RAPD MARKERS

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ABSTRACT

Molecular genetic fingerprints of medicinal species were developed using Randomly Amplified Polymorphic DNA (RAPD) marker to elucidate the genetic diversity among the 18 species. DNA was isolated using the CTAB method. The amplification was accomplished by using 10 primers and the specific PCR working program. Three decamer-primers generated 250 RAPD fragments, of which 232 fragments were polymorphic with 96.84% of polymorphism. Some of the RAPD markers were useful for species discrimination and identification. Most of the RAPD markers studied showed different level of genetic polymorphism. Amplified fragment sizes ranged from 300 to 5000 bp. Pairwise Nei and Li's similarity coefficient value ranged from 0.00-0.72 for 18 species of medicinal plants. A dendrogram was constructed based on the Unweighted pair group method using arithmetic averages. Cluster analysis of data using the UPGMA algorithm placed the 18 species of medicinal plants into four groups that are somewhat congruent with classification based on morphological characters proposed by earlier works. This analysis grouped all species into different clusters and clearly differentiated the medicinal plants into separate groups. This method of analysis can be helpful in selecting diverse parents and give broadness to the germplasm base of medicinal plants breeding programs in the future.

KEY WORDS: Medicinal plants, RAPD, Genetic Diversity.

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INTRODUCTION

The world is endowed with a rich wealth of medicinal plants. Herbs have been the principal form of medicine in ancient India. Vital herbs lost their importance due to pharmaceutics-d revolution. They are also becoming popular as people strive to live healthy in the face of chronic stress and pollution and to treat illness with medicines that work in concert with body's relied defense. Medicinal plants play a crucial role in the lives of rural people, in remote parts of developing countries with limited facilities for health care (Purohit and Prajapati, 2003).

Around 70,000 plant species, from lichens to flowering trees have been used for medicinal purposes. Many species are used in herbal medicines and is used in unrefined or semi-processed form, often as mixtures, which may also contain non-botanical ingredient. A few species are the sources of defined compounds used in the pharmaceutical industry. There is an international trade on medicinal plants used in herbal medicine and in the manufacture of pharmaceuticals. There is also a growing interest in obtaining samples of plant material and traditional knowledge about the uses of plants and also to explore commercial medicinal products. The scale of international trade in medicinal plants is difficult to assess, because of the paucity of reliable statistics and trade secrecy. Ancient Indian literature incorporates a broad definition of medicinal plants and considers "all" plant entities to be potential sources of medicinal substances. While all plant entities are potentially medicinal, only those plants are considered 'medicinal' whose medicinal use has already been discovered for human or animal application. Traditional medicinal plants have been used for human, veterinary and plant health. There are medical texts that deal with the treatment of cows; horses, elephants, and birds, there are also texts on subjects like Vriksh Ayurveda and Krishi Sastra that deal with the use of plants for controlling pests, treating plant diseases and as biofertilizers.

Conservation of Medicinal Plants

A taxon is endangered when it is not critically endangered but is facing a very high risk of extinction in the wild in the near future (Ravikumar and Ved, 2000).

RAPD, markers behaves as dominant genetic markers, meaning that in a segregating population the homozygote of the parental type from which a given RAPD, ISSR and SSR band is amplified cannot be distinguished from the heterozygote, because the heterozygote also produces a RAPD, band. The only unambiguously assigned genotype is the homozygote of the other parental type (no RAPD band). The segregating F2 population may therefore be scored as follows band present: AA or Ab; band absent: bb. Remembering this fact, it is easy to select the populations best suited for the construction of genetic maps with RAPD markers (Banerjee, N.S., Manoj, P. And Das, M.R., 1999).

MATERIALS AND METHODS

The materials required and methodology of the present work is carried out at the Department of Biotechnology, Acharya Institute of Technology, Karnataka and Plant molecular biology laboratory, Department of Horticulture, Hulimavu Biotechnology Centre, Govt of Karnataka, Bangalore, India. In the year 2010–2012. The materials used and methods followed in the study are presented here.

Materials: Fresh, young, disease free leaves of 18 medicinal plants *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 monierii, Piper longum, Piper nigrum, Clitoria ternatea, Aloe Vera, Stevia rebaudiana,* which were collected from the germplasm maintained at the different regions of Karnataka as medicinal plants germplasm conservation.

DNA Extraction

Fresh, young and disease free leaves of 6 different medicinal plants were collected and immediately kept on ice to reduce the nuclease activity. It was brought to the laboratory, weighed (2 g each), and frozen in liquid nitrogen and stored at 70ºC till further use. The DNA was extracted using the CTAB method (Porebski *et al*., 1997) with certain modifications. 2 g of fresh medicinal plant leaf material was ground into a fine powder using liquid nitrogen. The powder was then transferred to sterile centrifuge tubes and 12 ml of extraction buffer was added, mixed thoroughly and incubated at 65ºC in a water bath for one hour with intermittent shaking. The tubes were brought to room temperature and centrifuged at 8000 rpm for 10 min at 4ºC. The supernatant was transferred to new tubes, 6 ml of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly. The tubes were centrifuged at 8000 rpm for 10 min at 4ºC. The supernatant was transferred to new tubes and repeated the same steps twice. The DNA was then precipitated by adding half volume of 5M NaCl, an equal volume of chilled propanol and incubated at 4ºC over night. DNA was pelleted by centrifuging at 20,000 rpm for 12 min at 4ºC. The pellets were dried after adding 70% ethanol and 1 ml of TE buffer was added to which 20 µl of RNase was added. This was incubated at 37ºC for one hour and added 300 µl of saturated phenol. It was mixed, centrifuged at 8000 rpm for 10 min at 4ºC. The supernatant was transferred to another tube and repeated the same process by adding phenol: chloroform and chloroform respectively. The supernatant was treated with equal volume of isopropanol and incubated at 4ºC for overnight. The DNA was pelleted by centrifuging at 12000 rpm for 20 min. The pellet was washed with 70% ethanol and dried. Around 300 µl of TE buffer was added to dissolve the pellet and stored at 20ºC for further use.

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Table.1.Sequence information on RAPD oligonculeotide primers used for amplification and polymorphism study in 18 medicinal plants

S.No	RAPD Primers	Sequence (5'-3")
1	OPC - 7	GTCCCFACGA
2	OPL -11	ACGATGAGCC
3	OPO - 08	GCTCCAGTGT
4	OPAH - 15	CTACAGCGAG
5	OPAM - 20	ACCAACCAGG
6	OPAN - 01	ACTCCAGGTC
7	OPAO - 01	AAGACGACGG
8	OPAP - 20	CCCGGATACA
9	OPAN - 05	GGGTGCAGTT
10	OPAP - 10	TGGGTGATCC
11	OPAA - 01	AGACGGCTCC
12	OPAB - 01	CCGTCGGTAG
13	OPAB - 05	CCCGAAGCGA
14	OPAB - 14	AAGTGCGACC
15	OPAH - 13	TGAGTCCGCA
16	OPAF - 02	CAGCCGAGAA
17	OPAJ- 19	ACAGTGGCC
18	OPX - 20	CCCAGCTAGA
19	OPA - 08	GTGACGTAGG
20	OPD - 13	GGGGTGACGA

Table 2. Showing the thermal profile used for RAPD

Temperature	Time	Steps
93°C	2 min	Initial denaturation
5 cycles of 93°C	1 min	Denaturation
35°C	1 min	Annealing
72°C	2 min	Extension
72°C	7 min	Final extension
4°C	∞	Hold

Data Analysis

DNA binding patterns generated by RAPD were scored as '1' for the presence of band and '0' for its absence. All RAPD assays were performed twice and only the reproducible bands were scored. A similarity matrix was generated using a dendrogram based on distance matrix data sets by applying Wards method for cluster analysis using 'STATISTICA' 5.0 computer program.

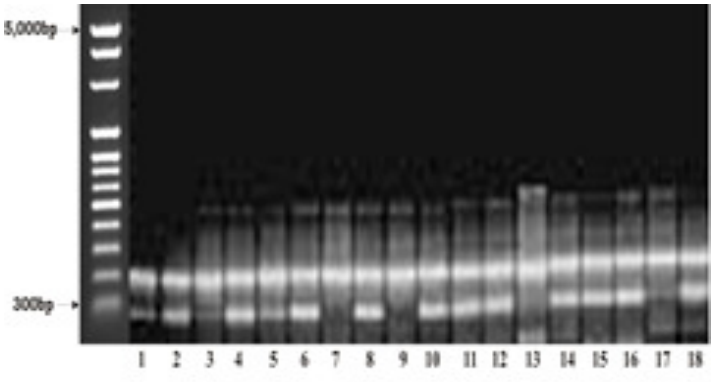
RESULTS

The genomic DNA of 18 medicinal plants viz, *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 monierii, Piper longum, Piper nigrum, Clitoria ternatea, Aloe vera, Stevia rebaudiana* were amplified with oligonucleotides primers.

RAPD analysis of medicinal plants using Primer OPAB– 05

The genomic DNA of 18 medicinal plants was amplified with decamer oligonucleotide primers such as OPAB-05 and as shown in Fig 1. The distinct and abundant RAPD fragments were recorded. The total numbers of bands generated were 51 RAPD gel profiles. The sizes of the RAPD bands were placed in between 300-5000 bp in length. The primer produced distinct polymorphic banding pattern in all the medicinal plant species, the number of RAPD bands per primer were 2.8 as expected to sexually reproduce plants. The RAPD bands distributed in the plant is important to know the value of breeding patterns in medicinal plants. (Banerjee, N.S., Manoj, P. and Das, M.R., 1999). The number of RAPD bands was produced to reveal Mendelian inherited character, and number scoring revealed medicinal characters. The banding patterns are important and distinct in medicinal plants. The polymorphism was very high and RAPD values were useful to distinguish between the medicinal plant species, apparently diverse elements such as diploid and other exotic species character. The identification of RAPD is very unique in plants, because medicinal value and coupled with highly cross – pollinated and revealed heterozygous character. In the present data the plants like 1 showed one band and the remaining had revealed 7-3 RAPD bands respectively. Further, 1 and 2 medicinal plants showed 1 and 3 RAPD bands due to amplification of primer with the genomic DNA of these plant species. However, it was observed that some of the plants viz., 11-12 have recorded 7 bands indicating diverse character compared to other plants. Polymorphic distribution as far as gene flow is concerned revealed high or low speciation. This has been used for various other calculations of medicinal plant breeding programs. Therefore, amplification of genomic DNA of these medicinal plants revealed moderate diversity among them.

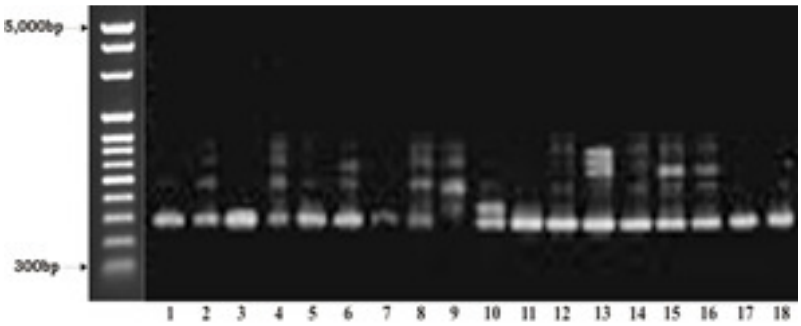
Fig 1. Gel profiles of 18 medicinal plants amplified with RAPD Primers –OPAB-05



RAPD analysis of 18 medicinal plants using Primer OPAB – 14

The data obtained in the present investigation revealed a total number of 111 RAPD bands. The genomic DNA of 18 medicinal plants amplified with OPAB –14 revealed both monomorphic and polymorphic RAPD bands. The distinct and abundant RAPD fragments were recorded. The total number of bands generated lie in between 300-5000bp in length. The primer produced medium low and high resolution of RAPD bands. The number of bands per primer was recorded at a maximum of 6.1 bands. However four bands were recorded in plants like 1-18 respectively. Despite, the plants revealed a total number of 111 bands, therefore the distribution of banding patterns is common, and one of the plants has revealed 6 RAPD bands due to the amplification of genomic DNA with primer OPAB –14. However, amplification showed very clear and distinct bands, and some of the medicinal plants like 1-18 have revealed four bands respectively. From this data it is possible to identify species specific band for medicinal plants for selection, in turn it helps for the cultivation of medicinal plants. The RAPD banding techniques are useful for selection multiplication and introgression of certain traits for breeding of medicinal plants as shown in Fig2.

Fig 2. Gel profiles of 18 medicinal plants amplified with RAPD Primers –OPAB-14



RAPD analysis of 18 medicinal plants using Primer OPAH -13

The data obtained in the present investigation revealed a total number of 88 RAPD bands. The genomic DNA of 18 medicinal plants amplified by OPAH -13 revealed both monomorphic and polymorphic RAPD bands. The distinct and abundant RAPD fragments were recorded. The total number of bands generated lie in between 300–5000bp in length. The primer produced medium low and high resolution of RAPD bands. The number of bands per primer was recorded at a maximum of 4.8 bands. However, four bands were recorded in plants like 1–18 respectively.

Despite, the plants revealed a total number of 88 bands, therefore the distribution of banding patterns is common, and one of the plants as revealed five RAPD bands due to the amplification of genomic DNA with primer OPAH -13. However, amplification showed very clear and distinct bands, and some of the medicinal plants from 1–8 have revealed 8 bands respectively. From this data it is possible to identify species specific band for medicinal plants for selection, in turn it helps for the cultivation of medicinal plants. The RAPD banding techniques are useful for selection multiplication and introgression of certain traits for breeding of medicinal plants as shown in Fig 3.

Fig3. Gel profiles of 18 medicinal plants amplified with RAPD Primers –OPAH-13

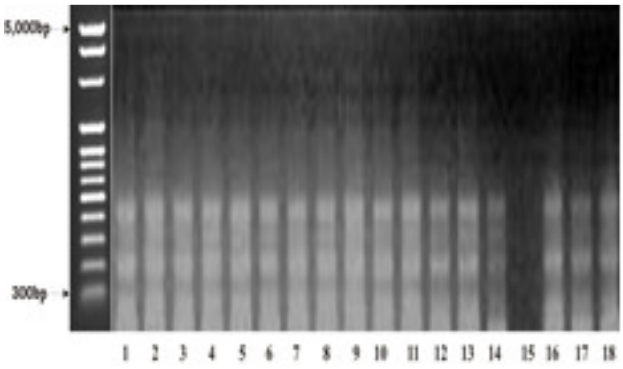


Fig 4. Dendrogram of 18 medicinal plants amplified with RAPD primer- OPAB-05

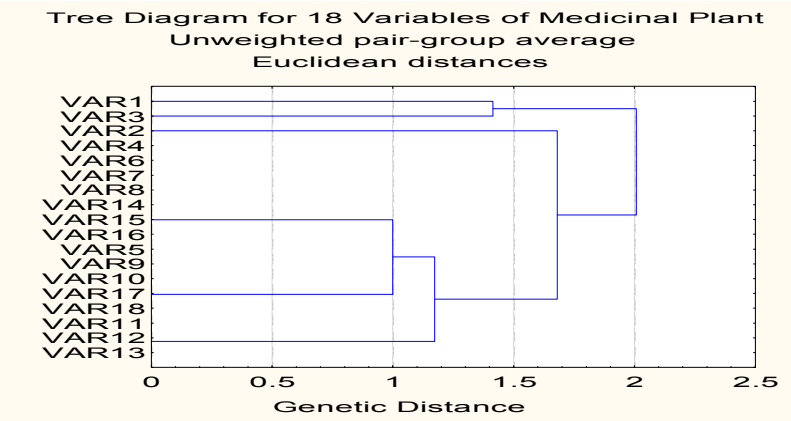


Fig 5. Dendrogram of 18 medicinal plants amplified with RAPD primer- OPAB-14

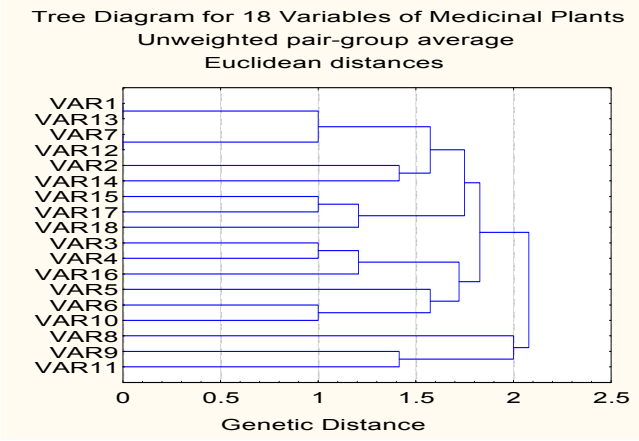
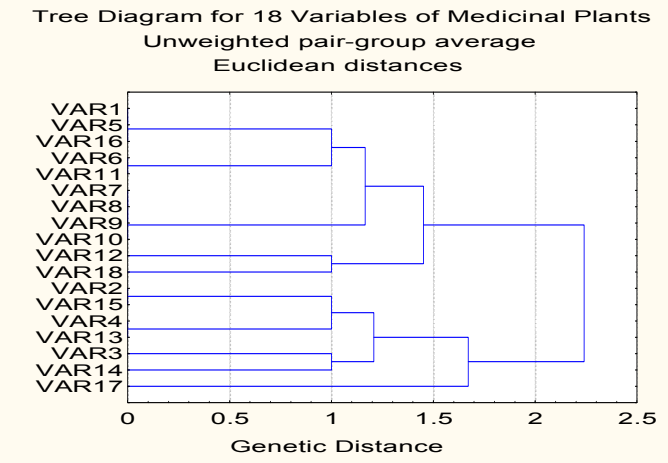


Fig 6. Dendrogram of 18 medicinal plants amplified with RAPD primer- OPAH-13



DISCUSSION

Genetic resources available for medicinal plant improvement are abundant within plant species. Even though a few species of medicinal plants occur naturally in India, many cultivated medicinal plant species do find their origin within the country especially India. 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 of sexual incompatibility characteristic of medicinal plant species. This fact suggests a close genetic or non-genetic relationship among the medicinal plants. The present study involves 18 medicinal plants with molecular characterization of RAPD, analysis for further supports this view. RAPD analysis of medicinal plants using Primer OPAB– 05. The results of the present investigation on genomic DNA of 18 medicinal plants viz , *Hemigraphis colorata, Marjorana hortensis, Artemisia vulgaris, Artemisia pallens, Ocimum sanctum, Ocimum basilicum, Ocimum ratissimum, Mentha piparita, Mentha citrata, Mentha spicata, Acorus Calamus, Centella asiatica, Bacopa monierii, Piper longum, Piper nigrum, Clitoria ternatea, Aloe Vera, Stevia rebaudiana* were amplified with oligonucleotides primers OPAB– 05 revealed total of 51 RAPD bands Fig 1 and the Dendrogram of 18 medicinal plants amplified with RAPD primer- OPAB– 05 shown in fig 4 **.** Similar observations were recorded by Girish Naik and Dandin 2006, Souframani and Gopalakrishna, 2004. Similar observations have also been made in other species at a cultivars level (Colombo *et al.,* 1998, Banerjee *et al.,* 1999, Das *et al.,* 1998). RAPD analyses of 18 medicinal plants using Primer OPAB –14 were amplified revealed a total of 111 RAPD bands. With an average of 6.1 bands per primer, all the 18 medicinal plants exhibited 10 RAPD bands respectively. Whereas some of the medicinal plants such as *Artemisia vulgaris, Mentha citrata and Piper longum* have expressed more than 10 RAPD bands per primer as shown in Fig 2 and the Dendrogram of 18 medicinal plants amplified with RAPD primer- OPAB– 14 shown in fig 5. Further, a similar observation was made by Awasthi *et al.,* (2004) Basha, S.D and Sujatha, M. 2007 in mulberry and medicinal plants. RAPD analysis of 18 medicinal plants using Primer OPAH -13.

CONCLUSION

The genomic DNA of 18 medicinal plants was amplified with decamer oligonucleotide primers In OPAB– 05 **primer** exhibited 51 bands with distinct and abundant RAPD fragments. Primer OPAB – 14 primer indicates 111bands with an average of 6.1 bands per primer. In the present investigation random polymorphic bands were seen to assess with different polymorphism. The minimum number clusters indicated gene flow in different medicinal plant species belonging to different family is very low.

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