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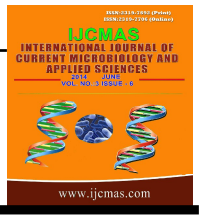

Original Research Article

Study on Genetic Diversity relationship some Medicinal plants using RAPD Molecular marker

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A B S T R A C T

Genetic relationships between eighteen medicinal were assayed with Random Amplified Polymorphic DNA (RAPD) markers, which distinguish individuals, as well as reflecting the inherent variation and interrelationships among the medicinal plants. 13 decamer RAPD primers were used in the present study. Over 77.23 reproducible bands were generated by RAPD primers, out of which, 77.23 polymorphic bands were identified, conferring 97.6% polymorphism. All the primers produced typical banding in each of the medicinal plants, suggesting the applicability of this test in medicinal plant identification. Most of the individuals of the test exhibited to have unique molecular genotype. Population genetics structure analysis of these species further revealed high genetic differentiation coefficients (Gst), the heterozygosity among populations (Ht) showed with the low gene flow (Nm) when the 1st cluster was paired with other medicinal plants On the basis of these parameters and the results of cluster analysis. It is concluded that three species can be considered as a separate group of medicinal plant, whereas the other four clusters may be grouped separately. A dendrogram was constructed using Euclidean distance methods. Based on the number of bands the of the medicinal plants were grouped to form1-4 clusters.

Keywords

RAPD, Genetic Diversity Medicinal plants

Introduction

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). Medicinal plants have curative properties due to complex chemical substances of different composition, found as secondary plant metabolites in one or more parts of these plants. The plant metabolites, according to their composition, are grouped as alkaloids, glycosides, corticosteroids, and essential oils and so on. Medicinal plants are a living and irreparable resource, exhaustible if overused and sustainable if used with care and wisdom. The importance of medicinal plants has been overlooked in the past, however, at present medicinal plants are looked not only as a source of affordable health care but also as a source of income.

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 international trade in 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. Molecular markers have particularly been suggested to be useful for confirmation of genetic fidelity in micro propagated tree species, where lifespan is quite long and performance of micro propagated plants could only be ascertained after their long juvenile stage in field conditions. In India also, studies have been conducted and research projects funded for research where lack of polymorphism shown through the use of molecular markers has been used to infer genetic fidelity. Recent advances in molecular genetics techniques and tools over the past decade have provided considerable impetus to research in this direction. Techniques using molecular markers based on DNA polymorphism are coming up at a rapid rate. However, molecular marker study of these medicinal plants was carried out by previous researchers with sporadic reports. and molecular marker studies in these medicinal plants, revealed a large gap, in view of the above the present investigation was undertaken on some of the medicinal plants such *Hemigraphis colorata, Marjorana hortensis, Artemisia vulgaris, Artemisia pallens, Ocimum sanctum, Ocimum basilicum, Ocimum hratissimum, Mentha piparita, Mentha citrate, Mentha spicata, Acorus calamus, Centella asiatica, Bacopa moninierii, Piper longum, Piper nigrum, Clitoria ternatea, Aloe vera, Stevia rebaudiana,* However the present investigation was carried out on studies on molecular characterization using molecular RAPD markers in some medicinal plants

Materials and Methods

The materials required and methodology of the present work is carried out at Department of Biotechnology, Acharya Institute of Technology, Karnataka and Plant molecular biology laboratory, Department of Horticulture, Hulimavu Biotechnology Centre, Govt of Karnataka, Bagalore, 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 hratissimum, Mentha piparita, Mentha citrate, Mentha spicata, Acorus calamus, Centella asiatica, Bacopa moninierii, 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 in ice to reduce the nuclease activity. It was brought to the

laboratory, weighed (2 gms 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. 2gms of fresh medicinal plants leaf material was ground into a fine powder using liquid nitrogen. The powder was then transferred to sterile centrifuge tubes and 12ml of extraction buffer was added, mixed thoroughly and incubated 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 10min 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, 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 1ml 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 8000rpm 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 20min. 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.

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 was constructed based on distance matrix data sets by applying Wards method for cluster analysis using 'STATISTICA' 5.0 computer program.

Results and Discussion

The genomic DNA of 18 medicinal plants viz , *Hemigraphis colorata, Marjorana hortensis, Artemisia vulgaris, Artemisia pallens, Ocimum sanctum, Ocimum basilicum, Ocimum hratissimum, Mentha piparita, Mentha citrate, Mentha spicata, Acorus calamus, Centella asiatica, Bacopa moninierii, Piper longum, Piper nigrum, Clitoria ternatea, Aloe vera, Stevia rebaudia* were amplified with oligonucleotides primers OPD-13 revealed total of 172 RAPD bands, however, the medicinal plants such as *Artemisia vulgaris, Artemisia pallen Mentha piparita, Mentha citrate Bacopa moninierii* and *Stevia rebaudia* have showed 9 RAPD bands respectively.

Further, oligonucleotides primers OPD -13 have showed 8-9 RAPD bands in each medicinal plant. The number and size of the amplification products varied depending up on the sequence of random primers and medicinal plants. The size of the amplified products ranged from 300-5000bp with an average of 9.5 bands per primer. The amplified products were generated by primers showing polymorphic bands Fig 1 and 14. The genomic were amplified with OPD-07 revealed a total of 104 RAPD bands with an average of 6 bands per primer with the primer sequence of

TTGGCACGGG..All the 18 medicinal plants exhibited 10 RAPD bands respectively. Whereas the some of the medicinal plants such as *Artemisia vulgaris Mentha citrate and Piper longum* have expressed more than 10 RAPD bands. Shown in Fig 2and 15. The primer OPL-11 shown 63 RAPD bands. An Out of these 63 RAPD bands of the medicinal plants plant such as Acorus calamu is revealed 10 RAPD bands. Whereas other medicinal have revealed 7-8 RAPD bands respectively as shown in fig 3 and 16. The genomic DNA of medicinal plants were amplified with the primer sequence OPO – 08 revealed a total of 89 RAPD bands. Out of these 89 RA PD bands indicating both monomorphic and polymorphic character with an average of 4.9 per primer.

A total of 89 bands amplified products were generated by random primer OPO – 08, as shown in the Fig 4 and 17. The primer OPAH -15 is generated 55 bands shown in the Fig. 5 and 18 with an average of 3.05 per primer. Of these 55 bands amplified products showing 99% fragments were polymorphic in pair wise comparison The highest number of RAPD bands was recorded in plants like 3, 4,9,10, and 16 and less bands was recorded in 1 and 2 plants species respectively. Primers such as OPAM– 2 and is shown in Fig 6 and 19. were generated 97 RAPD gel profiles. In the present data the plants like 3,4, 7,8,9,10,17 and 18 were recorded with 5-9 RAPD bands respectively. These medicinal plants revealed moderate diversity among them. OPAN – 01 primer as shown in Fig 7 and 20. Generated a total of 43 RAPD bands. However, some of the plant species was shown two bands of RAPD in 10, 11, 12, 13, 14. 15, 16, 17 and 18 respectively. Further some of the plant showed 3-4 RAPD bands respectively. Primer OPAO – 01 genomic DNA of 18 medicinal plants amplified with OPAO – 01 revealed 45 RAPD bands.

The both monomorphic and polymorphic RAPD bands. The number of bands per primer was recorded as 2.5 However, 2 to 4 bands were recorded in plants like 1 to 18 respectively. Despite, the other plants revealed a total number of 45 bands as shown in Fig 8 and 21. The RAPD profile generated by primer OPAP – 20 produced a total number of 41 bands, with 2.2 RAPD bands per primer..Therefore, the RAPD data was recorded in plants such as 1, 2,3,4,5,6,7,8 and 9 respectively. The genomic DNA of some of the varieties such as 10 to 18 plants was amplified with OPAP – 20 generated one RAPD bands per primer as shown in Fig 9 and 22. The RAPD profile generated by primer OPAN – 05 produced a total number of 46 bands.

The RAPD data was recorded in plants such as 1, 2,3,4,5,6,7,8, 9, 10, 11, 12, 13 and 14 revealed one band respectively. However the genomic DNA of 4th plant is not amplified with OPAN – 05. as shown in Fig 10and 23. Primer OPAP – 10 revealed a total number of 95 RAPD bands the number of bands per primer was recorded maximum of 5.2 bands. However, amplification showed very clear and distinct bands, and some of the medicinal plants like 7, 12, 15, 16 and 17 have revealed five bands respectively, as shown in Fig 11 and 24. Primer OPAA– 01 As shown in Fig 12 and 25. The distinct and abundant RAPD fragments were recorded. The total numbers of bands were generated 40 RAPD gel profiles In the present data, the plants like 3, 7, and 15 were recorded with 3 RAPD bands respectively. RAPD analysis of medicinal plants using Primer OPAB –

01 114 RAPD bands. The number of bands per primer was recorded maximum of 12.6 bands. However, three bands were recorded in plants like 1 to 18 respectively. Despite, the plants revealed a total number of 114 bands, nine bands respectively. From this data it is possible to identify species specific band for medicinal plants for selection,

Genetic resources available for medicinal plants improvement are abundant within plant species. Even though a few species of medicinal plants occur naturally in India, many cultivated medicinal plants species do find their origin with in the country especially India. Almost all the cultivated and naturaly occurring medicinal plants and 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 plants species. This fact suggests a close genetic or non genetic relationship among the medicinal plants. The present study involving 18 medicinal plants with molecular characterization, of RAPD, analysis for further supports this view. RAPD analysis of 18 medicinal plants using Primer OPD-13.

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 hratissimum, Mentha piparita, Mentha citrate, Mentha spicata, Acorus calamus, Centella asiatica, Bacopa moninierii, Piper longum, Piper nigrum, Clitoria ternatea, Aloe vera, Stevia rebaudia* were amplified with oligonucleotides primers OPD-13 revealed total of 172 RAPD bands, (Fig 1-14). Similar observations were recorded by Girish Naik and Dandin 2006, Souframani and Gopalakrishna, 2004. Similar observations have also made in other species at cultivars level (Colombo et al., 1998, Banerjee et al., 1999, Das et al., 1998,). RAPD analysis medicinal plants using Primer OPD-07 were amplified with OPD-07 revealed a total of 104 RAPD bands. With an average of 6 bands per primer, all the 18 medicinal plants exhibited 10 RAPD bands respectively. Whereas the some of the medicinal plants such as *Artemisia vulgaris Mentha citrate and Piper longum* have expressed more than 10 RAPD bands per primer. as shown in Fig 2-15. Further, similar observation was made by Awasthi et al., (2004) Basha, S.D and Sujatha, M. 2007 in mulberry and medicinal plants.

RAPD analysis of medicinal plants using Primer OPL-11. It is known from the literature that the molecular markers in medicinal plants is important observations (Girish Naik and Dandin 2002,). The genomic DNA of 18 medicinal plants was amplified with primer sequence of OPL – 11 produced 3.5 bands per primer. A total of 63 RAPD bands discrete amplified products were generated with the primer OPL – 11. Out of these 63 RAPD bands of the medicinal plants, the medicinal plant such as *Acorus calamu* is revealed 10 RAPD bands. OPL – 11 have revealed 7-8 RAPD bands respectively as shown in fig 3-16. The Primer OPO – O8. revealed a total of 89 RAPD bands. Out of these 89 RAPD bands indicating both monomorphic and polymorphic character in the medicinal plants like 1, 7, 8, 13, 15 and 18, significance of cross pollination is slow in these plants and out crossing is less as far as population genetics is considered. This study in accordance with (Balakrishna et.al, 2000, Aswathi et. al, 2004; Suryanarayan et.al, 2002;

Chikkaswamy et.al, 2007) Fig 4-17. RAPD analysis of medicinal plants using Primer OPAH – 15. A total of 13 decamer oligonucleotide primers were examined on the medicinal plants. All the random primers resulted in distinct polymorphic banding pattern. The results obtained in the primer OPAH – 15 are shown in the Fig.5-18. Similar observation made by (Souframanien and Gopalakrishna (2004), Srivastava et.al; 2004, Vijayan et.al,), with an average of 3.05 per primer with 55 bands. DNA of the plants revealed some common RAPD bands except species of medicinal plants like 1,2,3,4,7,8,13, and 14 revealed common banding patters , it clearly indicates that some of the plant species of medicinal plants is deviating from other plants as far as gene flow and elegant factor of RAPD sequence.

The highest number of RAPD bands was recorded in plants like 3, 4,9,10, and 16 and less bands was recorded in 1 and 2 plants species respectively. Primer OPAM– 20. and is shown in Fig 6-19. The distinct and abundant RAPD fragments were recorded. The total numbers of bands were generated 97 RAPD gel profiles. the number of RAPD bands per primer were 5.3. The RAPD bands distributed in plant is important to know the value of breeding pattern in medicinal plants. The number of RAPD bands was produced to reveals. In the present data the plants like 3,4, 7,8,9,10,17 and 18 were recorded with 5-9 RAPD bands respectively. Further, 1 and 2 medicinal plants was showed 5 RAPD bands due to amplification of primer with the genomic DNA of these plant species. The similar observation was made by (Colombo et.al, 1998, Das et.al, 1998;. The Primer OPAN – 01. as shown in Fig 7-20. A total of 43 RAPD bands were generated. With 2.3 markers per primer. However, some of the plant species was shown two bands of RAPD in 10, 11, 12, 13, and 14. 15, 16, 17 and 18 respectively. The distribution of RAPD bands linked and encoding to certain quantitative traits of medicinal plants. This hypothesis has been corroborated from Plomion et al. (1995). Primer OPAO – 01.The data obtained in the present investigation revealed a total number of 45 RAPD bands. The number of bands per primer was recorded as 2.5 However, 2 to 4 bands were recorded in plants like 1 to 18 respectively. As shown in Fig 8-21. Similar observation was made by (Colombo et.al, 1998; Das et.al, 1998 ;). Primer OPAP – 20. The RAPD profile generated by primer OPAP – 20 produced a total number of 41 bands with 2.2 RAPD bands per primer.

Therefore, the RAPD data was recorded in plants such as 1, 2,3,4,5,6,7,8 and 9 respectively. The genomic DNA of some of the varieties such as 10 to 18 plants was amplified with OPAP – 20 generated one RAPD bands per primer. medicinal plants using molecular markers as unique powerful tool for taxonomy of medicinal plant identification (Akito Kaga,et al., 1996, Bennett 1987, Bennett and Smith 1991) In this direction OPAP – 20 primers as showed low resolution of RAPD banding profiles to distinguish medicinal plants under differential speciation as shown in Fig 9-22. Primer OPAN – 05. Produced a total number of 46 bands, with 2 to 3 RAPD bands per primer. Therefore, the RAPD data was recorded in plants such as 1, 2,3,4,5,6,7,8, 9, 10, 11, 12, 13 and 14 revealed one band respectively. primers as showed low resolution of RAPD banding profiles to distinguish medicinal plants under differential speciation as shown in Fig 10-23. Similar data have been reported by (Darokar.M.P, 1998 and Das et.al, 1998).

Table.1. Sequence information of 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

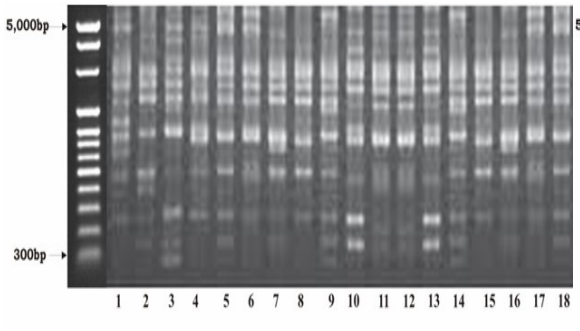

Fig.1: Gel profile 0f RAPD Primar OPD-13

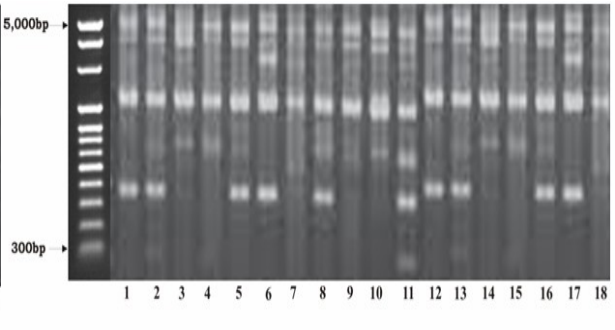

Fig.2: Gel profile 0f RAPD Primar OPD-07

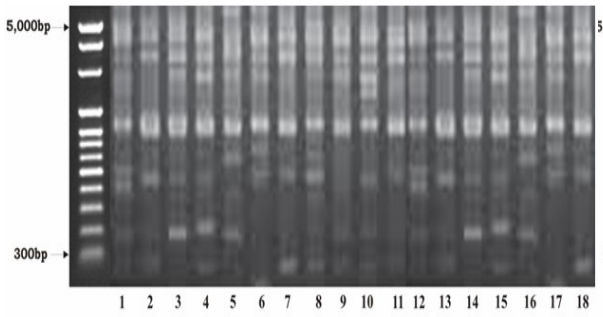

Fig.3: Gel profile 0f RAPD Primar OPL-11

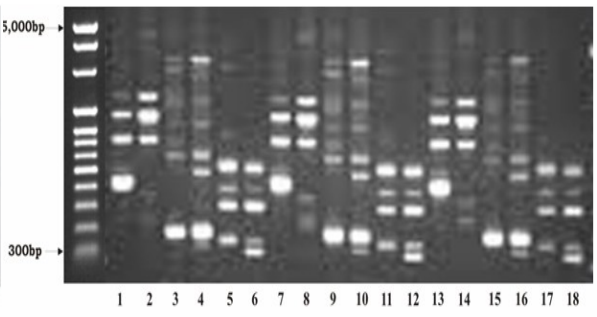

Fig.4: Gel profile 0f RAPD Primar OPO-08

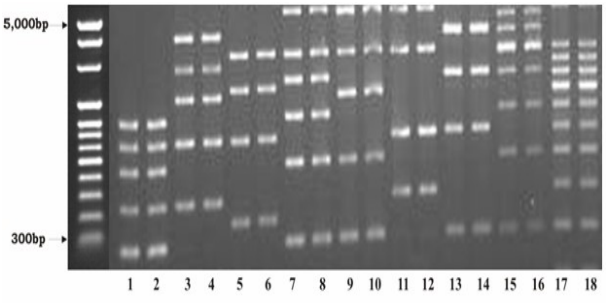

Fig.5: Gel profile 0f RAPD Primar OPAH-23

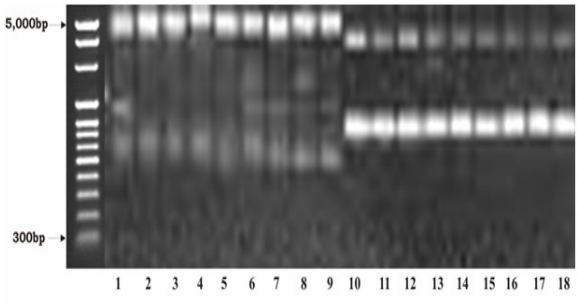

Fig.6: Gel profile 0f RAPD Primar OPAM-20

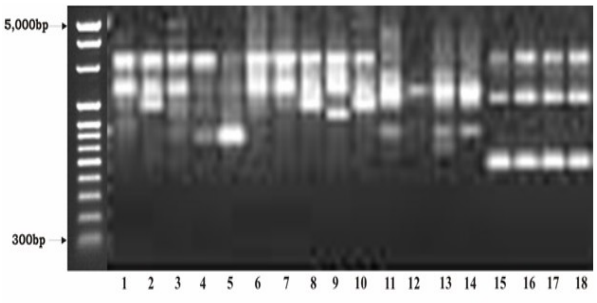

Fig.7: Gel profile 0f RAPD Primar OPAN-05

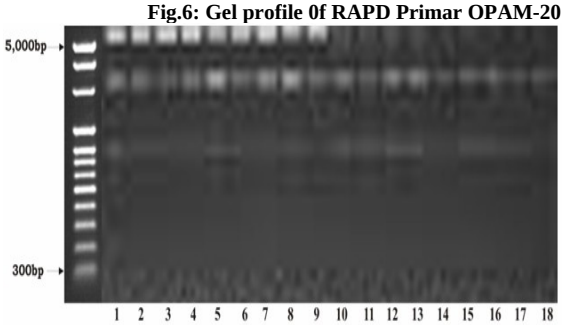

Fig.8: Gel profile 0f RAPD Primar OPAO-01

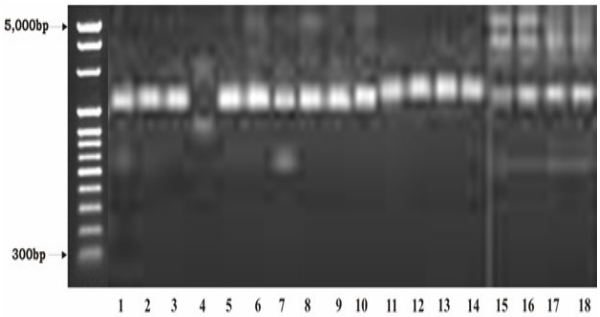

Fig.9: Gel profile 0f RAPD Primar OPAP-20

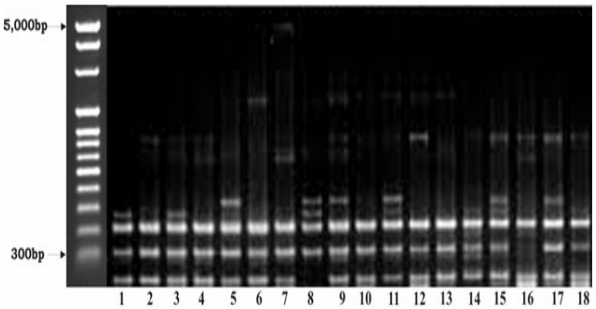

Fig.10: Gel profile 0f RAPD Primar OPAN-05

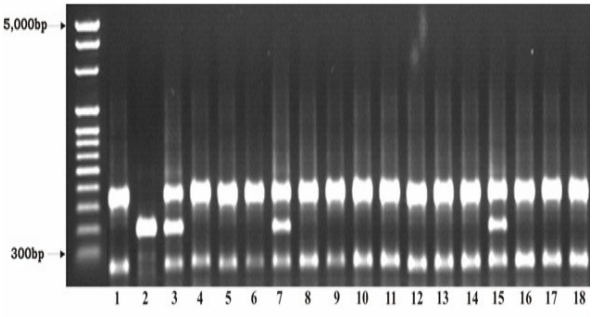

Fig.11: Gel profile 0f RAPD Primar OPAP-10

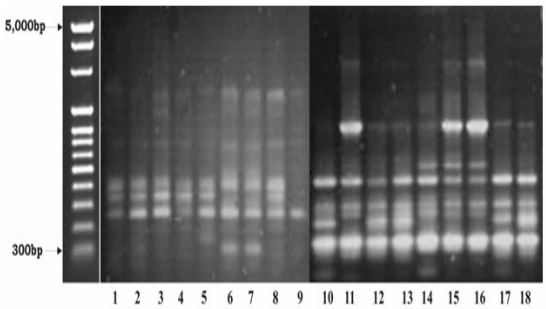

Fig.12: Gel profile 0f RAPD Primar OPAA-01

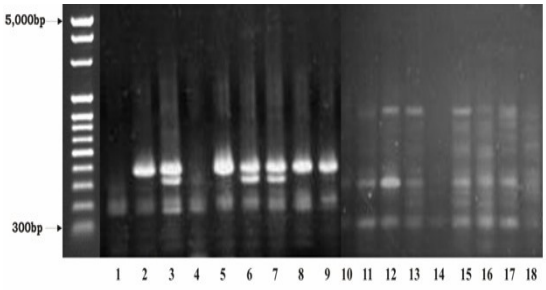

Fig.13: Gel profile 0f RAPD Primar OPAB-01

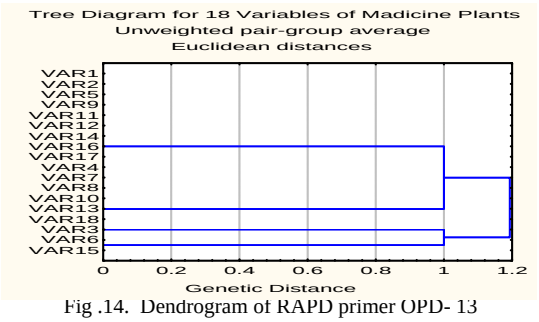

Fig .14. Dendrogram of RAPD primer OPD- 13

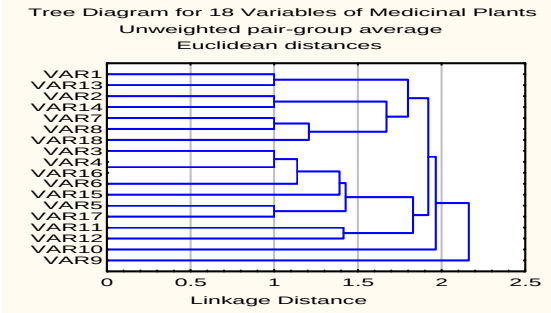

Fig.15. Dendrogram of RAPD primer OPD- 07

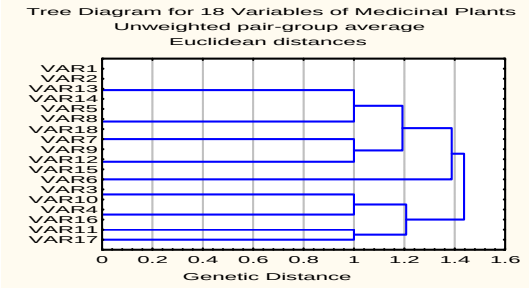

Fig.16 Dendrogram of RAPD primer OPL-11

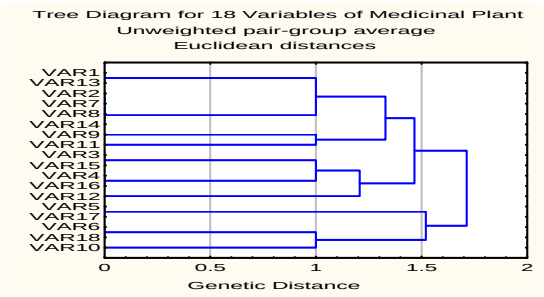

Fig 17. Dendrogram of RAPD primer OP0- 08

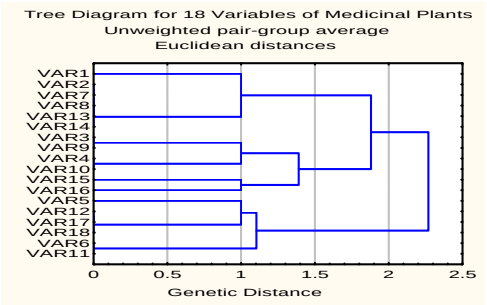

Fig 18. Dendrogram of RAPD primer OPAH- 15

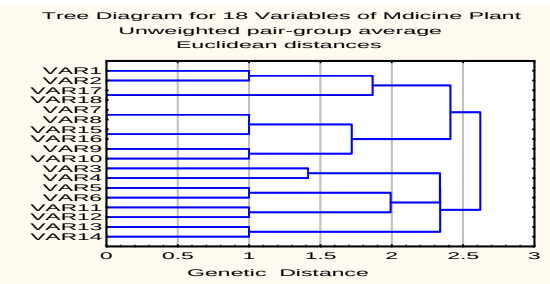

Fig 19. Dendrogram of RAPD primer OPAM- 20

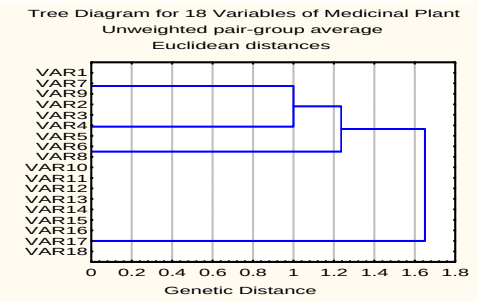

Fig 20. Dendrogram of RAPD primer- OPAN-01

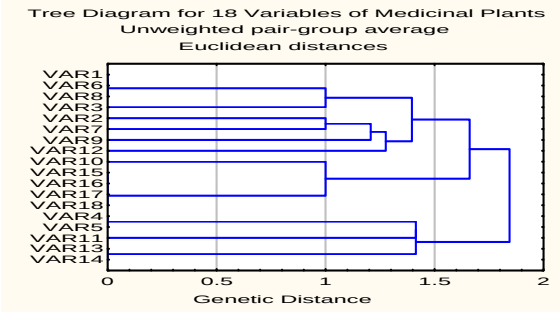

Fig 21. Dendrogram of RAPD primer- OPAO-01

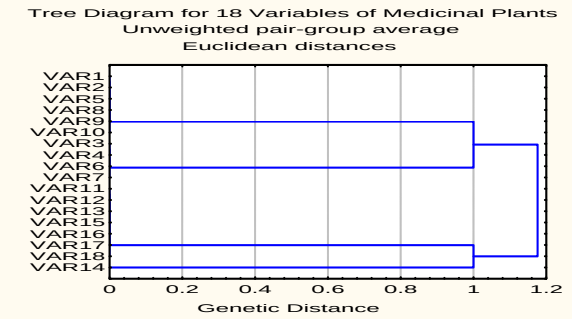

Fig 22. Dendrogram of RAPD primer- 0PAP-20

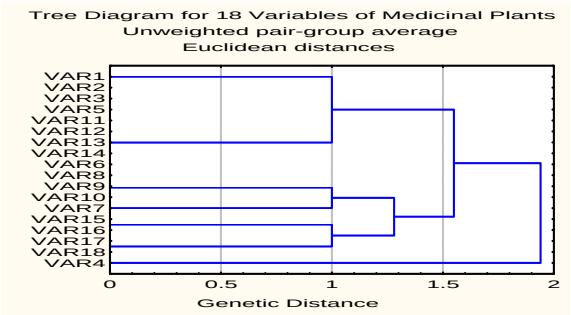

Fig 23. Dendrogram of RAPD primer- 0PAN-05

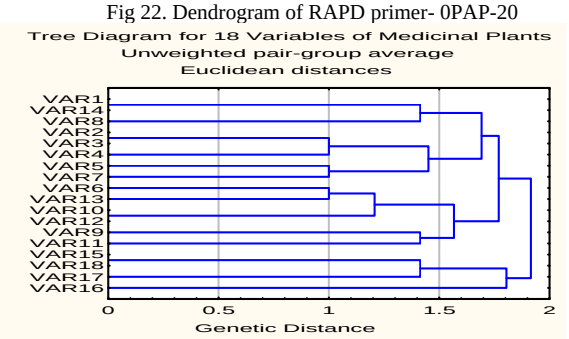

Fig 24. Dendrogram of RAPD primer- OPAP-10

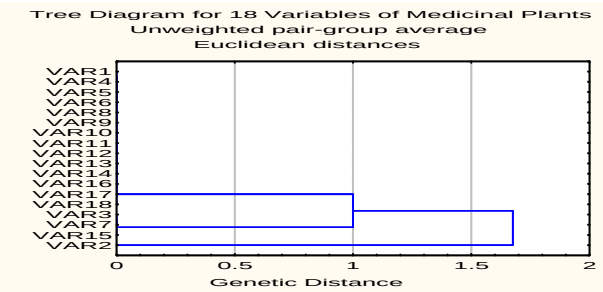

Fig 25. Dendrogram of RAPD primer- OPAA-01

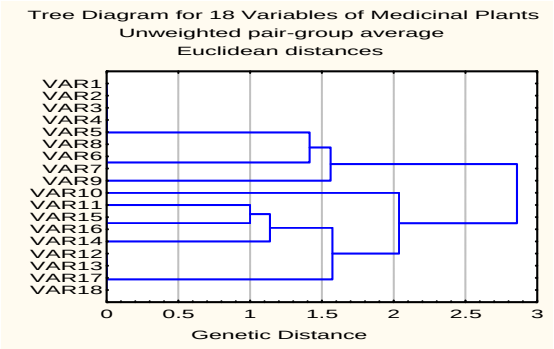

Fig 26. Dendrogra m of RAPD primer- OPAB-01

Further, RAPD analysis of medicinal plants using Primer OPAP – 10, revealed a total number of 95 RAPD bands. The banding patterns are common, and one of the plants as revealed 5 RAPD bands due to the amplification of genomic DNA with primer OPAP – 10. plants like 7, 12, 15, 16 and 17 have revealed five bands respectively. as shown in Fig 11-24. This finding is similar to (Das et al. 1998, Das et al. 2001). The Primer OPAA– 01. The data obtained in the present investigation was similar with respect to the other crop plants reported by (Hormaza 1995), Olive (Fabbri et al, 1995), Walnut (Neieise et al., 1998)The genomic DNA of 18 medicinal plants was amplified with decamer oligonucleotide primers such as OPAA–01 and is shown in Fig 12-25. The distinct and abundant RAPD fragments were recorded. The total numbers of bands were generated 40 RAPD gel profiles. Plants with Primer OPAB – 01. Shown 114 RAPD bands. The number of bands per primer was recorded maximum of 12.6 bands. However, three bands were recorded in plants like 1 to 18 respectively. Plants like 1 to 8 and 11 to 18 have revealed nine bands respectively as shown in Fig 13-26. This data is corroborated with the findings of (Krammer et al.,1992) and (Koller et

al.,1993) in Apple plants. In the present investigation revealed 1to 4 clusters indicated low gene flow in different medicinal plants species belonging to different family as reported by Stebbins evolution group of other plants

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