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Research Article

Analysis of genetic variability in patchouli cultivars (*Pogostemon cablin* Benth.) by using RAPD Markers

Kumara Swamy M[1, 2*], and Anuradha M[2, 3]

[1]Department of Biotechnology, Acharya Nagarjuna University, Nagarjunanagar, Guntur, India
[2]Rishi Foundation, #234, 10th C main, 1st Block, Jayanagar, Bangalore- 560011, India
[3]Padmashree Institute of Management and Sciences, Kommagatta, Kengeri, Bangalore- 560060
*Corresponding Author Email: swamy.bio@gmail.com

The genetic relationships among patchouli cultivars were determined by using Random Amplified Polymorphic DNA (RAPD) technology. Among 45 decamer random primers used for PCR reactions, 10 primers showed reproducible results. Out of 98 amplification products recorded, 16.7 per cent were monomorphic and 83.3 per cent were polymorphic. The highest dissimilarity (7.35) was detected between KSM 4 and 5 and the least 3.61 between KSM 2 and 3. Dendrogram constructed by cluster analysis of RAPD markers using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) produced two major clusters 'A' and 'B'. Cluster 'A' consisted of five cultivars that segregated into two sub clusters 'A1' and 'A2'. Overall, RAPD analysis revealed the existence of considerable genetic variations in patchouli cultivars. This information regarding genetic variability at the molecular level could be used to identify and develop genetically unique germplasm that complements existing cultivars.

Key words: *Pogostemon cablin*, RAPD marker, genetic variability, cluster analysis

Patchouli (*Pogostemon cablin* Benth.) belonging to the family Lamiaceae, is an aromatic herb cultivated on a commercial scale in India, Indonesia, Malaysia, China and Singapore. The commercial oil from patchouli is extensively used in perfumes and cosmetics (Hasegawa *et al*., 1992, Maheswari *et al*., 1993). The oil is widely used in the manufacture of soaps, scents, body lotions and detergents. It is been used to treat dysentery, diarrhea, colds without fevers, vomiting and nausea. The essential oil may be used to treat acne, dry skin, fungal infections, dermatitis, dandruff and eczema (Kalra *et al*., 2006). The fresh leaves can help in healing burns. In aromatherapy, it is used to calm nerves, control appetite, relieves depression, stress and lack of sexual interest (Bowel *et al*., 2002). It also possesses insecticidal, antibacterial and antifungal properties (Kukreja *et al*., 1990, Yang, 1996, Pattnaik *et al*., 1996). Fibrinolytic and anti thrombotic activity of this essential oil is also been reported (Sumi, 2003, Eunkyung *et al*., 2002).

Indian demand for patchouli oil is around 220 tonnes valued at 33 crores while global demand is to the tune of 1600 tonnes of oil per annum with a value of 240 crores (Vijaya Kumar, 2004). India is importing annually about 20 tonnes of pure patchouli oil and 100 tonnes of formulated oil which is

certainly a very huge quantity. Patchouli oil's growing demand can be understood as it can neither be replaced inorganically nor synthesized because of its complex molecular structure (Farooqi *et al*., 2001). Hence the only alternative is to cultivate the plant extensively.

Worldwide research activities are aiming at improving the plant quality. The increasing number of varieties and the importance of their choice make it necessary to strengthen user's guarantee concerning purity and identity. Hence, unambiguous characterization of varieties and conservation of elite germplasm is of prime importance. At present there are only few commercially grown varieties in patchouli (http://nhb.gov.in/bulletin_files/aromatic/patchouli/pat012.pdf). Many people have named the same lines according to their company or other local names. Hence, accurate identification of plant materials is essential for effective germplasm characterization.

Despite the commercial importance of the crop, genetic data on patchouli in India are scarce. The identification of different varieties/cultivars/germplasm based on morphological traits implies culture inspection at different stages and is not reliable because many traits are governed by complex genetic interactions. Molecular markers based on DNA sequences offer means of identification with much greater reliability than the morphological traits. RAPD remains one of the most extensively used molecular techniques due to its simplicity, low cost and high speed. Thus, RAPD markers have been successfully used in many crops in providing a convenient and rapid assessment of genetic diversity among different genotypes (Williams *et al*., 1990, Rafalski and Tingey, 1993, Ragot and Hoisington, 1993). RAPD markers have been already successfully used on other medicinal and aromatic crops (Kasaian *et al*., 2011, Salim Khan *et al*., 2010, Verma *et al*., 2009, Bharmauria *et al*., 2009, Padmalatha and Prasad, 2007). The present study therefore, was undertaken to study genetic diversity in patchouli cultivars. This might serve as a valuable tool for effective screening of genetic resources for future research and to improve and sustain genetic diversity of different patchouli varieties/cultivars. This would help in the identification and differentiation of various cultivars being cultivated.

Materials and methods

Plant material

The details of 6 patchouli cultivars selected for RAPD analysis are given in the table 1. All the cultivars were maintained in the herbal garden of Rishi Foundation, Bangalore, India. The fresh leaf sample collected were wiped with 70% alcohol and dried at 40^0 C for 24 hours. The dried leaves are then sealed in plastic bags and used later for DNA isolation.

Table 1. Details of patchouli cultivars used in the present study

S. No	Cultivar code	Cultivar details
1	KSM 1	Johor variety: Collected from the Aromatic section, Department of Horticulture, Gandhi Krishi Vignana Kendra (GKVK), Bangalore, India
2	KSM 2	Singapore variety: Collected from the Aromatic section, Department of Horticulture, Gandhi Krishi Vignana Kendra (GKVK), Bangalore, India
3	KSM 3	Simshreshta variety: Collected from the Aromatic section, Department of Horticulture, Gandhi Krishi Vignana Kendra (GKVK), Bangalore, India
4	KSM 4	Collected from Rishi Herbal Technologies Pvt Ltd, Bangalore, India
5	KSM 5	Collected from Rishi Herbal Technologies Pvt Ltd, Bangalore, India
6	KSM 6	Collected from Rishi Herbal Technologies Pvt Ltd, Bangalore, India

DNA extraction protocol

The method used here is a modification of the original Cetyl trimethyl ammonium bromide (CTAB) method outlined by Doyle and Doyle (1987). 500 mg of leaf powder is transferred to a sterile centrifuge tube containing 15ml of extraction buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 0.7MNaCl, 0.4M LiCl, 1% w/v CTAB, 1% w/v PVP and 0.25% beta mercaptoethanol) preheated at 65^0 C. The contents were then incubated in a water bath at 65^0 C for 30 minutes with intermittent shaking. After incubation the contents are brought to room temperature and 10ml of chloroform: isoamylalcohol (24:1) was added to the tube. The mixture is agitated thoroughly and centrifuged at 6000 rpm for 5 minutes. The aqueous phase is transferred to a new tube and centrifuged for 5minutes at 6000 rpm in order to pellet possible debris. The supernatant is then transferred to a new tube and an equivalent volume of isopropanol is added to the aqueous solution. The tube is swirled gently to precipitate DNA. The tube is then centrifuged for 5minutes at 6000rpm and the supernatant is withdrawn. The DNA pellet is washed with 70% ethanol and is air dried for 10minutes. DNA pellet is resuspended in 500µl of Tris-EDTA buffer (10mM tris-HCl and 1mM EDTA, pH 8.0). Concentration and quality of genomic DNA was verified by using gel electrophoresis and spectrophotometer.

Primer selection

Out of 140 primers (Operon Technologies, Alameda, CA, US) screened, 42 primers produced at least one band. During preliminary screening, 22 out of 42 primers yielding more than 6 bands were selected. Finally, 10 (out of 22) primers producing strong, intense and unambiguous bands were selected for fingerprinting patchouli cultivars. The selected primer details are shown in the table 2.

Table 2: Details of random primers used in RAPD analysis

S. No	Primer code	Sequence (5′ to 3′)
1	OPA02	5′ TGCCGAGCTG 3′
2	OPA11	5′ CAATCGCCGT 3′
3	OPC04	5′ CCGCATCTAC 3′
4	OPC07	5′ GTCCCGACGA 3′
5	OPD03	5′ GTCGCCGTCA 3′
6	OPF19	5′ CCTCTAGACC 3′
7	OPF08	5′ GGGATATCGG 3′
8	OPG05	5′ CTGAGACGGA 3′
9	OPG12	5′ CAGCTCACGA 3′
10	OPG17	5′ ACGACCGACA 3′

DNA amplification

Amplification was achieved by following the protocol outlined by Williams *et al*., (1990) with slight modifications. Polymerase reactions were carried out in a final volume of 25 µl reaction mixture containing 25 ng of template DNA, 10 p mol random decamer primer, 0.2 mM dNTPs, 2 U Taq polymerase (Genei, Bangalore, India) and 10 x PCR buffer (10 mM tris pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% gelatin, pH 9.0). Amplification was achieved in a Eppendorf Thermocycler programmed for initial denaturation at 95^0 C for 4 minutes, followed by 45 cycles; each cycle consisting of denaturation at 94^0 C for 1 minute, primer annealing at 35^0 C for 2 minutes, primer extention at 72^0 C for 2 minutes and a final extention of 10 minutes at 72^0 C. The PCR reactions were repeated 3 times, using the same conditions to check the repeatability of amplification products.

RAPD data analysis

Amplification of DNA fragments by random primers was scored "1" for the presence of the band and "0" for absence of the band and the data was converted into a matrix of binary data, where the presence of the band corresponded to value 1 and the absence to value 0. The genetic similarity was calculated using Squared Euclidean Distance

matrix based on RAPD markers amplified with the ten primers. The matrix was subjected to UPGMA (Unweighted Pair wise Methods with Arithmetic averages) cluster analysis to generate a dendrogram. All analyses were worked out using the software STATISTICA, version 3.0 (STATISTICA for Windows, Stat Soft Inc, Tulsa. OK, USA, 1996).

Results and Discussion

A total of 98 RAPD markers of size ranging from 80bp to 3kbp that were consistent, unambiguous and repeatable were produced from the selected 10 primers. These markers were used for fingerprinting and to estimate genetic diversity among the cultivars of patchouli. The number of markers or bands scored for each primer varied from 7 to 10 with an average of 9.6 bands per primer (Fig 1a-b). Out of 98 amplification products recorded, 16.7 per cent were monomorphic and 83.3 per cent were polymorphic bands (Table 3). The bands amplified were of uniform intensity and did not vary significantly with respect to the initial concentration of DNA. Such unique bands can be converted into genotype specific RAPD markers, which may be used for the identification of genotypes. This pool of primers yielded reasonable number of amplification products for all the cultivars examined. Hence, the result of the preliminary RAPD method is capable of revealing nuclear DNA variation in patchouli cultivars. The high number polymorphic markers detected in this study could be result of high diversity among the material used. The utility of RAPD markers in estimating genetic variability has been demonstrated in several studies on medicinal and aromatic plants (Kasaian *et al*., 2011, Salim Khan *et al*., 2010, Verma *et al*., 2009, Ganjewala, 2008, Bharmauria *et al*., 2009, Padmalatha and Prasad, 2007, Sangwan *et al*., 1999, Lal *et al*., 1997).

Table 3. Total number of amplified fragments, number of monomorphic and polymorphic bands generated by PCR using 10 selected primers

Primers	No of monomorphic bands	No of polymorphic bands	Polymorphism (%)	Total no of bands
1	0	12	100	12
2	0	11	100	11
3	4	6	60	10
4	0	10	100	10
5	2	7	77.7	9
6	0	10	100	10
7	4	4	50	8
8	1	6	85.7	7
9	2	7	77.7	9
10	3	7	70	10
Total	16	80	83.3	96

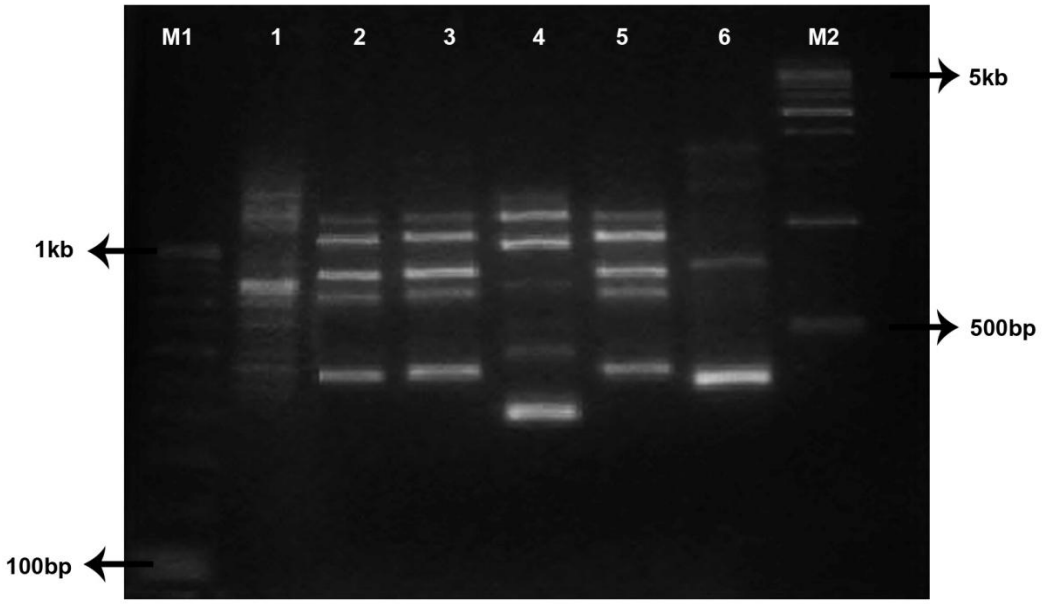

Fig 1a: RAPD banding pattern of patchouli cultivars generated by random primer OPC07. Lanes 1-6 contain the amplification profile obtained using the cultivars (KSM 1, KSM 2, KSM 3, KSM 4, KSM 5, KSM 6). Lane M1 contains 100 bp DNA ladder and M2 contains 500bp DNA ladder.

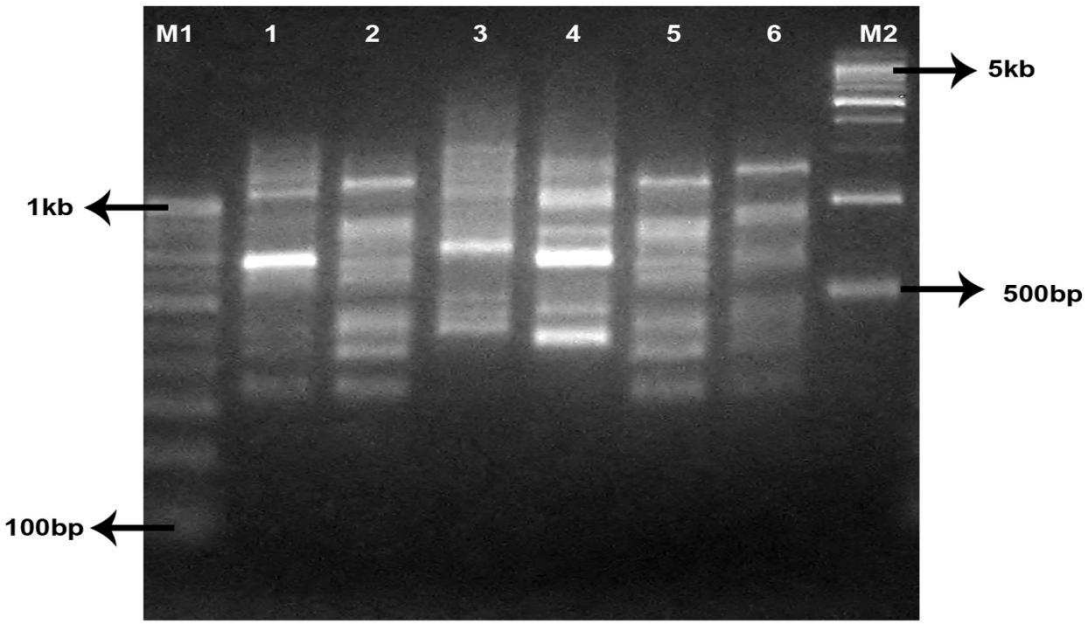

Fig 1b: RAPD banding pattern of patchouli cultivars generated by random primer OPG12. Lanes 1-6 contain the amplification profile obtained using the cultivars (KSM 1, KSM 2, KSM 3, KSM 4, KSM 5, KSM 6). Lane M1 contains 100 bp DNA ladder and M2 contains 500bp DNA ladder.

Table 4: Genetic distance matrix obtained for patchouli cultivars

	KSM 1	KSM 2	KSM 3	KSM 4	KSM 5	KSM 6
KSM 1	0					
KSM 2	5.29	0				
KSM 3	5.74	3.61	0			
KSM 4	6.24	6.24	6	0		
KSM 5	6.86	5.39	6.16	7.35	0	
KSM 6	6.24	5.20	5.48	6.63	5.83	0

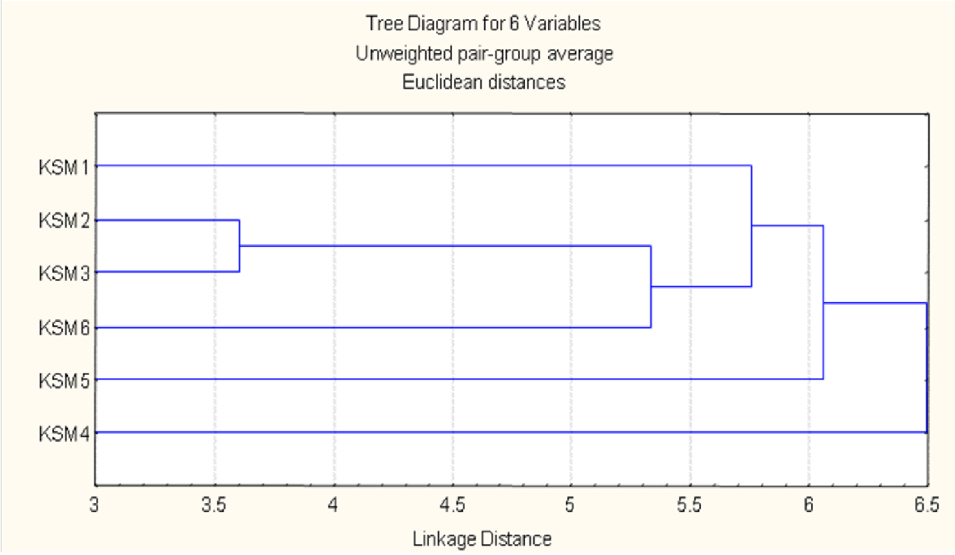

Fig 2: Dendrogram constructed based on Euclidean distances using the UPGMA clustering algorithm.

RAPD bands were manually scored from the gel profile, "1" for the presence and "0" for the absence and the binary data generated from all the profiles were used for statistical analysis. The dissimilarity matrix was computed using Squard Euclidean Distance (SED) that estimated all pair wise difference in the amplification product. The genetic distance matrix was ranged from 3.61 to 7.35 suggesting a narrow genetic base within the patchouli cultivars used in the present investigation. The highest dissimilarity (7.35) was detected between KSM 4 and 5 and the least 3.61 between KSM 2 and 3 (Table 4). The genetic distance among the cultivars clearly shows that significant genetic diversity exists among the patchouli cultivars. Hence, these cultivars are to be preserved as valuable genetic resources for breeding. The high genetic diversity present among these cultivars clearly suggests that they must have originated from genetically divergent parents or have a long history of adaptation to their respective micro-climatic regions. In the dendrogram (Fig 2), all 6 cultivars were divided into two major clusters 'A' and 'B'. Cluster 'A' consisted of five cultivars that segregated into two sub clusters 'A1' and 'A2' with four (KSM 1, 2, 3 & 6) and one (KSM 5) cultivar respectively. All the cultivars in the cluster 'A1' are related to each other. This is possible due to the similarity in their morphological and yield parameters. The KSM 5 was closely linked to KSM 4 which can be evidenced by the similarity in their morphology, yield and

chemical composition of the essential oil *viz*., patchouli alcohol content. The sub cluster 'A1' was divided into two minor clusters 'A1a' and 'A1b' at 5.75 linkage distance. The minor cluster 'A1a' consisted of one cultivar i.e., KSM 1. The minor cluster 'A1b' consisted of 3 cultivars segregated into two groups at 5.3 linkage distance. Group I of 'A1b' consisted of one cultivar, KSM 6. The cultivars, KSM 2 and 3 were closely linked at 3.61 linkage distance. Closeness of these cultivars may be due to the fact that they have originated from a single parent or a narrow genetic base. The major cluster 'B' consist only one cultivar (KSM 4) and was linked to cluster 'A' at 6.5 linkage distance. It is clear from the dendrogram that KSM 4 was very distinct from the rest of the cultivars, which might be due to its superior qualities with respect to morphology, yield and patchouli alcohol content. This cultivar might be an improved variety which is commercially available for mass cultivation.

This information regarding genetic variability at the molecular level could be used to identify and develop genetically unique germplasm that complements existing cultivars. Pan *et al*., (2006) have studied the genetic polymorphism and intra specific genetic differentiation of five plant populations of *Pogostemon cablin* in China. Four original plants of Xihuangcao (*P. cablin*) have been differentiated from each other by random amplified polymorphic DNA polymorphism (Chen *et al*., 2001). To the best of our knowledge in India, a little information is available for patchouli at the genetic level. This is the first report on the use of molecular markers for fingerprinting and evaluating genetic relationship of patchouli cultivars in India. Thus, RAPD method allowed us to access genetic diversity between patchouli cultivars. This assessment is fundamental because genetic diversity in the future could be exploited through molecular approaches or plant breeding techniques to improve patchouli cultivars for disease resistance or to increase essential oil yield.

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